REGULATION OF Ca^{2+} CHANNELS AND EXOCYTOSIS BY RECEPTORS FOR PROSTAGLANDIN E_2

By

Mark L. Jewell

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmacology

August, 2013

Nashville, Tennessee

Approved:	Date:
Kevin Currie	6/27/2013
Rich Breyer	6/27/2013
Aurelio Galli	6/27/2013
Dave Piston	6/27/2013
Heidi Hamm	6/27/2013

ACKNOWLEDGMENTS

I would like to acknowledge my sources of funding: NIH/NINDS:5T32 NS007491 Training Grant in Ion Channel and Transporter Biology, and funding granted to my mentor, Dr. Kevin Currie: American Heart Association [11GRNT7890031], and the National Institutes of Health [R01 NS052446].

My six-plus years in Nashville have been personally and professionally very rewarding. Maybe the best affirmation of that sentiment is my current lack of cynicism for basic molecular inquiry and my desire to keep contributing in the field. Like most feeling about various things this sentiment starts at home, with enthusiasm from my parents Bill and Barbara, my sister Andrea, and my Labrador, Betty. I married my wife Jolene, another Penn State/Vanderbilt alum, while researching this project. I have commented to friends on her remarkable comparatives to myself and others, but thought it should be published: smarter, prettier, kinder, more universally liked and respected, harder working, more focused, more patient, and so-on. Regarding this project and other places our careers intersect, she knows what the hell I'm talking about, why I'm talking about it, and why it matters.

A significant component of the educational energetics of graduate school involved informal conversation, and this was certainly the part I valued most. Specifically, I learned and improved as a student because others donated time. These conversations started as a rotation student in Ben Spiller's lab, and continued with organized data

meetings with Dr. Heidi Hamm, Dr. Randy Blakely, and a very engaging thesis committee. I don't have space to recount the topics, but want to acknowledge the countless bull sessions with Jason Downey, students and staff in the Breyer and Hamm labs, Dr. Breyer, Dr. Galli, our postdocs Lei Zhu and Rebecca Brindley, and research staff member Sarah McDavid.

Of course the most time and effort was contributed by Dr. Currie. My invitation to the lab seems risky in hindsight as I brought relatively little research experience and wanted to develop a project. His managerial approach is simple yet underused: invest in your workforce and make them successful. Kevin pulled up a chair and taught me every experimental technique in this document (except RT-PCR). Without exaggeration, I've walked into his office uninvited every day and started talking/drawing on the white board. He has proofed most of my talks/abstracts/posters/last minute efforts, and delivered only one (deserved) ass-chewing. I sincerely appreciate his work on these fronts.

Kevin was promoted recently, no doubt as result of great individual effort. His tenure was a tremendous relief knowing, as the only graduate student in a small lab, my work was significant enough to contribute to (rather than derail!) his achievement. Having a rooting section of employees is probably a sign you treat people well. The status of Kevin's lab will continue to rise, so new students should buy stock in the Currie Lab while prices are still reasonable, and before Kevin trades in the nuisance of graduate student mentorship for an all postdoc lab.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACh acetylcholine

ACTH adrenocorticotrophic hormone

ADP adenosine diphosphate

APW action potential waveform

ATP adenosine triphosphate

Ca^{2+} calcium

$Ca_v(x)$ voltage gated calcium channel subtype x

CaM calmodulin

CaMKII Ca^{2+}/ calmodulin-dependent protein kinase II

cAMP cyclic adenosine monophosphate

CHGA chromogranin A

DAG diacylglycerol

ECL extracellular loop

Epac exchange protein directly activated by cAMP

G protein guanine nucleotide binding proteins

GEF guanine nucleotide exchange factors

GIRK G protein gated inwardly rectifying potassium channels

GDP guanine diphosphate

GPCR G protein coupled receptor

GRK2 GPCR receptor kinase 2

GTP guanine triphosphate

IP3 inositol 1,4,5-trisphosphate

IL-1β interleukin 1-beta

LPS lipopolysaccharide

MRP4 multidrug resistance protein four

NOP nociception/orphanin FQ peptide receptor

NPY neuropeptide Y

NSAID non-steroidal anti-inflammatory drug

PACAP pituitary adenylate cyclase-activating polypeptide

PGT prostaglandin transporter

PMA phorbol 12-myristate 13-acetate

PIP_2 phosphatidylinositol 4,5-bisphosphate

PKA protein kinase A

PKC protein kinase C

PI3K phosphoinositide-3 kinase

PLC phospholipase C

PLD phospholipase D

PTX pertussis toxin

RGS regulators of G protein signaling

ROS reactive oxygen species

RRP readily releasable pool

PA phosphatidic acid

PC phosphatidylcholine

S1P sphingosine 1 phosphate receptor

SI sequence identity

SNAP25 synaptosomal associated protein 25

SNARE soluble n-ethylmaleimide sensitive factor attachment protein receptor

TRPM1 transient receptor potential cation channel subfamily M member 1

1. INTRODUCTION

The role of calcium channels in intercellular communication

Calcium dependent exocytosis underlies intercellular communication

Chemical synaptic transmission is fundamental for intercellular communication within the mammalian central and peripheral nervous systems. Voltage-gated calcium (Ca^{2+}) channels play pivotal roles in this process by coupling excitation (i.e. action potential firing) to secretion of neurotransmitters and hormones through entry of the ubiquitous second messenger Ca^{2+}. In this manner, Ca^{2+} ions facilitate cognition, endocrine homeostasis, muscle contraction, ion channel excitability, enzymatic activity, gene expression and cellular differentiation (Currie, 2010a). Accordingly, regulation of voltage-gated Ca^{2+} channels, and by extension the intensity / timing of neurosecretion is critical. Multiple protein-protein interactions and second messenger pathways converge on the channels to control the amount, location, and timing of Ca^{2+}entry, including: direct interaction with the exocytotic fusion machinery including the SNARE proteins syntaxin and SNAP25; phosphorylation by PKC, CaMKII, and other kinases; and complex feedback by Ca^{2+} itself mediated through calmodulin and other related calcium sensing proteins (for recent review see (Catterall and Few, 2008)). Another prominent control mechanism was first demonstrated ~ 30 years ago in chick sensory neurons (Dunlap and Fischbach, 1978; Dunlap and Fischbach, 1981), and involves inhibition of the Ca^{2+}

channels by G protein coupled receptors (GPCRs) (Currie, 2010a; Ikeda and Dunlap, 1999; Tedford and Zamponi, 2006) (Zamponi and Currie, 2013).

Calcium channel physiology

In mammals ten genes are known that encode pore forming α_1 subunits of voltage-gated Ca^{2+} channels. These are subdivided into three families based on sequence homology: four Ca_V1 members ($Ca_V1.1$ - $Ca_V1.4$; all L-type channels), three Ca_V2 members ($Ca_V2.1$, P/Q-type; $Ca_V2.2$, N-type; $Ca_V2.3$, R-type channels) and three Ca_V3 members ($Ca_V3.1$ - $Ca_V3.3$, all T-type channels) (Catterall et al., 2005; Ertel et al., 2000). Of these, the $Ca_V2.1$ and $Ca_V2.2$ channels are most closely coupled to neurotransmitter release, and as such are densely localized to brain regions rich in synaptic structures (Trimmer and Rhodes, 2004; Westenbroek et al., 1992; Westenbroek et al., 1995). Ca_V α_1 subunits are large (~175-225 kDa) proteins consisting of four homologous domains, each domain containing six transmembrane α-helical segments (S1-S6) (Figure 1) (Catterall, 2000). The channel adopts a tetrameric architecture, with the pore formed by the S5-S6 and intervening P-loop from each domain, while the S1-S4 segments comprise the voltage sensing regions (Figure 1). The intracellular N- and C-termini and the cytoplasmic loops connecting domains I-IV are all important regulatory regions targeted by a variety of proteins, including the $Ca_V\beta$ subunit, G proteins, SNARE proteins, calmodulin and protein kinases (Figure 1).

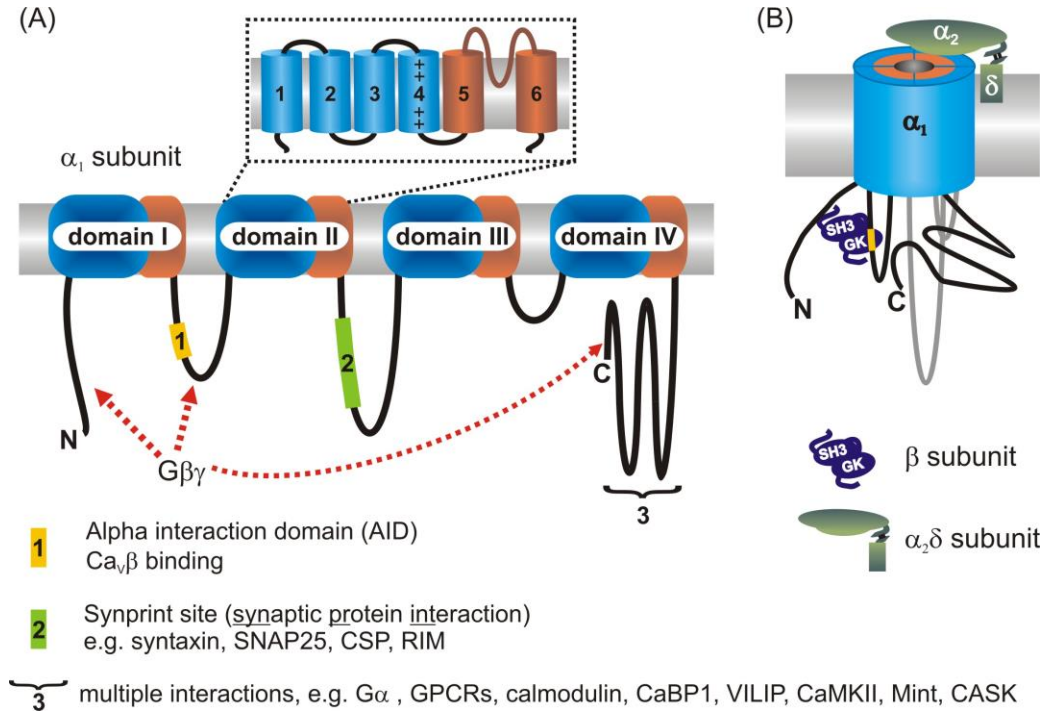

Figure 1: Topology, domain structure and subunit composition of voltage-gated Ca^{2+} channels. (A) Topology of the channel α_1 subunit. This pore forming subunit consists of four homologous repeats (domain I through domain IV), which fold to impart an overall tetrameric architecture to the channel. Each domain (see inset) has six transmembrane spanning α-helices (S1-S6) (blue or orange cylinders). S5, S6 and the P-loop connecting them comprise the 'pore domain' of the channel (colored orange), while S1-S4 (in particular S4 that has multiple charged residues) comprises the 'voltage sensor' (colored blue). The intracellular N- and C-termini and the cytoplasmic loops that connect domains I-IV are important for interaction with other proteins that modulate channel trafficking, stability and function including, the auxiliary β subunit, synaptic proteins, kinases, Gβγ, GPCRs, calmodulin and other Ca^{2+} binding proteins. **(B)** Cartoon depiction of the α_1 subunit along with auxiliary β and $\alpha_2\delta$ subunits. The α_1 subunit adopts a tetrameric architecture with the pore-forming region of each domain lining the aqueous pore, flanked by the four voltage-sensing domains. The β subunit is cytoplasmic and interacts through its guanylate kinase-like domain (GK) with the I-II linker of the α_1 subunit (at the α-interaction domain or AID). The $\alpha_2\delta$ subunit is largely extracellular and likely GPI-anchored to the plasma membrane.

The Ca_V1 and Ca_V2 families are high-voltage-activated (HVA) channels that require stronger membrane depolarization to activate relative to the low-voltage-activated Ca_V3 channels. These HVA channels are heteromultimeric complexes that, in addition to the pore forming α_1 subunit, contain auxiliary β and $\alpha_2\delta$ subunits (Catterall, 2000; Dolphin, 2012). The cytoplasmic $Ca_V\beta$ subunit binds to the Alpha Interaction Domain (or AID) on the I-II linker (Figure 1) (Opatowsky et al., 2004; Pragnell et al., 1994; Van Petegem et al., 2004). Four genes are known that encode $Ca_V\beta$ subunits (for reviews see (Buraei and Yang, 2010; Dolphin, 2003)). $\alpha_2\delta$ subunits are the product of a single gene that subsequently undergoes posttranslational cleavage into the α_2 and δ portions which are then reconnected by a disulfide bond (Klugbauer et al., 2003). The α_2 portion is extracellular while the δ portion is linked to the plasma membrane, potentially through a glycosylphosphatidylinositol (GPI) anchor (Davies et al., 2010). Four genes encode $\alpha_2\delta$ subunits. Both the β and $\alpha_2\delta$ subunits control trafficking / stability and modulate the biophysical properties of the channels (for reviews see (Arikkath and Campbell, 2003; Bauer et al., 2010; Buraei and Yang, 2010; Dolphin, 2012)). $Ca_V\beta$ also contributes to regulation of the channels by second messengers (Abiria and Colbran, 2010; Heneghan et al., 2009; Hermosilla et al., 2011; Suh et al., 2012) and, as detailed below, G proteins (Canti et al., 2000; Dresviannikov et al., 2009; Feng et al., 2001; Leroy et al., 2005; Zhang et al., 2008). An additional auxiliary subunit, the γ_1 subunit, has also been found to associate with $Ca_V1.1$ channels in skeletal muscle, and several neuronal isoforms including γ_2 (also called stargazin) have been identified in neurons (Chen et al., 2007). However, it remains unclear if these proteins constitute *bona fide* Ca^{2+} channel subunits

in neurons, where they have been shown to associate with and modulate glutamatergic AMPA receptors (Diaz, 2010).

Neuroendocrine Ca^{2+} channels: adrenal chromaffin cells

Adrenal glands comprise an outer cortex and an inner medulla; the former produces and secretes glucocorticoids and mineralocorticoids while the latter, primarily made up of chromaffin cells, secretes catecholamines. Chromaffin cells are derived from the neural crest (Huber et al., 2009), and act essentially as postganglionic sympathetic neurons but, rather than innervating a specific postsynaptic target, release catecholamines and a variety of other neuropeptides and hormones into the bloodstream. These transmitters then exert powerful control over the cardiovascular, endocrine, immune, and nervous systems, for example coordinating the "fight-or-flight" response to acute stress.

Chromaffin cells express $Ca_V2.1$, $Ca_V2.2$, and Ca_V1 channel subtypes (Fox et al., 2008; Garcia et al., 2006; Marcantoni et al., 2008). In the intact gland, chromaffin cells are directly innervated by splanchnic nerve terminals that release ACh (acetylcholine) and neuropeptide co-transmitters such as PACAP. This sympathetic drive depolarizes the chromaffin cells promoting Ca^{2+} influx through voltage-gated Ca^{2+} channels, subsequently triggering fusion of large dense core granules with the plasma membrane (Boarder et al., 1987; Douglas, 1968). Like neurons, target membrane SNARES (t-SNARES) syntaxin 1A and SNAP25 facilitate vesicle/membrane association by direct interaction with the vesicular SNARE (v-SNARE) synaptobrevin. Synaptotagmin,

through an N-terminal vesicular association and C-terminal Ca^{2+} binding C2 domains (C2A and C2B), "clamps" t-SNARES and prevents spontaneous exocytosis. Ca^{2+} binding the synaptotagmin C2 domain induces a *trans* to *cis* conformational change in the SNARE complex, and subsequent fusion of opposed vesicular and target membranes (Bai et al., 2004; Tang et al., 2006). As at presynaptic terminals, Ca^{2+} channels play a pivotal role in stimulus-secretion coupling in chromaffin cells and are an important target for regulation by GPCRs.

G protein coupled receptors and heterotrimeric G protein signaling

G protein coupled receptors

Eukaryotic life is sustained by highly organized response and adaptation to a chaotic array of chemical signals across phospholipid bilayers, often over long distances and on a millisecond time scale. In humans, physiological responses to sensory stimuli, (*i.e.* various odorants, tastes, pheromones and photons of light) and endogenous ligands (*e.g.* chemokines, calcium ion, neurotransmitters, various hormones) are mediated by modular signaling complexes with a common format: receptor, transducer, and effector (Gilman, 1987; Pierce et al., 2002). A massive family of cell surface receptors with seven transmembrane α helical domains, oriented with a large exofacial N-terminal ligand binding domain, intracellular C-terminal regulatory domain, three extracellular and three intracellular loops, provides the molecular framework for receiving these diverse signals.

Often cited as having over 800 distinct members, these receptors in humans can be classified into five main families based on phylogenetic analysis: rhodopsin, secretin, glutamate, adhesion, and frizzled/taste2. A majority are of these (> 700 total, >300 non-olfactory) are in the well-studied class A or group 1 rhodopsin family, with further segmentation into groups α-δ, each containing clusters (e.g. prostaglandin cluster, amine cluster, melatonin cluster etc.) (Fredriksson et al., 2003; Jassal et al., 2010; Oldham and Hamm, 2008).

Many, but not all (Sun et al., 2007), of the 7 transmembrane receptors are associated with an intracellular heterotrimeric protein complex regulated by a GTP-dependent intermediary (Rodbell et al., 1971). An activated receptor transduces signals through nucleotide binding cycle: dissociation of GDP, association with GTP, hydrolysis of GTP. Hence the name: *G protein* coupled receptors (Gilman, 1987). Heterotrimeric G proteins are named for their respective α subunit with four subfamilies recognized: G_s coupled receptors contain $G\alpha_s$ and activate adenylyl cyclase; G_q contain $G\alpha_q$ and activate phospholipase Cß, G_i contain $G\alpha_i$ and inhibit adenylyl cyclase while corresponding $G\beta\gamma$ subunits activate G protein coupled inwardly rectifying potassium channels (GIRK), and G_{12} couple to the activation of Rho guanine nucleotide exchange factors (Oldham and Hamm, 2006; Pierce et al., 2002)

All receptors across each of the five families present the canonical seven transmembrane architecture described above, however they possess little sequence identity in the transmembrane regions (<20% SI (Fredriksson et al., 2003; Katritch et al., 2013), and

vastly different N-terminal domains that, in combination with the three extracellular loops, form the ligand binding pocket. Rhodopsin, secretin, and taste/2 families of receptors generally have the shortest N-termini; adhesion family the longest: some up to 2800 amino acids with several EGF- and mucin-like domains likely involved in cell adhesion (Harmar, 2001; Hayflick, 2000; Stacey et al., 2000). Both glutamate and frizzled family receptors are intermediate in length. The ~200 amino acid frizzled family N-termini bind the secreted glycoprotein Wnt through a number of conserved cysteine residues (Fredriksson et al., 2003). Glutamate family N-termini form two lobes around a central cavity that closes around a ligand, earning the apt "venus fly trap" moniker (Kunishima et al., 2000) .

As previously mentioned, the Class A/rhodopsin family of receptors is the most extensively studied. Following the first three-dimensional crystal structure of rhodopsin in 2000 (Palczewski et al., 2000), the structural field has seen a relative explosion of progress, with 16 total structures as of 2012 (Katritch et al., 2013) Along with rhodopsin (Deupi et al., 2012; Nakamichi and Okada, 2006; Okada et al., 2004; Palczewski et al., 2000; Park et al., 2008; Scheerer et al., 2008), there is good structural coverage of α_{2A}-adrenergic (Dore et al., 2011; Hino et al., 2012; Jaakola et al., 2008; Lebon et al., 2011; Xu et al., 2011), and ß$_2$-adrenergic (Cherezov et al., 2007; Hanson et al., 2008; Rasmussen et al., 2011a; Wacker et al., 2010), among several others, and complete coverage of the four classes of opioid receptor (κ-, μ-, δ-, and NOP) (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012). Analysis of structural variations within rhodopsin subgroups reveals remarkable diversity in the extracellular

loop (ECL) regions, reflecting the diverse array of cognate ligands. For example, the opioid receptors share a β-hairpin in the ECL 2, a common peptide-binding motif, while the N-terminus and ECL 2 the lipid binding $S1P_1$ receptor form a lid and occlude the binding pocket (Rosen et al., 2013) Crystal structures of GPCRs in all four activation states: R (inactive), R'(inactive, agonist bound), R" (active), R* (active w/ Gα mimic) (Katritch et al., 2013), and additionally the structure of β_{2A} with Gαβγ (Rasmussen et al., 2011b) are providing insights not only on microdomain movements but also large scale conformational diversity where receptors can activate multiple signaling pathways in a ligand dependent manner. Early observations suggest movements of TM helix V and VI are important for G protein binding and activation, whereas movements of helix III, VII and VIII may signal independent of G proteins, possible through β-arrestin (Granier and Kobilka, 2012; Katritch et al., 2013). These studies could give rise to biased ligands that may stabilize or 'prefer' one pathway over another, although rational design of biased agonists is still in its infancy (Granier and Kobilka, 2012).

G protein coupled receptors are important drug targets

In humans, GPCRs are involved in the basic functioning of every tissue and organ system. Using evidence based gene finding approaches, it can be inferred from sequence similarities to known receptors that 1265 GPCRs are expressed in the human genome, a majority cognate for chemosensory ligands, while roughly 300-400 bind endogenous ligands (Insel et al., 2012). A reasonable consequence of finding receptors in a gene sequence is the occurrence of receptor 'orphans', receptors without an identified endogenous ligand, and the need for reverse pharmacological techniques to identify them.

There are over 100 “orphan” GPCRs that may bind endogenous ligands signaling possible unmet need in human pathophysiology, thus an opportunity for drug discovery (Chung et al., 2008).

Another point of relevance comes from examination of tissue specific expression of non-odorant GPCRs. The most comprehensive study to date by Regard et al. (Regard et al., 2008) quantified transcripts for 353 non-odorant GPCRs in 41 mouse tissues. A majority of receptors (238/353) were expressed in less than half of tested tissues, and high level expression was limited to tissues where each GPCR has a crucial physiological function (e.g. gamma-aminobutyric acid (GABA) in CNS, glucagon-like peptide receptor 1 (GLP-1) in pancreatic islets, angiotensin type 1A (Agtr1) in heart and blood vessels). This tissue specific, largely non-ubiquitous receptor expression/function not only helps predict roles for orphan receptors, but allows for selective, rational drug designs that target specific processes without bleeding over into off target organ systems, both features that make GPCRs attractive drug targets.

Drugs targeting GPCRs represent the largest class of pharmaceuticals

Over 50 years ago, before GPCRs were fully understood as distinct entities from their associated cellular transducers and enzymes, Sir James Black developed propranolol, an inhibitor of β adrenergic receptors (βARs). Subsequently, GPCRs have emerged as the single the most important drug target. A 2006 analysis by Overington (Overington et al., 2006) and colleagues rigorously distilled a list of over 21,000 marketed drug products into 1,357 unique drugs against 324 unique targets for all classes of approved drugs, a

vast majority of which are protein targets (1,065/1,357) on the surface of cells (60%). Of these, class I GPCRs constituted the largest targeted gene family (26.8% of marketed drugs). Of course, estimating druggable targets from the completed human genome has several complicating factors such as multimeric signaling complexes, clinically relevant drug promiscuity, conserved druggable domains, and heterodimerization of receptors; a conservative estimate suggests ~300 druggable Class 1 GPCRs (Russ and Lampel, 2005). While efficiencies in small molecular screening and the rise of monoclonal antibody therapeutics since 2006 has undoubtedly expanded the overall number of drug targets (and made it harder to define), as of 2012 40-50% of marketed drugs targeted GPCRs (Chen et al., 2012), and drugs targeting GPCRs occupied four of the top 15 best-selling pharmaceuticals in 2012. Interestingly, it is estimated only about 10% of the population of GPCRs are targeted (Jassal et al., 2010). Taken together, there is significant potential for growth targeting GPCRs.

G protein cycle/ subunit composition

At rest, G proteins bind GDP, and exist as a heterotrimer of α- β- and γ- subunits. Ligand binding and, simultaneously, receptor activation opens up a binding pocket on the intracellular face of the receptor for the Gα C-terminus. This high affinity G protein interaction with the activated receptor induces a conformational change in the nucleotide binding site on Gα that catalyzes the release of GDP. GTP rapidly binds Gα in its place, subsequently dissociating Gα-GTP and Gβγ from the receptor. Structural changes in Gα eliminate the Gβγ binding site allowing each liberated subunit to initiate downstream signaling cascades independently, targeting multiple effectors (McIntire, 2009;

Rosenbaum et al., 2009). Intrinsic GTPase activity of Gα terminates signaling and facilitates the reassociation of Gα-GDP with Gβγ, although RGS (regulator of G protein signaling) proteins accelerate GTPase activity (Hollinger and Hepler, 2002). In humans 16 genes encode Gα subunits, 5 genes encode Gβ, and 12 genes encode Gγ. As previously mentioned, heterotrimers are divided into classes based on sequence homology of the Gα subunit: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_t$ transducin. Each Gα subunit contains a GTPase domain and helical domain, and all but transducin are palmitylated to facilitate membrane localization (Chen and Manning, 2001) .

The G protein β and γ subunits form a constitutive heterodimer whose crystal structure is visually dominated by the propeller-like folding of the so-called WD40 sequence repeats of Gβ, with four-stranded β-sheets comprising the seven blades of the propeller (Figure 4B, red ribbon). The N-terminal region upstream of the propeller adopts an α-helical domain and serves as the interaction surface with Gγ (Wall et al., 1995) . The most relevant function of the Gγ subunit regarding Gβγ signaling may be to locate newly assembled Gβγ subunits to the plasma membrane through a Gγ, C-terminal targeted farnesyl or geranyl-geranyl group. Although it should be mentioned that Gγ prenylation is not necessary for Gβ and Gγ assembly per se, but rather seems to assure a functional Gβ subunit and promotes high affinity Gβ-effector interactions (Higgins and Casey, 1994; Iniguez-Lluhi et al., 1992)

In turn, Gβγ binding is required for targeting Gα subunits to the membrane. For $G\alpha_i$ and $G\alpha_z$ subunits, the co-translation addition of myristate (C14:0) to the N-terminal Gly^2

imparts weak association with the plasma membrane, where they can become more soundly attached by palmitoylation. Interestingly, Gβγ may have an overlapping role with palmitoylation for trapping $G\alpha_i$ subunits at the membrane (Chen and Manning, 2001). Alternatively, $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$ subunits undergo post-translational covalent addition of palmitate (C16:0) to a cysteine residue near their respective N-termini, and are not N-myristolated. Thus, membrane targeting by Gβγ may be equivalent to N-myristolation, and a requirement for concentrating $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$ subunits at the membrane (Evanko et al., 2000). The reason for apparent redundancy in targeting $G\alpha_i$ and $G\alpha_z$ subunits to the membrane by association with Gβγ, N-myristolation, and/or palmitoylation is unclear. However, there may be a relative abundance of $G_{i/o}$ -coupled heterotrimers compared with $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$ subtype (Clapham and Neer, 1997; Logothetis et al., 1987), thus placing stress on the membrane targeting system.

Additionally, Gβγ subunits are necessary for receptor mediated nucleotide exchange in the Gα subunit, possibly by presenting Gα in an orientation permissible for receptor interaction (Oldham and Hamm, 2006). Thus, Gα and Gβγ interaction is not an arbitrary mass action association, but an inter-subunit signaling motif crucial for GPCR function and effector selectivity.

Gβγ signaling

In addition to its role targeting Gα subunits to the membrane and facilitating nucleotide exchange, Gβγ activates a diverse and continually growing array of targets. Gβγ does not have a catalytic site, and undergoes no major structural rearrangements when dissociated

from Gα (Wall et al., 1995). As a result Gβγ generally activates effectors by bringing cytosolic proteins in contact with the membrane (AC, PLD, GRK2) or by causing some structural rearrangement (Ca^{2+} channels, GIRK channels) or possibly both (PLCβ) (Smrcka, 2008). Several of these targets can modulate exocytosis indirectly: phospholipase Cβ1,2,3, ε, η (PLC), phosphoinositide-3 kinase (PI3K), GPCR kinase 2 (GRK2), RGS proteins, microtubules, AC activation and inhibition, and other kinases and small GTPases. Other effectors are more directly involved the exocytotic process, including: phospholipase D (PLD), GIRK channels (G protein gated inwardly rectifying potassium channels), TRPM1 (transient receptor potential cation channel subfamily M member 1) channels, Ca_v channels, and SNARE proteins (Khan et al., 2013). The roles of each in exocytosis, and regulation by Gβγ, are described briefly below.

The production of phosphatidic acid (PA) spontaneously promotes membrane curvature in single-layer lipid micelles (Kooijman et al., 2003). PLD1 is activated by the secretagogue KCl in PC-12 cells and synthesis of its product, PA, is increased at the membrane. Inhibition of PLD1 by primary alcohols inhibits exocytosis, and knockdown of PLD1 inhibits membrane associated PA production (Vitale et al., 2001; Zeniou-Meyer et al., 2007). These results led to the hypothesis that PLD1 is important for granule fusion at a "late stage" of exocytosis. Gβγ ($\beta_1\gamma_1$ and $\beta_1\gamma_2$) negatively regulates PLD1 activity in vitro by binding to the N-terminal region of the enzyme containing the PX/PH domain (phox/pleckstrin homology domain), a common Gβγ binding domain, which presumably positions Gβγ for interference with the PLD1 catalytic domain (Preininger et al., 2006). The temporal aspects of PLD regulation of exocytosis have not yet been addressed, and

we have shown primary alcohols, a commonly used PLD inhibitor, also inhibit calcium entry into native chromaffin cells. Furthermore, the activity of endogenous PLD in native bovine chromaffin cells is limited (Currie Lab, unpublished data), casting some doubt on the hypothesized role of PLD in exocytosis.

In general, Gβγ dimers released from a wide variety of GPCRs can reduce membrane excitability by activating G protein gated inwardly rectifying potassium channels (GIRK channels) (Logothetis et al., 1987), inhibiting TRPM1 channels (Shen et al., 2012), and mediating voltage-dependent inhibition of Ca_v2 channels (Currie, 2010a), which will be detailed in subsequent sections. However Gβγ may regulate neurotransmission through direct interactions with the secretory apparatus (Silinsky, 1984). In lamprey reticulospinal motor- neurons, microinjection of Gβγ mimicked serotonergic-inhibition of excitatory post-synaptic currents (EPSCs) without affecting action-potential induced intracellular calcium transients. Cleavage of the C-terminal nine amino acids from SNAP-25 by botulinum toxin A (BoNT A) relieves this inhibition, suggesting the relevant Gβγ substrate in this system is SNAP-25 (Gerachshenko et al., 2005). Similar results on granule exocytosis were observed in permeablized PC-12 cells, where Gβγ ($\beta_1\gamma_1$ and $\beta_1\gamma_2$)- mediated inhibition was abolished by BoNT A (Blackmer et al., 2005). Indeed, *in vitro* binding assays indicate Gβγ can interact with syntaxin, SNAP-25B, VAMP2/synaptobrevin and may compete for synaptotagmin binding to the assembled SNARE complex in a calcium-dependent manner (Yoon et al., 2007). Currently, the relative inhibitory contribution, and relevant cellular context, of Gβγ binding to Ca^{2+} channels and SNARE proteins remains unclear, as very few studies have been extended

to intact mammalian systems. However, in rat adrenal chromaffin cells, the quantal size of individual exocytotic events induced by Ca^{2+} store mobilization is reduced by ATP. Notably, activation of PKC through the G_q-coupled muscarinic acetylcholine receptor reversed the inhibition, and will be discussed more below (Chen et al., 2005). Similarly, in bovine adrenal chromaffin cells, activation of P2Y receptors with ATP, μ-opioid receptors with DAMGO, or exogenous Gβγ application reduced the number and quantal size of exocytotic events elicited by a Ca^{2+} ionophore or by direct intracellular application of Ca^{2+} (Yoon et al., 2008). Taken together, these studies suggest an acute, Gβγ-mediated inhibitory effect on granule fusion dynamics subsequent to Ca^{2+} entry through Ca^{2+} channels- potentially through interactions with secretory proteins.

With respect to activating effectors, the significance of specific subtype combinations of $G\beta_x\gamma_x$ is not well understood (Smrcka, 2008). The exception being Gβγ subtypes containing γ_1, which are less potent at activating AC and PLC (Iniguez-Lluhi et al., 1992; Ueda et al., 1994). As previously mentioned, GTP binding to Gα induces structural changes that eliminate the Gβγ binding site, exposing the Gβγ effector binding surface. The propeller-like tertiary structure of Gβ is relatively flat, large, and topographically featureless and yet can selectively bind and activate dozens of effectors. Crystal structures of Gβγ in complex with GRK2 (Lodowski et al., 2003) and phosducin (Gaudet et al., 1996) suggest overlap with the binding sites for GαGDP. Chemical crosslinking studies using synthetic peptides from type II adenylyl cyclase (Chen et al., 1995) or PLCβ2 (Sankaran et al., 1998) also mapped interaction sites on Gβ near the Gα switch II binding region. An important study by Ford et al. implicates a common interface on Gβ

for both Gα and effector binding. Alanines were substituted for key residues on Gβ in the switch II and N-terminal interacting domains and subsequently Gβγ- dependent interactions with several effectors were increased, decreased or completely inhibited. This established a molecular "footprint" specific for each effector tested (Ford et al., 1998). Thus, a similar contact region on Gβ recognizes a complexity of targets

To specifically dissect the "footprint" on the face of Gβ, and identify a mechanism for this unique interaction with structurally distinct effectors, a random phage display peptide screen identified multiple peptides that bound the same 'hot spot' on Gβγ. One of these peptides, SIRK, blocked Gβγ interaction with PLCβ2 and PI3Kγ but left intact binding to AC and N-type Ca^{2+} channels, and a peptide derivative of SIRK, SIGK, binds the Gα-switch II region (Davis et al., 2005; Scott et al., 2001). These findings validate Gβγ may bind multiple effectors through conserved, energetically favorable residues and has led to the investigation of small molecules capable of selective interference with subsets of Gβγ effectors (Smrcka, 2013). For example the M119 class, including M119 and gallein, inhibited Gβγ interaction with GRK2, preventing βAR desensitization in an acute mouse model of heart failure (Casey et al., 2010). Similarly M119 and gallein block Gβγ-dependent PI3K activation preventing chemoattractant-dependent neutrophil migration, and inhibited whole animal inflammation (Lehmann et al., 2008). M119 blocked $G\beta_1\gamma_2$ activation of PLCβ2, PLCβ3, PI3Kγ, and binding to GRK2 whereas M201, another small molecule that binds a distinct Gβγ subsurface, potentiated activation of PLCβ3 and PI3Kγ (Bonacci et al., 2006).

Gβ and Gγ subtypes can exist in several combinations and regulate the activity of dozens of effectors with little selectivity intrinsic to subunit combinations. Yet pertussis toxin (PTX), which ADP ribosylates $G\alpha_i$ subunits and prevents receptor interaction, nucleotide exchange, and ultimately release of Gβγ subunits, prevents Gβγ regulation of several effectors, including N-type calcium channels (Ikeda, 1996). One explanation for the specificity of Gβγ subunits associated with G_i heterotrimers (i.e over G_s, or G_q heterotrimers), is that $G\alpha_i$ subunits may couple Gβγ subunits to effectors in signaling complexes, as is the case for GIRK channels (Huang et al., 1997).

Regulation of Ca^{2+} channels and exocytosis by G protein coupled receptors

Many neurotransmitters have cognate presynaptic GPCRs including GABA, acetylcholine, glutamate and several neuropeptides. Feedback inhibition by neurotransmitters was first demonstrated by Dunlap and Fischbach, who reported that norepinephrine reduced both duration of action potentials and amplitude of I_{Ca} in chick sensory neurons (Dunlap and Fischbach, 1978; Dunlap and Fischbach, 1981). This reduction in Ca^{2+} entry ultimately reduces neurotransmitter release, and is mediated by presynaptic G protein coupled receptors (GPCRs) (Hille, 1994).

GPCRs can recruit several pathways to inhibit Ca_V2 channels.

Several distinct signaling pathways recruited by GPCRs can converge on Ca^{2+} channels to inhibit their activity. Broadly speaking, these disparate mechanisms can be classified as either voltage-dependent or voltage-independent. *Voltage-dependent inhibition* is

widespread in both the central and peripheral nervous systems and is mediated by a single well defined mechanism involving direct binding of Gβγ to the α_1 subunit of Ca_V2 channels (Herlitze et al., 1996; Ikeda, 1996). As detailed below, this shifts the voltage-dependence of channel activation, and the inhibition can be reversed at depolarized membrane potentials. Hence the mechanism is dubbed "*voltage-dependent inhibition*". In contrast, *voltage-independent inhibition* lumps together several other mechanisms that generally develop more slowly and are mediated by a variety of distinct second messenger pathways including phosphorylation, lipid signaling, and channel trafficking (Elmslie, 2003; Hille, 1994; Michailidis et al., 2007; Roberts-Crowley et al., 2009). The common feature to all these pathways is the inability of strong membrane depolarization to overcome the inhibition. The prominence of voltage-independent inhibition is also more variable, but it seems particularly relevant for controlling somatic Ca^{2+} channels in sensory and sympathetic neurons.

Although there are a few exceptions (Currie and Fox, 2000; Elmslie, 1992; Zhu and Ikeda, 1994), Gβγ-mediated, voltage-dependent inhibition is usually elicited by $G_{i/o}$-coupled GPCRs and thus blocked by pertussis toxin. One explanation for this preferential involvement of $G_{i/o}$-coupled receptors is co-localization with the channels through adapter proteins like Homer (Kammermeier et al., 2000) or NHERF2 (Filippov et al., 2010). Direct interaction between GPCRs and the channels has also been reported, for example, metabotropic glutamate receptors with $Ca_V2.1$ (Kitano et al., 2003), and dopaminergic (D1 / D2) (Kisilevsky et al., 2008; Kisilevsky and Zamponi, 2008) or nociceptin (NOP) receptors (Altier et al., 2006; Beedle et al., 2004) with $Ca_V2.2$. Direct

interaction of GPCRs with the channels might also confer additional means of voltage-independent inhibition, such as agonist-mediated endocytosis of the channel/receptor complex (Altier et al., 2006) (but see (Murali et al., 2012)).

Characteristic features of voltage-dependent inhibition: the "willing-reluctant" model

Voltage-dependent inhibition exhibits several characteristic features that provide an identifying biophysical signature (Figure 2): the inhibition is diminished at depolarized membrane potentials; the voltage-dependence of activation is shifted to more depolarized potentials; the activation kinetics are slowed; a conditioning prepulse to depolarized potentials relieves most of the inhibition and normalizes channel kinetics (termed prepulse relief or prepulse facilitation). Voltage-dependent relief of the inhibition can also occur to some extent during more physiologically relevant stimuli such as high frequency trains of action potential-like waveforms (Brody et al., 1997; Currie and Fox, 2002; Park and Dunlap, 1998; Tosetti et al., 1999; Williams et al., 1997; Womack and McCleskey, 1995). In this case the magnitude of facilitation increases with stimulation frequency, and in turn this might contribute to short term synaptic plasticity at some synapses (Brody and Yue, 2000).

As first proposed by Bean (Bean, 1989), these characteristic features have been incorporated into models in which the channels exhibit two functional gating states, "willing' and "reluctant" (Bean, 1989; Carabelli et al., 1996; Colecraft et al., 2000; Elmslie et al., 1990; Lee and Elmslie, 2000). In the absence of Gβγ, the channels

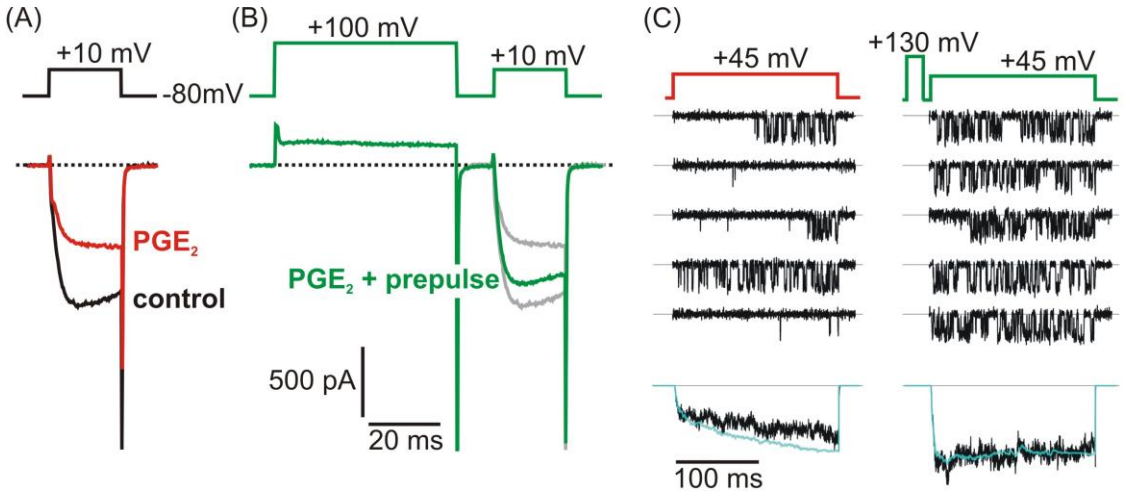

Figure 2: Hallmark features of voltage-dependent inhibition. **(A, B)** Example of "whole cell" patch clamp recording of I_{Ca} from an adrenal chromaffin cell. Prostaglandin E_2 (PGE_2) acts through $G_{i/o}$-coupled EP3 receptors to inhibit I_{Ca}. The inhibition displays hallmarks of voltage-dependent inhibition: peak amplitude was reduced, activation kinetics were slowed, and in the continued presence of agonist both of these effects were reversed by a conditioning prepulse to +100 mV (green trace) (panel B). **(C)** Example showing voltage-dependent inhibition of single N-type Ca^{2+} channel currents (with permission from Colecraft, Brody and Yue (2001) (Colecraft et al., 2001)). Recombinant $Ca_V2.2$ channel currents were recorded in the "on-cell" patch clamp configuration with GPCR agonist included in the patch-pipette to elicit tonic inhibition of the channels in the membrane patch (left panel). A conditioning prepulse (to +130 mV) was used to reverse this inhibition (right panel). Five representative current sweeps are shown, along with an ensemble (pseudo macroscopic) current at the bottom of each panel. Inhibited "reluctant" channels display substantially longer latency (time to first channel opening) upon membrane depolarization. The conditioning prepulse normalizes channel gating to that seen under control conditions.

predominantly populate the "willing" state, whilst binding of Gβγ favors the "reluctant" state. Voltage-dependent relief of the inhibition, for example by a depolarizing prepulse, is thought to reflect transient dissociation of Gβγ from the channels with a concomitant shift from "reluctant" to "willing" gating states (Figure 3). Although dissociation of Gβγ is not the only mechanism that could underlie facilitation, it is supported by analyses of prepulse relief as a function of agonist or Gβγ concentration. When the concentration of Gβγ was increased, the rate of relief during the prepulse (i.e. dissociation of Gβγ) was not altered. However, the rate of reinhibition following the prepulse was faster, as predicted for rebinding of the Gβγ (Delmas et al., 1998; Elmslie and Jones, 1994; Golard and Siegelbaum, 1993; Zamponi and Snutch, 1998). The concentration-dependence and monoexponential kinetics of reinhibition were also consistent with a bimolecular interaction of a single Gβγ dimer with the channel (Zamponi and Snutch, 1998). Gβγ dissociation also occurs (albeit more slowly) with moderate membrane depolarization, manifest as the slowed activation kinetics of whole cell I_{Ca} (Figure 2A).

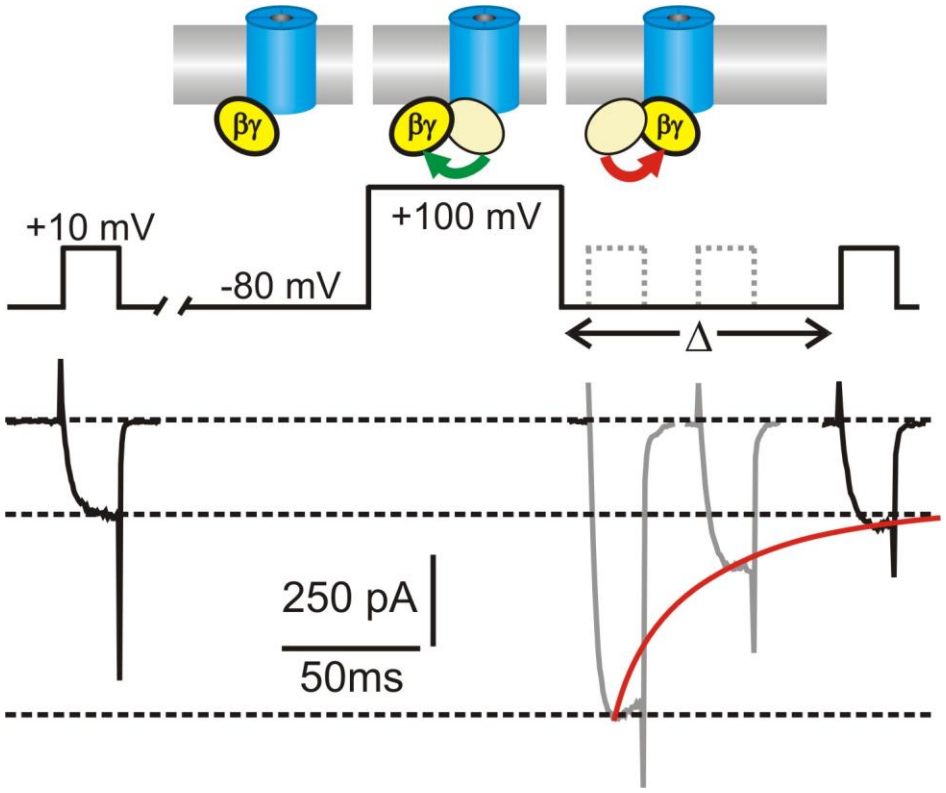

Figure 3: Voltage-dependent relief of inhibition reflects transient dissociation of Gβγ from the channel. The currents shown were recorded from recombinant $Ca_V2.2$ channels expressed with β1b, α2δ in HEK293 cells. Gβγ was co-expressed and produced tonic inhibition of I_{Ca} that was reversed by a conditioning prepulse to +100 mV. As illustrated by the cartoon, prepulse facilitation is thought to reflect dissociation of Gβγ from an inhibitory binding site on the channel at the depolarized membrane potential. Upon return to the hyperpolarized membrane potential, Gβγ rebinds to, and re-inhibits, the channel. The timecourse of this re-inhibition can be investigated by varying the interval between the prepulse and test pulse (Δ). Re-inhibition of I_{Ca} is well fit with a single exponential (red line) and the rate is faster as the local concentration of Gβγ increases.

Single channel investigation of voltage-dependent inhibition

Using the "cell-attached" ("on-cell") recording configuration, several studies showed that agonist must be included in the patch pipette to elicit inhibition (Bernheim et al., 1991; Elmslie and Jones, 1994; Forscher et al., 1986). Thus, only GPCRs localized close to the channels (within the membrane patch under the pipette) can couple to and inhibit the channels in that patch. When agonist is bath applied (i.e. outside the patch pipette), the GPCRs on the rest of the cell membrane are activated but do not inhibit the channels, showing that the signaling pathway is "membrane delimited" and does not involve diffusible intracellular messengers. Single channel recordings also reveal the characteristic gating shifts associated with voltage-dependent inhibition (Figure 2C). Upon membrane depolarization, the latency (delay) to first channel opening was increased with little effect on other single channel parameters (Carabelli et al., 1996; Patil et al., 1996). Thus, the inhibited ("reluctant") channels appear essentially silenced, unable to open until Gβγ dissociated and the channels shift to the "willing" state. Subsequently it has been reported that $Ca_V2.2$ but not $Ca_V2.1$ channels can display very brief channel openings from the "reluctant" state (i.e. without Gβγ unbinding), although the probability of such events was low (Colecraft et al., 2000; Lee and Elmslie, 2000).

Alteration of gating currents by Gβγ

Gating currents of voltage-gated channels are not due to ionic flux through the channel pore, but rather reflect movement of the charged voltage-sensor domain of the channels in response to membrane potential changes. Expression of recombinant $Ca_V2.2$ in HEK293 cells enables recording of these gating currents in isolation as the cells lack

other endogenous voltage-gated channels. Using this approach it was found that Gβγ reduced the amplitude, and shifted the voltage-dependence of gating currents to more depolarized potentials (Jones and Elmslie, 1997), again consistent with the channels entering a "reluctant" state. G proteins also produced a significant separation in the voltage-dependent activation of gating current and ionic current (Jones and Elmslie, 1997). Together these data suggest that Gβγ binding slows movement of the voltage-sensor and uncouples this movement from opening of the channels. Similar modulation of gating currents by G proteins has also been reported in rat sympathetic neurons (Hernandez-Ochoa et al., 2007; Rebolledo-Antunez et al., 2009).

Gβγ modulates channel inactivation

In addition to these dominant effects on channel activation, evidence shows that Gβγ can also modulate *inactivation* of $Ca_V2.2$ channels (McDavid and Currie, 2006; Weiss et al., 2007). Although the precise molecular correlates remain somewhat unclear, fast voltage-dependent inactivation might involve a "hinged lid" mechanism in which the pore is occluded by the intracellular loop connecting domains I and II of the α_1-subunit (Stotz and Zamponi, 2001; Tadross et al., 2010) (but see (Findeisen and Minor, 2009)). The I-II linker is also important for binding Gβγ (De Waard et al., 2005; De Waard et al., 1997; Herlitze et al., 1997; Schiff et al., 2000) (Figure 1) (see below for more discussion), so it is feasible that this could disrupt movement or interaction of this putative inactivation gate with other channel domains. Ca_V2 channels can also inactivate from intermediate closed state(s) favored during trains of brief repetitive stimuli (Patil et al., 1996). Gβγ could also reduce the cumulative inactivation throughout a stimulus train by reducing the

probability that the channels populate the state from which inactivation is preferred. In addition to voltage-dependent mechanisms, the channels can also undergo Ca^{2+}-dependent inactivation mediated through calmodulin interaction with the C-terminus of the channel (Lee et al., 1999; Lee et al., 2003; Liang et al., 2003; Peterson et al., 1999; Zuhlke et al., 1999). The reduction of Ca^{2+}-dependent inactivation by Gβγ (McDavid and Currie, 2006) might therefore result from fewer channels opening and a diminished "global" Ca^{2+} signal, or through more complex interactions perhaps including binding of Ca^{2+}-calmodulin to Gβγ which has been reported to occur at least *in vitro* (Liu et al., 1997).

$Ca_V2.2$ channels are more susceptible to Gβγ-mediated inhibition than $Ca_V2.1$ channels.

Ca^{2+} entry through $Ca_V2.1$ and/or $Ca_V2.2$ channels triggers neurotransmitter release at most synapses, and both of these channels are inhibited by Gβγ. However, the magnitude of inhibition is greater for N-type ($Ca_V2.2$) than for P/Q-type ($Ca_V2.1$) I_{Ca} (Bourinet et al., 1996; Currie and Fox, 1997; Zhang et al., 1996). Reversal of $Ca_V2.2$ inhibition during high frequency bursts of action potentials is more sensitive to changes in the action potential amplitude and duration, and overall occurs to a lesser extent than for $Ca_V2.1$ (Currie and Fox, 2002). These differences are consistent with higher affinity binding of Gβγ to $Ca_V2.2$. Indeed, although the apparent affinity of Gβγ for the two channel types is similar at hyperpolarized or very depolarized potentials, there is a significant divergence at moderately depolarized potentials (< +30 mV) (Colecraft et al., 2000). These data all suggest that GPCR mediated inhibition of neurotransmission would be more effective at

synapses expressing $Ca_V2.2$ compared to those expressing $Ca_V2.1$ channels. Furthermore, although changes in the relative contribution of the two channel types might have little effect on transmitter release *per se*, it could significantly change neuromodulation by GPCRs (Brody and Yue, 2000; Cao and Tsien, 2005; Inchauspe et al., 2007).

Regions of the calcium channel that mediate inhibition by Gβγ.

Cumulative evidence from a variety of approaches (mutagesis, chimeric channels, peptide mimetics/blockers) suggests that multiple sites on the α1 subunit of the channel comprise a binding pocket for Gβγ. Two distinct binding sites for Gβγ have been reported on the I-II linker (De Waard et al., 1997; Herlitze et al., 1997; Tedford et al., 2010; Zamponi et al., 1997). The first site has a consensus sequence for Gβγ binding found in phopholipase C β2 and type 2 adenylyl cyclase (QXXER). This site (containing QQIER in all three Ca_V2 channel members) overlaps with the binding site for the $Ca_V\beta$ subunit (the AID) (Pragnell et al., 1994; Van Petegem et al., 2004). A second site further along the I-II linker has also been identified (De Waard et al., 2005). *In vitro* binding assays between Gβγ and the I-II linker peptide show high affinity interactions (20 - 60 nM) (Bell et al., 2001; De Waard et al., 1997; Zamponi et al., 1997), although this is reduced somewhat by the presence of a $Ca_V\beta$ subunit (Zhang et al., 2008). Peptides based on both sites diminish voltage-dependent inhibition of the channels and point mutations introduced into the sites can either reduce or enhance inhibition (Tedford et al., 2010). PKC can reduce voltage-dependent inhibition of $Ca_V2.2$ channels (N-type) I_{Ca} (Barrett and Rittenhouse, 2000; Bertaso et al., 2003; Simen et al., 2001; Swartz, 1993; Zamponi et al., 1997), and this has been linked to phosphorylation of Thr^{422} on the I-II linker (of the rat

$Ca_V2.2$), close to the second Gβγ binding site (Hamid et al., 1999; Zamponi et al., 1997). Of note, phosphorylation of Thr^{422} disrupts the inhibition of I_{Ca} mediated by $G\beta_1$, but not other Gβ subunits (Cooper et al., 2000), and two residues on Gβ1 (Asn^{35} and Asn^{36}) have been shown to underlie this difference (Doering et al., 2004). These data also suggest that Thr^{422} on the rat $Ca_V2.2$ I-II linker and Gβγ come into close proximity with one another during inhibition. Evidence implicating the I-II linker has been less clear in some other studies. For example, chimeric channels in which the I-II linker of $Ca_V1.2$ was introduced into the $Ca_V2.2$ backbone were still inhibited (Agler et al., 2005; Canti et al., 1999; Zhang et al., 1996).

The N-terminus of $Ca_V2.2$ has also been identified as crucial for voltage-dependent inhibition (Agler et al., 2005; Canti et al., 1999; Simen and Miller, 1998; Stephens et al., 1998). Evidence for this emerged from the finding that a short splice variant of $Ca_V2.3$ channels with a truncated N-terminus was not inhibited by Gβγ, whereas a splice variant with an intact N-terminus was (Page et al., 1998). The Dolphin lab further demonstrated that truncating the N-terminal 55 amino acids of $Ca_V2.2$ prevented voltage-dependent inhibition, whereas introducing the $Ca_V2.2$ N-terminus into the $Ca_V1.2$ backbone conferred modest inhibition onto these normally resistant channels (Canti et al., 1999; Page et al., 1998). An eleven amino acid stretch of the N-terminus (residues 45-55) predicted to form an α-helix (Page et al., 2010) seems critical for Gβγ-mediated inhibition, especially residues S48, R52 and R54, with I49 involved to a lesser extent (Canti et al., 1999). The Yue lab demonstrated that Gβγ interacts directly with the N-terminus and also showed that the N-terminus (residues 56-95) directly binds to the I-II

linker from $Ca_V2.2$ but not $Ca_V1.2$ (Agler et al., 2005). Thus, the N-terminus may contribute to a binding pocket for Gβγ and, through intra-molecular interaction with the I-II linker, serve as an "inhibitory module" that mediates the functional shift from willing to reluctant gating states. A recent study reported that peptides based on the N-terminus (residues 45-55 of rat $Ca_V2.2$) or AID of the channel (377-393 of rat $Ca_V2.2$) suppressed I_{Ca} and transmitter release from superior cervical ganglion neurons, and diminished Gβγ-mediated inhibition (Bucci et al., 2011). It was concluded that peptide interaction with the channels partially recapitulated and occluded the shifts in channel gating produced by Gβγ.

The C-terminus of the channel has also been reported to play an important role in modulation of $Ca_V2.3$ (Qin et al., 1997). However, large parts of the C-terminus can be deleted in $Ca_V2.2$ channels with little impact on the extent of voltage-dependent inhibition (Furukawa et al., 1998; Hamid et al., 1999). Thus, it might play a modulatory role in $Ca_V2.2$ channel regulation, perhaps by increasing the affinity of Gβγ binding (Li et al., 2004). Of note, the C-terminus does bind a number of other proteins including calmodulin, CaMKII, PKC, and Gα subunits (Catterall and Few, 2008; Evans and Zamponi, 2006), which could facilitate crosstalk between Gβγ-mediated inhibition and other signaling pathways (Bertaso et al., 2003; Simen et al., 2001).

To summarize, it appears that the binding site for Gβγ is comprised from multiple sites on the N-terminus, I-II linker, and perhaps the C-terminus of the channel. Upon binding of Gβγ, the N-terminus (residues 56-95) interacts with the proximal one-third of the I-II-

linker (see Figure 4). This (and perhaps other interactions) presumably underlies the shift in channel gating from "willing" to "reluctant".

Regions of Gβγ implicated in the inhibition of Ca_V2 channels

Several studies have also used mutagenesis approaches to identify residues on Gβγ involved in inhibiting Ca_V2 channels. High resolution crystal structures are available showing that Gβ exhibits a seven blade β-propeller structure with an α-helical N-terminus that binds Gγ (Gaudet et al., 1996; Lambright et al., 1996; Lodowski et al., 2003; Sondek et al., 1996; Wall et al., 1995) (see Figure 4). In the heterotrimer, binding of Gα to Gβ masks a protein interaction "hot spot" that contains overlapping subsets of residues involved in many effector protein interactions (Figure 4B) (Smrcka, 2008). Most mutagenesis studies that disrupt inhibition of Ca_V2 channels have identified residues on this Gα interacting surface (Agler et al., 2005; Ford et al., 1998; McDavid and Currie, 2006; Tedford et al., 2006) (Figure 4B). Three residues on the opposite face of $G\beta_1$ have also been implicated (Doering et al., 2004; Mirshahi et al., 2002; Tedford et al., 2006), while two (Asn^{35} and Asn^{36}) appear to underlie the ability of PKC to antagonize inhibition of $Ca_V2.2$ by $G\beta_1$ (Doering et al., 2004). Less is known about the role of Gγ, although it has been reported that different isoforms of Gγ can influence the extent of inhibition (Blake et al., 2001; Zhou et al., 2000) .

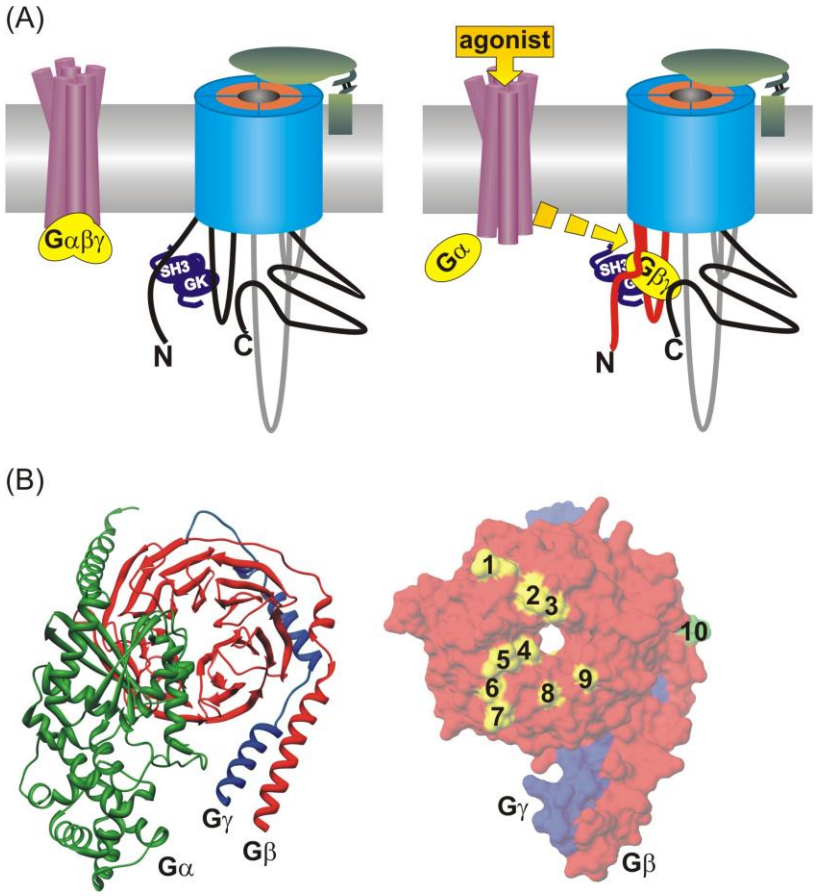

Figure 4: Cartoon model depicting the molecular interactions that underlie Gβγ-mediated inhibition of Ca_V2 channels. **(A)** Depicts a channel, GPCR, and heterotrimeric G protein under basal conditions (no agonist; left panel). Upon GPCR stimulation (right panel), Gβγ dissociates and is free to interact with effector proteins including Ca_V2 channels. Mutagenesis and other approaches suggest the Gβγ binding pocket is comprised from multiple sites on the N-terminus, I-II linker, and probably C-terminus of the channel. Gβγ binding promotes interaction of the N-terminus "inhibitory module" with the initial one-third of the I-II-linker. This (and perhaps other interactions) shifts the channels to reluctant gating states and results in functional inhibition. Although not required for inhibition per se, binding of a $Ca_V\beta$ subunit to the AID on the I-II linker is necessary for voltage-dependent reversal of the inhibition by strong depolarizations. **(B)** *Left panel*: Ribbon diagram showing the structure of a heterotrimeric G protein ($G\alpha_i$ - green; $G\beta_1$ - red; and $G\gamma_2$ blue). Many effectors bind to a protein interaction "hot spot" on the surface of Gβ that is masked by Gα in the heterotrimer. Activation by a GPCR results in dissociation of Gα and unmasking of this effector interaction face of Gβγ. *Right panel*: Molecular surface rendering of the Gα interacting face of Gβγ. Mutagenesis of the residues marked in yellow has been reported to disrupt inhibition of Ca_V2 channels. (1 = L55; 2 = K57; 3 = W332; 4 = M101; 5 = L117; 6 = M119; 7 = T143; 8 = D186; 9 = D228). Residues marked in green (10 = N35, N36) are involved in crosstalk between Gβ1 and PKC phosphorylation of $Ca_V2.2$. Images were generated using the UCSF Chimera package (Pettersen et al., 2004; Sanner et al., 1996) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco using data reported by Wall et al (Wall et al., 1995) (PDB ID: 1GP2).

Influence of the calcium channel β subunit on Gβγ-mediated inhibition.

$Ca_V\beta$ subunits exert a variety of effects on Ca^{2+} channels from trafficking, modulation of channel kinetics, and recruitment of signaling complexes (for reviews see (Buraei and Yang, 2010; Dolphin, 2012). It has also been shown that the magnitude and kinetics of voltage-dependent inhibition depends on the subtype of $Ca_V\beta$ and G protein β subunit involved (Canti et al., 2000; Feng et al., 2001). Recent evidence from the Dolphin and Yang labs show that binding of $Ca_V\beta$ to the $Ca_V\alpha_1$ subunit is not required for Gβγ-mediated inhibition *per se*, but is required for voltage-dependent reversal of that inhibition (Dresviannikov et al., 2009; Leroy et al., 2005; Meir et al., 2000; Zhang et al., 2008).

The Dolphin lab introduced a mutation (W391A) into the AID on the I-II linker of $Ca_V2.2$ channels which reduces $Ca_V\beta$ subunit binding affinity by ~1000 fold. (Leroy et al., 2005). Altered gating kinetics and reduced current density (due to disrupted trafficking) confirmed the channels lacked a $Ca_V\beta$ subunit. The magnitude of inhibition evoked by D2 dopamine receptors or exogenous Gβγ was similar to that seen in wild-type channels, however prepulse reversal of the inhibition was lost in the W391A mutant. Mutation of an additional two residues shown to be essential for Gβγ-mediated inhibition (R52A and R54A on the N-terminus) abolished this voltage-independent inhibition in W391A channels. When the β_{2a} subunit was expressed with the W391A channels rather than the β_{1b} subunit, voltage-dependent relief of the Gβγ mediated inhibition was restored. This was attributed to palmitoylation of the β_{2a} subunit at two N-terminal cysteine residues, because when these were mutated voltage-dependent relief was lost

(i.e. the data resembled β_{1b}). The authors proposed that palmitoylation effectively increases the local plasma membrane concentration of β_{2a} and thereby promotes low affinity interaction with the α_1 subunit. A follow up study demonstrated essentially the same findings, intact inhibition but loss of voltage-dependent reversal, in cells transfected with wild type $Ca_V2.2$ and $\alpha_2\delta$ (but without $Ca_V\beta$) (Dresviannikov et al., 2009).

The Yang lab investigated inhibition of $Ca_V2.1$ channels and chose to mutate $Ca_V\beta$ to reduce the affinity for the AID (Zhang et al., 2008). The channels were expressed in Xenopus oocytes and macroscopic currents recorded from giant inside-out patches. Due to the reduced binding affinity of the mutant $Ca_V\beta$ subunit, washing the cytoplasmic face of the patches resulted in loss of binding which was confirmed by the expected shifts in channel kinetics compared to wild type. These channels lacking $Ca_V\beta$ were still inhibited by application of $G\beta\gamma$, but prepulse reversal of this inhibition was abolished.

The $Ca_V\beta$ subunit consists of SH3 and GK domains separated by a variable HOOK region (Buraei and Yang, 2010; Dolphin, 2003). Expression of the isolated GK domain (which binds the AID) was sufficient to confer voltage-dependent reversal of $G\beta\gamma$-mediated inhibition (Dresviannikov et al., 2009; Zhang et al., 2008). It has been reported that the AID adopts a random coil and that binding of $Ca_V\beta$ induces an α-helical conformation that extends back to the interface with domain I (Arias et al., 2005; Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). Disruption of this α-helical structure by introducing seven glycines between the AID and IS6 did not prevent inhibition by $G\beta\gamma$, but did prevent voltage-dependent reversal in the presence of $Ca_V\beta$

(Zhang et al., 2008). Overall, it appears that Gβγ binds to a pocket formed by the N-terminus, I-II linker and perhaps C-terminus of the channel. In doing so, it promotes interaction of the channel N-terminus and I-II-linker, disrupts voltage-sensor movement and coupling to channel activation, thereby shifting the channels from "willing" to "reluctant". With strong depolarization, a rigid α-helix produced by binding of $Ca_V\beta$ to the AID might relay movement of the voltage-sensor / activation gate downstream to the I-II linker to alter the binding pocket, resulting in unbinding of Gβγ and reversal of the inhibition.

Influence of SNAREs and other synaptic proteins on Gβγ-mediated inhibition

The SNARE proteins syntaxin 1A and SNAP25 can bind directly to Ca_V2 channels via the synaptic protein interaction (synprint) site on the domain II-III linker of the channel (Figure 1) (Bezprozvanny et al., 1995; Rettig et al., 1996; Sheng et al., 1994; Sheng et al., 1997; Wiser et al., 1997; Zhong et al., 1999). This has several consequences, such as helping to ensure efficient stimulus-secretion coupling by targeting the channels near to vesicle release sites (Mochida et al., 1996). Binding of syntaxin-1 results in a hyperpolarizing shift in voltage dependent inactivation of Ca_V2 channels, which is reversed with the further addition of SNAP -25 (Bergsman and Tsien, 2000; Bezprozvanny et al., 1995; Davies et al., 2011; Jarvis and Zamponi, 2001) (for review see (Davies and Zamponi, 2008)). This might serve to effectively shunt extracellular Ca^{2+} entry through Ca_V2 channels associated with a docked vesicle/t-SNARE complex, and

inhibit Ca^{2+} entry through incomplete signaling complexes. Gβγ also binds syntaxin 1, but at a site distinct from that for Ca_V2 channels (Davies et al., 2011; Jarvis et al., 2002). This promotes tonic inhibition, presumably by colocalizing Gβγ and $Ca_V2.2$ channels (Davies et al., 2011; Jarvis et al., 2002; Jarvis et al., 2000). In contrast, even though syntaxin 1B binds both Gβγ and the channel, it does not promote tonic inhibition (Lu et al., 2001), perhaps suggesting a difference in the spatial orientation of the syntaxin/Gβγ complex relative to the channel. Botulinum neurotoxin C, which cleaves syntaxin, diminished inhibition of Ca^{2+} channels in neuronal preparations supporting the notion that this interaction is physiologically important (Silinsky, 2005; Stanley and Mirotznik, 1997). Cysteine string protein (CSP) also interacts with G proteins and the synprint site to promote Gβγ-mediated inhibition.

Interaction with other synaptic proteins might diminish rather than enhance Gβγ-mediated inhibition of Ca^{2+} channels. For example, RIMs (rab3 interacting molecules) have emerged as important organizers of the presynaptic active zone (Sudhof, 2012), and can bind Ca^{2+} channels directly, or through interaction with RIM binding proteins or the $Ca_V\beta$ subunit (Gandini and Felix, 2012; Han et al., 2011; Hibino et al., 2002; Kaeser et al., 2011; Kiyonaka et al., 2007; Uriu et al., 2010). Coexpression of Rim1 with $Ca_V2.2$ in HEK293 cells has complex effects and promotes "deinhibition" (recovery from inhibition during depolarization) perhaps in part through dramatic slowing of channel inactivation (Weiss et al., 2011). It has also been reported that stargazin (aka the Ca^{2+} channel γ2 subunit), although not covalently bound to the channel complex, scavenges Gβγ in Xenopus oocytes to reduce inhibition of $Ca_V2.2$ channels (Tselnicker et al., 2010). And,

as already noted PKC can reduce voltage-dependent inhibition of $Ca_V2.2$ channels (Barrett and Rittenhouse, 2000; Bertaso et al., 2003; Simen et al., 2001; Swartz, 1993; Zamponi et al., 1997), likely through phosphorylation of the channel I-II linker (Hamid et al., 1999; Zamponi et al., 1997), or perhaps in some cases through phosphorylation of the GPCR (Wu et al., 2002).

Exocytosis from adrenal chromaffin cells

Experimental advantages inherent to chromaffin cells

Different combinations of pore forming and auxiliary channel subunits (Catterall, 2000; Ertel et al., 2000; Yokoyama et al., 2005), all of which are subject to alternate mRNA splicing (Flucher and Tuluc, 2011; Gray et al., 2007; Liao et al., 2005; Lieb et al., 2012) or RNA editing (Huang et al., 2012), result in substantial functional diversity of Ca^{2+} channels. Recording from recombinant channels in heterologous expression systems is one powerful tool to investigate ion channel function, and has proven invaluable for assigning specific traits to a particular subunit, or structure-function studies involving mutagenesis. However, it can be challenging to precisely reconstitute all aspects of native Ca^{2+} currents and recording the downstream physiological consequences (i.e. transmitter exocytosis) may not be possible. Recording endogenous channels provides more physiological context, but can be complicated by the presence of multiple channel types, auxiliary subunits, and so on. Typically, neuronal Ca^{2+} currents are recorded from the cell soma, due to the inaccessibility and small size of the presynaptic terminal. For the same reason, in most cases presynaptic transmitter release is monitored indirectly, for example

by recording post-synaptic electrical responses or by optical approaches that track labeled synaptic vesicles. Therefore, directly relating channel function to transmitter release can be a challenge. There are a few specialized synapses that are amenable to direct electrical recording of presynaptic channels, for example the Calyx of Held in the auditory brain stem (Schneggenburger and Forsythe, 2006).

As previously mentioned, Ca_V2 channels are expressed in chromaffin cells of the adrenal medulla (Currie, 2010b; Fox et al., 2008; Garcia et al., 2006). The small (~10-15 μm), spherical cells are electrically compact and well suited for patch clamp electrophysiology to not only record ion channel currents, but also membrane capacitance which precisely reflects the surface area of the cell so one can track exocytosis and endocytosis with millisecond time resolution (Fenwick et al., 1982) (Borges et al., 2008; Gillis, 2000; Yao et al., 2012) (Figure 5). Direct electrochemical monitoring of catecholamine release is also possible using carbon fiber amperometry (Borges et al., 2008; Travis and Wightman, 1998; Wightman et al., 1991). With suitable stimulation protocols transient amperometric current "spikes" can be resolved, each of which can be analyzed to determine the amount and kinetics of catecholamine release from individual vesicular fusion events (Machado et al., 2008; Mosharov and Sulzer, 2005) (Figure 5). These approaches can also be combined with other techniques including electron microscopy, fluorescent imaging, and photorelease of "caged" Ca^{2+}. The ability to deliver precisely controlled stimuli, and simultaneously record ion channel activity and exocytosis / transmitter release from the same cellular compartment enables direct cause-and-effect assessment of mechanisms that control neurosecretion. It also enables dissection of the various steps in the

exocytotic process, and how those are altered in response to neuromodulators. Of course, there are differences between chromaffin cells and neurons (as is also the case between different types of neurons) (Neher, 2006). For example, catecholamines are stored and released from large dense core granules rather than small synaptic like vesicles. Nonetheless, chromaffin cells provide both a physiologically important system and powerful cellular model to investigate neurosecretion and its modulation by GPCRs (Currie, 2010b).

"Fight or Flight"/chromaffin cell secretory function

The physiological response to metabolic or emotional stressors involves the coordinated activation of the hypothalamic-pituitary-adrenocortical (HPA) axis and the sympathoadrenal system (Goldstein, 2010). Functionally, at the level of the splanchnic-adrenal synapse, this is represented as shift from low-frequency action-potential firing (basal sympathetic tone) to higher frequency "burst mode" stress firing, which increases the output of acetylcholine (ACh) and the neuropeptide co-transmitter PACAP (Smith and Eiden, 2012). This sympathetic drive depolarizes chromaffin cells, promoting Ca^{2+} influx through voltage-gated Ca^{2+} channels, and subsequently triggers fusion of large dense core granules with the plasma membrane, thus releasing catecholamines and other peptide transmitters into circulation. PACAP is hypothesized to be a crucial player for the prolonged secretory response associated with stress firing, as its application induces robust secretion by initiating Ca^{2+} entry through T-type (Hill et al., 2011), 2-APB sensitive (Mustafa et al., 2007), N-, P/Q- and L-type Ca^{2+} channels (O'Farrell and Marley, 1997) while bypassing sodium-channel based action-potentials (Mustafa et al., 2007)

associated with nicotinic ACh receptor activation. Furthermore, PACAP upregulates mRNA for tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT), both involved in catecholamine biosynthesis, as well as biosynthetic enzymes for neuropeptides co-secreted with catecholamine (Ait-Ali et al., 2010). Chromaffin cells contain ~10,000 to 20,000 granules divided into five pools based on kinetics of release, physical association with the membrane, and sensitivity to various regulatory co-factors: the immediately releasable pool (IRP), readily releasable pool (RRP), docked vesicle pool (DRP), unprimed pool (UPP), and reserve pool (RSP). As a result chromaffin cells provide a supply of catecholamine that is both rapidly available and physiologically inexhaustible. It is unclear the exact number of vesicles in each pool, but the vesicles docked and primed at the membrane represent a minority of granules, likely numbering in the hundreds, with the rest associated with the cytoskeleton in the reserve pool (Garcia et al., 2006). The RRP can be estimated by identifying membrane capacitance (ΔCm) plateaus presumably corresponding to a decline in secretory rate under sustained depolarization or caged calcium release. A single granule fusion is known to contribute a ΔCm of about 1.3 fF thus can be correlated to total granule fusion. The maximum size of the RRP in bovine chromaffin cells was estimated as ~34 granules by Gillis et al., using a novel "paired pulse" methodology. The authors interpret the decline of exocytosis triggered by two short (100 ms) depolarizations as an exhaustion of the RRP, assuming no pool replenishment occurs with a brief interpulse interval, and calcium entry is constant during each pulse (Gillis et al., 1996). Using this methodology, the authors demonstrated that activation of PKC with the diacylglycerol mimetic PMA increased the

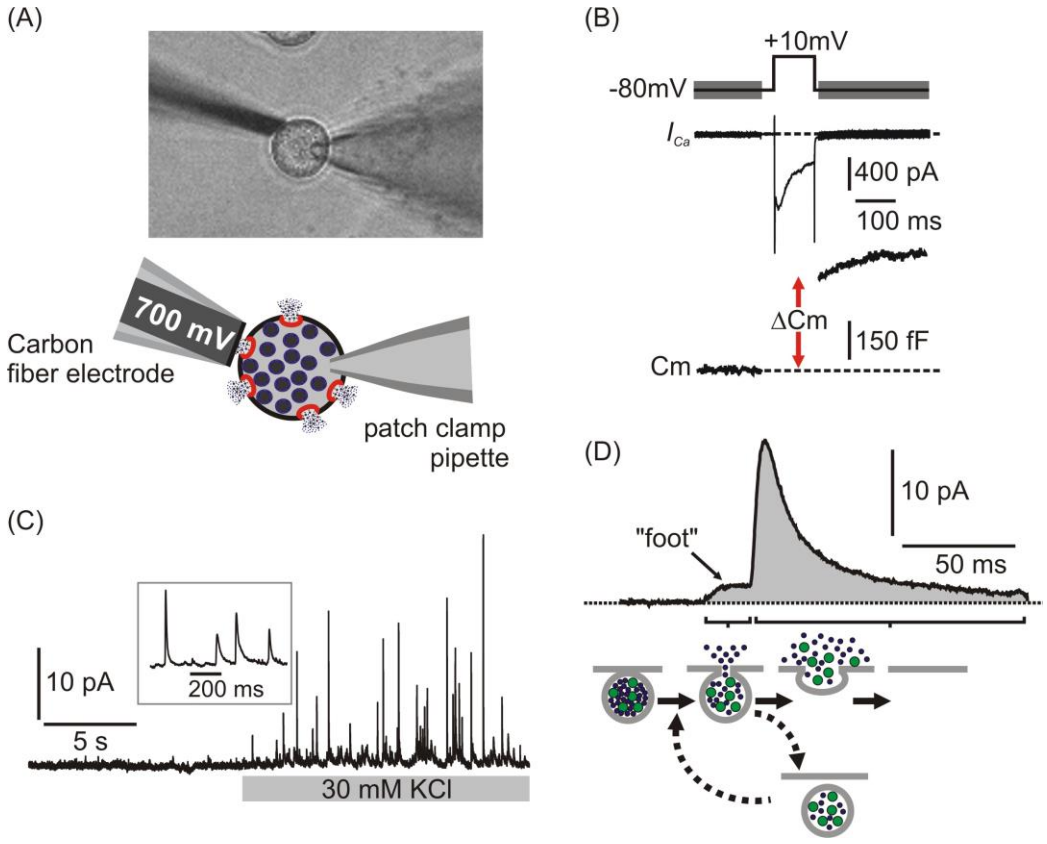

Figure 5: Adrenal chromaffin cells are well suited for investigating stimulus-secretion coupling at the cellular level. **(A)** Photograph and cartoon depiction of a single chromaffin cell with a patch clamp pipette and carbon fiber amperometry electrode in position. **(B)** Vesicle fusion (exocytosis) and recycling (endocytosis) can be tracked as changes in membrane capacitance (Cm) using patch clamp electrophysiology. The upper trace represent the voltage-command applied to the cell, including a sine wave (grey box) superimposed on the holding potential. The step depolarization evoked an inward Ca^{2+} current (I_{Ca}, middle trace), which in turn evoked vesicular exocytosis detected as a jump in membrane capacitance (ΔCm, bottom trace). **(C)** Direct electrochemical detection of catecholamine exocytosis by carbon fiber amperometry.The example shows the amperometric current from the carbon fiber electrode elicited from a non-voltage-clamped chromaffin cell by 30 mM KCl. Each upward "spike" is due to catecholamine release from a single vesicular fusion event. The inset shows a few spikes on an extended time scale. **(D)** An amperometric spike due to oxidation of catecholamines released during a single vesicular fusion event is shown. The charge of the spike (integral – grey shading) is directly proportional to the number of catecholamine molecules released. Other kinetic features of the spike can also be analyzed. Some spikes (~ 1 in 3) display a smaller amplitude plateau or pre-spike "foot" that is thought to reflect release of catecholamine through a narrow fusion pore. As illustrated in the cartoon, the fusion pore may then expand irreversibly resulting in full collapse of the vesicle into the plasma membrane (solid arrows), or may open transiently resulting in partial emptying of the vesicular content and rapid recycling of the vesicle

size of the RRP to ~130 granules. Using caged calcium release to estimate pool size instead of paired step depolarizations predicts a larger RRP, and may in fact be targeting both the IRP and RRP (Gillis et al., 1996; Heinemann et al., 1994; Horrigan and Bookman, 1994). Replenishment of membrane- associated, release competent pools with vesicles from the reserve pool can be measured as recovery from depletion and has been shown to rely on $[Ca^{2+}]_i$ (Smith et al., 1998; von Ruden and Neher, 1993). Taken together Ca^{2+} demonstrates a broad and temporally complex role in regulating the secretory process, both in the late stage triggering of exocytosis and maintaining supply of release competent vesicles.

Role in disease

Adrenal chromaffin cells are a major sympathetic output of the peripheral nervous system, responsible for most of the circulating epinephrine, and to a lesser extent norepinephrine (collectively referred to as catecholamines). By binding to adrenergic receptors, catecholamines maintain cardiovascular homeostasis. However in response to physiological stress catecholamines are the primary effector in coordinating "fight or flight" sympathetic activation.

Chromaffin cell dense core granules contain remarkably high concentrations of catecholamine (~600 mM), ATP (~150 mM) and Ca^{2+} (40 mM) (Videen et al., 1992), along with neuropeptide Y (NPY) and the endogenous opioid enkephalin. Accordingly, transport of catecholamine from the cytosol into granules presents a solubility problem (Kim and Loh, 2005). Catecholamine aggregation with the acidic polypeptide

chromogranin A (CHGA) may facilitate granulogenesis by forming a gel-like storage complex with catecholamines and ATP that relieves intra-granular osmotic pressure (Machado et al., 2008; Mahapatra et al., 2005). Accompanying its role in granulogenesis, CMGA is also a prohormone that gives rise to a number of bioactive peptides that correlate with disease. For example: pancreastatin (dysglycemic peptide, elevated in diabetes) (Gayen et al., 2009), the nAChR antagonist catestatin (diminished in hypertension), and CHGA itself is elevated in essential hypertension (Mahapatra, 2008; Mahapatra et al., 2005). Interestingly Aβ peptides are co-released from chromaffin granules along with APP and α- β- and γ-secretases, molecules involved in the pathogenesis of Alzheimers disease. (Toneff et al., 2013). Understanding the full impact of the so called adrenal 'secretome' is in its early stages.

Under basal sympathetic electrical input, catecholamines are selectively released from a restricted fusion pore. During electrical stimulation that matches acute stress, dense-core granules undergo full fusion with the membrane, co-releasing catecholamines along with the peptidergic granule core (Fulop et al., 2005). Certainly catecholamines and bioactive peptides have divergent physiological effects on cardiovascular, endocrine and nervous systems, thus regulation of the fusion pore by second messenger pathways provides both regulatory richness and important pathophysiological targets. For example PKC promotes fusion pore dilation and full granule collapse (Chen and Levine, 2005; Fulop and Smith, 2006) whereas GPCR dependent Gβγ activation may shift the mode of exocytosis toward smaller fusion events that release less catecholamine, and potentially occlude neuropeptide release (Chen et al., 2005; Yoon et al., 2008).

The homeostatic role of sympathoadrenal output can be observed by removing chromaffin cell granular components. CMGA$^{-/-}$ mice have severe morphological defects in chromaffin granules leading to increased circulating catecholamine, NPY and elevated blood pressure. Blood pressure can be rescued with exogenous catestatin, a cleavage product of CMGA (Mahapatra et al., 2005). CMGA$^{-/-}$ animals also display increased insulin sensitivity in liver, owing to the loss of pancreastatin (Gayen et al., 2009). Rats lacking a functional adrenal gland had pronounced hypotension when challenged with endotoxin, an effect rescued with endogenous NPY(Evequoz et al., 1988). Mice that cannot make epinephrine (PNMT $^{-/-}$) are hypertensive during exercise, and become hyperglycemic and insulin resistant on a high fat diet, suggesting an emerging role for adrenal output in the development of metabolic syndrome (Ziegler et al., 2012).

Increased catecholamine levels are observed in patients with acute decompensated heart failure. While initially compensatory, this sympathetic overdrive leads to cardiac remodeling and eventual myocyte death (Feldman et al., 2008; Mudd and Kass, 2008). Adrenal GRK2 is upregulated in rodent models of heart failure, leading to downregulation of auto-inhibitory α2 adrenergic receptors, resulting in increased catecholamine secretion (Lymperopoulos et al., 2007). The same group used adrenal targeted gene-transfer approaches to inhibit GRK2, and as a consequence significantly reduced circulating catecholamine (Lymperopoulos et al., 2008) and improved cardiac function in a post-myocardial infarction model of heart failure (Lymperopoulos et al., 2010).

Intra-adrenal paracrine signaling

Regulation of synthetic enzymes for PGE_2

Sympathoadrenal epinephrine outflow in response to physiological stressors like hypoglycemia, hemorrhagic hypotension, emotional stress, exercise beyond an anaerobic threshold, and shock is highly correlated with plasma adrenocorticotrophic hormone (ACTH) levels, suggesting a possible role for ACTH in coordinating adrenocortical-adrenomedullary responses to stress, or indicating intra-adrenal interactions between cortical cells and chromaffin cells (Goldstein, 2010). Certainly catecholamines stimulate steroidogenesis and corticosteroid release from the adrenal cortex, demonstrated by the finding that tyrosine hydroxylase knockout mice had hypofunctional cortical cells and decreased circulated cortisol (Bornstein et al., 2000). Conversely, ACTH and glucocorticoids control epinephrine biosynthesis by regulating PNMT (phenylethanolamine N-methyltransferase) activity (Wurtman and Axelrod, 1965). Blood flow has been described as centripetal from the cortex to the medulla through a network of arterioles in the connective tissue, which branch and distribute blood to the cortex through a network of sinousoids, and to medulla through medullary arteries. (Bornstein et al., 1997), (Ehrhart-Bornstein et al., 1998). However the impact of blood flow on medullar-cortical counter regulation is unclear.

In an animal model of systemic inflammation, infusion of LPS (lipopolysaccharide) induced the cortical expression of the proinflammatory mediator IL-1β (interleukin-1β), its receptor IL-1βR, and synthetic enzymes for PGE_2: Cox-2 and mPGES1, both in

resident and newly infiltrating immune cells (macrophages and dendritic cells). Injection of IL-1β also induced its own cortical expression, and the expression of Cox-2. Because IL-1β stimulates cortisol release, and IL-1βR was only found expressed on immune cells, the authors suggest perhaps prostaglandin production ultimately regulates cortisol release during an immune challenge (Engstrom et al., 2008) (Deak, 2008). Interestingly, Cox-1, Cox-2, and mPGES-1, were constitutively expressed in the adrenal medulla along with dense expression of receptors for $PGE_{2,}$ EP1 and EP3, shown by *in situ* hybridization (Engstrom et al., 2008). Given the close association of cortex and medulla, the functional effects that increased local production of PGE_2 may have on medullar output remain unclear (Figure 6).

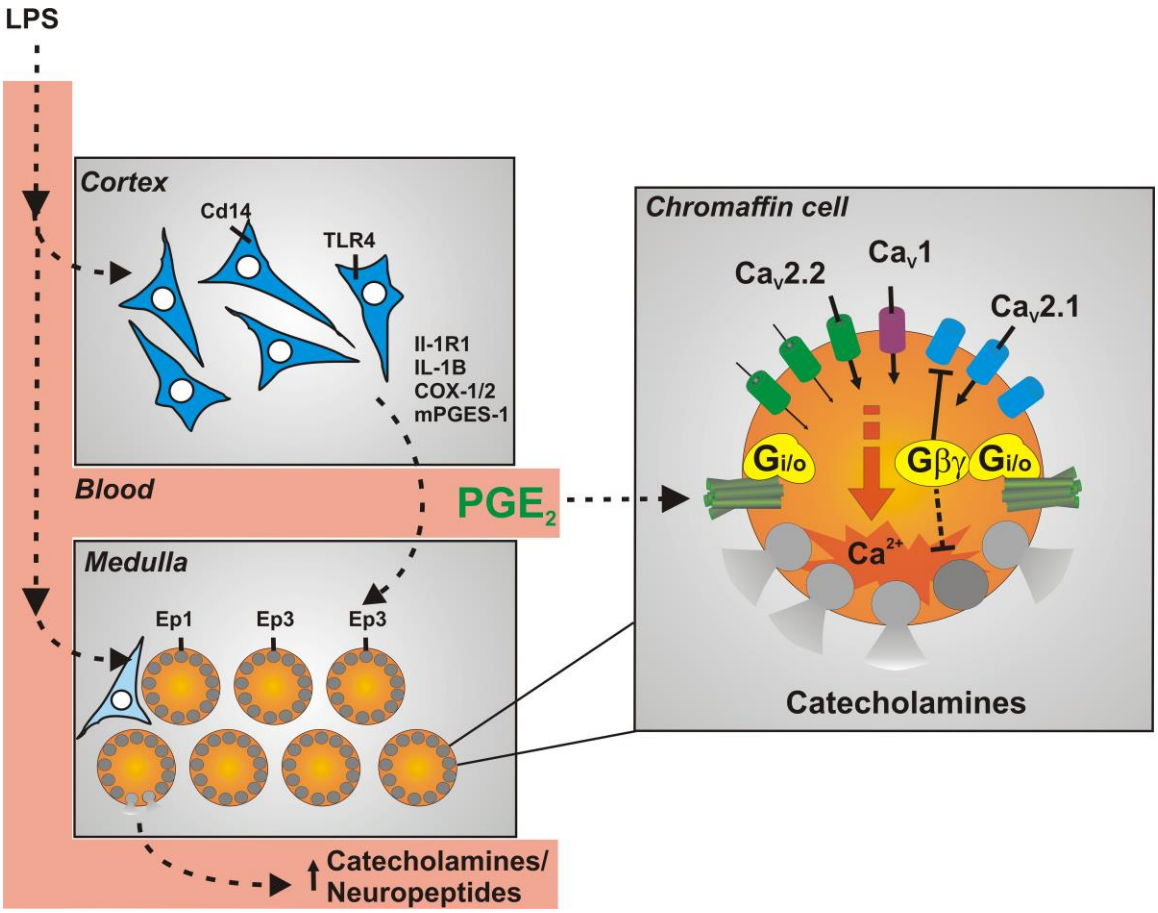

Figure 6: Intra-adrenal paracrine signaling in response to immune challenge. Schematic depicting potential cortex-to-adrenal signaling pathways and relative predicted movement of PGE_2. Systemic injection of LPS induces the production of IL-1β, its receptor IL-1βR, and synthetic enzymes for PGE_2: Cox-2 and mPGES1 in the adrenal cortex. Presumably LPS binds its cognate receptors, toll-like receptors (TLR4) and CD-14 on resident macrophages and dendrites (blue cells, top panel) to increase the expression of IL-1β, which in turn increases the expression of Cox-2 and mPGES1. The functional effect of increased prostaglandin production "downstream" on adrenal chromaffin cells is unknown. Chromaffin cells express EP1 and EP3 receptors (bottom panel) and N-, P/Q-, and L-type calcium channels that trigger exocytosis of catecholamine and other neuropeptides directly into the circulation. How PGE_2 may regulate catecholamine release from individual chromaffin cells is unclear.

Prostaglandins and the EP family of GPCRs

PGE_2 is the most widely expressed prostanoid, formed by cyclooxygenase (COX1/2) induced arachidonic acid metabolism to PGH_2, which is converted to PGE_2 by tissue specific synthases (PGE_2 synthase) (Figure 7). PGE_2 produces a myriad of biological effects including inflammation, tumorigenesis in a number of cancers (Chen and Smyth, 2011; Rundhaug et al., 2011; Wu et al., 2010) uterine and smooth muscle contraction, inhibition of gastric acid secretion, modulation of neurotransmitter release and sodium and water reabsorption in the kidney (Grantham and Orloff, 1968).

PGE_2 mediates its effects in an autocrine or paracrine fashion on specific Class A, rhodopsin-like GPCRs designated EP receptors (Breyer et al., 2001; Regan, 2003; Sugimoto and Narumiya, 2007). Four EP receptor subtypes: EP1, EP2, EP3 and EP4 give rise to diverse, and sometimes contradictory functional effects of PGE_2. Adding to the functional repertoire, multiple isoforms of EP1 and EP3 are generated by alternative splicing. (Coleman et al., 1994; Schmid et al., 1995). Therefore the diverse array of PGE_2 actions depends on the physiological context and expression of specific EP receptors in each respective tissue.

Studying agonist-induced changes in second messenger signaling has led to the characterization of signal transduction pathways for each EP receptor (Hirata and Narumiya, 2011). Stimulation of mouse EP1 expressed in CHO cells leads to a robust increase in intracellular calcium concentration $[Ca^{2+}]_i$ and to a lesser extent phosphotidylinositol hydrolysis and IP_3 generation, both independent of phospholipase C

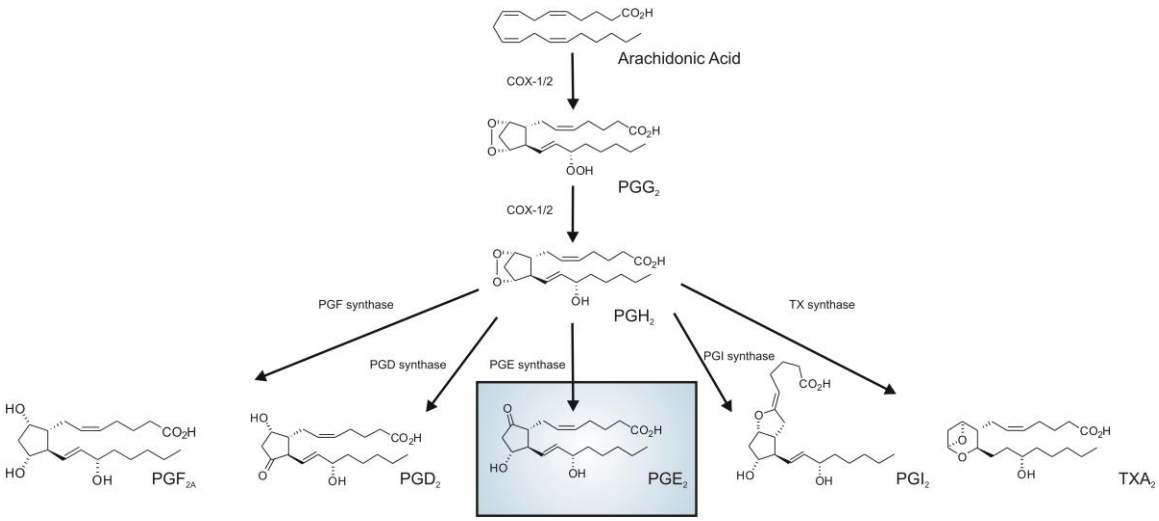

Figure 7: Prostanoid biosynthesis. Cyclooxygenase isozymes (COX-1 or COX-2) catalyzse a two-step synthesis of the prostanoid precursor PGH_2. Arachidonic acid (AA) is cyclized to a PGG_2 intermediate, then reduced in the peroxidase active site to PGH_2. The expression of COX isozymes and cell specific PG synthases are highly regulated.

(Katoh et al., 1995). It is accepted that activating EP1 increases $[Ca^{2+}]_i$ and produces smooth muscle contraction, however only recent work by Ji *et al.* shows human EP1 produces PI turnover as well as a pertussis toxin-sensitive activation of PI3K, indicating an association with both G_q and $G_{i/o}$ (Ji et al., 2010). Similarly, a rat EP1 splice variant has been characterized that fails to increase $[Ca^{2+}]_i$ and when expressed in CHO cells, antagonizes the effects of EP4 receptor activation (Okuda-Ashitaka et al., 1996).

The EP2 and EP4 subtypes couple to G_s and activate AC, converting ATP to cAMP. cAMP signals through the canonical activation of PKA, and the novel target Epac (exchange protein directly activated by cAMP), which impacts numerous physiological processes through its own 'signalosome' (Schmidt et al., 2013). Additionally EP4 can activate ERK 1/2 through PI3K activation (Regan, 2003).

The EP3 receptors were initially characterized as smooth muscle constrictors, decreasing cAMP. Alternative splicing of the C-terminus generates three known mouse EP3 splice variants: $EP3_\alpha$, $EP3_\beta$ and $EP3_\gamma$ (Irie et al., 1993), that vary in G protein coupling and agonist dependent desensitization. In the mouse, $EP3_\alpha$ and $EP3_\beta$ signal through G_i mediated inhibition of cAMP. Both bind agonist with similar affinities but $EP3_\alpha$ has been shown to desensitize and downregulate. $EP3_\gamma$ is coupled to both stimulation and inhibition of cAMP (Negishi et al., 1993). Additionally all three EP3 mouse splice variants increase $[Ca^{2+}]_i$ and IP_3 generation, and signal through RhoA (Macias-Perez et al., 2008)' (Aoki et al., 1999; Katoh et al., 1995). Bovine and human EP3 splice variants have also been identified (Namba et al., 1993; Negishi et al., 1989; Schmid et al., 1995) (Figure 8).

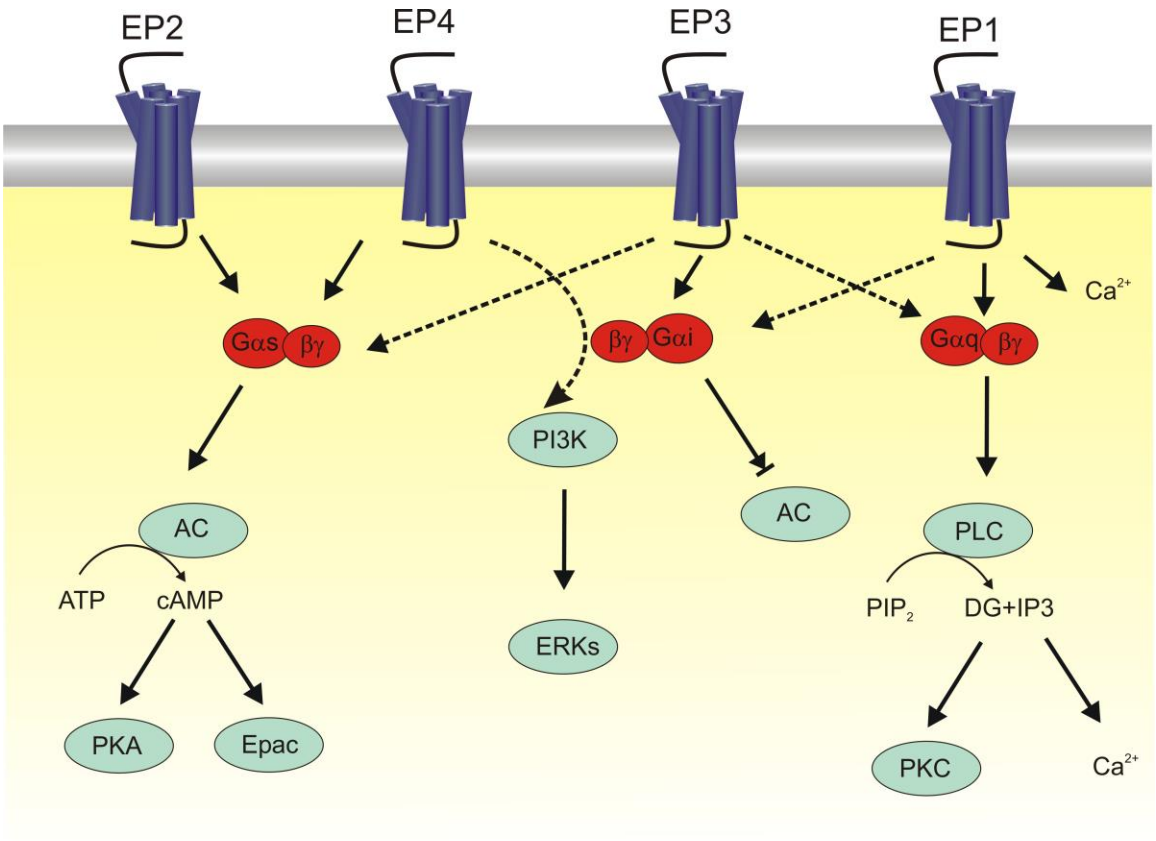

Figure 8: Promiscuous coupling of EP receptors to intracellular signal transduction pathways. Commonly observed signaling pathways shown with dark arrows. The EP3 receptor, though primarily "inhibitory", interacts with a greater diversity of G proteins due to C-terminal splicing (not shown but represented as dashed arrows). Additionally each EP3 splice variant can signal to RhoA (not shown).

The impact of PGE_2 on adrenal function

Studies focusing on PGE_2 augmentation of adrenal catecholamine secretion have largely been conducted in bovine chromaffin cells or whole glands, measuring bulk catecholamine release, and have yielded inconsistent results. 1 μM PGE_2 application concomitant with ouabain stimulation of bovine chromaffin cells for up to 30 min was shown to enhance catecholamine secretion due to IP_3 and DAG production, indicating PLC activation, with maximal DAG production to 1.5 fold basal occurring at a concentration of 10 μM PGE_2 (Negishi et al., 1990). Given the K_d for PGE_2 binding to EP subtypes ranges from 1-40 nM, these concentrations are quite high. Potentiation of ACh stimulated secretion was also reported in perfused dog adrenals, but independent of $[Ca^{2+}]_i$ elevations (Yamada et al., 1988). Other work suggests selective EP1 activation increases $[Ca^{2+}]_i$ from caffeine and ryanodine dependent stores independent of IP_3 production (Shibuya et al., 1999), consistent with multiple functional enzyme couplings of EP1 (Figure 8). Karaplis and colleagues report PGE_2 (1-10 nM) inhibits nicotine stimulated catecholamine release, whereas higher concentrations (1 μM) stimulated spontaneous release in bovine adrenal monolayers (Karaplis et al., 1989). Consistent with these findings, M&B28767, an EP3 specific agonist, inhibited dopamine release from PC-12 cells stably expressing the $EP3_\beta$ receptor variant (Nakamura et al., 1998). Clearly the functional effects of EP receptor activation in chromaffin cells depend on the stimulation paradigm and receptor subtype present. The effect of physiologically relevant PGE_2 concentrations on quantal exocytosis, assayed using sensitive electrophysiological techniques to resolve individual fusion events has not been investigated. Similarly, physiological stimulation paradigms have not been employed.

Specific Questions

Sympathetic stress increases firing rates at the splanchnic-adrenal synapse, resulting in sustained depolarization and secretion of catecholamine from chromaffin cells (Smith and Eiden, 2012). Similarly, systemic inflammation increases local production of Cox-2 and mPGES1, likely resulting in increased PGE_2 production (Engstrom et al., 2008). The cellular consequences of PGE_2 action are primarily mediated by binding four cognate GPCRs (EP1-EP4) with the net functional effect reflecting a balance of stimulatory and inhibitory receptors. EP1 and EP3 receptors are expressed in the adrenal medulla (Breyer et al., 1993; Engstrom et al., 2008; Namba et al., 1993; Shibuya et al., 1999) along with the synthetic enzymes for PGE_2 (Engstrom et al., 2008; Ichitani et al., 2001). Thus the components are in place for local modulation of chromaffin cells by PGE_2. We hypothesize that local PGE_2 production in the adrenal gland serves to regulate stimulus secretion coupling in adrenal chromaffin cells.

Specific Questions:

1. Does PGE_2 regulate Ca^{2+} signaling in adrenal chromaffin cells?

2. If we change the cellular context by modeling sympathetic stress firing, does the physiological impact of PGE_2 on chromaffin cells change?

2. GENERAL MATERIALS AND METHODS

Cell preparation and culture

Male mice (6-12 weeks old), wild type, EP3 receptor knockout (Zhang et al 2011; manuscript submitted to Prostaglandins and Other Lipid Mediators) or EP1 receptor knockouts (Guan et al., 2007)), all on C57BL/6 background were euthanized using carbon dioxide followed by cervical dislocation. Adrenal glands were quickly harvested and placed in ice cold Magnesium Free Locke's solution containing (in mM):153 NaCl, 6 KCl, 2 $NaH_2PO_4.7H_20$, 1 $NaH_2PO_4.H_20$, 10 Glucose, 10 HEPES (Figure 9). The glands were trimmed of fat and the cortex dissected from the medullae. The medullae were incubated for 10 minutes at 37° C in a papain digestion solution (2.5 mg/ml papain, Genlantis, San Diego, CA), followed by another 10 minute incubation at 37° C in collagenase P (3 U/ml, Roche Diagnostics, Indianapolis, IN.). Tissues were washed 2X with Locke's and transferred to growth medium consisting of: DMEM / F12/GlutaMAX (catalogue # 10565) (Invitrogen, Carlsbad, CA) supplemented with Hyclone defined fetal bovine serum (10%) (Fisher Scientific, Pittsburgh, PA) and penicillin (100 unit/ml) / streptomycin (100 μg/ml), (Sigma Aldrich, St. Louis, MO). Tissues were then triturated with a 2 ml fire polished glass pipette coated in growth medium and allowed to settle. The cell containing supernatant was removed and plated on glass coverslips thinly coated in growth factor reduced Matrigel (BD Biosciences, Bedford, MA). Cells were allowed to settle and adhere to the coverslips for 2 hours before 2ml of growth medium was added to

the coverslips. Cells were maintained at 37° C in a humidified, 5% CO_2 atmosphere and used 1-3 days post-isolation. Each cell preparation was from a single mouse. All experimental studies were approved by the IACUC of Vanderbilt University Medical Center.

Electrophysiology

Electrodes were pulled from borosilicate glass capillary tubes (World Precision Instruments, Sarasota, FL), coated with dental wax (Electron Microscopy Sciences, Hatfield, PA) and fire polished to a final resistance of 1.8-3 MΩ when filled with a CsCl-based internal solution. Cells were voltage-clamped in the whole-cell configuration using an Axopatch 200B amplifier, Digidata1400A interface and PClamp10 software (Molecular Devices, Sunnyvale, CA). Analog data were filtered at 2 kHz and digitized at 20 μs/point (50 kHz). Data were analyzed using PClamp10, OriginPro software (OriginLab Corp, Northampton MA) and GraphPad Prism (version 5, GraphPad Software Inc., San Diego, CA). For perforated whole- cell recording configuration the pipette tip was filled with amphotericin-free solution and then backfilled with solution that contained ~0.5 mg/ml amphotericin-B (Calbiochem, Carlsbad CA). After forming a cell-attached seal, series resistance was monitored to assess the progress of perforation. Typically, series resistance <10-15 MΩ was achieved within 5-15 minutes, and cells that did not show good perforation within this time frame were discarded. I_{Ca} was activated by brief 20-100 ms step depolarizations to a predetermined peak (10-30 mV) from a

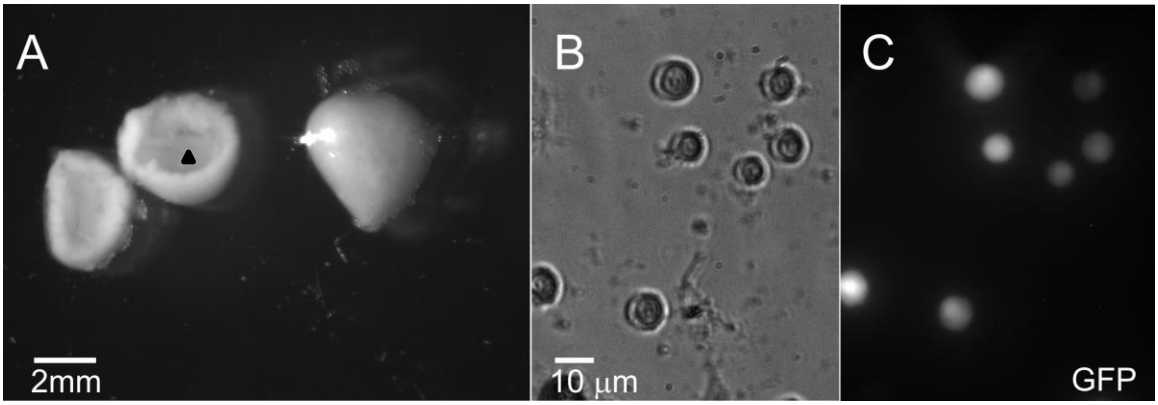

Figure 9: Preparation of chromaffin cells from mouse adrenal glands. (A) A single intact mouse adrenal gland (right) imaged under a Nikon stereoscope with a bifurcated fiber optic illuminator attachment. The bisected gland (left) reveals the relative volume of the inner, chromaffin cell containing medulla (black arrow), compared to the outer cortex. The darker contrast of the medulla under a bright field microscope aids in the removal of the cortex. Trimmed medullae were digested, dissociated, and plated on matrigel-coated coverslips as described in detail in the Materials and Methods section. **(B)** Individual chromaffin cells imaged with an inverted fluorescent microscope at 400X total magnification. Cells were isolated from a transgenic mouse carrying the gene for green fluorescent protein (GFP) under the control of the tyrosine hydroxylase (TH) promoter (courtesy of Dr. Danny Winder). TH is a key synthetic enzyme for catecholamine biosynthesis and is not expressed in cortical cells, fibroblasts, endothelial cells or various immune cells found in the adrenal gland, thus was used to verify isolation of chromaffin cells **(C)** The same field of cells as (B) imaged with a GFP filter, confirming our protocol is largely homogeneous for chromaffin cells.

holding potential of -80 mV, or by trains of action potential-like waveforms loaded as a stimulus file (-80-45 mV, 16 ms duration). Data were subjected to linear capacitance and leak subtraction using standard P/N protocols. When determining the inhibition of I_{Ca} produced by PGE_2 (100 nM) cells in which the current amplitude decreased <10% were designated as "non-responders" and reported as such in the results section. Only "responders" (i.e. >10% current decrease) were included when calculating mean percent inhibition. The presence of both responders and non-responders was consistently observed in multiple cell preparations, but we did not investigate any other possible differences between the two sets of cells in this study. To calculate an EC_{50} for inhibition of I_{Ca} by PGE_2 data were fit with a Boltzmann function of the form: Y = Ymax / (1 + 10^(Log EC_{50} - X)); where Y = % inhibition of I_{Ca} and X is the concentration of PGE_2. The Hill slope was assumed to be 1 and the curve fit with the least squares method in Prism5 software. Goodness of fit was indicated by $R^2 = 0.97$.

Nicotinic ACh receptor currents were activated by bath perfusion for 45 s with 30-100 μM carbachol. This enabled multiple reproducible responses to be obtained from the same cell. The delay in current activation (see Figure 12A) was due to the "dead space" in the perfusion system. The amplitude of the sustained inward current activated by carbachol was determined by calculating the mean current amplitude over a 5 s period starting 30 s after carbachol application. The mean current amplitude over this 5 s period was determined for each cell and then data pooled. The current amplitude was also calculated (mean over a 5 s period) at the end of the drug application to determine the extent to which the response declined.

Changes in membrane capacitance (ΔCm) were monitored in the perforated whole-cell recording configuration using a HEKA EPC10 amplifier in combination with PatchMaster data acquisition software (HEKA Electronik). The software lock-in module was used to implement the "sine + d.c." approach for estimating Cm. A sine wave (1 kHz, 20 mV peak - peak) was imposed on the holding potential of -80 mV and the assumed reversal potential was set to 0 mV. Membrane conductance was simultaneously calculated by the software and any cells that showed simultaneous changes in Cm and Gs were discarded. Cells were stimulated by two step depolarizations (to +10 mV, 100 ms duration) separated by 100 ms. The stimulus was repeated every three minutes. Membrane capacitance was averaged over a 50 ms period before the stimulus (baseline) and again 50 ms after the end of the stimulus to calculate ΔCm. After two control responses cells were exposed to 100 nM PGE_2 during the third response. The second control response was typically of equal or greater magnitude than the first and if this was not the case the cell was discarded due to concern about "rundown". For data analysis, cells were divided into those that responded to PGE_2 with decrease in I_{Ca} amplitude >10% (group-1), and those in which I_{Ca} was not inhibited (group-2) (see results for more discussion). Data are reported as mean ± standard error of the mean and statistical significance was determined using paired or independent Student's t test as appropriate.

Amperometry

ProCFE Carbon Fiber Microelectrodes were purchased prefabricated from Dagan Corporation (Minneapolis, MN.). Electrodes were backfilled with 3 M KCl and fixed to a custom electrode holder on a standard NPI 500 MΩ low-noise headstage. The 5μM diameter carbon fiber was maneuvered to the side of the cell to easily confirm contact without depressing the membrane. Although the manufacturer allows 5-10 cells/electrode without fouling or increases in noise, fibers were discarded after no more than three uses. A +700 mV potential was applied to the carbon fiber using a NPI VA-10x voltammetric and amperometric amplifier (NPI electronic gmbH, Tamm, Germany). Amperometric currents were continuously sampled over two stimulation windows (denoted S1 and S2 in chapter 4 Results) at 10 kHz, and filtered at 2 kHz with a low-pass hardware filter on the NPI amplifier.

Amperometric currents were digitized with a National Instruments BNC-2090 board (National Instruments Corporation, Austin, TX.) and acquired using the WinEDR Strathclyde Electrophysiological Recorder (Strathclyde Institute of Pharmacy and Biomedical Sciences, Glascow, Scotland). Peak finding and kinetic parameters were analyzed using a custom macro for Igor Pro (Wavemetrics, Lake Oswego, OR.) developed by Eugene Mosharov and David Sulzer at Columbia University. With this program an additional 150 Hz filter was applied to the current (I) and 300 Hz to the first derivative of the current (dI/dt). Events were detected using an initial threshold of 4X the $SD_{dI/dt}$ in a spike free "control" region of the trace, with an absolute undifferentiated

current cutoff of 4 pA. After initial peak finding, events were confirmed by visual inspection. Overall secretory activity was assessed by counting all spikes ≥ 4 pA, which was the minimum threshold ensuring discrimination from noise. For quantal size determination and other kinetic parameters, spikes with a rise time ≥10 ms and an amplitude ≤ 4 pA were excluded, so only "fast" quantal events occurring near the fiber were analyzed. Further analysis and spike sorting was conducted using OriginPro spreadsheet software (OriginLab, Northhampton, MA) and Graphpad Prism (GraphPad software, San Diego, CA.). To take into account cell to cell variability in both the number and size of individual events as they factor in statistical weight, numbers of events for each cell and stimulation window were reported as an average (median or mean). Subsequently averages were pooled and subjected to non-parametric statistical tests as spike parameters do not follow a normal Guassian distribution. Pooled data are reported as mean ± standard error of the mean and statistical significance was determined using paired or independent Student's t test, ANOVA, or Dunnett's multiple comparison post test as appropriate.

$[Ca^{2+}]_i$ Measurements

Free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in cells loaded with the fluorescent Ca^{2+} indicator Fura-2 (Molecular Probes, Eugene OR). Cells were washed twice with HEPES-buffered Hanks Balanced Salt Solution (HBSS) and incubated for 30-45 minutes with 3 μM Fura-2 AM at 37°C. Cells were then washed in Fura-free solution for 30-60 minutes before recording. For recording, the coverslip with the cells attached

was transferred to a recording chamber and mounted on the stage of a Nikon TE2000 fluorescence microscope. The recording chamber had a volume of ~300-400 μL and was continually perfused with fresh solution from gravity-fed reservoirs at a flow rate of ~4 ml/min. An InCyt IM2 fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH) was used to monitor $[Ca^{2+}]_i$. Cells were alternately excited at wavelengths of 340 nm and 380 nm and emission at 510 nm detected using a pixelfly digital camera as detailed previously (Dzhura et al., 2006). Ratios were collected every 2 s throughout the experiment and converted to $[Ca^{2+}]_i$ using an *in vitro* calibration curve, generated by adding 15.8 μM Fura-2 pentapotassium salt to solutions from a calibration kit containing 1mM $MgCl_2$ and known concentrations of Ca^{2+} (0–1350 nM) (Molecular Probes, Eugene OR). One or two cells in the field of view were selected in each experiment and after a 2-minute baseline were exposed to 1μM PGE_2 for 3-minutes and subsequently to a 50 mM KCl containing solution (by replacing an equimolar amount of NaCl in our standard extracellular solution - see below). This was done as a positive control for the assay - to ensure the cells were loaded with Fura-2 and responded to calcium elevations. It also served as a means to identify any non-excitable (i.e. non-chromaffin) cells as these typically fail to respond to KCl. Cells that had an unstable baseline or failed to respond robustly to KCl (>300 nM elevation) were excluded from analysis. Data analysis was performed using OriginPro software (OriginLab Corporation, Northampton, MA).

RT-PCR

Total RNA was prepared from mouse adrenal and kidney tissue using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from total RNA with ABI High Capacity Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA) and amplified using specific primers: EP1: 5'-TTAACCTGAGCCTAGCGGATG-3' (sense primer, nucleotides 13-37), 5'-CGCTGAGCGTATTGCACACTA -3' (antisense primer, nucleotides 662-682), EP2: 5'-CCTGGGACATGGTGCTTTAT-3' (sense primer, nucleotides 1404-1423), 5'-GGTGGCCTAAGTATGGCAAA -3' (antisense primer, nucleotides 1797-1816), EP3c: 5'-CGCCGTCTCGCAGTC-3' (sense primer, nucleotides 849-863), EP3αβ: 5'- TGTGTCGTCTTGCCCCCG -3' (antisense primer, nucleotides 1362-1379), EP3γ: 5'- TGTGGCTTCATTCCTTGCCCA -3' (antisense primer, nucleotides 1572 -1592), EP4: 5'-GGTCATCTTACTCATCGCCACCTCTC -3' (sense primer, nucleotides 1027-1052, 5'-TCCCACTAACCTCATCCACCAACAG -3 (antisense primer, nucleotides 1538-1562), GAPDH: 5' GGCATTGCTCTCAATGACAA-3' (sense primer, nucleotides 942-961) 5'-TGTGAGGGAGATGCTCAGTG-3' (antisense primer, nucleotides 1122-1141).

Drugs and Solutions

For whole cell and perforated whole cell patch clamp experiments, cells were perfused at a rate of ~ 4 ml/min with external solution consisting of (in mM): 136 NaCl, 2 KCl, 1 $MgCl.6H_20$, 10 Glucose, 10 HEPES, 10 $CaCl.2H_20$, pH 7.3 osmolarity ~ 305. For

amperometry experiments, control solutions consisted of (in mM): 145 NaCl, 2 KCl, 1 $MgCl.6H_20$, 10 Glucose, 10 HEPES, 2 $CaCl.2H_20$, pH 7.3 osmolarity ~ 305. Exocytosis was stimulated with a 30 mM KCl solution consisting of (in mM): 117 NaCl, 30 KCl, 1 $MgCl.6H_20$, 10 Glucose, 10 HEPES, 2 $CaCl.2H_20$, pH 7.3 osmolarity ~ 305. For all amperometry experiments cells were perfused at a rate of ~4 ml/min from gravity fed reservoirs. All drugs were diluted (≥1000X) and perfused in these extracellular solutions unless otherwise noted. For whole-cell recordings electrodes were filled with internal solution containing (in mM):110 CsCl, 10 EGTA, 20 HEPES, 4 $MgCl_2$, 0.35 GTP, 4 ATP, 14 creatine phosphate, pH 7.3 osmolarity ~305. The free calcium concentration in this solution is estimated to be very low (<1nM) (http://maxchelator.stanford.edu). For perforated whole-cell recording electrode tips were filled with internal solution containing (in mM): 145 Glutamic Acid, 10 HEPES, 10 NaCl, 1 TEA-Cl, pH 7.3, osmolarity 309, and backfilled with internal solution containing Amphotericin B (Calbiochem, San Diego, CA) at a final concentration of 0.53 mM, prepared from a 100X stock solution in DMSO every two hours. PGE_2 (Cayman Chemical Company, Ann Arbor, MI.) and Sulprostone (Sigma-Aldrich, St. Louis, MO.) were prepared as 10 mM stock solutions in ethanol and DMSO, respectively, and frozen until day of use. DG-041 was synthesized in the Vanderbilt Institute of Chemical Biology Chemical Synthesis Core. Pertussis toxin (Calbiochem, San Diego, CA) was prepared as a 100 µg/µL stock in water and applied 24 hours prior to experiment in cell culture medium at 300 ng/µL. Carbachol (Calbiochem, San Diego, CA.) and TTX (Alomone Labs, Jerusalem, Israel) were prepared in sterile water as 100 mM and 1 mM stocks respectively and diluted on the day of use. Nitrendipine (ICN Biomedicals Inc., Aurora, OH.) was prepared as a 10

mM stock solution in ethanol and diluted to 10 μM in external solution. Stock solutions of ω-conotoxin GVIA (100 μM) (Alomone Labs, Jerusalem, Israel) and ω-agatoxin IVA (10 μM) (BACHEM Bioscience Inc., King of Prussia, PA.) were prepared in standard extracellular solution and diluted to final working concentrations of 1.5 μM and 400 nM respectively on the day of use. SC51322 (Cayman Chemical Company, Ann Arbor, MI.) was prepared as a 10mM stock in ethanol and frozen until day of use. Gallein (Tocris Bioscience, Bristol, UK.) and Phosducin C-terminal peptide (Anaspec, Fremont, CA.) were prepared as 50mM and 300 μM stock solutions in DMSO, respectively.

3. REGULATION OF CALCIUM CHANNELS AND EXOCYTOSIS IN MOUSE ADRENAL CHROMAFFIN CELLS BY PROSTAGLANDIN EP3 RECEPTORS

Abstract

Prostaglandin E_2 (PGE_2) controls numerous physiological functions through a family of cognate G protein coupled receptors (EP1-EP4). Targeting specific EP receptors might be therapeutically useful and reduce side effects associated with non-steroidal anti-inflammatory drugs and selective cyclooxygenase-2 inhibitors that block prostanoid synthesis. Recently, systemic immune challenge and inflammatory cytokines were shown to increase expression of the synthetic enzymes for PGE_2 in the adrenal gland. Catecholamines and other hormones, released from adrenal chromaffin cells in response to Ca^{2+} influx through voltage-gated Ca^{2+} channels, play central roles in homeostatic function and the coordinated stress response. However, chronic elevation of circulating catecholamines contributes to the pathogenesis of hypertension and heart failure. Here we investigated the EP receptor(s) and cellular mechanisms by which PGE_2 might modulate chromaffin cell function. PGE_2 did not alter resting intracellular [Ca^{2+}] or the peak amplitude of nicotinic acetylcholine receptor currents, but did inhibit Ca_V2 voltage-gated Ca^{2+} channel currents (I_{Ca}). This inhibition was voltage-dependent and mediated by pertussis toxin-sensitive G proteins, consistent with a direct Gβγ subunit-mediated mechanism common to other $G_{i/o}$-coupled receptors. mRNA for all four EP receptors was detected, but using selective pharmacological tools and EP receptor knockout mice we

demonstrated that EP3 receptors mediate the inhibition of I_{Ca}. Finally, changes in membrane capacitance showed that Ca^{2+}-dependent exocytosis was reduced in parallel with I_{Ca}. To our knowledge this is the first study of EP receptor signaling in mouse chromaffin cells and identifies a molecular mechanism for paracrine regulation of neuroendocrine function by PGE_2.

Introduction

Catecholamines and other hormones released from adrenal chromaffin cells help maintain normal homeostatic function and play central roles in the coordinated response to acute stressors, for example during "fight-or-flight" sympathetic activation. Elevation of circulating catecholamines is closely correlated with hypertension and is a hallmark of chronic heart failure. Indeed, specifically targeting the adrenal gland to inhibit catecholamine outflow *in vivo* improved cardiac function in rat models of heart failure, suggesting adrenal hormone release as a potential therapeutic target (Lymperopoulos et al., 2008). In situ, acetylcholine (ACh) released from splanchnic nerve fibers activates nicotinic ACh receptors on the chromaffin cell causing membrane depolarization, opening of voltage-gated calcium channels, and influx of calcium that subsequently triggers exocytosis (Boarder et al., 1987). Thus, as with neurons and other excitable cells, voltage-gated calcium channels play pivotal roles in chromaffin cell function. The cells also express a variety of G protein coupled receptors (GPCRs) that orchestrate complex regulation of stimulus-secretion coupling. For example, chromaffin cells express autoreceptors for ATP (P2Y receptors), catecholamines (α-adrenergic) and enkephalin

(μ-opioid receptors) that couple to $G_{i/o}$ -type G proteins and mediate autocrine/paracrine inhibition of catecholamine release through inhibition of voltage-gated calcium channels (Albillos et al., 1996; Currie and Fox, 1996; Harkins and Fox, 2000; Powell et al., 2000; Ulate et al., 2000) and other downstream targets (Chen et al., 2005; Yoon et al., 2008).

In this study we investigated the effects of prostaglandin E_2 (PGE_2) on chromaffin cells. PGE_2 is produced in a variety of cell types through metabolism of arachidonic acid by cyclooxygenase (COX-1 or COX-2) and prostaglandin E synthases. It acts in an autocrine / paracrine manner, primarily through binding to a family of cognate GPCRs (EP1-EP4 receptors) (Breyer et al., 2001), to control a variety physiological functions including: protection of the gastric mucosa, renal function, inflammation, pain, blood pressure, and secretion of hormones and neurotransmitters. Non-steroidal anti-inflammatory drugs such as aspirin and selective COX-2 inhibitors reduce production of PGE_2, but also disrupt synthesis of other prostanoids and can cause serious side effects. One strategy to reduce these unwanted side-effects is to identify specific cellular functions of EP receptors and develop EP receptor subtype-selective drugs. For example, characterization of EP receptor signaling in smooth muscle suggests EP1 receptors could be targeted for antihypertensive treatment, and an EP3 receptor antagonist (DG-041) is under investigation for treatment of atherothrombosis (Guan et al., 2007; Heptinstall et al., 2008).

EP1 and EP3 receptors are expressed in the adrenal medulla (Breyer et al., 1993; Engstrom et al., 2008; Namba et al., 1993; Shibuya et al., 1999) along with the synthetic

enzymes for PGE_2 (Engstrom et al., 2008; Ichitani et al., 2001). Moreover, systemic immune challenge or circulating cytokines rapidly recruit dendritic cells and macrophages to the adrenal gland, increase the expression of COX-2 and PGE synthase, and presumably local PGE_2 production (Engstrom et al., 2008). Thus the components are in place for local modulation of chromaffin cells by PGE_2, but previous studies present confusing and contradictory findings: PGE_2 has been reported to increase (Karaplis et al., 1989; Marley et al., 1988; Yamada et al., 1988; Yokohama et al., 1988) or decrease (Karaplis et al., 1989) adrenal catecholamine release, elevate intracellular calcium levels (Mochizuki-Oda et al., 1991; Shibuya et al., 1999), or inhibit voltage-gated calcium channels (Currie et al., 2000). In sympathetic neurons, which are closely related to chromaffin cells, PGE_2 can modulate nicotinic ACh receptors (Du and Role, 2001; Tan et al., 1998) as well as voltage-gated calcium channels (Ikeda, 1992). Thus PGE_2 may alter calcium signaling and exocytosis in chromaffin cells by multiple pathways and potentially through multiple EP receptors.

The goal of this study was to define the receptors and mechanisms by which PGE_2 modulates calcium signaling in adrenal chromaffin cells using a combination of pharmacological tools and EP receptor knockout mice. We show that PGE_2 did not alter the peak amplitude of nicotinic ACh receptor currents or resting intracellular [Ca^{2+}], but potently inhibited Ca_V2 voltage-gated calcium channel currents (I_{Ca}) (EC_{50} = 5.5 nM). Although mRNA for all four EP receptor subtypes is expressed in the mouse adrenal gland, our data unequivocally show that EP3 receptors mediate this inhibition of I_{Ca}. PGE_2 also decreased the change in membrane capacitance in response to membrane

depolarization / calcium entry, suggesting that the number of secretory vesicles undergoing exocytosis was reduced. To our knowledge this is the first study of EP receptor signaling in mouse chromaffin cells and identifies a cellular / molecular mechanism for paracrine regulation of neuroendocrine function by PGE_2.

Results

PGE_2 inhibits I_{Ca} in mouse adrenal chromaffin cells

Given the pivotal roles of voltage-gated calcium channels in stimulus-secretion coupling and many other cellular functions we initially tested whether PGE_2 inhibited the calcium channel currents (I_{Ca}) in mouse chromaffin cells. The cells were voltage-clamped at -80 mV and stimulated with a 20 ms step-depolarization to evoke I_{Ca} every 10 s (Figure 10A). Application of 100 nM PGE_2 produced a significant and reversible inhibition of peak I_{Ca} amplitude (Figure 10A) in approximately 76% of cells tested under similar experimental conditions (conventional whole-cell recording, n = 37 of 49 cells; N = 9 mice). The inhibition of I_{Ca} was concentration dependent (Figure 10B) and the data fit well with a Boltzmann function that yielded a maximal inhibition of 40% and an EC_{50} of 5.5 nM consistent with the low nM affinities reported for PGE_2 binding to EP receptors (Breyer et al., 2001).

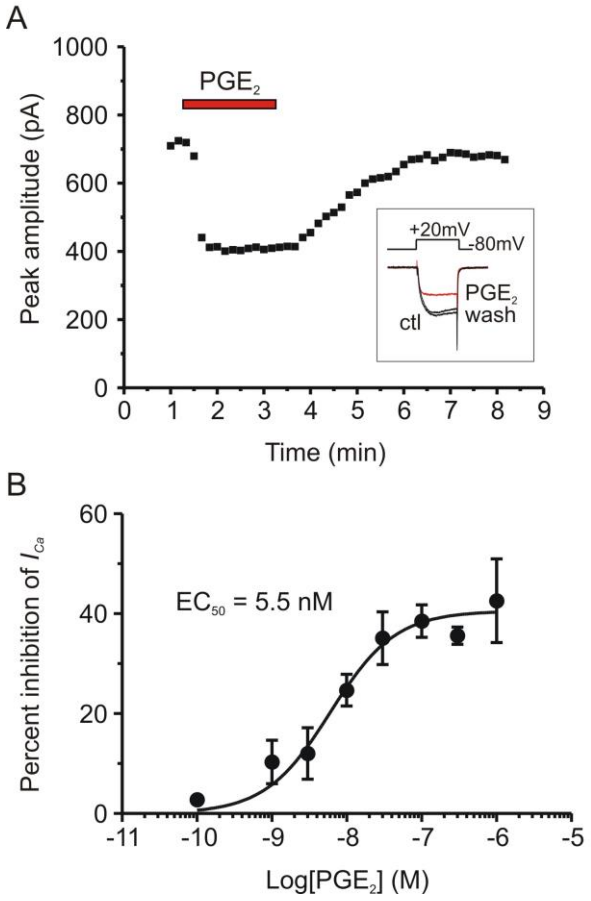

Figure 10: PGE_2 inhibits I_{Ca} in mouse adrenal chromaffin cells. (A) Peak amplitude of I_{Ca} is plotted against time in a representative cell. The cell was voltage-clamped in the whole-cell configuration and stimulated with a 20 ms step-depolarization from -80 mV to +20 mV every 10 s. Application of PGE_2 (100 nM) (indicated by horizontal bar) produced robust, reversible inhibition of I_{Ca}. Inset: shows the voltage command (upper) and three representative current traces before (ctl), during (PGE_2), and after washout of PGE_2 (wash). **(B)** Log $_{10}$ concentration response curve plotting percent inhibition of I_{Ca} to varying concentrations of PGE_2. Each cell was exposed to three increasing concentrations of PGE_2, with 10 nM being common to all experiments (n = 4-16 cells). The indicated fit was to a Boltzmann function with a Hill slope = 1 (see methods) and yielded an EC_{50} of 5.5 nM.

The inhibition of I_{Ca} by PGE_2 is voltage-dependent and mediated by pertussis toxin-sensitive G proteins

G protein coupled receptors (GPCRs) inhibit I_{Ca} by several different mechanisms, but perhaps the most widespread and best understood pathway is mediated by direct binding of G protein βγ subunits to P/Q-type ($Ca_V2.1$) and N-type ($Ca_V2.2$) channels (Currie, 2010a). Although there are exceptions, in most cases this pathway involves GPCRs that couple to pertussis toxin-sensitive $G_{i/o}$-type G proteins. We and others have previously shown that P2Y purinergic receptors and μ-opioid receptors utilize this pathway to produce autocrine/paracrine inhibition of I_{Ca} in chromaffin cells (Albillos et al., 1996; Currie and Fox, 1996; Powell et al., 2000). To determine if PGE_2 acts through a $G_{i/o}$-coupled GPCR we incubated isolated mouse chromaffin cells with pertussis toxin (300 ng/mL) for ~24-hours prior to whole-cell recording. Control cells were from the same cell preparations and were recorded on the same days as the pertussis toxin treated cells. As shown in Figure 11A, the inhibition of I_{Ca} produced by PGE_2 was virtually abolished in pertussis toxin treated cells (2 ± 2.6%, n = 6 compared to 31 ± 7.1%, n = 7, in control cells; $p < 0.002$). As a positive control we also used the P2Y receptor agonist ATP (100 μM), as this is known to inhibit I_{Ca} via pertussis toxin-sensitive G proteins in chromaffin cells (Currie and Fox, 1996). The inhibition produced by ATP was also significantly reduced (29 ± 8.5 %, n = 7 in control cells compared to 7 ± 5.4 %, n = 6, in pertussis toxin treated cells; $p < 0.05$). These data confirmed that PGE_2 acts though a $G_{i/o}$-coupled GPCR to inhibit I_{Ca}.

A defining biophysical signature of direct Gβγ-mediated inhibition of N- and P/Q-type channels is reversal by a strongly depolarizing voltage-step. This reversal is thought to reflect transient dissociation of Gβγ from the channel at the depolarized membrane potential (for review see (Currie, 2010a). Therefore, we used a prepulse facilitation protocol to determine if PGE_2 utilized this mechanism to inhibit I_{Ca} in mouse chromaffin cells. Figure 11B illustrates a representative voltage command (upper) and current trace (lower). The cell was stimulated by two identical test pulses (P1 and P2), the second of which was preceded by a 50 ms step to +120 mV. PGE_2 significantly reduced the amplitude of I_{Ca} during both P1 and P2 (Figure 11C), but the prepulse (immediately preceding P2) significantly reduced this inhibition from 43 ± 6 % during P1 to 13 ± 3% during P2 (n = 6; $p < 0.001$) (Figure 11D). Thus the inhibition of I_{Ca} produced by PGE_2 was largely voltage-dependent although there was also a voltage-independent component to the inhibition (the residual inhibition seen during P2) consistent with what has been reported previously for P2Y and opioid receptors.

Voltage-dependent inhibition of I_{Ca} by other GPCRs preferentially targets the Ca_V2 family of calcium channels, in particular P/Q-type ($Ca_V2.1$) and N-type ($Ca_V2.2$) channels (Currie, 2010a). Mouse chromaffin cells are known to express Ca_V2 channels (P/Q-type and N-type and R-type channels) and also members of the Ca_V1 family (L-type channels) (Garcia et al., 2006). Consistent with previous reports we found that nitrendipine, a dihydropyridine antagonist of L-type channels, blocked 41 ± 5 % (n = 7) of the whole-cell current. We did not systematically dissect the channel types comprising the non-L-type current, but previous reports indicate the majority is carried by N- and

P/Q-type channels, with 10-20% accounted for by R-type and perhaps T-type channels (Garcia et al., 2006). After block of N-type ($Ca_V2.2$) and P/Q-type ($Ca_V2.1$) channels by pre-incubation with ω-conotoxin GVIA (1.5 μM) and ω-agatoxin IVA (400 nM) respectively, the inhibition by PGE_2 was dramatically reduced (7 ± 1%; n = 6; $p < 0.05$) confirming that N- and P/Q-type channels are the main target for this pathway.

Short-term application of PGE_2 did not alter peak nicotinic acetylcholine receptor currents

In situ, chromaffin cells are directly innervated by cholinergic splanchnic nerve fibers. Activation of nicotinic acetylcholine receptors (nAChRs) on the chromaffin cells causes membrane depolarization, activation of voltage-gated calcium channels and influx of calcium that triggers exocytosis. It has been reported that PGE_2 modulates nicotinic acetylcholine receptors in sympathetic neurons (Du and Role, 2001; Tan et al., 1998), although we are not aware of any similar studies in chromaffin cells. However, it has been shown that inhibition of nAChRs in chromaffin cells can reduce cytosolic calcium elevations and catecholamine release elicited by cholinergic stimuli (Dzhura et al., 2006). Thus PGE_2 could indirectly alter calcium channels and calcium signaling in chromaffin cells by modulating nAChR. To test this possibility cells were voltage-clamped at a holding potential of -80 mV in the perforated whole-cell recording configuration and the bath was continuously perfused with fresh extracellular recording solution. Nicotinic ACh receptor currents were evoked by application of 100 μM carbachol for 45 seconds (Figure 12A). Under these conditions the inward current was primarily due to the relatively non-desensitizing nACh receptors found in chromaffin cells.

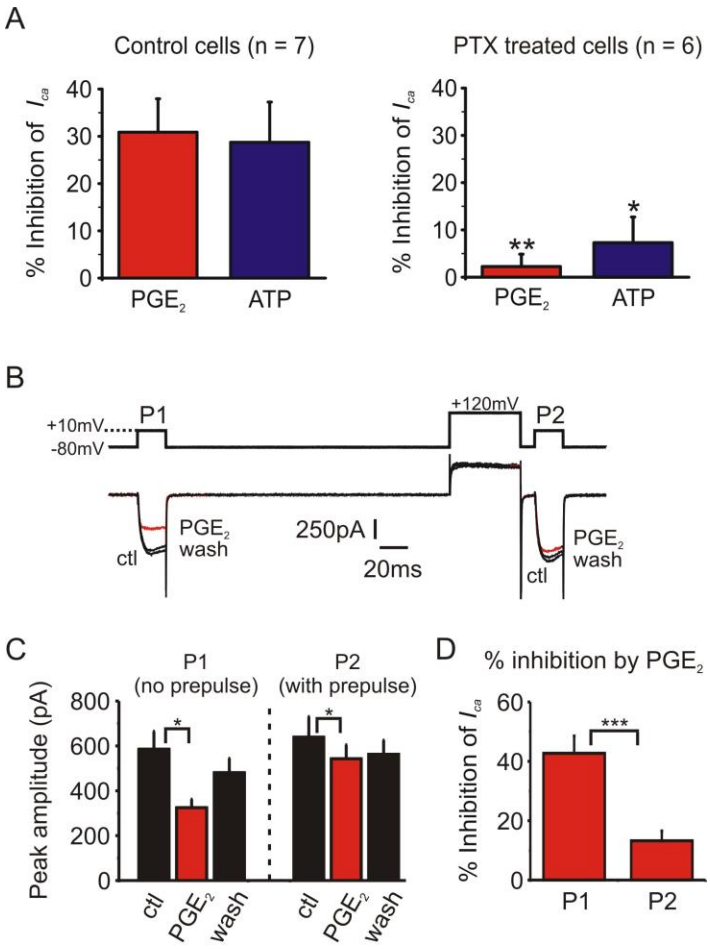

Figure 11: The inhibition of I_{Ca} by PGE_2 is voltage-dependent and mediated by pertussis toxin-sensitive G proteins. (A) The percent inhibition of I_{Ca} produced by 100 nM PGE_2 or 100 μM ATP for control cells (left panel) and cells treated with 300 ng/mL pertussis toxin (PTX) for ~24-hours prior to whole-cell recording of I_{Ca}. Control and pertussis toxin-treated cells were from the same cultures, and recordings were alternated on the same day. PTX treatment significantly reduced the inhibition by PGE_2 (** $p < 0.002$) and ATP (* $p < 0.05$). **(B)** The inhibition of I_{Ca} by PGE_2 was voltage-dependent. The upper trace illustrates the voltage command for the prepulse facilitation protocol. Cells were stimulated by two identical test pulses (P1 and P2, 20 ms step to +10 mV, separated by 300 ms), but the second pulse (P2) was preceded by a 50 ms step to +120 mV. Three representative currents are superimposed (lower trace), showing I_{Ca} before (ctl), during application of 100 nM PGE_2 (PGE_2), and after washout (wash). The prepulse to +120 mV reversed most of the inhibition of I_{Ca} produced by PGE_2. **(C)** Bar chart summarizing the mean peak amplitude of I_{Ca} in six cells like that shown in panel B during the first pulse (P1-no prepulse) and the second pulse (P2- with prepulse) (* $P < 0.05$; $n = 6$). **(D)** The percent inhibition by PGE_2 of I_{Ca} elicited by P1 (without a prepulse) and P2 (with a prepulse) (*** $P < 0.001$; $n = 6$).

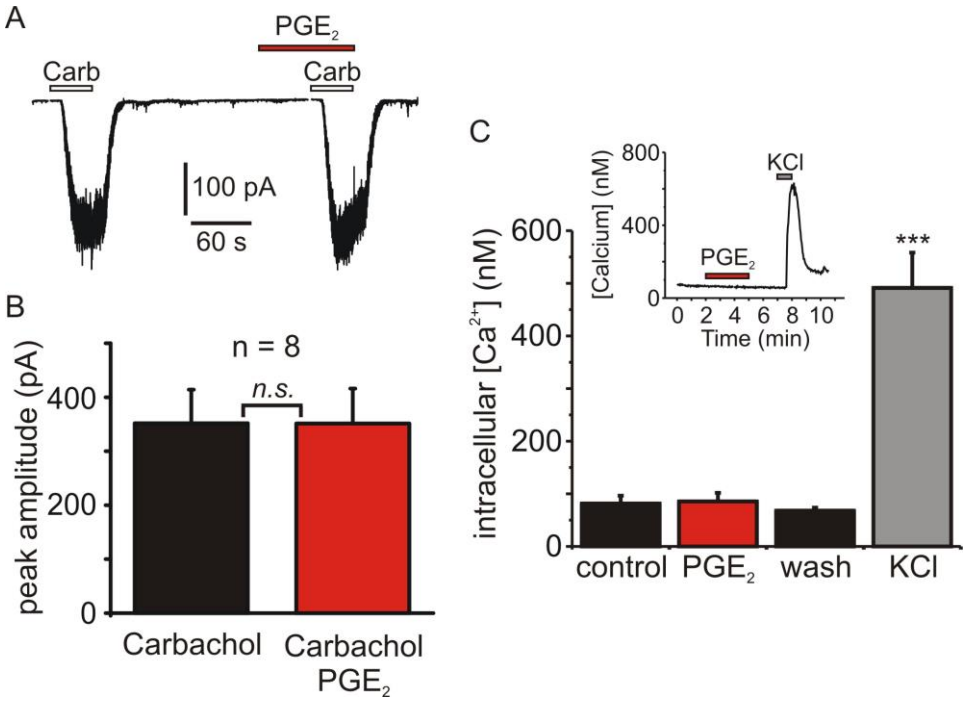

Figure 12: PGE_2 does not alter peak nicotinic acetylcholine receptor currents or resting $[Ca^{2+}]_i$ in mouse chromaffin cells. (A) Representative recording of nicotinic acetylcholine receptor (nAChR) currents evoked by two applications of carbachol (100 µM) in the absence (left) or presence (right) of 100 nM PGE_2. Drug application is indicated by the horizontal bars. Cells were voltage-clamped at -80 mV in the perforated whole-cell recording configuration. **(B)** Bar chart showing that PGE_2 had no effect on the mean amplitude of the nAChR currents evoked by carbachol (n = 8 cells). **(C)** Ratiometric imaging of FURA2 loaded chromaffin cells. Inset shows a representative recording from a single cell plotting estimated $[Ca^{2+}]_i$ against time (sampling rate 0.5Hz). The cell was exposed to 1µM PGE_2 for three minutes and then to 50 mM KCl to depolarize the membrane and elicit Ca^{2+} entry through voltage-gated Ca^{2+} channels (positive control). The main chart shows mean $[Ca^{2+}]_i$ before (control), during (PGE_2) and after washout (wash) of 1µM PGE_2, and the response to 50mM KCl. (n = 9 cells from 7 independent experiments).

After washout of carbachol the cells were allowed to recover for six minutes before exposure to 100 nM PGE_2 and a second application of 100 μM carbachol (in the continued presence of PGE_2) (Figure 12A). Acute application of PGE_2 had no effect on the mean peak inward current evoked by carbachol (351 ± 65 pA in the presence of PGE_2 compared to 352 ± 63 pA before application of PGE_2; n = 8) (Figure 12B). The inward current response did decay slightly during continued application of carbachol (9 ± 2% in control conditions) and this was significantly increased in the presence of PGE_2 (21 ± 3%; $p < 0.01$).

Short-term application of PGE_2 did not elevate basal intracellular calcium concentration

Previous reports indicated that relatively high concentrations of PGE_2 ranging from 200 nM to 1 μM can directly elevate intracellular calcium concentration ($[Ca^{2+}]_i$) either by activating a Ca^{2+} influx pathway or by releasing Ca^{2+} from intracellular stores (Mochizuki-Oda et al., 1991; Shibuya et al., 1999). However, in the experiments investigating the effects of PGE_2 on nAChR currents, PGE_2 did not alter the holding current of chromaffin cells voltage-clamped at -80 mV (see Figure 12A) suggesting that PGE_2 did not activate an inward calcium current. We also used Fura-2 imaging to determine if PGE_2 could elevate resting $[Ca^{2+}]_i$ in individual mouse chromaffin cells. Our data showed that an acute (3-minute) application of 1 μM PGE_2 had no effect on $[Ca^{2+}]_i$ (82 ± 15 nM before and 85 ± 16 nM during application of PGE_2 ; n = 9 cells from 7 independent experiments) (Figure 12C).

mRNA for all four EP receptor subtypes was detected in mouse adrenal tissue

The data presented above demonstrated that PGE_2 acts through a $G_{i/o}$-coupled GPCR to inhibit I_{Ca} in mouse chromaffin cells. Of the four known receptors for PGE_2, termed EP1-EP4, only EP3 typically couples to $G_{i/o}$-type G proteins, although it has been reported recently that EP1 receptors might also couple to $G_{i/o}$ at least in some cell types (Ji et al., 2010). Both EP3 and EP1 receptors have been reported previously in the adrenal medulla (Breyer et al., 1993; Engstrom et al., 2008; Namba et al., 1993).

To determine which EP receptors were expressed in the mouse adrenal gland we used RT-PCR. The adrenal gland was isolated as described in the methods section and kidney tissue, which expresses all four EP receptor subtypes, was isolated in parallel as a positive control. The adrenal cortex was dissected from the gland leaving the adrenal medulla for RNA isolation, however small traces of cortex were likely present. Three known splice variants of the EP3 receptor are found in mice: $EP3_\alpha$, $EP3_\beta$ and $EP3_\gamma$ (Breyer et al., 2001; Irie et al., 1993). These splice variants differ in their C-terminal tail and can exhibit different downstream signaling pathways and agonist dependent desensitization in heterologous expression systems. We detected mRNA for all three EP3 receptor splice variants in the mouse adrenal tissue (Figure 13A). We also detected mRNA for the EP1, EP2 and EP4 receptors (Figure 13B). GAPDH was used as an internal standard and amplified in all tissues (data not shown).

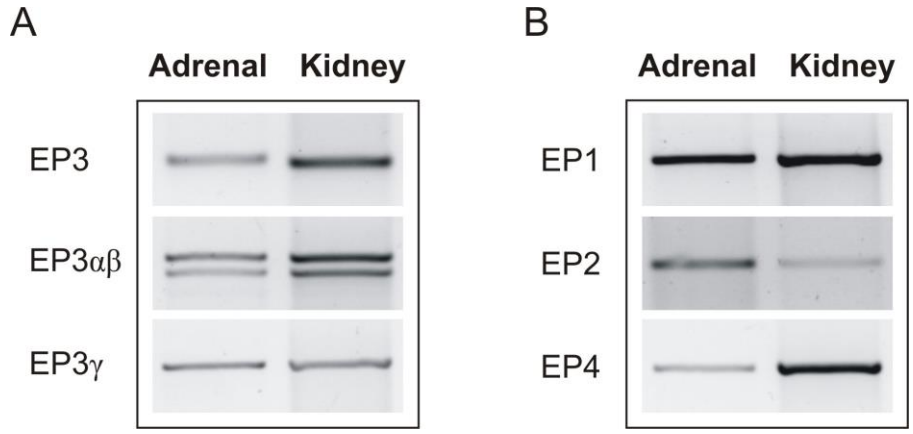

Figure 13: EP receptor mRNA expressed in mouse adrenal tissue. (A) RT-PCR was used to detect expression of the EP3 receptor in mouse adrenal tissue (left), and kidney tissue (right) that was isolated in parallel as a positive control. The upper panel used primers common to all splice variants of the EP3 receptor. The lower two panels used primers selective for the splice variants. The forward primers for EP3α and EP3β are identical so the fragments run in the same lane (middle panel): the top band corresponds with the expected amplicon size of EP3α, and the bottom band EP3β . **(B)** In addition to EP3, EP1 (top), EP2 (middle) and EP4 (bottom) mRNA was amplified. All samples from figures A and B expressed the internal standard GAPDH (data not shown). Data shown are representative of three replicate experiments on tissue from three different mice.

Pharmacological evidence that EP3 receptors mediate the inhibition of I_{Ca} by PGE_2

We showed that the inhibition of I_{Ca} was abolished in pertussis toxin treated cells (Figure 11A), so is mediated by $G_{i/o}$-coupled GPCRs. While we detected mRNA for all four EP receptor subtypes in the mouse adrenal gland (Figure 13), generally only EP3 receptors couple to $G_{i/o}$ (Breyer et al., 2001). Therefore, we used EP receptor subtype selective agonists and antagonists to investigate the involvement of EP3 receptors. First we used the selective EP1/EP3 receptor agonist sulprostone (Figure 14A, B). In these experiments we used perforated whole-cell recordings to maintain endogenous calcium buffering of the chromaffin cells. The inhibition produced by 100 nM PGE_2 (43 ± 6%, n = 15) was similar to that in conventional whole-cell recording. Sulprostone (100 nM) significantly reduced the amplitude of I_{Ca} in six-out-of-seven cells by 41 ± 9% (n = 6) and this was not significantly different from the inhibition produced by 100 nM PGE_2 under the same recording conditions (p = 0.89).

It has been reported that DG-041 is a selective, non-competitive antagonist of EP3 receptors (Heptinstall et al., 2008). Cells were stimulated every 10 seconds with a 20-ms step depolarization to evoke I_{Ca}. DG-041 (30 nM) was applied to the cells for ~2 minutes before application of PGE_2 (100 nM). DG-041 alone had little effect on I_{Ca} but completely blocked the inhibition produced by PGE_2 (2.0 ± 2.2 %, n = 9; Figure 14C, D). Subsequent applications of PGE_2 after several minutes of washout of DG-041 also produced no inhibitory effect, suggesting DG-041 is functionally irreversible over the time course of our experiments. As a control we used ATP (100 μM) to activate P2Y

receptors in the presence of DG-041. ATP inhibited I_{Ca} by 26 ± 4.7 % (n = 4) (Figure 14D**)**, similar to the inhibition produced by ATP in the absence of DG-041 (29 ± 8.5 %; Figure 11A**).** This suggests DG-041 selectively blocked PGE_2 and the downstream signaling pathways responsible for voltage-dependent inhibition of I_{Ca} were intact.

The inhibition of I_{Ca} produced by PGE_2 was abolished in cells isolated from EP3 receptor knockout mice

The pharmacological data presented above strongly implicated EP3 receptors in the inhibition of I_{Ca} by PGE_2. However, it was still possible that other receptors could play a role. For example, DG-041 is reported to be a selective noncompetitive antagonist of EP3 receptors but off-target effects of the compound have not been widely studied. Therefore, to unequivocally identify the receptor subtype involved, we isolated chromaffin cells from EP3 receptor knockout mice ($EP3^{-/-}$ mice). As shown in Figure 15, the inhibition of I_{Ca} by PGE_2 was abolished in cells isolated from $EP3^{-/-}$ mice. In the same cells 100 μM ATP significantly reduced the amplitude of I_{Ca} by 33 ± 7 % (n = 6, $p < 0.05$), indicating the effect of the knockout was selective for PGE_2 and did not perturb G protein mediated inhibition of I_{Ca} by other receptors. Similarly, in perforated whole- cell recording PGE_2 did not inhibit I_{Ca} in cells isolated from $EP3^{-/-}$ mice (1 ± 4% inhibition; n = 6).

As a complementary approach, we tested the ability of sulprostone (an EP1/EP3 selective agonist) to inhibit I_{Ca} in cells isolated from EP1 receptor knockout mice. Under these

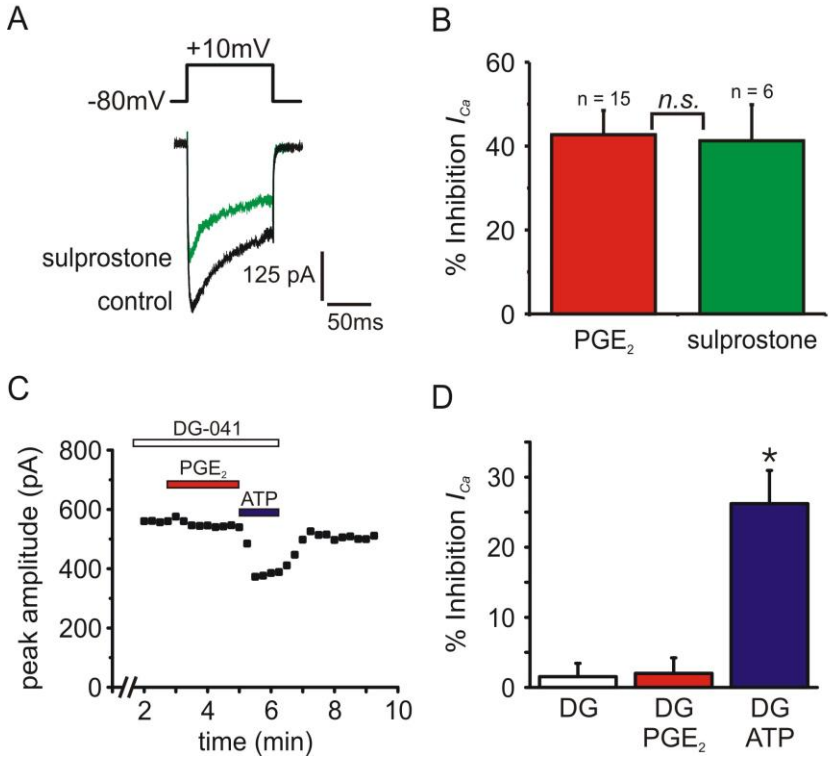

Figure 14: Pharmacological evidence that EP3 receptors mediate the inhibition of I_{Ca} by PGE_2. (A) The selective EP1/EP3 receptor agonist sulprostone inhibits I_{Ca}. Representative voltage command (upper) and I_{Ca} (lower) recorded in presence and absence of sulprostone (100 nM), obtained in the perforated whole-cell recording configuration. **(B)** Bar chart illustrating the mean percent inhibition of I_{Ca} produced by PGE_2 (100 nM) or sulprostone (100 nM). The inhibition produced by the two agonists was not significantly different. **(C, D)** DG-041, a selective EP3 receptor antagonist, blocked the inhibition of I_{Ca} produced by PGE_2. **(C)** Experimental time course in a representative cell plotting peak amplitude of I_{Ca} against time. DG-041 (30 nM) was applied ~ 2 minutes before PGE_2 (100 nM) and completely blocked the inhibition of I_{Ca}, but had no effect on the inhibition produced by the P2Y receptor agonist ATP (100 μM). **(D)** Bar chart summarizing the percent inhibition of I_{Ca} by application of 30 nM DG-041 alone (DG), and in the presence of either 100 nM PGE_2 (DG + PGE_2; n = 9) or 100 μM ATP (DG + ATP; n = 4). DG-041 prevented the inhibition produced by PGE_2 but not that produced by ATP.

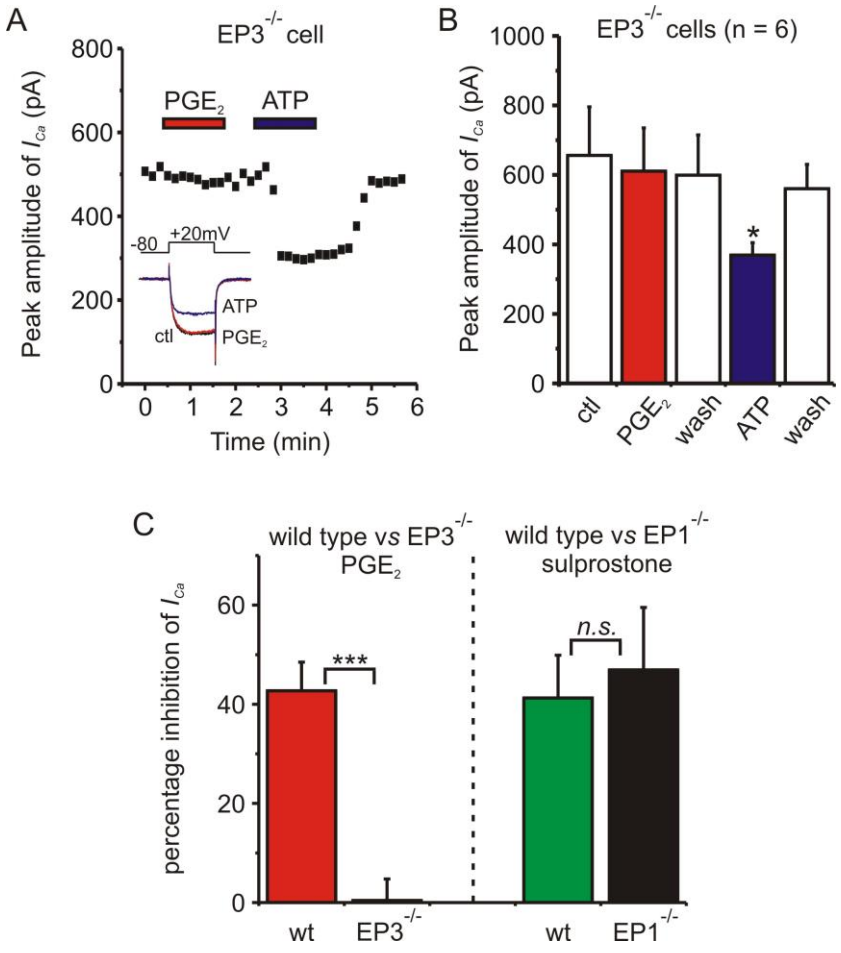

Figure 15: The inhibition of I_{Ca} produced by PGE_2 was abolished in cells isolated from EP3 receptor knockout mice. (A) Experimental time course plotting peak amplitude of I_{Ca} vs. time from a representative cell isolated from an EP3 receptor knockout mouse ($EP3^{-/-}$ cells). I_{Ca} was recorded in the conventional whole-cell configuration and elicited every 10 s with a 20 ms step-depolarization from -80 mV to +20 mV. The cell was exposed first to 100 nM PGE_2 and subsequently to 100 μM ATP (to activate P2Y receptors) as indicated by the horizontal bars. PGE_2 had no effect on I_{Ca} recorded from $EP3^{-/-}$ chromaffin cells whereas the inhibition produced by P2Y receptors remained intact. The inset shows three superimposed currents recorded before application of PGE_2 (ctl), during application of PGE_2 and during application of ATP. **(B)** Bar chart plotting the effects of PGE_2 and ATP on the mean peak amplitude of I_{Ca} in $EP3^{-/-}$ chromaffin cells (* $p < 0.05$; n = 6). **(C)** Data obtained from wild type and EP receptor knockout mice using perforated whole-cell recording. *Left panel:* mean percent inhibition of I_{Ca} produced by PGE_2 in cells isolated from wild type (wt) (n = 15) vs. EP3 receptor knockout mice ($EP3^{-/-}$) (n = 6) (*** $p < 0.001$). *Right panel:* percent inhibition of I_{Ca} produced by sulprostone (an EP1/EP3 selective agonist) in cells isolated from wild type mice (wt) (n = 6) vs. EP1 receptor knockout mice ($EP1^{-/-}$) (n = 4). (Wild type data is from the same cells shown in Figure 14B).

conditions any effect of sulprostone can be attributed to EP3 receptor signaling as the EP1 receptors are absent. Sulprostone inhibited I_{Ca} in these $EP1^{-/-}$ chromaffin cells by 47 ± 12 % (n = 4), an effect that was not significantly different from that seen in chromaffin cells from wild type mice (41 ± 9 %; n = 6). Taken together, our data using pharmacological approaches and knockout mice demonstrate the inhibition of I_{Ca} by PGE_2 is mediated solely by EP3 receptors.

Effects of PGE_2 on Ca^{2+}-dependent exocytosis

Ca^{2+} influx through voltage-gated calcium channels is the primary trigger for fusion of large dense core vesicles with the plasma membrane (i.e. Ca^{2+}-dependent exocytosis). Inhibition of I_{Ca} is thought to an important mechanism that controls neurosecretion and a number of GPCRs inhibit I_{Ca} and exocytosis in parallel in adrenal chromaffin cells (Currie, 2010b; Garcia et al., 2006). Membrane capacitance precisely reflects the surface area of a cell and transiently increases when secretory vesicles fuse with the plasma membrane. The magnitude of this increase (ΔCm) reflects the number of vesicles that have undergone exocytosis. We used perforated whole-cell recordings to measure I_{Ca} and ΔCm evoked by two 100 ms steps from -80mV to +10mV (Figure 16A) in chromaffin cells isolated from wild type mice. As already noted, cells could be separated into two groups based on the response of I_{Ca} to application of PGE_2. In this particular series of experiments PGE_2 (100 nM) inhibited the peak amplitude of I_{Ca} in seven-out-of-twelve cells (group-1) by 41 ± 10% (n = 7; $p < 0.005$) but had no effect in the remaining five cells (group-2) (3 ± 4% decrease; n = 5; p = 0.41) (Figure 16B). Application of 100 nM PGE_2 significantly reduced ΔCm in group-1 (those cells in which I_{Ca} was inhibited) from

153 ± 40 fF to 68 ± 16 fF (n = 7, $p < 0.03$). ΔCm was also significantly smaller during application of PGE_2 in group-2, even though I_{Ca} was not reduced in these cells (Figure 1617C). This might reflect other pathways recruited by PGE_2 to control exocytosis independent of I_{Ca} and/or time-dependent rundown of the exocytotic response, but these possibilities will require further investigation. Notably, the inhibition of ΔCm was significantly greater in group-1 cells (in which I_{Ca} was also reduced) compared to group-2 cells (49 ± 7 %, n = 7 compared to 24 ± 4%, n = 5; $p < 0.02$) (Figure 16C) consistent with the idea that inhibition of Ca^{2+} entry by PGE_2 leads to a parallel inhibition exocytosis as reported for other $G_{i/o}$-coupled GPCRs (Harkins and Fox, 2000; Powell et al., 2000; Ulate et al., 2000).

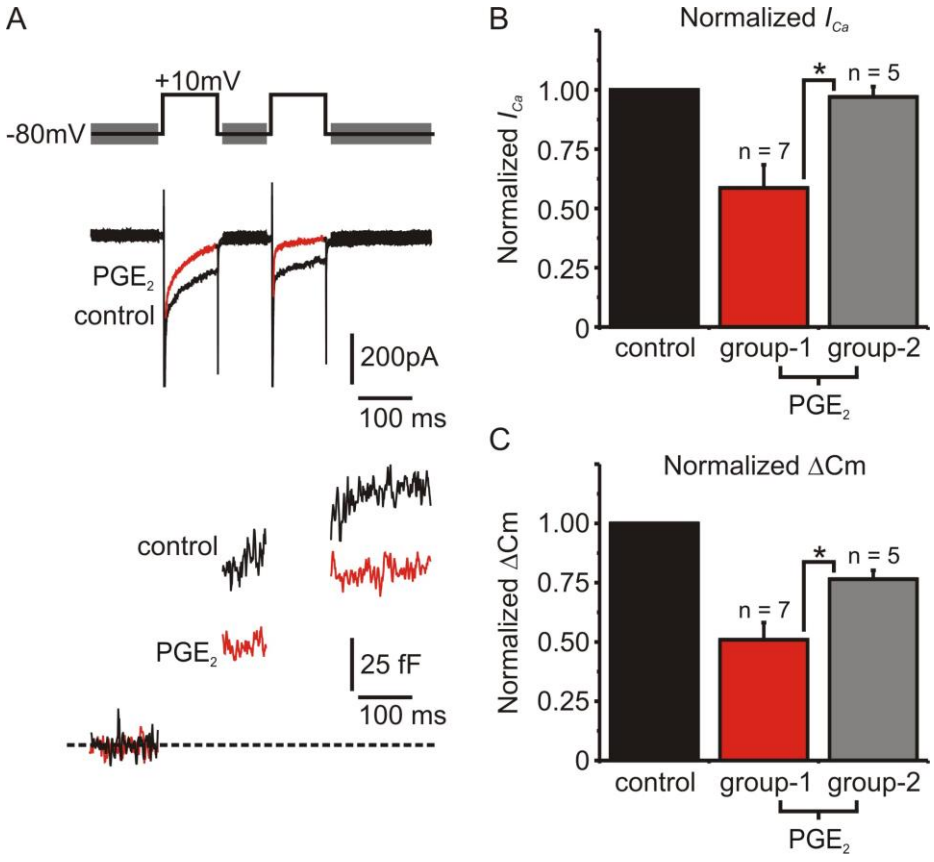

Figure 16: Parallel inhibition of I_{Ca} and Ca^{2+}-dependent exocytosis by PGE_2. Perforated whole- cell recording was used to measure I_{Ca} and membrane capacitance (Cm) in chromaffin cells isolated from wild-type mice. **(A)** Voltage command (top panel), I_{Ca} (middle panel), and membrane capacitance (lower panel) recorded from a representative cell. Two superimposed recordings are shown in the absence (control) and presence of 100 nM PGE_2. The stimulus (top panel) consisted of two step-depolarizations (100 ms duration) from -80 to +10 mV. A 1 kHz sine wave was superimposed on the holding potential to calculate membrane capacitance (see methods for details) and this was interrupted during the step-depolarizations as indicated. **(B)** Peak amplitude of I_{Ca} in the presence of PGE_2 was normalized to control I_{Ca} amplitude in the same cell (open bar- control). Cells were separated into two groups based on the response of I_{Ca} to application of PGE_2: group-1 (black bar; n = 7 / 12 cells) in which PGE_2 significantly reduced the amplitude of I_{Ca}, and group-2 (grey bar; n = 5/12 cells) in which PGE_2 did not inhibit I_{Ca} (* denotes $p < 0.05$ comparing group-1 and group-2 in the presence of PGE_2). **(C)** The change in membrane capacitance (ΔCm) in response to stimulation in the presence of PGE_2 was normalized to ΔCm in control conditions in the same cell. Black bar (group-1) shows data from cells in which I_{Ca} was inhibited (7/12 cells) and the grey bar (group-2) from cells in which I_{Ca} was not inhibited. ΔCm was reduced in both groups but the inhibition was significantly greater in group-1 compared to group-2 (* $p < 0.05$) (i.e. in those cells in which I_{Ca} was also reduced).

Discussion

Previous work has suggested that PGE_2 might modulate adrenal chromaffin cells, although the effects and EP receptors involved remained unclear. The synthetic enzymes for PGE_2 are present in the adrenal medulla, and cholinergic stimulation leads to release of prostaglandins from the intact adrenal gland (Ramwell et al., 1966). Furthermore, a recent *in vivo* analysis showed that systemic immune challenge or circulating cytokines rapidly recruited dendritic cells and macrophages to the adrenal gland and increased the expression of COX-2 and PGE synthase (Engstrom et al., 2008). Thus, periods of inflammation or stress might boost production of PGE_2 within the adrenal gland. We previously reported that PGE_2 inhibited I_{Ca} in bovine chromaffin cells, although the receptor(s) and detailed mechanisms were not determined (Currie et al., 2000). In contrast, others reported that PGE_2 stimulated calcium influx (Mochizuki-Oda et al., 1991) or released calcium from a ryanodine sensitive intracellular store, an effect attributed to EP1 receptors (Shibuya et al., 1999). It has also been reported that PGE_2 inhibited nicotinic ACh receptors in sympathetic neurons (Tan et al., 1998). To our knowledge this has not been tested in chromaffin cells, but if it were to occur it could reduce cholinergic excitation / membrane depolarization and thereby opening of voltage-gated calcium channels.

In the current chapter we report that PGE_2 inhibits I_{Ca} in mouse chromaffin cells through pertussis toxin-sensitive G proteins. The inhibition was voltage-dependent (reversed by strong membrane depolarization) and preferentially targeted Ca_V2 calcium channels (N-

and P/Q-type channels). Thus, PGE_2 mimicked agonists of other $G_{i/o}$-coupled GPCRs including P2Y receptors that inhibit I_{Ca} in chromaffin cells (Albillos et al., 1996; Currie and Fox, 1996; Powell et al., 2000). The inhibition by PGE_2 bore all the hallmarks of that mediated by Gβγ subunit binding to the calcium channels (for reviews see (Currie, 2010a)). To unequivocally identify the EP receptor subtype(s) involved we used cells isolated from knockout mice that lack either the EP3 or EP1 receptors. To complement this genetic approach we used selective pharmacological tools including a recently described EP3 receptor antagonist, DG-041 (Heptinstall et al., 2008). As this compound was not readily available it was made in the Vanderbilt Institute for Chemical Biology Chemical Synthesis Core. Our data provide conclusive evidence that EP3 receptors mediated the inhibition of I_{Ca} by PGE_2 in chromaffin cells.

It should be noted that PGE_2 inhibited I_{Ca} in approximately three-quarters of cells tested, presumably reflecting expression of the EP3 receptor in this subpopulation of cells. In the rodent adrenal medulla 70-80% of chromaffin cells express phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts norepinephrine to epinephrine, so are termed "adrenergic" (Verhofstad et al., 1985). The remainder lack PNMT and are termed "noradrenergic". There is evidence for differential expression of GPCRs in adrenergic vs. noradrenergic cells (Renshaw et al., 2000), so it is interesting to speculate that EP3 receptor expression might be limited to the adrenergic cells and preferentially modulate epinephrine release. Further work will be required to determine if this is the case. It is also noteworthy that the EP3 receptor undergoes alternative splicing, leading to sequence diversity in the cytoplasmic C-terminus (Breyer et al., 2001)). In recombinant

systems all the splice variants couple to $G_{i/o}$-type G proteins, but can also couple differentially to other effectors including G_s, and G_{12}-RhoA. Because extracellular ligand binding is not altered, pharmacological distinction of the EP3 splice variants is not possible. Four alternatively spliced variants of the EP3 receptor have been identified in a bovine chromaffin cell library (Namba et al., 1993), and we detected mRNA for all three mouse splice variants ($EP3_\alpha$, $EP3_\beta$ and $EP3_\gamma$) in the adrenal gland using RT-PCR (Figure 13). However, a more detailed molecular analysis will be required to determine which splice variants are expressed in chromaffin cells and if additional non-$G_{i/o}$-coupled signaling pathways are recruited.

In contrast to some previous reports (Mochizuki-Oda et al., 1991; Shibuya et al., 1999), we found no evidence that PGE_2 can directly elevate $[Ca^{2+}]_i$ (Figure 12C). PGE_2 had no effect on the holding current needed to voltage-clamp cells at -80 mV (i.e. did not open / close any channels) (Figure 12A), and had no effect on resting $[Ca^{2+}]_i$ in Fura-2 loaded cells (Figure 12C). It is possible there are species differences in the expression of EP receptor subtypes as none of the previous studies used mice. We also found that PGE_2 had no effect on the peak amplitude of whole-cell nicotinic ACh receptor currents evoked by bath application of carbachol for 45 s (Figure 12A, B). However, we did note that there was a modest increase in current decay during the sustained application of carbachol in the presence of PGE_2 ($21 \pm 3\%$; $p < 0.01$ compared to $9 \pm 2\%$). Although "non-desensitizing" $\alpha3\beta4$* containing channels predominate in chromaffin cells, several other nicotinic receptor subunits are expressed in a species dependent manner, including the rapidly desensitizing $\alpha7$ subunit (Lopez et al., 1998; Sala et al., 2008). In chick

sympathetic neurons PGE_2 inhibited the whole-cell nicotinic current, but closer analysis revealed opposing effects on different channel subtypes. In particular, the dominant 36pS channel was inhibited, but a 23 pS channel likely mediated by α7 containing receptors was potentiated by PGE_2 (Du and Role, 2001). Further studies using fast, brief agonist applications will be needed to fully address the effects of PGE_2 on nicotinic receptors, but our data do suggest that the predominant non-desensitizing nAChR current, (α3β4* receptors) is likely not a major target for PGE_2 modulation of chromaffin cell function.

Previous studies investigating the effects of PGE_2 on catecholamine secretion have all used large populations of cultured chromaffin cells or intact adrenal gland preparations and present somewhat inconclusive findings. In some cases PGE_2 inhibited release (Karaplis et al., 1989), while others report that PGE_2 potentiated release (Marley et al., 1988; Yamada et al., 1988; Yokohama et al., 1988). As discussed above, high concentrations of PGE_2 used in some of these studies raise the possibility of non-EP receptor involvement. Our data demonstrate for the first time that EP3 receptors utilize the same mechanism as P2Y, α2-adrenergic, and μ-opioid receptors to inhibit voltage-gated calcium channels in chromaffin cells. Typically such inhibition of I_{Ca} by GPCRs is paralleled by an inhibition of Ca^{2+}-dependent exocytosis, the mechanism that underlies vesicular catecholamine release (Harkins and Fox, 2000; Powell et al., 2000; Ulate et al., 2000). Exocytosis can be monitored in individual cells by tracking changes in membrane capacitance (ΔCm) that precisely reflect the surface area of a cell. The magnitude of ΔCm reflects the number of vesicles that have undergone exocytosis. As previously reported for other GPCRs application of PGE_2 led to a robust inhibition of ΔCm (49 ± 7%) that

paralleled inhibition of I_{Ca} (41 ± 10%) (Figure 16). There was also a modest decrease in ΔCm (24 ± 4%) in cells that showed no inhibition of I_{Ca}. This might reflect a time-dependent rundown of the exocytotic response or the possibility that other mechanisms are recruited to control secretion. Gβγ-mediated inhibition of catecholamine release independent from I_{Ca} modulation has been reported for other $G_{i/o}$-coupled receptors (Chen et al., 2005; Yoon et al., 2008). We also detected mRNA for EP1, 2 and 4 receptors in the mouse adrenal gland, in addition to all three splice variants of the EP3 receptor (Figure 13). Further detailed investigations will be required to determine if these receptors are expressed in chromaffin cells and what functional impact they might have. However, our data clearly show that the inhibition of ΔCm was significantly greater when I_{Ca} was also reduced (49 ± 7 %, n = 7 compared to 24 ± 4%, n = 5; $p < 0.02$) (Figure 16C) supporting the idea that inhibition of Ca^{2+} entry by PGE_2 leads to a parallel inhibition exocytosis as reported for other $G_{i/o}$-coupled GPCRs (Harkins and Fox, 2000; Powell et al., 2000; Ulate et al., 2000).

To summarize, there is growing interest in developing subtype selective EP receptor drugs as therapeutic agents for a variety of disorders, so identitying the physiological roles distinct receptors play will be important for interpreting and predicting the impact of these drugs. We have used a powerful combination of pharmacology and cells isolated from receptor knockout mice to demonstrate that prostaglandin EP3 receptors inhibit I_{Ca} in adrenal chromaffin cells and that this results in a parallel inhibition of Ca^{2+}-dependent exocytosis. To our knowledge this is the first study of EP receptor signaling in mouse

chromaffin cells and identifies a cellular / molecular mechanism for paracrine regulation of neuroendocrine function by PGE_2.

4. POTENTIATION OF ADRENAL CATECHOLAMINE RELEASE BY PGE_2: A NOVEL ROLE FOR "INHIBITORY" G PROTEINS?

Abstract

PGE_2 is the most widely expressed prostanoid, exerting its functional effects in an autocrine or paracrine manner. The net functional effect of PGE_2 may depend on the relative expression of specific EP receptors. We have shown previously that PGE_2 functions through the $G_{i/o}$-coupled EP3 receptor to inhibit I_{Ca} and exocytosis evoked by short step depolarizations. To define the physiological impact of PGE_2 on adrenal catecholamine release during sustained stress depolarization, we employ two electrical stimulus models: action potential waveforms (APW) delivered at 15 Hz, whereby cells are voltage clamped and I_{Ca} is quantified, or chemical depolarization with a 30 mM KCl containing extracellular solution, while evoked quantal catecholamine release is measured amperometrically. We show the inhibitory contribution of PGE_2 on I_{Ca} is negligible as Ca^{2+} channels inactivate, revealing a PGE_2 mediated potentiation of the secretory response during a sustained depolarization that mimics acute stress. The potentiation resulted from a significant increase in number of vesicles fusing, with little increase in single vesicle content, and was abolished by pertussis toxin treatment, consistent with a $G_{i/o}$–coupled G protein signaling pathway. Additionally, treatment with two different Gβγ inhibitors (gallein or phosducin-like C terminus peptide) blocked the inhibition. Furthermore, we show the potentiation is prevented by the selective EP3

inhibitor DG-041 or in chromaffin cells isolated from $EP3^{-/-}$ mice. Thus during brief stimuli that mimic basal sympathetic tone, EP3 receptors suppress exocytosis by mediating voltage dependent inhibition of I_{Ca}. During sustained stress depolarization, EP3 receptors potentiate catecholamine release by a non-canonical mechanism that involves Gβγ subunits from $G_{i/o}$ -coupled receptors. Taken together, our data reveal a novel molecular framework for context-dependent modulation of catecholamine secretion by the inflammatory mediator PGE_2, and identify a novel signaling pathway through which "inhibitory" G-proteins can potentiate neuroendocrine hormone secretion.

Introduction

Chromaffin cells of the adrenal medulla release epinephrine, norepinephrine, and a cocktail of bioactive peptides that modulate hemodynamics and metabolism in response to physiological stress. ACh released onto chromaffin cells by preganglionic sympathetic afferents activates nicotinic ACh receptors, triggering membrane depolarization and opening of voltage-gated Ca^{2+} channels (Boarder et al., 1987; Ehrhart-Bornstein et al., 1998). Ca^{2+} entry induces a conformational change in the SNARE complex that ultimately triggers exocytosis (Catterall and Few, 2008), thus regulation of intracellular Ca^{2+} homeostasis is crucial for appropriate tuning of adrenomedullar outflow.

Intra-adrenal autocrine/paracrine signaling is coordinated by cell-surface G protein coupled receptors (GPCRs) that regulate chromaffin cell activity. Our group and others have shown that voltage-gated Ca^{2+} channels and exocytosis evoked by short step-

depolarizations or low frequency trains of depolarizations are inhibited in parallel by activation of auto-inhibitory receptors for ATP (P2Y-receptors), enkephalin (μ-opioid receptors) or catecholamines (α-adrenergic) (Albillos et al., 1996; Currie and Fox, 1996; Harkins and Fox, 2000; Powell et al., 2000; Ulate et al., 2000). This type of inhibition is attributed to direct binding of activated Gβγ subunits to an intracellular binding pocket on the α_1 subunit of the channel, and mediated almost entirely by $G_{i/o}$ -coupled G proteins (Ikeda, 1996; Zamponi and Currie, 2013). A strong depolarizing prepulse reverses the inhibition (facilitation) by transiently dissociating Gβγ from the channel, thus the inhibition is referred to as 'voltage-dependent' (Currie and Fox, 1996). In addition to facilitation by a conditioning prepulse, the inhibition mediated by ATP or enkephalin is relieved with sustained chemical depolarization (Ulate et al., 2000) or trains of action potential-like waveforms (APW) (Currie and Fox, 2002; Womack and McCleskey, 1995). As such, activation of $G_{i/o}$ -coupled GPCRs during sustained depolarization may have minimal physiological impact.

Conversely, activation of PLC-coupled receptors, with endogenous ligands histamine or bradykinin, potentiates catecholamine release evoked by step-depolarizations or sustained chemical depolarization in bovine chromaffin cells (Bauer et al., 2007; Marom et al., 2011). G_q -coupled GPCRs activate PLCβ, mobilizing inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate PIP_2. IP3 raises $[Ca^{2+}]_i$ by releasing Ca^{2+} from internal stores which may activate PKC and potentiate release on its own during depolarizing trains (Smith, 1999). DAG is a well-known modulator of synaptic plasticity in all types of synapses (Zucker and Regehr, 2002), and

acts in conjunction with Ca^{2+} to enhance presynaptic release by a PKC dependent pathway (Barclay et al., 2003; Wierda et al., 2007) or by direct activation of the vesicle priming protein Munc-13 (Rhee et al., 2002). These stimulatory pathways downstream of PLC activation are conserved in chromaffin cells and both Munc13 and PKC are likely involved in potentiation of exocytosis (Bauer et al., 2007; Gillis et al., 1996).

Sympathetic stress at the splanchnic-adrenal synapse increases the rate of acetylcholine output as well as the neuropeptide co-transmitter PACAP, resulting in sustained depolarization and secretion of catecholamine from chromaffin cells (Smith and Eiden, 2012). Similarly, systemic inflammation modeled by the injection of LPS increases local expression of Cox-2 and mPGES1, likely resulting in PGE_2 production (Engstrom et al., 2008). The cellular consequences of PGE_2 action are primarily mediated by binding four cognate GPCRs (EP1-EP4) with the net functional effect reflecting a balance of stimulatory and inhibitory receptors. We have shown previously by using genetic and pharmacological tools in isolated mouse adrenal chromaffin cells that PGE_2 functions through the EP3 receptor to inhibit I_{Ca} and exocytosis evoked by short action potentials. The inhibition was pertussis-toxin sensitive and reversed by a strong depolarizing prepulse, suggestive of direct $G_{i/o}$ -coupled Gβγ subunit binding to the channel (Jewell et al., 2011). Thus, PGE_2 uses the same mechanistic framework as $G_{i/o}$ -coupled autoreceptors for ATP and enkephalin.

Previous reports suggest strong expression of EP1 and EP3 receptors in adrenal medulla (Breyer et al., 1993; Engstrom et al., 2008; Shibuya et al., 1999) and we have identified

mRNA for all four EP receptor subtypes (EP1-EP4) in adrenal tissue. The goal of this study is to define the action of PGE_2 on chromaffin cells during sustained stress depolarization. Given the diverse expression of EP receptor subtypes on chromaffin cells, and relief of $G_{i/o}$ -mediated inhibition during sustained depolarizations, we hypothesize that the net functional impact of PGE_2 during a strong stimulation will depend on the recruitment of distinct signaling pathways, and will reveal functional diversity reliant on cellular context.

Results

Inhibition of I_{Ca} by PGE_2 does not persist during sustained depolarization

To define the physiological impact of PGE_2 on adrenal catecholamine release during sustained stress depolarization, we employ two electrical stimulus models: action potential waveforms (APW) delivered at 15 Hz, whereby cells are voltage clamped and I_{Ca} is quantified, or chemical depolarization with a 30 mM KCl containing extracellular solution, while evoked quantal catecholamine release is measured amperometrically. We have shown previously that PGE_2 inhibits I_{Ca} and exocytosis evoked by short step-depolarizations. The effect was pertussis toxin sensitive and reversed by a strongly depolarizing voltage step; characteristic of direct G protein βγ subunit binding to the channel ((Jewell et al., 2011)/Chapter 3). Generally voltage-dependent inhibition targets Ca_V2 family members $Ca_V2.1$ (P/Q- type) and $Ca_V2.2$ (N-type), and we showed previously that the inhibition by PGE_2 preferentially targets N- and P/Q- type Ca^{2+} channels over L-type channels in both mouse and bovine chromaffin cells, although the

relative contribution of each channel subtype differs between species (Currie and Fox, 2002; Jewell et al., 2011). The physiological impact of $G_{i/o}$–coupled receptor activation during longer depolarizations is unclear as the inhibition of both N- and P/Q- type calcium channels can be reversed during trains of action potentials (Currie and Fox, 2002; Womack and McCleskey, 1995) and cumulative inactivation is reduced (McDavid and Currie, 2006). As a consequence, inhibition of I_{Ca} became negligible during sustained stimulus trains (McDavid and Currie, 2006).

To determine the effect of PGE_2 on I_{Ca} during sustained stimulation, cells were patch clamped and stimulated with 15 Hz trains of APW as described previously (Currie and Fox, 2002). Briefly, action potentials were stimulated in chromaffin cells by short current injections and recorded in the standard whole- cell configuration. A typical action potential was used as a template for generating APW commands used in voltage clamp. The duration of the APW at half maximal amplitude was 4 ms, and the holding and peak potential were adjusted to -80 mV and +45 mV, respectively (Figure 17A). For the experiment, cells were voltage clamped in the perforated whole-cell recording configuration and I_{Ca} was evoked with two trains of APW (15 Hz for 90 s) separated by an intervening period of 210 s (Figure 17B, middle panel). Application of PGE_2 or vehicle control occurred in the intervening window, 120 s prior to S2 (Figure 17B, top panel). Individual currents (1350 total, 15/sec, 90 sec total) in S1 and S2 were normalized to the first current in the S1 train. The amplitude of I_{Ca} showed a rapid decline during the first ~6 seconds of the S1 train (Figure 17C, D, black trace), reaching a stable plateau at ~ 30% of maximum that was maintained for the remainder of the train**.** In control cells, I_{Ca}

evoked by the second train (S2) was almost identical to S1, decaying at a similar rate and reaching a similar plateau (Figure 17C**)** (amplitude of I_{Ca} at end of S1 train = 178 ± 20 pA n=4 vs. end of S2 train = 198 ± 21 pA n=3). In the presence of PGE_2, the amplitude of I_{Ca} during S2 was smaller than that of S1 for only the first ~5s (Figure 17D, inset) after which the currents reached a sustained plateau of similar amplitude (amplitude of I_{Ca} at the end of S1 = 208 ± 65 pA, n=3 vs end of S2 = 211 ± 86 pA n=3). Previous reports suggest trains of APW may potentiate catecholamine release by Ca^{2+} dependent PKC activation (Smith, 1999), or by altering the relative coupling of Ca_V1 current (L-type) to catecholamine release (Polo-Parada et al., 2006). We don't specifically dissect the Ca^{2+} channel subtype contribution to the currents or the exocytotic response, but the primary difference between the S2 segment in CTL and PGE_2 treated cells is for the first ~6-8 seconds, indicating PGE_2 is ineffective at regulating whole-cell current during sustained stimulation.

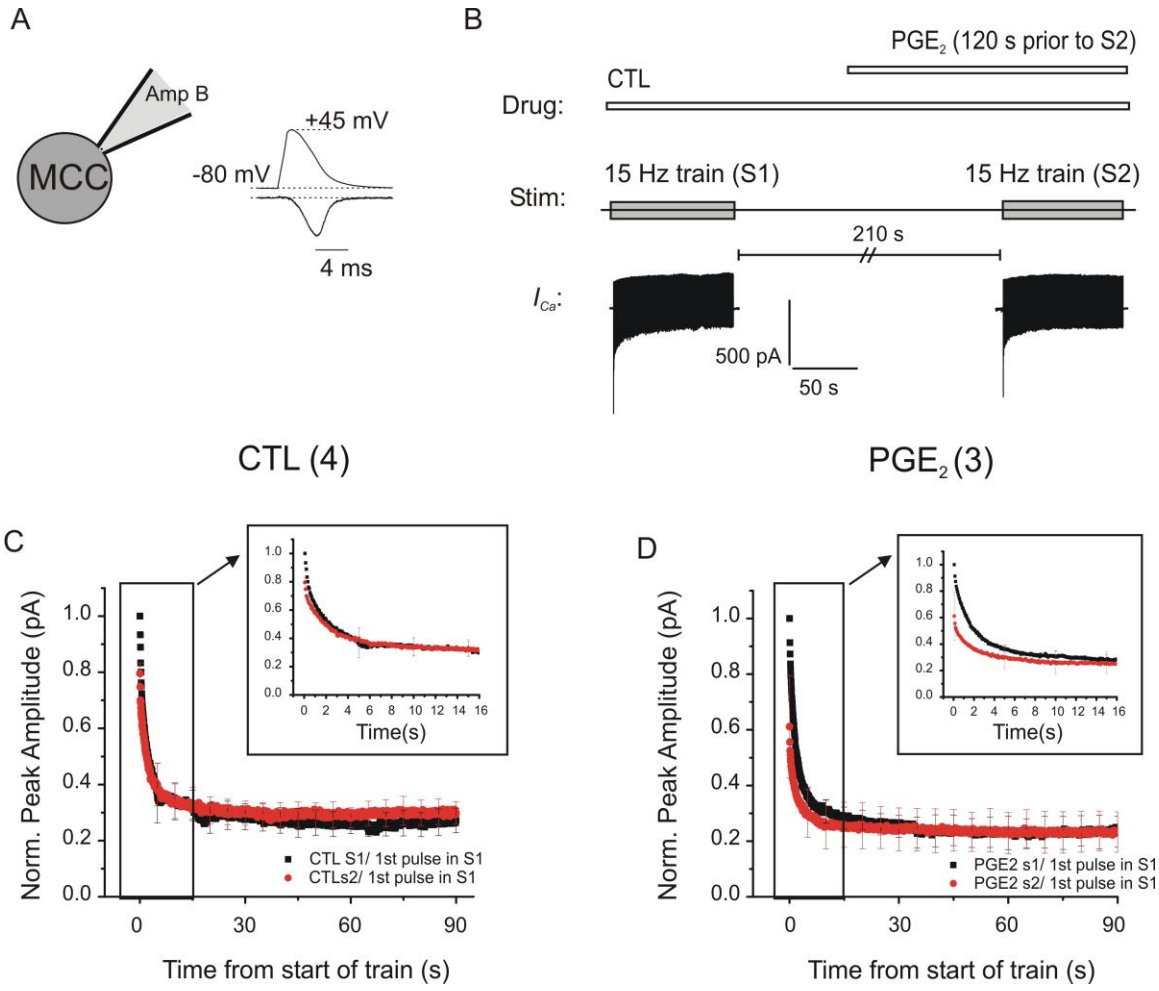

Figure 17: Inhibition of I_{Ca} by PGE_2 does not persist during sustained depolarization: (A) Chromaffin cells were recorded in the amphotericin B perforated whole-cell recording configuration (left) and stimulated with trains of APW. The whole cell I_{Ca} elicited by a single APW is shown **(**right). **(B)** Cells were stimulated with two trains of APW (15Hz of 90 s; S1 and S2-middle) separated by a 210 s recovery window. The schematic indicates timing of drug application (top), stimulation paradigm (middle) and a representative current recording for a control cell (bottom). **(C)** Individual currents in S1 and S2 were normalized to the first current in the S1 train and plotted over each 90 s stimulation window. The amplitude of I_{Ca} was very similar during both S1 and S2 trains. It declined rapidly during the first ~6-8 seconds of stimulation and reached a stable plateau (~30% of maximum) that was maintained for the duration of the train. The inset shows the first 16 s of stimulation on an expanded time frame **(D).** In the presence of PGE_2, the amplitude of I_{Ca} during S2 was smaller than that of S1 for only the first ~5 s (see inset) after which the currents reached a sustained plateau of similar amplitude. In both CTL (panel C) and PGE_2 treated cells (panel D), the amplitudes of I_{Ca} evoked by the last APW of the trains were not significantly different. Thus the effect of PGE_2 on I_{Ca} amplitude only persists for a brief 6-8 second window at the start of the APW train.

PGE_2 potentiates exocytosis during cellular conditions that mimic stress firing

Chromaffin cells constitutively express synthetic enzymes for PGE_2, however in an animal model of systemic inflammation, expression of Cox-2 and mPGES are enhanced, suggesting increased production of adrenal PGE_2 consequent to a physiological stress response (Engstrom et al., 2008). We have detected mRNA for all four EP receptor subtypes (EP1-EP4 Figure 13). So while the net functional impact of PGE_2 during brief depolarizations may solely rely on EP3, PGE_2 may recruit additional cellular architecture during sustained depolarization that ultimately represents a shift in the balance of various EP subtypes. To assay the effect of PGE_2 on sustained depolarizations, we used carbon fiber amperometry as described in MATERIALS AND METHODS. Figure 18A depicts the experimental setup. Briefly, a carbon fiber held at a positive potential was positioned next to individual chromaffin cells, the cells were depolarized with 30 mM KCl for two 90-second stimulation periods (S1 and S2) separated by an inter-stimulation interval of 210 seconds. KCl was used to avoid dialyzing the cell, thus serves as a non-invasive model of stress stimulation. Catecholamines released in the vicinity of the fiber are oxidized and exocytotic activity is recorded as amperometric current 'spikes' (Figure 18A inset**)**, each individual spike representing the catecholamine content from a single vesicle fusing with the membrane. Cells were either controls (treated with vehicle before and during S2) or treated with 100 nM PGE_2. This paired experimental design means that each cell serves as its own control by comparing secretion evoked during S2 to that evoked during S1. This helps account for the well-documented variability of secretory responses detected by amperometry. Furthermore, normalizing the data within each cell (the S2/S1 ratio) reports the "change in secretion" and permits comparison across

different experimental preparations and treatment groups. With this design the duration of the inter-stimulation interval is vitally important to ensure recovery between S1 and S2, and as shown in Figure 18 the S2/S1 ratio in control cells was close to 1.

The secretory response was quantified in a number of ways. First, to obtain a measure of the overall amount of catecholamine secretion, we calculated the total charge (sum of AUC) of all the amperometric spikes evoked during S1 or S2. In control cells the total charge in S2 was slightly reduced compared to S1 but this was not statistically significant (92.9 ± 24.5 pC Vs. 79.8 ± 20.8 pC; n=11; $p = 0.2$) (Figure 18B). In contrast, treatment with PGE_2 prior to the S2 stimulation significantly potentiated catecholamine secretion from 65 ± 26.7 pC during S1 to 113.6 ± 36.6 pC during S2 (n=7; $p < 0.02$) (Figure 18C). We also compared the S2/S1 ratio for PGE_2 treated cells and control cells. In PGE_2 treated cells there was a nearly 2.5 fold potentiation of catecholamine release whereas there was virtually no change in secretion in control cells (Figure 18D) (S2/S1 ratio for PGE_2 was 2.45 ± 0.51, n=7 compared to 0.94 ± 0.8, n=11 for controls; $p<0.005$). The potentiation of total catecholamine secretion could reflect more vesicles fusing with the plasma membrane, a larger quantal content of individual vesicles, or a combination of both these effects. As shown in Figure 18E, we found that the number of secretory events (amperometric spikes) was significantly potentiated by PGE_2 compared to controls (S2/S1 ratio = 0.88 ± 0.07, n=11 for controls and 1.67 ± 0.26, n=6 for PGE_2 treated cells; $p < 0.005$). We also calculated the median charge of the individual amperometric spikes which is proportional to the amount of catecholamine released from each vesicle.

Although there was a small increase in spike charge during the S2 train, this was not significantly different in PGE_2 treated cells compared to control cells (Figure 18F).

To summarize, PGE_2 did not elicit secretion directly, but significantly potentiated evoked catecholamine release during a sustained stress depolarization. The potentiation is manifest by more vesicles fusing with the plasma membrane, rather than larger vesicles.

The PLC inhibitor U73122 and its unreactive analogue U73343 blocks stimulus-secretion coupling

The EP1 receptor is highly expressed in the adrenal medulla (Breyer et al., 1993; Engstrom et al., 2008). EP1 $G_q\alpha$ subunits activate PLCβ, mobilizing inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2). IP3 raises $[Ca^{2+}]_i$ by releasing Ca^{2+} from internal stores and DAG enhances exocytosis by binding to several cellular effectors (Barclay et al., 2003; Rhee et al., 2002; Wierda et al., 2007; Zucker and Regehr, 2002). It is well established that PLCβ activation increases the size of the readily releasable pool of secretory vesicles (RRP) (Bauer et al., 2007; Gillis et al., 1996), resulting in a potentiation in the number of secretory events, but not the size of individual events. Because the functional consequences of PLCβ activation were consistent with our results in Figure 18, we predicted PLCβ was a reasonable downstream mediator of the potentiation. We tested the widely used PLC inhibitor U73122 and its inactive analogue U73343.

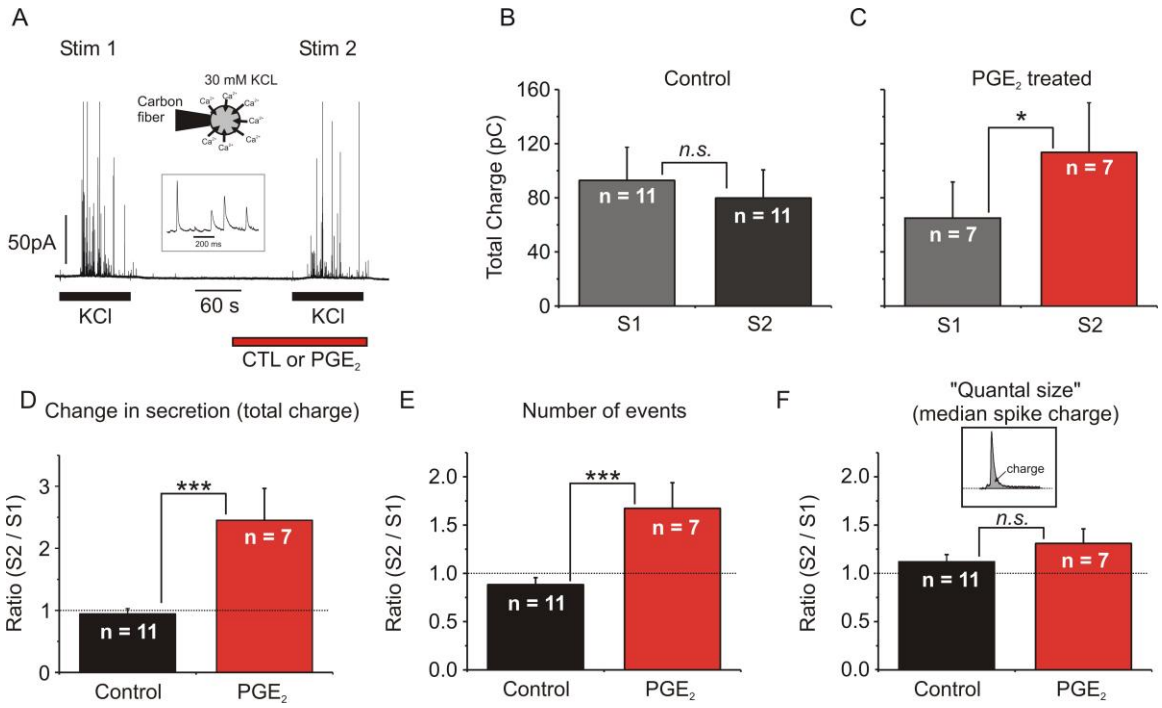

Figure 18: PGE_2 potentiates exocytosis during cellular conditions that mimic stress firing. (A) Representative amperometric current from a control cell showing experimental setup (cartoon inset) and timing of S1 and S2 KCl depolarizations (90 s each) in relationship to PGE_2 application (120 s). **(B, C)** Total amperometric charge (the sum of all spikes) during each stimulation period (total area under the curve for S1 and S2) was calculated and is reported as mean ± SE. In control cells evoked secretion was similar in both rounds of stimulation and the total charge during S2 was not significantly different from S1 (79.8 ± 20.8 pC vs 92.9 ± 24.5 pC, n=11; p = 0.2; paired t-test; **(B)**). Treating cells with PGE_2 prior to the S2 stimulation significantly potentiated catecholamine secretion (total during S2 = 113.6 ± 36.6 pC compared to 65 ± 26.7 pC during S1; n=7; p < 0.02; paired t-test; **(C)**). **(D)** The change in secretion in each cell was calculated as the S2/S1 ratio for total amperometric charge. In controls cells secretion showed little change (S2/S1 ratio = 0.94 ± 0.8, n=11), whereas PGE_2 produced a substantial potentiation (2.45 ± 0.51, n=7) (*** p<0.005; unpaired t-test). **(E)** The number of fusion events (amperometric spikes) showed little change in control cells (S2/S1 ratio = 0.88 ± 0.07, n=11) but was significantly potentiated by PGE_2 (S2/S1 ratio = 1.67 ± 0.26, n=6; *** p<0.005, unpaired t-test). **(F)** The "quantal size" or amount of catecholamine released from each vesicular fusion event (median spike charge) was not significantly altered by PGE_2 treatment compared to controls.

U73122 (3 μM) completely abolished the secretory response evoked by KCl in S2 in control cells (Figure 19A) or PGE_2 treated cells (Figure 19B). Surprisingly, the inactive analogue U73343 (3 μM) had the same effect, a complete block of exocytosis not rescued by application of PGE_2. Several groups have reported that U73122 has off target effects and potentially acts as a weak agonist of PLC, opens ion channels and increases intracellular calcium concentration (Horowitz et al., 2005; Mogami et al., 1997), however none of these off target effects would block exocytosis. U73122 is an N-substituted maleimide, like N-ethylmaleimide, and therefore it has been suggested that U73122 inactivates its targets by irreversible alkylation of –SH groups, covalently attaching itself to its target (useful discussion in (Horowitz et al., 2005)). U73343 is structurally identical except for one double bond, thus is unreactive. We show the complete block of exocytosis (Figure 19) is supported by both U73122 and the 'unreactive' U73343, hence may be independent of the capacity for alkylation. The exact mechanism of action warrants further examination, however we discontinued use of U73122 due to significant off target effects.

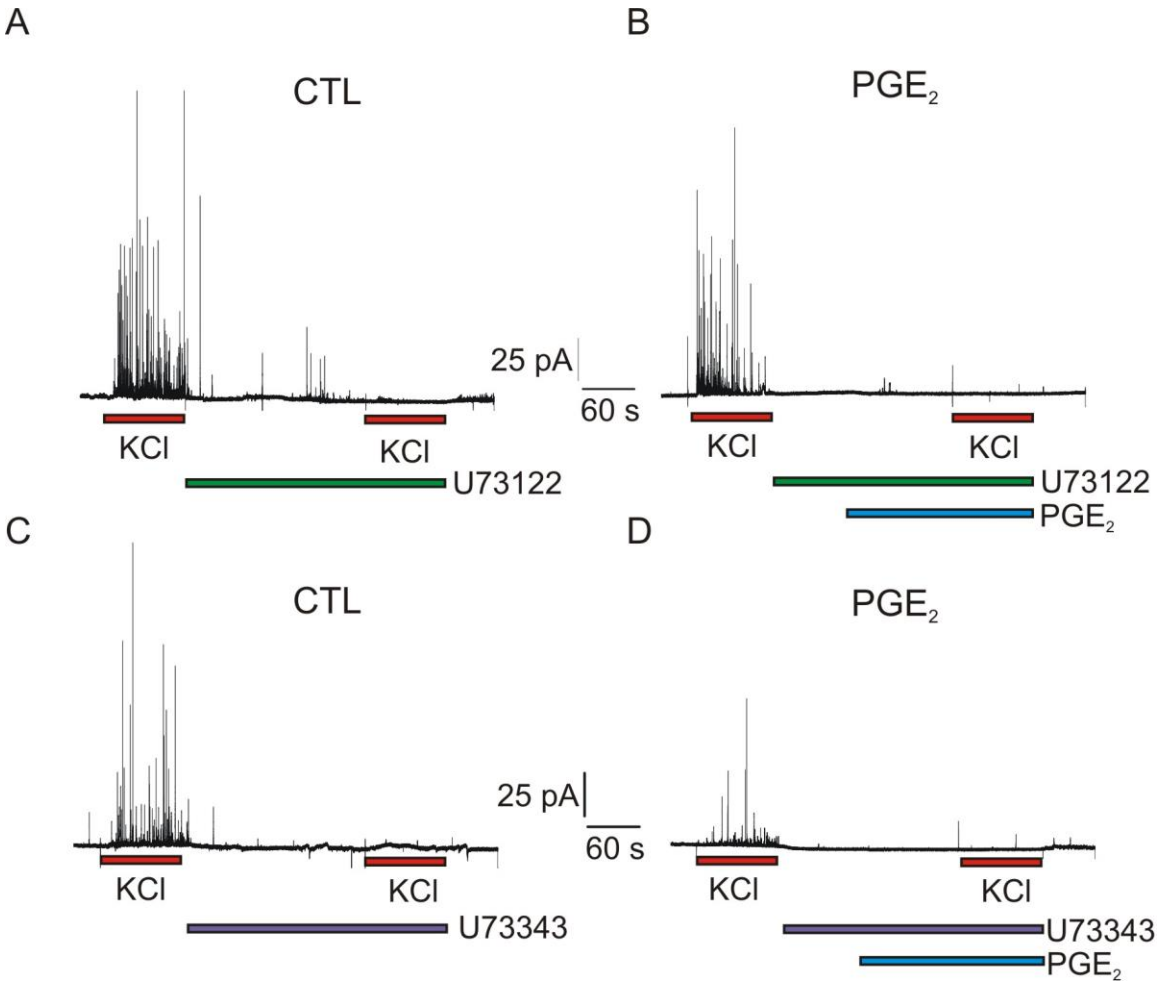

Figure 19: The PLC inhibitor U73122 blocks stimulus-secretion coupling by a non-enzymatic mechanism: Amperometric current traces showing the experimental design and relative timing of drug application in four representative cells. The two consecutive red bars in each trace labeled KCl represent S1 and S2. **(A)** The PLC inhibitor U73122 abolished the secretory response in the KCl evoked S2 stimulation window in control cells. **(B)** Application of PGE_2 was unable to rescue the secretory response in S2. **(C)** The inactive analogue U73343 also blocks the secretory response in both control and PGE_2 treated cells **(D)**.

PGE_2 does not elevate intracellular calcium concentration

Activation of EP1 receptors raises intracellular calcium concentration ($[Ca^{2+}]_i$) (Funk et al., 1993; Hata and Breyer, 2004; Shibuya et al., 1999; Watabe et al., 1993) and stimulates IP production (Ji et al., 2010) consistent with coupling to G_q receptors. PGE_2 does not alter resting $[Ca^{2+}]_i$ (Figure 12) yet may potentiate KCl mediated Ca^{2+} transients which would be expected to potentiate exocytosis. Similarly cAMP may raise $[Ca^{2+}]_i$ by facilitating L-type channels. We used Fura-2 ratiometric imaging to test if PGE_2 potentiates the KCl stimulated rise in $[Ca^{2+}]_i$ over stimulation periods relevant to our amperometry experiments (Figure 20A). Two 90-second KCl applications depolarize the cell and raise $[Ca^{2+}]_i$. A 2-minute bath application of 100 nM PGE_2 prior to S2 did not alter the magnitude of KCl evoked $[Ca^{2+}]_i$ transients (KCl: 388 ± 56 vs KCL/PGE_2 : 376 ± 55, n=16, Figure 20B). PGE_2 application in the inter stimulation window caused a slight dip and recovery in baseline cytosolic Ca^{2+} (Figure 20B) that was not seen in all cells, and not reflected on the cell averages (Figure 20B). While this data is inconsistent with PGE_2 activation of EP1 receptors, we also tested the EP1 selective antagonist SC-51322 using amperometry. SC-51322 (100 nM) was applied after S1, but before PGE_2 application, and remained in the bath for the duration of the experiment. Blocking EP1 receptors pharmacologically did not significantly alter the PGE_2 mediated potentiation (Figure 20C).

We have also detected mRNA for EP2 and EP4 receptors, which typically raise cAMP (Breyer et al., 2001). Several groups have reported cAMP dependent facilitation of I_{Ca}, and cAMP increased unitary quantal size during brief depolarizations. Significant

increases in calcium entry would be reflected in our fura-2 imaging, and we measured no significant change in median quantal size in PGE_2 treated cells (Figure 18F). Also, we previously isolated L-type currents by pharmacological block of N- and P/Q-type channels and found the inhibition by PGE_2 was abolished, suggesting PGE_2 does not signal through cAMP. Taken together these results cast doubt the PGE_2 mediated potentiation of catecholamine release during sustained depolarization is mediated by EP1, EP2 or EP4 receptor activation.

PGE_2 mediated potentiation is pertussis-toxin sensitive and abolished by pharmacological and genetic targeting of the EP3 receptor

We have shown previously that during short step depolarizations, PGE_2 signals through EP3 receptors to produce voltage-dependent inhibition of Ca_V2 currents through activation of pertussis toxin sensitive $G_{i/o}$ –coupled G proteins (Jewell et al., 2011). PGE_2 had no modulatory effect on intracellular calcium concentration in our fura-2 imaging that would be expected downstream of IP3 turnover (EP1) or production of cAMP (EP2/EP4). To determine whether PGE_2 potentiation of the secretory response acts through a $G_{i/o}$ –coupled GPCR, we pre-treated cells with 300 ng/ml pertussis toxin for 24 hours prior to amperometric recording. As shown in Figure 21A, the number of secretory events (amperometric spikes) in pertussis-toxin treated cells was not potentiated by application of PGE_2 (S1 200 ± 39, n=5 vs S2 142 ± 63, n=5). The average number of S1 events was not altered by PTX treatment, and was consistent with S1 in our controls (CTL S1: 206 ± 39, n=11 vs PTX S1: 200 ± 39, n=5).

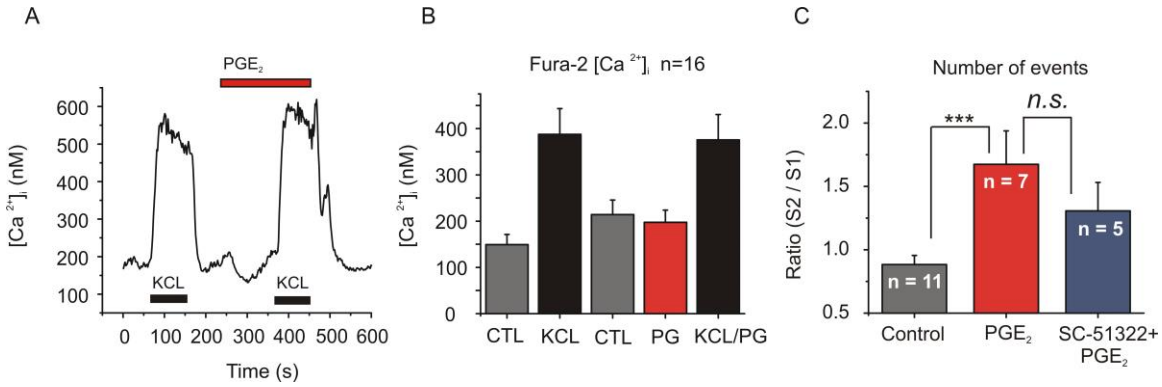

Figure 20: PGE_2 does not elevate intracellular calcium concentration. (A) KCl evoked calcium transients from a representative fura-2 loaded cell. The fluorescent response to KCl was converted to $[Ca^{2+}]_i$ using an in vitro calibration curve, as described in METHODS, and plotted against time. The experimental time course and drug application windows mirrored our amperometry experiments. **(B)** A 2-minute application of 100 nM PGE_2 did not alter the magnitude of KCl evoked $[Ca^{2+}]_i$ transients (KCL: 387.6 ± 55.7, n=16 vs KCL/PGE_2 : 375.7 ± 54.7). **(C)** PGE_2 potentiates the number of fusion events compared to control (S2/S1 ratio: 0.88 ± 0.07, n=11 to 1.67 ± 0.26, n=6; $p<0.005$; unpaired t-test), while pre-application of the selective EP1 inhibitor SC-51322 does not significantly alter the potentiation (1.67 ± 0.26, n=6 to 1.31 ± 0.22, n=5).

This data demonstrates, rather unexpectedly, that PGE_2 mediated potentiation occurs through a $G_{i/o}$-coupled G protein receptor which are thought to typically inhibit secretion.

To confirm these results we use pharmacological and genetic approaches to target the EP3 receptor. We tested the capacity of the selective, non-competitive antagonist of EP3 receptors, DG-041 (reported on in Chapter 3 and elsewhere (Heptinstall et al., 2008)), to block PGE_2 mediated potentiation of the secretory response. DG-041 (30 nM) was applied subsequent to S1 KCL, and ~90 seconds prior to PGE_2 application, and remained on the cells through the S2 stimulation window. DG-041 blocked the potentiation of the S2/S1 ratio by PGE_2 (Figure 21B), and was not significantly different than control (S2/S1 CTL: 0.88 ± 0.07, n=11 vs DG-041 1.06 ± 0.17, n=5). While this data strongly implicates the EP3 receptor, we tested PGE_2 in chromaffin cells isolated from $EP3^{-/-}$ mice. The secretory ratio (S2/S1) of PGE_2 treated $EP3^{-/-}$ cells was not significantly different than CTL. These results unequivocally validate the EP3 receptor as the target for PGE_2 -based enhancement of the secretory response.

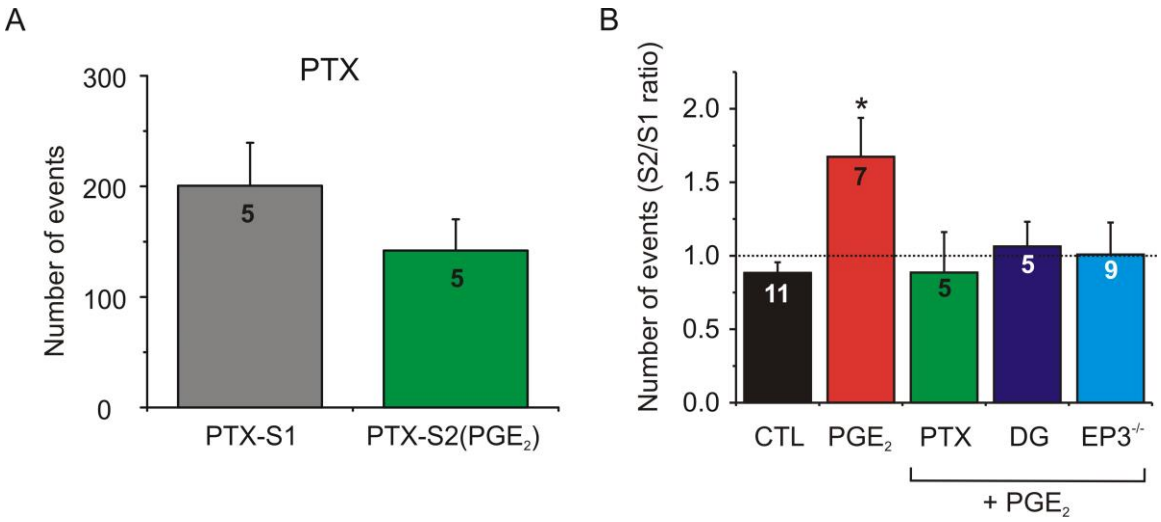

Figure 21: **PGE_2 mediated potentiation is pertussis-toxin sensitive and abolished by pharmacological and genetic targeting of the EP3 receptor. (A)** PGE_2 mediated potentiation was blocked in pertussis-toxin treated cells. The number of secretory events during PGE_2 treatment (S2) is not significantly different than S1 (S1 200 ± 39, n=5 vs S2 142 ± 63, n=5). **(B)** PGE_2 -mediated potentiation of the secretory response is blocked in cells treated with DG-041 or cells isolated from $EP3^{-/-}$ mice (S2/S1: PGE_2 1.67 ± 0.26, n=7; DG-041 1.06 ± 0.17, n=5; $EP3^{-/-}$ 1.01 ± 0.22, n=9; ANOVA p=0.04) and both DG-041treated and $EP3^{-/-}$ cells were not significantly different than control (S2/S1 CTL: 0.88 ± 0.07, n=11 vs DG-041 1.06 ± 0.17, n=5; vs $EP3^{-/-}$ 1.01 ± 0.22; Dunnett's multiple comparison post- test).

PGE_2 mediated potentiation is dependent on Gβγ subunits

Of the four main G protein families, the relative expression of $G_{i/o}$ heterotrimers is relatively high. Activation of $G_{i/o}$-coupled receptors may be the primary means of activating Gβγ mediated signaling processes (Clapham and Neer, 1997; Wettschureck and Offermanns, 2005). Consequently pertussis- toxin treatment, which ADP ribosylates $G\alpha_i$ subunits and prevents receptor interaction and subsequent release of Gβγ, blocks a number of Gβγ subunit regulated process, including N-type calcium channel inhibition (Ikeda, 1996). To test if PGE_2 dependent potentiation is mediated by release of Gβγ subunits, we used the cell-permeant small molecule Gβγ inhibitor gallein. Gallein has been shown to disrupt protein-protein interactions between Gβγ and GRK2 (Casey et al., 2010) and PI3K (Lehmann et al., 2008), in both instances inhibiting Gβγ dependent activation. However the gallein specificity against Gβγ interaction with relevant exocytotic effectors remains unclear.

We initially tested whether 10 μM gallein blocked voltage-dependent inhibition of whole-cell I_{Ca} in chromaffin cells. Cells were voltage clamped at -80 mV and I_{Ca} was evoked by two consecutive 100 ms steps to +10 mV every two minutes (Figure 22A). ATP is known to activate $G_{i/o}$ –coupled P2Y receptors and inhibit I_{Ca} through pertussis toxin sensitive G proteins in bovine chromaffin cells (Currie and Fox, 1996). After two control stimulations, application of 100 μM ATP produced a significant inhibition of I_{Ca} (second control: 434 ± 43 vs first ATP : 197± 18 pA; paired t-test; $p<0.05$; Figure 22B). A 30 minute pre-incubation with 10 μM gallein did not block ATP mediated inhibition of I_{Ca}, and in fact was quantitatively very similar (second control: 422 ± 30 vs first ATP :

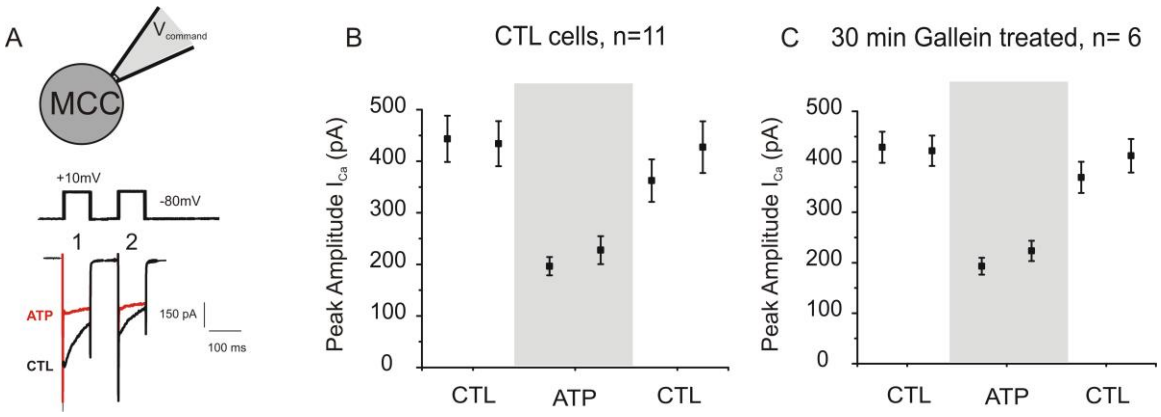

Figure 22: Gallein does not perturb G protein mediated inhibition of I_{Ca} (A) Cartoon depiction of experimental setup (top). The voltage command (middle) consisted of two consecutive 100 ms step-depolarizations from -80 mV to +10 mV(1 and 2). Evoked I_{Ca} in the absence (CTL), and presence, of 100 µM ATP (bottom). Peak amplitude was measured from the first depolarization (1), as I_{Ca} inactivated during the second depolarization (2). **(B)** Peak amplitude plotted against drug application. Cells were stimulated every two minutes, application of ATP subsequent to two control stimulation significantly inhibited peak amplitude of I_{Ca}. **(C)** Pre-treating cells for 30 minutes with Gβγ inhibitor gallein (10 µM) did not block ATP mediated inhibition of I_{Ca}, or have any obvious off-target effects on peak amplitude of controls or recovery from inhibition.

193 ± 17 pA; paired t-test; $p<0.05$; Figure 22C). From these results we conclude gallein does not perturb G protein mediated inhibition of I_{Ca}, or have any remarkable off-target effect on Ca^{2+} flux through voltage-gated Ca^{2+} channels.

To determine if the potentiation of secretion mediated by PGE_2 is a functional effect of Gβγ subunit release from activated EP3 receptors, and subsequent downstream signaling, we used carbon fiber amperometry. Cells were depolarized with 30 mM KCl for two 90-second stimulation periods (S1 and S2), identical to the experimental setup described in Figure 18A. We pre-incubated cells for 15 minutes in two different concentrations of gallein (10 μM and 50 μM). As a complimentary approach we also pre-incubated cells with the membrane permeable, phosducin-like C-terminal peptide (1 μM), which is derived from the inhibitory domain (C-terminal residues 168-195) of the ubiquitous Gβγ inhibitor phosducin-like protein (Chang et al., 2000). We find the PGE_2 mediated potentiation of exocytosis is significantly blocked by pre-treatment with gallein (10 and 50 μM) or phosducin-like C-terminal peptide (1 μM compared to vehicle treated controls (1/5000 DMSO) (Figure 23A,B). Three representative amperometric current traces in Figure 23A show evoked secretion and the timing of PGE_2 application prior to S2. Figure 23B reports the S2/S1 ratios for PGE_2 treatment, showing that both inhibitors significantly reduced PGE_2 potentiation. Notably, in control cells (no PGE_2, lower panel), the evoked secretory spikes in S2/S1 were not significantly altered by pre-treatment with DMSO (vehicle), phosducin-like C-terminal peptide, or 10 μM gallein, however 50 μM gallein reduced spikes evoked in S1 compared to DMSO controls (quantitative data not shown). In conclusion, we have defined a non-canonical regulatory framework by which

$G_{i/o}$ –coupled EP3 receptors activate Gβγ subunits and potentiate the secretory response during depolarizations that mimic acute stress.

Discussion

PGE_2 is the most widely expressed prostanoid, exerting its functional effects in an autocrine or paracrine manner on four GPCRs designated EP1, EP2, EP3 and EP4 (Breyer et al., 2001; Regan, 2003; Sugimoto and Narumiya, 2007). The net functional effect of PGE_2 may depend on the relative expression of specific EP receptors, a useful example being the well-studied impact of PGE_2 on rodent vascular tone. EP1 and EP3 receptors constrict vascular beds by increasing $[Ca^{2+}]_i$ or decreasing cAMP, respectively, while EP2 and EP4 increase cAMP and are known vasodilators. Infusion of PGE_2 decreased mean arterial pressure (MAP) in wild type animals, but the effect was reversed in EP2 $^{-/-}$ animals as MAP increased. Intravenous infusion of EP3 or EP1/ EP3 selective agonists increased MAP, while EP1 selective antagonists lowered MAP in spontaneously hypertensive rats. Ultimately, while the net functional effect of PGE_2 was to lower blood pressure, the vasoconstrictor effect mediated by EP1 and EP3 is "uncovered" by genetic elimination of EP2 (Guan et al., 2007; Zhang et al., 2000). Previous reports suggest strong expression of EP1 and EP3 receptors in adrenal medulla (Breyer et al., 1993; Engstrom et al., 2008; Shibuya et al., 1999) and we have identified mRNA for all four EP

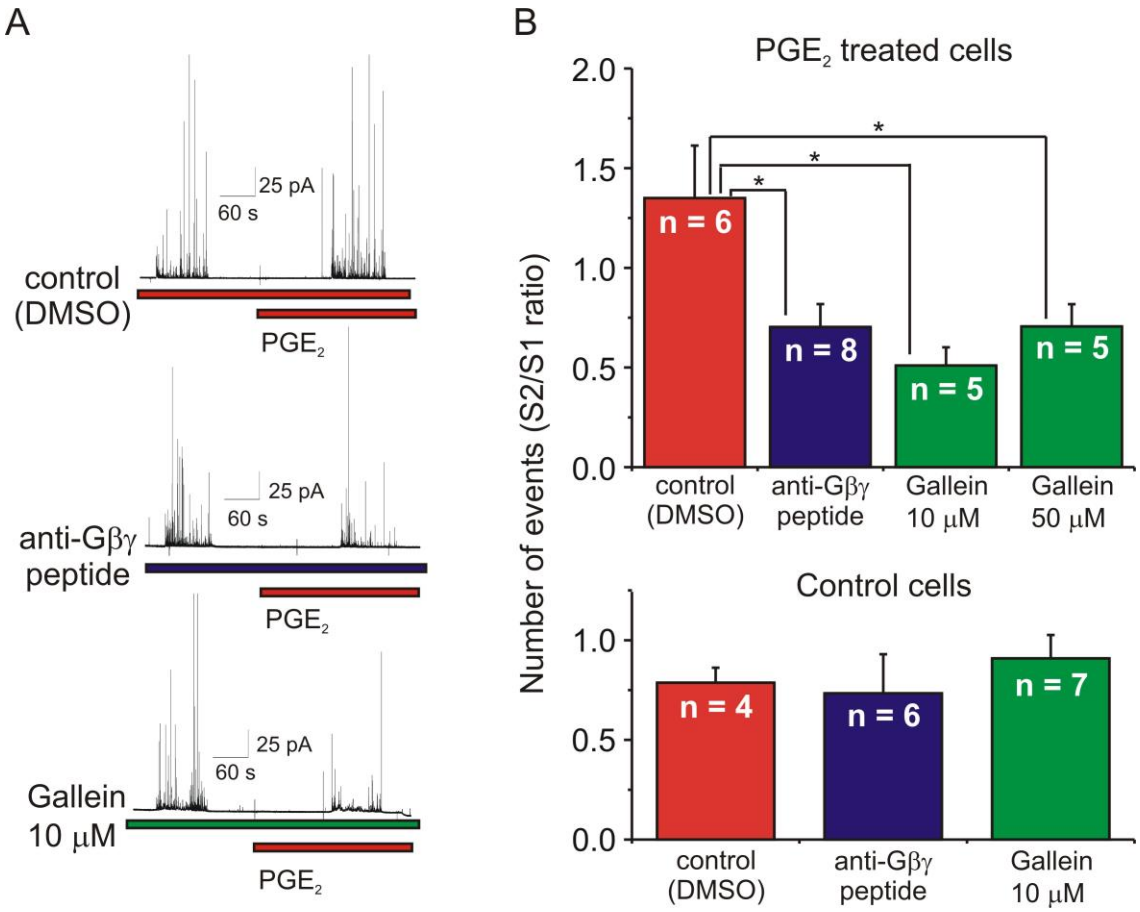

Figure 23: Gβγ inhibitors block potentiation mediated by PGE_2 Three representative amperometric current traces are shown in **(A)**: (top) pre-incubation with vehicle control (DMSO), (middle) pre-incubation with phosducin-like C-terminal peptide, and (bottom) 10 μM gallein. The timing of PGE_2 application (100 nM) is displayed; KCl application is inferred by evoked amperometric spikes. The evoked secretory spikes in S1 were not significantly altered by pre-treatment with DMSO, phosducin-like C-terminal peptide, or 10 μM gallein. **(B)** Change in secretion (S2/S1) in PGE_2 treated cells (upper panel). Pre-treatment with 1/5000 DMSO (vehicle) did not alter PGE_2 mediated potentiation (1.35 ± 0.26,n=6). However, pre-treatment with phosducin-like C-terminal peptide (0.70 ± 0.33, n=8), 10 μM gallein (0.51 ± 0.20, n=5) and 50 μM gallein (0.70 ± 0.25, n=5) significantly reduced the potentiation (ANOVA, overall p=0.011, Dunnett's post test ,* denotes $p < 0.05$). In control cells (no PGE_2, lower panel), the S2/S1 ratio was not altered by pretreatment with DMSO, phosducin-like peptide or 10 μM gallein (ANOVA p=0.67).

receptor subtypes (EP1-EP4) in adrenal tissue. PGE_2 inhibits voltage gated Ca^{2+} channels and exocytosis in parallel during short step depolarizations (2-100 ms depolarizing steps). Furthermore the inhibition is mediated solely by EP3 receptors coupled to $G_{i/o}$-associated Gβγ subunits (Chapter 3). We hypothesized that by changing the cellular context, and evoking exocytosis with sustained depolarizations resembling the physiological stress response, we would "uncover" additional signaling modalities with potential therapeutic use for tuning adrenal output. In the present study we have dissected the molecular mechanism by which PGE_2 modulates chromaffin cell function.

PGE_2 potentiates exocytosis during sustained depolarizations that mimic stress firing

In the present study we show PGE_2 produces a robust potentiation of the secretory response during a sustained depolarization that mimics acute stress. The potentiation resulted from a significant increase in the numbers of vesicles fusing with the membrane, rather than augmentation of quantal size. Moreover PGE_2 application did not raise intracellular calcium concentration, or directly elicit release of catecholamine, suggesting membrane potential was not appreciably altered by PGE_2.

We used a paired experimental design to account for the well-documented variability of secretory responses detected by amperometry. By comparing secretion evoked during S2 to that evoked during S1, each cell serves as its own control and normalizing the data within each cell (the S2/S1 ratio) reports the "change in secretion". With this design, the duration of the inter-stimulation interval is vitally important to ensure recovery between S1 and S2. Alternatively Ca^{2+} entry associated with S1 may potentiate S2 because refilling of the readily releasable pool of vesicles is Ca^{2+} dependent (von Ruden and

Neher, 1993). The S2/S1 ratio in control cells was close to 1, suggesting 210 second inter-stimulation interval provided adequate time for recovery and for PGE_2 to exert its physiological effect.

The effect of PGE_2 on intracellular calcium and the potential implication of signaling through EP1/EP2/EP4

The EP1 selective antagonist SC-51322 in our secretion assay did not significantly alter the PGE_2 mediated potentiation. Furthermore, PGE_2 did not alter the magnitude of KCl evoked $[Ca^{2+}]_i$ transients which is inconsistent with PGE_2 activation of EP1 receptors, which have been shown to raise $[Ca^{2+}]_i$ (Hata and Breyer, 2004; Shibuya et al., 1999) and stimulate IP production (Ji et al., 2010).

As previously mentioned we detected mRNA for EP2 and EP4 receptors, which typically raise cAMP (Breyer et al., 2001). Application of the membrane permeable cAMP analogue 8-CPT-cAMP to chromaffin cells potentiates L-type currents in single channel recordings (Carabelli et al., 2001). Similarly, in perforated whole cell recordings I_{Ca} and exocytosis evoked by short 100-ms step depolarizations are potentiated by 8-CPT-cAMP or activation of β1-adrenoreceptors, and reversed by a PKA inhibitor (Carabelli et al., 2003). Notably, the potentiation of exocytosis was attributed to an increase in quantal size of individual release events, which we do not observe. Furthermore any potentiation of L-type currents would likely be reflected in the fura-2 imaging. As outlined previously and in Chapter 3, PGE_2 does not modulate pharmacologically isolated L-type channels, suggesting PGE_2 does not raise cAMP enough to observe any measurable functional outcomes in mouse chromaffin cells.

Interestingly, activation of G_s-coupled D1-like dopamine receptors facilitate calcium channels by a cAMP dependent mechanism (Artalejo et al., 1990), and D1 agonists potentiated secretion evoked by subsecond pressure injected puffs of 60 mM KCl in bovine chromaffin cells (Villanueva and Wightman, 2007). However, D1 receptor agonists inhibited catecholamine release stimulated by a 10-minute application of 55 mM KCl (Dahmer and Senogles, 1996). These results raise questions whether G_s would potentiate exocytosis under sustained depolarizations that were used in this study. Collectively we find it unlikely that PGE_2 mediated potentiation of catecholamine release during sustained depolarization is mediated by EP1, EP2 or EP4 receptor activation.

PGE_2 mediated potentiation is dependent on pertussis toxin sensitive $G\beta\gamma$ subunits associated with the EP3 receptor

We show the potentiation is prevented by the selective EP3 inhibitor DG-041 or in chromaffin cells isolated from $EP3^{-/-}$ mice. Thus during brief stimuli that mimic basal sympathetic tone, EP3 receptors suppress exocytosis by mediating voltage-dependent inhibition of I_{Ca}, but during sustained stimulation this inhibition shifts to potentiation. Alternative splicing of the EP3 C-terminus generates three known splice variants (α-, β-, γ-) that vary in G protein coupling. Activation of $EP3_\alpha$ and $EP3_\beta$ were initially characterized to inhibit cAMP (Negishi et al., 1993), so we first tested pertussis toxin to characterize the pathway downstream of EP3. Pertussis toxin abolished the PGE_2-mediated potentiation, suggesting the involvement of $G_{i/o}$ –coupled G protein signaling, as did two Gβγ inhibitors, gallein or phosducin-like C terminus peptide. Gallein is a cell-permeant small molecule shown previously to disrupt protein-protein interactions

between Gβγ and GRK2 (Casey et al., 2010) and PI3K (Lehmann et al., 2008), but its full inhibitory repertoire is not well understood. Gallein is related in structure and efficacy to several molecules in the M119 class of compounds that compete for binding with a fluorescein isothiocyanate-labeled $G\alpha_i$ to the Gα switch II binding surface on Gβγ (Bonacci et al., 2006; Casey et al., 2010). This so called "hotspot" mediates interaction with a number of Gβγ effectors, including stimulatory contacts with PLCβ (Bonacci et al., 2006). On the surface, the finding that gallein potentially binds a region on Gβγ known to activate PLCβ and also blocks PGE_2 mediated potentiation of exocytosis is circumstantial evidence that the final mediator of the potentiation is downstream of PLCβ activation. However, another molecule in the M119 class, M201, binds a subsurface in the hotspot and instead of inhibiting the interaction, potentiates PLCβ2 activation by Gβγ, an indication of the functional complexity of the switch II binding surface on Gβγ (Bonacci et al., 2006; Ford et al., 1998). Preliminary to targeting Gβγ with gallein in our secretion assay, we tested if pre-incubation with 10 μM gallein blocked voltage-dependent inhibition of whole-cell I_{Ca} in chromaffin cells. ATP produced a significant and reversible inhibition of I_{Ca} in gallein treated cells (Figure 22C). This demonstrated Gβγ interactions with Ca_V2 channels are not perturbed by gallein, making it a potentially useful tool for dissecting G protein mediated aspects of the secretory response distinct from Ca^{2+} channels. To our knowledge this is the first characterization of this nature. Interestingly, by blocking Gβγ signaling (Figure 23B), the secretion ratio (S2/S1) drops below the ratio of ~1 we observed for S2/S1 control cells (CTL S2/S1: 0.94 ±0.8, n=11; Figure 18D, Figure 21B) and for PTX treated cells (PTX S2/S1 0.89 ± 0.27; Figure 21B). As mentioned previously, a rise in cAMP through G_s –coupled receptor activation

would be expected to potentiate L-type currents, which is not observed. However we have not investigated the impact of Gα subunit mediated inhibition of cAMP in any of our assays, and this effect is certainly occluded by our potentiation. Two recent reports outline cAMP- independent inhibitory effects of $G_{i/o}$ Gα subunits. NPY inhibition of insulin secretion from intact islets is pertussis toxin sensitive, but did not block the insulin potentiating effects of the Gβγ activating peptide mSIRK, indicative of an inhibitory pathway distinct from Gβγ. Furthermore the inhibition was independent of $[Ca^{2+}]_i$, and downstream of cAMP, suggesting the inhibitory effect of NPY is through non-canonical $G_{i/o}$ -inhibitory signaling mediated through the Gα subunit (Schwetz et al., 2013). Similarly, norepinephrine inhibited filling of the readily releasable pool of secretory granules in INS 832/13 β-cells, and the inhibition was blocked by $G\alpha_i$ inhibitory peptide (Zhao et al., 2010). This effect did not involve Gα mediated disruption of the cAMP/PKA pathway (which can increase the RRP) because $[cAMP]_i$ was buffered in the patch pipette solution for these experiments suggesting a cAMP independent inhibitory effect of $G\alpha_i$. Although we see a potentiation, we cannot rule out cAMP dependent or independent inhibitory EP3 signaling through $G_{i/o}\alpha$.

Targets of Gβγ

Whether the PLC inhibitor U73122 inactivates PLC by irreversible alkylation, and the specific target by which U73122 and the control U73343 block secretion warrants further study. Regardless, based on our observation that potentiation of catecholamine release by PGE_2 is manifest by more vesicles fusing with the plasma membrane, rather than larger vesicles, PLC activation by Gβγ remains a potential mechanism. Currently there are 13 identified isoforms of PLC divided into six families: PLC β-, γ-,δ-,ε-,ζ-, and η-, and Gβγ

can activate β-,ε-, and η- (Khan et al., 2013). PLC mediated production of IP3/Ca^{2+} and DAG can activate at least 12 different protein kinase C subtypes (PKC), which may phosphorylate several critical exocytotic effectors (Staal et al., 2008). Consequently PLC is emerging as a regulatory node as its downstream products produce great functional diversity necessary for organizing signals from a large number of GPCRs.

Pertussis toxin blocks receptor-mediated IP3 generation in leukocytes and mast cells but fails to have an effect in hepatocytes or cardiac myocytes (Gilman, 1987). This suggests both $G_{i/o}$- and $G_{q/11}$ -coupled receptors must participate in PLCβ activation, however no pertussis toxin sensitive Gα subunits have been shown to activate PLC (Clapham and Neer, 1997). In vitro, synergistic activation of PLCβ3 by Gβγ dimers and G_qα produces a ten fold increase in the Ca^{2+} response compared to either subunit alone, thus displaying allosteric synergism for regulation of PLCβ3 (Philip et al., 2010), however this was not supported in any other PLCβ isoform (1,2 and 4).

Others have shown G_qα and Gβγ can synergistically activate PLCβ2 (Wu et al., 1993), furthermore PLC activity is enhanced by intracellular Ca^{2+} release from internal stores (Horowitz et al., 2005). Thus KCl evoked Ca^{2+} transients in our amperometry experiments (Figure 20) may serve as the minimum threshold for activation of PLCβ, which is enhanced by activation of βγ subunits, resulting in a quantifiable effect on the secretory response. Certainly G_qα has a higher binding affinity for PLCβ (Khan et al., 2013), but we may be compensating for an absence EP1/ G_qα release by activating Gβγ through EP3 concomitant with increasing $[Ca^{2+}]_i$. In transfected Cos-7 cells, fMet-Leu-

Phe (fMLP)- receptors (Jiang et al., 1996) and adenosine A1 receptors (Tomura et al., 1997) couple to pertussis toxin-sensitive generation of IP, suggestive of $G_{i/o}$ mediated activation of PLC. Whether the potentiation of catecholamine release by PGE_2 is mediated by Gβγ activation of PLC requires further study, however to our knowledge this has not been shown previously in a secretory system. It is known that inhibition of phosphoinositide 3 kinases (PI3K) in chromaffin cells blocked secretion and prevented cortical actin disassembly (Chasserot-Golaz et al., 1998). Similarly F-actin disassembly has been shown to favor full fusion of chromaffin granules (Doreian et al., 2009). P13K and cytoskeletal components are regulated by Gβγ, and their involvement in EP3 - mediated potentiation requires further investigation.

During sustained stress depolarization, EP3 receptors potentiate catecholamine release by a non-canonical mechanism that involves Gβγ subunits from $G_{i/o}$ -coupled receptors (Figure 24/Chapter 4). We believe the contextual shift in the function of an EP3, $G_{i/o}$-coupled pathway is a novel characterization, but it was unexpected. A broad literature describes how $G_{i/o}$- coupled Gβγ activation generally reduces membrane excitability in several systems by activating GIRK channels (Logothetis et al., 1987), inhibiting TRPM1 channels (Shen et al., 2012), targeting SNARES to inhibit synaptic transmission (Gerachshenko et al., 2005), and/or mediating voltage-dependent inhibition of Ca_V2 channels at the synapse and in neuroendocrine cells (Zamponi and Currie, 2013). In chromaffin cells, activation of $G_{i/o}$ –coupled P2Y receptors, μ-opioid receptors or exogenous Gβγ application reduced the number and quantal size of amperometric events evoked by ionomycin or direct application of Ca^{2+} in the patch pipette (Yoon et al.,

2008). It was proposed that Gβγ may bind exocytotic machinery and shift the mode of exocytosis to a preference for smaller events. Notably in these experiments the evoked secretion was markedly smaller (by ~25%) than our KCl evoked secretion. Whether the potentiation is restricted to EP3 or supported by other $G_{i/o}$ –coupled receptors needs to be investigated further, but it is clear that cellular context does matter.

Taken together, we have outlined a molecular framework for the context-dependent, bimodal regulation of adrenal catecholamine release by the $G_{i/o}$-coupled EP3 receptor. Additionally we identify a novel behavior for $G_{i/o}$ associated βγ subunits not previously observed in the regulation of stimulus secretion coupling.

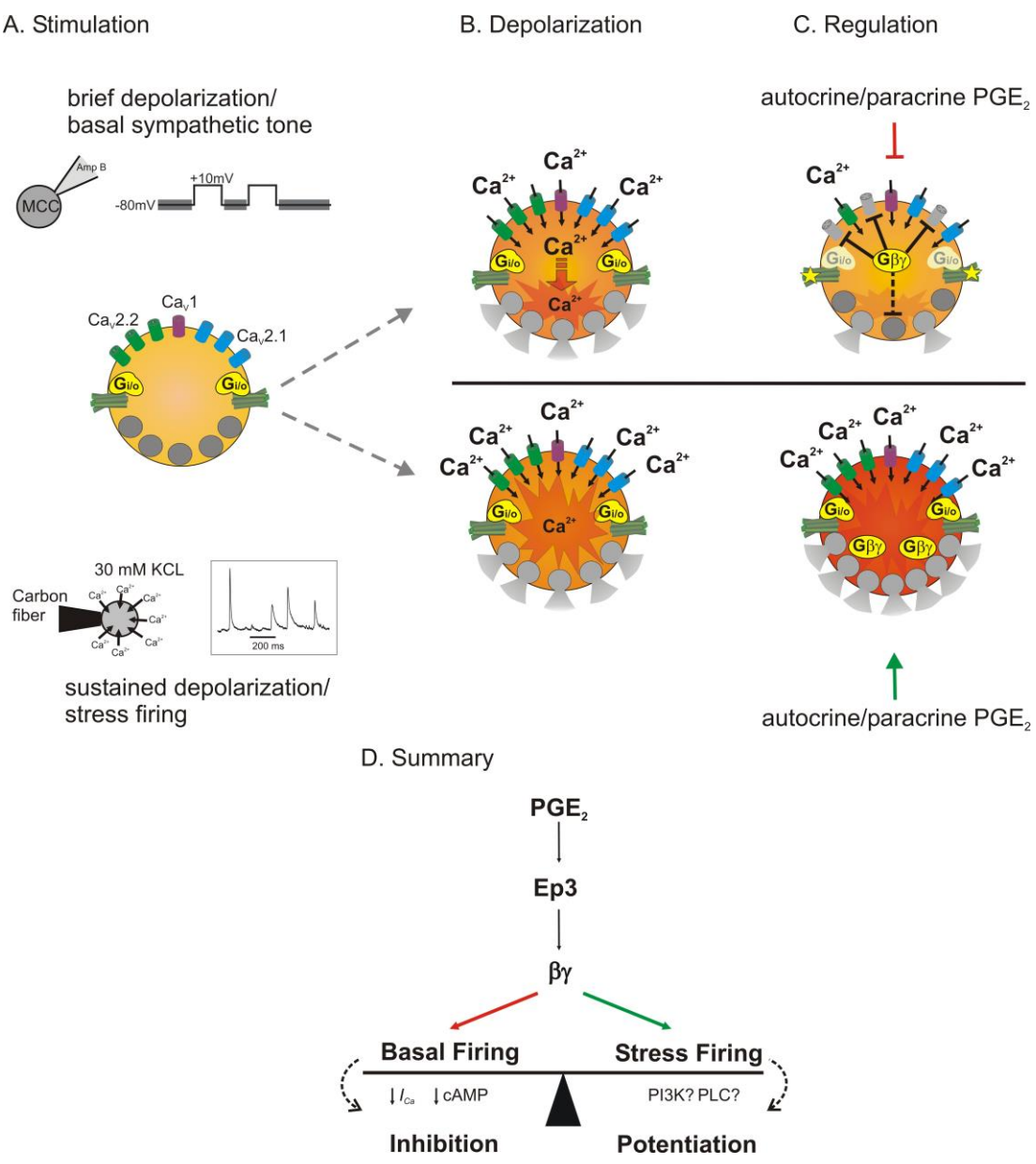

Figure 24: Bimodal regulation of adrenal catecholamine release by PGE_2: (A) Chromaffin cells are directly innervated by cholinergic sympathetic afferents that release Acetylcholine (ACh). Low frequency sympathetic nerve activity (basal) was modeled using step depolarizations (Ch. 3/top panel). Sympathetic stress at the splanchnic-adrenal synapse increases the rate of acetylcholine output resulting in sustained depolarization and increased secretion of catecholamine (bottom panel). These conditions were modeled with sustained application of 30 mM KCl (Ch. 4). **(B)** Activation of cell surface nicotinic ACh receptors depolarizes the membrane and opens voltage-gated Ca^{2+} channels, subsequently triggering exocytosis. **(C, D)** PGE_2 suppresses adrenal output during brief step depolarizations or low frequency trains of APW that mimic basal sympathetic tone (Top panel). The EP3 receptor supports voltage-dependent inhibition of I_{Ca} and exocytosis, by activating Gβγ subunits from the $G_{i/o}$ coupled EP3 receptor. During sustained stress depolarization, PGE_2 potentiates catecholamine release, by an unexpected mechanism involving the same molecular components as inhibition:Gβγ subunits liberated from the $G_{i/o}$ -coupled EP3 receptor (bottom). This regulatory shift may serve to augment circulating neuropeptides co-released with catecholamine during stress firing. Model summarized in **(D).**

5. GENERAL DISCUSSION AND FUTURE DIRECTIONS

General Discussion

A broad variety pharmaceutical agents in clinically important drug classes target stimulus secretion coupling: sulfonyurea antidiabetic drugs, opioids, non-opioid anesthetics, antidepressants, anti-arrhythmics and antihypertensives. Might pharmacological augmentation of adrenal catecholamine release provide potential therapeutic benefit? The intra-adrenal signaling pathway referenced in Chapter 1, Figure 6, was initiated with an i.v. injection of 25 μg/kg LPS in rat: a dose associated with moderate, but not severe, endotoxemia. The net functional consequence was an increase in cortical expression of synthetic enzymes for PGE_2, and presumably PGE_2, at the cortical/medullar interface. The authors hypothesize prostaglandin production ultimately regulates cortisol release during an immune challenge. Hypotension is a feature of endotoxemia, and both hypotensive and non-hypotensive doses of endotoxin elicit elevated circulating catecholamine (Burnier et al., 1988; Evequoz et al., 1988). It is interesting to speculate, based on the findings here, that adrenal PGE_2 production during systemic stress may connect the inflammatory immune response to increased sympathetic output from chromaffin cells, in an effort to maintain cardiovascular homeostasis. In support of this idea, pretreatment with indomethacin, an NSAID, prior to a nonhypotensive dose of endotoxin reduced plasma catecholamine levels (Burnier et al., 1988), and the endotoxin induced blood pressure fall in biadrenalectomized rats is prevented by epinephrine

infusion (Evequoz et al., 1988). However other reports suggest the vascular and myocardial responsiveness to catecholamine may be reduced by endotoxin administration (Bhagat et al., 1970).

If PGE_2 tunes adrenal output through the EP3 receptor in response to endotoxic stress, the logical extension to human therapeutics would be management of hemodynamics in the clinical setting of severe sepsis and septic shock. The transition from systemic inflammatory response syndrome to sepsis, and subsequently septic shock occurs in a setting of circulatory abnormalities that lead to tissue hypoxia. While specific treatment regimens are multifactorial, hemodynamic optimization and the general prevention cardiovascular collapse significantly improves outcomes (Rivers et al., 2008). The significant benefit of intact adrenal medullar function in this setting may simply be cardiovascular maintenance rather than an explicit therapeutic target, per se. Epinephrine is the primary catecholamine released from chromaffin cells, and has the highest potency of any endogenous agonists for β2 receptors. As a result, while exogenous epinephrine increases heart rate and systolic blood pressure, it has little effect on diastolic pressure and is rarely administered as a pressor. In clinical settings, epinephrine use is generally limited to treatment of anaphylaxis (Nowak et al., 2013).

Targeting adrenal output, potentially through EP3, in the pathogenesis of heart failure (Lymperopoulos et al., 2008) and metabolic syndrome (Ziegler et al., 2012), may be more therapeutically useful. Both diseases are progressive, where maladaptive signaling leads to progression of disease and symptoms over time. The long-term consequence of PGE_2

signaling in chromaffin cells was not investigated, and may provide insight on relevance of the pathway. Additionally, many components of the adrenal 'secretome' are bioactive and along with catecholamine, are correlated with disease. It remains uncharacterized how EP signaling may regulate peptide hormone synthesis, co-packaging with catecholamines (granulogenesis), and release.

Future Directions

We have outlined a molecular framework for the regulation of adrenal catecholamine release by receptors for prostaglandin E_2, specifically the $G_{i/o}$-coupled EP3 receptor. In the process we identified a stimulatory behavior for $G_{i/o}$ associated βγ subunits not previously observed in the regulation of stimulus secretion coupling. We outline the *potential* for bimodal, EP3-mediated regulation of catecholamine release, however the fundamental question underlying future experiments will be: Is bimodal, EP3-mediated regulation a *relevant* mechanism at the systems or organismal level for regulating adrenal output? Initially we intend to further characterize the pathway by identifying the molecular effector of Gβγ. In addition to activation of PLCβ discussed in Chapter 4, Gβγ subunits from $G_{i/o}$- receptors are known to activate PI3K (Kamal et al., 2011; Stoyanov et al., 1995). Neuroendocrine exocytosis is sensitive to the PI3K inhibitor LY294002 (Meunier et al., 2005; Wen et al., 2008), as PI3K may modulate granule priming and potentiate exocytosis through both PKA and Akt dependent pathways (Mori et al., 2004). Preliminary data from our lab suggests PGE_2 mediated potentiation of exocytosis is blocked by LY294002, however more work is needed to characterize this pathway. Along

with identifying a cellular target, four lines of inquiry directed at the relevance of this mechanism will be determining 1) the source of PGE_2, 2) the scale of the effect 3) the prevalence in other $G_{i/o}$ coupled receptors and 4) the long-term consequences of PGE_2 application.

Source of PGE_2

The first arm of bimodal regulation by EP3 was described in Chapter 3. We proposed that during brief stimuli, EP3 receptors suppress secretion through inhibition of I_{Ca}, but we provide background leading into Ch. 4 that systemic immune challenge is thought to boost local production of prostaglandin E_2. Therefore the implication is that PGE_2 is a paracrine signal of cortical origin produced in response to physiological stress and likely accompanied by increased sympathetic firing. However the origin of PGE_2 is an important unanswered question. Cox-1, Cox-2, and mPGES-1, are constitutively expressed in the adrenal medulla, therefore autocrine, or chromaffin- to -chromaffin regulation is possible. We propose a model that during brief stimuli, EP3 receptors suppress secretion through inhibition of I_{Ca}, but during sustained stimuli EP3 receptors potentiate evoked catecholamine secretion through a distinct pathway which also involves Gβγ subunits liberated from $G_{i/o}$-type G proteins. It follows that demonstrating both pathways work together in a physiological setting will help demonstrate regulatory relevance. In pre-synaptic neurons, voltage dependent inhibition of Ca^{2+} channels suppresses exocytosis contributing to short-term plasticity at the synapse. It is interesting to speculate that during basal sympathetic firing rates, autocrine PGE_2 is produced in chromaffin cells and subsequently transported out of the cell where it activates auto-

receptors or receptors on nearby cells, ultimately inhibiting catecholamine release by binding to EP3 receptors. This is mechanistically similar to the physiological function of ATP and enkephalin binding P2Y and μ-opioid receptors. However during stress activation/increased sympathetic input, paracrine PGE_2 is produced and potentiates release. Recent work similarly aimed to identify the role of EP signaling and enzymatic source of PGE_2 in subfornical organ (SFO) neurons, which may mediate slow pressor Ang II hypertension through production of reactive oxygen species (ROS). The authors show COX-1 derived PGE_2 was increased in SFO neurons and not surrounding brain regions, and elicited ROS production and ultimately hypertension through the EP1 receptors (Cao et al., 2012). Interestingly, prostaglandins are poorly membrane permeable. Prostaglandin uptake is thought to be mediated by the prostaglandin transporter (PGT), whereas multidrug resistance protein four (MRP4) mediates release of PGE_2 from cells. We used RT-PCR to detect expression of MRP4, but not PGT, in mouse adrenal tissue, suggesting the components are in place for chromaffin-chromaffin, or autocrine inhibition (Figure 25). Demonstrating both arms of our bimodal regulation work in tandem in a physiological setting, potentially increasing the functional range of sympathetic input/ adrenal output, will help show relevance of the model.

Scale of effect

High frequency splanchnic input to the adrenal gland has been proposed to desensitize nACh receptors on a scale of minutes, thus the non-cholinergic peptide transmitter PACAP may predominate as the primary stress mediator of exocytosis (Smith and Eiden,

2012; Stroth et al., 2011). Therefore chemical depolarization with KCl may closely model the physiological setting. PACAP application to adrenal slices has been shown to increase cell-to-cell electrical coupling via regulation of gap-junctions (Hill et al., 2011). Investigating EP signaling in a more tissue selective manner, e.g. adrenal slices, would be a useful model to uncover tissue specific influences in sustained stress depolarization. Using the same rational we conducted preliminary experiments sampling blood via cardiac puncture and measuring circulating catecholamine in wild type, $EP1^{-/-}$, $EP3^{-/-}$, and $EP1/3^{-/-}$ animals. Our results were highly variable, as is reported across different sampling methodologies (Grouzmann et al., 2003). An interesting approach would revisit circulating catecholamine levels in animals with pharmacological or genetic targeting of EP receptors, employing chronic catheterization to normalize reported variability.

Is Gβγ mediated bimodal regulation supported by other $G_{i/o}$ –coupled receptors?

Certainly $G_{i/o}$ - receptors for ATP (P2Y-receptors), enkephalin (μ-opioid receptors) or catecholamines (α-adrenergic) (Albillos et al., 1996; Currie and Fox, 1996; Harkins and Fox, 2000; Powell et al., 2000; Ulate et al., 2000) support voltage-dependent inhibition of I_{Ca} and exocytosis, however potentiation has not been shown. In addition to ATP and enkephalin, chromaffin granules contain NPY and dopamine which may modulate exocytosis. It is notable that activation of G_s-coupled D1-like dopamine receptors facilitate calcium channels by a cAMP dependent mechanism (Artalejo et al., 1990), and D1 agonists expectedly potentiated secretion evoked by short depolarizations (Villanueva and Wightman, 2007).Yet, D1 receptor agonists inhibited catecholamine release stimulated by a 10-minute application of 55 mM KCl (Dahmer and Senogles, 1996). One

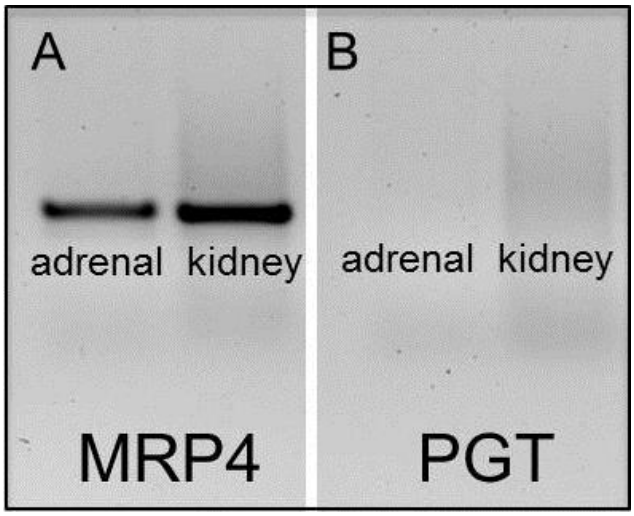

Figure 25: MRP4 export pump mRNA expressed in mouse adrenal tissue. (A) RT-PCR was used to detect expression of the MRP4 receptor in mouse adrenal tissue (left), and kidney tissue (right) that was isolated in parallel as a positive control. **(B)** PGT mRNA was not amplified. All samples from A and B expressed the internal standard GAPDH (data not shown)

could speculate the source of the ligand, regardless of its GPCR receptor coupling, may determine the net functional effect during a stress response. Similar to ascertaining the scale of our effect, broadening the scope of the model imparts relevance.

Long-term consequences of PGE_2 application

By necessity, our current studies outlined in Ch. 3 and 4 measure Ca^{2+} activation and exocytosis with precise temporal resolution. This is crucial for coupling activation of receptor mediated second messenger pathways to distinct cellular cascades in stimulus secretion coupling. However many diseases that implicate adrenal function are chronic, progressive, inflammatory states where maladaptive signaling leads to worsening of symptoms over time. Investigating cellular changes due to chronic PGE_2, and on a larger scale, characterizing adrenal function in $EP^{-/-}$ animals will be an important approach to determine if EP3-mediated, bimodal regulation is relevant *in vivo*.

Final Remarks

Dissecting subcellular GPCR signaling has intellectual value, but also makes therapeutic sense as most drugs targeting GPCRs lack selectivity and display complex pharmacology. This may doom a potential drug on adverse side effects, or conversely, may have clinical relevance and contribute to the desired efficacy by acting on multiple GPCRs. (Hopkins et al., 2006; Overington et al., 2006). Several studies highlight significant risk of broadly targeting prostanoids with NSAIDS (Gurwitz et al., 1994; Laine et al., 2003; Nussmeier et al., 2005), thus specifically targeting EP receptors may be the practical approach.

Overall, this work has increased the understanding of basic molecular instructions inherent to EP receptor function and control of neuroendocrine release.

REFERENCES

Abiria SA and Colbran RJ (2010) CaMKII associates with CaV1.2 L-type calcium channels via selected beta subunits to enhance regulatory phosphorylation. *J Neurochem* **112**(1): 150-161.

Agler HL, Evans J, Tay LH, Anderson MJ, Colecraft HM and Yue DT (2005) G protein-gated inhibitory module of N-type (ca(v)2.2) ca2+ channels. *Neuron* **46**(6): 891-904.

Ait-Ali D, Samal B, Mustafa T and Eiden LE (2010) Neuropeptides, growth factors, and cytokines: a cohort of informational molecules whose expression is up-regulated by the stress-associated slow transmitter PACAP in chromaffin cells. *Cellular and molecular neurobiology* **30**(8): 1441-1449.

Albillos A, Carbone E, Gandia L, Garcia AG and Pollo A (1996) Opioid inhibition of Ca2+ channel subtypes in bovine chromaffin cells: selectivity of action and voltage-dependence. *The European journal of neuroscience* **8**(8): 1561-1570.

Altier C, Khosravani H, Evans RM, Hameed S, Peloquin JB, Vartian BA, Chen L, Beedle AM, Ferguson SS, Mezghrani A, Dubel SJ, Bourinet E, McRory JE and Zamponi GW (2006) ORL1 receptor-mediated internalization of N-type calcium channels. *Nature neuroscience* **9**(1): 31-40.

Aoki J, Katoh H, Yasui H, Yamaguchi Y, Nakamura K, Hasegawa H, Ichikawa A and Negishi M (1999) Signal transduction pathway regulating prostaglandin EP3 receptor-induced neurite retraction: requirement for two different tyrosine kinases. *The Biochemical journal* **340 (Pt 2)**: 365-369.

Arias JM, Murbartian J, Vitko I, Lee JH and Perez-Reyes E (2005) Transfer of beta subunit regulation from high to low voltage-gated Ca2+ channels. *FEBS letters* **579**(18): 3907-3912.

Arikkath J and Campbell KP (2003) Auxiliary subunits: essential components of the voltage-gated calcium channel complex. *Curr Opin Neurobiol* **13**(3): 298-307.

Artalejo CR, Ariano MA, Perlman RL and Fox AP (1990) Activation of facilitation calcium channels in chromaffin cells by D1 dopamine receptors through a cAMP/protein kinase A-dependent mechanism. *Nature* **348**(6298): 239-242.

Bai J, Wang CT, Richards DA, Jackson MB and Chapman ER (2004) Fusion pore dynamics are regulated by synaptotagmin*t-SNARE interactions. *Neuron* **41**(6): 929-942.

Barclay JW, Craig TJ, Fisher RJ, Ciufo LF, Evans GJ, Morgan A and Burgoyne RD (2003) Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. *The Journal of biological chemistry* **278**(12): 10538-10545.

Barrett CF and Rittenhouse AR (2000) Modulation of N-type calcium channel activity by G-proteins and protein kinase C. *The Journal of general physiology* **115**(3): 277-286.

Bauer CS, Tran-Van-Minh A, Kadurin I and Dolphin AC (2010) A new look at calcium channel alpha2delta subunits. *Curr Opin Neurobiol* **20**(5): 563-571.

Bauer CS, Woolley RJ, Teschemacher AG and Seward EP (2007) Potentiation of exocytosis by phospholipase C-coupled G-protein-coupled receptors requires the

priming protein Munc13-1. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**(1): 212-219.
Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* **340**(6229): 153-156.
Beedle AM, McRory JE, Poirot O, Doering CJ, Altier C, Barrere C, Hamid J, Nargeot J, Bourinet E and Zamponi GW (2004) Agonist-independent modulation of N-type calcium channels by ORL1 receptors. *Nature neuroscience* **7**(2): 118-125.
Bell DC, Butcher AJ, Berrow NS, Page KM, Brust PF, Nesterova A, Stauderman KA, Seabrook GR, Nurnberg B and Dolphin AC (2001) Biophysical properties, pharmacology, and modulation of human, neuronal L-type (alpha(1D), Ca(V)1.3) voltage-dependent calcium currents. *Journal of neurophysiology* **85**(2): 816-827.
Bergsman JB and Tsien RW (2000) Syntaxin modulation of calcium channels in cortical synaptosomes as revealed by botulinum toxin C1. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**(12): 4368-4378.
Bernheim L, Beech DJ and Hille B (1991) A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. *Neuron* **6**(6): 859-867.
Bertaso F, Ward RJ, Viard P, Milligan G and Dolphin AC (2003) Mechanism of action of Gq to inhibit G beta gamma modulation of CaV2.2 calcium channels: probed by the use of receptor-G alpha tandems. *Molecular pharmacology* **63**(4): 832-843.
Bezprozvanny I, Scheller RH and Tsien RW (1995) Functional impact of syntaxin on gating of N-type and Q-type calcium channels. *Nature* **378**(6557): 623-626.
Bhagat B, Cavanagh D, Merrild BN, Rana MW and Rao PS (1970) Noradrenaline and tyramine action on isolated atrial muscle of endotoxin-treated guinea-pigs. *British journal of pharmacology* **39**(4): 688-695.
Blackmer T, Larsen EC, Bartleson C, Kowalchyk JA, Yoon EJ, Preininger AM, Alford S, Hamm HE and Martin TF (2005) G protein betagamma directly regulates SNARE protein fusion machinery for secretory granule exocytosis. *Nature neuroscience* **8**(4): 421-425.
Blake BL, Wing MR, Zhou JY, Lei Q, Hillmann JR, Behe CI, Morris RA, Harden TK, Bayliss DA, Miller RJ and Siderovski DP (2001) G beta association and effector interaction selectivities of the divergent G gamma subunit G gamma(13). *The Journal of biological chemistry* **276**(52): 49267-49274.
Boarder MR, Marriott D and Adams M (1987) Stimulus secretion coupling in cultured chromaffin cells. Dependency on external sodium and on dihydropyridine-sensitive calcium channels. *Biochemical pharmacology* **36**(1): 163-167.
Bonacci TM, Mathews JL, Yuan C, Lehmann DM, Malik S, Wu D, Font JL, Bidlack JM and Smrcka AV (2006) Differential targeting of Gbetagamma-subunit signaling with small molecules. *Science (New York, NY)* **312**(5772): 443-446.
Borges R, Camacho M and Gillis KD (2008) Measuring secretion in chromaffin cells using electrophysiological and electrochemical methods. *Acta physiologica (Oxford, England)* **192**(2): 173-184.
Bornstein SR, Ehrhart-Bornstein M and Scherbaum WA (1997) Morphological and functional studies of the paracrine interaction between cortex and medulla in the adrenal gland. *Microscopy research and technique* **36**(6): 520-533.

Bornstein SR, Tian H, Haidan A, Bottner A, Hiroi N, Eisenhofer G, McCann SM, Chrousos GP and Roffler-Tarlov S (2000) Deletion of tyrosine hydroxylase gene reveals functional interdependence of adrenocortical and chromaffin cell system in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **97**(26): 14742-14747.

Bourinet E, Soong TW, Stea A and Snutch TP (1996) Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. *Proceedings of the National Academy of Sciences of the United States of America* **93**(4): 1486-1491.

Breyer MD, Jacobson HR, Davis LS and Breyer RM (1993) In situ hybridization and localization of mRNA for the rabbit prostaglandin EP3 receptor. *Kidney Int* **44**(6): 1372-1378.

Breyer RM, Bagdassarian CK, Myers SA and Breyer MD (2001) Prostanoid receptors: subtypes and signaling. *Annual review of pharmacology and toxicology* **41**: 661-690.

Brody DL, Patil PG, Mulle JG, Snutch TP and Yue DT (1997) Bursts of action potential waveforms relieve G-protein inhibition of recombinant P/Q-type Ca2+ channels in HEK 293 cells. *The Journal of physiology* **499 (Pt 3)**: 637-644.

Brody DL and Yue DT (2000) Relief of G-protein inhibition of calcium channels and short-term synaptic facilitation in cultured hippocampal neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**(3): 889-898.

Bucci G, Mochida S and Stephens GJ (2011) Inhibition of synaptic transmission and G protein modulation by synthetic CaV2.2 Ca(2)+ channel peptides. *The Journal of physiology* **589**(Pt 13): 3085-3101.

Buraei Z and Yang J (2010) The beta subunit of voltage-gated Ca2+ channels. *Physiological reviews* **90**(4): 1461-1506.

Burnier M, Waeber B, Aubert JF, Nussberger J and Brunner HR (1988) Effects of nonhypotensive endotoxemia in conscious rats: role of prostaglandins. *The American journal of physiology* **254**(3 Pt 2): H509-516.

Canti C, Bogdanov Y and Dolphin AC (2000) Interaction between G proteins and accessory subunits in the regulation of 1B calcium channels in Xenopus oocytes. *The Journal of physiology* **527 Pt 3**: 419-432.

Canti C, Page KM, Stephens GJ and Dolphin AC (1999) Identification of residues in the N terminus of alpha1B critical for inhibition of the voltage-dependent calcium channel by Gbeta gamma. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **19**(16): 6855-6864.

Cao X, Peterson JR, Wang G, Anrather J, Young CN, Guruju MR, Burmeister MA, Iadecola C and Davisson RL (2012) Angiotensin II-dependent hypertension requires cyclooxygenase 1-derived prostaglandin E2 and EP1 receptor signaling in the subfornical organ of the brain. *Hypertension* **59**(4): 869-876.

Cao YQ and Tsien RW (2005) Effects of familial hemiplegic migraine type 1 mutations on neuronal P/Q-type Ca2+ channel activity and inhibitory synaptic transmission. *Proceedings of the National Academy of Sciences of the United States of America* **102**(7): 2590-2595.

Carabelli V, Giancippoli A, Baldelli P, Carbone E and Artalejo AR (2003) Distinct potentiation of L-type currents and secretion by cAMP in rat chromaffin cells. *Biophysical journal* **85**(2): 1326-1337.

Carabelli V, Hernandez-Guijo JM, Baldelli P and Carbone E (2001) Direct autocrine inhibition and cAMP-dependent potentiation of single L-type Ca2+ channels in bovine chromaffin cells. *The Journal of physiology* **532**(Pt 1): 73-90.

Carabelli V, Lovallo M, Magnelli V, Zucker H and Carbone E (1996) Voltage-dependent modulation of single N-Type Ca2+ channel kinetics by receptor agonists in IMR32 cells. *Biophysical journal* **70**(5): 2144-2154.

Casey LM, Pistner AR, Belmonte SL, Migdalovich D, Stolpnik O, Nwakanma FE, Vorobiof G, Dunaevsky O, Matavel A, Lopes CM, Smrcka AV and Blaxall BC (2010) Small molecule disruption of G beta gamma signaling inhibits the progression of heart failure. *Circulation research* **107**(4): 532-539.

Catterall WA (2000) Structure and regulation of voltage-gated Ca2+ channels. *Annu Rev Cell Dev Biol* **16**: 521-555.

Catterall WA and Few AP (2008) Calcium channel regulation and presynaptic plasticity. *Neuron* **59**(6): 882-901.

Catterall WA, Perez-Reyes E, Snutch TP and Striessnig J (2005) International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. *Pharmacological reviews* **57**(4): 411-425.

Chang M, Zhang L, Tam JP and Sanders-Bush E (2000) Dissecting G protein-coupled receptor signaling pathways with membrane-permeable blocking peptides. Endogenous 5-HT(2C) receptors in choroid plexus epithelial cells. *The Journal of biological chemistry* **275**(10): 7021-7029.

Chasserot-Golaz S, Hubert P, Thierse D, Dirrig S, Vlahos CJ, Aunis D and Bader MF (1998) Possible involvement of phosphatidylinositol 3-kinase in regulated exocytosis: studies in chromaffin cells with inhibitor LY294002. *J Neurochem* **70**(6): 2347-2356.

Chen CA and Manning DR (2001) Regulation of G proteins by covalent modification. *Oncogene* **20**(13): 1643-1652.

Chen EP and Smyth EM (2011) COX-2 and PGE2-dependent immunomodulation in breast cancer. *Prostaglandins & other lipid mediators* **96**(1-4): 14-20.

Chen J, DeVivo M, Dingus J, Harry A, Li J, Sui J, Carty DJ, Blank JL, Exton JH, Stoffel RH and et al. (1995) A region of adenylyl cyclase 2 critical for regulation by G protein beta gamma subunits. *Science (New York, NY)* **268**(5214): 1166-1169.

Chen L, Jin L and Zhou N (2012) An update of novel screening methods for GPCR in drug discovery. *Expert opinion on drug discovery* **7**(9): 791-806.

Chen RS, Deng TC, Garcia T, Sellers ZM and Best PM (2007) Calcium channel gamma subunits: a functionally diverse protein family. *Cell Biochem Biophys* **47**(2): 178-186.

Chen X and Levine JD (2005) Epinephrine-induced excitation and sensitization of rat C-fiber nociceptors. *The journal of pain : official journal of the American Pain Society* **6**(7): 439-446.

Chen XK, Wang LC, Zhou Y, Cai Q, Prakriya M, Duan KL, Sheng ZH, Lingle C and Zhou Z (2005) Activation of GPCRs modulates quantal size in chromaffin cells through G(betagamma) and PKC. *Nature neuroscience* **8**(9): 1160-1168.

Chen YH, Li MH, Zhang Y, He LL, Yamada Y, Fitzmaurice A, Shen Y, Zhang H, Tong L and Yang J (2004) Structural basis of the alpha1-beta subunit interaction of voltage-gated Ca2+ channels. *Nature* **429**(6992): 675-680.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK and Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science (New York, NY)* **318**(5854): 1258-1265.

Chung S, Funakoshi T and Civelli O (2008) Orphan GPCR research. *British journal of pharmacology* **153 Suppl 1**: S339-346.

Clapham DE and Neer EJ (1997) G protein beta gamma subunits. *Annual review of pharmacology and toxicology* **37**: 167-203.

Colecraft HM, Brody DL and Yue DT (2001) G-protein inhibition of N- and P/Q-type calcium channels: distinctive elementary mechanisms and their functional impact. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **21**(4): 1137-1147.

Colecraft HM, Patil PG and Yue DT (2000) Differential occurrence of reluctant openings in G-protein-inhibited N- and P/Q-type calcium channels. *The Journal of general physiology* **115**(2): 175-192.

Coleman RA, Smith WL and Narumiya S (1994) International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacological reviews* **46**(2): 205-229.

Cooper CB, Arnot MI, Feng ZP, Jarvis SE, Hamid J and Zamponi GW (2000) Cross-talk between G-protein and protein kinase C modulation of N-type calcium channels is dependent on the G-protein beta subunit isoform. *The Journal of biological chemistry* **275**(52): 40777-40781.

Currie KP (2010a) G protein inhibition of CaV2 calcium channels. *Channels (Austin)* **4**(6): 497-509.

Currie KP (2010b) Inhibition of Ca2+ channels and adrenal catecholamine release by G protein coupled receptors. *Cellular and molecular neurobiology* **30**(8): 1201-1208.

Currie KP, Zhou Z and Fox AP (2000) Evidence for paracrine signaling between macrophages and bovine adrenal chromaffin cell Ca(2+) channels. *Journal of neurophysiology* **83**(1): 280-287.

Currie KPM and Fox AP (1996) ATP serves as a negative feedback inhibitor of voltage-gated Ca2+ channel currents in cultured bovine adrenal chromaffin cells. *Neuron* **16**(5): 1027-1036.

Currie KPM and Fox AP (1997) Comparison of N- and P/Q-type voltage-gated calcium channel current inhibition. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **17**(12): 4570-4579.

Currie KPM and Fox AP (2000) Voltage-dependent, pertussis toxin insensitive inhibition of calcium currents by histamine in bovine adrenal chromaffin cells. *Journal of neurophysiology* **83**(3): 1435-1442.

Currie KPM and Fox AP (2002) Differential facilitation of N- and P/Q-type calcium channels during trains of action potential-like waveforms. *The Journal of physiology* **539**(Pt 2): 419-431.

Dahmer MK and Senogles SE (1996) Dopaminergic inhibition of catecholamine secretion from chromaffin cells: evidence that inhibition is mediated by D4 and D5 dopamine receptors. *J Neurochem* **66**(1): 222-232.

Davies A, Kadurin I, Alvarez-Laviada A, Douglas L, Nieto-Rostro M, Bauer CS, Pratt WS and Dolphin AC (2010) The alpha2delta subunits of voltage-gated calcium channels form GPI-anchored proteins, a posttranslational modification essential for function. *Proceedings of the National Academy of Sciences of the United States of America* **107**(4): 1654-1659.

Davies JN, Jarvis SE and Zamponi GW (2011) Bipartite syntaxin 1A interactions mediate CaV2.2 calcium channel regulation. *Biochemical and biophysical research communications* **411**(3): 562-568.

Davies JN and Zamponi GW (2008) Old proteins, developing roles: The regulation of calcium channels by synaptic proteins. *Channels (Austin)* **2**(2): 130-138.

Davis TL, Bonacci TM, Sprang SR and Smrcka AV (2005) Structural and molecular characterization of a preferred protein interaction surface on G protein beta gamma subunits. *Biochemistry* **44**(31): 10593-10604.

De Waard M, Hering J, Weiss N and Feltz A (2005) How do G proteins directly control neuronal Ca2+ channel function? *Trends in pharmacological sciences* **26**(8): 427-436.

De Waard M, Liu H, Walker D, Scott VE, Gurnett CA and Campbell KP (1997) Direct binding of G-protein betagamma complex to voltage-dependent calcium channels. *Nature* **385**(6615): 446-450.

Deak T (2008) Immune cells and cytokine circuits: toward a working model for understanding direct immune-to-adrenal communication pathways. *Endocrinology* **149**(4): 1433-1435.

Delmas P, Brown DA, Dayrell M, Abogadie FC, Caulfield MP and Buckley NJ (1998) On the role of endogenous G-protein beta gamma subunits in N-type Ca2+ current inhibition by neurotransmitters in rat sympathetic neurones. *The Journal of physiology* **506 (Pt 2)**: 319-329.

Deupi X, Edwards P, Singhal A, Nickle B, Oprian D, Schertler G and Standfuss J (2012) Stabilized G protein binding site in the structure of constitutively active metarhodopsin-II. *Proceedings of the National Academy of Sciences of the United States of America* **109**(1): 119-124.

Diaz E (2010) Regulation of AMPA receptors by transmembrane accessory proteins. *The European journal of neuroscience* **32**(2): 261-268.

Doering CJ, Kisilevsky AE, Feng ZP, Arnot MI, Peloquin J, Hamid J, Barr W, Nirdosh A, Simms B, Winkfein RJ and Zamponi GW (2004) A single Gbeta subunit locus controls crosstalk between PKC and G protein regulation of N-type calcium channels. *The Journal of biological chemistry*.

Dolphin AC (2003) Beta subunits of voltage-gated calcium channels. *J Bioenerg Biomembr* **35**(6): 599-620.

Dolphin AC (2012) Calcium channel auxiliary alpha2delta and beta subunits: trafficking and one step beyond. *Nat Rev Neurosci* **13**(8): 542-555.

Dore AS, Robertson N, Errey JC, Ng I, Hollenstein K, Tehan B, Hurrell E, Bennett K, Congreve M, Magnani F, Tate CG, Weir M and Marshall FH (2011) Structure of the adenosine A(2A) receptor in complex with ZM241385 and the xanthines XAC and caffeine. *Structure (London, England : 1993)* **19**(9): 1283-1293.

Doreian BW, Fulop TG, Meklemburg RL and Smith CB (2009) Cortical F-actin, the exocytic mode, and neuropeptide release in mouse chromaffin cells is regulated

by myristoylated alanine-rich C-kinase substrate and myosin II. *Molecular biology of the cell* **20**(13): 3142-3154.
Douglas WW (1968) Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *British journal of pharmacology* **34**: 451-469.
Dresviannikov AV, Page KM, Leroy J, Pratt WS and Dolphin AC (2009) Determinants of the voltage dependence of G protein modulation within calcium channel beta subunits. *Pflugers Archiv : European journal of physiology* **457**(4): 743-756.
Du C and Role LW (2001) Differential modulation of nicotinic acetylcholine receptor subtypes and synaptic transmission in chick sympathetic ganglia by PGE(2). *Journal of neurophysiology* **85**(6): 2498-2508.
Dunlap K and Fischbach GD (1978) Neurotransmitters decrease the calcium ocmponent of sensory neurone action potentials. *Nature* **276**(5690): 837-839.
Dunlap K and Fischbach GD (1981) Neurotransmitters decrease the calcium conductance activated by depolarization of embryonic chick sensory neurones. *The Journal of physiology* **317**: 519-535.
Dzhura EV, He W and Currie KP (2006) Linopirdine modulates calcium signaling and stimulus-secretion coupling in adrenal chromaffin cells by targeting M-type K+ channels and nicotinic acetylcholine receptors. *The Journal of pharmacology and experimental therapeutics* **316**(3): 1165-1174.
Ehrhart-Bornstein M, Hinson JP, Bornstein SR, Scherbaum WA and Vinson GP (1998) Intraadrenal interactions in the regulation of adrenocortical steroidogenesis. *Endocrine reviews* **19**(2): 101-143.
Elmslie KS (1992) Calcium current modulation in frog sympathetic neurones: multiple neurotransmitters and G proteins. *The Journal of physiology* **451**: 229-246.
Elmslie KS (2003) Neurotransmitter modulation of neuronal calcium channels. *J Bioenerg Biomembr* **35**(6): 477-489.
Elmslie KS and Jones SW (1994) Concentration dependence of neurotransmitter effects on calcium current kinetics in frog sympathetic neurones. *The Journal of physiology* **481 (Pt 1)**: 35-46.
Elmslie KS, Zhou W and Jones SW (1990) LHRH and GTP-gamma-S modify calcium current activation in bullfrog sympathetic neurons. *Neuron* **5**(1): 75-80.
Engstrom L, Rosen K, Angel A, Fyrberg A, Mackerlova L, Konsman JP, Engblom D and Blomqvist A (2008) Systemic immune challenge activates an intrinsically regulated local inflammatory circuit in the adrenal gland. *Endocrinology* **149**(4): 1436-1450.
Ertel EA, Campbell KP, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E, Schwartz A, Snutch TP, Tanabe T, Birnbaumer L, Tsien RW and Catterall WA (2000) Nomenclature of voltage-gated calcium channels. *Neuron* **25**(3): 533-535.
Evanko DS, Thiyagarajan MM and Wedegaertner PB (2000) Interaction with Gbetagamma is required for membrane targeting and palmitoylation of Galpha(s) and Galpha(q). *The Journal of biological chemistry* **275**(2): 1327-1336.
Evans RM and Zamponi GW (2006) Presynaptic Ca2+ channels--integration centers for neuronal signaling pathways. *Trends in neurosciences* **29**(11): 617-624.
Evequoz D, Waeber B, Aubert JF, Fluckiger JP, Nussberger J and Brunner HR (1988) Neuropeptide Y prevents the blood pressure fall induced by endotoxin in conscious rats with adrenal medullectomy. *Circulation research* **62**(1): 25-30.

Feldman DS, Elton TS, Sun B, Martin MM and Ziolo MT (2008) Mechanisms of disease: detrimental adrenergic signaling in acute decompensated heart failure. *Nat Clin Pract Cardiovasc Med* **5**(4): 208-218.

Feng ZP, Arnot MI, Doering CJ and Zamponi GW (2001) Calcium channel beta subunits differentially regulate the inhibition of N-type channels by individual Gbeta isoforms. *The Journal of biological chemistry* **276**(48): 45051-45058.

Fenwick EM, Marty A and Neher E (1982) Sodium and calcium channels in bovine chromaffin cells. *The Journal of physiology* **331**: 599-635.

Filippov AK, Simon J, Barnard EA and Brown DA (2010) The scaffold protein NHERF2 determines the coupling of P2Y1 nucleotide and mGluR5 glutamate receptor to different ion channels in neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**(33): 11068-11072.

Findeisen F and Minor DL, Jr. (2009) Disruption of the IS6-AID linker affects voltage-gated calcium channel inactivation and facilitation. *The Journal of general physiology* **133**(3): 327-343.

Flucher BE and Tuluc P (2011) A new L-type calcium channel isoform required for normal patterning of the developing neuromuscular junction. *Channels (Austin)* **5**(6): 518-524.

Ford CE, Skiba NP, Bae H, Daaka Y, Reuveny E, Shekter LR, Rosal R, Weng G, Yang CS, Iyengar R, Miller RJ, Jan LY, Lefkowitz RJ and Hamm HE (1998) Molecular basis for interactions of G protein betagamma subunits with effectors. *Science (New York, NY)* **280**(5367): 1271-1274.

Forscher P, Oxford GS and Schulz D (1986) Noradrenaline modulates calcium channels in avian dorsal root ganglion cells through tight receptor-channel coupling. *The Journal of physiology* **379**: 131-144.

Fox AP, Cahill AL, Currie KP, Grabner C, Harkins AB, Herring B, Hurley JH and Xie Z (2008) N- and P/Q-type Ca2+ channels in adrenal chromaffin cells. *Acta physiologica (Oxford, England)* **192**(2): 247-261.

Fredriksson R, Lagerstrom MC, Lundin LG and Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Molecular pharmacology* **63**(6): 1256-1272.

Fulop T, Radabaugh S and Smith C (2005) Activity-dependent differential transmitter release in mouse adrenal chromaffin cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**(32): 7324-7332.

Fulop T and Smith C (2006) Physiological stimulation regulates the exocytic mode through calcium activation of protein kinase C in mouse chromaffin cells. *The Biochemical journal* **399**(1): 111-119.

Funk CD, Furci L, FitzGerald GA, Grygorczyk R, Rochette C, Bayne MA, Abramovitz M, Adam M and Metters KM (1993) Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype. *The Journal of biological chemistry* **268**(35): 26767-26772.

Furukawa T, Nukada T, Mori Y, Wakamori M, Fujita Y, Ishida H, Fukuda K, Kato S and Yoshii M (1998) Differential interactions of the C terminus and the cytoplasmic I-II loop of neuronal Ca2+ channels with G-protein alpha and beta gamma subunits.

I. Molecular determination. *The Journal of biological chemistry* **273**(28): 17585-17594.

Gandini MA and Felix R (2012) Functional interactions between voltage-gated Ca(2+) channels and Rab3-interacting molecules (RIMs): new insights into stimulus-secretion coupling. *Biochimica et biophysica acta* **1818**(3): 551-558.

Garcia AG, Garcia-De-Diego AM, Gandia L, Borges R and Garcia-Sancho J (2006) Calcium signaling and exocytosis in adrenal chromaffin cells. *Physiological reviews* **86**(4): 1093-1131.

Gaudet R, Bohm A and Sigler PB (1996) Crystal structure at 2.4 angstroms resolution of the complex of transducin betagamma and its regulator, phosducin. *Cell* **87**(3): 577-588.

Gayen JR, Saberi M, Schenk S, Biswas N, Vaingankar SM, Cheung WW, Najjar SM, O'Connor DT, Bandyopadhyay G and Mahata SK (2009) A novel pathway of insulin sensitivity in chromogranin A null mice: a crucial role for pancreastatin in glucose homeostasis. *The Journal of biological chemistry* **284**(42): 28498-28509.

Gerachshenko T, Blackmer T, Yoon EJ, Bartleson C, Hamm HE and Alford S (2005) Gbetagamma acts at the C terminus of SNAP-25 to mediate presynaptic inhibition. *Nature neuroscience* **8**(5): 597-605.

Gillis KD (2000) Admittance-based measurement of membrane capacitance using the EPC-9 patch-clamp amplifier. *Pflugers Archiv : European journal of physiology* **439**(5): 655-664.

Gillis KD, Mossner R and Neher E (1996) Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules. *Neuron* **16**(6): 1209-1220.

Gilman AG (1987) G proteins: transducers of receptor-generated signals. *Annual review of biochemistry* **56**: 615-649.

Golard A and Siegelbaum SA (1993) Kinetic basis for the voltage-dependent inhibition of N-type calcium current by somatostatin and norepinephrine in chick sympathetic neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **13**(9): 3884-3894.

Goldstein DS (2010) Adrenal responses to stress. *Cellular and molecular neurobiology* **30**(8): 1433-1440.

Granier S and Kobilka B (2012) A new era of GPCR structural and chemical biology. *Nature chemical biology* **8**(8): 670-673.

Granier S, Manglik A, Kruse AC, Kobilka TS, Thian FS, Weis WI and Kobilka BK (2012) Structure of the delta-opioid receptor bound to naltrindole. *Nature* **485**(7398): 400-404.

Grantham JJ and Orloff J (1968) Effect of prostaglandin E1 on the permeability response of the isolated collecting tubule to vasopressin, adenosine 3',5'-monophosphate, and theophylline. *The Journal of clinical investigation* **47**(5): 1154-1161.

Gray AC, Raingo J and Lipscombe D (2007) Neuronal calcium channels: splicing for optimal performance. *Cell calcium* **42**(4-5): 409-417.

Grouzmann E, Cavadas C, Grand D, Moratel M, Aubert JF, Brunner HR and Mazzolai L (2003) Blood sampling methodology is crucial for precise measurement of plasma catecholamines concentrations in mice. *Pflugers Archiv : European journal of physiology* **447**(2): 254-258.

Guan Y, Zhang Y, Wu J, Qi Z, Yang G, Dou D, Gao Y, Chen L, Zhang X, Davis LS, Wei M, Fan X, Carmosino M, Hao C, Imig JD, Breyer RM and Breyer MD (2007) Antihypertensive effects of selective prostaglandin E2 receptor subtype 1 targeting. *The Journal of clinical investigation* **117**(9): 2496-2505.

Gurwitz JH, Avorn J, Bohn RL, Glynn RJ, Monane M and Mogun H (1994) Initiation of antihypertensive treatment during nonsteroidal anti-inflammatory drug therapy. *JAMA : the journal of the American Medical Association* **272**(10): 781-786.

Hamid J, Nelson D, Spaetgens R, Dubel SJ, Snutch TP and Zamponi GW (1999) Identification of an integration center for cross-talk between protein kinase C and G protein modulation of N-type calcium channels. *The Journal of biological chemistry* **274**(10): 6195-6202.

Han Y, Kaeser PS, Sudhof TC and Schneggenburger R (2011) RIM determines Ca(2)+ channel density and vesicle docking at the presynaptic active zone. *Neuron* **69**(2): 304-316.

Hanson MA, Cherezov V, Griffith MT, Roth CB, Jaakola VP, Chien EY, Velasquez J, Kuhn P and Stevens RC (2008) A specific cholesterol binding site is established by the 2.8 A structure of the human beta2-adrenergic receptor. *Structure (London, England : 1993)* **16**(6): 897-905.

Harkins AB and Fox AP (2000) Activation of purinergic receptors by ATP inhibits secretion in bovine adrenal chromaffin cells. *Brain research* **885**(2): 231-239.

Harmar AJ (2001) Family-B G-protein-coupled receptors. *Genome biology* **2**(12): REVIEWS3013.

Hata AN and Breyer RM (2004) Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacology & therapeutics* **103**(2): 147-166.

Hayflick JS (2000) A family of heptahelical receptors with adhesion-like domains: a marriage between two super families. *Journal of receptor and signal transduction research* **20**(2-3): 119-131.

Heinemann C, Chow RH, Neher E and Zucker RS (1994) Kinetics of the secretory response in bovine chromaffin cells following flash photolysis of caged Ca2+. *Biophysical journal* **67**(6): 2546-2557.

Heneghan JF, Mitra-Ganguli T, Stanish LF, Liu L, Zhao R and Rittenhouse AR (2009) The Ca2+ channel beta subunit determines whether stimulation of Gq-coupled receptors enhances or inhibits N current. *The Journal of general physiology* **134**(5): 369-384.

Heptinstall S, Espinosa DI, Manolopoulos P, Glenn JR, White AE, Johnson A, Dovlatova N, Fox SC, May JA, Hermann D, Magnusson O, Stefansson K, Hartman D and Gurney M (2008) DG-041 inhibits the EP3 prostanoid receptor--a new target for inhibition of platelet function in atherothrombotic disease. *Platelets* **19**(8): 605-613.

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T and Catterall WA (1996) Modulation of Ca2+ channels by G-protein beta gamma subunits. *Nature* **380**(6571): 258-262.

Herlitze S, Hockerman GH, Scheuer T and Catterall WA (1997) Molecular determinants of inactivation and G protein modulation in the intracellular loop connecting

domains I and II of the calcium channel alpha1A subunit. *Proceedings of the National Academy of Sciences of the United States of America* **94**(4): 1512-1516.
Hermosilla T, Moreno C, Itfinca M, Altier C, Armisen R, Stutzin A, Zamponi GW and Varela D (2011) L-type calcium channel beta subunit modulates angiotensin II responses in cardiomyocytes. *Channels (Austin)* **5**(3): 280-286.
Hernandez-Ochoa EO, Garcia-Ferreiro RE and Garcia DE (2007) G protein activation inhibits gating charge movement in rat sympathetic neurons. *American journal of physiology Cell physiology* **292**(6): C2226-2238.
Hibino H, Pironkova R, Onwumere O, Vologodskaia M, Hudspeth AJ and Lesage F (2002) RIM binding proteins (RBPs) couple Rab3-interacting molecules (RIMs) to voltage-gated Ca(2+) channels. *Neuron* **34**(3): 411-423.
Higgins JB and Casey PJ (1994) In vitro processing of recombinant G protein gamma subunits. Requirements for assembly of an active beta gamma complex. *The Journal of biological chemistry* **269**(12): 9067-9073.
Hill J, Chan SA, Kuri B and Smith C (2011) Pituitary adenylate cyclase-activating peptide (PACAP) recruits low voltage-activated T-type calcium influx under acute sympathetic stimulation in mouse adrenal chromaffin cells. *The Journal of biological chemistry* **286**(49): 42459-42469.
Hille B (1994) Modulation of ion-channel function by G-protein-coupled receptors. *Trends in neurosciences* **17**(12): 531-536.
Hino T, Arakawa T, Iwanari H, Yurugi-Kobayashi T, Ikeda-Suno C, Nakada-Nakura Y, Kusano-Arai O, Weyand S, Shimamura T, Nomura N, Cameron AD, Kobayashi T, Hamakubo T, Iwata S and Murata T (2012) G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody. *Nature* **482**(7384): 237-240.
Hirata T and Narumiya S (2011) Prostanoid receptors. *Chemical reviews* **111**(10): 6209-6230.
Hollinger S and Hepler JR (2002) Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. *Pharmacological reviews* **54**(3): 527-559.
Hopkins AL, Mason JS and Overington JP (2006) Can we rationally design promiscuous drugs? *Current opinion in structural biology* **16**(1): 127-136.
Horowitz LF, Hirdes W, Suh BC, Hilgemann DW, Mackie K and Hille B (2005) Phospholipase C in living cells: activation, inhibition, Ca2+ requirement, and regulation of M current. *The Journal of general physiology* **126**(3): 243-262.
Horrigan FT and Bookman RJ (1994) Releasable pools and the kinetics of exocytosis in adrenal chromaffin cells. *Neuron* **13**(5): 1119-1129.
Huang CL, Jan YN and Jan LY (1997) Binding of the G protein betagamma subunit to multiple regions of G protein-gated inward-rectifying K+ channels. *FEBS letters* **405**(3): 291-298.
Huang H, Tan BZ, Shen Y, Tao J, Jiang F, Sung YY, Ng CK, Raida M, Kohr G, Higuchi M, Fatemi-Shariatpanahi H, Harden B, Yue DT and Soong TW (2012) RNA editing of the IQ domain in Ca(v)1.3 channels modulates their Ca(2)(+)-dependent inactivation. *Neuron* **73**(2): 304-316.
Huber K, Kalcheim C and Unsicker K (2009) The development of the chromaffin cell lineage from the neural crest. *Auton Neurosci* **151**(1): 10-16.
Ichitani Y, Holmberg K, Maunsbach AB, Haeggstrom JZ, Samuelsson B, De Witt D and Hokfelt T (2001) Cyclooxygenase-1 and cyclooxygenase-2 expression in rat

kidney and adrenal gland after stimulation with systemic lipopolysaccharide: in situ hybridization and immunocytochemical studies. *Cell and tissue research* **303**(2): 235-252.

Ikeda SR (1992) Prostaglandin modulation of Ca2+ channels in rat sympathetic neurones is mediated by guanine nucleotide binding proteins. *The Journal of physiology* **458**: 339-359.

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. *Nature* **380**(6571): 255-258.

Ikeda SR and Dunlap K (1999) Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. *Adv Second Messenger Phosphoprotein Res* **33**: 131-151.

Inchauspe CG, Forsythe ID and Uchitel OD (2007) Changes in synaptic transmission properties due to the expression of N-type calcium channels at the calyx of Held synapse of mice lacking P/Q-type calcium channels. *The Journal of physiology* **584**(Pt 3): 835-851.

Iniguez-Lluhi JA, Simon MI, Robishaw JD and Gilman AG (1992) G protein beta gamma subunits synthesized in Sf9 cells. Functional characterization and the significance of prenylation of gamma. *The Journal of biological chemistry* **267**(32): 23409-23417.

Insel PA, Snead A, Murray F, Zhang L, Yokouchi H, Katakia T, Kwon O, Dimucci D and Wilderman A (2012) GPCR expression in tissues and cells: are the optimal receptors being used as drug targets? *British journal of pharmacology* **165**(6): 1613-1616.

Irie A, Sugimoto Y, Namba T, Harazono A, Honda A, Watabe A, Negishi M, Narumiya S and Ichikawa A (1993) Third isoform of the prostaglandin-E-receptor EP3 subtype with different C-terminal tail coupling to both stimulation and inhibition of adenylate cyclase. *European journal of biochemistry / FEBS* **217**(1): 313-318.

Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, Ijzerman AP and Stevens RC (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science (New York, NY)* **322**(5905): 1211-1217.

Jarvis SE, Barr W, Feng ZP, Hamid J and Zamponi GW (2002) Molecular determinants of syntaxin 1 modulation of N-type calcium channels. *The Journal of biological chemistry* **277**(46): 44399-44407.

Jarvis SE, Magga JM, Beedle AM, Braun JE and Zamponi GW (2000) G protein modulation of N-type calcium channels is facilitated by physical interactions between syntaxin 1A and G betagamma. *Journal of Biological Chemistry* **275**: 6388-6394.

Jarvis SE and Zamponi GW (2001) Interactions between presynaptic Ca2+ channels, cytoplasmic messengers and proteins of the synaptic vesicle release complex. *Trends in pharmacological sciences* **22**(10): 519-525.

Jassal B, Jupe S, Caudy M, Birney E, Stein L, Hermjakob H and D'Eustachio P (2010) The systematic annotation of the three main GPCR families in Reactome. *Database : the journal of biological databases and curation* **2010**: baq018.

Jewell ML, Breyer RM and Currie KP (2011) Regulation of calcium channels and exocytosis in mouse adrenal chromaffin cells by prostaglandin EP3 receptors. *Molecular pharmacology* **79**(6): 987-996.

Ji R, Chou CL, Xu W, Chen XB, Woodward DF and Regan JW (2010) EP1 prostanoid receptor coupling to G i/o up-regulates the expression of hypoxia-inducible factor-1 alpha through activation of a phosphoinositide-3 kinase signaling pathway. *Molecular pharmacology* **77**(6): 1025-1036.

Jiang H, Kuang Y, Wu Y, Smrcka A, Simon MI and Wu D (1996) Pertussis toxin-sensitive activation of phospholipase C by the C5a and fMet-Leu-Phe receptors. *The Journal of biological chemistry* **271**(23): 13430-13434.

Jones SW and Elmslie KS (1997) Transmitter modulation of neuronal calcium channels. *J Membr Biol* **155**(1): 1-10.

Kaeser PS, Deng L, Wang Y, Dulubova I, Liu X, Rizo J and Sudhof TC (2011) RIM proteins tether Ca2+ channels to presynaptic active zones via a direct PDZ-domain interaction. *Cell* **144**(2): 282-295.

Kamal FA, Smrcka AV and Blaxall BC (2011) Taking the heart failure battle inside the cell: small molecule targeting of Gbetagamma subunits. *Journal of molecular and cellular cardiology* **51**(4): 462-467.

Kammermeier PJ, Xiao B, Tu JC, Worley PF and Ikeda SR (2000) Homer proteins regulate coupling of group I metabotropic glutamate receptors to N-type calcium and M-type potassium channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**(19): 7238-7245.

Karaplis AC, Funk CD and Powell WS (1989) Binding of prostaglandin E2 to cultured bovine adrenal chromaffin cells and its effect on catecholamine secretion. *Biochimica et biophysica acta* **1010**(3): 369-376.

Katoh H, Watabe A, Sugimoto Y, Ichikawa A and Negishi M (1995) Characterization of the signal transduction of prostaglandin E receptor EP1 subtype in cDNA-transfected Chinese hamster ovary cells. *Biochimica et biophysica acta* **1244**(1): 41-48.

Katritch V, Cherezov V and Stevens RC (2013) Structure-function of the G protein-coupled receptor superfamily. *Annual review of pharmacology and toxicology* **53**: 531-556.

Khan SM, Sleno R, Gora S, Zylbergold P, Laverdure JP, Labbe JC, Miller GJ and Hebert TE (2013) The expanding roles of Gbetagamma subunits in G protein-coupled receptor signaling and drug action. *Pharmacological reviews* **65**(2): 545-577.

Kim T and Loh YP (2005) Chromogranin A: a surprising link between granule biogenesis and hypertension. *The Journal of clinical investigation* **115**(7): 1711-1713.

Kisilevsky AE, Mulligan SJ, Altier C, Iftinca MC, Varela D, Tai C, Chen L, Hameed S, Hamid J, Macvicar BA and Zamponi GW (2008) D1 receptors physically interact with N-type calcium channels to regulate channel distribution and dendritic calcium entry. *Neuron* **58**(4): 557-570.

Kisilevsky AE and Zamponi GW (2008) D2 dopamine receptors interact directly with N-type calcium channels and regulate channel surface expression levels. *Channels (Austin)* **2**(4): 269-277.

Kitano J, Nishida M, Itsukaichi Y, Minami I, Ogawa M, Hirano T, Mori Y and Nakanishi S (2003) Direct interaction and functional coupling between metabotropic glutamate receptor subtype 1 and voltage-sensitive Cav2.1 Ca2+ channel. *The Journal of biological chemistry* **278**(27): 25101-25108.

Kiyonaka S, Wakamori M, Miki T, Uriu Y, Nonaka M, Bito H, Beedle AM, Mori E, Hara Y, De Waard M, Kanagawa M, Itakura M, Takahashi M, Campbell KP and Mori Y (2007) RIM1 confers sustained activity and neurotransmitter vesicle anchoring to presynaptic Ca2+ channels. *Nature neuroscience* **10**(6): 691-701.

Klugbauer N, Marais E and Hofmann F (2003) Calcium channel alpha2delta subunits: differential expression, function, and drug binding. *J Bioenerg Biomembr* **35**(6): 639-647.

Kooijman EE, Chupin V, de Kruijff B and Burger KN (2003) Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. *Traffic (Copenhagen, Denmark)* **4**(3): 162-174.

Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H and Morikawa K (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* **407**(6807): 971-977.

Laine L, Connors LG, Reicin A, Hawkey CJ, Burgos-Vargas R, Schnitzer TJ, Yu Q and Bombardier C (2003) Serious lower gastrointestinal clinical events with nonselective NSAID or coxib use. *Gastroenterology* **124**(2): 288-292.

Lambright DG, Sondek J, Bohm A, Skiba NP, Hamm HE and Sigler PB (1996) The 2.0 A crystal structure of a heterotrimeric G protein. *Nature* **379**(6563): 311-319.

Lebon G, Warne T, Edwards PC, Bennett K, Langmead CJ, Leslie AG and Tate CG (2011) Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. *Nature* **474**(7352): 521-525.

Lee A, Wong ST, Gallagher D, Li B, Storm DR, Scheuer T and Catterall WA (1999) Ca2+/calmodulin binds to and modulates P/Q-type calcium channels. *Nature* **399**(6732): 155-159.

Lee A, Zhou H, Scheuer T and Catterall WA (2003) Molecular determinants of Ca(2+)/calmodulin-dependent regulation of Ca(v)2.1 channels. *Proceedings of the National Academy of Sciences of the United States of America* **100**(26): 16059-16064.

Lee HK and Elmslie KS (2000) Reluctant gating of single N-type calcium channels during neurotransmitter-induced inhibition in bullfrog sympathetic neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**(9): 3115-3128.

Lehmann DM, Seneviratne AM and Smrcka AV (2008) Small molecule disruption of G protein beta gamma subunit signaling inhibits neutrophil chemotaxis and inflammation. *Molecular pharmacology* **73**(2): 410-418.

Leroy J, Richards MW, Butcher AJ, Nieto-Rostro M, Pratt WS, Davies A and Dolphin AC (2005) Interaction via a key tryptophan in the I-II linker of N-type calcium channels is required for beta1 but not for palmitoylated beta2, implicating an additional binding site in the regulation of channel voltage-dependent properties. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**(30): 6984-6996.

Li B, Zhong H, Scheuer T and Catterall WA (2004) Functional role of a C-terminal Gbetagamma-binding domain of Ca(v)2.2 channels. *Molecular pharmacology* **66**(3): 761-769.

Liang H, DeMaria CD, Erickson MG, Mori MX, Alseikhan BA and Yue DT (2003) Unified mechanisms of Ca2+ regulation across the Ca2+ channel family. *Neuron* **39**(6): 951-960.

Liao P, Yong TF, Liang MC, Yue DT and Soong TW (2005) Splicing for alternative structures of Cav1.2 Ca2+ channels in cardiac and smooth muscles. *Cardiovascular research* **68**(2): 197-203.

Lieb A, Scharinger A, Sartori S, Sinnegger-Brauns MJ and Striessnig J (2012) Structural determinants of CaV 1.3 L-type calcium channel gating. *Channels (Austin)* **6**(3).

Liu M, Yu B, Nakanishi O, Wieland T and Simon M (1997) The Ca2+-dependent binding of calmodulin to an N-terminal motif of the heterotrimeric G protein beta subunit. *The Journal of biological chemistry* **272**(30): 18801-18807.

Lodowski DT, Pitcher JA, Capel WD, Lefkowitz RJ and Tesmer JJ (2003) Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gbetagamma. *Science (New York, NY)* **300**(5623): 1256-1262.

Logothetis DE, Kurachi Y, Galper J, Neer EJ and Clapham DE (1987) The beta gamma subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. *Nature* **325**(6102): 321-326.

Lopez MG, Montiel C, Herrero CJ, Garcia-Palomero E, Mayorgas I, Hernandez-Guijo JM, Villarroya M, Olivares R, Gandia L, McIntosh JM, Olivera BM and Garcia AG (1998) Unmasking the functions of the chromaffin cell alpha7 nicotinic receptor by using short pulses of acetylcholine and selective blockers. *Proceedings of the National Academy of Sciences of the United States of America* **95**(24): 14184-14189.

Lu Q, AtKisson MS, Jarvis SE, Feng ZP, Zamponi GW and Dunlap K (2001) Syntaxin 1A supports voltage-dependent inhibition of alpha1B Ca2+ channels by Gbetagamma in chick sensory neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **21**(9): 2949-2957.

Lymperopoulos A, Rengo G, Funakoshi H, Eckhart AD and Koch WJ (2007) Adrenal GRK2 upregulation mediates sympathetic overdrive in heart failure. *Nat Med* **13**(3): 315-323.

Lymperopoulos A, Rengo G, Gao E, Ebert SN, Dorn GW, 2nd and Koch WJ (2010) Reduction of sympathetic activity via adrenal-targeted GRK2 gene deletion attenuates heart failure progression and improves cardiac function after myocardial infarction. *The Journal of biological chemistry* **285**(21): 16378-16386.

Lymperopoulos A, Rengo G, Zincarelli C, Soltys S and Koch WJ (2008) Modulation of adrenal catecholamine secretion by in vivo gene transfer and manipulation of G protein-coupled receptor kinase-2 activity. *Mol Ther* **16**(2): 302-307.

Machado DJ, Montesinos MS and Borges R (2008) Good practices in single-cell amperometry. *Methods in molecular biology (Clifton, NJ)* **440**: 297-313.

Macias-Perez IM, Zent R, Carmosino M, Breyer MD, Breyer RM and Pozzi A (2008) Mouse EP3 alpha, beta, and gamma receptor variants reduce tumor cell proliferation and tumorigenesis in vivo. *The Journal of biological chemistry* **283**(18): 12538-12545.

Mahapatra NR (2008) Catestatin is a novel endogenous peptide that regulates cardiac function and blood pressure. *Cardiovascular research* **80**(3): 330-338.

Mahapatra NR, O'Connor D T, Vaingankar SM, Hikim AP, Mahata M, Ray S, Staite E, Wu H, Gu Y, Dalton N, Kennedy BP, Ziegler MG, Ross J and Mahata SK (2005) Hypertension from targeted ablation of chromogranin A can be rescued by the human ortholog. *The Journal of clinical investigation* **115**(7): 1942-1952.

Manglik A, Kruse AC, Kobilka TS, Thian FS, Mathiesen JM, Sunahara RK, Pardo L, Weis WI, Kobilka BK and Granier S (2012) Crystal structure of the micro-opioid receptor bound to a morphinan antagonist. *Nature* **485**(7398): 321-326.

Marcantoni A, Carabelli V, Comunanza V, Hoddah H and Carbone E (2008) Calcium channels in chromaffin cells: focus on L and T types. *Acta physiologica (Oxford, England)* **192**(2): 233-246.

Marley PD, Bunn SJ and Livett BG (1988) Prostanoid responses of bovine adrenal medullary cells: lack of effect of opioids. *European journal of pharmacology* **145**(2): 173-181.

Marom M, Birnbaumer L and Atlas D (2011) Membrane depolarization combined with Gq-activated G-protein-coupled receptors induce transient receptor potential channel 1 (TRPC1)- dependent potentiation of catecholamine release. *Neuroscience* **189**: 132-145.

McDavid S and Currie KP (2006) G-proteins modulate cumulative inactivation of N-type (Cav2.2) calcium channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **26**(51): 13373-13383.

McIntire WE (2009) Structural determinants involved in the formation and activation of G protein betagamma dimers. *Neurosignals* **17**(1): 82-99.

Meir A, Bell DC, Stephens GJ, Page KM and Dolphin AC (2000) Calcium channel beta subunit promotes voltage-dependent modulation of alpha 1 B by G beta gamma. *Biophysical journal* **79**(2): 731-746.

Meunier FA, Osborne SL, Hammond GR, Cooke FT, Parker PJ, Domin J and Schiavo G (2005) Phosphatidylinositol 3-kinase C2alpha is essential for ATP-dependent priming of neurosecretory granule exocytosis. *Molecular biology of the cell* **16**(10): 4841-4851.

Michailidis IE, Zhang Y and Yang J (2007) The lipid connection-regulation of voltage-gated Ca(2+) channels by phosphoinositides. *Pflugers Archiv : European journal of physiology* **455**(1): 147-155.

Mirshahi T, Mittal V, Zhang H, Linder ME and Logothetis DE (2002) Distinct sites on G protein beta gamma subunits regulate different effector functions. *The Journal of biological chemistry* **277**(39): 36345-36350.

Mochida S, Sheng ZH, Baker C, Kobayashi H and Catterall WA (1996) Inhibition of neurotransmission by peptides containing the synaptic protein interaction site of N-type Ca2+ channels. *Neuron* **17**(4): 781-788.

Mochizuki-Oda N, Mori K, Negishi M and Ito S (1991) Prostaglandin E2 activates Ca2+ channels in bovine adrenal chromaffin cells. *J Neurochem* **56**(2): 541-547.

Mogami H, Lloyd Mills C and Gallacher DV (1997) Phospholipase C inhibitor, U73122, releases intracellular Ca2+, potentiates Ins(1,4,5)P3-mediated Ca2+ release and directly activates ion channels in mouse pancreatic acinar cells. *The Biochemical journal* **324 (Pt 2)**: 645-651.

Mori Y, Higuchi M, Masuyama N and Gotoh Y (2004) Adenosine A2A receptor facilitates calcium-dependent protein secretion through the activation of protein kinase A and phosphatidylinositol-3 kinase in PC12 cells. *Cell structure and function* **29**(4): 101-110.

Mosharov EV and Sulzer D (2005) Analysis of exocytotic events recorded by amperometry. *Nature methods* **2**(9): 651-658.

Mudd JO and Kass DA (2008) Tackling heart failure in the twenty-first century. *Nature* **451**(7181): 919-928.

Murali SS, Napier IA, Rycroft BK and Christie MJ (2012) Opioid-related (ORL1) receptors are enriched in a subpopulation of sensory neurons and prolonged activation produces no functional loss of surface N-type calcium channels. *The Journal of physiology* **590**(Pt 7): 1655-1667.

Mustafa T, Grimaldi M and Eiden LE (2007) The hop cassette of the PAC1 receptor confers coupling to Ca2+ elevation required for pituitary adenylate cyclase-activating polypeptide-evoked neurosecretion. *The Journal of biological chemistry* **282**(11): 8079-8091.

Nakamichi H and Okada T (2006) Local peptide movement in the photoreaction intermediate of rhodopsin. *Proceedings of the National Academy of Sciences of the United States of America* **103**(34): 12729-12734.

Nakamura K, Katoh H, Ichikawa A and Negishi M (1998) Inhibition of dopamine release by prostaglandin EP3 receptor via pertussis toxin-sensitive and -insensitive pathways in PC12 cells. *J Neurochem* **71**(2): 646-652.

Namba T, Sugimoto Y, Negishi M, Irie A, Ushikubi F, Kakizuka A, Ito S, Ichikawa A and Narumiya S (1993) Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature* **365**(6442): 166-170.

Negishi M, Ito S and Hayaishi O (1989) Prostaglandin E receptors in bovine adrenal medulla are coupled to adenylate cyclase via Gi and to phosphoinositide metabolism in a pertussis toxin-insensitive manner. *The Journal of biological chemistry* **264**(7): 3916-3923.

Negishi M, Ito S and Hayaishi O (1990) Involvement of protein kinase C in prostaglandin E2-induced catecholamine release from cultured bovine adrenal chromaffin cells. *The Journal of biological chemistry* **265**(11): 6182-6188.

Negishi M, Sugimoto Y, Irie A, Narumiya S and Ichikawa A (1993) Two isoforms of prostaglandin E receptor EP3 subtype. Different COOH-terminal domains determine sensitivity to agonist-induced desensitization. *The Journal of biological chemistry* **268**(13): 9517-9521.

Neher E (2006) A comparison between exocytic control mechanisms in adrenal chromaffin cells and a glutamatergic synapse. *Pflugers Archiv : European journal of physiology* **453**(3): 261-268.

Nowak R, Farrar JR, Brenner BE, Lewis L, Silverman RA, Emerman C, Hays DP, Russell WS, Schmitz N, Miller J, Singer E, Camargo CA, Jr. and Wood J (2013) Customizing Anaphylaxis Guidelines for Emergency Medicine. *The Journal of emergency medicine*.

Nussmeier NA, Whelton AA, Brown MT, Langford RM, Hoeft A, Parlow JL, Boyce SW and Verburg KM (2005) Complications of the COX-2 inhibitors parecoxib and

valdecoxib after cardiac surgery. *The New England journal of medicine* **352**(11): 1081-1091.

O'Farrell M and Marley PD (1997) Multiple calcium channels are required for pituitary adenylate cyclase-activating polypeptide-induced catecholamine secretion from bovine cultured adrenal chromaffin cells. *Naunyn-Schmiedeberg's archives of pharmacology* **356**(5): 536-542.

Okada T, Sugihara M, Bondar AN, Elstner M, Entel P and Buss V (2004) The retinal conformation and its environment in rhodopsin in light of a new 2.2 A crystal structure. *Journal of molecular biology* **342**(2): 571-583.

Okuda-Ashitaka E, Sakamoto K, Ezashi T, Miwa K, Ito S and Hayaishi O (1996) Suppression of prostaglandin E receptor signaling by the variant form of EP1 subtype. *The Journal of biological chemistry* **271**(49): 31255-31261.

Oldham WM and Hamm HE (2006) Structural basis of function in heterotrimeric G proteins. *Quarterly reviews of biophysics* **39**(2): 117-166.

Oldham WM and Hamm HE (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nature reviews Molecular cell biology* **9**(1): 60-71.

Opatowsky Y, Chen CC, Campbell KP and Hirsch JA (2004) Structural analysis of the voltage-dependent calcium channel beta subunit functional core and its complex with the alpha 1 interaction domain. *Neuron* **42**(3): 387-399.

Overington JP, Al-Lazikani B and Hopkins AL (2006) How many drug targets are there? *Nature reviews Drug discovery* **5**(12): 993-996.

Page KM, Canti C, Stephens GJ, Berrow NS and Dolphin AC (1998) Identification of the amino terminus of neuronal Ca2+ channel alpha1 subunits alpha1B and alpha1E as an essential determinant of G-protein modulation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **18**(13): 4815-4824.

Page KM, Heblich F, Margas W, Pratt WS, Nieto-Rostro M, Chaggar K, Sandhu K, Davies A and Dolphin AC (2010) N terminus is key to the dominant negative suppression of Ca(V)2 calcium channels: implications for episodic ataxia type 2. *The Journal of biological chemistry* **285**(2): 835-844.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science (New York, NY)* **289**(5480): 739-745.

Park D and Dunlap K (1998) Dynamic regulation of calcium influx by G-proteins, action potential waveform, and neuronal firing frequency. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **18**(17): 6757-6766.

Park JH, Scheerer P, Hofmann KP, Choe HW and Ernst OP (2008) Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature* **454**(7201): 183-187.

Patil PG, de Leon M, Reed RR, Dubel S, Snutch TP and Yue DT (1996) Elementary events underlying voltage-dependent G-protein inhibition of N-type calcium channels. *Biophysical journal* **71**(5): 2509-2521.

Peterson BZ, DeMaria CD, Adelman JP and Yue DT (1999) Calmodulin is the Ca2+ sensor for Ca2+ -dependent inactivation of L-type calcium channels. *Neuron* **22**(3): 549-558.

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC and Ferrin TE (2004) UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **25**(13): 1605-1612.

Philip F, Kadamur G, Silos RG, Woodson J and Ross EM (2010) Synergistic activation of phospholipase C-beta3 by Galpha(q) and Gbetagamma describes a simple two-state coincidence detector. *Current biology : CB* **20**(15): 1327-1335.

Pierce KL, Premont RT and Lefkowitz RJ (2002) Seven-transmembrane receptors. *Nature reviews Molecular cell biology* **3**(9): 639-650.

Polo-Parada L, Chan SA and Smith C (2006) An activity-dependent increased role for L-type calcium channels in exocytosis is regulated by adrenergic signaling in chromaffin cells. *Neuroscience* **143**(2): 445-459.

Powell AD, Teschemacher AG and Seward EP (2000) P2Y purinoceptors inhibit exocytosis in adrenal chromaffin cells via modulation of voltage-operated calcium channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**(2): 606-616.

Pragnell M, De Waard M, Mori Y, Tanabe T, Snutch TP and Campbell KP (1994) Calcium channel beta-subunit binds to a conserved motif in the I-II cytoplasmic linker of the alpha 1-subunit. *Nature* **368**(6466): 67-70.

Preininger AM, Henage LG, Oldham WM, Yoon EJ, Hamm HE and Brown HA (2006) Direct modulation of phospholipase D activity by Gbetagamma. *Molecular pharmacology* **70**(1): 311-318.

Qin N, Platano D, Olcese R, Stefani E and Birnbaumer L (1997) Direct interaction of gbetagamma with a C-terminal gbetagamma-binding domain of the Ca2+ channel alpha1 subunit is responsible for channel inhibition by G protein-coupled receptors. *Proceedings of the National Academy of Sciences of the United States of America* **94**(16): 8866-8871.

Ramwell PW, Shaw JE, Douglas WW and Poisner AM (1966) Efflux of prostaglandin from adrenal glands stimulated with acetylcholine. *Nature* **210**(5033): 273-274.

Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, Schnapp A, Konetzki I, Sunahara RK, Gellman SH, Pautsch A, Steyaert J, Weis WI and Kobilka BK (2011a) Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature* **469**(7329): 175-180.

Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah ST, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skiniotis G, Weis WI, Sunahara RK and Kobilka BK (2011b) Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* **477**(7366): 549-555.

Rebolledo-Antunez S, Farias JM, Arenas I and Garcia DE (2009) Gating charges per channel of Ca(V)2.2 channels are modified by G protein activation in rat sympathetic neurons. *Arch Biochem Biophys* **486**(1): 51-57.

Regan JW (2003) EP2 and EP4 prostanoid receptor signaling. *Life sciences* **74**(2-3): 143-153.

Regard JB, Sato IT and Coughlin SR (2008) Anatomical profiling of G protein-coupled receptor expression. *Cell* **135**(3): 561-571.

Renshaw D, Thomson LM, Michael GJ, Carroll M, Kapas S and Hinson JP (2000) Adrenomedullin receptor is found exclusively in noradrenaline-secreting cells of the rat adrenal medulla. *J Neurochem* **74**(4): 1766-1772.

Rettig J, Sheng ZH, Kim DK, Hodson CD, Snutch TP and Catterall WA (1996) Isoform-specific interaction of the alpha1A subunits of brain Ca2+ channels with the presynaptic proteins syntaxin and SNAP-25. *Proceedings of the National Academy of Sciences of the United States of America* **93**(14): 7363-7368.

Rhee JS, Betz A, Pyott S, Reim K, Varoqueaux F, Augustin I, Hesse D, Sudhof TC, Takahashi M, Rosenmund C and Brose N (2002) Beta phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. *Cell* **108**(1): 121-133.

Rivers EP, Coba V and Whitmill M (2008) Early goal-directed therapy in severe sepsis and septic shock: a contemporary review of the literature. *Current opinion in anaesthesiology* **21**(2): 128-140.

Roberts-Crowley ML, Mitra-Ganguli T, Liu L and Rittenhouse AR (2009) Regulation of voltage-gated Ca2+ channels by lipids. *Cell calcium* **45**(6): 589-601.

Rodbell M, Birnbaumer L, Pohl SL and Krans HM (1971) The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanylnucleotides in glucagon action. *The Journal of biological chemistry* **246**(6): 1877-1882.

Rosen H, Stevens RC, Hanson M, Roberts E and Oldstone MB (2013) Sphingosine-1-Phosphate and Its Receptors: Structure, Signaling, and Influence. *Annual review of biochemistry*.

Rosenbaum DM, Rasmussen SG and Kobilka BK (2009) The structure and function of G-protein-coupled receptors. *Nature* **459**(7245): 356-363.

Rundhaug JE, Simper MS, Surh I and Fischer SM (2011) The role of the EP receptors for prostaglandin E2 in skin and skin cancer. *Cancer metastasis reviews* **30**(3-4): 465-480.

Russ AP and Lampel S (2005) The druggable genome: an update. *Drug discovery today* **10**(23-24): 1607-1610.

Sala F, Nistri A and Criado M (2008) Nicotinic acetylcholine receptors of adrenal chromaffin cells. *Acta physiologica (Oxford, England)* **192**(2): 203-212.

Sankaran B, Osterhout J, Wu D and Smrcka AV (1998) Identification of a structural element in phospholipase C beta2 that interacts with G protein betagamma subunits. *The Journal of biological chemistry* **273**(12): 7148-7154.

Sanner MF, Olson AJ and Spehner JC (1996) Reduced surface: an efficient way to compute molecular surfaces. *Biopolymers* **38**(3): 305-320.

Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP and Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* **455**(7212): 497-502.

Schiff ML, Siderovski DP, Jordan JD, Brothers G, Snow B, De Vries L, Ortiz DF and Diverse-Pierluissi M (2000) Tyrosine-kinase-dependent recruitment of RGS12 to the N-type calcium channel. *Nature* **408**(6813): 723-727.

Schmid A, Thierauch KH, Schleuning WD and Dinter H (1995) Splice variants of the human EP3 receptor for prostaglandin E2. *European journal of biochemistry / FEBS* **228**(1): 23-30.

Schmidt M, Dekker FJ and Maarsingh H (2013) Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions. *Pharmacological reviews* **65**(2): 670-709.

Schneggenburger R and Forsythe ID (2006) The calyx of Held. *Cell and tissue research* **326**(2): 311-337.

Schwetz TA, Ustione A and Piston DW (2013) Neuropeptide Y and somatostatin inhibit insulin secretion through different mechanisms. *American journal of physiology Endocrinology and metabolism* **304**(2): E211-221.

Scott JK, Huang SF, Gangadhar BP, Samoriski GM, Clapp P, Gross RA, Taussig R and Smrcka AV (2001) Evidence that a protein-protein interaction 'hot spot' on heterotrimeric G protein betagamma subunits is used for recognition of a subclass of effectors. *The EMBO journal* **20**(4): 767-776.

Shen Y, Rampino MA, Carroll RC and Nawy S (2012) G-protein-mediated inhibition of the Trp channel TRPM1 requires the Gbetagamma dimer. *Proceedings of the National Academy of Sciences of the United States of America* **109**(22): 8752-8757.

Sheng ZH, Rettig J, Takahashi M and Catterall WA (1994) Identification of a syntaxin-binding site on N-type calcium channels. *Neuron* **13**(6): 1303-1313.

Sheng ZH, Yokoyama CT and Catterall WA (1997) Interaction of the synprint site of N-type Ca2+ channels with the C2B domain of synaptotagmin I. *Proceedings of the National Academy of Sciences of the United States of America* **94**(10): 5405-5410.

Shibuya I, Tanaka K, Uezono Y, Ueta Y, Toyohira Y, Yanagihara N, Izumi F and Yamashita H (1999) Prostaglandin E2 induces Ca2+ release from ryanodine/caffeine-sensitive stores in bovine adrenal medullary cells via EP1-like receptors. *J Neurochem* **73**(5): 2167-2174.

Silinsky EM (1984) On the mechanism by which adenosine recptor activation inhibits the release of acetylcholine from motor nerve endings. *Journal of Physiology* **346**: 243-256.

Silinsky EM (2005) Modulation of calcium currents is eliminated after cleavage of a strategic component of the mammalian secretory apparatus. *The Journal of physiology* **566**(Pt 3): 681-688.

Simen AA, Lee CC, Simen BB, Bindokas VP and Miller RJ (2001) The C terminus of the Ca channel alpha1B subunit mediates selective inhibition by G-protein-coupled receptors. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **21**(19): 7587-7597.

Simen AA and Miller RJ (1998) Structural features determining differential receptor regulation of neuronal Ca channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **18**(10): 3689-3698.

Smith C (1999) A persistent activity-dependent facilitation in chromaffin cells is caused by Ca2+ activation of protein kinase C. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **19**(2): 589-598.

Smith C, Moser T, Xu T and Neher E (1998) Cytosolic Ca2+ acts by two separate pathways to modulate the supply of release-competent vesicles in chromaffin cells. *Neuron* **20**(6): 1243-1253.

Smith CB and Eiden LE (2012) Is PACAP the major neurotransmitter for stress transduction at the adrenomedullary synapse? *Journal of molecular neuroscience : MN* **48**(2): 403-412.
Smrcka AV (2008) G protein betagamma subunits: central mediators of G protein-coupled receptor signaling. *Cell Mol Life Sci* **65**(14): 2191-2214.
Smrcka AV (2013) Molecular targeting of Galpha and Gbetagamma subunits: a potential approach for cancer therapeutics. *Trends in pharmacological sciences* **34**(5): 290-298.
Sondek J, Bohm A, Lambright DG, Hamm HE and Sigler PB (1996) Crystal structure of a G-protein beta gamma dimer at 2.1A resolution. *Nature* **379**(6563): 369-374.
Staal RG, Hananiya A and Sulzer D (2008) PKC theta activity maintains normal quantal size in chromaffin cells. *J Neurochem* **105**(5): 1635-1641.
Stacey M, Lin HH, Gordon S and McKnight AJ (2000) LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors. *Trends in biochemical sciences* **25**(6): 284-289.
Stanley EF and Mirotznik RR (1997) Cleavage of syntaxin prevents G-protein regulation of presynaptic calcium channels. *Nature* **385**(6614): 340-343.
Stephens GJ, Canti C, Page KM and Dolphin AC (1998) Role of domain I of neuronal Ca2+ channel alpha1 subunits in G protein modulation. *The Journal of physiology* **509 (Pt 1)**: 163-169.
Stotz SC and Zamponi GW (2001) Structural determinants of fast inactivation of high voltage-activated Ca(2+) channels. *Trends in neurosciences* **24**(3): 176-181.
Stoyanov B, Volinia S, Hanck T, Rubio I, Loubtchenkov M, Malek D, Stoyanova S, Vanhaesebroeck B, Dhand R, Nurnberg B and et al. (1995) Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science (New York, NY)* **269**(5224): 690-693.
Stroth N, Holighaus Y, Ait-Ali D and Eiden LE (2011) PACAP: a master regulator of neuroendocrine stress circuits and the cellular stress response. *Annals of the New York Academy of Sciences* **1220**: 49-59.
Sudhof TC (2012) The presynaptic active zone. *Neuron* **75**(1): 11-25.
Sugimoto Y and Narumiya S (2007) Prostaglandin E receptors. *The Journal of biological chemistry* **282**(16): 11613-11617.
Suh BC, Kim DI, Falkenburger BH and Hille B (2012) Membrane-localized beta-subunits alter the PIP2 regulation of high-voltage activated Ca2+ channels. *Proceedings of the National Academy of Sciences of the United States of America* **109**(8): 3161-3166.
Sun Y, McGarrigle D and Huang XY (2007) When a G protein-coupled receptor does not couple to a G protein. *Molecular bioSystems* **3**(12): 849-854.
Swartz KJ (1993) Modulation of Ca2+ channels by protein kinase C in rat central and peripheral neurons: disruption of G protein-mediated inhibition. *Neuron* **11**(2): 305-320.
Tadross MR, Ben Johny M and Yue DT (2010) Molecular endpoints of Ca2+/calmodulin- and voltage-dependent inactivation of Ca(v)1.3 channels. *The Journal of general physiology* **135**(3): 197-215.

Tan W, Du C, Siegelbaum SA and Role LW (1998) Modulation of nicotinic AChR channels by prostaglandin E2 in chick sympathetic ganglion neurons. *Journal of neurophysiology* **79**(2): 870-878.

Tang J, Maximov A, Shin OH, Dai H, Rizo J and Sudhof TC (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. *Cell* **126**(6): 1175-1187.

Tedford HW, Kisilevsky AE, Peloquin JB and Zamponi GW (2006) Scanning mutagenesis reveals a role for serine 189 of the heterotrimeric G-protein beta 1 subunit in the inhibition of N-type calcium channels. *Journal of neurophysiology* **96**(1): 465-470.

Tedford HW, Kisilevsky AE, Vieira LB, Varela D, Chen L and Zamponi GW (2010) Scanning mutagenesis of the I-II loop of the Cav2.2 calcium channel identifies residues Arginine 376 and Valine 416 as molecular determinants of voltage dependent G protein inhibition. *Mol Brain* **3**(1): 6.

Tedford HW and Zamponi GW (2006) Direct G protein modulation of Cav2 calcium channels. *Pharmacological reviews* **58**(4): 837-862.

Thompson AA, Liu W, Chun E, Katritch V, Wu H, Vardy E, Huang XP, Trapella C, Guerrini R, Calo G, Roth BL, Cherezov V and Stevens RC (2012) Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature* **485**(7398): 395-399.

Tomura H, Itoh H, Sho K, Sato K, Nagao M, Ui M, Kondo Y and Okajima F (1997) Betagamma subunits of pertussis toxin-sensitive G proteins mediate A1 adenosine receptor agonist-induced activation of phospholipase C in collaboration with thyrotropin. A novel stimulatory mechanism through the cross-talk of two types of receptors. *The Journal of biological chemistry* **272**(37): 23130-23137.

Toneff T, Funkelstein L, Mosier C, Abagyan A, Ziegler M and Hook V (2013) Beta-amyloid peptides undergo regulated co-secretion with neuropeptide and catecholamine neurotransmitters. *Peptides*.

Tosetti P, Taglietti V and Toselli M (1999) Action-potential-like depolarizations relieve opioid inhibition of N-type Ca2+ channels in NG108-15 cells. *Pflugers Archiv : European journal of physiology* **437**(3): 441-448.

Travis ER and Wightman RM (1998) Spatio-temporal resolution of exocytosis from individual cells. *Annu Rev Biophys Biomol Struct* **27**: 77-103.

Trimmer JS and Rhodes KJ (2004) Localization of voltage-gated ion channels in mammalian brain. *Annual review of physiology* **66**: 477-519.

Tselnicker I, Tsemakhovich VA, Dessauer CW and Dascal N (2010) Stargazin modulates neuronal voltage-dependent Ca(2+) channel Ca(v)2.2 by a Gbetagamma-dependent mechanism. *The Journal of biological chemistry* **285**(27): 20462-20471.

Ueda N, Iniguez-Lluhi JA, Lee E, Smrcka AV, Robishaw JD and Gilman AG (1994) G protein beta gamma subunits. Simplified purification and properties of novel isoforms. *The Journal of biological chemistry* **269**(6): 4388-4395.

Ulate G, Scott SR, Gonzalez J, Gilabert JA and Artalejo AR (2000) Extracellular ATP regulates exocytosis in inhibiting multiple Ca(2+) channel types in bovine chromaffin cells. *Pflugers Archiv : European journal of physiology* **439**(3): 304-314.

Uriu Y, Kiyonaka S, Miki T, Yagi M, Akiyama S, Mori E, Nakao A, Beedle AM, Campbell KP, Wakamori M and Mori Y (2010) Rab3-interacting molecule gamma isoforms lacking the Rab3-binding domain induce long lasting currents but block neurotransmitter vesicle anchoring in voltage-dependent P/Q-type Ca2+ channels. *The Journal of biological chemistry* **285**(28): 21750-21767.

Van Petegem F, Clark KA, Chatelain FC and Minor DL, Jr. (2004) Structure of a complex between a voltage-gated calcium channel beta-subunit and an alpha-subunit domain. *Nature* **429**(6992): 671-675.

Verhofstad AA, Coupland RE, Parker TR and Goldstein M (1985) Immunohistochemical and biochemical study on the development of the noradrenaline- and adrenaline-storing cells of the adrenal medulla of the rat. *Cell and tissue research* **242**(2): 233-243.

Videen JS, Mezger MS, Chang YM and O'Connor DT (1992) Calcium and catecholamine interactions with adrenal chromogranins. Comparison of driving forces in binding and aggregation. *The Journal of biological chemistry* **267**(5): 3066-3073.

Villanueva M and Wightman RM (2007) Facilitation of quantal release induced by a D1-like receptor on bovine chromaffin cells. *Biochemistry* **46**(12): 3881-3887.

Vitale N, Caumont AS, Chasserot-Golaz S, Du G, Wu S, Sciorra VA, Morris AJ, Frohman MA and Bader MF (2001) Phospholipase D1: a key factor for the exocytotic machinery in neuroendocrine cells. *The EMBO journal* **20**(10): 2424-2434.

von Ruden L and Neher E (1993) A Ca-dependent early step in the release of catecholamines from adrenal chromaffin cells. *Science (New York, NY)* **262**(5136): 1061-1065.

Wacker D, Fenalti G, Brown MA, Katritch V, Abagyan R, Cherezov V and Stevens RC (2010) Conserved binding mode of human beta2 adrenergic receptor inverse agonists and antagonist revealed by X-ray crystallography. *Journal of the American Chemical Society* **132**(33): 11443-11445.

Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG and Sprang SR (1995) The structure of the G protein heterotrimer Gi alpha 1 beta 1 gamma 2. *Cell* **83**(6): 1047-1058.

Watabe A, Sugimoto Y, Honda A, Irie A, Namba T, Negishi M, Ito S, Narumiya S and Ichikawa A (1993) Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor. *The Journal of biological chemistry* **268**(27): 20175-20178.

Weiss N, Sandoval A, Kyonaka S, Felix R, Mori Y and De Waard M (2011) Rim1 modulates direct G-protein regulation of Ca(v)2.2 channels. *Pflugers Archiv : European journal of physiology* **461**(4): 447-459.

Weiss N, Tadmouri A, Mikati M, Ronjat M and De Waard M (2007) Importance of voltage-dependent inactivation in N-type calcium channel regulation by G-proteins. *Pflugers Archiv : European journal of physiology* **454**(1): 115-129.

Wen PJ, Osborne SL, Morrow IC, Parton RG, Domin J and Meunier FA (2008) Ca2+-regulated pool of phosphatidylinositol-3-phosphate produced by phosphatidylinositol 3-kinase C2alpha on neurosecretory vesicles. *Molecular biology of the cell* **19**(12): 5593-5603.

Westenbroek RE, Hell JW, Warner C, Dubel SJ, Snutch TP and Catterall WA (1992) Biochemical properties and subcellular distribution of an N-type calcium channel alpha 1 subunit. *Neuron* **9**(6): 1099-1115.

Westenbroek RE, Sakurai T, Elliott EM, Hell JW, Starr TV, Snutch TP and Catterall WA (1995) Immunochemical identification and subcellular distribution of the alpha 1A subunits of brain calcium channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **15**(10): 6403-6418.

Wettschureck N and Offermanns S (2005) Mammalian G proteins and their cell type specific functions. *Physiological reviews* **85**(4): 1159-1204.

Wierda KD, Toonen RF, de Wit H, Brussaard AB and Verhage M (2007) Interdependence of PKC-dependent and PKC-independent pathways for presynaptic plasticity. *Neuron* **54**(2): 275-290.

Wightman RM, Jankowski JA, Kennedy RT, Kawagoe KT, Schroeder TJ, Leszczyszyn DJ, Near JA, Diliberto EJ, Jr. and Viveros OH (1991) Temporally resolved catecholamine spikes correspond to single vesicle release from individual chromaffin cells. *Proceedings of the National Academy of Sciences of the United States of America* **88**(23): 10754-10758.

Williams S, Serafin M, Muhlethaler M and Bernheim L (1997) Facilitation of N-type calcium current is dependent on the frequency of action potential-like depolarizations in dissociated cholinergic basal forebrain neurons of the guinea pig. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **17**(5): 1625-1632.

Wiser O, Tobi D, Trus M and Atlas D (1997) Synaptotagmin restores kinetic properties of a syntaxin-associated N-type voltage sensitive calcium channel. *FEBS letters* **404**(2-3): 203-207.

Womack MD and McCleskey EW (1995) Interaction of opioids and membrane potential to modulate Ca2+ channels in rat dorsal root ganglion neurons. *Journal of neurophysiology* **73**(5): 1793-1798.

Wu D, Katz A and Simon MI (1993) Activation of phospholipase C beta 2 by the alpha and beta gamma subunits of trimeric GTP-binding protein. *Proceedings of the National Academy of Sciences of the United States of America* **90**(11): 5297-5301.

Wu H, Wacker D, Mileni M, Katritch V, Han GW, Vardy E, Liu W, Thompson AA, Huang XP, Carroll FI, Mascarella SW, Westkaemper RB, Mosier PD, Roth BL, Cherezov V and Stevens RC (2012) Structure of the human kappa-opioid receptor in complex with JDTic. *Nature* **485**(7398): 327-332.

Wu WK, Sung JJ, Lee CW, Yu J and Cho CH (2010) Cyclooxygenase-2 in tumorigenesis of gastrointestinal cancers: an update on the molecular mechanisms. *Cancer letters* **295**(1): 7-16.

Wu X, Kushwaha N, Albert PR and Penington NJ (2002) A critical protein kinase C phosphorylation site on the 5-HT(1A) receptor controlling coupling to N-type calcium channels. *The Journal of physiology* **538**(Pt 1): 41-51.

Wurtman RJ and Axelrod J (1965) Adrenaline synthesis: control by the pituitary gland and adrenal glucocorticoids. *Science (New York, NY)* **150**(3702): 1464-1465.

Xu F, Wu H, Katritch V, Han GW, Jacobson KA, Gao ZG, Cherezov V and Stevens RC (2011) Structure of an agonist-bound human A2A adenosine receptor. *Science (New York, NY)* **332**(6027): 322-327.

Yamada S, Morita K, Dohi T and Tsujimoto A (1988) A modulating role of prostaglandins in catecholamine release by perfused dog adrenal glands. *European journal of pharmacology* **146**(1): 27-34.

Yao LH, Rao Y, Varga K, Wang CY, Xiao P, Lindau M and Gong LW (2012) Synaptotagmin 1 is necessary for the Ca2+ dependence of clathrin-mediated endocytosis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**(11): 3778-3785.

Yokohama H, Tanaka T, Ito S, Negishi M, Hayashi H and Hayaishi O (1988) Prostaglandin E receptor enhancement of catecholamine release may be mediated by phosphoinositide metabolism in bovine adrenal chromaffin cells. *The Journal of biological chemistry* **263**(3): 1119-1122.

Yokoyama CT, Myers SJ, Fu J, Mockus SM, Scheuer T and Catterall WA (2005) Mechanism of SNARE protein binding and regulation of Cav2 channels by phosphorylation of the synaptic protein interaction site. *Molecular and cellular neurosciences* **28**(1): 1-17.

Yoon EJ, Gerachshenko T, Spiegelberg BD, Alford S and Hamm HE (2007) Gbetagamma interferes with Ca2+-dependent binding of synaptotagmin to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. *Molecular pharmacology* **72**(5): 1210-1219.

Yoon EJ, Hamm HE and Currie KP (2008) G protein betagamma subunits modulate the number and nature of exocytotic fusion events in adrenal chromaffin cells independent of calcium entry. *Journal of neurophysiology* **100**(5): 2929-2939.

Zamponi GW, Bourinet E, Nelson D, Nargeot J and Snutch TP (1997) Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. *Nature* **385**(6615): 442-446.

Zamponi GW and Currie KP (2013) Regulation of CaV2 calcium channels by G protein coupled receptors. *Biochimica et biophysica acta* **1828**(7): 1629-1643.

Zamponi GW and Snutch TP (1998) Modulation of voltage-dependent calcium channels by G proteins. *Curr Opin Neurobiol* **8**(3): 351-356.

Zeniou-Meyer M, Zabari N, Ashery U, Chasserot-Golaz S, Haeberle AM, Demais V, Bailly Y, Gottfried I, Nakanishi H, Neiman AM, Du G, Frohman MA, Bader MF and Vitale N (2007) Phospholipase D1 production of phosphatidic acid at the plasma membrane promotes exocytosis of large dense-core granules at a late stage. *The Journal of biological chemistry* **282**(30): 21746-21757.

Zhang JF, Ellinor PT, Aldrich RW and Tsien RW (1996) Multiple structural elements in voltage-dependent Ca2+ channels support their inhibition by G proteins. *Neuron* **17**(5): 991-1003.

Zhang Y, Chen YH, Bangaru SD, He L, Abele K, Tanabe S, Kozasa T and Yang J (2008) Origin of the voltage dependence of G-protein regulation of P/Q-type Ca2+ channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**(52): 14176-14188.

Zhang Y, Guan Y, Schneider A, Brandon S, Breyer RM and Breyer MD (2000) Characterization of murine vasopressor and vasodepressor prostaglandin E(2) receptors. *Hypertension* **35**(5): 1129-1134.

Zhao Y, Fang Q, Straub SG, Lindau M and Sharp GW (2010) Noradrenaline inhibits exocytosis via the G protein betagamma subunit and refilling of the readily

releasable granule pool via the alpha(i1/2) subunit. *The Journal of physiology* **588**(Pt 18): 3485-3498.

Zhong H, Yokoyama CT, Scheuer T and Catterall WA (1999) Reciprocal regulation of P/Q-type Ca2+ channels by SNAP-25, syntaxin and synaptotagmin. *Nature neuroscience* **2**(11): 939-941.

Zhou JY, Siderovski DP and Miller RJ (2000) Selective regulation of N-type Ca channels by different combinations of G-protein beta/gamma subunits and RGS proteins. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**(19): 7143-7148.

Zhu Y and Ikeda SR (1994) VIP inhibits N-type Ca2+ channels of sympathetic neurons via a pertussis toxin-insensitive but cholera toxin-sensitive pathway. *Neuron* **13**(3): 657-669.

Ziegler MG, Elayan H, Milic M, Sun P and Gharaibeh M (2012) Epinephrine and the metabolic syndrome. *Current hypertension reports* **14**(1): 1-7.

Zucker RS and Regehr WG (2002) Short-term synaptic plasticity. *Annual review of physiology* **64**: 355-405.

Zuhlke RD, Pitt GS, Deisseroth K, Tsien RW and Reuter H (1999) Calmodulin supports both inactivation and facilitation of L-type calcium channels. *Nature* **399**(6732): 159-162.