Regulation of calcium channels and exocytosis in mouse adrenal chromaffin cells
by prostaglandin EP3 receptors

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Non-standard abbreviations: DG-041, (2E)-3-[l-[(2,4-dichlorophenyl)methyl]-5-fluoro-3-methyl-lH-indol-7-yl]-N-[(4,5-dichloro-2-thienyl)sulfonyl]-2-propenamide
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$_{v}$2 voltage-gated Ca$^{2+}$ channel currents ($I_{Ca}$). This inhibition was voltage-dependent and mediated by pertussis toxin-sensitive G proteins, consistent with a direct G$\beta$G$_{\gamma}$ 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$_2$. 

Mol # 68569
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\textsubscript{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\textsubscript{2} (PGE\textsubscript{2}) on chromaffin cells. PGE\textsubscript{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 PGE₂, 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 PGE₂ (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 PGE₂ production (Engstrom et al., 2008). Thus the components are in place for local modulation of chromaffin cells by PGE₂, but previous studies present confusing and contradictory findings: PGE₂ 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, PGE2 can modulate nicotinic ACh receptors (Du and Role, 2001; Tan et al., 1998) as well as voltage-gated calcium channels (Ikeda, 1992). Thus PGE2 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 PGE2 modulates calcium signaling in adrenal chromaffin cells using a combination of pharmacological tools and EP receptor knockout mice. We show that PGE2 did not alter the peak amplitude of nicotinic ACh receptor currents or resting intracellular [Ca$^{2+}$], but potently inhibited CaV2 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 $I_{Ca}$. PGE2 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 PGE2.
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 NaH2PO4·7H2O, 1 NaH2PO4·H2O, 10 Glucose, 10 HEPES. The glands were trimmed of fat and the cortex dissected from the medullae. The medullae were incubated for 15 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% CO₂ 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 holding potential of -80 mV. Data were subjected to linear capacitance and leak subtraction using standard P/N protocols. When determining the inhibition of \( I_{Ca} \) produced by PGE2 (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 PGE2 data were fit with a Boltzmann function of the form: \( Y = Y_{\text{max}} / \)
(1 + 10^\alpha(\text{Log} \text{ EC}_{50} - X)); where Y = \% inhibition of \text{IC}_{\alpha} \text{ and } X \text{ is the concentration of PGE}_2. \text{ The } \text{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 \mu M carbachol. This enabled multiple reproducible responses to be obtained from the same cell. The delay in current activation (see Fig 3A) 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 (\Delta C_m) 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 C_m. 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 C_m and G_s 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 \Delta C_m. After two
control responses cells were exposed to 100 nM PGE2 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 PGE2 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.

**[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’-GGTGGCCTAAAGTGATGGGACA -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’-GGTCATCTTACTCGGCACCTCTC -3’ (sense primer, nucleotides 1027-1052, 5’-
TCCCACTAACCCTCATCCACCAACAG -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**

Cells were perfused at a rate of ~ 4 ml/min with external solution consisting of (in mM): 136 NaCl, 2 KCl, 1 MgCl.6H2O, 10 Glucose, 10 HEPES, 10 CaCl.2H2O, pH 7.3 osmolarity ~ 305. All drugs were diluted (≥1000X) and perfused in this extracellular solution unless otherwise noted. For whole-cell recordings electrodes were filled with internal solution containing (in mM):110 CsCl, 10 EGTA, 20 HEPES, 4 MgCl2, 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. PGE2 (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.
Results

PGE₂ 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 PGE₂ 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 (Fig 1A). Application of 100 nM PGE₂ produced a significant and reversible inhibition of peak $I_{Ca}$ amplitude (Fig 1A) 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 (Fig 1B) and the data fit well with a Boltzmann function that yielded a maximal inhibition of 40% and an EC₅₀ of 5.5 nM consistent with the low nM affinities reported for PGE₂ binding to EP receptors (Breyer et al., 2001).

The inhibition of $I_{Ca}$ by PGE₂ 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 (Caᵥ₂.₁) and N-type (Caᵥ₂.₂) channels (Currie, 2010a). Although there are exceptions, in most cases this pathway involves GPCRs that couple to pertussis toxin-sensitive G₁₅/₁₆-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 2A, the inhibition of $I_{Ca}$ produced by PGE$_2$ was virtually abolished in pertussis toxin treated cells ($2 \pm 2.6\%$, $n = 6$ compared to $31 \pm 7.1\%$, $n = 7$, in control cells; $p < 0.002$). As a positive control we also used the P2Y receptor agonist ATP (100 $\mu$M), 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 ($29 \pm 8.5\%$, $n = 7$ in control cells compared to $7 \pm 5.4\%$, $n = 6$, 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 G$\beta\gamma$-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 G$\beta\gamma$ 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 2B 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 (Fig 2C), but the prepulse (immediately preceding P2) significantly reduced this inhibition from $43 \pm 6\%$ during P1 to $13 \pm 3\%$ during P2 ($n = 6$; $p < 0.001$) (Fig 2D). 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 CaV2 family of calcium channels, in particular P/Q-type (CaV2.1) and N-type (CaV2.2) channels (Currie, 2010a). Mouse chromaffin cells are known to express CaV2 channels (P/Q-type and N-type and R-type channels) and also members of the CaV1 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 (CaV2.2) and P/Q-type (CaV2.1) channels by pre-incubation with ω-conotoxin GVIA (1.5 μM) and ω-agatoxin IVA (400 nM) respectively, the inhibition by PGE2 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.

**Acute application of PGE2 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 PGE2 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 (Fig 3A). Under these conditions the inward current was primarily due to the relatively non-desensitizing nACh receptors found in chromaffin cells. 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) (Fig 3A). 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) (Fig 3B). 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).

**Acute 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 Fig 3A) suggesting that PGE2 did not activate an inward calcium current. We also used Fura-2 imaging to determine if PGE2 could elevate resting [Ca\(^{2+}\)], in individual mouse chromaffin cells. Our data showed that an acute (3-minute) application of 1 μM PGE2 had no effect on [Ca\(^{2+}\)], (82 ± 15 nM before and 85 ± 16 nM during application of PGE2; n = 9 cells from 7 independent experiments) (Fig 3C).

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

The data presented above demonstrated that PGE2 acts through a G\(_{i/o}\)-coupled GPCR to inhibit \(I_{Ca}\) in mouse chromaffin cells. Of the four known receptors for PGE2, 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\(_{α}\), EP3\(_{β}\) and EP3\(_{γ}\) (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 (Fig 4A).
We also detected mRNA for the EP1, EP2 and EP4 receptors (Fig 4B). GAPDH was used as an internal standard and amplified in all tissues (data not shown).

**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 (Fig 2A), so is mediated by $G_{i/o}$-coupled GPCRs. While we detected mRNA for all four EP receptor subtypes in the mouse adrenal gland (Fig 4), 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 (Fig 5A, 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; Fig 5C, 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) (Fig 5D), similar to the inhibition produced by ATP in the absence of DG-041 (29 ± 8.5 %; Fig 2B). This suggests DG-041 selectively blocked PGE2 and the downstream signaling pathways responsible for voltage-dependent inhibition of \( I_{Ca} \) were intact.

The inhibition of \( I_{Ca} \) produced by PGE2 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 PGE2. 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 6, the inhibition of \( I_{Ca} \) by PGE2 was abolished in cells isolated from EP3\(^{-/-}\) mice. In the same cells 100 \( \mu \)M 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 PGE2 and did not perturb G protein mediated inhibition of \( I_{Ca} \) by other receptors. Similarly, in perforated whole-cell recording PGE2 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 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 ($\Delta C_m$) reflects the number of vesicles that have undergone exocytosis. We used perforated whole-cell recordings to measure $I_{Ca}$ and $\Delta C_m$ evoked by two 100 ms steps from -80mV to +10mV (Fig 7A) 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 $\pm$ 10% (n = 7; p < 0.005) but had no effect in the remaining five cells (group-2) (3 $\pm$ 4% decrease; n = 5; p = 0.41) (Fig 7B). Application of 100 nM PGE$_2$ significantly reduced $\Delta C_m$ in group-1 (those cells in which $I_{Ca}$ was inhibited) from 153 $\pm$ 40 fF to 68 $\pm$ 16 fF (n = 7, p < 0.03). $\Delta C_m$ was also significantly smaller during application of PGE$_2$ in group-2, even though $I_{Ca}$ was not reduced in these cells (Fig 7C). 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 $\Delta C_m$ was significantly greater in group-1 cells (in which $I_{Ca}$ was also reduced) compared to group-2 cells (49 $\pm$ 7 %, n = 7 compared to 24 $\pm$ 4%, n = 5; p < 0.02) (Fig
7C) consistent with the idea that inhibition of Ca\(^{2+}\) entry by PGE\(_2\) leads to a parallel inhibition of exocytosis as reported for other G\(_\alpha\)-coupled GPCRs (Harkins and Fox, 2000; Powell et al., 2000; Ulate et al., 2000).
Discussion

Previous work has suggested that PGE2 might modulate adrenal chromaffin cells, although the effects and EP receptors involved remained unclear. The synthetic enzymes for PGE2 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 PGE2 within the adrenal gland. We previously reported that PGE2 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 PGE2 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 PGE2 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 paper we report that PGE2 inhibited $I_{Ca}$ in mouse chromaffin cells through pertussis toxin-sensitive G proteins. The inhibition was voltage-dependent (reversed by strong membrane depolarization) and preferentially targeted CaV2 calcium channels (N- and P/Q-type channels). Thus, PGE2 mimicked agonists of other Gi/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 G$\beta$G 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 murine splice variants (EP3\(_{\alpha}\), EP3\(_{\beta}\) and EP3\(_{\gamma}\)) in the adrenal gland using RT-PCR (Fig 4). 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 \([\text{Ca}^{2+}]_i\) (Fig 3C). 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) (Fig 3A), and had no effect on resting \([\text{Ca}^{2+}]_i\) in Fura-2 loaded cells (Fig 3C). 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 (Fig 3A, 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 ± 3%; \(p < 0.01\) compared to 9 ± 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 \(\alpha7\) 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 PGE2 modulation of chromaffin cell function.

Previous studies investigating the effects of PGE2 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 PGE2 inhibited release (Karaplis et al., 1989), while others report that PGE2 potentiated release (Marley et al., 1988; Yamada et al., 1988; Yokohama et al., 1988). As discussed above, high concentrations of PGE2 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 ($\Delta C_m$) that precisely reflect the surface area of a cell. The magnitude of $\Delta C_m$ reflects the number of vesicles that have undergone exocytosis. As previously reported for other GPCRs application of PGE2 led to a robust inhibition of $\Delta C_m$ (49 ± 7%) that paralleled inhibition of $I_{Ca}$ (41 ± 10%) (Fig 7). There was also a modest decrease in $\Delta C_m$ (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 (Fig 4). 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 ($49 \pm 7\%$, $n = 7$ compared to $24 \pm 4\%$, $n = 5$; $p < 0.02$) (Fig 7C) 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 identifying 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$_2$. 


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Authorship contribution:

*Participated in research design*: Jewell, Breyer, Currie.

*Conducted experiments*: Jewell.

*Contributed new reagents or analytic tools*: Breyer.

*Performed data analysis*: Jewell, Currie.

*Wrote or contributed to the writing of the manuscript*: Jewell, Breyer, Currie.

*Other*: Breyer and Currie acquired funding for the research.
References:


Footnotes:

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Legends for figures

Figure 1: PGE2 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 PGE2 (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 (PGE2), and after washout of PGE2 (wash). B) Log_{10} concentration response curve plotting percent inhibition of $I_{Ca}$ to varying concentrations of PGE2. Each cell was exposed to three increasing concentrations of PGE2, 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.

Figure 2: The inhibition of $I_{Ca}$ by PGE2 is voltage-dependent and mediated by pertussis toxin-sensitive G proteins. A) The percent inhibition of $I_{Ca}$ produced by 100 nM PGE2 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 PGE2 (** p < 0.002) and ATP (* p < 0.05). B) The inhibition of $I_{Ca}$ by PGE2 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 PGE2 (PGE2), and after washout (wash). The prepulse to +120 mV reversed most of the inhibition of $I_{Ca}$ produced by PGE2. (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 PGE2 of $I_{Ca}$ elicited by P1 (without a prepulse) and P2 (with a prepulse) (*** P < 0.001; n = 6).

Figure 3: PGE2 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 PGE2. 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 PGE2 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 PGE2 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 (PGE2) and after washout (wash) of 1µM PGE2, and the response to 50mM KCl. (n = 9 cells from 7 independent experiments).

Figure 4: 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.

**Fig 5: Pharmacological evidence that EP3 receptors mediate the inhibition of $I_{Ca}$ by PGE2.**  

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 PGE2 (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 PGE2.  

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 PGE2 (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 PGE2 (DG + PGE2; n = 9) or 100 μM ATP (DG + ATP; n = 4). DG-041 prevented the inhibition produced by PGE2 but not that produced by ATP.
Fig. 6: 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 fig 5B).

Fig. 7: 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 PGE2. 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 PGE2 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 PGE2: group-1 (black bar; n = 7/12 cells) in which PGE2 significantly reduced the amplitude of $I_{Ca}$, and group-2 (grey bar; n = 5/12 cells) in which PGE2 did not inhibit $I_{Ca}$ (* denotes p < 0.05 comparing group-1 and group-2 in the presence of PGE2). **C)** The change in membrane capacitance ($\Delta C_m$) in response to stimulation in the presence of PGE2 was normalized to $\Delta C_m$ 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. $\Delta C_m$ 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).
Figure 1

A

Peak amplitude (pA)

Time (min)

0 1 2 3 4 5 6 7 8 9

PGE$_2$

B

Percent inhibition of $I_{Ca}$

EC$_{50}$ = 5.5 nM

Log[PGE$_2$] (M)
Figure 4

A

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