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Title: Prostaglandin-induced activation of nociceptive neurons via direct interaction with TRPA1

Thomas E. Taylor-Clark, Bradley J. Undem, Donald W. MacGlashan Jr., Srinivas Ghatta, Michael J. Carr, and M. Allen McAlexander

Johns Hopkins University, Department of Medicine, 5501 Hopkins Bayview Circle, Baltimore, MD 21224 (T.E.T.-C., B.J.U., D.W.M.)

GlaxoSmithKline Pharmaceuticals, Respiratory and Inflammation Centre of Excellence for Drug Discovery, Target Discovery, 709 Swedeland Rd, King of Prussia, PA 19406 (S.G., M.J.C., M.A.M.)

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Corresponding author:

Bradley J. Undem, Ph.D., Professor of Medicine

Johns Hopkins Medical Institutions

Johns Hopkins Asthma and Allergy Center

3A.44

Baltimore, MD 21224

Phone: (410)550-2160

Fax: (410)550-2130

Email: bundem@jjhmi.edu

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Abbreviations: PG, prostaglandin; 15dPGJ$_2$; 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; AITC, allyl isothiocyanate; Caps, capsaicin; COX, cyclooxygenase; RR, Ruthenium Red; I-RTX, Iodoresiniferatoxin; HC-030031, 1,2,3,6-tetrahydro-1,3-dimethyl-N-[4-(1-methylethyl)phenyl]-2,6-; hTRPA1-HEK, HEK cells stably transfected with human TRPA1 channels; nt-HEK, non-transfected HEK cells.

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Abstract

Inflammation contributes to pain hypersensitivity through multiple mechanisms. Among the most well-characterized of these is the sensitization of primary nociceptive neurons by arachidonic acid metabolites such as prostaglandins through G protein-coupled receptors. However, in light of the recent discovery that the nociceptor-specific ion channel TRPA1 can be activated by exogenous electrophilic irritants through direct covalent modification, we reasoned that electrophilic carbon-containing A- and J-series prostaglandins, metabolites of prostaglandin E_2 and D_2 (PGE_2 and PGD_2), respectively, would excite nociceptive neurons through direct activation of TRPA1. Consistent with this prediction, the PGD_2 metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($15dPGJ_2$), activated heterologously expressed human TRPA1 (hTRPA1-HEK), as well as a subset of chemosensitive mouse trigeminal neurons. $15dPGJ_2$'s effects on neurons were blocked by both the non-selective TRP channel blocker ruthenium red and the TRPA1 inhibitor HC-030031, but unaffected by the TRPV1 blocker iodo-resiniferatoxin. In whole-cell patch clamp studies on hTRPA1-HEK cells, $15dPGJ_2$ evoked currents similar to equimolar AITC in the nominal absence of calcium, suggesting a direct mechanism of activation. Consistent with the hypothesis that TRPA1 activation required reactive electrophilic moieties, A- and J-series prostaglandins and the isoprostane 8-iso prostaglandin A_2 evoked calcium influx in hTRPA1-HEK cells with similar potency and efficacy. Notably, this effect was not mimicked by their non-electrophilic precursors, PGE_2 and PGD_2, or PGB_2, which differs from prostaglandin A_2 (PGA_2) only in that its electrophilic carbon is rendered unreactive through steric hindrance. Taken together, these data suggest a novel mechanism through which reactive prostanoids may activate nociceptive neurons independent of prostaglandin receptors.

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Introduction

Peripheral inflammation induces the formation of prostaglandins (PGs), both centrally and peripherally, which contribute to pain sensation and sensitivity (Burian and Geisslinger, 2005; Woolf and Costigan, 1999). During inflammation, a superfamily of phospholipase A_2 enzymes hydrolyzes membrane phospholipids to release arachidonic acid, which is subsequently converted by cyclooxygenases (COXs) into PGH_2. Through the actions of tissue-specific isomerases, a variety of prostaglandins are formed from this intermediate; for example, PGE_2, PGD_2 and PGI_2. The contribution of prostaglandins to inflammatory pain is extensively documented and is demonstrated by the analgesic properties of COX inhibitors (Burian and Geisslinger, 2005). Research has followed into the specific mechanisms and pathways through which prostaglandins contribute to inflammation and nociception and many prostaglandin receptors (including those for PGE_2, PGD_2 and PGI_2) have been demonstrated on sensory nerves (Jenkins et al., 2001; Moriyama et al., 2005).

The non-selective cation channel *transient receptor potential* (TRP) A1 is primarily expressed in small diameter, nociceptive neurons (Hjerling-Leffler et al., 2007; Katsura et al., 2006; Story et al., 2003), where its activation likely contributes to the perception of noxious stimuli and inflammatory hyperalgesia (Bautista et al., 2006; Kwan et al., 2006; McNamara et al., 2007). Multiple mechanisms converge to regulate TRPA1's function, as it can be activated by inflammatory mediators through Gq/PLC pathways (Bandell et al., 2004; Jordt et al., 2004), by intracellular calcium directly (Doerner et al., 2007; Zurborg et al., 2007) and by plant-derived compounds such as carvacrol (Xu et al., 2006) and Δ9-THC (Jordt et al., 2004). In addition, recent studies have described a novel mechanism through which allyl isothiocyanate (AITC) and other electrophilic irritants can activate TRPA1 by covalently binding to cysteine residues within the cytosolic N-terminus of the channel (Hinman et al., 2006; Macpherson et al., 2007).

Inflammation can lead to the formation of electrophilic compounds in vivo (Stamatakis and Perez-Sala, 2006). Specifically, cyclopentenone ring-containing A- and J-series prostaglandins are formed as nonenzymatic dehydration products of PGE_2 and PGD_2, respectively (Fitzpatrick and Waynalda, 1981; Herlong and Scott, 2006). While these molecules have been studied due to their effects on cell growth,

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cytokine production, chemotaxis and cytotoxicity (Gayarre et al., 2006; Herlong and Scott, 2006), their role in nociception remains largely unexplored. We hypothesize here that certain prostanoids may participate in nociception independently of prostaglandin receptors. Specifically, we address the hypothesis that prostanoids containing electrophilic moieties may stimulate nociceptive sensory nerves via direct activation of TRPA1, suggesting a novel mechanism through which inflammation stimulates nociceptive pathways.

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Materials and Methods

Dissociation of mouse trigeminal neurons: The methods were modified from those previously described (Taylor-Clark et al., 2005). All experiments were approved by the Johns Hopkins Animal Care and Use Committee. Briefly, male C57BI6 mice (20-40 g) were euthanized by CO_2 overdose and the trigeminal ganglia rapidly dissected and cleared of adhering connective tissue. The medial portion of the ganglia was isolated (contains neurons that innervate the upper airways) and incubated in 2 mg/mL collagenase type 1A and 2 mg/mL dispase II in 2 mL Ca^{2+}-free, Mg^{2+}-free HBSS (18 hours, 4°C; then 10 min, 37°C). Neurons were dissociated by trituration, washed by centrifugation, resuspended in L-15 medium containing 10% FBS and then transferred onto circular 25 mm glass coverslips (Bellco Glass Inc, Vineland, NJ) coated with poly-D-lysine (0.1 mg/mL) and laminin (5 μg/mL, 25 μL per coverslip). Coverslips were used within 24 hours.

HEK293 cell culture: In addition to wild-type HEK293 cells, cells stably expressing human TRPA1 (hTRPA1-HEK (Hill and Schaefer, 2007)) or human TRPV1 (hTRPV1-HEK (Hayes et al., 2000)) were used in the current study. Cells were maintained in an incubator (37˚C, 5% CO_2) in DMEM (containing 110 mg/L pyruvate) supplemented with 10% FBS and 500 μg/mL Geneticin as a selection agent. Cells were removed from their culture flasks by treatment with Accutase (Sigma), then plated onto poly-lysine-coated cover slips (BD Biosciences, Bedford) and incubated at 37˚C for >1 h before experimentation.

Calcium imaging: HEK293-covered coverslips were loaded with Fura 2 acetyoxymethyl ester (Fura-2 AM; 8 μM) (Molecular Probes, Carlsbad, Calif) in DMEM (containing 110 mg/L pyruvate) supplemented with 10% FBS and incubated (40 min, 37°C, 5% CO_2). Neuron-covered coverslips were loaded with Fura-2 AM (8 μM) in L-15 media containing 20% FBS and incubated (40 min, 37°C). For imaging, the coverslip was placed in a custom-built chamber (bath volume of 600 μL) and superfused at 4 mL/min with Locke solution (34°C) for 15 minutes before each experiment by an infusion pump.

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Changes in intracellular free calcium concentration (intracellular $[Ca^{2+}]_{free}$) were measured by digital microscopy (Universal; Carl Zeiss, Inc, Thornwood, NY) equipped with in-house equipment for ratiometric recording of single cells. The field of cells was monitored by sequential dual excitation, 352 and 380 nm, and the analysis of the image ratios used methods previously described to calculate changes in intracellular $[Ca^{2+}]_{free}$ (MacGlashan, 1989). The ratio images were acquired every 6 seconds. Superfused buffer was stopped 30 seconds before each drug application, when 300 μL buffer was removed from the bath and replaced by 300 μL of 2X test agent solution added between image acquisitions. Following treatments, neurons were exposed to KCl (30 seconds, 75 mM) to confirm voltage sensitivity. At the end of experiments, both neurons and HEK cells were exposed to ionomycin (30 seconds, 1 μM) to obtain a maximal response.

Patch-clamp experiments: Conventional whole cell patch-clamp recordings were performed at room temperature (21-24°C) using a Multiclamp 700B amplifier and pCLAMP 9 software (Molecular Devices, Sunnyvale, CA). Pipettes (3-5.5 MΩ) fabricated from borosilicate glass (Sutter Instruments, Novato) were filled with an internal solution composed of (mM): 140 CsCl, 4 $MgCl_2$, 10 HEPES, and 5 EGTA; pH was adjusted to 7.2 with CsOH. Cover slips were superfused continuously during recording with an external solution composed of (in mM): 140 NaCl, 2 $MgCl_2$, 5 CsCl, 10 HEPES, and 10 D-Glucose (pH adjusted to 7.4 with NaOH) and gassed with 95% O_2 – 5% CO_2. Only cells with <10 MΩ series resistances were used and compensated up to 80%. Currents were sampled at 500 Hz, and recordings were filtered at 10 kHz. The membrane potential was held at 0 mV prior to exposing the cell to a series of voltage ramps (-80 to +80 mV over 500 ms). Data were analyzed using clampfit software and transferred to Excel spreadsheets or GraphPad Prism 4 for further analysis.

Chemicals: Stock solutions (200X+) of all agonists were dissolved in 100% ethanol and 100% DMSO for HC-030031. Prostaglandins were purchased from Cayman Chemicals (Ann Arbor, MI). Fura 2AM was purchased from Molecular Probes (Carlsbad, CA). 1,2,3,6-tetrahydro-1,3-dimethyl-N-[4-(1-methylethyl)phenyl]-2,6- (also known as HC-030031 (McNamara et al., 2007)) was purchased from

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ChemBridge (San Diego, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All drugs were diluted fresh on the day of experiment, except for PGD_2 and PGE_2, which were diluted 5 minutes prior to each application in order to minimize their non-enzymatic dehydration (Ito et al., 1988).

Statistics: For the analysis of Fura-2 AM loaded cells, the measurement software converted ratiometric information to intracellular $[Ca^{2+}]_{free}$ using a default set of Tsien parameters (Grynkiewicz et al., 1985) particular to this instrumentation and a broad selection of cells. We did not specifically calibrate the relationship between ratiometric data and absolute calcium concentration, choosing instead to use the default parameters provided and relate all measurements to the peak ionomycin response in each viable cell. This effectively provided the needed cell-to-cell calibration for enumerating individual neuronal responses. Only cells that had a robust response to ionomycin (>400 nM) were included in analyses (maximum HEK cell apparent $[Ca^{2+}]_{free}$ response to ionomycin: 1406 nM (standard deviation of 202 nM, 2784 cells); maximum neuronal apparent $[Ca^{2+}]_{free}$ response to ionomycin: 1149 nM (standard deviation of 374 nM, 396 cells)). At each time point for each cell data was presented as the percentage change in intracellular $[Ca^{2+}]_{free}$, normalized to ionomycin: $response_x = 100 \text{ X } ([Ca^{2+}]_x-[Ca^{2+}]_{bl})/([Ca^{2+}]_{max}-[Ca^{2+}]_{bl})$, where $[Ca^{2+}]_x$ was the apparent $[Ca^{2+}]_{free}$ of the cell at a given time point, $[Ca^{2+}]_{bl}$ was the cell's mean baseline apparent $[Ca^{2+}]_{free}$ measured over 120s, and $[Ca^{2+}]_{max}$ was the cell's peak apparent $[Ca^{2+}]_{free}$ during ionomycin treatment. For the neuronal experiments, neurons were defined as 'responders' to a given compound if the mean response was greater than the mean baseline plus 2 X the standard deviation. Only neurons that responded to KCl were included in analyses. All data are mean ± standard error of the mean. Unpaired t-tests were used for statistical analysis when appropriate. A p value of less than 0.05 was taken as significant.

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Results

In order to test the hypothesis that endogenously occurring prostaglandins containing electrophilic groups might activate TRPA1 on native nociceptive neurons, we used calcium imaging to examine the responses of dissociated mouse trigeminal neurons to the dehydration product of PGD_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15dPGJ$_2$, 100 μM), the TRPA1 agonist allyl isothiocyanate (AITC, 100 μM) and the TRPV1 agonist capsaicin (1 μM). All three compounds robustly activated trigeminal neurons, with 50% (81/162) responding to 15dPGJ$_2$, 49% (388/794) responding to AITC and 46% (339/730) responding to capsaicin (Fig. 1A, B and C, respectively). In general, responses to all agonists tended to wane over time, and in some neurons this happened in the continued presence of the agonist.

Since 15dPGJ$_2$ possesses electrophilic moieties that may activate TRPA1, and most (86%) trigeminal neurons that responded to 15dPGJ$_2$ also responded to TRPV1 and/or TRPA1 agonists, we used the selective TRPV1 blocker iodoresiniferatoxin (I-RTX), the non-selective TRP channel blocker ruthenium red (RR) and the selective inhibitor of TRPA1 channel responses HC-030031 (McNamara et al., 2007) to determine whether these agents activated trigeminal neurons through TRP-dependent pathways. I-RTX, at a concentration (1 μM) that abolished capsaicin sensitivity (0/108 responsive neurons; Fig. 1C), did not reduce the responses of native neurons to 15dPGJ$_2$ or AITC (Fig. 1A and B, respectively). By contrast, RR (30 μM) reduced the response to all three agonists, as only 8% (7/86) of neurons responded to 15dPGJ$_2$, 6% (8/138) responded to AITC and 9% (5/53) responded to capsaicin (Fig. 1A, B and C, respectively). In the remaining minority of neurons that retained TRP agonist sensitivity in the presence of RR, the magnitude of their responses was dramatically reduced ($p<0.005$). 10 μM HC-030031 reduced the number of 15dPGJ$_2$-responsive neurons by 50% and inhibited the response magnitude of these responders by 54% (data not shown). In the presence of 100 μM HC-030031, only 8% (7/87) of neurons responded to 15dPGJ$_2$, and the magnitude of the responses of the remaining minority of neurons that retained 15dPGJ$_2$ sensitivity was dramatically reduced ($p<0.005$, Fig. 1A). Although the peak mean response to AITC was unaffected by the selective TRPA1 inhibitor, the

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area under the mean response versus time curve was significantly reduced (by 46%, $p<0.005$) and the time to peak response was increased from 24 seconds to 120 seconds in the presence of HC-030031 (Fig. 1B). HC-030031 (100 μM) did not reduce the response of capsaicin-sensitive native neurons (Fig. 1C).

In further support of the hypothesis that $15dPGJ_2$ and AITC share activation pathways in trigeminal neurons, pretreatment with AITC (100 μM) for 2 minutes, followed by a 3 minute washout, caused a marked desensitization to subsequent application of either AITC (100 μM) or $15dPGJ_2$ (100 μM), but did not alter the percentage of neurons responding or the maximal response to 1μM capsaicin (Fig. 2A-B).

In order to confirm that $15dPGJ_2$ activates TRPA1, we examined the response to this agonist in HEK293 cells stably transfected with human TRPA1 (hTRPA1-HEK). As predicted, hTRPA1-HEK cells in calcium imaging assays responded robustly to 100 μM AITC (68 ±1.5% of ionomycin, n=332), whereas 100 μM AITC failed to activate non-transfected HEK cells (nt-HEK; 0.5 ±0.1% of ionomycin, n=331). $15dPGJ_2$ (1-100 μM) also robustly activated hTRPA1-HEK cells (74 ±1.7% of ionomycin, n=236) and this response was absent in hTRPA1-HEK cells pretreated with RR (30 μM) (6.4 ±0.6% of ionomycin, n=241) and in nt-HEK cells (2.4 ±0.2% of ionomycin, n=317, Fig. 3A-B).

In addition to calcium imaging assays, we performed whole cell patch clamp recordings on hTRPA1-HEK cells, in which, as noted in Materials & Methods, calcium was excluded from the bath and pipette solutions and the chelator EGTA was added to the pipette solution to minimize the impact of calcium-dependent signaling pathways and any confounding direct effects of calcium on channel activity (Doerner et al., 2007; Zurborg et al., 2007). As predicted, AITC activated TRPA1 channels in these cells, with 10 μM AITC evoking outwardly rectifying currents with peak current densities of -57.4 + 16.2 pA/pF and 199.6 + 48.3 pA/pF at -70 and 70mV, respectively (n=6) in hTRPA1-HEK cells exposed to 500ms voltage ramps from -80 to +80 mV (Fig. 4A & E). Consistent with our hypothesis that $15dPGJ_2$ directly activates TRPA1, voltage ramps in the presence of 10 μM $15dPGJ_2$ evoked outwardly rectifying currents (Fig. 4B-C) of a magnitude (peak current densities of -42.8 + 10.9 pA/pF and 131.7 + 18.5 pA/pF

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at -70 and 70mV, respectively; n=8; Fig. 4E) similar to those evoked by the same concentration of AITC. Furthermore, we sought to gauge the selectivity of $15dPGJ_2$ in activating nociceptive transducers by applying it to HEK293 cells stably-transfected with human TRPV1 channels (hTRPV1-HEK). Using the same voltage ramp protocol that we employed in hTRPA1-HEK cells, we observed small baseline currents with peak densities of -0.8 + 0.6 pA/pF and 18.3 + 6.6 pA/pF at -70 and 70 mV, respectively (n=6) in hTRPV1-HEK cells. Exposing these cells to 10 μM $15dPGJ_2$ (n=5) did not appreciably alter baseline currents (peak densities of -0.0 + 0.5 pA/pF and 16.3 + 4.8 pA/pF at -70 and 70 mV, respectively) after $\geq$5 min treatment (Fig. 4D-E). By contrast, capsaicin (1 or 3 μM), applied at the end of experiments as a positive control for cell viability and transgene expression, evoked outwardly-rectifying currents with peak densities of -12.2 + 3.4 pA/pF and 151.3 + 33.8 pA/pF at -70 and 70mV, respectively (n=8; Fig. 4D-E).

Since a subset of known exogenous activators of TRPA1 (e.g., AITC) and $15dPGJ_2$ possess electrophilic moieties, we used calcium imaging to investigate the role of α,β unsaturated carbonyls in the TRPA1 activation caused by products downstream of COX activity (Fig. 5). Consistent with the hypothesis that electrophilic moieties are required for activation of TRPA1 both PGA_2 (100 μM), an electrophilic dehydration product of PGE_2, and Δ^{12}-PGJ_2 (100 μM), an electrophilic intermediate in the metabolism of PGD_2 to $15dPGJ_2$, activated hTRPA1-HEK cells but not nt-HEK cells in a similar manner to $15dPGJ_2$. However, PGD_2 and PGE_2, which do not contain any electrophilic carbons, failed to activate hTRPA1-HEK cells at a concentration of 100 μM. Interestingly, PGB_2 (100 μM), which is nearly identical in structure to PGA_2, with the exception that its reactive double bond is sterically hindered (Ohno et al., 1990), caused at most a trivial activation of hTRPA1-HEK cells (4.0±0.5% of ionomycin). Finally, 100 μM 8-iso PGA_2, a COX-independent product of oxidative stress-induced peroxidation of arachidonic acid, with an identical electrophilic group to PGA_2, also activated hTRPA1-HEK cells but not nt-HEK cells in a similar manner to PGA_2. Dose response curves (1, 10 and 100 μM) were constructed in both hTRPA1-HEK cells and dissociated mouse trigeminal neurons for those prostanoids that activated

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the TRPA1 channel (Figs. 5B and 5C). Activation of hTRPA1-HEK cells and neurons followed the same rank order: 15dPGJ$_2$ > Δ^{12}-PGJ$_2$ > PGA$_2$ = 8-iso PGA$_2$, suggesting that these agents increase calcium in neurons through the same mechanism that is activated in hTRPA1-HEK cells.

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Discussion

Recent studies have shown that AITC, cinnamaldehyde and other exogenous irritants with electrophilic groups directly activate TRPA1 (Hinman et al., 2006; Macpherson et al., 2007; McNamara et al., 2007). Our work expands upon these studies by demonstrating that electrophilic molecules that are produced downstream of COX activity during inflammation can also directly activate the channel.

Trigeminal neurons responded to 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($15dPGJ_2$) with an increase in intracellular calcium, and this response was blocked by the non-selective TRP channel blocker ruthenium red and the TRPA1 inhibitor HC-030031, but not by the TRPV1 blocker I-RTX. In addition, AITC selectively desensitized the neurons' response to subsequent exposure to AITC and $15dPGJ_2$ but had no effect on subsequent capsaicin-induced responses. Taken together, these results suggest that $15dPGJ_2$ activates TRPA1-containing ion channels on trigeminal neurons.

Our observation that $15dPGJ_2$, like AITC, causes calcium influx in hTRPA1-HEK cells but not nt-HEK cells, provides direct evidence that $15dPGJ_2$ activates TRPA1. Since TRPA1 can be indirectly stimulated through increases in intracellular calcium (Doerner et al., 2007; Zurborg et al., 2007), it is possible that $15dPGJ_2$ activated TRPA1 as a consequence of uncharacterized mechanism(s) leading to elevated intracellular calcium. This is unlikely, however, as $15dPGJ_2$ activated TRPA1 in whole cell patch-clamp studies carried out at room temperature in the nominal absence of calcium. In addition, our assays detected essentially no $15dPGJ_2$-mediated increase in intracellular calcium in nt-HEK cells.

Prostanoids that contain one or two electrophilic carbons ($15dPGJ_2$, Δ^{12}-PGJ_2, 8-iso PGA_2 and PGA_2) each effectively activated trigeminal neurons and TRPA1-expressing HEK cells with a similar rank order ($15dPGJ_2 > \Delta^{12}$-$PGJ_2 >$ 8-iso $PGA_2 = PGA_2$). By contrast, their structurally-related precursors which lack electrophilic carbons (PGD_2, PGE_2) failed to cause more than a trivial activation of TRPA1. Critically, PGB_2, which is structurally identical to PGA_2 with the exception that its reactive double bond spans the 8 and 12 carbons and is thus sterically hindered, also failed to activate TRPA1. Collectively, these results confirm the absolute requirement of reactive electrophilic moieties for TRPA1 activation by

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these COX metabolites. Taken together, the findings that $15dPGJ_2$ directly activated TRPA1 and that electrophilic carbons were necessary for TRPA1 activation by A- and J-series prostanoids are consistent with the model of TRPA1 activation through direct covalent modification described by Hinman et al (2006) and Macpherson et al (2007) for AITC and related irritants.

Prostaglandins such as PGE_2 and PGD_2 are elevated at sites of inflammation (Davies et al., 1984; Woolf and Costigan, 1999) and can be produced by multiple cell types including nociceptive neurons themselves (Chopra et al., 2000; Vesin et al., 1995), as well as cells such as epithelial cells (Folkerts and Nijkamp, 1998) and mast cells (Roberts et al., 1979), which are commonly found in close proximity to nerves. While rigorously quantifying the extent to which PGE_2 and PGD_2 are converted into A- and J-series prostaglandins, respectively, is challenging due their reactivity, μM levels of $15dPGJ_2$ have been detected in inflammatory exudates in a rat pleurisy model (Gilroy et al., 1999) and in lymph nodes of micedisplaying delayed type hypersensitivity reactions in response to methylated bovine serum albumin sensitization and challenge (Trivedi et al., 2006). These levels were dramatically decreased by treatment with the COX-2 inhibitor NS398 (Gilroy et al., 1999) or targeted deletion of hematopoietic PGD_2 synthase (Trivedi et al., 2006). Finally, there is at least preliminary evidence that production of A- and J-series prostanoids may also occur in humans (Blanco et al., 2005; Chen et al., 1999).

Unlike the other prostanoids tested in this study, which require COX activity for their production, the isoprostane 8-iso PGA_2 is produced non-enzymatically though oxidative metabolism of membrane phospholipids (Roberts and Morrow, 2002). Thus, 8-iso PGA_2 activates TRPA1, mimicking the activation that we and others have previously described for 4-hydroxynonenal (Taylor-Clark et al., 2007; Trevisani et al., 2007), another product of oxidative metabolism of membrane phospholipids. Thus, these data suggest that a variety of reactive electrophiles produced downstream of COX activation and/or lipid peroxidation may contribute to nociception by directly activating TRPA1 at local sites of inflammation.

In summary, we demonstrate evidence of a novel mechanism through which a subset of reactive metabolites of PGD_2 and PGE_2 may stimulate nociceptive neurons through direct activation of TRPA1.

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Footnotes

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Reprint request to B. Undem, 5501 Hopkins Bayview Circle, Baltimore, MD 21224. bundem@jhmi.edu

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Fig. 1. 15dPGJ$_2$ mimics the effect of AITC on trigeminal neurons. (A) Mean ± S.E.M. Ca^{2+} responses of dissociated trigeminal neurons responding to 15dPGJ$_2$ (100 μM) in the presence of vehicle (81 sensitive neurons out of 162 tested), the TRPV1 inhibitor iodo-resiniferatoxin (I-RTX, 1 μM, 59/108), the non-selective TRP inhibitor ruthenium red (RR, 30 μM, 7/86) or the TRPA1 inhibitor HC-030031 (HC, 100 μM, 7/87). (B) Mean ± S.E.M. Ca^{2+} responses of dissociated trigeminal neurons responding to AITC (100 μM) in the presence of vehicle (388/794), I-RTX (1 μM, 37/108), RR (30 μM, 8/138) or HC (100 μM, 37/87). (C) Mean ± S.E.M. Ca^{2+} responses of dissociated trigeminal neurons responding to capsaicin (Caps, 1 μM), in the presence of vehicle (339/730), I-RTX (1 μM, 0/108), RR (30 μM, 5/53) or HC (100 μM, 37/87). Blocked line denotes the duration of agonist treatment: 15dPGJ$_2$ and AITC were applied for 120s, capsaicin for 60s. All neurons responded to KCl (75 mM) applied immediately prior to ionomycin.

Fig. 2. Responses to 15dPGJ$_2$ and AITC in trigeminal neurons are inhibited by AITC pretreatment. Ca^{2+} responses to AITC (100 μM), 15dPGJ$_2$ (100 μM) and capsaicin (Caps, 1 μM) without AITC pretreatment (white columns, data account for 794, 162 and 62 neurons, respectively) or following AITC (100 μM) pretreatment (black columns, data account for 50 neurons). (A) Percentage of neurons responding to either AITC, 15dPGJ$_2$ or capsaicin. (B) Magnitude of the Ca^{2+} responses of neurons that responded to either AITC, 15dPGJ$_2$ or capsaicin. Data represent the maximum net increase in normalized Ca^{2+} response (during the 120s drug treatment taken from mean neuron response versus time curves) above the mean response over 120s prior to AITC, 15dPGJ$_2$ or capsaicin treatment (as appropriate) *** Significant decrease in the Ca^{2+} responses following AITC pretreatment ($p<0.001$).

Fig. 3. 15dPGJ$_2$ activates TRPA1 channels. (A) Representative Fura 2AM ratiometric image of Ca^{2+} responses of hTRPA1-HEK cells to putative endogenous TRPA1 agonist 15dPGJ$_2$ (100 μM). (B) Mean ± S.E.M. Ca^{2+} responses of hTRPA1-HEK cells to putative endogenous TRPA1 agonist 15dPGJ$_2$ (1, 10 and 100 μM). Legend denotes the number of cells analyzed. 15dPGJ$_2$ was applied for 60s (blocked line).

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Black line denotes responses of hTRPA1-HEK cells, red line denotes responses of hTRPA1-HEK cells in the presence of ruthenium red (RR, 30 μM), broken line denotes responses of nt-HEK cells.

Fig. 4. 15dPGJ$_2$ evokes currents in TRPA1- but not TRPV1-expressing HEK cells. (A) Current-voltage relation of AITC (10 μM)-evoked whole-cell currents in hTRPA1-HEK cells, representative of 6 separate experiments. (B) 15dPGJ$_2$ (10 μM) evokes whole-cell currents in hTRPA1-HEK similar to AITC, representative of 8 separate experiments. Current-voltage traces are represented as the responses to 10 consecutive voltage ramps during basal conditions or after maximum agonist-evoked response had been reached. (C) Timecourse of 15dPGJ$_2$-evoked currents at -70 and 70 mV in a hTRPA1-HEK cell. (D) Capsaicin (1 μM), but not 10 μM 15dPGJ$_2$, evokes currents in hTRPV1-HEK cells (n=5). (E) Mean + S.E.M. data demonstrating the effects of 10μM 15dPGJ$_2$ on current densities at -70 and 70 mV in hTRPA1-HEK and hTRPV1-HEK cells relative to the two known channel agonists, AITC and capsaicin, respectively (n=5-8).

Fig. 5. Electrophilic nature of putative agonists is critical for TRPA1 activity. (A) Maximal Ca^{2+} responses of hTRPA1-HEK and nt-HEK cells to prostanoids (all 100 μM): 15dPGJ$_2$, PGD$_2$, PGE$_2$, PGB$_2$, PGA$_2$, 8-iso PGA$_2$ and Δ^{12}-PGJ$_2$. All hTRPA1-HEK data (black columns) comprise >170 cells, all nt-HEK data (white columns) comprise >240 cells. All drugs were applied for 60s. Asterisk on prostanoid structures denote reactive carbonyl groups: PGD$_2$, PGE$_2$ (lacking group); PGB$_2$ (sterically-hindered group); and 15dPGJ$_2$, PGA$_2$, 8-iso PGA$_2$ and Δ^{12}-PGJ$_2$ (free groups). (B) Dose-response relationships of Ca^{2+} responses of hTRPA1-HEK cells for 15dPGJ$_2$, Δ^{12}-PGJ$_2$, PGA$_2$ and 8-iso PGA$_2$ (1, 10 and 100 μM) (data comprise >170 cells). Data from (A) and (B) represent the maximum response during the 60s agonist treatment taken from mean cell response versus time curves (with S.E.M. for that data point). (C) Dose-response relationships (1, 10 and 100 μM) of Ca^{2+} responses of prostanoid-sensitive trigeminal neurons for 15dPGJ$_2$ (69 sensitive neurons out of 139), Δ^{12}-PGJ$_2$ (29/136), PGA$_2$ (13/105) and 8-iso

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PGA_2 (20/91). Data represent the maximum response during the 120s drug treatment taken from mean cell response versus time curves (with S.E.M. for that data point). All neurons responded to KCl (75 mM).

FIGURE 1

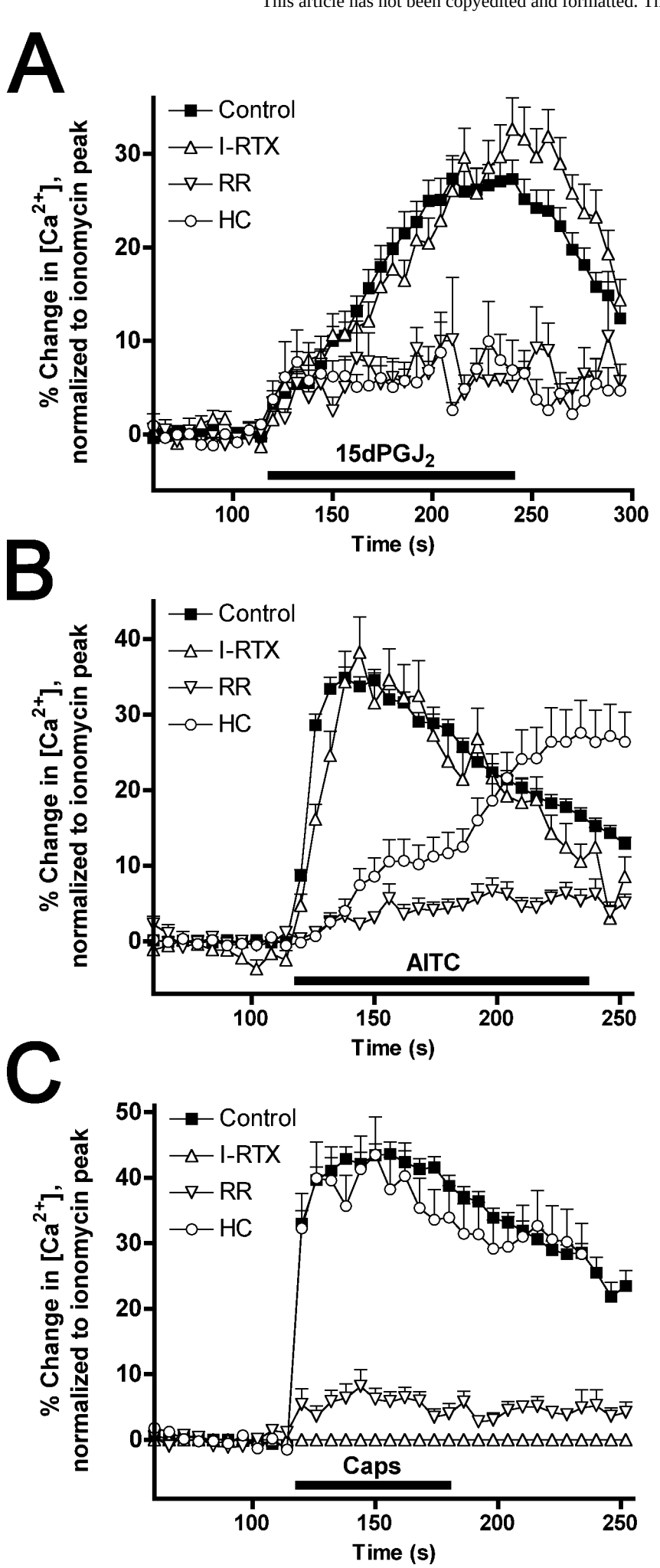


FIGURE 2

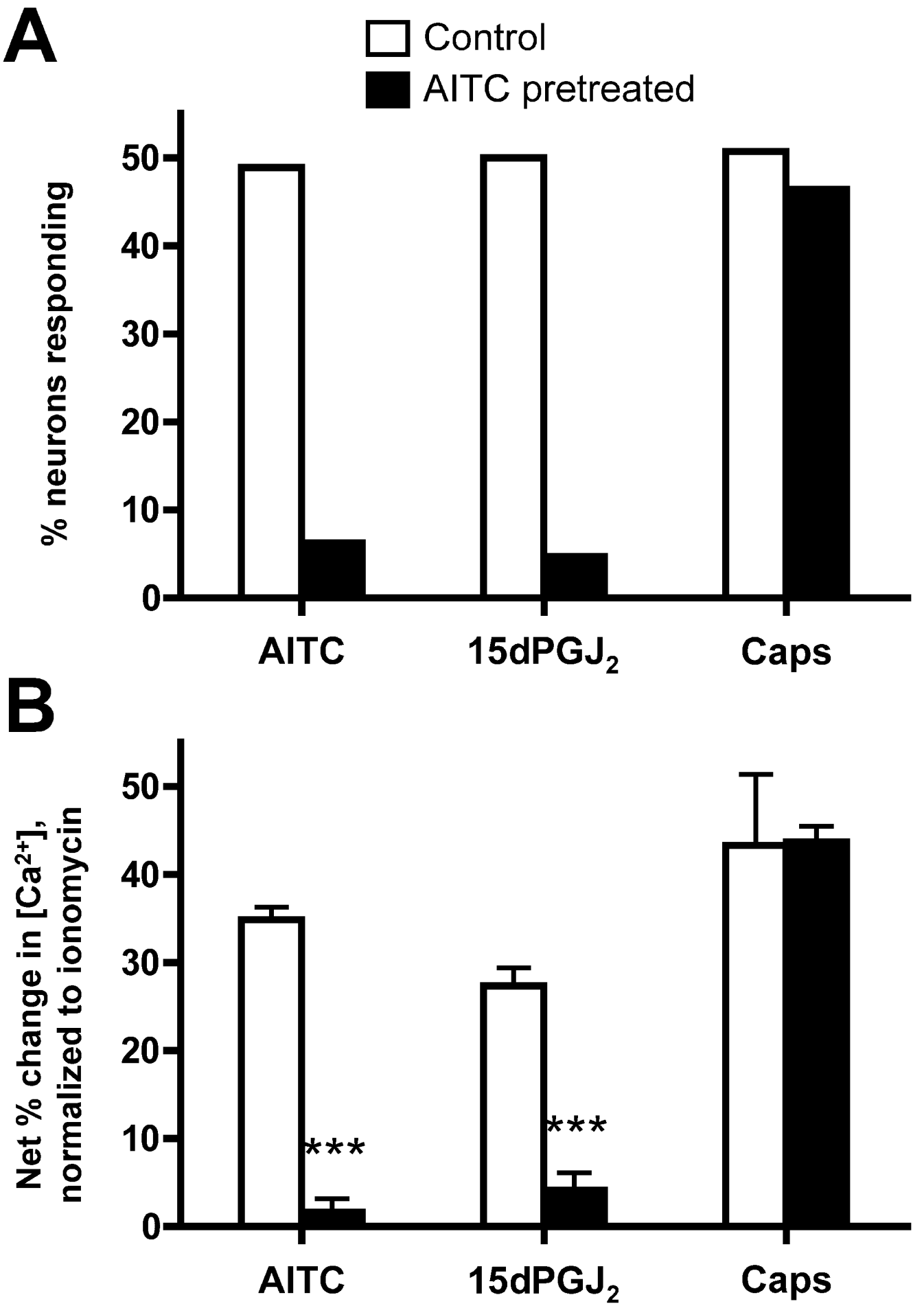


FIGURE 3

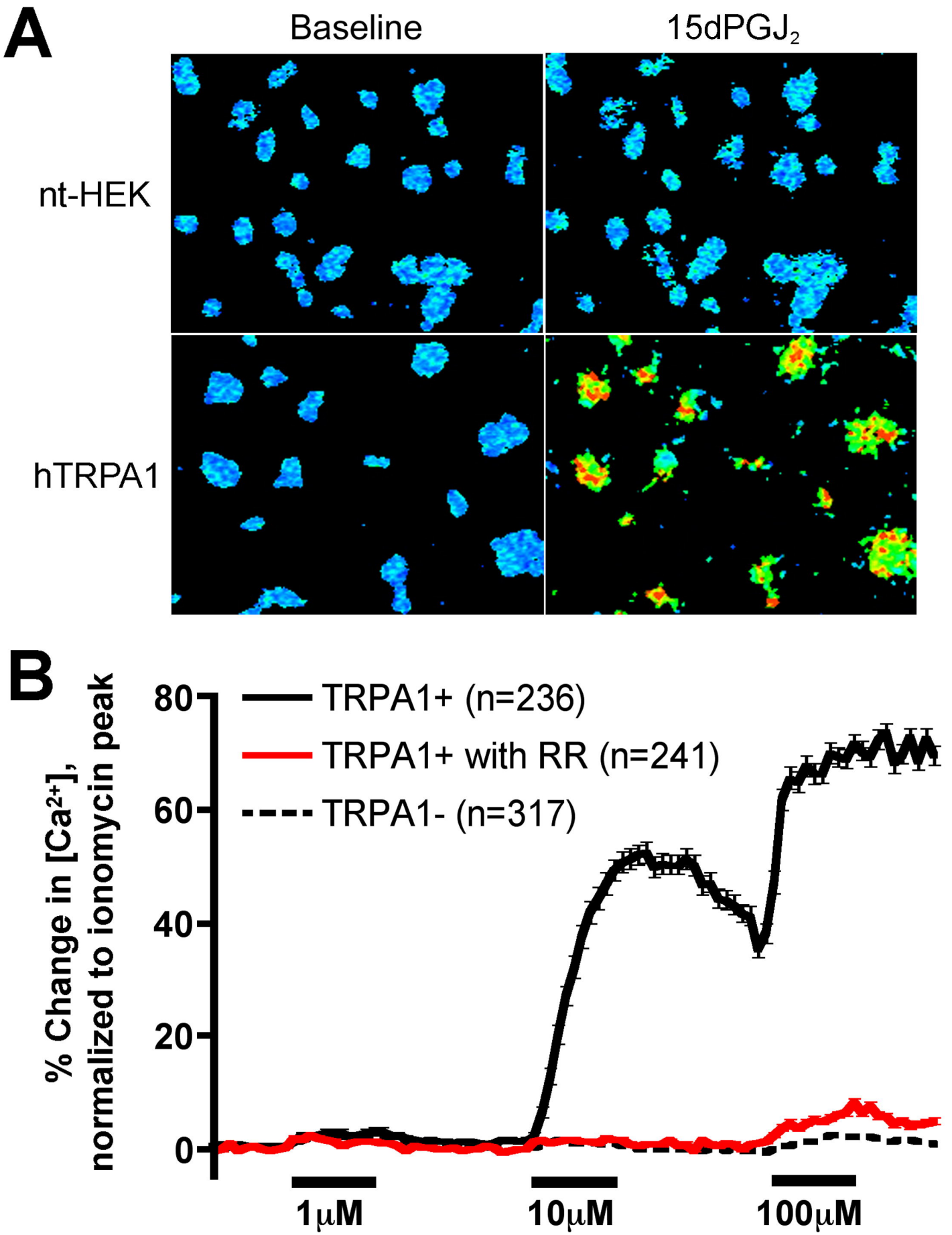

A
Baseline
15dPGJ2
nt-HEK
hTRPA1
B
% Change in [Ca2+], normalized to ionomycin peak
80
60
40
20
0
TRPA1+ (n=236)
TRPA1+ with RR (n=241)
TRPA1- (n=317)
1μM
10μM
100μM

FIGURE 4

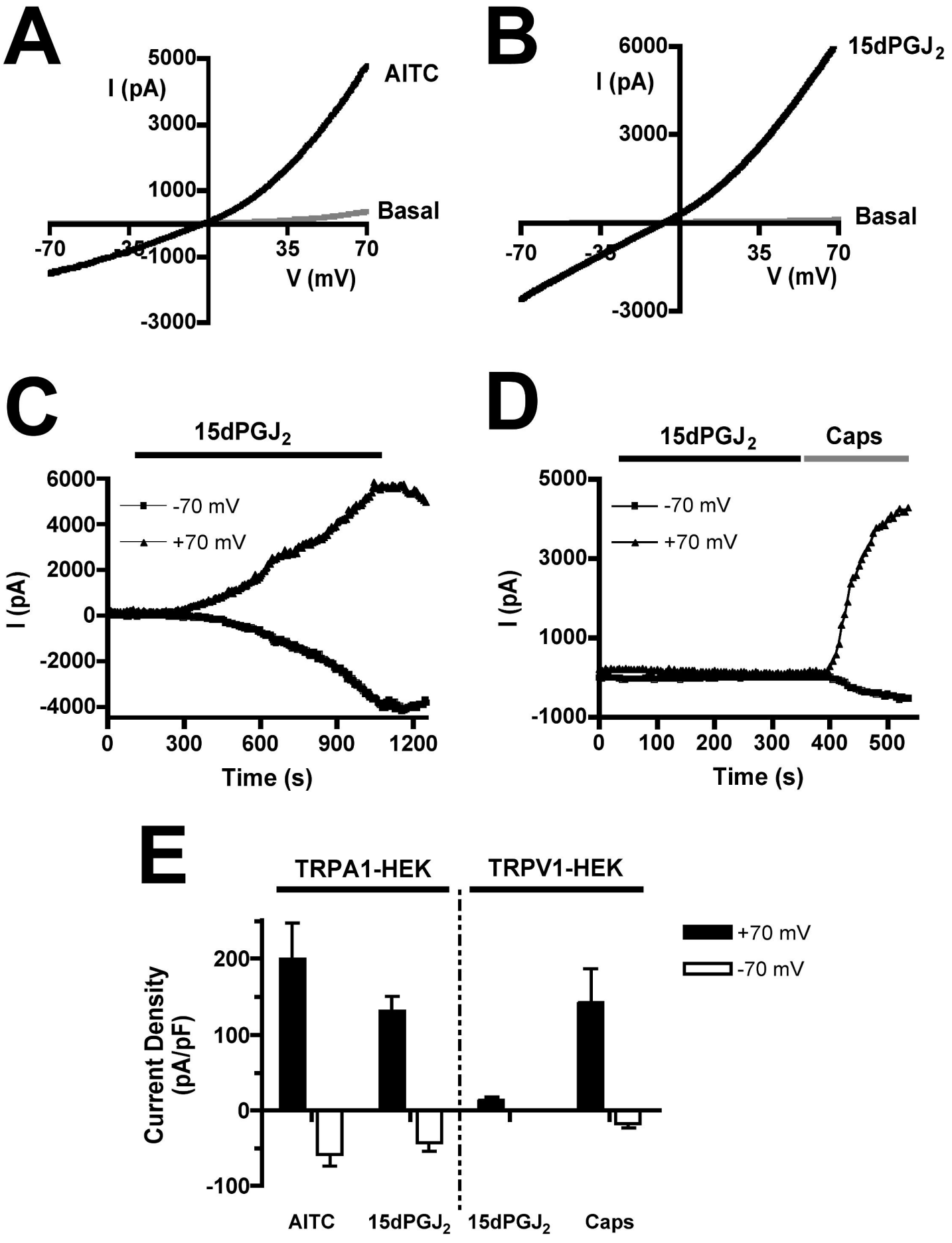
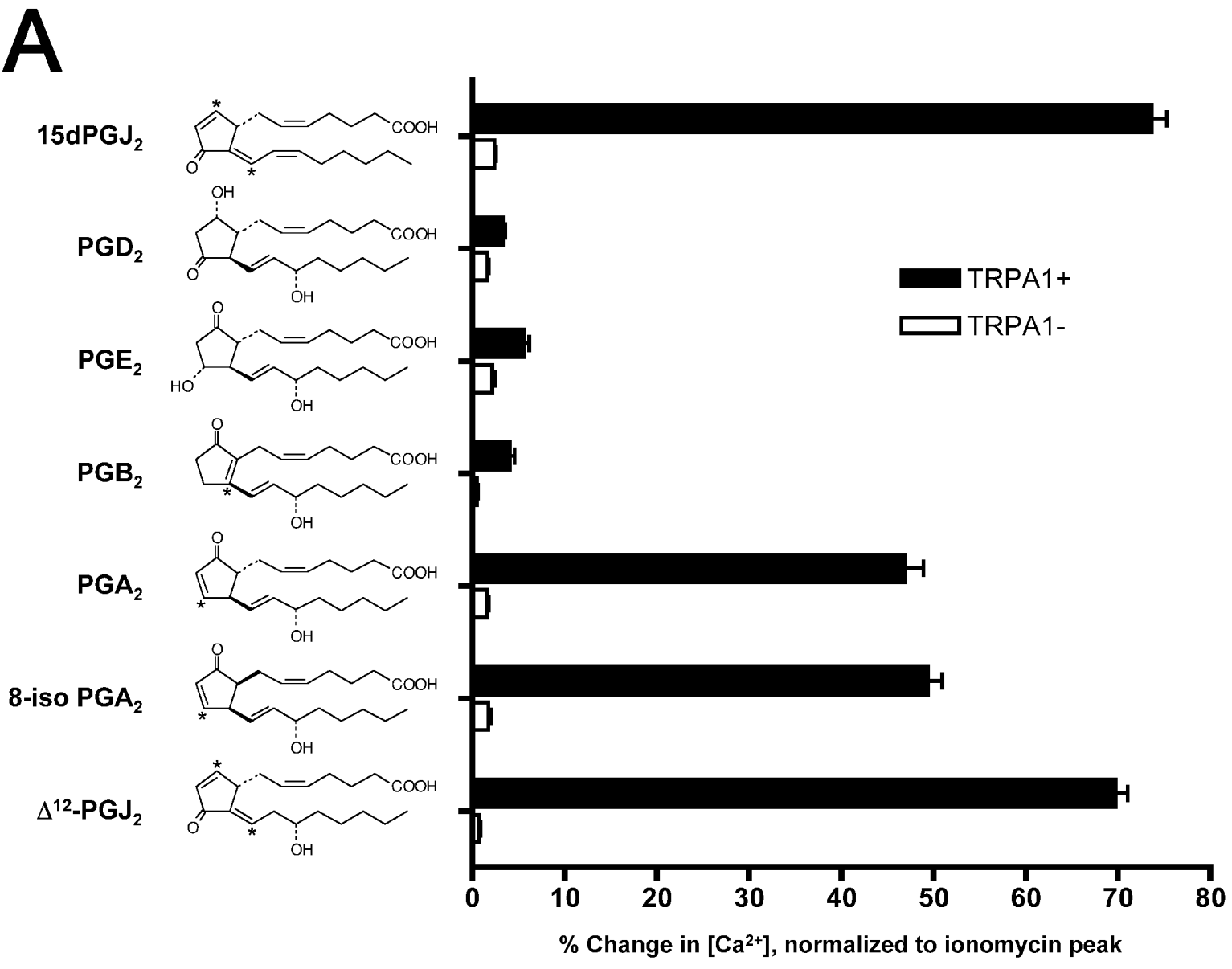
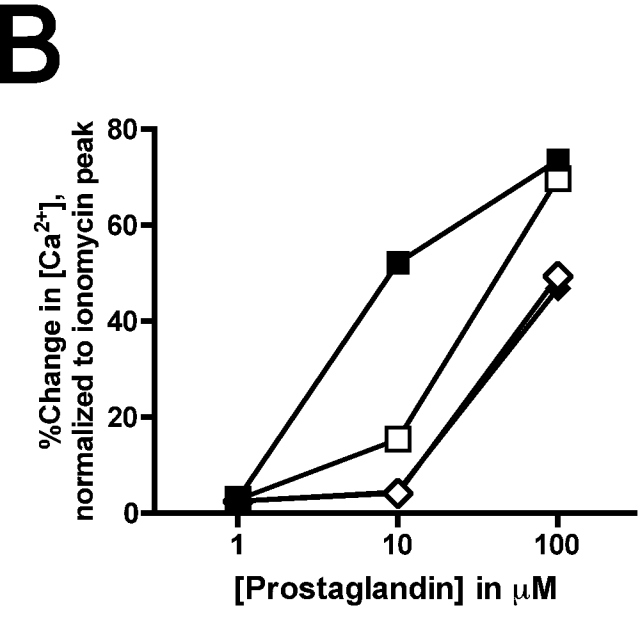


FIGURE 5


A
15dPGJ2
PGD2
PGE2
PGB2
PGA2
8-iso PGA2
Δ12-PGJ2
COOH
OH
HO
TRPA1+
TRPA1-
0
10
20
30
40
50
60
70
80
% Change in [Ca2+], normalized to ionomycin peak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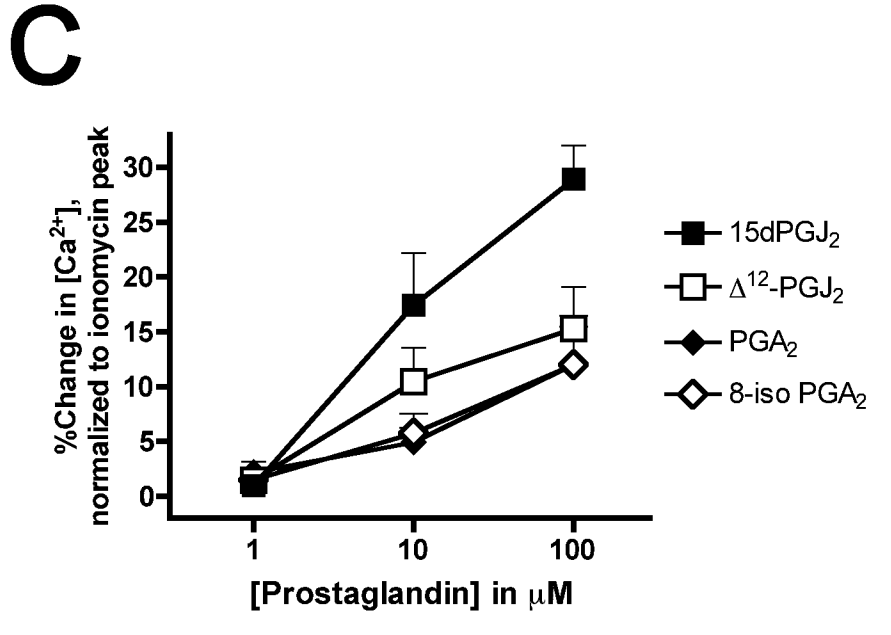
%Change in [Ca2+], normalized to ionomycin peak
0
20
40
60
80
1
10
100
[Prostaglandin] in μM


C
%Change in [Ca2+], normalized to ionomycin peak
0
5
10
15
20
25
30
1
10
100
[Prostaglandin] in μM
15dPGJ2
Δ12-PGJ2
PGA2
8-iso PGA2