Prostaglandin E₂ Inhibits Histamine-Evoked Ca\(^{2+}\) Release in Human Aortic Smooth Muscle Cells through Hyperactive cAMP Signaling Junctions and Protein Kinase A

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ABSTRACT

In human aortic smooth muscle cells, prostaglandin E₂ (PGE₂) stimulates adenylyl cyclase (AC) and attenuates the increase in intracellular free Ca\(^{2+}\) concentration evoked by activation of histamine H₁ receptors. The mechanisms are not resolved. We show that cAMP mediates inhibition of histamine-evoked Ca\(^{2+}\) signals by PGE₂. Exchange proteins activated by cAMP were not required, but the effects were attenuated by inhibition of cAMP-dependent protein kinase (PKA). PGE₂ had no effect on the Ca\(^{2+}\) signals evoked by protease-activated receptors, heterologously expressed muscarinic M₃ receptors, or by direct activation of inositol 1,4,5-trisphosphate (IP₃) receptors by photolysis of caged IP₃. The rate of Ca\(^{2+}\) removal from the cytosol was unaffected by PGE₂, but PGE₂ attenuated histamine-evoked IP₃ accumulation. Substantial inhibition of AC had no effect on the concentration-dependent inhibition of Ca\(^{2+}\) signals by PGE₂ or butaprost (to activate EP₂ receptors selectively), but it modestly attenuated responses to EP₂ receptors, activation of which generated less cAMP than EP₃ receptors. We conclude that inhibition of histamine-evoked Ca\(^{2+}\) signals by PGE₂ occurs through “hyperactive signaling junctions,” wherein cAMP is locally delivered to PKA at supersaturating concentrations to cause uncoupling of H₁ receptors from phosphodiesterase C. This sequence allows digital signaling from PGE₂ receptors, through cAMP and PKA, to histamine-evoked Ca\(^{2+}\) signals.

Introduction

Ca\(^{2+}\) and cAMP are ubiquitous intracellular messengers that regulate most cellular behaviors. The versatility of these messengers depends on both the spatiotemporal organization of the changes in their concentration within cells (Cooper and Tabbasum, 2014) and on interactions between them [see references in Tovey et al. (2008)]. These interactions are important in many smooth muscles, where increases in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) stimulate contraction, but receptors that stimulate formation of cAMP usually cause relaxation. The clinical importance is clear from the widespread use of β-agonists to provide symptomatic relief from asthma (Morgan et al., 2014). In vascular smooth muscle (VSM), too, cAMP attenuates the contractile responses mediated by many receptors that evoke Ca\(^{2+}\) signals (Morgado et al., 2012). This inhibition is assumed to be mediated by cAMP-dependent protein kinase (PKA) (Murthy, 2006), but there are also PKA-independent effects of cAMP (Spicuzza et al., 2001). At least some of these effects may be through exchange proteins activated by cAMP (EPACs), probably EPAC 1, which is abundant in blood vessels particularly within endothelial cells (Roscioni et al., 2011).

Histamine and prostaglandin E₂ (PGE₂) are two important inflammatory mediators. Their effects on VSM, which include regulation of proliferation (Yau and Zahradka, 2003) and vascular tone (Toda, 1987; Jadhav et al., 2004), are mediated by direct interactions with VSM and indirectly through release of autocrine signals from other cells (Norel, 2007). Histamine, PGE₂ and their receptors are also implicated in vascular pathology, including inflammation (Norel, 2007),...
Materials and Methods

Materials. H89, NKH 477, 8-Br-cAMP, and 8-Br-cGMP were from R&D Systems (Abingdon, Oxford, UK). 9-(4-amino-2-methyl-6-pyridinium)imino)propyl)adenine (A-83-01), forskolin, IBMX, and PGE2 were from Alomone Laboratories (Jerusalem, Israel). PAR1 peptide, histamine dihydrochloride, (st-Ht31P) were from Promega (Southampton, UK). Thapsigargin was from Stratech Scientific Ltd (Newmarket, Suffolk, UK). Other reagents were from Sigma-Aldrich, sources specified previously (Pantazaka et al., 2013) or identified in this section.

Cultures of Human Aortic Smooth Muscle Cells. Human ASMC from the American Tissue Culture Collection (Manassas, VA) or Dr. Trevor Littlewood (Boyle et al., 2002) were cultured as described (Pantazaka et al., 2013). Ethical approval for the latter was obtained from NHS Trust. Cells were derived from four Caucasian patients (males aged 23, 52, and 54, and a female aged 58), who died of causes unrelated to cardiovascular pathologies. Cells were used between passages two and six.

Measurements of \([\text{Ca}^{2+}]_{\text{i}}\). Histamine-evoked changes in \([\text{Ca}^{2+}]_{\text{i}}\) were recorded from cell populations using confluent cultures of ASMC grown in 96-well plates and loaded with Fluo-4 or Fluo-8. Experiments were performed in HEPES-buffered saline (HBS) at 20°C. HBS had the following composition (mM): NaCl 135, KCl 5.9, MgCl2 1.2, CaCl2 1.5, glucose 11.5, and HEPES 11.6 (pH 7.3). Fluorescence was recorded using a FlexStation 3 fluorescence plate-reader (MDS Analytical Technologies, Wokingham, UK) and calibrated to \([\text{Ca}^{2+}]_{\text{i}}\) as described (Pantazaka et al., 2013).

For measurements of \([\text{Ca}^{2+}]_{\text{i}}\), in single cells, confluent cultures of ASMC grown on poly-l-lysine-coated coverslips (22-mm diameter) were loaded with Fura-2 in HBS containing Fura-2 AM (4 µM), probenecid (2.5 mM), and pluronic F127 (0.02% v/v) for 1 hour at 20°C. Fluorescence (detected at ∼510 nm with alternating excitation at 340 and 380 nm) was recorded using an Olympus IX71 inverted fluorescence microscope and Luca (electron-multiplying charge-coupled device) EMCCD Andor Technology, Belfast, UK camera. After correction for background fluorescence, determined by addition of iomycin (1–5 µM) in HBS containing MnCl2 (1 mM), fluorescence ratios (F340/F380) were calibrated to \([\text{Ca}^{2+}]_{\text{i}}\) (Tovey et al., 2003).

Measurements of Intracellular cAMP. Confluent cultures of ASMC grown in 24-well plates and labeled with \(^{3}H\)-adenine were incubated under conditions that replicated those used for measurements of \([\text{Ca}^{2+}]_{\text{i}}\). Reactions were terminated by aspiration of medium and addition of ice-cold trichloroacetic acid (5% v/v, 1 ml). After 30 minutes on ice, \(^{3}H\)-cAMP was separated from other \(^{3}H\)-labeled adenine nucleotides (Pantazaka et al., 2013).

Expression of PKI and M3 Muscarinic Receptors. Plasmids encoding PKI (pRSV-PKI-v2) and its inactive form (pRSV-mut PKI-v2) were from Addgene (cat. no. 45066 and cat. no. 45067; Cambridge, MA) (Day et al., 1989); they were C-terminally tagged with mCherry. Plasmid encoding the human M3 muscarinic acetylcholine receptor was from the cDNA Resource Centre (cat. no. MAAK0300000) (Ford et al., 2002). The three constructs were each recombined into BacMam pCMV-DEST. Bacmids were then prepared, and virus was produced from medium-infected S99 cells according to the manufacturer’s instructions (Thermo Fisher Scientific, Runcorn, UK). ASMC were infected at a multiplicity of infection (MOI) of ~50 and used after 96 hours.

Flash Photolysis of Caged IP3. Confluent cultures of ASMC grown on poly-l-lysine-coated imaging dishes (35-mm diameter with a 7-mm glass insert; MatTek Corporation, Ashland, MA) were loaded (45 minutes, 20°C) with a membrane-permeant form of caged IP3 (ci-IP3PM, 1 mM) in HBS with probenecid (2.5 mM) and pluronic F127 (0.02% v/v). Fluo-4 AM (4 µM) was then added and after 45 minutes at 20°C, the medium was replaced with HBS containing only probenecid. After a further 45 minutes, this medium was replaced with HBS. Cells were illuminated with a 488-nm diode-based solid-state laser, and emitted fluorescence (500–550 nm) was captured with an EMCCD camera. Three UV flashes (each ~1-millisecond duration; <345 nm, 3000 µJ, 300 V, ~170 J) from a JML-C2 Xe flash-lamp (Rapid OptoElectronic, Hamburg, Germany) allowed photolysis of caged IP3 (ci-IP3PM). Responses are reported as F/F0, where F0 and F are fluorescence intensities corrected for background recorded from the same region of interest immediately before (F0) and after stimulation (F).

Measurements of IP3 and PLC Activity. ASMC in 12-well plates were cultured until confluent. The medium was then supplemented with d-myog-[^3]H]-inositol (10 µCi/ml) for 48 hours at 37°C. After washing, cells were incubated at 20°C in HBS with LiCl (10 mM) for 5 minutes before stimulation. Reactions were terminated by aspirating medium and adding cold HClO4 (1 ml, 0.6 M) containing phytic acid (0.2 mg/ml). After 30 minutes, the acid-extract was removed, the cells were scraped into 50 mM Tris at pH 7 (400 µl), and the pooled extract and cells were centrifuged (10,000g, 2 minutes, 4°C). The supernatant was neutralized using K2CO3 (1 M) with EDTA (5 mM). \(^{3}H\)-inositol phosphates were separated using ion-exchange columns.

For assays of IP3 mass, ASMC in 75-cm² flasks were stimulated, the medium was removed, and the incubations were terminated by scraping cells into ice-cold ethanol (1 ml). After 30 minutes, extracts were dried and suspended in 300 µl of Tris-EDTA medium (TEM: 50 mM Tris, 1 mM EDTA, pH 8.3). Equilibrium-competition binding using \(^{3}H\)-IP3 (4.5 nM, 19.3 Ci/nmol), rat cerebellar membranes (10 µg) and cell extract (20–100 µl) in a final volume of 200 µl of TEM was used to determine the IP3 content of the extracts (Rossi et al., 2009).
In Immunoblotting. Confluent ASMC in 75-cm² flasks or six-well plates were stimulated and then scraped into cold phosphate-buffered saline supplemented with Triton-X-100 (1% w/v), protease inhibitors (one mini-tablet per 10 ml; Roche Applied Science, Burgess Hill, UK), and phosphatase inhibitors (10 μl/mg; Sigma-Aldrich). Scraped cells were disrupted by ∼30 passages through a 28-gauge needle and sonicated (3 × 10 seconds). Proteins were separated by SDS-PAGE (NuPAGE 4%–12% Bis-Tris gels; Invitrogen, Paisley, UK) and transferred to a polyvinylidene difluoride membrane (iBlot; Invitrogen). Membranes were washed (5 minutes) with Tris-buffered saline (TBS: 137 mM NaCl, 20 mM Tris, pH 7.6), blocked by incubation in TBS containing 0.1% Tween-20 (TBS-T) and 5% (w/v) nonfat milk powder (1 hour, 20°C), and then washed with TBS-T (3 × 5 minutes). Blots were incubated for 12 hours at 4°C with primary antibody (1:1000) in TBS-T with 5% (w/v) nonfat milk powder. After further washing (3 × 5 minutes), bands were detected using ECL Prime (GE Healthcare, Chalfont St Giles, UK) and quantified using GeneTools (1 hour, 20°C), and then washed with TBS-T (3 minutes), bands were detected using ECL Prime (GE Healthcare, Chalfont St Giles, UK) and quantified using GeneTools (1 hour, 20°C).

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proteins involved in PKG signaling in VSM are downregulated in culture (Lincoln et al., 2006, and references therein). 8-Br-cGMP (pIC50 4.50, n = 5) partially inhibited Ca2+ signals evoked by a submaximal concentration of histamine, but the maximal inhibition was less than half that evoked by PGE2 or 8-Br-cAMP (Fig. 3A and B). Furthermore, and in contrast to the effects of 8-Br-cAMP (Fig. 2C), 8-Br-cGMP did not inhibit the Ca2+ signals evoked by a maximal histamine concentration (Fig. 2D). Prolonged incubation with IBMX (20 minutes, 1 mM), a nonselective inhibitor of PDEs, inhibited histamine-evoked Ca2+ signals, but the inhibition (33% ± 3%, n = 4) was less than that caused by PGE2 (56% ± 3%) (Fig. 3C). More importantly, a maximal concentration of PGE2 similarly inhibited histamine-evoked Ca2+ signals in

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Inhibition of histamine-evoked Ca2+ signals by PGE2 is mediated by cAMP. (A) Ca2+ signals evoked by histamine (3 μM, black bar) alone or with PGE2 (10 μM, added 5 minutes before and then during stimulation with histamine). Results show means ± range from two wells on a single plate; they are typical of results from at least four independent experiments. (B) Effects of PGE2 on cAMP accumulation (measured after 5 minutes) and inhibition of the peak Ca2+ signals evoked by histamine (3 μM). Results, as percentages of maximal inhibition (Ca2+) or stimulation (cAMP), are means ± S.E.M. from six and four experiments, respectively. This panel includes some data for cAMP measurements that were published previously (Pantazaka et al., 2013). (C) Effect of NKH 477 (100 μM) on the peak Ca2+ signal evoked by histamine. (D) Concentration-dependent effects of forskolin on the peak Ca2+ signals evoked by histamine (1 mM). (E) Effect of PGE2 (10 μM), NKH 477 (100 μM), or both on the peak Ca2+ signals evoked by histamine (as percentages of the maximal response). NKH 477, forskolin, or PGE2 were added 5 minutes before and then during stimulation with histamine. Results show means ± S.E.M. from four (C and D) or three (E) independent plates with one to three wells analyzed from each. Ct denotes control.

| Drug            | Maximal Inhibition | pIC50 | n  |
|-----------------|--------------------|-------|----|
| PGE2 (5 min)    | 64 ± 4             | 9.01  | 5  |
| Butaprost (5 min)| 61 ± 1             | 7.28  | 6  |
| L902,688 (5 min)| 76 ± 4             | 9.35  | 5  |
| 8-Br-cAMP (20 min)| 85 ± 3            | 2.98 ± 0.20 | 3 |
| 6-Bnz-cAMP (20 min)| 64 ± 5         | 3.73 ± 0.14 | 5 |
| Forskolin (5 min)| 57 ± 6             | 6.24 ± 0.11 | 4 |
| NKH 477 (5 min) | 53 ± 4             | 5.50 ± 0.18 | 5 |
the presence and absence of IBMX (Fig. 3C), demonstrating that the effects of PGE2 are not mediated by inhibition of PDEs. We conclude that inhibition of histamine-evoked Ca\[^{2+}\] signals by PGE2 is not mediated by inhibition of PDEs and consequent accumulation of cGMP.

**Histamine and 8-Br-cAMP Stimulate PKA-Mediated Protein Phosphorylation in Different Microenvironments.** Most effects of cAMP are mediated by PKA, EPACs, or cyclic nucleotide-regulated plasma membrane cation channels (Gloerich and Bos, 2010; Cooper and Tabbasum, 2014).
The latter cannot mediate the effects of cAMP on IP3-evoked Ca\(^{2+}\) release, nor are EPACs responsible. We therefore assessed the role of PKA.

Immunoblotting with an antiserum that recognizes sequences phosphorylated by PKA showed that maximally effective concentrations of PGE\(_2\) or 8-Br-cAMP stimulated similar levels of protein phosphorylation in ASMC and their effects were nonadditive (Fig. 4A). The phosphorylation was mimicked by 6-Bnz-cAMP but not by the EPAC-selective analog 8-pCPT-2'-O-Me-cAMP (Fig. 4A). PGE\(_2\)-evoked protein phosphorylation was attenuated by inhibition of either PKA (with H89) or AC [with 1 mM SQ 22536 with 200 \(\mu\)M DDA (SQ/DDA)] (Fig. 4B).

Maximal concentrations of PGE\(_2\) and 8-Br-cAMP caused phosphorylation of the same proteins (Fig. 4A), but the two stimuli differed in their susceptibility to PKA inhibitors. Rp-8-CPT-cAMPS, an inhibitor of PKA that competes with cAMP by binding to the regulatory subunit of PKA, abolished the phosphorylation evoked by 8-Br-cAMP but only partially inhibited that evoked by PGE\(_2\) (Fig. 4C). Conversely, H89, which inhibits PKA (and other kinases) by competing for the ATP-binding site, abolished the phosphorylation evoked by PGE\(_2\) but caused lesser inhibition of the response to 8-Br-cAMP (Fig. 4C). Similar results were obtained when an antiserum to phospho-VASP was used to assess PKA-mediated phosphorylation (Supplemental Fig. S2).

These results suggest that PKA activated by PGE\(_2\) may be exposed to high local concentrations of cAMP, which might then effectively compete with the inhibitor Rp-8-CPT-cAMPS. Conversely, PKA activated by 8-Br-cAMP, which would probably be uniformly distributed within the cell, may be less susceptible to inhibition by H89.

Inhibition of Histamine-Evoked Ca\(^{2+}\) Signals by PGE\(_2\) Is Attenuated by Inhibition of PKA. Inhibition of histamine-evoked Ca\(^{2+}\) signals by PGE\(_2\) or 8-Br-cAMP was inhibited by Rp-8-CPT-cAMPS (1 mM), which reduced the sensitivity to PGE\(_2\) (decreasing the pIC\(_{50}\) for PGE\(_2\) from 8.68 ± 0.17 to 8.26 ± 0.05, \(\Delta pIC_{50} = 0.4 ± 0.1\), where \(\Delta pIC_{50} = pIC_{50}\)control - pIC\(_{50}\)Hp-8-CPT-cAMPS) and 8-Br-cAMP (\(\Delta pIC_{50} = 0.6 ± 0.1\)) without affecting the maximal inhibition (Fig. 5, A and B). These results are consistent with Rp-8-CPT-cAMPS competitively inhibiting cAMP binding to PKA and thereby attenuating the effects of cAMP on Ca\(^{2+}\) signals.

H89 (10 \(\mu\)M) also attenuated the inhibition of histamine-evoked Ca\(^{2+}\) signals by 8-Br-cAMP (\(\Delta pIC_{50} = 1.13 ± 0.18\) \(n = 3\)) and PGE\(_2\) (Fig. 5, A and B). In keeping with our analyses of protein phosphorylation (Fig. 4C), the inhibition of histamine-evoked Ca\(^{2+}\) signals by maximal concentrations of PGE\(_2\) were less effectively inhibited by H89 than were the effects of maximal concentrations of 8-Br-cAMP (compare Fig. 5, A and B).

PKI inhibits PKA by competing with its peptide substrates. We could not achieve effective inhibition of PKA-mediated protein phosphorylation with myristoylated-PKI (10 \(\mu\)M, 20 minutes, data not shown). But using a baculovirus, we achieved expression of PKI in 90% of cells, and this caused 49% ± 6% (\(n = 3\)) inhibition of the VASP phosphorylation evoked by PGE\(_2\) (100 nM) (Supplemental Fig. S3). Expression of an inactive PKI (mut PKI) had no effect on PGE\(_2\)-evoked protein phosphorylation (Supplemental Fig. S3). The effects of H89 and PKI on the inhibition of histamine-evoked...
Ca\(^{2+}\) signals by PGE\(_2\) were similar: Each substantially reduced the maximal inhibition without significantly affecting the IC\(_{50}\) for PGE\(_2\) (Fig. 5, A and C). The effects of H89 on inhibition of histamine-evoked Ca\(^{2+}\) signals by selective agonists of EP\(_2\) (butaprost) and EP\(_4\) (L902,688) receptors were similar to those observed with PGE\(_2\) (Supplemental Fig. S4). We conclude that inhibition of histamine-evoked Ca\(^{2+}\) signals by PGE\(_2\) is mediated by cAMP and requires PKA (Fig. 5D).

PGE\(_2\) Does Not Inhibit Ca\(^{2+}\) Release Evoked by Direct Activation of IP\(_3\)Rs. The rate at which [Ca\(^{2+}\)]\(_i\) recovered from the peak Ca\(^{2+}\) signal evoked by histamine was unaffected by PGE\(_2\) (half-times for recovery were 19 ± 1 and 17 ± 1 seconds, after histamine alone or with PGE\(_2\), respectively; n = 11) (Supplemental Fig. S5). This suggests that the attenuated Ca\(^{2+}\) signals do not result from PGE\(_2\) stimulating Ca\(^{2+}\) extrusion from the cytosol.

We used flash-photolysis of ci-IP\(_3\) to activate IP\(_3\)R directly in Fluo-4-loaded ASMC. Single-cell analyses of ASMC established that most cells (99% ± 1%, from 12 fields) responded to histamine (1 mM) with an increase in [Ca\(^{2+}\)]\(_i\), and that two successive challenges with histamine evoked indistinguishable Ca\(^{2+}\) signals (Fig. 6, A and B). PGE\(_2\) reduced the peak amplitude of the Ca\(^{2+}\) signal evoked by a second histamine challenge by 28% ± 4% (n = 65 cells), without significantly affecting the number of cells that responded (91% ± 8% and 83% ± 7% for control and PGE\(_2\)-treated cells, respectively) (Fig. 6, C and D). These results confirm that under the conditions used for uncaging ci-IP\(_3\), PGE\(_2\) inhibits histamine-evoked Ca\(^{2+}\) signals.

ASMC loaded with ci-IP\(_3\) responded to UV flashes with rapid increases in Fluo-4 fluorescence (F/F\(_0\), see Materials and Methods). The amplitudes of these signals were less than those evoked by a maximal concentration of histamine (Fig. 6, E and F), confirming that responses to photolysis of ci-IP\(_3\) were not saturated. Although cells responded similarly to successive histamine challenges (Fig. 6, A and B), the response to a second photolysis of ci-IP\(_3\) was smaller than the first (Fig. 6G), presumably because each stimulus depleted a fraction of the ci-IP\(_3\). We therefore used two methods to assess the effects of PGE\(_2\) on the Ca\(^{2+}\) signals evoked by photolysis of ci-IP\(_3\). Cells were either stimulated twice with a UV stimulus, and the amplitude of the second response (with or without PGE\(_2\)) was compared with the first response for each cell (R2/R1) (Fig. 6, G–I), or cells were stimulated once with UV flashes alone or in the presence of PGE\(_2\) (Fig. 6J). Both analyses concur in demonstrating that PGE\(_2\) has no significant effect on the Ca\(^{2+}\) signals evoked by IP\(_3\) (Fig. 6, G–J). The results with ci-IP\(_3\) therefore demonstrate that PGE\(_2\) does not affect the...
interactions of IP3 with IP3R. Furthermore, because the peak IP3-evoked Ca2+ signals were unaffected by PGE2 under conditions where it attenuates responses to histamine (Fig. 6, I and J), the results provide additional evidence that PGE2 does not stimulate Ca2+ removal from the cytosol.

The product of ci-IP3 photolysis is an active but modified form of IP3 (D-2,3-O-isopropylidene-myo-inositol 1,4,5-trisphosphate) (Dakin and Li, 2007) that is not a substrate for IP3 3-kinase and may differ from IP3 in its rate of dephosphorylation. Our results do therefore exclude the possibility that PGE2 may accelerate degradation of IP3. These results suggest that PGE2 attenuates histamine-evoked Ca2+ signals by inhibiting IP3 formation, stimulating IP3 degradation, and/or disrupting IP3 delivery to IP3Rs.

**PGE2 Attenuates Histamine-Evoked Accumulation of IP3.** Using an assay that reports PLC activity (stimulation after blocking inositol monophosphate degradation by Li+), histamine (1 mM, 30 minutes) stimulated a small accumulation of 3H-inositol phosphates in ASMC. Although the response was modestly attenuated by PGE2 (10 μM), the effect was not statistically significant (Fig. 7A). Using an IP3R-based bioassay that detects only (1,4,5)IP3, histamine stimulated IP3 accumulation, and the response was attenuated by PGE2, although the latter again failed to achieve statistical significance (Fig. 7B). Using an IP3 sensor (Gulyas et al., 2015), but the signals were too small to resolve reliably any inhibitory effect of PGE2. Available genetically encoded IP3 sensors are known to have limited dynamic range and limited capacity to resolve small changes in intracellular IP3 concentration (Miyamoto and Mikoshiba, 2017).

We assessed the responses of ASMC to other stimuli (ATP, bradykinin, carbachol, phenylephrine, and thrombin) that might be expected to evoke Ca2+ signals through receptors that stimulate Gq (results not shown). Only thrombin reproducibly evoked substantial increases in [Ca2+]. Thrombin is a protease that cleaves the type 1 protease-activated receptor (PAR1) to unmask an N-terminal ligand. Thrombin and the PAR1 peptide itself evoked concentration-dependent increases in [Ca2+] in ASMC (Fig. 7C). In parallel analyses, PGE2 (10 μM, 5 minutes) attenuated the Ca2+ signals evoked by histamine without affecting those evoked by PAR1 peptide (Fig. 7D). Although the maximal increase in [Ca2+] evoked by PAR1 peptide was larger than that evoked by histamine, with concentrations of histamine and the PAR1 peptide that evoked comparable increases in [Ca2+], only the response to histamine was inhibited by PGE2 (Fig. 7E). After heterologous expression of human muscarinic M3 acetylcholine receptors in ASMC, carbachol evoked a concentration-dependent (pEC50 = 7.72 ± 0.04, n = 3) increase in [Ca2+], with a maximal increase (272 ± 31 nM, n = 3) comparable to that evoked by histamine (204 ± 13 nM, Fig. 2A). However, the responses to carbachol were unaffected by PGE2 (Fig. 7F). These results, demonstrating that PGE2 selectively inhibits the Ca2+ signals evoked by histamine, suggest that the inhibition probably does not arise downstream of PLC.

**Fig. 5.** Inhibition of histamine-evoked Ca2+ signals by PGE2 or 8-Br-cAMP requires PKA. (A and B) Effects of PGE2 (5 minutes) or 8-Br-cAMP (20 minutes) on the peak Ca2+ signals evoked by histamine (3 μM) alone or with H89 (10 μM, 20 minutes) or Rp-8-CPT-cAMPS (1 mM, 20 minutes). (C) Effects of PGE2 (5 minutes) on the Ca2+ signals evoked by histamine (3 μM) in cells infected with baculovirus expressing PKI or its inactive form (mut PKI). Results (A–C) are means ± S.E.M. from three experiments with two to three wells in each. (D) The results suggest that PKA mediates the inhibition of histamine-evoked Ca2+ signals by PGE2.
Fig. 6. PGE2 does not inhibit Ca\textsuperscript{2+} signals evoked by direct activation of IP\textsubscript{3} receptors. (A–D) Typical fluorescence traces from single Fura-2-loaded ASMC sequentially stimulated with histamine (1 mM) alone (A) or with PGE\textsubscript{2} (10 \mu M) (C). Summary results (means ± S.E.M., 65 cells from six independent fields) show peak amplitudes of the first and second responses to histamine in the absence (B) or presence (D) of PGE\textsubscript{2}. (E) Typical fluorescence traces from single Fluo-4-loaded ASMC stimulated with a UV flash to photolyse ci-IP\textsubscript{3} (red trace) or histamine (1 mM, black trace). (F) Summary results (means ± S.E.M. from 35 and 118 cells, for histamine and ci-IP\textsubscript{3} respectively) show peak responses. (G and H) Three typical fluorescence traces from single ASMC stimulated twice with UV flashes (arrowheads) alone (G) or with PGE\textsubscript{2} (10 \mu M) (H). (I) Summary results (means ± S.E.M. from 50–68 cells) show relative amplitudes of the peak responses (R2/R1). (J) Summary results (means ± S.E.M. from 36–43 cells) show F/F\textsubscript{0} (see Materials and Methods) for cells stimulated with a UV flash alone or with PGE\textsubscript{2} (10 \mu M added 5 minutes before flash). *P < 0.05, relative to the response to histamine alone (D, paired Student’s t test) or to the UV flash alone (F, unpaired Student’s t test).
Histamine-Evoked Ca\textsuperscript{2+} Signals Are Inhibited by Local cAMP Signals. Inhibitors of AC (SQ/DDA) attenuated PGE\textsubscript{2}-evoked cAMP formation (by 79% ± 2%, n = 4) (Fig. 8A) and protein phosphorylation (Fig. 4B). However, SQ/DDA had no effect on the inhibition of histamine-evoked Ca\textsuperscript{2+} signals evoked by PGE\textsubscript{2} or butaprost (Fig. 8, B and C). Although cAMP mediates the inhibition of Ca\textsuperscript{2+} signals by PGE\textsubscript{2}, the response to a maximal concentration of PGE\textsubscript{2} might survive substantial inhibition of AC because it stimulates formation of more cAMP than needed to maximally inhibit Ca\textsuperscript{2+} signals (Fig. 1B). However, the same argument cannot account for the lack of effect of SQ/DDA on responses to submaximal concentrations of PGE\textsubscript{2}. How might a submaximal response to PGE\textsubscript{2} be unaffected by substantial inhibition of cAMP formation and PKA activity (Fig. 4B; Fig. 8, A and B)? A possible explanation is that SQ 22356 and DDA, related inhibitors that bind to the ATP-binding site of AC (Brand et al., 2013), selectively inhibit subtypes of AC distinct from those that mediate the effects of PGE\textsubscript{2}. Available antibodies do not allow quantitative assessment of the expression of AC
subtypes, but QPCR analysis shows that human ASMC express similar amounts (~30%) of AC3, AC7, AC9, some AC6 (~10%), and detectable AC4 (~2%) (Supplemental Fig. S6). AC9 probably does not mediate the effects of PGE2 on Ca$^{2+}$ signals because AC9 is insensitive to forskolin and NKH 477 (Seifert et al., 2012), which mimic the effects of PGE2 on Ca$^{2+}$ signals (Fig. 1, C–E; Fig. 2D). Among the remaining ACs expressed in ASMC, SQ22536 and DDA probably have some selectivity for AC6 over AC3 and AC7 despite some inconsistent reports (Pierre et al., 2009; Seifert et al., 2012). From analyses of individual AC isoforms, maximally effective concentrations of SQ 22356 (and other P-site inhibitors) inhibit catalytic activity by only ~80% [Brand et al. (2013), but see Onda et al. (2001)]. This is similar to the ~80% inhibition of PGE2-evoked cAMP accumulation by SQ/DDA in ASMC (Fig. 8A), suggesting that the incomplete inhibition observed in ASMC probably does not reflect the unperturbed activity of SQ/DDA-insensitive ACs. Furthermore, the effects
of PGE$_2$ on protein phosphorylation in ASMC are inhibited by SQ/DDA (Fig. 4B), again suggesting that the ACs activated by PGE$_2$ are inhibited. We conclude that the lack of effect of SQ/DDA on PGE$_2$-mediated inhibition of histamine-evoked Ca$^{2+}$ signals is probably not the result of ineffective inhibition of an SQ/DDA-resistant subtype of AC.

To account for the results with SQ/DDA, we suggest that cAMP is delivered locally to PKA at concentrations more than sufficient to fully inhibit Ca$^{2+}$ signals. The concentration-dependent effects of PGE$_2$ might then result from recruitment of these “hyperactive” cAMP signaling junctions, rather than from increased activity within individual junctions (Fig. 8F). This interpretation is consistent with analyses of the effects of SQ/DDA on the inhibition of Ca$^{2+}$ signals by selective activation of EP$_4$ receptors. Although activation of EP$_2$ and EP$_4$ receptors causes similar maximal inhibition of histamine-evoked Ca$^{2+}$ signals, EP$_4$ receptors cause less stimulation of AC (Pantazaka et al., 2013). This suggests that EP$_4$ receptors may less effectively saturate the cAMP signaling junctions. Whereas inhibition of AC with SQ/DDA had no effect on the inhibition of histamine-evoked Ca$^{2+}$ signals by PGE$_2$ or butaprost (to selectively activate EP$_2$ receptors), the sensitivity to L902,688, a selective agonist of EP$_4$ receptors, was modestly reduced by SQ/DDA (ΔpIC$_{50}$ = 0.32 ± 0.10, n = 5) (Fig. 8B-E). This observation supports our suggestion that the subtype(s) of AC that link prostanoid receptors to inhibition of Ca$^{2+}$ signals are sensitive to SQ/DDA. Furthermore, these results are consistent with the scheme shown in Fig. 8F, where we suggest that cAMP is locally delivered within “hyperactive” signaling junctions at concentrations more than sufficient to maximally activate the PKA that inhibits Ca$^{2+}$ signals.

We considered whether AKAPs, which are widely implicated in assembling PKA with its regulators and effectors (Smith et al., 2017), might contribute to organization of the cAMP signaling through PKA that leads to inhibition of histamine-evoked Ca$^{2+}$ signals. A membrane-permeant peptide that disrupts association of AKAPs with PKA (st-Ht31) but not its inactive analog (st-Ht31P), significantly attenuated the protein phosphorylation evoked by PGE$_2$, but neither peptide affected the concentration-dependent inhibition of histamine-evoked Ca$^{2+}$ signals by PGE$_2$ (Supplemental Fig. S7). These results suggest that AKAPs are probably not important components of the signaling pathway from PGE$_2$ to inhibition of Ca$^{2+}$ signals.

**Discussion**

In human ASMC, the IP$_3$-mediated Ca$^{2+}$ signals evoked by activation of H$_1$ histamine receptors are attenuated by PGE$_2$. Several lines of evidence show that this inhibition is mediated by cAMP. The concentration-effect relationships for regulation of AC and Ca$^{2+}$ signals by PGE$_2$ are consistent with cAMP lying upstream of Ca$^{2+}$ in the signaling pathway (Fig. 1B), direct activation of AC or membrane-permeant analogs of cAMP mimic PGE$_2$, and maximal concentrations of these drugs are not additive (Figs. 1 and 2). Our conclusion that cAMP mediates the inhibition of Ca$^{2+}$ signals in human ASMC is consistent with evidence that many receptors, via stimulation of AC, attenuate Ca$^{2+}$ signaling in smooth muscle, including VSM (Morgado et al., 2012). Inhibition of histamine-evoked Ca$^{2+}$ signals by PGE$_2$ does not require activation of EPACs (Fig. 2, D and E). The inhibition is not mediated by accumulation of cGMP after inhibition of PDEs since neither cGMP nor inhibition of PDEs effectively mimicked PGE$_2$ (Fig. 3). Inhibition of histamine-evoked Ca$^{2+}$ signals by PGE$_2$ or 8-Br-cAMP was attenuated by inhibition of PKA using H89, PKI, or Rp-8-CPT-cAMPS (Fig. 4). We conclude that inhibition of histamine-evoked Ca$^{2+}$ signals by PGE$_2$ is (at least largely) mediated by PKA (Fig. 5D).

PKA can enhance Ca$^{2+}$ removal from the cytosol by stimulating Ca$^{2+}$ pumps (Tada and Toyofuku, 1998) or the Na$^+$/Ca$^{2+}$ exchanger (Karashima et al., 2007). However, accelerated removal of cytosolic Ca$^{2+}$ does not mediate inhibition of histamine-evoked Ca$^{2+}$ signals by PGE$_2$ in human ASMC (Supplemental Fig. S5). Nor would this mechanism be consistent with the lack of effect of PGE$_2$ on the Ca$^{2+}$ signals evoked by stimulation of endogenous PAR1 or heterologously expressed M3 muscarinic receptors (Fig. 7, D-F). Cyclic AMP has been proposed to inhibit IP$_3$-evoked Ca$^{2+}$ release (Bai and Sanderson, 2006), but PKA (IP$_3$R1 and IP$_3$R2) and cAMP (IP$_3$R1-3) more often potentiate responses to IP$_3$ (Taylor, 2017). However, under conditions where PGE$_2$ inhibited histamine-evoked Ca$^{2+}$ signals, it had no effect on the sensitivity of IP$_3$Rs to IP$_3$ (Fig. 6). Steps linking receptors to PLC can also be inhibited by cAMP (see references in Yang et al. (1999)). Although two different assays suggested that PGE$_2$ attenuated histamine-evoked PLC activity in human ASMC, neither analysis demonstrated a statistically significant effect (Fig. 7, A and B). However, the lack of effect of PGE$_2$ on the Ca$^{2+}$ signals evoked by PAR1 and muscarinic M3 receptors (Fig. 7, D–F) suggests that the inhibition of histamine-evoked Ca$^{2+}$ signals by cAMP/PKA is probably the result of uncoupling of H$_1$ histamine receptors from G$_{q/11}$. PKA has been reported to phosphorylate H$_2$ histamine receptors (Kawakami et al., 2003; Horio et al., 2004), but the functional consequences have not been thoroughly examined (Miyoshi et al., 2006). We conclude that in human ASMC, PGE$_2$, through EP$_2$ and EP$_4$ receptors (Pantazaka et al., 2013), stimulates AC, leading to formation of cAMP and uncoupling of histamine from stimulation of PLC, most probably by PKA-mediated phosphorylation of H$_1$ receptors.

Cyclic AMP can be locally delivered to intracellular targets (Zaccolo, 2011; Cooper and Tabbassum, 2014). AKAPs play prominent roles in targeting cAMP through PKA to specific cellular responses (Smith et al., 2017), but our results suggest that AKAPs probably do not contribute to inhibition of histamine-evoked Ca$^{2+}$ signals by PGE$_2$ (Supplemental Fig. S7). Our results do, however, reveal an additional complexity in the pathways linking PGE$_2$ to inhibition of histamine-evoked Ca$^{2+}$ signals. Although cAMP mediates this inhibition, the concentration-dependent effects of PGE$_2$ were insensitive to substantial inhibition of AC (Fig. 8). These results and analyses of the effects of selective activation of EP$_2$ and EP$_4$ receptors lead to the scheme shown in Fig. 8F. We suggest that communication between EP receptors and the PKA that inhibits histamine-evoked IP$_3$ formation is mediated by delivery of cAMP within signaling junctions. Activation of a junction allows local delivery of a supersaturating concentration of cAMP to PKA, allowing each junction to function as a robust on-off switch. We suggest that the concentration-dependent effects of PGE$_2$ arise from recruitment of these junctions and not from graded activity within individual junctions. Such digital
signaling from receptors to intracellular targets via hyperactive junctions (Fig. 8F) allows robust and reliable communication, and may be a general feature of signaling by diffusible intracellular messengers (Tovey et al., 2008).

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Authorship Contributions

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