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8-isoprostaglandin E_2 activates Ca^{2+}-dependent K^+ current via cyclic AMP signaling pathway in murine renal artery

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Abstract

The inhibitory pathway of 8-isoprostaglandin E_2 was investigated in murine renal arterial smooth muscle. K^+ current was augmented in a concentration-dependent fashion, with an average increase of 123±28% (n=6) following application of 10^{-5} M 8-isoPGE$_2$. This augmentation was observed in the presence of 4-aminopyridine (4-AP, 10^{-3} M) but not that of charybdotoxin (ChTx, 10^{-7} M). Fluorimetric recordings showed marked concentration-dependent increase of cytosolic Ca^{2+} levels by 8-isoPGE$_2$, while an enzyme-linked immunosorbent assay (ELISA)-based cyclic AMP assay showed increased cAMP levels by 10^{-7} M 8-isoPGE$_2$ challenge. The isoprostane-induced augmentation was prevented by the ryanodine receptor blocker ruthenium red (10^{-5} M) or the adenylate cyclase blocker SQ 22536 (10^{-4} M). The protein kinase A (PKA) inhibitor H89 (10^{-5} M) inhibited resting K^+ currents (78±5%, n=5) but did not prevent 8-isoPGE$_2$ from augmenting the remaining K^+ current. We conclude that 8-isoPGE$_2$ enhances Ca^{2+}-dependent K^+ currents in murine renal artery through a cAMP-dependent pathway which may involve internally sequestered Ca^{2+}.

Keywords: Isoprostane; K^+ channel; Cyclic AMP; Protein kinase A; Rho/Rho-kinase

1. Introduction

Isoprostanes are a group of metabolic products of membrane-derived arachidonic acid, formed in vivo by nonenzymatic free radical-catalyzed peroxidation. Although they are frequently used as markers of oxidative stress, it is becoming increasingly important to also recognize that isoprostanes exert potent biological activities on a variety of cell types including smooth muscle (Crocowski et al., 2002; Cracowski and Ormezzano, 2004; Janssen, 2001, 2004; Morrow, 1995). For example, we have previously described powerful constrictor effects in airway and pulmonary vascular preparations, mediated through activation of thromboxane A_2 selective prostanoid (TP) and/or prostaglandin E_2 selective prostanoid (EP) receptors (Catalli and Janssen, 2004; Janssen et al., 2000, 2001; Janssen and Tazzeo, 2002). More recently, we have also described relaxations exerted by isoprostanes, primarily those of the E-ring series, via activation of EP receptors (Catalli et al., 2002; Janssen et al., 2000; Zhang et al., 2003). This property and several others (e.g., the chemical nature and cellular origin of the isoprostanes) closely mirror that of endothelium-derived hyperpolarizing factor, prompting us to propose that isoprostanes may be candidate molecules for this biologically important autacoid (Janssen, 2002). Furthermore, given that isoprostanes can act through prostanoid TP, EP and $F_{2\alpha}$-selective (FP) receptors, we have hypothesized that isoprostanes may exert this endothelium-derived hyperpolarizing factor (EDHF)-like function through activation of prostacyclin-selective prostanoid (IP) receptors. The downstream signaling events underlying isoprostane-evoked vasodilations have not yet been examined.

In this study, we examined the electrophysiological effects of the E-ring isoprostane 8-isoPGE$_2$ in a murine

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renal artery preparation. The arterial smooth muscle cells were pretreated with a prostanoid TP-receptor blocker (ICI 192605, added in perfusing Ringer's solution) to block its potential excitatory effect. We found this autacoid activates Ca^{2+}-dependent K^+ current via elevation of cytosolic levels of cAMP, but apparently not via activation of cAMP-dependent protein kinase A.

2. Materials and methods

2.1. Isolation of smooth muscle cells from mouse renal arteries

Adult (6–8 months old) Balb/c mice were purchased from the Charles River Laboratories, Montreal, Quebec. After euthanizing with carbon-dioxide suffocation, the right and left renal arteries were carefully dissected under microscope. The arteries were cleaned of loosely adherent connective tissue in oxygenated Kreb's solution at 4 °C. The tissues were minced and transferred to an enzyme solution containing collagenase and elastase (described in the Chemicals and solutions). The tissues were incubated with the enzymes in a 37 °C bath for 45 min, then carefully triturated to liberate free smooth muscle cells. The resulting cell suspension was stored at 4 °C and used within 8 h. Individual cells were allowed to adhere to the glass bottom of a 1 ml recording chamber, and then were superfused with Ringer's solution at a rate of 2 ml/min.

2.2. Patch clamp electrophysiology

Membrane potassium currents were recorded at room temperature using the nystatin-perforated patch configuration. The pipette tip was back-filled with nystatin-free electrode solution, and the remainder of the pipette filled with nystatin-containing electrode solution (0.3 mg/ml) for membrane perforation. All experimental recordings were made in smooth muscle cells that were phase-dense and appeared relaxed. Micropipettes were made from borosilicate glass capillary tubing (Sutter Instrument Co, Novato, CA) using a programmable puller (model P-87, Sutter Instrument Co, Novato, CA) and fire polished (MF-830, Narishige, Japan). Tip resistance was adjusted to a range of 3 to 5 MΩ by heat polishing. Access resistances ranged from 8 to 34 MΩ (70% compensation applied), and cell capacitance ranged from 9 to 28 pF. Membrane currents were filtered at 1 kHz and sampled at 2 kHz. Current signals were converted from analog to digital format (DigiData 1200, Axon Instruments, Foster City, CA), and stored on the computer. Acquisition and analysis of data were accomplished using Axopatch 200B and pCLAMP8 software (Axon Instruments, Foster City, CA). A micro-puffer (Picospritzer™ II, General Valve Corp, Fairfield, NJ) was used for drug application.

Pipettes were sealed to the cell using a negative pressure at 0 mV. Holding potential was set at −70 mV. After a suitable access resistance (<40 MΩ) was gained, membrane potential was stepped (350 ms pulse duration) from −60 to +50 mV in 10 mV increments at 3 s intervals. In most experiments, current–voltage relationships were recorded before and after drug application. The K^+ currents were also measured during step depolarization to either +30 or +50 mV, delivered at 10 s intervals. The data were later plotted and analyzed using Clampfit 8.0 and SigmaPlot 2000 software.

2.3. Cyclic AMP assays

A commercially available kit (Cayman Chemicals; Ann Arbor, MI) was used to determine whether 8-isoPGE$_2$ increased intracellular levels of cAMP. Briefly, forty segments of renal arteries were dissected from twenty mice, and evenly distributed in five Eppendorf tubes each containing 1 ml Kreb's solution. The tissues were challenged with 10^{-7} and 10^{-5} M 8-isoPGE$_2$ under different conditions, and compared with negative controls [Kreb's solution alone, or supplemented with 0.5 mM 3-isobutyl-1-methylxanthine and 10^{-6} M ICI 192605] and positive control (tissues stimulated with 10^{-5} M forskolin and 0.5 mM 3-isobutyl-1-methylxanthine). The tissues were incubated in 37 °C bath for 20 min, and quickly transferred to dry ice to stop the biochemical reaction. The samples were collected and homogenized to extract cAMP with phosphate buffer containing 7.5% trichloroacetic acid (neutralized with $CaCO_3$). The cAMP was then detected by enzyme-linked immunosorbent assay, as per kit instructions.

2.4. $[Ca^{2+}]_I$ fluorimetry

Murine renal arteries were digested and dissociated as described above; the tissue digest was centrifuged (200/*g* for 1 min at room temperature), the pellet re-suspended in Ringer's buffer and cells incubated with fluo-4 AM cell permeant (2 μM, dissolved in dimethyl sulphoxid containing 0.1% pluronic F-127; 37 °C; 30 min). Cells were placed in the recording chamber of a custom-built confocal microscope. Cells studied were chosen on the basis of morphology (spindle-shaped; relaxed; absence of blebs; phase-bright) and responsiveness to 10 mM caffeine (applied by micropipette brought into vicinity of the cell using a hydraulic micromanipulator). Dye was excited using 488 nm light from a 20 mW photodiode laser (Coherent Technologies; CA) and emitted light images (>500 nm) were acquired at 1 Hz. Fluorescence intensities from regions-of-interest (central, non-nuclear regions of cell) were saved and plotted against time.

2.5. Chemicals and solutions

Digestion solution contained collagenase (type F, from *Clostridium histolyticum*, 1 mg/ml), elastase (type IV, from porcine pancreas, 0.25 mg/ml), and bovine serum albumin

Table 1
Pharmacological sensitivities of baseline currents and 8-isoPGE$_2$-evoked currents

	Blocker-induced change in baseline current	8-isoPGE$_2$-induced change of current in the presence of blocker
Control	N/A	+123±28% (n=6)
TEA (1 mM)	−68±7% (n=4)[a]	Not done
4-AP (1 mM)	−15±5% (n=3)	+109±55% (n=4)
ChTx (10^{-7} M)	−57±9% (n=6)[a]	+16±11% (n=4)[b]
Ruth red (10^{-5} M)	−43±13% (n=4)[a]	0% (n=2)
H89 (10^{-5} M)	−78±5% (n=5)[a]	+160±31% (n=3)
Y27 (10^{-5} M)	−26±8% (n=6)	+22±10% (n=4)[b]
SQ 22536 (10^{-4} M)	+9±3% (n=4)	+6±4% (n=4)[b]

[a] Significantly different from control baseline current.
[b] Significantly different from 8-isoPGE$_2$-evoked response in the absence of blocker.

(1 mg/ml), in Ca^{2+}- and Mg^{2+}-free Hanks' solution. Electrode solution had the following components (in mM): 140 KCl, 1 $MgCl_2$, 0.4 $CaCl_2$, 20 HEPES, and 1 EGTA (pH=7.2). The nystatin solution was prepared as 30 mg/ml stock in dimethyl sulphoxid, then freshly diluted to a final concentration of 0.3 mg/ml in electrode solution. The cells were constantly perfused using standard Ringer's solution which contained the following (in mM): 130 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, and 10 D-glucose (pH=7.4).

8-isoPGE$_2$ was purchased from Cayman Chemical (Ann Arbor, MI). *N*-[2-(*p*-bromocinnamyl-amino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89) was purchased from Upstate Cell Signaling Solution (Charlottesville, VA). 9-(Tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ 22536) was purchased from Calbiochem (Hornby, ON). *trans*-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride (Y27632) was obtained from Tocris Cookson Inc. (Ellisville, MO). Fluo-4 AM cell permeant was purchased from Molecular Probes, Inc. (Eugene OR). HEPES, $MgCl_2$ and KCl were purchased from BioShop Canada Inc. (Burlington, ON). D-glucose was purchased from Merck Inc. (Darmstadt, Germany). DMSO was obtained from Caledon Laboratories Ltd. (Georgetown, ON). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

2.6. Statistical analysis

All values are expressed as mean±S.E.M. unless otherwise indicated. Statistical comparison was performed using two-tailed paired Student's *t*-test. Differences were considered significant when $P<0.05$.

3. Results

3.1. Basal potassium currents in murine renal artery

We first examined the basal potassium currents in freshly isolated smooth muscle cells from murine renal artery using several selective potassium channel blockers. These currents were significantly reduced by tetraethylammonium (TEA; 1 mM; n=4) and ChTx (10^{-7} M;

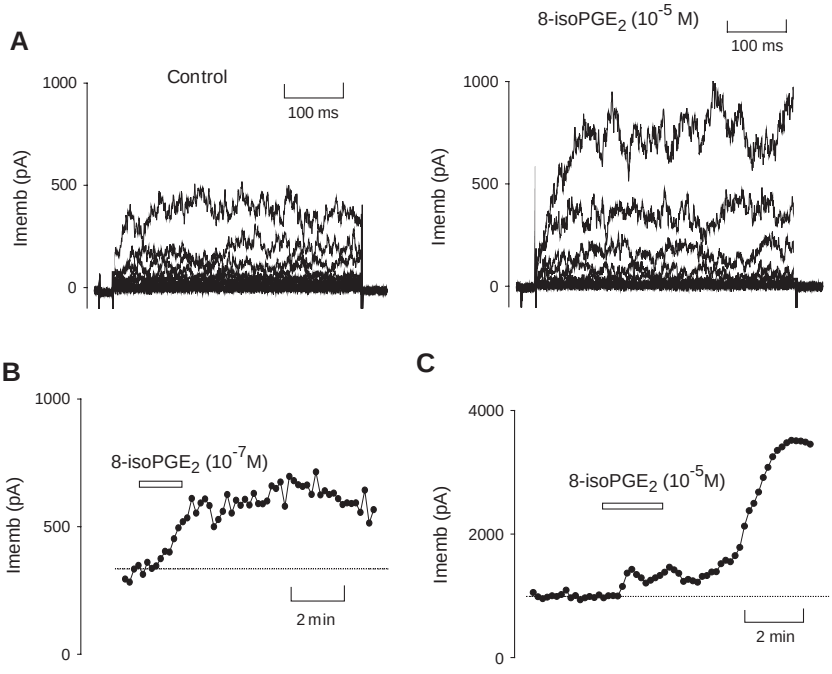

Fig. 1. Effect of 8-isoPGE$_2$ on K^+ currents in murine renal artery. (A) K^+ currents were evoked by depolarizing pulses from −60 to +50 mV (350 ms duration) in the absence (left panel) and presence (right panel) of 10^{-5} M 8-isoPGE$_2$. (B) and (C) Single depolarizing pulses (to +30 mV; 850 ms duration, delivered at 20 s intervals) were used to evaluate the time course of changes evoked by 8-isoPGE$_2$ (10^{-7} and 10^{-5} M, respectively).

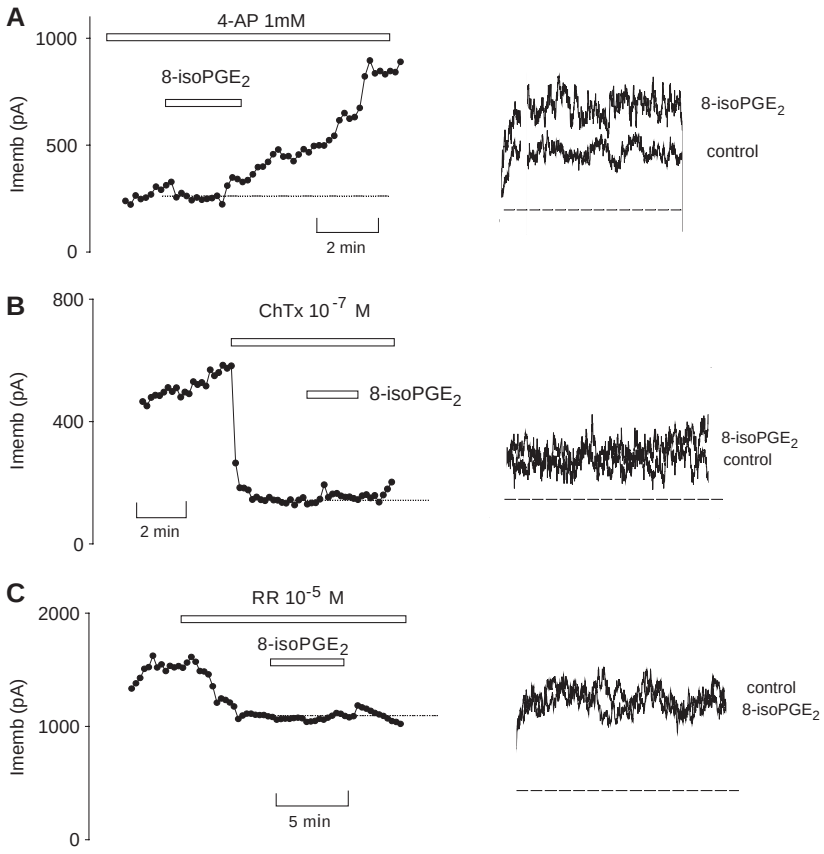

Fig. 2. Effect of selective K^+ channel blockers on isoprostane-evoked currents. 8-isoPGE$_2$ and the channel blockers were applied by different micro-puffers placed close to the testing cell. Dotted lines in each figure indicate magnitude of K^+ currents immediately before application of the isoprostane. 8-isoPGE$_2$ (10^{-5} M) augmented K^+ current in the presence of the Kv blocker 4-AP (1 mM) (A), but not in the presence of the K_{Ca} antagonist charybdotoxin (10^{-7} M) (B). In cell treated with the ruthenium red (10^{-5} M), 8-isoPGE$_2$ didn't augment K^+ currents (C). The original recording traces were presented in the right panels.

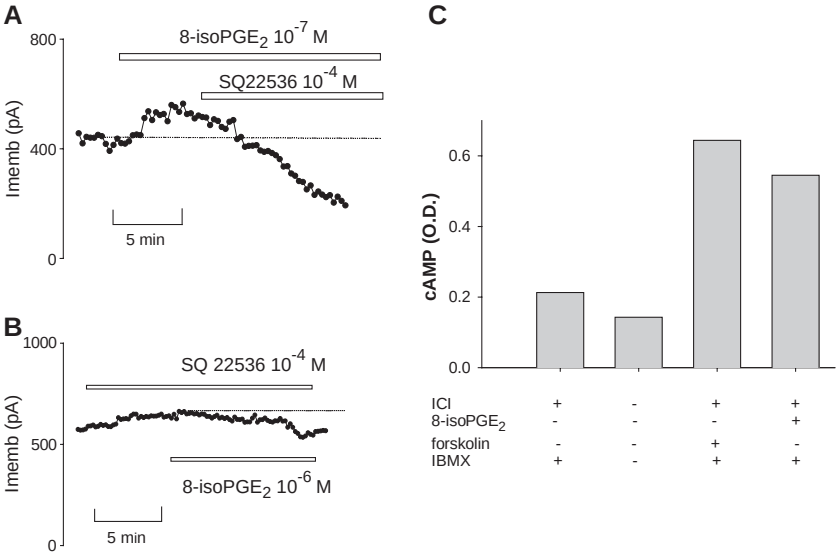

Fig. 3. Role of cAMP in 8-isoPGE$_2$-evoked response. 8-isoPGE$_2$ and SQ 22536 (adenylate cyclase inhibitor) were delivered by micro-puffers placed near to the experimental cell at a testing pulse of +30 mV. The 8-isoPGE$_2$-induced change in K^+ current was reversed (A) or prevented (B) by SQ 22536 (10^{-4} M). (C) Tissues were incubated with ICI 192605 for 20 min, followed by 20 min incubation with other reagents as indicated, then were subjected to ELISA for accumulation of cAMP: 8-isoPGE$_2$ (10^{-7}M) elevated cAMP levels to nearly the same extent as forskolin.

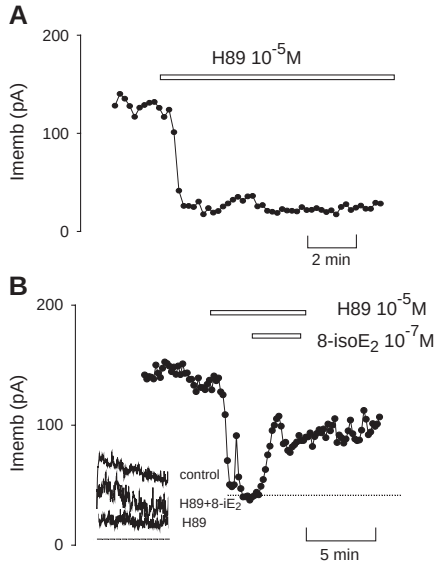

Fig. 4. Effects of H89 on basal and 8-isoPGE$_2$-induced K^+ currents. All reagents were delivered through micro-puffers after a steady control current level was reached at a testing pulse of +30 mV. H89 (10^{-5} M) partially suppressed basal K^+ current (A), but did not prevent 8-isoPGE$_2$ from activating further K^+ current (B). The original experimental recording was presented as inset (B).

n=6), but not by 4-AP (1 mM; n=3) (Table 1; Fig. 2A; B), suggesting that voltage-dependent K^+ channels contribute very little to basal membrane current in these cells, and that Ca^{2+}-dependent K^+ channels are primarily involved. Consistent with this, we also found that basal membrane currents were significantly reduced by the ryanodine receptor blocker ruthenium red (10^{-4} M; n=4) (Table 1; Fig. 2C).

3.2. Electrophysiological response to 8-isoPGE$_2$

In murine renal arterial smooth muscle cells pretreated with the prostanoid TP receptor antagonist ICI 192605 (10^{-6} M), application of 8-isoPGE$_2$ resulted in marked and statistically significant augmentation of membrane K^+ current. Fig. 1A summarizes the family of K^+ currents evoked in one cell by incrementing step depolarizations delivered before (left panel) and after (right panel) application of 10^{-6} M 8-isoPGE$_2$, resulting in augmentation of 88%. Fig. 1B and C illustrates the time-course of these changes in two different cells depolarized repeatedly with a single step command (to +50 mV, from holding potential of −70 mV; 850 ms duration). In particular, augmentation of the K^+ current was not instantaneous, but developed gradually over the course of several minutes, continuing even after the application of the isoprostane had ended. On average, 8-isoPGE$_2$ (10^{-5} M) augmented the magnitude of membrane current evoked by depolarization to +50 mV by 123±28% above baseline (n=6, P<0.05) (Table 1), the maximal effect occurring 6 to 8 min after onset of drug application.

To characterize the subtype of the 8-isoPGE$_2$-augmented current, we also examined the response to the isoprostane following pretreatment with the selective K^+ channel blockers. In the presence of the voltage-gated K^+ channel blocker 4-AP (1 mM), 8-isoPGE$_2$ continued to augment K^+ current (Fig. 2A); on average, the magnitudes of K^+ currents evoked at +50 mV were augmented above baseline by 109 ±55% (n=4) (Table 1). In the presence of ChTx (10^{-7} M), on the other hand, the effect of 8-isoPGE$_2$ was virtually abolished (16±11% augmentation, n=4) (Fig. 2B; Table 1). Likewise, 8-isoPGE$_2$ showed no effect on K^+ current in cells pretreated with ryanodine receptor blocker ruthenium red (10^{-5} M) (Fig. 2C; Table 1).

3.3. Signaling pathway underlying isoprostane-evoked electrophysiological response

Our laboratory and others have previously shown that relaxant effects of isoprostanes involve the cyclic AMP-mediated signal transduction pathway. To test this hypothesis in the present study, we used the adenylate cyclase inhibitor SQ 22536 (10^{-4} M). This pharmacological tool had no statistically significant effect on basal K^+ currents, but eliminated the electrophysiological response to 8-isoPGE$_2$ (Fig. 3A; Table 1), consistent with a role for adenylate cyclase in the isoprostane-response.

We also measured cAMP accumulation directly using a commercially available ELISA kit (see Methods): levels of cAMP were nearly tripled in cells treated with 10^{-7} M 8-isoPGE$_2$, almost matching the increase caused by the

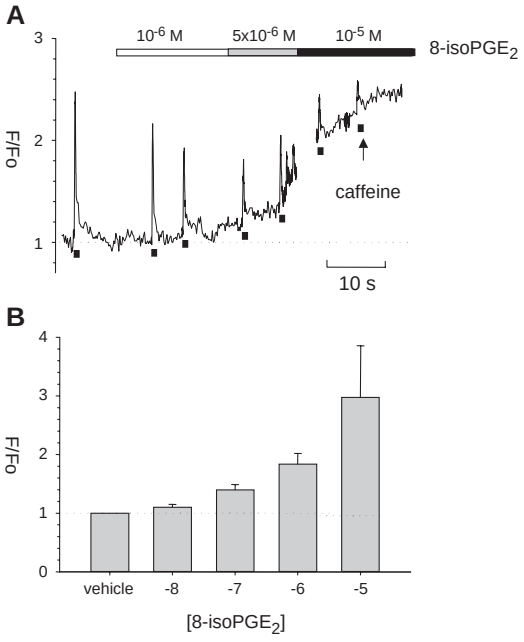

Fig. 5. Effect of isoprostane on cytosolic [Ca^{2+}]. (A) In a cell loaded with the fluorimetric dye fluo-4 and pretreated with ICI 192605, 8-isoPGE$_2$ elevated [Ca^{2+}] in a concentration-dependent fashion. Caffeine (10 mM) was also applied at various times, as indicated. (B) Concentration–response relationship for 8-isoPGE$_2$-induced changes in [Ca^{2+}]$_i$ (n=5).

adenylate cyclase activator forskolin (10^{-5} M), compared to the control (Fig. 3).

Finally, we examined the role of protein kinase A, the downstream effector of cAMP, using H89 (10^{-5} M), a compound which inhibits PKA (as well as Rho-kinase) (Davies et al., 2000). Curiously, H89 did markedly suppress basal current (Fig. 4A; Table 1) but did not prevent the response to 8-isoPGE$_2$ (Fig. 4B; Table 1).

The effect of H89 on basal current surprised us, given that SQ 22536 had no effect on this parameter. Since H89 also inhibits Rho-kinase, we compared its effect to that of Y27632, a highly selective Rho-associated protein kinase inhibitor (Uehata et al., 1997; Yoshii et al., 1999). Similar to the effect of H89, Y27632 (10^{-5} M) showed a tendency to suppress basal membrane currents, though this effect was not statistically significant; subsequent application of 8-isoPGE$_2$ (10^{-5} M) still evoked a membrane current response, albeit significantly smaller than that observed in control cells (Table 1).

3.4. Ca^{2+} imaging

8-isoPGE$_2$ caused a marked elevation of intracellular levels of $[Ca^{2+}]_i$ in murine renal arterial myocytes pretreated with ICI 192605 and loaded with Fluo-4. The effect showed a concentration-dependent fashion, with a threshold concentration in the submicromolar range and peak at 10^{-5} M. Caffeine (10 mM) trigged the release of Ca^{2+} from sarcoplasmic reticulum, and was applied intermittently as an indication of cell viability during the experiment. When 10^{-5} M 8-isoPGE$_2$ was applied into the chamber, the intracellular $[Ca^{2+}]$ was increased to 250% of the base level. The results are summarized in Fig. 5.

4. Discussion

In this study, we demonstrate that submicromolar concentrations of 8-isoPGE$_2$ augment membrane K^+ currents in the murine renal artery (with TP receptors blocked to prevent any of its potential excitatory effects). This is expected to cause membrane hyperpolarization (not examined here), leading to vasodilation. The potassium channels involved in this response are apparently Ca^{2+}-dependent in nature, given that the responses were blocked by TEA or by charybdotoxin, but not by 4-AP. Furthermore, we found the isoprostane directly elevated $[Ca^{2+}]$ in ICI 192605-pretreated cells. Finally, the isoprostane-evoked potassium current responses were sensitive to ruthenium red, suggesting that the Ca^{2+} that regulates their function derives from the sarcoplasmic reticulum.

We also probed the signaling pathway underlying the response to 8-isoPGE$_2$. Our previous studies of isoprostane-evoked relaxant responses (Catalli et al., 2002) prompted us to first examine the possibility that cAMP transduces this effect. Consistent with this, we found endogenous levels of cAMP to be markedly increased by 8-isoPGE$_2$, and the potassium current response to be abolished by the adenylate cyclase inhibitor SQ 22536. Surprisingly, however, the electrophysiological response was not affected by the PKA inhibitor H89, suggesting a lack of involvement of this kinase. The link between cAMP, Ca^{2+} and the K^+ channels is at present unclear: it might be that cAMP acts directly on the K^+ channel (as it does elsewhere) or on some Ca^{2+}-homeostatic mechanism of the intracellular Ca^{2+}-store. Further experiments are required to clarify this aspect of the signaling pathway.

There continues to be extensive debate regarding the molecular nature of EDHF. In fact, it may be that there are many different molecular forms of EDHF. We have hypothesized that isoprostanes might represent one of these forms (Janssen, 2002). The current study is consistent with such a role in that the isoprostanes can clearly exert a hyperpolarizing effect on the membrane via activation of BK_{Ca} current, ultimately leading to vasodilation. We further hypothesize that this effect might involve IP receptors; unfortunately, blockers for IP receptors are not available, preventing us from directly testing this hypothesis.

The observation that H89 inhibited basal K^+ currents might suggest that on-going activity within the cAMP/PKA-signaling pathway stimulates K^+ channels at rest. However, this explanation is at odds with our finding that the adenylate cyclase inhibitor SQ 22536 did not affect baseline K^+ currents. Instead, H89 is also known to inhibit Rho-activated kinase (Davies et al., 2000). Consistent with that explanation, we also found baseline currents to be suppressed by the selective Rho-kinase inhibitor Y27632. Thus, it would appear that baseline currents (predominantly Ca^{2+}-dependent K^+-currents, given their sensitivity to charybdotoxin and ruthenium red) are under regulation in some way by Rho/Rho-kinase.

In summary, our results suggest that 8-isoPGE$_2$ augments Ca^{2+}-dependent K^+-currents activity via the cAMP-mediated signaling pathway in a manner which does not involve PKA activity. These data are consistent with the possibility that isoprostanes might represent one of the EDHFs.

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