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# VASODILATORY AND ELECTROPHYSIOLOGICAL ACTIONS OF 8-*iso* PROSTAGLANDIN $E_2$ IN PORCINE CORONARY ARTERY

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Running title: **Isoprostane-evoked electrophysiological responses**

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Non-standard abbreviations:

$EC_{50}$ - half-maximally effective concentration

EDCF - endothelium-derived contracting factor

EDHF - endothelium-derived hyperpolarizing factor

ICI 192605 - 4(Z)-6-[(2,4,5 cis)2-(2-chlorophenyl)-4-(2-hydroxyphenyl)1,3-dioxan-5-yl] hexenoic acid

8-*iso* PG - 8-*iso* prostaglandin

L-NNA- N-ω-nitro-L-arginine

TP - thromboxane $A_2$-selective prostanoid receptor

**Abstract**

We examined the effects of several E-ring and F-ring isoprostanes on mechanical and electrophysiological activity in porcine coronary artery. Several isoprostanes evoked concentration-dependent contractions, with 8-*iso* $PGE_2$ being the most potent (-log $EC_{50}$ of 6.9±0.1): this excitatory effect has been described in detail elsewhere and was not examined further here. 8-*iso* $PGE_2$ evoked dose-dependent relaxations in tissues preconstricted with the $TXA_2$-agonist U46619 ($10^{-6}$ M), with an $EC_{50}$ of 6.0±0.1 (n=5). 8-*iso* $PGE_1$ and 8-*iso* $PGF_{2\beta}$ also evoked relaxations (albeit with lower potency), while the other F-ring isoprostanes (8-*iso* $PGF_{1\alpha}$ , 8-*iso* $PGF_{1\beta}$, and 8-*iso* $PGF_{2\alpha}$) were largely ineffective in this respect. The potency and efficacy of 8-*iso* $PGE_2$ in reversing tone were not dependent upon the concentration of U46619 used to preconstrict the tissues ($10^{-8}$ to $10^{-6}$ M) , indicating a lack of U46619-induced functional antagonism of these responses. 8-*iso* $PGE_2$ was able to completely relax tissues which had been denuded of endothelium (as indicated by loss of responsiveness to bradykinin). 8-*iso* $PGE_2$-evoked relaxations were markedly reduced by elevating the $K^+$ equilibrium potential using 30 mM KCl, and abolished by 60 mM KCl; they were also sensitive to charybdotoxin ($10^{-7}$ M) but not to 4-aminopyridine (1 mM). 8-*iso* $PGE_2$ also caused membrane hyperpolarization and augmentation of outward $K^+$ current. We conclude that 8-*iso* prostaglandin $E_2$ acts directly on the smooth muscle to increase $K^+$ conductance, leading to membrane hyperpolarization and vasodilation.

Isoprostanes are a novel class of arachidonic acid metabolites generated by oxygen free radical-mediated peroxidation of membrane phospholipids (Janssen, 2001). As such, they have been used clinically and experimentally as markers for many disease states in which oxidative stress is a prominent feature, including myocardial and renal ischemia-reperfusion injury (Mobert and Becker, 1998; Takahashi *et al.*, 1992), atherosclerosis (Pratico *et al.*, 1997), pulmonary hypertension (Christman *et al.*, 1998), and hypercholesterolemia (Oguogho *et al.*, 1999).

They are now recognized to also have powerful effects on mechanical activity in vascular smooth muscle. Many have described contractile responses to isoprostanes in a wide variety of arterial beds, generally via stimulation of thromboxane $A_2$ receptors (TP receptors), which then enhance the $Ca^{2+}$-sensitivity of the contractile apparatus through some mechanism that is largely dependent on tyrosine kinase activation (Janssen, 2001). They may also cause vasoconstriction through an action on $PGE_2$-selective (EP) receptors coupled to release of internally sequestered $Ca^{2+}$ (Janssen and Tazzeo, 2002). More recently, some have identified important vasodilatory actions of isoprostanes (Janssen *et al.*, 2001; Jourdan *et al.*, 1997; Janssen *et al.*, 2000); however, the signaling mechanisms underlying those inhibitory responses have not been investigated. Likewise, the vascular electrophysiological actions of isoprostanes — both excitatory and inhibitory — have also been unexplored.

In general, many vasodilators act indirectly via the endothelium, causing the latter to release prostacyclin, nitric oxide, and/or one or more endothelium-derived hyperpolarizing factors (EDHFs). Much is known about the properties and actions of EDHF, but there is still considerable

debate regarding its identity (McGuire *et al.*, 2001; Busse *et al.*, 2002). In the porcine coronary artery, vasodilators such as bradykinin stimulate production of reactive oxygen species by cytochrome P450 enzymes in the endothelium (Fleming *et al.*, 2001), and trigger a series of events which result in activation of large conductance $Ca^{2+}$-dependent $K^+$ currents and vasodilation (Pomposiello *et al.*, 1999; Hayabuchi *et al.*, 1998b; Barlow and White, 1998). One of these events appears to include release of a cyclooxygenase-independent metabolite of arachidonic acid (Chataigneau *et al.*, 1998; Cowan and Cohen, 1991; Cowan and Cohen, 1992; Hayabuchi *et al.*, 1998a; Hecker *et al.*, 1994; Weintraub *et al.*, 1995), one which is not a cannabinoid (Chataigneau *et al.*, 1998; Pomposiello *et al.*, 1999; Grainger and Boachie-Ansah, 2001). While many acknowledge that cytochrome P450 plays a key role in the production of EDHF(s) in this tissue (Bauersachs *et al.*, 1994; Busse *et al.*, 2002; Fleming *et al.*, 2001), it is not clear whether the vasoactive metabolite is an epoxyeicosatrienoic acid or some oxygen free radical which could in turn lead to generation of isoprostanes. In fact, it is possible that epoxyeicosatrienoic acids, isoprostanes and reactive oxygen species could all collectively play the role of EDHF in this tissue.

In this study, we explored the mechanisms by which isoprostanes exert inhibitory effects on porcine coronary artery (outer diameter 0.5 to 1.0 mm). We examined the effects of two E-ring isoprostanes (8-*iso* $PGE_1$ and 8-*iso* $PGE_2$) and four F-ring isoprostanes (8-*iso* $PGF_{1\alpha}$ , 8-*iso* $PGF_{1\beta}$, 8-*iso* $PGF_{2\alpha}$ and 8-*iso* $PGF_{2\beta}$) using standard organ bath, intracellular microelectrode, and patch-clamp electrophysiological techniques.

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## MATERIALS AND METHODS

**Tissues.** Porcine hearts were transported from a local abattoir in ice-cold standard Krebs buffer; segments of the left descending coronary artery (0.5 to 1.0 mm outer diameter) were excised and used immediately or refrigerated for use the following day.

**Muscle baths.** Intact tissues were mounted as ring segments (3-4 mm long) in standard organ baths for recording of mechanical activity (as described elsewhere (Janssen *et al.*, 2000; Janssen *et al.*, 2001)) and bathed at 37°C in Krebs buffer (NaCl, 116; KCl, 4.2; $CaCl_2$, 2.5; $NaH_2PO_4$, 1.6; $MgSO_4$, 1.2; $NaHCO_3$, 22; D-glucose, 11) supplemented with indomethacin ($10^{-5}$ M) and bubbled to maintain pH at 7.4. Where indicated, the endothelium was intentionally damaged by gently rubbing the lumen of the tissues using a wood splinter. While the endothelium was clearly affected by this procedure (as indicated by the reduction in bradykinin-evoked responses; Fig. 2), the smooth muscle *per se* appeared not to be seriously damaged, since the magnitude of contractions evoked by $10^{-6}$ M U46619 were 0.92±0.21 g and 0.74±0.16 g in endothelium-intact and -denuded tissues, respectively (n=6 for both). After a 60-90 minute equilibration period, tissue viability was assessed using 60 mM KCl, after which tissues were washed and preload adjusted to 0.4 - 0.5 grams and L-NNA added; experiments were commenced 30 minutes later. Isometric changes in tension were digitized (2 samples per second) and recorded on-line (DigiMed System Integrator, MicroMed, Louisville, KY) for subsequent analysis using Origin 6.0 software (Microcal software, Inc).

**Intracellular microelectrode recordings.** Porcine coronary arterial segments were slipped over a cannula with adventitia outwards, or were cut open and pinned out with adventitia upwards. These were superfused with Krebs buffer (composition above; supplemented with $10^{-5}$ M indomethacin) at 37°C at a rate of 3 ml/min. Cells were impaled with microelectrodes having tip resistance of 30-100 MΩ when filled with 3 M KCl. Membrane potentials were amplified (Duo

773, World Precision Instruments, Sarasota, FL) and digitized at 5 Hz using SigmaPlot 2000 software (SPSS Inc., Chicago, IL).

**Patch clamp electrophysiology.** Intact tissues were minced and incubated for 30-60 minutes with collagenase (Sigma Chemicals, blend F; 0.9 U/ml) and elastase (Sigma Chemicals, type IV, 12.5 U/ml) and incubated at 37°C for 1 hour, then gently triturated to liberate individual myocytes. The single cells were allowed to settle and adhere to the bottom of a recording chamber (1ml volume), were superfused at room temperature with standard Ringer's solution (130 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 10 D-glucose, pH 7.4) containing the thromboxane receptor antagonist ICI 192605, and were studied within 8 hour after dissociation. Patch clamp recordings were made in cells that were phase-dense and appeared relaxed. The majority of recordings were made using the nystatin-perforated configuration of the whole-cell patch-clamp technique (Hamill *et al.*, 1981) and pipettes with tip resistance of 3-5 MΩ when filled with standard electrode solution (140 KCl, 1 $MgCl_2$, 0.4 $CaCl_2$, 20 HEPES, 1 EGTA, and 150 U/ml nystatin, pH 7.2). The current-voltage relationship of the membrane currents was examined using a series of incrementing step depolarizations (10 mV increments from holding potential of -70 mV; 1 s duration). Membrane currents were amplified, filtered at 1 kHz and sampled at 2 kHz using an Axopatch 200B amplifier and pCLAMP8 software (Axon Instruments, Foster City, CA). The current-voltage relationship of outward currents were compared before and after application of 8-*iso* $PGE_2$; the time-course of the changes exerted by 8-*iso* $PGE_2$ was followed using depolarizing pulses to +30 mV (from the holding potential of -70 mV) delivered at 15 second intervals. In a variation of this approach, an excised vesicle (total capacitance of >3 pF) was formed by gently removing the electrode while maintaining a tight seal (electrode solution as described above), after which unitary outward currents were recorded as described above.

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**Chemicals and solvents** Isoprostanes were purchased from Cayman Chemicals (Ann Arbor, MI, USA); all other chemicals were obtained from Sigma Chemical Company. 10mM stock solutions were prepared in absolute EtOH (isoprostanes, U46619) or distilled water (bradykinin, L-NNA). Dilutions of these were made in physiological medium; the maximal bath concentration of EtOH did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity.

**Statistics** The half-maximum effective concentration ($EC_{50}$) for the isoprostanes were interpolated from individual concentration-effect curves as described previously (Janssen *et al.*, 2000; Janssen *et al.*, 2001). Mechanical responses to isoprostanes were standardized relative to responses to either 60 mM KCl or to $10^{-6}$ M U46619, as indicated, and are reported as mean ± S.E.M. ANOVA (with Neuman-Keuls *post hoc* test) analyses were performed using SigmaStat software (SPSS Inc., Chicago, IL). $P<0.05$ was considered statistically significant; n refers to the number of animals.

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## RESULTS

Mechanical effects of isoprostanes

In porcine coronary arteries pretreated with indomethacin and L-NNA (to block synthesis of prostanoids and nitric oxide, respectively), isoprostanes evoked contractions in a concentration-dependent manner (Fig. 1). In particular, 8-*iso* $PGE_2$, 8-*iso* $PGE_1$, and 8-*iso* $PGF_{2\alpha}$ were the most potent, with negative log $EC_{50}$ (half-maximally effective concentrations) of 6.9±0.1, 6.6±0.1 and 6.3±0.1, respectively. These excitatory effects of isoprostanes have been examined in detail in numerous vascular beds (Janssen, 2001) including the coronary artery (Kromer and Tippins, 1999; Mobert *et al.*, 1997) and been shown to involve TP receptors: we did not characterize these effects any further in this study.

Following preconstriction of the tissues with the thromboxane mimetic U46619 ($10^{-6}$ M; sufficient to saturate the TP receptors), three of the isoprostane molecules studied reversed U46619-induced tone in a concentration-dependent fashion (Fig. 2 and 3A): 8-*iso* $PGE_2$ was the most potent (negative log $EC_{50}$ of 6.0±0.1; n=5), while 8-*iso* $PGE_1$ and 8-*iso* $PGF_{2\beta}$ were somewhat less so (negative log $EC_{50}$ values of 5.5±0.1; n=5). The other F-ring isoprostanes, on the other hand, were largely ineffective in this respect, evoking less than 10% reversal of tone at the highest concentration tested (Fig. 3A).

In a separate set of experiments, we re-examined 8-*iso* $PGE_2$-relaxations under conditions in which the TP receptors were not already maximally stimulated ($10^{-8}$ and $10^{-7}$ M U46619 elicited an increase in tone of 0.69±0.17 and 0.85±0.19 g, respectively, compared to the 1.21±0.14 g response evoked by $10^{-6}$ U46619). Following submaximal stimulation, addition of 8-*iso* $PGE_2$ led to further contraction at submicromolar concentrations, followed by complete reversal of tone at higher concentrations (Figure 3B): there were no significant differences with respect to potency

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($EC_{50}$) or efficacy (% reversal of tone) of 8-*iso* $PGE_2$ in producing these relaxations at any of the [U46619] used to preconstrict the tissues (Table 1).

Endothelial-dependence of 8-*iso* $PGE_2$-evoked relaxations

Many vasodilators (*e.g.*, bradykinin) mediate their effects via an action on the endothelium, causing the latter to release EDHF. To test whether isoprostanes also act here in such an endothelium-dependent fashion, we examined the responses to 8-*iso* $PGE_2$ in indomethacin/L-NNA-treated tissues which had been intentionally denuded of endothelium; bradykinin ($10^{-7}$ M) was used as a functional assay for endothelial integrity. 8-*iso* $PGE_2$ was able to completely reverse U46619-tone ($10^{-6}$ M) even in tissues which had lost all responsiveness to bradykinin (Fig. 2). On average, relaxations evoked by bradykinin ($10^{-7}$ M) were 18±10% (n=6) in intentionally denuded tissues, but 106±8% (n=5) in tissues which were intended to be left intact. The corresponding responses to 8-*iso* $PGE_2$ ($10^{-5}$ M) in the very same tissues, however, were 73±22% and 115±14%, respectively, indicating that the latter are not dependent on the functional integrity of the endothelium. Thus, we conclude that 8-*iso* $PGE_2$ does not act via the endothelium (*i.e.*, to stimulate EDHF release), but rather through a receptor found on the vascular smooth muscle cells.

Role of membrane hyperpolarization in 8-*iso* $PGE_2$-evoked responses

To test whether isoprostane-evoked relaxations were dependent on membrane hyperpolarization and $K^+$ channels, we first investigated the sensitivity of the 8-*iso* $PGE_2$-evoked relaxations to high millimolar concentrations of potassium chloride: under this experimental condition, the potassium equilibrium potential is elevated such that membrane hyperpolarization does not occur even if potassium channels do open. Tissues were maximally stimulated with

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U46619 ($10^{-6}$ M) in the presence or absence of KCl (the latter did not evoke further tone above that elicited by U46619). Relaxations evoked by 8-*iso* $PGE_2$ ($10^{-6}$ and $10^{-5}$ M) were markedly and significantly reduced in the presence of 30 mM KCl, and were abolished in the presence of 60 mM KCl ($p<0.05$; Fig. 4). Thus, the relaxant response to 8-*iso* $PGE_2$ requires hyperpolarization of the membrane.

Intracellular microelectrodes were used to record the hyperpolarization which accompanies this relaxant response. In the presence of indomethacin and L-NNA ($10^{-5}$ and $10^{-4}$ M, respectively), resting membrane potential was -60.0±1.9 mV (n=11). Upon addition of a single bolus of 8-*iso* $PGE_2$ ($3x10^{-5}$ M), the membrane potential briefly depolarized by 5.5±1.0 mV, then exhibited a larger and sustained hyperpolarization of 16.6±1.9 mV (to -71.9±1.9 mV; n=3; $p<0.05$).

Finally, pharmacological blockers were employed to test the pharmacological sensitivities of the 8-*iso* $PGE_2$-evoked responses. Tissues were pretreated with 4-aminopyridine (1 mM) or with charybdotoxin ($10^{-7}$ M) 20 minutes prior to evaluating the response to $10^{-6}$ M 8-*iso* $PGE_2$: in the presence of 4-aminopyridine, there was a nonsignificant trend for reduced relaxations, while charybdotoxin completely abolished them (Fig. 5).

8-*iso* $PGE_2$ augments outward $K^+$ conductances

Patch-clamp electrophysiological techniques were employed to examine more candidly whether or not 8-*iso* $PGE_2$ activated outward potassium conductances. Step depolarizations (10 mV increments) from a holding potential of -70 mV were used to examine the current-voltage relationship of outward $K^+$ conductances before and after application of 8-*iso* $PGE_2$, while test pulses to +30 mV (from the holding potential of -70 mV; 1 sec duration, delivered at 15 second intervals) were used to monitor the time-course of any 8-*iso* $PGE_2$-evoked changes. Step

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depolarizations evoked large outwardly rectifying potassium currents (Fig. 6): these have been characterized in detail elsewhere (Balwierczak *et al.*, 1995; Barlow and White, 1998) and found to represent primarily large conductance $Ca^{2+}$-dependent potassium currents. 8-*iso* $PGE_2$ ($10^{-5}$ M) caused a marked augmentation of these currents at all potentials tested (*i.e.*, the current-voltage relationship was displaced to more negative potentials; Fig. 6B); this augmentation was maximal after 2-3 minutes of application (Fig. 6C). On average, the magnitude of currents evoked using depolarizing pulses to +30 mV were significantly increased to 122±8% of control (n=5; $p<0.05$) by 8-*iso* $PGE_2$. When large conductance $Ca^{2+}$-dependent $K^+$ conductances were blocked using charybdotoxin ($10^{-7}$ M), however, 8-*iso* $PGE_2$ did not cause an increase in depolarization-evoked $K^+$ currents (n=3). There was no evidence of activation of any inward current by 8-*iso* $PGE_2$.

Finally, Figure 7 shows discontinuous recordings of membrane current obtained from an excised vesicle using the nystatin-perforated patch configuration, including activation of unitary conductances of approximately 14 pA during application of 8-*iso* $PGE_2$ ($10^{-5}$ M) from a puffer pipette. The driving force on potassium under these conditions is ≈115 mV, given that the $K^+$ equilibrium potential is -85 mV and the membrane potential is held at +30 mV. As such, unitary currents of this magnitude arise from opening of channels with a conductance of 122 pS. Others have found that, in the porcine coronary artery, peroxide stimulates $Ca^{2+}$-dependent $K^+$ channels with a unitary conductance of 119 pS (Barlow and White, 1998).

## DISCUSSION

There have been many reports of the excitatory actions of isoprostanes and the receptor-effector coupling pathways underlying them in vascular, airway and gastrointestinal smooth muscles (Janssen *et al.*, 2000; Janssen *et al.*, 2001;Janssen, 2001; Kromer and Tippins, 1999; Mobert *et al.*, 1997). However, their inhibitory effects on smooth muscle have been largely unexplored: only two groups have described their relaxant effects in pulmonary vasculature (Janssen *et al.*, 2001; Jourdan *et al.* 1997) and airway smooth muscle (Janssen *et al.*, 2000); and none have examined the signaling pathways involved. Moreover, there have been no studies of the electrophysiological actions (neither excitatory nor inhibitory) of any isoprostane.

Here, we describe in detail the relaxations and electrophysiological effects which are evoked by E-ring isoprostanes, but not their F-ring counterparts, in porcine coronary artery; in fact, 8-*iso* $PGE_2$ achieves this effect with a similar potency and greater efficacy than that of anandamide, another compound which has recently received a great deal of attention with respect to the regulation of vascular smooth muscle function (Chataigneau *et al.*, 1998; Grainger and Boachie-Ansah, 2001; Harris *et al.*, 2002; White and Hiley, 1997; Zygmunt *et al.*, 1997). We have also observed similar relaxations in mesenteric and bronchial arteries, but found cerebral arteries to only constrict in response to any of the isoprostanes tested (data not shown). Clearly, then, this vasodilatory response to isoprostanes is both compound- and tissue-specific.

The inhibitory response in the coronary artery is completely independent of a functional endothelium, indicating that isoprostanes do not act by releasing some other EDHF. Instead, they appear to act directly on the smooth muscle. Furthermore, this direct action is clearly receptor-mediated, since certain isoprostanes were highly effective while others were completely ineffective. In particular, the E-ring compounds were generally far better vasodilators than the F-ring molecules:

these two different subgroups of isoprostanes differ solely with respect to whether the second carbon of the central cyclopentane ring features a ketone or a hydroxyl group, respectively. Also, 8-*iso* $PGF_{2\beta}$ could evoke large relaxations (albeit at relatively high concentrations) while 8-*iso* $PGF_{2\alpha}$ did not: these two compounds differ only in the orientation of a hydroxyl group on the cyclopentane ring. As such, the marked compound-related specificity in the responsiveness of this tissue speaks toward a receptor-mediated mechanism, rather than non-specific changes such as altered membrane fluidity, redox state or effects of vehicle. We did not characterize the receptor(s) involved in mediating this response. However, many believe that isoprostanes act through prostanoid receptors (Janssen, 2001): thus, it is possible that these actions are exerted through the same inhibitory receptors which are activated by $PGE_2$ or $PGI_2$.

Despite the great number of studies addressing the biological actions of EDHF, there is still debate as to its identity (McGuire *et al.*, 2001; Busse *et al.*, 2002). Candidate molecular species have been proposed, each being met simultaneously with support and dispute. Several lines of evidence have prompted us to suggest that isoprostanes might be an EDHF (Janssen, 2002). However, two critical pieces of evidence were lacking at that time.

First, the electrophysiological actions of isoprostanes had been completely unexplored prior to that earlier study. Here we show that the relaxant response evoked by 8-*iso* $PGE_2$ is accompanied by and dependent upon membrane hyperpolarization and augmentation of outward $K^+$ currents. Our observation that 8-*iso* $PGE_2$ activates outward unitary currents of approximately 120 pS, and that the isoprostane-relaxations are sensitive to charybdotoxin, both suggest that the $K^+$ channel involved is of a large conductance $Ca^{2+}$-dependent variety: a full pharmacological and electrophysiological characterization of this current is beyond the scope of the present study. Nonetheless, these findings are consistent with our hypothesis that isoprostanes might mediate EDHF effects.

Second, it will be necessary to show that the endothelium synthesizes and releases one or more of the vasodilatory isoprostanes upon stimulation with an appropriate agonist (*e.g.*, substance P or bradykinin), an endeavour which is also beyond the scope of the present study. Others have shown that the endothelium can release isoprostanes (Watkins *et al.*, 1999). While this has generally been viewed as a result of membrane damage, it is entirely possible that the endothelium might do so in a carefully controlled, enzymatically-driven fashion. For example, free radicals and reactive oxygen species are produced by cyclooxygenase, cytochrome P450, lipoxygenase, nitric oxide synthase, and NADPH oxidase (Matoba *et al.*, 2000; Fulton *et al.*, 1997; Fleming *et al.*, 2001; Thannickal and Fanburg, 2000), which in turn are under direct regulation by the endothelial cell. This could explain some of the reports that EDHF is sensitive to inhibitors of P450 (Bauersachs *et al.*, 1994; Hecker *et al.*, 1994; Adeagbo, 1997), or EDCF to COX inhibitors (Yang *et al.*, 1991), as well as the apparent insensitivity of EDHF/EDCF to free radical scavengers when they are applied extracellularly (Rodriguez-Martinez *et al.*, 1998).

In theory, dozens (if not hundreds) of isoprostane species and their metabolites may exist (Janssen, 2001), but only a handful of these have been tested to date; in fact, most studies of isoprostane pharmacology and pathophysiology focus solely on 8-*iso* $PGF_{2\alpha}$. It may be that the physiologically-relevant isoprostane(s) may be ones which are not yet commercially available. In the present study, we found 8-*iso* $PGE_2$ to be the best vasodilator molecule among the 6 which were tested, but this relaxant effect was masked by its excitatory actions at TP receptors: an isoprostane which would be a better candidate for EDHF would be one which does not stimulate TP-receptors (we have previously identified several of these (Janssen *et al.*, 2000; Janssen *et al.*, 2001) and/or is much more potent at inhibitory receptors.

Thus, isoprostanes are capable of exerting both excitatory and inhibitory actions on smooth

muscle, depending on the particular isoprostane and tissue being tested. In this study, 8-*iso* $PGE_2$ in particular caused excitation at submicromolar concentrations, likely via activation of TP receptors (Janssen, 2001), but relaxation at slightly higher concentrations. Isoprostanes have been the subject of investigation for only a little over one decade, and for most of that time they have been viewed primarily as breakdown products of lipid peroxidation. Recently, however, there has been a growing interest in their biological actions, particularly in the context of oxidative pathophysiology; as such, they have been elevated from being merely markers of oxidative stress to being a novel class of inflammatory mediator (Janssen, 2001). Now it may even be possible that isoprostanes serve a physiological role in the regulation of vascular smooth muscle tone by the endothelium.

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## FOOTNOTES

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## FIGURE LEGENDS

Figure 1 Isoprostane-evoked contractions Mean concentration-response relationships for contractions evoked by 6 different isoprostanes or vehicle (EtOH), as indicated. Responses are standardized as a % of the response evoked by 60 mM KCl. n=4-6.

Figure 2 8-*iso* $PGE_2$ evokes relaxations. After constriction with U46619 ($10^{-6}$ M), bradykinin (Bk; $10^{-7}$ M) evoked relaxations in intact preparations **(A)** but not in those which had been intentionally denuded of endothelium **(B)**; 8-*iso* $PGE_2$, however, relaxed both preparations in a concentration-dependent fashion.

Figure 3 Concentration-dependence of isoprostane relaxations **(A)** Mean concentration-response relationships were derived for the 6 isoprostanes (as indicated) in endothelium-intact tissues preconstricted with U46619 ($10^{-6}$ M). Responses were standardized as percent reversal of U46619-induced tone. n=5 for all. **(B)** In a second set of tissues, ring segments were preconstricted with U46619 ($10^{-8}$ or $10^{-7}$; n=5 for each) before re-examining the responses to 8-*iso* $PGE_2$ ($10^{-9}$-$10^{-5}$ M); responses were standardized as a per cent of the U46619-induced tone existing immediately prior to addition of 8-*iso* $PGE_2$. Inset: mean magnitudes of contractions evoked by U46619.

Figure 4 Dependence of 8-*iso* $PGE_2$-evoked relaxation upon membrane hyperpolarization Mean concentration-relaxation relationships for 8-*iso* $PGE_2$ in tissues preconstricted with U46619 ($10^{-6}$ M) in the presence of 0 mM (□; n=14), 30 mM (▲; n=7), or 60 mM KCl (■; n=4). *, $p<0.05$; **, $p<0.01$

Figure 5 Pharmacological sensitivity of 8-*iso* $PGE_2$-induced relaxations. Tissues were pretreated with vehicle, 4-aminopyridine (1 mM) or charybdotoxin ($10^{-7}$ M), then constricted with U46619 ($10^{-6}$ M) prior to evaluating the relaxant response to 8-*iso* $PGE_2$ ($10^{-6}$ M). * indicates significantly different ($p<0.05$) from the control response.

Figure 6 8-*iso* $PGE_2$ augments macroscopic $K^+$ currents. **(A)** Outward membrane currents were evoked by incrementing step depolarizations (10 mV increments from holding potential of -70 mV; 1 s duration); these were augmented following application of 8-*iso* $PGE_2$ ($10^{-5}$ M). The current-voltage relationship of these currents is given in **(B)**. The time-course of this augmentation is given in **(C)**, which shows mean peak membrane currents evoked by pulses to +30 mV (delivered at 15 second intervals) during the application of 8-*iso* $PGE_2$.

Figure 7 8-*iso* $PGE_2$ activates unitary large conductance $K^+$ currents. Membrane current trace from an outside-out patch studied using the nystatin-perforated patch configuration and the same voltage protocol as that in Fig. . The first 100 milliseconds of data from each step have been blanked from each trace to remove the capacitive transients. No unitary events were noted before application of 8-*iso* $PGE_2$ ($10^{-5}$ M); however, unitary events of 14 pA in amplitude were apparent shortly thereafter. Portions of these data have been expanded below to more clearly reveal these unitary events.

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TABLE 1 Potency and efficacy of 8-*iso* $PGE_2$ under different levels of precontracted state

| [U46619] ‡ | Potency ($EC_{50}$) | Efficacy (% reversal of tone) § |
|---|---|---|
| 37536 | 5.6±0.1 | 71±50 |
| 37535 | 5.7±0.1 | 79±19 |
| 37534 | 6.0±0.1 | 83±17 |

‡ concentration of U46619 used to preconstrict the tissues prior to addition of 8-*iso* $PGE_2$

§ tone existing immediately prior to addition of 8-*iso* $PGE_2$ was defined as 100%, and maximal reversal of tone was scaled accordingly

Index terms:

coronary artery

vascular smooth muscle

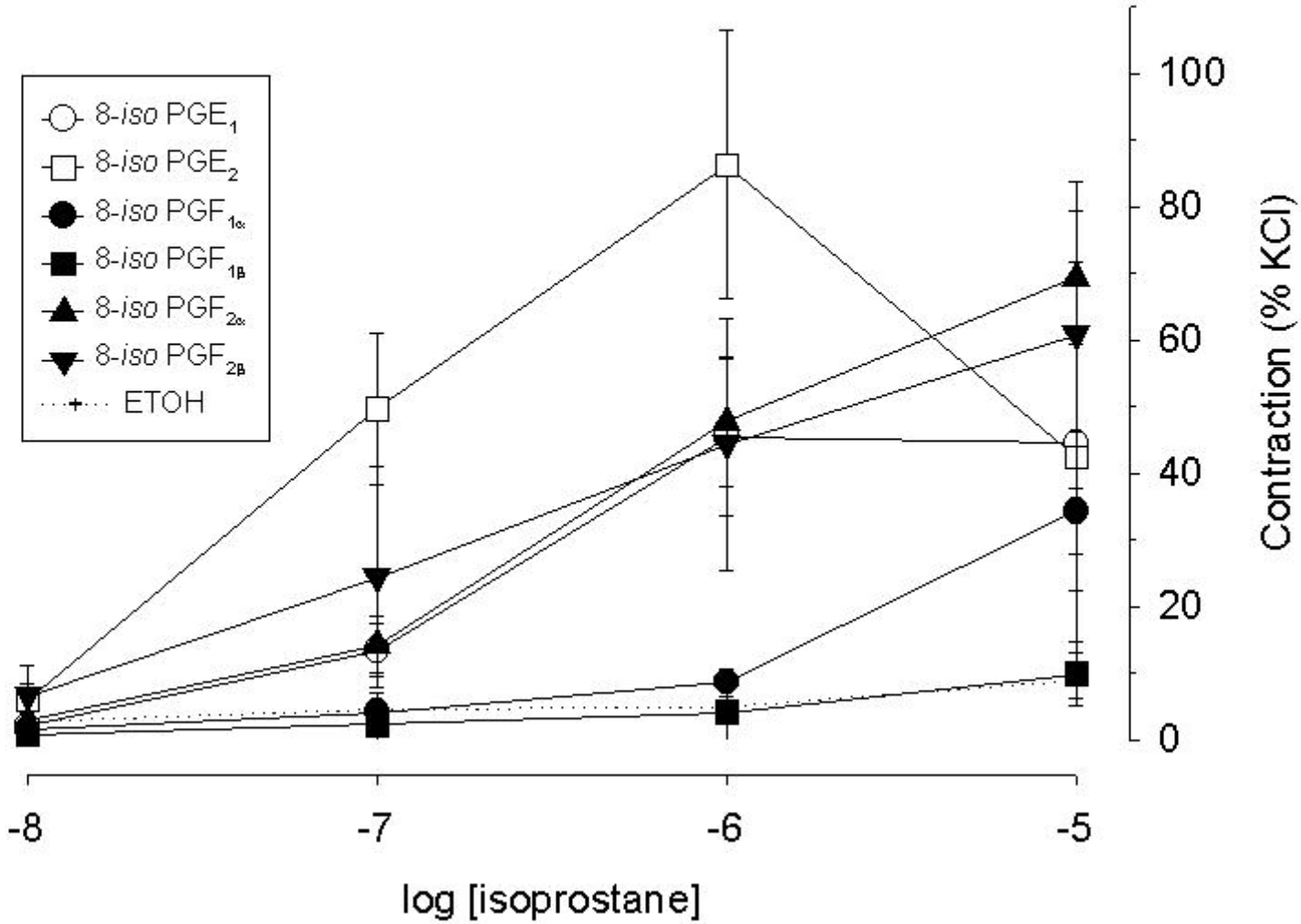
isoprostanes

relaxation

prostanoid receptors

endothelium, endothelium-derived hyperpolarizing factor

FIGURE 1


8-iso PGE1
8-iso PGE2
8-iso PGF1α
8-iso PGF1β
8-iso PGF2α
8-iso PGF2β
ETOH
100
80
60
40
20
0
Contraction (% KCl)
-8
-7
-6
-5
log [isoprostane]

# FIGURE 2

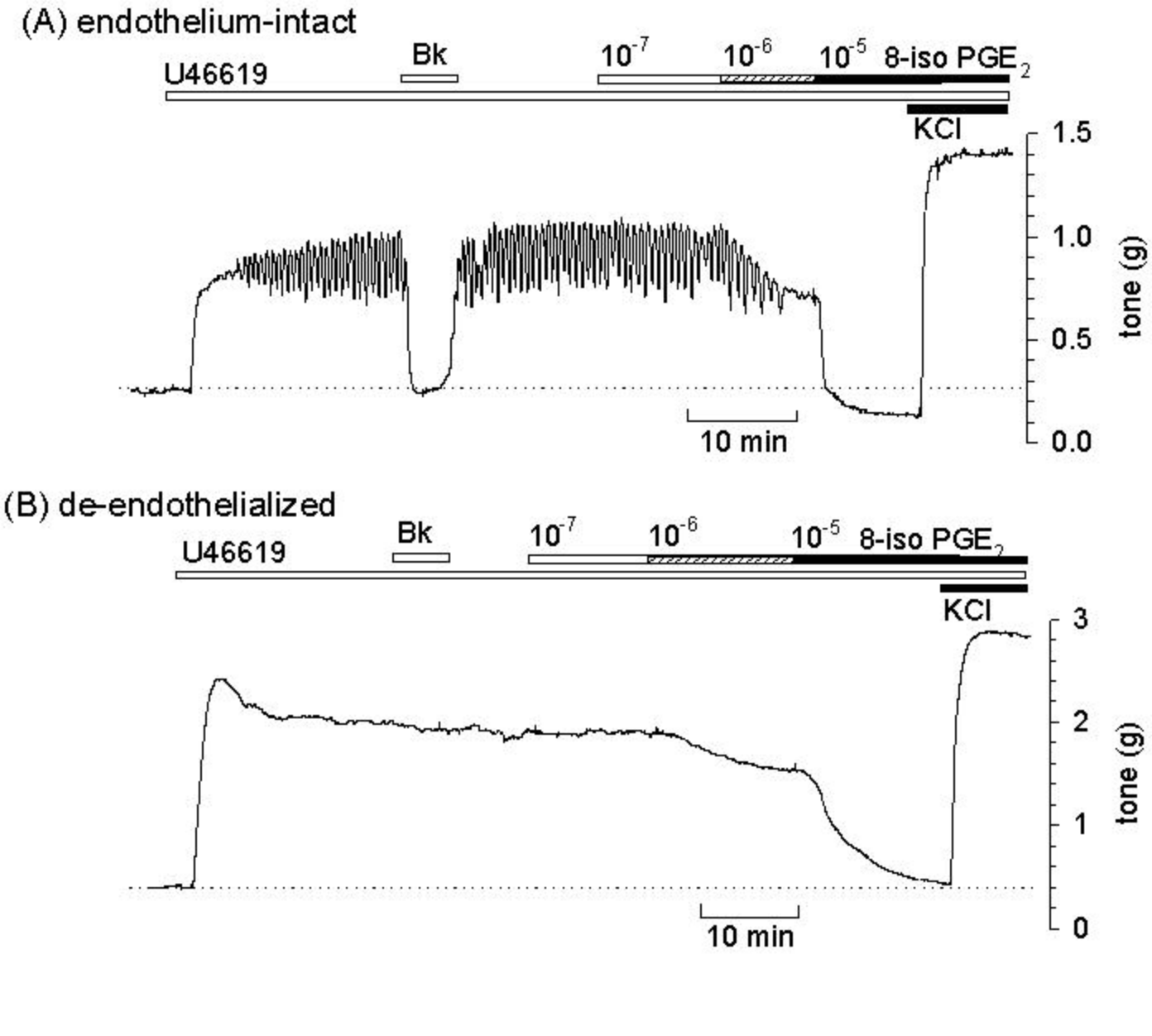

(A) endothelium-intact
U46619
Bk
10-7
10-6
10-5
8-iso PGE2
KCl
1.5
1.0
0.5
0.0
tone (g)
10 min
(B) de-endothelialized
U46619
Bk
10-7
10-6
10-5
8-iso PGE2
KCl
3
2
1
0
tone (g)
10 min

**FIGURE 3**

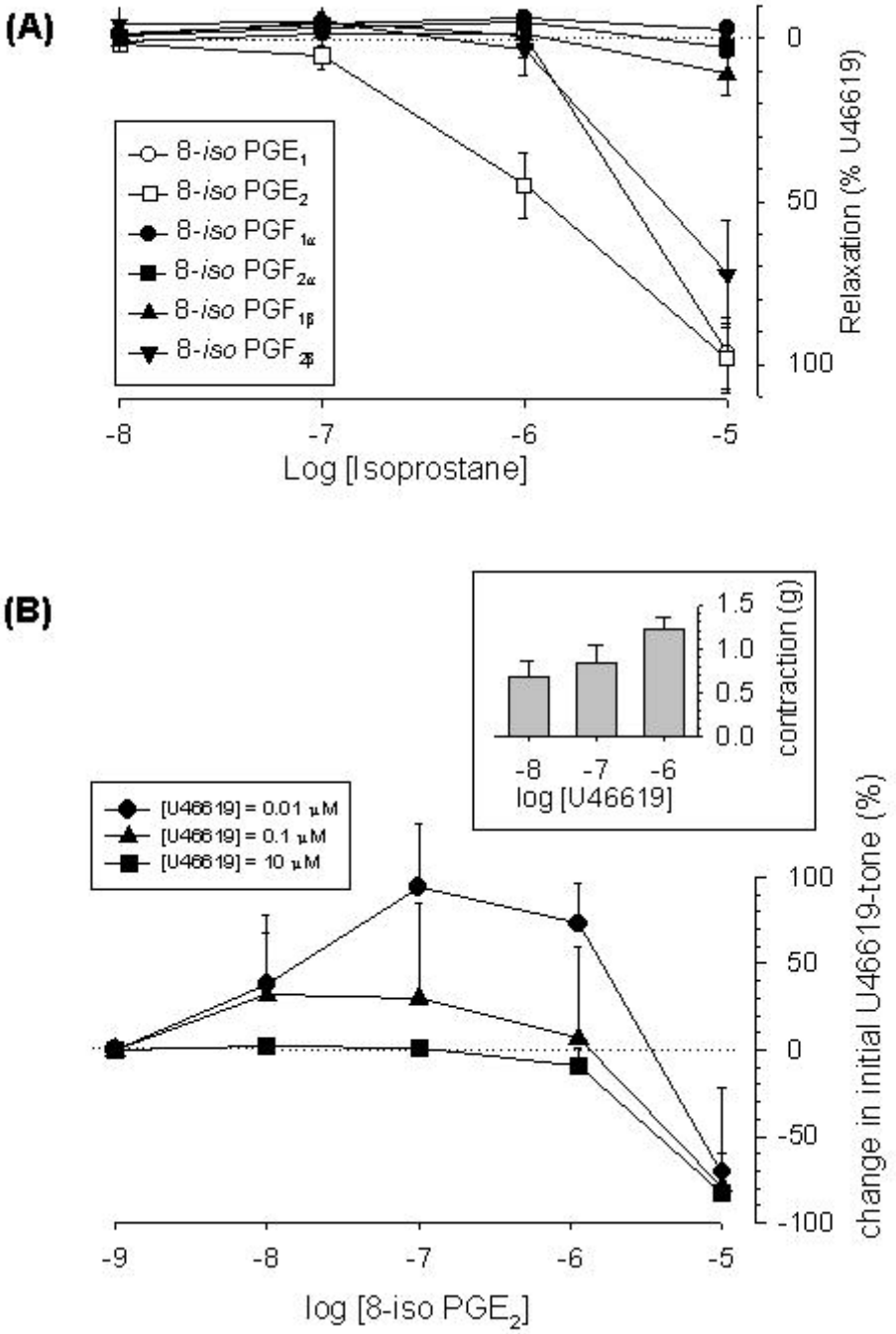
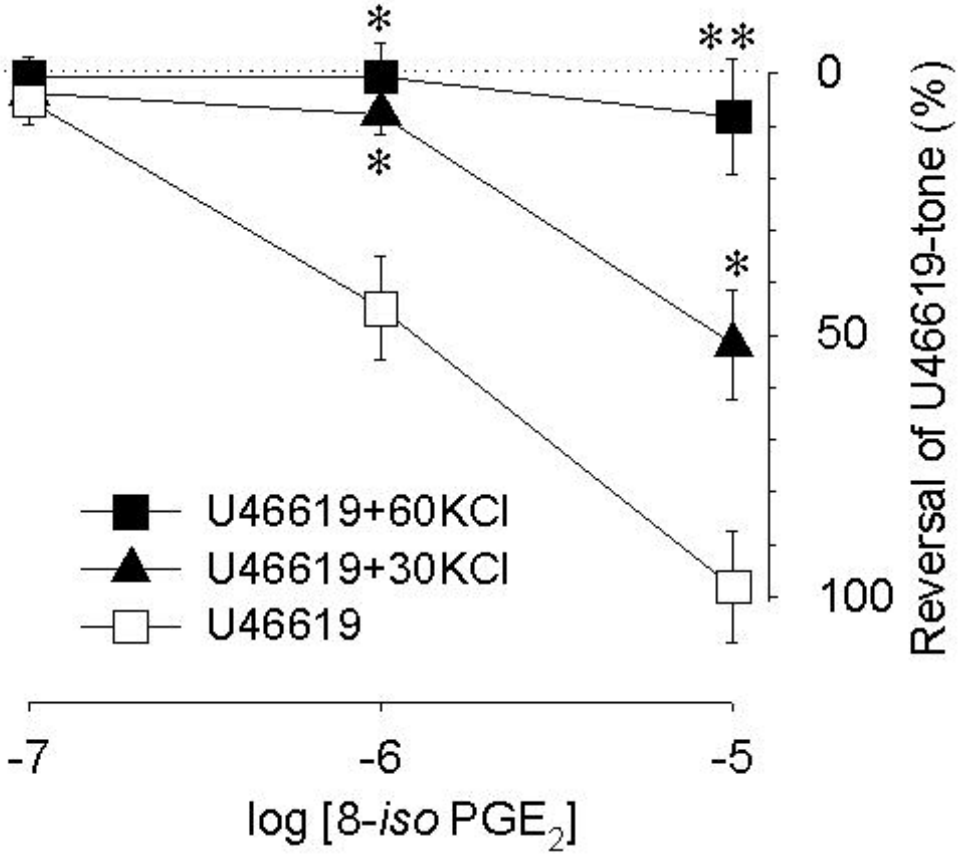

(A)
8-iso PGE1
8-iso PGE2
8-iso PGF1α
8-iso PGF2α
8-iso PGF1β
8-iso PGF2β
0
50
100
Relaxation (% U46619)
-8
-7
-6
-5
Log [Isoprostane]
(B)
1.5
1.0
0.5
0.0
contraction (g)
-8
-7
-6
log [U46619]
[U46619] = 0.01 μM
[U46619] = 0.1 μM
[U46619] = 10 μM
100
50
0
-50
-100
change in initial U46619-tone (%)
-9
-8
-7
-6
-5
log [8-iso PGE2]

FIGURE 4


*
**
*
*
0
50
100
Reversal of U46619-tone (%)
U46619+60KCl
U46619+30KCl
U46619
-7
-6
-5
log [8-iso PGE2]

# FIGURE 5

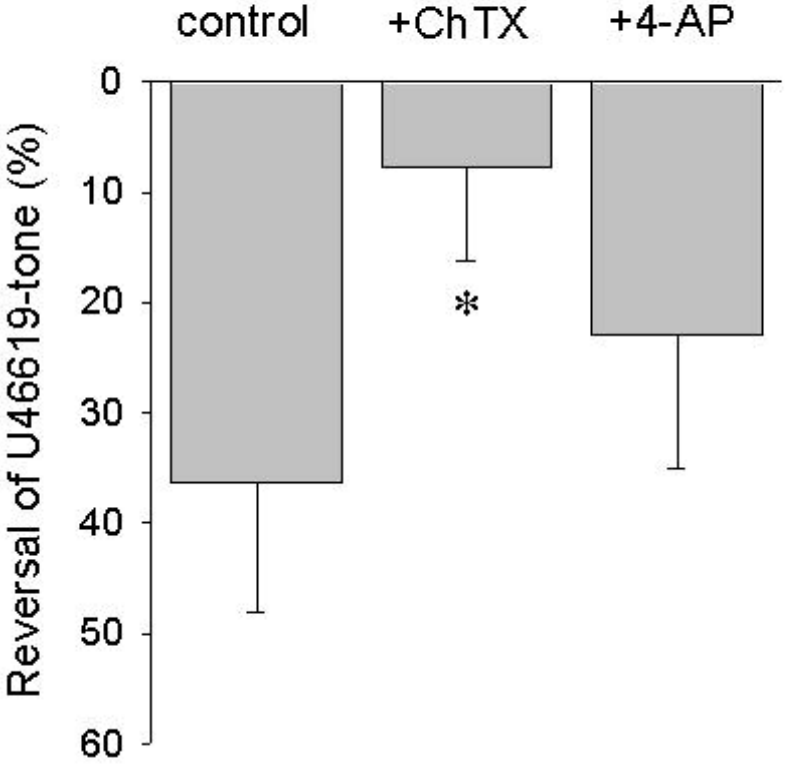
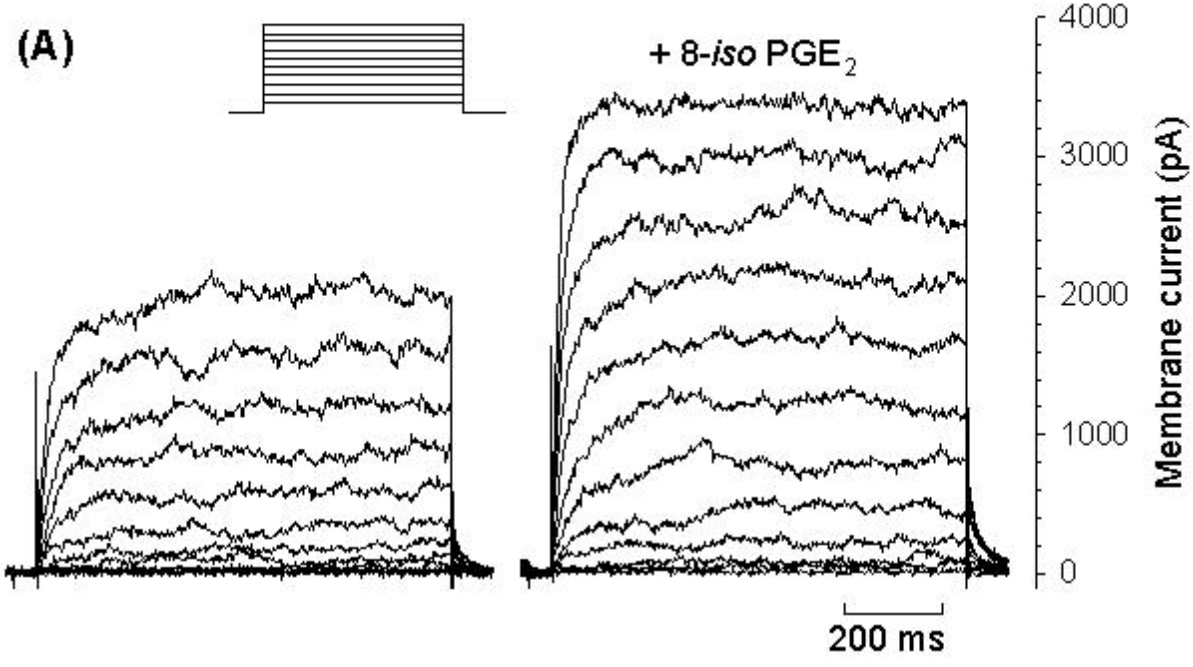
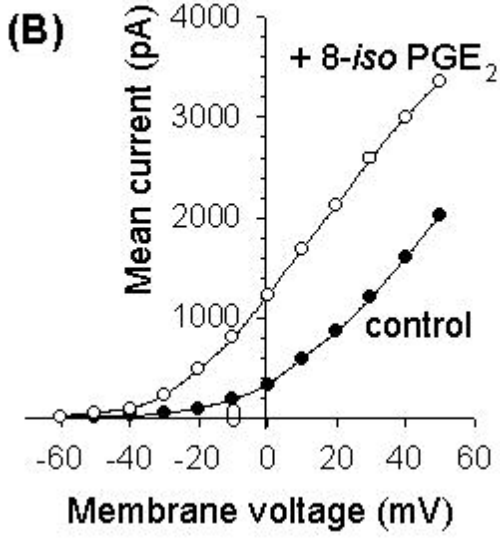


FIGURE 6


(A)
+ 8-iso PGE2
4000
3000
2000
1000
0
Membrane current (pA)
200 ms


(B)
Mean current (pA)
4000
3000
2000
1000
+ 8-iso PGE2
control
-60 -40 -20 0 20 40 60
Membrane voltage (mV)

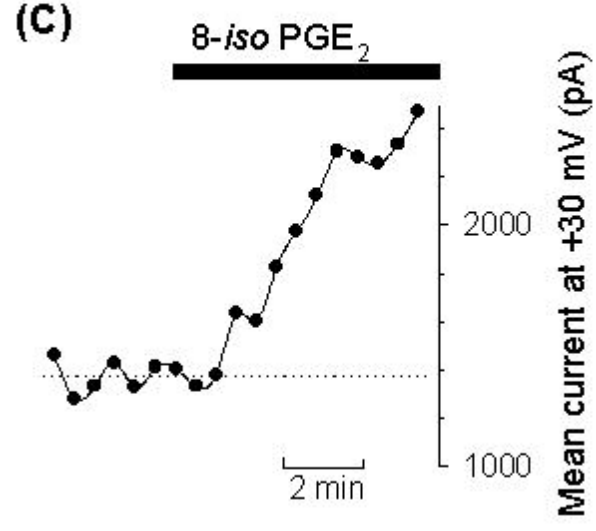

(C)
8-iso PGE2
2000
1000
2 min
Mean current at +30 mV (pA)

FIGURE 7