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# Role of $Cl^-$ currents in rat aortic smooth muscle activation by prostaglandin $F_{2\alpha}$

Jihua Jiang[a], Peter H. Backx[a,*], Hwee Teoh[b], Michael E. Ward[b]

[a] *The Heart and Stroke/Richard Lewar Centre of Excellence in Cardiovascular Research, Department of Physiology and Medicine and Division of Cardiology, University Health Network, 150 College Street, FitzGerald Building, University of Toronto, Toronto, Ontario, Canada M5S 3E2*
[b] *Department of Critical Care and Division of Respirology, St. Michael's Hospital, University of Toronto, Toronto, Ontario, Canada M5S 3E2*

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**Abstract**

The aim of this study was to determine the role of $Cl^-$ channel activation in prostaglandin $F_{2\alpha}$-stimulated aortic contraction and in membrane depolarization during stimulation with prostaglandin $F_{2\alpha}$ in an aortic smooth muscle cell line (A7r5). The $Cl^-$ channel antagonists 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), indanyloxyacetic acid-94 (IAA-94) and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) were found to decrease ($P<0.05$) the maximum tension generated by rat thoracic aortic segments during stimulation with prostaglandin $F_{2\alpha}$ and to shift the concentration–response relationship to the right. In the presence of Nifedipine and Cesium, rat aorta-derived A7r5 smooth muscle cells demonstrated outwardly rectifying voltage-dependent currents that were inhibited by NPPB, IAA-94 and DIDS. Both inward and outward currents were enhanced ($P<0.05$) following addition of prostaglandin $F_{2\alpha}$ (4 μmol/l, final concentration) to the bath solution and this increase was completely inhibited by NPPB. In the absence of Cesium, the addition of prostaglandin $F_{2\alpha}$ (4 μmol/l) to the extracellular bath solution either depolarized or hyperpolarized the cell membrane depending on the equilibrium potential for $Cl^-$ ions. Our results indicate that altered $Cl^-$ conductance is an important mechanism mediating membrane depolarization and contraction of aortic smooth muscle cells during stimulation with prostaglandin $F_{2\alpha}$. Given the significant role that prostaglandin $F_{2\alpha}$ and its biologically active isomers, the $F_2$ isoprostanes, play in the control of vascular tone during hypoxic and oxidative stress in the systemic circulation, alterations in $Cl^-$ channel function and expression may represent an important mechanism in the pathogenesis of abnormal blood flow regulation in disease states.


*Keywords:* $Cl^-$ channel; Smooth muscle; Systemic circulation; Blood flow regulation; Hypoxia

## 1. Introduction

Depolarization of the cell membrane is an important mechanism in agonist activation of vascular smooth muscle contraction. Since this determines the capacity to regulate vascular tone through the release of endogenous vasoconstrictors, the mechanisms by which agonists initiate smooth muscle depolarization have been the subject of extensive research. The majority of these studies have focused on the role of voltage-activated, $Ca^{2+}$-sensitive and ATP-dependent $K^+$ channels (Salter and Kozlowski, 1998). A smaller body of work, driven by the observation that the equilibrium potential for $Cl^-$ ($E_{Cl}$) is positive relative to the resting membrane potential of smooth muscle cells (Aickin and Brading, 1982), suggests that activation of $Cl^-$ efflux may also contribute to depolarization by driving the membrane potential toward $E_{Cl}$. Transmembrane $Cl^-$ flux in smooth muscle cells is mediated by a group of voltage-dependent channels (ClCs) (Yamazaki et al., 1998). Although the precise functional roles of each of these ClC isoforms remain unclear, volume-activated $Cl^-$ channels have been implicated in the arterial myogenic response to increases in transmural pressure (Doughty and Langton, 2001) and activation of $Ca^{2+}$-dependent $Cl^-$ currents contributes to smooth muscle depolarization during stimulation with some agonists (Van Renterghem and Lazdunski, 1994).

Prostaglandin $F_{2\alpha}$ (prostaglandin $F_{2\alpha}$) and its biologically active isomers, the $F_2$ isoprostanes, are produced in the systemic circulation by endothelial cells activated by hypoxia (Arnould et al., 2001) and during periods of oxidative

* Corresponding author. Tel.: +1-416-946-8112; fax: +1-416-864-5922.
*E-mail address:* p.backx@utoronto.ca (P.H. Backx).

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stress (Mervaala et al., 2001). They, therefore, play an important role in the regulation of vascular tone in a number of vascular disorders and during reductions in systemic oxygen delivery. Their effects are mediated by two specific receptor subtypes ($FP_A$ and $FP_B$) (Srinivasan et al., 2002) and they are weak agonists at the shared thromboxane $A_2$/prostaglandin $H_2$ (TP) receptor (Cracowski et al., 2002). The ionic mechanisms that link smooth muscle cell activation to stimulation of these receptors by prostaglandin $F_{2\alpha}$ remain unknown; however, $Cl^-$ currents have been shown to be activated by prostaglandin $F_{2\alpha}$ and to mediate membrane depolarization in fibroblasts (De Roos et al., 1997) and in FP receptor expressing *Xenopus* oocytes (Anthony et al., 2002). We hypothesized, therefore, that changes in $Cl^-$ conductance may also be an important determinant of the systemic vascular response to this agonist. Accordingly, this study was carried out to determine the functional role of $Cl^-$ channel activation in prostaglandin $F_{2\alpha}$-stimulated aortic contraction and whether $Cl^-$ currents contribute to membrane depolarization during stimulation with prostaglandin $F_{2\alpha}$ in an aortic smooth muscle cell line (A7r5).

## 2. Methods

### 2.1. Functional studies

The effect of $Cl^-$ channel inhibition on prostaglandin $F_{2\alpha}$-induced contraction was determined in thoracic aortic segments from male Sprague–Dawley rats (200–250 g). All animal procedures were approved by the institutional animal care committee. Thoracic aortas were excised immediately after decapitation, denuded of endothelium by gentle abrasion of the luminal surface and sectioned into 4-mm rings. Segments were mounted in organ bath myographs containing Krebs–Henseleit solution (KHS, in mmol/l: 120 NaCl, 25 $NaHCO_3$, 11.1 glucose, 4.76 KCl, 1.18 $MgSO_4$, 1.18 $KH_2PO_4$, 2.5 $CaCl_2$) aerated with 95% $O_2$/5% $CO_2$) and equilibrated at 37 °C for 1 h under a resting tension of 2 g. Failure of acetylcholine (1 μmol/l) to elicit relaxation following contraction with phenylephrine (1 μmol/l) was taken as evidence of functional endothelial ablation (Auer and Ward, 1998). Phenylephrine and acetylcholine were washed out, tension was allowed to return to baseline and rings from each rat were incubated for a further 60 min in the presence of one of the $Cl^-$ channel antagonists 4,4′-diisothiocyanostilbene-2, 2′-disulfonic acid, sodium (DIDS, 93 μmol/l, Sigma), indanyloxyacetic acid (IAA-94, 93 μmol/l. Research Biochemical International), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB, 54 μmol/l, Sigma,) or vehicle alone (dimethyl sulphoxide, DMSO, 0.07% volume/volume, Caledon). Concentration–response relationships were then generated by the addition of prostaglandin $F_{2\alpha}$ ($10^{-10}$–$10^{-5}$ mol/l, Sigma) to the organ baths in a cumulative fashion. The concentrations of DIDS, IAA94 and NPPB used were those that in preliminary experiments were found to be the minimum effective concentrations (minimum concentration above which no further inhibitory effect was observed). Upon completion of the experiments, rings were dried overnight at 50 °C and weighed in order to express tension as g/mg dry weight.

### 2.2. Electrophysiological studies

#### 2.2.1. Cell preparation

Rat aortic smooth muscle-derived A7r5 cells were purchased from the American Type Culture Collection and cultured in Dulbecco′s modified Eagle medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a fully humidified 5% $CO_2$ atmosphere. Cells used in our studies were subcultured to passage 6, dispersed at low density and cultured on glass coverslips for whole-cell patch-clamp recordings.

#### 2.2.2. Whole cell recordings

Membrane potential and $Cl^-$ currents were recorded by the patch-clamp method, (Axopatch 200B and Digidata 1320 Series, Axon Instruments, USA). The external electrode was an Ag–AgCl wire encased in a 3 mol/l KCl agar bridge. The series resistance of the heat polished glass pipette was 6–8 MΩ when sealed onto the cell and was typically compensated by 70%. In voltage-clamp studies to record $Cl^-$ currents, cells were held at −50 mV and voltage steps ranging from −130 to 70 mV were applied for 400 ms. The current-clamp method was used to record the membrane potential. Current and voltage traces were sampled at 10 kHz and filtered at 2 kHz. The bath chamber (0.5 ml volume) was superfused with drug-containing or solvent-containing solution at 4 ml/min. Cells were dialyzed for 2 min before the addition of drug or $Cl^-$ concentration alterations.

#### 2.2.3. Bath and pipette solutions

In order to isolate $Cl^-$ currents the bath solution contained (in mmol/l): 135 NaCl, 5 CsCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 5 HEPES–NaOH buffer (pH 7.4). For measurement of membrane potential 5 mmol/l CsCl was replaced with 5 mmol/l KCl. Nifedipine (1.0 μmol/l, final concentration) was added to all of bath solutions to block $Ca^{2+}$ currents. The pipette solution used for measurement of $Cl^-$ currents contained (in mM): CsCl 125, $MgCl_2$ 1, Mg-ATP 5, EGTA10, HEPES–CsOH buffer 10 (pH 7.2). The pipette solution used for measurement of membrane potential was (in mmol/l): 125 KCl, 1 $MgCl_2$, 5 Mg-ATP, 10 EGTA, 10 HEPES–KOH buffer (pH 7.2). EGTA was added to the pipette solution to chelate $Ca^{2+}$ to minimize $Ca^{2+}$-activated $Cl^-$ currents. CsCl was added to both the bath and pipette solutions to block $K^+$ channel conductance during measurement of $Cl^-$ currents. The $Cl^-$ concentration of the bath solutions was altered by equimolar substitution of NaCl with aspartate sodium. The $Cl^-$ concentration of the pipette solution was altered by equimolar substitution of KCl with aspartate potassium.

*2.2.4. Drugs*

Prostaglandin $F_{2\alpha}$ and DIDS were dissolved in bath solution. Nifedipine (Sigma) and NPPB were dissolved in DMSO to make stock solutions of 100 mmol/l. IAA-94 was dissolved in anhydrous ethyl alcohol to make a stock solution of 50 mmol/l. The minimum effective concentrations of NPPB, IAA-94 and DIDS in this cell model were found to be 100 μmol/l, 1 mmol/l and 100 μmol/l, respectively, and were used in the subsequent experiments. Similar dilutions of the solvents alone in the bath solutions had no effect on membrane potential or $Cl^-$ currents. All concentrations given are final molar concentrations in the bath chamber.

*2.3. Western blotting*

To confirm ClC expression in A7r5 cells at the same passage as those used for whole cell patch clamp recordings, Western blot analysis was carried out using the ClC-specific antisera currently available from commercial sources. Cells subcultured to passage 6 were treated with lysis buffer (50 mmol/l Tris (pH 8), 150 mmol/l NaCl, 0.1% sodium dodecyl sulfate (SDS), 1 mol/l phenylmethylsulphonyl fluoride, 1 μg/ml Aprotinin, 1 μg/ml NP40, 0.5% Sodium deoxycholate). Lysates were centrifuged and protein concentration in the supernatant was determined by the Lowry assay. Appropriate volumes of extraction buffer to produce constant protein loading in each lane were mixed with SDS loading buffer, to a final concentration of 0.25 mol/l Tris (pH 6.8), 2% SDS, 5% mercaptoethanol, 0.03% bromophenol blue, 11.25% glycerol. Samples were heated to 95 °C for 5 min, then loaded and electrophoresed at a constant voltage of 125 V with running buffer of the following composition: 0.025 mol/l Tris, 0.192 mol/l glycine, 0.1% SDS using 4–12% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) minigels (Helixx, Mississauga). Separated proteins were transferred to polyvinylidene difluoride membranes in 0.025 mol/l Tris, 0.192 mol/l glycine, 20% methanol transfer buffer under a constant voltage of 100 V. Membranes were blocked with 5% skimmed-milk in TTBS (Tris-buffered saline with 0.1% Tween-20). Polyclonal antibodies specific for ClC-1, ClC-2 and ClC-3 were from Chemicon International, Temecula, CA. Donkey anti-rabbit-horse radish peroxidase secondary antibody was from Amersham (Oakville, Ontario). All antibodies were diluted in blocking buffer. Conditions for the various steps of the Western blotting procedures were optimized for the individual ClC in preliminary experiments. Blots were developed using enhanced chemiluminescence (Amersham).

*2.4. Data analysis*

Unless otherwise stated, results are presented as mean ± S.E.M. for $n$ number of samples with $P<0.05$ representing statistical significance. Paired means were compared by two-tailed Student's $t$ test. Differences among multiple means were evaluated by analysis of variance (ANOVA) and, when overall differences were detected, individual means were compared post-hoc using Bonferroni's test.

## 3. Results

The effects of $Cl^-$ channel inhibition with DIDS, IAA94 and NPPB on contractile responses of aortic rings to prostaglandin $F_{2\alpha}$ are illustrated in Fig. 1. Table 1 presents the maximum tensions and $EC_{50}$ values during prostaglandin $F_{2\alpha}$-induced contraction in the presence and absence of these agents. In rings incubated with $Cl^-$ channel inhibitors, maximum tension is decreased and the concentration–response curve is shifted to the right ($EC_{50}$ is increased) compared to rings incubated with vehicle alone. These results support the hypothesis that $Cl^-$ channel activation plays an important role in the mediating the contractile response to prostaglandin $F_{2\alpha}$.

The ability of prostaglandin $F_{2\alpha}$ to activate $Cl^-$ channels was examined using voltage-clamp recordings of cultured rat smooth muscle cells (A7r5). Fig. 2A shows typical current recordings in response to voltage steps ranging between −130 and +70 mV for 400-ms duration in the presence of Nifedipine and $Cs^+$ to eliminate $Ca^{2+}$ and $K^+$ currents when the equilibrium $Cl^-$ potential ($E_{Cl}$) is −3.3 mV. The average current–voltage relationship for these conditions is illustrated in Fig. 2C (solid circles). To test whether these currents originate from $Cl^-$ channels,

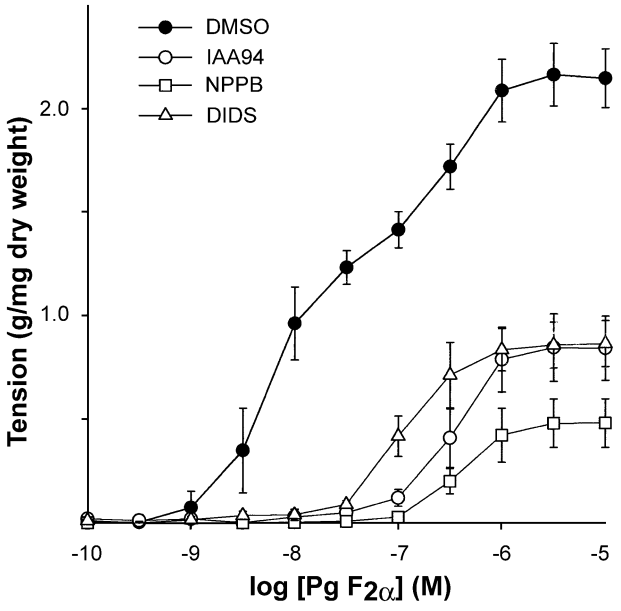


Fig. 1. Concentration–response curves for prostaglandin $F_{2\alpha}$ in endothelium-denuded rat aortic rings following incubation with the $Cl^-$ channel inhibitors NPPB, IAA94 and DIDS and with vehicle (DMSO) alone. All three agents decreased maximum tension and shifted the concentration–response curve to the right.

Table 1
Effect of $Cl^-$ channel inhibition on contractile response to prostaglandin $F_{2\alpha}$

| Drug treatment | Maximum tension (g/mg dry weight) | $-\log EC_{50}$ (mmol/l) |
|---|---|---|
| DMSO | $2.18 \pm 0.15$ | $7.89 \pm 0.31$ |
| DIDS | $0.85 \pm 0.16^a$ | $6.55 \pm 0.07^a$ |
| IAA94 | $0.49 \pm 0.12^a$ | $6.32 \pm 0.11^a$ |
| NPPB | $0.89 \pm 0.1^a$ | $6.89 \pm 0.10^a$ |

[a] $P<0.05$ for difference from DMSO treated group.

the $Cl^-$ channel blocker NPPB was applied. Fig. 2B and C (open circles) demonstrates that NPPB was able to eliminate more than 70% of the membrane conductance. Similar effects were observed after treatment with IAA94 (Fig. 3A) and DIDS (Fig. 3B), establishing that $Cl^-$ currents are responsible for the bulk of the observed current in these cells. The functional significance of these $Cl^-$ currents in these cells is demonstrated in Fig. 4 which illustrates the effect of altering $E_{Cl}$ on the currents recorded in the presence of Nifedipine and $Cs^+$. Reducing the external $Cl^-$ concentration from 146 to 20 mmol/l resulted in a rightward shift of the $I-V$ curve with the reversal potential shifting ($P<0.01$, $n=7$) from $-8.7 \pm 2.3$ mV ($E_{Cl}=-3.3$ mV) to $21.8 \pm 3.3$ mV

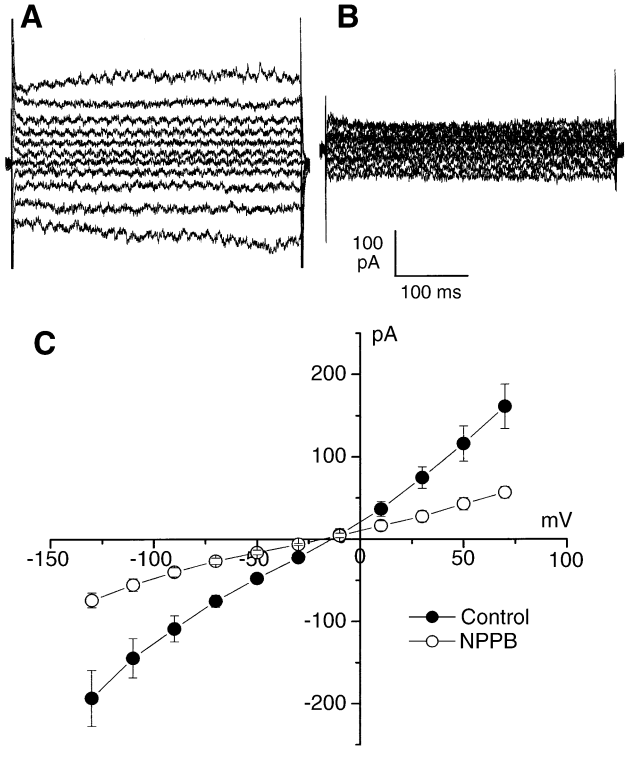


Fig. 2. Effect of NPPB on $Cl^-$ currents. (A) Typical current recordings in the absence (A) and presence (B) of NPPB (100 μM) using the same voltage protocols illustrated in Fig. 3. (C) Current–voltage relationships ($n=8$ per group): (●) control, (○) 5 min after administration of NPPB 100 μM. Both inward and outward currents are decreased ($P<0.01$) in NPPB-treated cells.

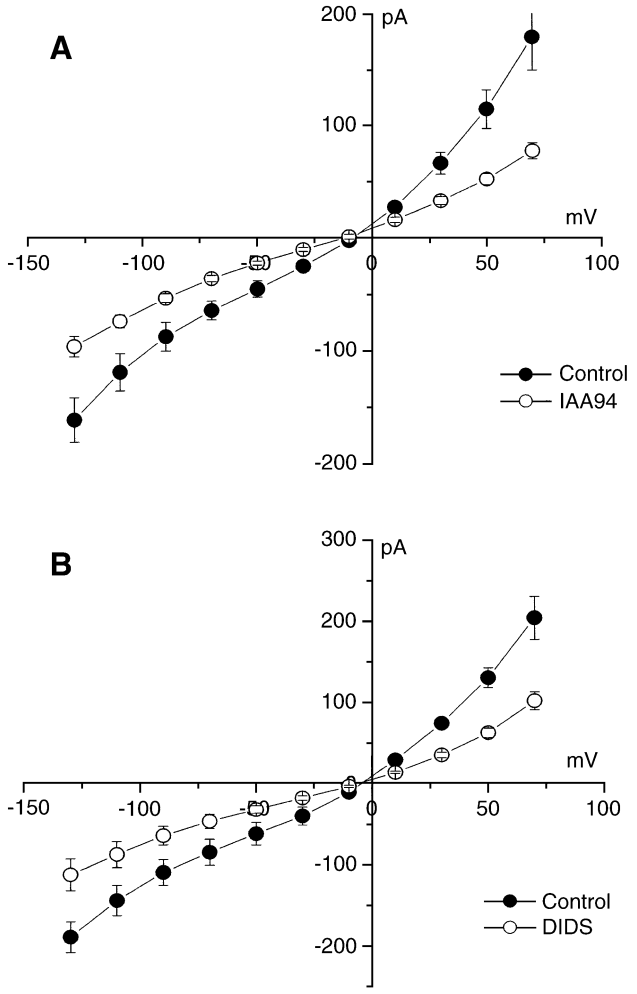


Fig. 3. Effect of IAA-94 and DIDS on $Cl^-$ currents. (A) (●) control, (○) 15 min after administration of IAA-94 100 μM. IAA94 inhibited both outward and inward $Cl^-$ currents ($n=6$ per group, $P<0.01$ for both). (B) (●) control, (○) 15 min after administration of DIDS 1 mM. DIDS inhibited both outward and inward currents ($n=6$ per group, $P<0.01$ for both).

($E_{Cl}=43$ mV) while also slightly increasing the conductance (slope of the current–voltage relationship) at negative voltages.

The voltage-clamp studies above establish that $Cl^-$ channels contribute to transmembrane currents in cultured smooth muscle cells. To assess whether $Cl^-$ currents could play a role in the smooth muscle cell activation (illustrated in Fig. 1) during stimulation with prostaglandin $F_{2\alpha}$, we examined effects of prostaglandin $F_{2\alpha}$ (4 μmol/l, final concentration) on $Cl^-$ currents. As shown in Fig. 5, prostaglandin $F_{2\alpha}$ increased both inward and outward currents in the presence of Nifedipine and $Cs^+$ (compare solid circles with open circles). In a separate set of experiments ($n=4$) pretreatment of A7r5 cells with NPPB (100 μmol/l) completely abolished the prostaglandin $F_{2\alpha}$-induced increase in $Cl^-$ current. (Fig. 5, solid triangles).

To establish that the increased $Cl^-$ currents induced by prostaglandin $F_{2\alpha}$ can regulate the membrane potential,

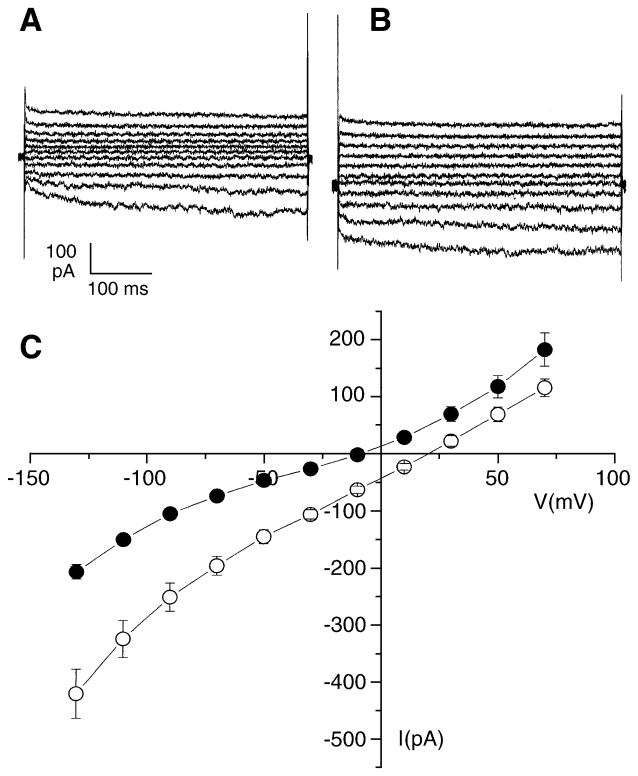


Fig. 4. Effect of decreasing extracellular $Cl^-$ ion concentration on $Cl^-$ conductance. (A) Typical current recordings under control conditions (bath $Cl^-$ ion concentration = 146 mM) for voltage steps ranging from − 130 to + 70 mV for a duration of 400 ms from a holding potential of − 40 mV. (B) Sample current traces, recorded under the same conditions as in (A), after 5 min of superfusion with bath solution containing 20 mM $Cl^-$ ion. (C) Current–voltage relationships ($n = 6$): (●) control, (○) superfusion with 20 mM $Cl^-$ ion-containing bath solution. Reducing extracellular $Cl^-$ concentration shifts the reversal potential of the $Cl^-$ conductance to the right ($P < 0.05$).

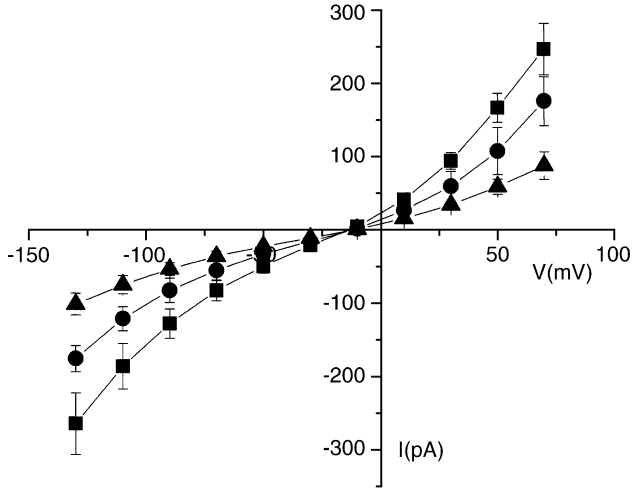


Fig. 5. Effects of prostaglandin $F_{2\alpha}$ on $Cl^-$ currents. (●) Control ($n = 7$); (○) 5 min after administration of prostaglandin $F_{2\alpha}$ 4 μM ($n = 7$) both outward and inward currents are increased ($P < 0.01$ for both). ▲ In the presence of NPPB 100 μM ($n = 5$) the prostaglandin $F_{2\alpha}$-induced enhancements of both currents were abolished ($P < 0.01$ compared to prostaglandin $F_{2\alpha}$ alone).

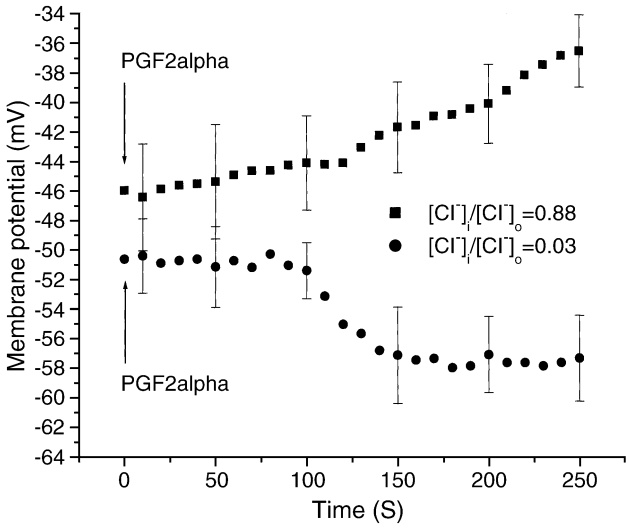


Fig. 6. Effect of prostaglandin $F_{2\alpha}$ (4 μM) on membrane potential in the presence of 127 (■) or 5 (●) mM $Cl^-$ ion-containing pipette solution. With the 127 mM $Cl^-$ solution, prostaglandin $F_{2\alpha}$ depolarized the cell membrane. With the 5 mM $Cl^-$ pipette solution, prostaglandin $F_{2\alpha}$ hyperpolarized the cell membrane.

and thus contraction, we applied 4 μmol/l prostaglandin $F_{2\alpha}$ while measuring membrane potential (Fig. 6). As expected from the results in Fig. 5, application of 4 μmol/l prostaglandin $F_{2\alpha}$ caused membrane depolarization when the pipette solution contained 127 mmol/l $Cl^-$ and $E_{Cl}$ was positive relative to the resting membrane potential ($E_{Cl} = -3.3$ mV). As can be seen, the prostaglandin $F_{2\alpha}$ effect took several minutes to develop, which is similar to the time course of the changes in the $Cl^-$ current observed in our voltage-clamp studies (data not shown). In contrast, when $E_{Cl}$ was reduced by decreasing pipette solution $Cl^-$ concentration to 5 mmol/l ($E_{Cl} = -79.1$ mV), application of prostaglandin $F_{2\alpha}$ elicited hyperpolarization.

To investigate the molecular basis for the $Cl^-$ conductance observed in cultured rat smooth muscle cells, we performed Western Blots on total protein extracts from A7r5 cells (passage 6) using antibodies raised against ClC-1, ClC-2 and ClC-3 channel proteins. As illustrated in Fig. 7, all three of these proteins could be detected in A7r5 cells at the same passage as was used in the electrophysiological studies.

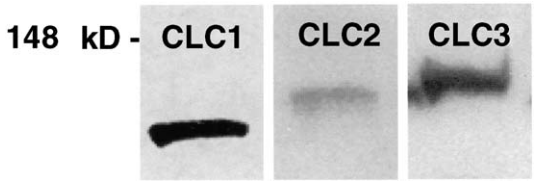


Fig. 7. Western blots demonstrating the presence of ClC-1, ClC-2 and ClC-3 protein expression in A7r5 cells subcultured at passage 6. Experiments were repeated thrice for each channel.

## 4. Discussion

In this study we found that in rat aortic rings the maximum tension generated during prostaglandin $F_{2\alpha}$-stimulated contraction is decreased and the concentration–response relationship is shifted to the right (increased $EC_{50}$) after treatment with the $Cl^-$ channel antagonists NPPB, IAA94 and DIDS. In rat aorta-derived A7r5 smooth muscle cells, we detected ClC-1, ClC-2 and ClC-3 proteins and found that $Cl^-$ currents contribute to the regulation of their transmembrane potential. When intra- and extracellular ion concentrations were such that $E_{Cl}$ is greater than the resting membrane potential, stimulation of A7r5 cells with prostaglandin $F_{2\alpha}$ activates both inward and outward $Cl^-$ currents and causes depolarization of the cell membrane. In contrast, after reducing intracellular $Cl^-$ concentration and thus $E_{Cl}$, stimulation of A7r5 cells with prostaglandin $F_{2\alpha}$ causes hyperpolarization.

Prostaglandin $F_{2\alpha}$ plays a number of important physiological and pathophysiological roles. It is released in response to hypoxia and influences inflammatory cell recruitment (Arnould et al., 2001) as well as endothelial and smooth muscle cell proliferatiion (Dorn et al., 1992; Yura et al., 1999). Endothelium-derived prostaglandin $F_{2\alpha}$, acting either directly or through potentiation of the response to other endogenous vasocontrictors (Rapoport, 1993), regulates human umbilical (De Morase et al., 1997) and penile arterial smooth muscle tone (Angulo et al., 2002) and fluctuations in prostaglandin $F_{2\alpha}$ production contribute to estrus phase-dependent changes in systemic vascular reactivity (Zamorano et al., 1995). Its biologically active isomers, the $F_2$ isoprostanes, are produced both enzymatically (Watkins et al., 1999) and by free-radical-catalyzed lipid peroxidation (Morrow et al., 1992) in atherosclerotic lesions (Gniwotta et al., 1997) and during coronary ischemia-reperfusion injury (Delanty et al., 1997) and participate in the alterations in vascular tone that accompany these conditions.

Little is known about the ionic mechanisms that mediate the contractile response to prostaglandin $F_{2\alpha}$. In rat arterial smooth muscle it is inhibited by removal of $Na^+$ from the extracellular milieu possibly through effects on $Na^+/Ca^{2+}$ exchange (Rapoport et al., 2000) and by sulfonylureas, which block ATP-sensitive $K^+$ channel activity (Zhang et al., 1991). The role of $Cl^-$ channels in prostaglandin $F_{2\alpha}$-induced vascular smooth muscle contraction has not been previously investigated. In *Xenopus* oocytes, which express $Ca^{2+}$-dependent $Cl^-$ channels, stimulation of transfected FP receptors with prostaglandin $F_{2\alpha}$ activates both inwardly and outwardly rectifying $Cl^-$ currents (Anthony et al., 2002), suggesting that $Cl^-$ ion flux may play a role in the response to this agonist in cells that endogenously express these receptors. This was supported a subsequent study in a fibroblast cell line in which prostaglandin $F_{2\alpha}$-induced depolarization was found to be enhanced by reducing extracellular $Cl^-$ concentration and abolished by $Ca^{2+}$-dependent $Cl^-$ channel antagonists (De Roos et al., 1997). Our current results confirm and extend these previous observations since we demonstrate, for the first time, that prostaglandin $F_{2\alpha}$ activates $Cl^-$ currents in vascular smooth muscle cells; that these currents contribute to the change in transmembrane potential which accompanies stimulation with this agonist; and that $Cl^-$ currents play an important role in the contractile response which it elicits in arterial segments.

Understanding of the functional role of the $Cl^-$ channels expressed by smooth muscle cells continues to evolve. The development of pressure-sensitive, myogenic, tone in rat cerebral arteries is associated with increased $Cl^-$ efflux (Doughty and Langton, 2001) and in rabbit mesenteric and rat cerebral arteries (Yamazaki et al., 1998; Nelson et al., 1997) this tone is inhibited by the nonselective $Cl^-$ channel inhibitors DIDS and IAA94. Tone was insensitive, however, to antagonists that demonstrate relative selectivity for $Ca^{2+}$-dependent $Cl^-$ channels (Nelson et al., 1997) suggesting that activation of a volume-sensitive depolarizing $Cl^-$ current by cell deformation may initiate myogenic contraction in these vessels. $Cl^-$ channel antagonists have previously been shown to inhibit smooth muscle contraction in response to a number of agonists including norepinephrine (Lamb and Barna, 1997) and endothelin-1 (Klocckers and Iserberg, 1991). The hypothesis advanced to explain these findings is that release of $Ca^{2+}$ from the intracellular store through the inositol triphosphate/diacylglycerol pathway activates the $Ca^{2+}$-dpendent $Cl^-$ current, thus depolarizing the cell membrane. Elevation of intracellular $Ca^{2+}$ also activates $Ca^{2+}$-dependent $K^+$ channels, which hyperpolarize the membrane. The outcome, either contraction or relaxation, is determined by the type of channel that plays the dominant role (Dai and Zhang, 2001). To the extent that the receptors that mediate the effects of prostaglandin $F_{2\alpha}$ also initiate smooth muscle contraction through activation of intracellular $Ca^{2+}$ release (Kurata et al., 1993), our current finding that $Cl^-$ channel antagonists inhibit prostaglandin $F_{2\alpha}$-induced aortic contraction is consistent with this explanation. Our electrophysiological data indicate that in A7r5 cells the $Cl^-$ current predominates when $E_{Cl}$ is positive relative to the resting membrane potential, as it has been reported to be in smooth muscle cells under physiological conditions (Aickin and Brading, 1982), since stimulation with prostaglandin $F_{2\alpha}$ elicits depolarization. When $E_{Cl}$ is reduced, decreasing the influence of depolarizing $Cl^-$ current, application of prostaglandin $F_{2\alpha}$ hyperpolarizes the cell membrane.

The biophysical and genotypic characterization of the channels that conduct transmembrane $Cl^-$ flux has been impeded by the lack of specific pharmacological tools reflecting their high level of sequence and structural homology. Since this group of channels is ubiquitously expressed, it is likely that multiple ClC isoforms are present in smooth muscle and are activated either directly by $Ca^{2+}$ released from intracellular stores or as a secondary reinforcing

response to the initial change in membrane potential. By Western blotting we were able to detect ClC-1, ClC-2 and ClC-3 proteins in A7r5 smooth muscle cells confirming the presence of voltage-regulated channels capable of conducting both inward and outward $Cl^-$ currents. Outwardly rectifying $Cl^-$ currents have been recorded in a number of tissues and play roles in volume regulation and signal transduction (Duan et al., 1997; Garber, 1992). Recently, a volume-sensitive outwardly rectifying $Cl^-$ current was shown, in guinea pig cardiac and canine pulmonary vascular myocytes, to be completely abrogated by intracellular dialysis with anti-ClC-3 antibody (Duan et al., 2001). In view of these observations and our present finding that in smooth muscle cells that express ClC-3 protein, prostaglandin $F_{2\alpha}$ exerts its effect through modulation of an outwardly rectifying $Cl^-$ current, we speculate that this isoform may play an additional role in the regulation of membrane potential during agonist activation.

Prostaglandin $F_{2\alpha}$ and its isomers the F2 isoprostanes are produced in the systemic circulation in response to hypoxic and oxidative stress. In the present study, we demonstrate that activation of $Cl^-$ efflux is an important mechanism mediating smooth muscle cell depolarization and contraction in response to prostaglandin $F_{2\alpha}$. It is of interest, therefore, that alteration of redox status has recently been shown to modulate $Cl^-$ channel conductance in retinal pigment epithelial cells (Wills et al., 2000). The possibility that the release of vasoconstrictor prostanoids and the activity of the ionic channels that mediate the smooth muscle response to activation of their receptors may be co-regulated through redox-dependent mechanisms, therefore, merits further investigation. Promoter sequences in the 5′ flanking regions of the mouse ClC-1 (AJ539382), ClC-3 (AF347681) and ClC-5 (Tanaka et al., 1999), rat ClC-2 (Chu, 1999) and human ClCA2 (AF114429), ClC-6 and ClC-7 (Kornak et al., 1999) genes have been published or reported to GenBank. Analysis of these regions (Quandt et al., 1995) indicates the presence of consensus transcription factor elements corresponding to the aryl hydrocarbon receptor nuclear translocator (ARNT) required by hypoxia inducible factor-1α to enhance transcription (Wiesener et al., 2001) and for activating protein 1 (AP1) which mediates hypoxic activation of tyrosine hydroxylase expression (Norris and Milhorn, 1995). Changes in ClC expression may, therefore, be a determinant of the response to locally released prostanoid vasoconstrictors in hypoxic vascular beds. Studies to evaluate the functionality of these putative transcription factor binding sites and to determine the role of such synergistic regulation in preserving the capacity to autoregulate the systemic circulation and/or its pathophysiological role in limiting blood flow to hypoxic regions are now indicated.

We have shown that $Cl^-$ channel activation is an important mechanism mediating vascular smooth muscle contraction in response to prostaglandin $F_{2\alpha}$. Given the significant role that prostanoid vasoconstrictors of this class play in regulating vascular tone during hypoxic and oxidative stress in the systemic circulation, it is likely that alterations in $Cl^-$ channel function and expression represent an important and overlooked mechanism in the pathogenesis of abnormal blood flow regulation in disease states. The development of pharmacological agents which target $Cl^-$ channel activity, therefore, may lead to new therapeutic approaches in the management of vascular dysregulation in disorders such as atherosclerosis (Delanty et al., 1997) and coronary ischemia (Gniwotta et al., 1997) and in the vital organ dysfunction that accompanies reductions in systemic oxygen delivery due to shock or respiratory failure.

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