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Modulation of volume-sensitive chloride current by noradrenaline in rabbit portal vein myocytes

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The effect of noradrenaline on the volume-sensitive chloride current ($I_{Cl(swell)}$) was studied with conventional whole-cell recording techniques in freshly dispersed isolated smooth muscle cells of the rabbit portal vein. In the absence of receptor antagonists, noradrenaline produced an increase in the amplitude of $I_{Cl(swell)}$ in some cells and a decrease in others. In the presence of the β-adrenoceptor antagonist propranolol, noradrenaline increased $I_{Cl(swell)}$ and in the presence of the α_1-adrenoceptor antagonist prazosin, noradrenaline reduced $I_{Cl(swell)}$. The phospholipase C (PLC) inhibitor U73122 reduced the amplitude of $I_{Cl(swell)}$ whereas the inactive analogue U73343 had no effect. The phorbol esters phorbol-12-myristate-13-acetate (PMA) and phorbol-12,13-dibutyrate (PDBu) increased the amplitude of $I_{Cl(swell)}$ by approximately 60 and 100 %, respectively, in a voltage-independent fashion. Inhibitors of protein kinase C (PKC) chelerythrine and calphostin-C decreased the amplitude of $I_{Cl(swell)}$ in a concentration-dependent but voltage-independent manner. Bath application of 8-Br-cAMP decreased $I_{Cl(swell)}$ by about 60 % whereas the inhibitor of protein kinase A (PKA) KT5720 increased the amplitude of $I_{Cl(swell)}$ by approximately 80–90 %. In the presence of propranolol, chelerythrine prevented the increase of $I_{Cl(swell)}$ by noradrenaline; in the presence of prazosin, KT5720 blocked the inhibitory action of noradrenaline. The results show that in rabbit portal vein myocytes noradrenaline enhances $I_{Cl(swell)}$ by acting on α_1-adrenoceptors and reduces $I_{Cl(swell)}$ by stimulating β-adrenoceptors. The data suggest that the potentiating and inhibitory effects of noradrenaline are mediated, respectively, by PKC and PKA.

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A volume-sensitive chloride current ($I_{Cl(swell)}$) has been recorded in many cell types and several physiological roles have been proposed for this conductance including volume regulation and cell proliferation (e.g. see Okada, 1997). Recently $I_{Cl(swell)}$ has also been identified in vascular smooth muscle cells (Yamazaki *et al.* 1998; Greenwood & Large, 1998) and it has been suggested that $I_{Cl(swell)}$ may also be involved in controlling vascular contractility (Greenwood & Large, 1998; Nelson, 1998; Greenwood & Large, 1999; Graves *et al.* 2000). This proposed function is based on the fact that in smooth muscle the chloride equilibrium potential (E_{Cl}, about −20 to −30 mV) is much more positive than the resting membrane potential. Consequently, activation of $I_{Cl(swell)}$ depolarises the membrane potential and increases the open probability of voltage-dependent calcium channels resulting in an influx of Ca^{2+}.

The possible involvement of $I_{Cl(swell)}$ in vascular contraction was based primarily on pharmacological studies in which it was found that inhibitors of $I_{Cl(swell)}$ blocked the myogenic response in rat cerebral arteries (Nelson *et al.* 1997); Cl^- efflux has also been recorded during the myogenic response (Doughty & Langton, 2001). In addition the contraction of rat coronary arteries produced by inhibition of nitric oxide (NO) synthesis was also blocked by antagonists of $I_{Cl(swell)}$ (Graves *et al.* 2000). These observations suggested that $I_{Cl(swell)}$ may contribute to vascular contraction in addition to its other proposed roles, such as cell proliferation. It is well established that agents that cause smooth muscle contraction also stimulate mitogenesis (e.g. see Tolloczko *et al.* 2000).

Previously we demonstrated that the vasodilator substance NO has a dual effect on $I_{Cl(swell)}$ in rabbit portal vein myocytes. NO increased the amplitude of $I_{Cl(swell)}$ by cGMP-dependent phosphorylation while NO decreased $I_{Cl(swell)}$ in a cGMP-independent manner (Ellershaw *et al.* 2000). In the present work we have investigated the effects of the sympathetic neuro-effector transmitter noradrenaline on $I_{Cl(swell)}$ in rabbit portal vein cells. The results show that noradrenaline acts on α-adrenoceptors to increase $I_{Cl(swell)}$, an effect which is mimicked by phorbol esters and blocked by inhibitors of protein kinase C (PKC) and therefore may be mediated by PKC. In addition there is a β-adrenoceptor-mediated decrease of $I_{Cl(swell)}$ which is blocked by an inhibitor of protein kinase A (PKA) and which is similar to the effect of 8-Br-cAMP implicating a role for PKA in the inhibitory effect of noradrenaline.

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METHODS

Cell preparation

New Zealand White rabbits (2–3 kg) were killed by injection of a lethal dose of sodium pentobarbitone (120 mg kg^{-1} I.V.) into the ear vein in accordance with Home Office regulations. Portal veins were excised, cleaned of fat and connective tissue and the exposed muscle sheet was cut into strips which were then immersed in physiological salt solution (PSS) containing 100 μM $CaCl_2$ at 37 °C. Single smooth muscle cells were prepared by first incubating the tissue with protease Type 14 (0.2 mg ml^{-1}, Sigma, UK) for 5 min. The tissue was then washed in PSS, containing 100 μM Ca^{2+}, and incubated for a further 10 min in collagenase Type 4 (1 mg ml^{-1}, Sigma, UK). The digested tissue was then triturated using a wide bore Pasteur pipette in order to liberate single smooth muscle cells. Isolated cells were transferred to PSS containing 0.75 mM $CaCl_2$, placed on cover slips for storage at 4 °C and were used within 6 h of isolation. All experiments were carried out at room temperature (21–24 °C).

Electrophysiological recording

Membrane currents were recorded with a List LM PCA amplifier using the whole-cell patch clamp technique. All voltage protocols were generated by the CED (Cambridge, UK) Voltage Clamp program and evoked currents were analysed using the corresponding CED analysis package, filtering at 3 kHz and sampling at a rate of 5 kHz. Further analysis and graphics were produced using Microcal Origin (Northampton, USA). Changes in junction potentials between the pipette and bath solutions were minimised by use of a KCl–agar bridge linking the main chamber to a side bath in which the reference electrode was located. The voltage-dependent characteristics of the hypotonicity-activated current were investigated by applying a voltage-ramp every 15 s. The ramp protocol consisted of stepping the voltage from the holding potential of −50 to −100 mV for 50 ms followed by continuously changing the voltage from −100 to +100 mV at a rate of 250 mV s^{-1} in isotonic PSS and hypotonic solutions. The leak-subtracted current voltage (*I–V*) relationship of the volume-sensitive chloride current was calculated by subtracting the control *I–V* curve in isotonic solution from that in hypotonic solution when the current had reached equilibrium.

Solutions

Normal PSS used for dissection contained (mM): NaCl 126, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 1.5, Hepes 10, glucose 11 and was adjusted to pH 7.2 with NaOH. To remove contaminating K^+ currents experiments were performed using K^+-free internal and external solutions. The normal K^+-free extracellular solution was composed of (mM): NaCl 126, $MgCl_2$ 1.2, $CaCl_2$ 1.5, Hepes 10, glucose 11 and was adjusted to pH 7.2 with NaOH. Nicardipine (5 μM) was also included to inhibit voltage-dependent Ca^{2+} currents. In all experiments the K^+-free pipette solution contained (mM): CsCl 126, $MgCl_2$ 1.2, Hepes 10, glucose 11, and the pH was adjusted to 7.2 with CsOH. In the present study the volume-sensitive chloride current was activated by substituting normal K^+-free PSS, i.e. with 126 mM NaCl, with an external solution in which the NaCl concentration was reduced to 75 mM. This procedure evokes $I_{Cl(swell)}$ which has no cationic contribution (Ellershaw *et al.* 2000) and is due to cell swelling and not a change in ionic strength (Greenwood & Large, 1998). To eliminate any contamination from Ca^{2+}-activated Cl^- currents 10 mM EGTA was added to the pipette solution in most experiments although in some experiments 1 mM EGTA was used for comparison. There was no difference in the results obtained with 1 and 10 mM EGTA. As noradrenaline evokes a non-selective cation current (I_{cat}) in these cells (Helliwell & Large; 1996) we performed experiments to determine the influence of I_{cat} in these experiments by including 1 mM $CdCl_2$ in the bathing solution which totally blocks I_{cat} (A. S. Aromolaran & W. A. Large, unpublished data). In other experiments we replaced external NaCl and pipette CsCl with *N*-methyl-D-glucamine (NMDG) Cl to block all non-selective cation conductances.

Chemicals

Calphostin-C, phorbol-12-myristate-13-acetate (PMA), phorbol-12,13-dibutyrate (PDBu), 8-Br-cAMP, U73122, U73443 and KT5720 were purchased from Calbiochem (La Jolla, CA, USA). Noradrenaline bitartrate, isoprenaline hydrochloride, prazosin hydrochloride, phenylephrine hydrochloride, chelerythrine chloride, cadmium chloride, *N*-methyl-D-glucamine (NMDG) and 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS) were purchased from Sigma (Poole, UK). Reagents were dissolved in dimethylsulphoxide which at the highest concentration used (0.1 %) had no effect on $I_{Cl(swell)}$.

Statistics

All data are shown as the mean ± S.E.M. of *n* cells in at least three rabbits. Student's *t* test was used to compare mean values and statistical significance was set at $P < 0.05$.

RESULTS

Effect of noradrenaline on $I_{Cl(swell)}$

Previously it has been shown that application of hypotonic solution to rabbit portal vein myocytes produces cell swelling and the simultaneous activation of a volume-sensitive Cl^- current, designated $I_{Cl(swell)}$, which is sustained during the continued presence of the hypotonic solution (Greenwood & Large, 1998; Ellershaw *et al.* 2000). On application of 75 mM NaCl hypotonic solution, the maximum width of the cell increased from 12 ± 1 μm to 16 ± 2 μm ($n = 16$). First we investigated the effects of noradrenaline on evoked $I_{Cl(swell)}$ in rabbit portal vein myocytes.

In the absence of pharmacological receptor antagonists, bath-applied noradrenaline (10 μM) produced a complex range of effects. In some cells (3 out of 8 cells) $I_{Cl(swell)}$ was increased by noradrenaline (Fig. 1*A*) and this effect was not voltage dependent (Fig. 1*B*). The maximum potentiation by noradrenaline was 35–45 % and occurred within 5–10 min. In other cells (3 out of 8 cells) noradrenaline decreased $I_{Cl(swell)}$ and interestingly in some cells this reduction was associated with oscillation of $I_{Cl(swell)}$ (Fig. 1*C*). The maximum inhibition of $I_{Cl(swell)}$ produced by 10 μM noradrenaline was about 50 % and was not voltage dependent (Fig. 1*D*). In two of eight cells 10 μM noradrenaline produced no net effect on membrane conductance. As it has been shown that both α- and β-adrenoceptors are present in the rabbit portal vein (Holman *et al.* 1968) it is possible that pathways elicited by activation of these receptors might modulate $I_{Cl(swell)}$.

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Consequently further experiments were conducted in the presence of propranolol, an antagonist of β-adrenoceptors, or prazosin, an antagonist of α_1-adrenoceptors.

In the presence of 1 μM propranolol, application of noradrenaline to the bathing solution produced an increase in the amplitude of $I_{Cl(swell)}$ which reached a plateau after approximately 5–10 min. An example of this effect is shown in Fig. 2*A* where it can be seen that the noradrenaline-induced increase was reversed on washout of the drug. The increase of $I_{Cl(swell)}$ by noradrenaline was concentration dependent (Fig. 2*B*) and was reversible on removal from the bathing solution. The concentration producing 50 % of the maximum effect (EC_{50}), measured from Fig. 2*B*, was approximately 5 μM. The potentiating effect of noradrenaline on $I_{Cl(swell)}$ was not voltage dependent and the increase produced by 10 μM noradrenaline was 65 ± 9 % ($n = 5$) at −50 mV and 62 ± 9 % ($n = 5$) at +100 mV.

The potentiating effect of noradrenaline was not due to a change in cell size. Under hypotonic conditions maximum cell width was 15 ± 2 μm and following application of 10 μM noradrenaline, the maximum cell width was 15 ± 2 μm ($n = 5$). Similarly, in all further experiments agents which had an effect on the amplitude of $I_{Cl(swell)}$ did not alter cell size.

In the presence of 100 nM prazosin, noradrenaline always inhibited $I_{Cl(swell)}$ with a similar time course to the excitatory effect. An example of a typical trace is shown in Fig. 2*C*. This inhibitory effect was also concentration dependent (Fig. 2*D*) and the concentration producing 50 % of maximum inhibition (IC_{50}) was approximately 1.5 μM. The inhibitory effect of noradrenaline was voltage-independent and the inhibition produced by 10 μM noradrenaline was 64 ± 4 % ($n = 6$) at −50 mV and 65 ± 4 % ($n = 6$) at +100 mV. The inhibition produced by noradrenaline was, at least partially, reversible on washout of this agent. The inhibitory effect of noradrenaline was not due to a change in cell size. Under hypotonic conditions maximum cell width was 16 ± 2 μm and following application of noradrenaline the maximum cell width was 16 ± 2 μm ($n = 5$).

It has been stipulated that activation of $I_{Cl(swell)}$ requires internal ATP (reviewed by Strange *et al.* 1996; Nilius *et al.*

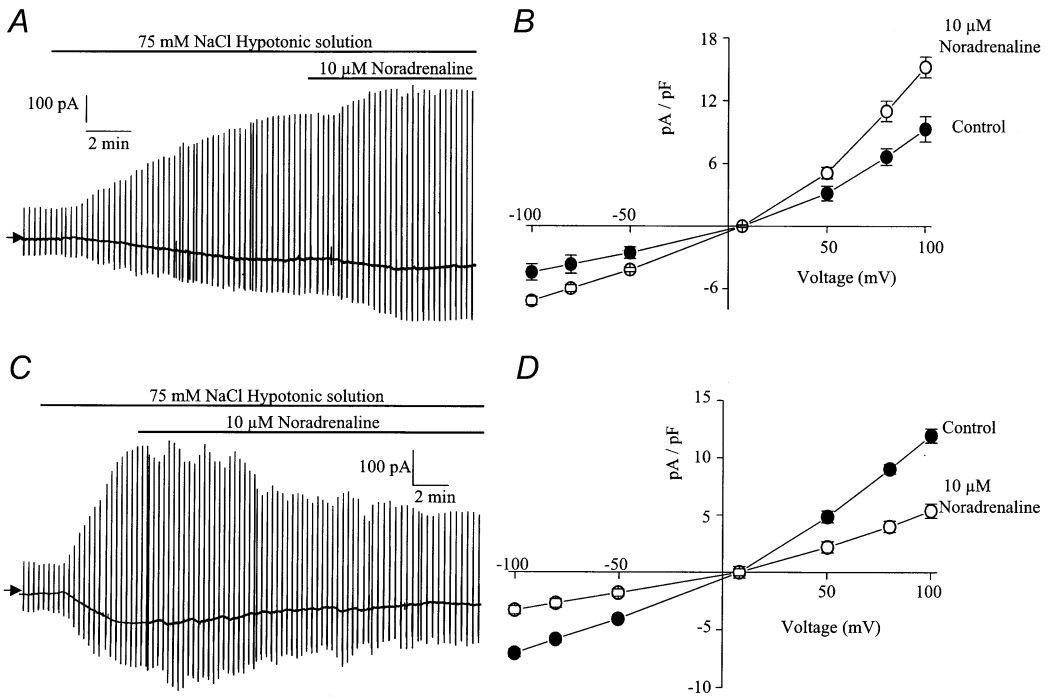

Figure 1. The effect of noradrenaline on $I_{Cl(swell)}$

A, representative trace illustrating the increase of $I_{Cl(swell)}$ produced by 10 μM noradrenaline. In all traces vertical deflections represent the ramp protocol from a holding potential of −50 mV and in this and subsequent figures the arrow to the left of the trace indicates zero current level. *B*, leak-subtracted current–voltage relationship (see Methods) of $I_{Cl(swell)}$ evoked by 75 mM NaCl (●) and in the same hypotonic solution containing 10 μM noradrenaline (○). In this and subsequent figures mean current densities were calculated as pA pF^{-1}. Each point is the mean ± S.E.M. of 5 cells. *C*, representative trace illustrating the inhibitory effect of 10 μM noradrenaline on $I_{Cl(swell)}$ in another cell. Note the oscillation of $I_{Cl(swell)}$ as it is inhibited. *D*, leak-subtracted current–voltage relationship of $I_{Cl(swell)}$ evoked by 75 mM NaCl (●) and in the same hypotonic solution containing 10 μM noradrenaline (○). Each point is the mean ± S.E.M. of 6 cells.

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1997; Okada, 1997). The present experiments were carried out without ATP in the patch-pipette solution since it is apparent that rabbit portal vein myocytes generate sufficient endogenous ATP to sustain phosphorylation. Thus, for example, contractile agents such as noradrenaline and caffeine induce contraction of myocytes that have been dialysed with ATP-free pipette solution for periods of up to 60 min. However we carried out a few experiments on the effect of noradrenaline with 1 mM ATP in the pipette solution. The enhancement of $I_{Cl(swell)}$ produced by 10 μM noradrenaline, when 1 mM ATP was present in the pipette, was 66 ± 10 % (n = 5) at −50 mV and 67 ± 10 % (n = 5) at +100 mV. The inhibitory effect of 10 μM noradrenaline on the amplitude of $I_{Cl(swell)}$ in the presence of 1 mM ATP in the pipette, was 62 ± 6 % (n = 5) at −50 mV and 62 ± 6 % (n = 5) at +100 mV. These values are not significantly different from those obtained with ATP-free pipette solution.

Noradrenaline had no effect on the resting conductance recorded under isotonic conditions. In isotonic solutions the current amplitudes at −50 mV and +100 mV were 1.0 ± 0.07 and 5.7 ± 0.2 pA pF^{-1} (n = 5), respectively. When 10 μM noradrenaline was added to the bathing solution the current amplitudes were 1.1 ± 0.1 and 5.7 ± 0.4 pA pF^{-1} (n = 5) at −50 and +100 mV, respectively. Similarly, in all further experiments none of the drugs investigated had an effect on the amplitude of $I_{Cl(swell)}$ under isotonic conditions.

However, under isotonic conditions application of 10 μM noradrenaline sometimes activated a small 'noisy' current (about 10–30 pA in amplitude at −50 mV) which appeared to be the non-selective cation current I_{cat} previously described (e.g. Helliwell & Large, 1996; Byrne & Large, 1988). Noradrenaline did not appear to evoke I_{cat} under hypotonic solutions. Nevertheless we carried out experiments in which I_{cat} was blocked to determine whether the increase of $I_{Cl(swell)}$ produced by noradrenaline was due to the concomitant activation of I_{cat}. With 1 mM Cd^{2+} in the bathing solution, which totally blocks I_{cat} (A. S. Aromolaran & W. A. Large, unpublished data), 10 μM noradrenaline increased $I_{Cl(swell)}$ by 60 ± 12 and 59 ± 11 % at, respectively, −50 and +100 mV (n = 8). In other experiments where NMDG chloride was used to replace both external NaCl and internal CsCl 10 μM noradrenaline increased $I_{Cl(swell)}$ by 57 ± 11 and 62 ± 8 % at, respectively, −50 and +100 mV (n = 5). In the latter conditions it is probable that no cation conductance could be recorded and the quantitative data on the effect of noradrenaline on $I_{Cl(swell)}$ are similar to the values without Cd^{2+} or NMDG. Consequently the enhanced conductance produced by noradrenaline is due solely to an effect on $I_{Cl(swell)}$ and not due to co-activation of a cation conductance.

It can be concluded that the biphasic effect of noradrenaline is due to activation of α_1-adrenoceptors to

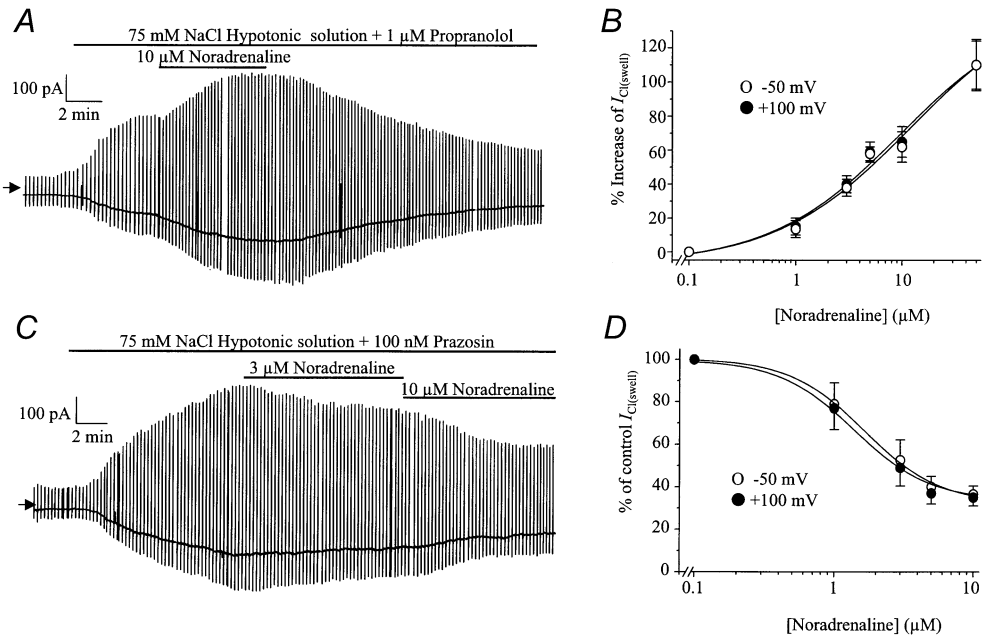

Figure 2. The effect of noradrenaline on $I_{Cl(swell)}$ in the presence of propranolol or prazosin

A, representative trace illustrating the increase of $I_{Cl(swell)}$ produced by 10 μM noradrenaline in the presence of 1 μM propranolol. *B*, concentration–effect curve of the noradrenaline-induced increase of $I_{Cl(swell)}$ at −50 mV (○) and +100 mV(●). Each point is the mean ± S.E.M. of 5 cells. *C*, representative trace illustrating the inhibitory effect of 3 and 10 μM noradrenaline on $I_{Cl(swell)}$ in the presence of prazosin (100 nM). *D*, concentration–effect curve of the noradrenaline-induced inhibition of $I_{Cl(swell)}$ at −50 mV (○) and +100 mV (●). The vertical axis represents the percentage of the current before noradrenaline was added. Each point is the mean ± S.E.M. of 6 cells.

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increase $I_{Cl(swell)}$ whereas the inhibitory effect of noradrenaline is mediated by β-adrenoceptor stimulation. It should be noted that in three populations of cells in the presence of propranolol, noradrenaline up to 50 μM did not increase $I_{Cl(swell)}$. We have no explanation for these negative results but these cells were not used for our studies.

Effect of U73122 and IP_3 on $I_{Cl(swell)}$

α_1-Adrenoceptor stimulation is linked to the phosphoinositide cascade via activation of phosphatidylinositol phospholipase C (PLC). This leads to the production of the second messengers diacylglycerol (DAG), with subsequent activation of protein kinase C (PKC), and the Ca^{2+}-releasing messenger inositol 1,4,5-trisphosphate (IP_3). Therefore we investigated the effect of U73122, an inhibitor of PLC, on $I_{Cl(swell)}$. Application of 1 μM U73122 to the bathing solution produced slow inhibition of $I_{Cl(swell)}$ which reached its maximum effect after approximately 10 min and a typical record is shown in Fig. 3*A*. The effect of U73122 was voltage-independent (Fig. 3*B*) and the inhibition produced by 1 μM U73122 was 49 ± 3 % (n = 5) at −50 mV and 50 ± 4 % (n = 5) at +100 mV. In five cells application of 2 μM U73343, the inactive analogue of U73122, had no effect on $I_{Cl(swell)}$. A typical trace is shown in Fig. 3*C* and it can be seen that subsequent addition of U73122 produced its normal inhibitory effect. This result suggests that during activation of $I_{Cl(swell)}$ there is tonic PLC activity that enhances the amplitude of this current.

In normal isotonic conditions inclusion of 100 μM IP_3 in the pipette solution had no effect on the resting current. Furthermore, under hypotonic conditions the normalised amplitudes of $I_{Cl(swell)}$ at −50 and +100 mV were, respectively, 3 ± 0.3 pA pF^{-1} (n = 6) and 12 ± 0.7 pA pF^{-1} (n = 6) in control conditions. Inclusion of 100 μM IP_3 in the pipette solution had no effect on the development of evoked $I_{Cl(swell)}$ and the mean current amplitudes at −50 and +100 mV were, respectively, 3.7 ± 0.8 pA pF^{-1} (n = 6) and 13 ± 0.8 pA pF^{-1} (n = 6). These data suggest that the IP_3 arm of the signalling cascade is not responsible for the noradrenaline-dependent increase of $I_{Cl(swell)}$.

Effect of the phorbol esters PMA and PDBu on $I_{Cl(swell)}$

In the next series of experiments we investigated the possible role of PKC in the stimulation of $I_{Cl(swell)}$ by studying the effects of phorbol esters, which are known to directly activate PKC, on $I_{Cl(swell)}$. The addition of the phorbol ester PMA (100 nM–1 μM) to the bathing solution increased the amplitude of $I_{Cl(swell)}$ in all cells and a representative trace showing the effect of 1 μM PMA is shown in Fig. 4*A*. This effect was not voltage dependent (Fig. 4*B*) and the maximum enhancement produced by

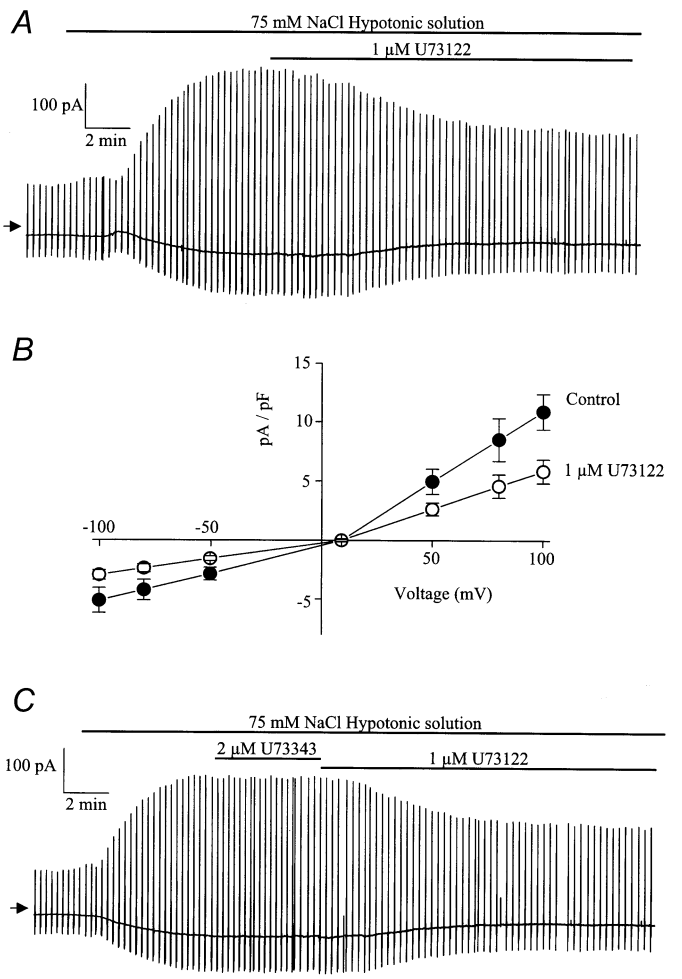

Figure 3. Effect of U73122 on $I_{Cl(swell)}$

A, representative trace illustrating inhibitory effect of 1 μM U73122 on $I_{Cl(swell)}$. *B*, leak-subtracted current–voltage relationship of $I_{Cl(swell)}$ evoked by 75 mM NaCl (●) and in the same hypotonic solution containing 1 μM U73122 (○) shown in *A*. Each point is the mean ± S.E.M. of 5 cells *C*, representative trace illustrating the effects of 2 μM U73343 (inactive analogue) and 1 μM U73122 on $I_{Cl(swell)}$.

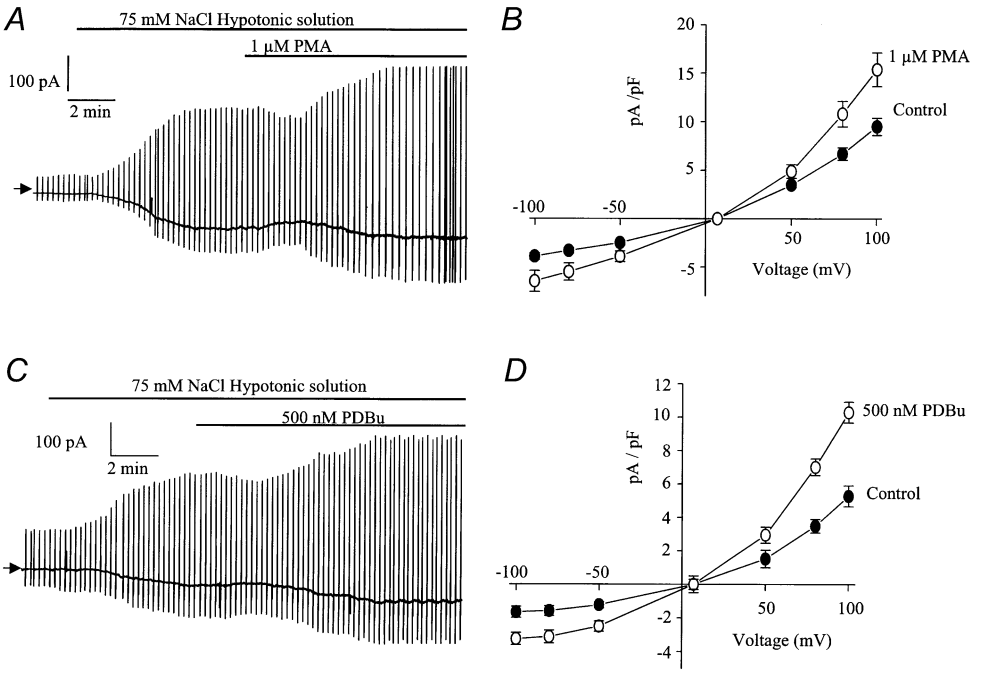

Figure 4. Effect of phorbol esters on $I_{Cl(swell)}$

A, representative trace illustrating the excitatory effect of 1 μM PMA on $I_{Cl(swell)}$. *B*, leak-subtracted mean current–voltage relationship of $I_{Cl(swell)}$ evoked by 75 mM NaCl (●) and in the same hypotonic solution containing 1 μM PMA (○). Each point is the mean ± S.E.M. of 5 cells. *C*, representative trace illustrating the excitatory effect of 500 nM PDBu on $I_{Cl(swell)}$. *D*, leak-subtracted mean current–voltage relationship of $I_{Cl(swell)}$ evoked by 75 mM NaCl (●) and in the same hypotonic solution containing 500 nM PDBu (○). Each point is the mean ± S.E.M. of 6 cells.

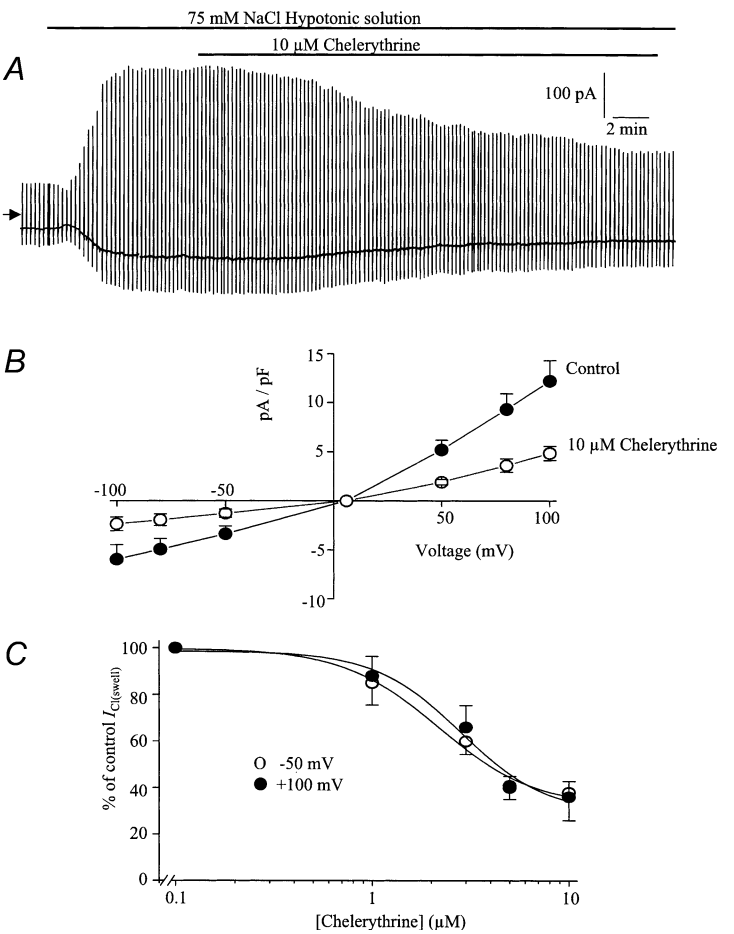

Figure 5. Effect of chelerythrine on $I_{Cl(swell)}$

A, typical cell showing the inhibition of $I_{Cl(swell)}$ by 10 μM chelerythrine. *B*, leak-subtracted mean current–voltage relationship of $I_{Cl(swell)}$ evoked by 75 mM NaCl (●) and in the same hypotonic solution containing 10 μM chelerythrine (○). Each point is the mean ± S.E.M. of 5 cells. *C*, concentration–effect curve of the chelerythrine-induced inhibition of $I_{Cl(swell)}$ at −50 mV (○) and +100 mV (●). Each point is the mean ± S.E.M. of 5 cells.

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100 nM PMA was 41 ± 6 % (n = 5) at −50 mV and 46 ± 7 % (n = 5) at +100 mV. The effect of PMA was concentration dependent and 1 μM increased $I_{Cl(swell)}$ by 55 ± 4 % (n = 5) at −50 mV and by 67 ± 7 % (n = 5) at +100 mV. Application of another phorbol ester, PDBu (100–500 nM), to the bathing solution also produced an increase in the amplitude of the current (Fig. 4*C*) with a similar time course to PMA and showed no voltage dependence (Fig. 4*D*). The enhancement of $I_{Cl(swell)}$ by 500 nM PDBu was 108 ± 15 % (n = 6) at −50 mV and 94 ± 7 % at +100 mV (n = 6). Interestingly, application of PMA or PDBu to the bathing solution produced a small transient reduction in the amplitude of $I_{Cl(swell)}$ prior to the sustained enhancement of the current. This was a consistent observation but was not investigated further in this study.

In the presence of 1 mM Cd^{2+}, which abolishes I_{cat}, application of 500 nM PDBu to the bathing solution increased the amplitude of $I_{Cl(swell)}$ by 80 ± 9 and 82 ± 12 % (n = 4) at, respectively, −50 and +100 mV.

Inhibition of $I_{Cl(swell)}$ by chelerythrine and calphostin-C

Since phorbol esters augmented $I_{Cl(swell)}$ we subsequently studied the effects of the PKC inhibitors chelerythrine and calphostin-C on $I_{Cl(swell)}$. Application of chelerythrine (1–10 μM) to the bathing solution produced slow inhibition of $I_{Cl(swell)}$ which took approximately 15 min to reach its maximum effect. A typical effect of 10 μM chelerythrine is shown in Fig 5*A*. The inhibitory effect of chelerythrine was voltage-independent (Fig. 5*B*) and the maximum inhibition of $I_{Cl(swell)}$ by 10 μM chelerythrine was 63 ± 10 % (n = 5) at −50 mV and 65 ± 7 % (n = 5) at +100 mV (Fig. 5*C*). From Fig. 5*C* the estimated IC_{50} of chelerythrine on $I_{Cl(swell)}$ was approximately 2 μM. Similarly, calphosin-C (1–10 μM) also inhibited $I_{Cl(swell)}$ with a similar time course to chelerythrine and a representative trace is shown in Fig. 6*A*. The inhibitory effect of calphostin-C was also voltage-independent (Fig. 6*B*) and the maximal inhibition of $I_{Cl(swell)}$ produced by 10 μM calphostin-C was 58 ± 7 (n = 5) at −50 mV and 58 ± 6 % (n = 5) at +100 mV. The IC_{50} for the inhibitory effect of calphostin-C on $I_{Cl(swell)}$ was approximately 1 μM (Fig. 6*C*).

Both the effects of phorbol esters and PKC inhibitors had long time courses which prevented a study of their reversibility as it was usually difficult to keep the whole-cell configuration for more than 30–45 min.

Effect of isoprenaline on $I_{Cl(swell)}$

The above data with pharmacological receptor antagonists imply that the noradrenaline-induced inhibition of $I_{Cl(swell)}$

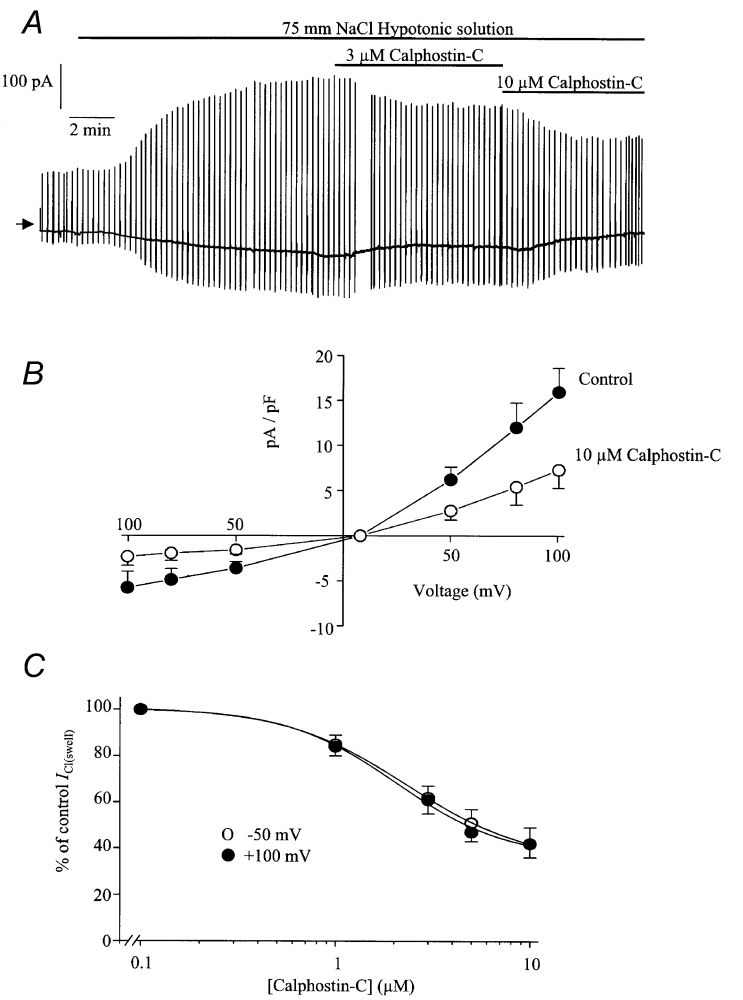

Figure 6. Effect of calphostin-C on $I_{Cl(swell)}$

A, typical cell showing the inhibition of $I_{Cl(swell)}$ by 3 and 10 μM calphostin-C. *B*, leak-subtracted mean current–voltage relationship of $I_{Cl(swell)}$ evoked by 75 mM NaCl (●) and in the same hypotonic solution containing 10 μM calphostin-C (○). Each point is the mean ± S.E.M of 5 cells. *C*, concentration–effect curve of the calphostin-C-induced inhibition of $I_{Cl(swell)}$ at −50 mV (○) and +100 mV (●). Each point is the mean ± S.E.M. of 5 cells.

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was a result of β-adrenoceptor activation. To confirm this possibility we investigated the effect of isoprenaline, a selective agonist of β-adrenoceptors, on $I_{Cl(swell)}$. Application of isoprenaline (1–10 μM) to the bathing solution (no prazosin present) produced slow inhibition of $I_{Cl(swell)}$ which reached a plateau within approximately 10 min (Fig. 7*A*) and was not voltage dependent (Fig. 7*B*). The maximum inhibition produced by 10 μM isoprenaline was 55 ± 6 % at −50 mV and 53 ± 5 % at +100 mV (n = 6). These results confirm that β-adrenoceptor stimulation inhibits $I_{Cl(swell)}$.

Effect of 8-Br-cAMP and KT5720 on $I_{Cl(swell)}$

Many of the effects of β-adrenoceptor stimulation in smooth muscle are due to the activation of adenylate cyclase and subsequent increase in intracellular concentration of cAMP (Lincoln & Fisher-Simpson, 1984). Therefore it is possible that the inhibition of $I_{Cl(swell)}$ by noradrenaline in the presence of prazosin and isoprenaline was cAMP dependent. We therefore investigated the effect of the membrane permeable synthetic analogue of cAMP, 8-Br-cAMP, on evoked $I_{Cl(swell)}$. Application of 100 μM 8-Br-cAMP to the bathing solution produced slow inhibition of $I_{Cl(swell)}$ and a representative trace of the effect of 100 μM 8-Br-cAMP is shown in Fig. 8*A*. The inhibitory effect of 8-Br-cAMP showed no voltage dependence (Fig. 8*B*) and the inhibition produced by 100 μM 8-Br-cAMP was 62 ± 6 % (n = 6) at −50 mV and 61 ± 5 % (n = 6) at +100 mV. Many of the effects of cAMP are mediated by a cAMP-dependent protein kinase and therefore we studied the effect of the selective cAMP-dependent protein kinase inhibitor, KT5720, on evoked $I_{Cl(swell)}$. Application of 1 μM KT5720 to the bathing solution produced a marked increase in evoked $I_{Cl(swell)}$ reaching a plateau approximately 7 min after application. An example of a typical trace is shown in Fig 8*C*. The effect of 1 μM KT5720 was not voltage dependent (Fig. 8*D*) and increased $I_{Cl(swell)}$ by 89 ± 10 % (n = 5) at −50 mV and by 84 ± 8 % (n = 5) at +100 mV.

Role of PKC and PKA on the effects of noradrenaline

The above data suggest that the excitatory effects of noradrenaline on $I_{Cl(swell)}$ may be mediated via PKC. Therefore we investigated the effects of chelerythrine against the action of noradrenaline in the presence of propranolol. Due to the slow time course of these agents, chelerythrine was applied to the hypotonic bathing solution during the development of $I_{Cl(swell)}$ and noradrenaline was applied immediately the current reached the peak value. A concentration of 50 μM noradrenaline was used in order to produce a near maximal increase in the amplitude of $I_{Cl(swell)}$. In this series of experiments application of 50 μM noradrenaline to the bathing solution increased the amplitude of $I_{Cl(swell)}$ by 110 ± 15 % at −50 mV and by 111 ± 14 % (n = 7) at +100 mV. However, when cells were pre-treated with 10 μM chelerythrine, the increase of $I_{Cl(swell)}$ to 50 μM noradrenaline was 9 ± 3 % at −50 mV and 10 ± 3 % at +100 mV (n = 7). Therefore chelerythrine markedly inhibited the potentiating effect of noradrenaline on $I_{Cl(swell)}$.

We also investigated the effects of KT5720 against the inhibitory action of noradrenaline in the presence of prazosin. Application of 1 μM KT5720 to the bathing solution produced an increase in the amplitude of $I_{Cl(swell)}$ of 77 ± 8 % at −50 mV and 75 ± 9 % at +100 mV (n = 5). Subsequent application of 10 μM noradrenaline in the presence of KT5720 produced little inhibition of $I_{Cl(swell)}$. The inhibition produced by 10 μM noradrenaline in the

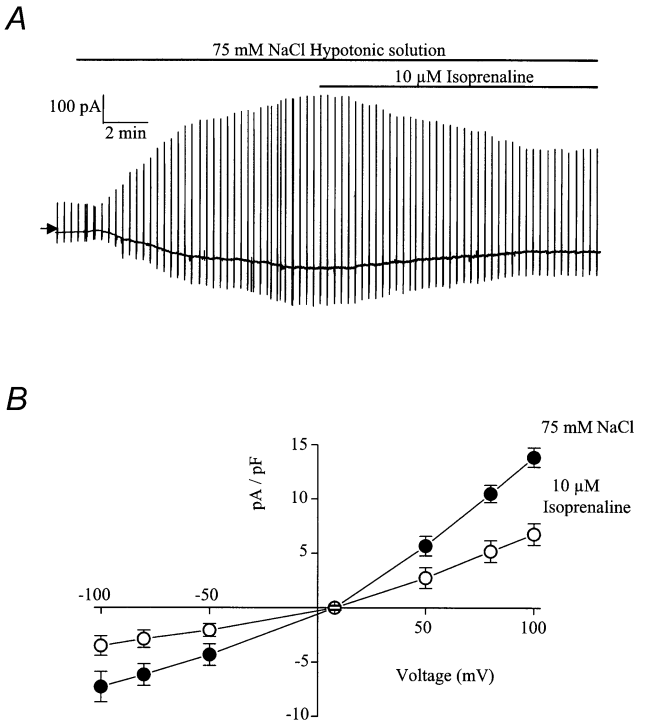

Figure 7. The effect of isoprenaline on $I_{Cl(swell)}$

A, representative trace illustrating the inhibitory effect of 10 μM isoprenaline on $I_{Cl(swell)}$. *B*, leak-subtracted current–voltage relationship of $I_{Cl(swell)}$ evoked by 75 mM NaCl (●) and in the same hypotonic solution containing 10 μM isoprenaline (○). Each point is the mean ± S.E.M. of 6 cells.

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presence of 1 μM KT5720 was 5 ± 2 % at −50 mV and 7 ± 2 % at +100 mV (n = 5). Therefore the inhibitory action of noradrenaline was inhibited by KT5720.

DISCUSSION

The present study shows that noradrenaline modulates $I_{Cl(swell)}$ in rabbit portal vein smooth muscle cells by two distinct pathways. In the absence of pharmacological antagonists noradrenaline increased the amplitude of $I_{Cl(swell)}$ in some cells and decreased the current in others. In the presence of the β-adrenoceptor antagonist propranolol, noradrenaline enhanced $I_{Cl(swell)}$ while in the presence of the selective α_1-adrenoceptor antagonist prazosin, noradrenaline always reduced $I_{Cl(swell)}$. It is important to emphasise that the increase in current produced by noradrenaline was not due to co-activation of I_{cat}. In the presence of 1 mM Cd^{2+}, which totally blocks I_{cat}, the increase of $I_{Cl(swell)}$ was similar to that observed in the absence of Cd^{2+}. Therefore the increase in current is solely due to an increase in $I_{Cl(swell)}$. These results show that the increase and decrease of $I_{Cl(swell)}$ caused by noradrenaline are mediated by, respectively, α_1- and β-adrenoceptors in these vascular myocytes. The present study does not relate the changes in amplitude of $I_{Cl(swell)}$ to single channel conductance, open channel probability and/or channel number which is the subject of future work.

Modulation of $I_{Cl(swell)}$ by noradrenaline

The modulation of $I_{Cl(swell)}$ by noradrenaline was dependent on the class of adrenoceptor stimulated. Stimulation of α_1-adrenoceptor augmented $I_{Cl(swell)}$ that was blocked by the PKC inhibitors chelerythrine and calphostin-C and suggests that the noradrenaline-dependent enhancement of $I_{Cl(swell)}$ is mediated by PKC. This hypothesis is supported by the observation that in the absence of noradrenaline, inhibitors of PKC decreased $I_{Cl(swell)}$ and activators of PKC increased the conductance. In comparison inclusion of IP_3 in the pipette did not affect the amplitude of $I_{Cl(swell)}$.

The inhibitory effect of noradrenaline was produced by β-adrenoceptor stimulation and was blocked by the PKA inhibitor KT5720 which suggests that this inhibitory action is mediated by a PKA-dependent pathway. In addition, in the absence of noradrenaline the cell permeable analogue of cAMP, 8-bromo cAMP, decreased the amplitude of $I_{Cl(swell)}$ whereas the PKA inhibitor KT5720 increased $I_{Cl(swell)}$. Previously we have shown that cGMP-dependent protein kinase increases the amplitude of $I_{Cl(swell)}$ (Ellershaw *et al.* 2000). The present study shows that PKC also increases this conductance whereas PKA decreases the amplitude of $I_{Cl(swell)}$. Thus in rabbit portal vein smooth muscle cells it appears that $I_{Cl(swell)}$ is the target of complex kinase regulation. In addition, the ability of PLC, PKC and PKA inhibitors to modulate $I_{Cl(swell)}$ in the absence of noradrenaline suggests

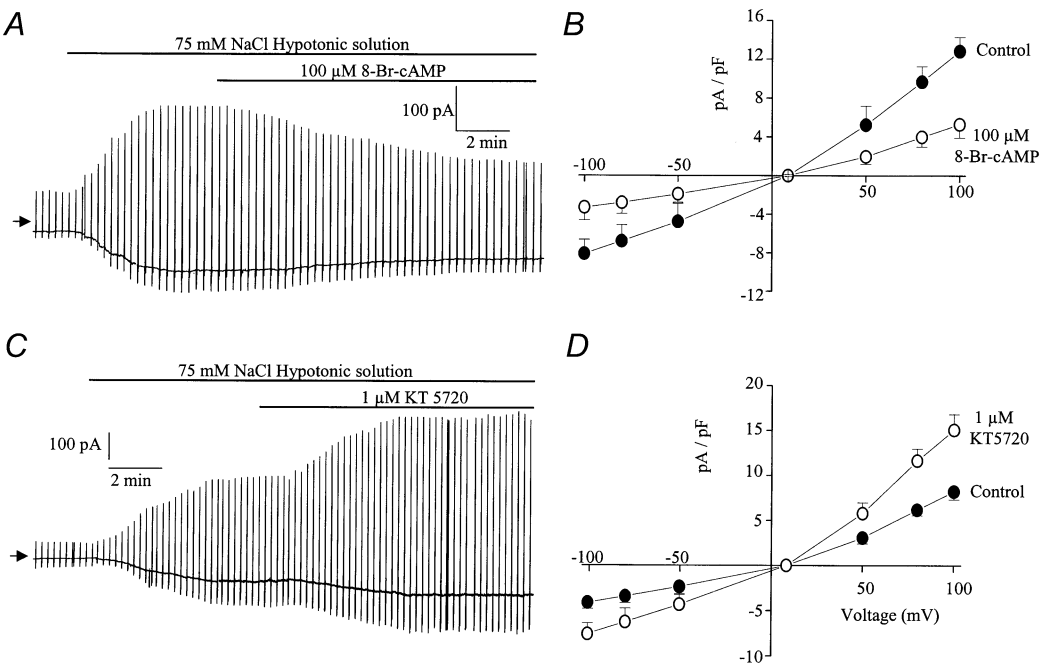

Figure 8. Effect of 8-Br-cAMP and KT5720 on $I_{Cl(swell)}$

A, representative trace illustrating the inhibitory effect of 100 μM 8-Br-cAMP on $I_{Cl(swell)}$. *B*, leak-subtracted current–voltage relationship of $I_{Cl(swell)}$ evoked by 75 mM NaCl (●) and in the same hypotonic solution containing 100 μM 8- Br-cAMP (○). Each point is the mean ± S.E.M. of 6 cells. *C*, representative trace illustrating the increase of $I_{Cl(swell)}$ induced by 1 μM KT5720. D, leak-subtracted current–voltage relationship of $I_{Cl(swell)}$ evoked by 75 mM NaCl (●) and in the same hypotonic solution containing 1 μM KT5720 (○). Each point is the mean ± S.E.M. of 5 cells.

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that hypotonic swelling of rabbit portal vein myocytes increases the activity of these kinases. Moreover, in the absence of noradrenaline, the PLC inhibitor U73122 also decreased the amplitude of $I_{Cl(swell)}$ which suggests that there is tonic PLC activity during activation of $I_{Cl(swell)}$.

Comparison of regulation of $I_{Cl(swell)}$ in other tisssues

There have been no previous reports on the effect of adrenoceptor stimulation on $I_{Cl(swell)}$ in smooth muscle. However there have been numerous reports regarding phosphorylation reactions and $I_{Cl(swell)}$ in many different cell types and there is much conflicting data on this subject (e.g. see reviews by Okada, 1997; Strange *et al.* 1996). For example, stimulation of PKC by phorbol esters decreases $I_{Cl(swell)}$ in rabbit and guinea-pig cardiac cells (Duan *et al.* 1995, 1999) but increases $I_{Cl(swell)}$ in canine atrial cells (Du & Sorota, 1999). More pertinently to the present work in smooth muscle, it was shown in canine colonic smooth muscle that phorbol esters reduced $I_{Cl(swell)}$. Moreover the PKC inhibitor chelerythrine activated the current in isotonic conditions (Dick *et al.* 1998). Clearly these latter results are in marked contrast to the present work in vascular smooth muscle where chelerythrine had no effect in isotonic solutions and decreased $I_{Cl(swell)}$ which illustrates the complex modulation of this conductance in different types of smooth muscle and other cell types. Overall the data on PKC modulation of native $I_{Cl(swell)}$ appears conflicting and dependent on the cell type studied.

Similarly there are conflicting results regarding PKA modulation of $I_{Cl(swell)}$ in other classes of cell. In agreement with the present work it was concluded that PKA-induced phosphorylation reduced $I_{Cl(swell)}$ in chick (Hall *et al.* 1995) and in mammalian cardiac cells (Du & Sorota, 1997, Nagasaki *et al.* 2000). In contrast, increasing intracellular levels of cAMP potentiated the amplitude of $I_{Cl(swell)}$ by a PKA-independent pathway in canine atrial cells and in human epithelial cells (Shimizu *et al.* 2000). At present these contradictory data cannot be explained but may be related to the different physiological functions of $I_{Cl(swell)}$ in these diverse cell types.

Conclusions

In freshly dispersed rabbit portal vein myocytes noradrenaline stimulates α-adrenoceptors to increase $I_{Cl(swell)}$ via a PKC-dependent mechanism whereas β-adrenoceptor activation reduces the amplitude of $I_{Cl(swell)}$ by a PKA-dependent mechanism. The fact that different kinases modulate $I_{Cl(swell)}$ in different ways shows that phosphorylation is a crucial determinant of $I_{Cl(swell)}$ activity. However, comparison with other smooth muscle preparations and other cell types illustrates that there is complex regulation of this conductance by receptor transduction pathways.

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