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ARTICLE *in* BRITISH JOURNAL OF PHARMACOLOGY · FEBRUARY 2003

Impact Factor: 4.84 · DOI: 10.1038/sj.bjp.0705000 · Source: PubMed

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Anomalous effect of anthracene-9-carboxylic acid on calcium-activated chloride currents in rabbit pulmonary artery smooth muscle cells

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1 Ca^{2+}-activated Cl^- currents ($I_{Cl(Ca)}$) evoked by K^+-free pipette solutions containing 500 nM Ca^{2+} were recorded in rabbit pulmonary artery smooth muscle cells. A voltage step protocol in which the cells were stepped to +70 mV and then to −80 mV produced outward and inward Cl^- currents respectively that exhibited distinctive voltage- and time-dependent kinetics that remained consistent for the recording period.

2 Application of the Cl^- channel inhibitor anthracene-9-carboxylic acid (A-9-C, 500 μM), produced a small inhibition of the maximum outward Cl^- current at +70 mV ($21 \pm 10\%$) but augmented the amplitude of the instantaneous inward relaxation at −80 mV by $321 \pm 34\%$ ($n = 12$).

3 The current recorded in the absence and presence of A-9-C reversed at the theoretical Cl^- equilibrium potential and the reversal potential was shifted by about −40 mV upon replacement of external chloride ion by the more permeant anion thiocyanate. Currents in the absence and presence of A-9-C were similarly affected by 100 μM niflumic acid.

4 Augmentation of the inward current at −80 mV by A-9-C required prior depolarization, i.e. A-9-C did not simply activate a Cl^- current at negative membrane potentials. Moreover the degree of augmentation was independent of the internal Ca^{2+} for concentrations between 100 nM and 1 μM Ca^{2+}.

5 The data from the present study confirm previous observations that the inhibitory effect of Cl^- channel blockers is modified when $[Ca^{2+}]_i$ is maintained at higher than normal resting concentrations.

British Journal of Pharmacology (2003) **138,** 31–38. doi:10.1038/sj.bjp.0705000

Keywords: Vascular smooth muscle; calcium-activated chloride current; anthracene 9 carboxylic acid

Abbreviations: A-9-C, anthracene-9-carboxylic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-tetra-acetic acid; $I_{Cl(Ca)}$, calcium-activated chloride current; DCDPC, dichloro-diphenylamine 2-carboxylic acid; HEPES, 4-(2-hydroxy-ethyl)-1-piperazine ethanesulphonic acid; NFA, niflumic acid; TEA, tetraethyl ammonium

Introduction

In smooth muscle cells calcium-activated chloride currents ($I_{Cl(Ca)}$) evoked by transient increments in intracellular calcium, generated by either release from intracellular Ca^{2+} stores or by Ca^{2+} influx through voltage-dependent Ca^{2+} channels, are blocked by a range of chemically dissimilar agents (Hogg *et al*., 1993; 1994a, b, Large & Wang, 1996). Out of the agents considered to be chloride channel blockers niflumic acid (NFA) is the most potent in smooth muscle cells with an IC_{50} against spontaneously occurring $I_{Cl(Ca)}$ of 2 μM (Hogg *et al*., 1994a). The action of NFA was moderately voltage dependent, and was associated with a modification of the decay kinetics of spontaneous $I_{Cl(Ca)}$ currents (Hogg *et al*., 1994a). The structurally related compound dichloro-diphenylamine 2-carboxylic acid (DCDPC) had similar effects on the decay of spontaneous Ca^{2+}-activated chloride currents (Greenwood & Large, 1998). These data suggested that both NFA and its congeners act as open channel blockers of $I_{Cl(Ca)}$. However, when the Cl^- channel is stimulated by a sustained level of calcium supplied by the pipette solution the effect of NFA is altered markedly (Piper *et al*., 2002). In pulmonary artery myocytes when $I_{Cl(Ca)}$ were activated by pipette solutions containing 500 nM Ca^{2+} 100 μM NFA did not abolish $I_{Cl(Ca)}$. Instead NFA augmented the resting $I_{Cl(Ca)}$ at the holding potential of −50 mV and altered the voltage-dependent kinetics of the channel. Moreover, upon washout of NFA, we observed a large increase in $I_{Cl(Ca)}$ amplitude at all potentials. Similar results were obtained with DCDPC (Piper *et al*., 2002). In comparison, DIDS, which does not inhibit Ca^{2+}-activated Cl^- channels by a mechanism consistent with open channel block (Hogg *et al*., 1994b), simply inhibited the $I_{Cl(Ca)}$ activated by 500 nM Ca^{2+} (Piper *et al*., 2002).

Anthracene-9-carboxylic acid (A-9-C) is an agent chemically unrelated to NFA (Wangemann *et al*., 1986) that blocks spontaneous $I_{Cl(Ca)}$ in rabbit portal vein smooth muscle (Hogg *et al*., 1993, 1994b). A-9-C also inhibits $I_{Cl(Ca)}$ evoked as a consequence of Ca^{2+} influx through voltage-dependent channels in various smooth muscle cell types including oesophageal (Akbarali & Giles, 1993), urethral (Cotton *et al*., 1997) anococcygeal (Wayman *et al*., 1997) and lymphatic smooth muscle cells (Toland *et al*., 2000). The inhibitory

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effect of A-9-C is distinguished by being highly voltage-dependent. Hogg *et al.* (1994b) showed that the IC_{50} for inhibition of spontaneous $I_{Cl(Ca)}$ in rabbit portal vein myocytes was 300 μM at -50 mV and 90 μM at $+50$ mV. A-9-C also modified the decay kinetics of spontaneous $I_{Cl(Ca)}$ (Hogg *et al.*, 1993) suggesting that despite being chemically dissimilar to niflumic acid and DCDPC A-9-C may also act as an open channel blocker of $I_{Cl(Ca)}$.

The aim of the present study was to investigate the effect of A-9-C on $I_{Cl(Ca)}$ elicited by pipette solutions containing 500 nM Ca^{2+} in pulmonary artery myocytes to determine if the anomalous effects of NFA and DCDPC were unique to these antagonists. A voltage step protocol established in previous studies (Greenwood *et al.*, 2001; Piper *et al.*, 2002) was used to investigate the effect of A-9-C at different voltages.

Methods

Isolation of vascular myocytes

Cells were prepared from the main pulmonary artery isolated from New Zealand white rabbits (2–3 kg) that had been killed by an overdose of sodium pentobarbitone as approved under Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. After dissection and removal of connective tissue the artery was rubbed with a moist cotton bud to remove endothelial cells. The tissue was then cut into small strips and incubated in a physiological salt solution (PSS) containing 50 μM $CaCl_2$, 1 mg ml^{-1} papain, 0.15 mg ml^{-1} dithiothreitol and 2 mg ml^{-1} bovine serum albumin and stored overnight at 4°C. The next day the test tube containing the tissue in enzyme solution was placed in a water bath at 37°C for 5 min and cells were released by gentle agitation with a wide bore pasteur pipette. Cells were stored at 4°C and used within 6 h.

Electrophysiology

All currents were recorded in the whole cell voltage clamp mode using CED software and List amplifier (Heka Limited, Darmstadt, Germany). Analysis was performed using the applicable CED software as well as Origin (Microcal, Northampton, MA, U.S.A.). In most experiments $I_{Cl(Ca)}$ were evoked by pipette solutions containing 500 nM Ca^{2+} as this concentration of Ca^{2+} generates large and robust Cl^- currents in pulmonary artery smooth muscle cells (Greenwood *et al.*, 2001; Piper *et al.*, 2002). The pipette solution contained (mM): TEA-Cl 20; CsCl 106; HEPES 5; BAPTA 10; MgATP 3; $GTPNa_2$ 0.2; $MgCl_2$ 0.42 and pH was set to 7.2 by addition of CsOH. Free [Ca^{2+}] was set at 500 nM by the addition of 7.8 mM $CaCl_2$ determined by the EQCAL buffer program. In the experiments where different $[Ca^{2+}]_i$ were used the pipette solution was enriched by 2.1 mM, 4.2 mM and 8.8 mM $CaCl_2$ to give final $[Ca^{2+}]_i$ of 20 nM, 100 nM and 1 μM, respectively. The external solution contained (mM): NaCl 126; HEPES 10; pH 7.4, glucose 11; $CaCl_2$ 1.8; $MgCl_2$ 1.2; TEA-Cl 10 and 4-aminopyridine 5. All enzymes and anthracene-9-carboxylic acid (A-9-C) were purchased from Sigma Chemical Company (Poole, Dorset, U.K.).

Voltage step protocol

The voltage step protocol used in these experiments was adapted from previous studies using non-smooth muscle cell types (Arreola *et al.*, 1996; Nilius *et al.*, 1997) and has been used in two previous studies of $I_{Cl(Ca)}$ in smooth muscle cells (Greenwood *et al.*, 2001; Piper *et al.*, 2002). Cells were held at a holding potential of -50 mV and the voltage-dependent properties of $I_{Cl(Ca)}$ were studied by depolarizing initially to $+70$ mV for 1.5 s. This resulted in the generation of an instantaneous outward current followed by a relaxation of the outward current to a higher level that was described adequately by a single exponential function and has been proposed to be due to a change in the binding affinity of the channel for Ca^{2+}, as well as an alteration in the channel open time equilibrium (see Arreola *et al.*, 1996 for full discussion). Following the depolarizing pulse the cell was then stepped to -80 mV for 750 ms. This resulted in an exponentially declining inward current due to the closure of the channels opened by the depolarizing pulse. The different properties of they currents elicited by this protocol are highlighted in Figure 1A. In the experiments on the Ca^{2+}-dependence of the effects of A-9-C the standard protocol was modified slightly with cells being depolarized to $+90$ mV from a holding potential of -50 mV to account for the smaller currents evoked by the lower [Ca^{2+}]. The voltage-dependence of the open channels was investigated by stepping the cell to different test potentials between -100 mV and $+60$ mV after the initial depolarizing step (Arreola *et al.*, 1996; Greenwood *et al.*, 2001). This protocol also allowed the voltage dependence of current decay to be assessed. In experiments where the reversal potential of the evoked current was determined the anion equilibrium potential was shifted to more negative potentials by replacement of the extracellular NaCl by NaSCN (e.g. Amédée *et al.*, 1990; Greenwood & Large, 1999; Greenwood *et al.*, 2001). Junction potentials were minimized by the use of a KCl containing agar bridge between bath and reference electrode.

Statistical analysis

In the text data are expressed as mean $\pm$ standard error of the mean. Data were compared by two-tailed, paired or unpaired Student's *t*-tests as appropriate (applied using Microsoft Excel software).

Results

Dialysis of pulmonary artery smooth muscle cells with a K^+ free pipette solution containing 500 nM Ca^{2+} evoked a stable inward current at the holding potential of -50 mV (I_{hold}) that had a mean steady state amplitude of -52 ± 12 pA ($n=12$). This current has been shown by previous studies (Greenwood *et al.*, 2001; Piper *et al.*, 2002) to be a Cl^- current that is activated at physiological membrane potentials by intracellular calcium concentrations greater than 100 nM. Similar to the previous reports, $I_{Cl(Ca)}$ elicited in the present study exhibited time- and voltage-dependent properties that were revealed by depolarisation from -50 mV to $+70$ mV (see Figure 1A). Stepping to $+70$ mV produced an instantaneous current ($I_{inst+70\ mV}$) that was followed by the development of an outward current during the voltage step

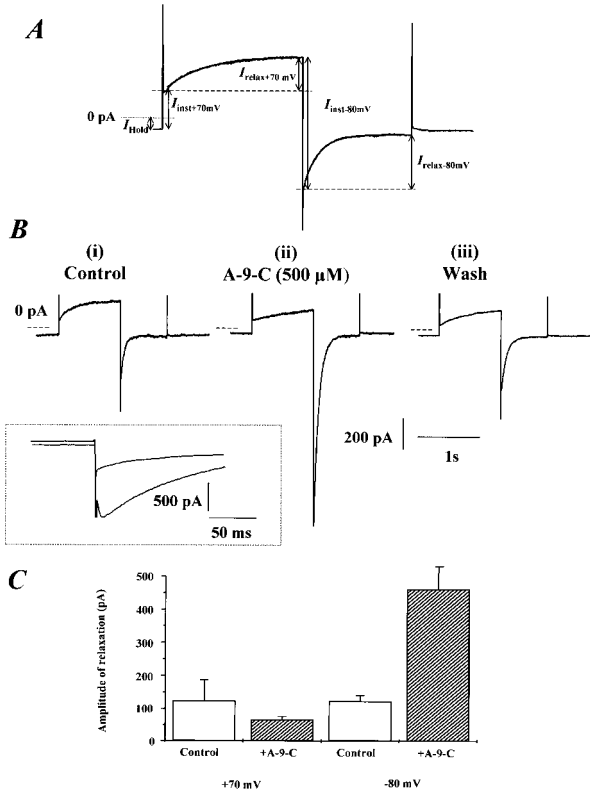

Figure 1 Effect of A-9-C on $I_{Cl(Ca)}$ stimulated by 500 nM $[Ca^{2+}]_i$. (A) shows typical whole-cell current recorded from a rabbit pulmonary artery myocyte at a holding potential of −50 mV. The arrows illustrate the current measurements discussed in the text: instantaneous current at +70 mV ($I_{inst+70\ mV}$), outward current relaxation at +70 mV ($I_{relax+70\ mV}$), instantaneous current on stepping to −80 mV ($I_{inst-80\ mV}$) and the inward current relaxation at −80 mV ($I_{relax-80\ mV}$). (B) recording of current from a second pulmonary artery cell before (i), during the application of A-9-C (500 μM; ii) and after A-9-C was removed (iii). A section of traces Bi and Bii is shown alongside on an expanded scale (enclosed by the box); the dotted line represents zero current. (C) plot of amplitude of current relaxation at +70 mV or −80 mV in the absence (open; $n=12$), or presence of A-9-C (hatched; $n=12$). Columns represent mean data±s.e.mean, downward error bars have been omitted for clarity.

($I_{relax+70\ mV}$). Upon repolarization to −80 mV, a large instantaneous current ($I_{inst-80\ mV}$) was recorded that declined over the course of the step ($I_{relax-80\ mV}$). After an initial period of stabilization the amplitude of the current at −50 mV and the kinetics of the voltage-dependent relaxations were reproducible for the duration of the experiment under control conditions.

A-9-C inhibits spontaneous Cl^- currents at −50 mV with an IC_{50} of 300 μM (Hogg *et al*., 1994b). In comparison I_{hold} at −50 mV evoked by 500 nM Ca^{2+} was only reduced from −52±13 pA to −43±11 pA ($n=12$; $P<0.01$, paired *t*-test) by the application of 500 μM A-9-C. Figure 1B shows that there was a greater effect of 500 μM A-9-C on the current developed at +70 mV with the mean outward relaxation ($I_{relax+70\ mV}$) decreasing from 123±63 pA to 66±10 pA ($P<0.01$, paired *t*-test; Figure 1C). Even more strikingly, Figure 1B shows that A-9-C markedly augmented the amplitude of the inward current recorded upon repolarization to −80 mV. The mean amplitude of the inward 'tail' current at −80 mV ($I_{relax-80\ mV}$) was −122±19 pA and −461±65 pA (Figure 1C; $P<0.01$, paired *t*-test) in the absence and presence of 500 μM A-9-C, respectively ($n=12$). In the presence of A-9-C there was an initial growth phase to the inward current recorded upon repolarization to −80 mV (see inset in Figure 1B) that was never present under control conditions and was not observed in our previous study when NFA was applied (Piper *et al*., 2002). These data show that A-9-C is a relatively ineffective blocker of $I_{Cl(Ca)}$ evoked by a sustained increase in $[Ca^{2+}]_i$ at negative potentials. In comparison to NFA, which effectively abolished voltage-dependent transitions concomitant with a drastic slowing of the current decay at −80 mV, A-9-C enhanced the amplitude of the current relaxation at −80 mV but had no significant effect on the kinetics of the current decay (mean time constant for the exponential decay at −80 mV was 68±5 ms and 79±6 ms in the absence and presence of A-9-C, respectively). However, there was a marked prolongation of the tail current decay at less negative potentials (e.g. control mean time constant at −20 mV was 81±14 ms compared to 172±15 ms in 500 μM A-9-C; $P<0.01$, paired *t*-test). The effect of A-9-C at all potentials was readily reversible and current levels returned gradually to control levels with progressive washout of A-9-C (see Figure 1B).

To investigate the effect of A-9-C further we studied the concentration-dependence of the A-9-C effects. Figure 2 shows an example of an experiment where concentrations of A-9-C between 10 μM and 500 μM were applied to a pulmonary artery cell. It was noted that with concentrations of A-9-C between 30 and 100 μM potentiation of inward current on stepping to −80 mV was apparent, while there was no significant effect on outward current at +70 mV (Figure 2A) until the concentration of A-9-C was raised to 300–500 μM. The mean data from four such experiments is shown in Figure 2B, which highlights the difference in concentration required to produce potentiation of the inward current at −80 mV compared to the inhibitory effect at +70 mV.

Is the current in the presence of A-9-C generated by the same channel as the control current?

We have characterized extensively the control current elicited by pipette solutions of known $[Ca^{2+}]_i$ in pulmonary artery cells (Greenwood *et al*., 2001). Moreover, we have shown that the current recorded in the presence of NFA reverses at the theoretical Cl^- equilibrium potential (E_{Cl}) and is shifted to significantly more negative potentials when the extracellular Cl^- is replaced by the more permeant anion, thiocyanate (Piper *et al*., 2002). We performed similar experiments to confirm that the current recorded in the presence of A-9-C was carried by the same channels as the control current. Figure 3A shows families of currents evoked by a two-pulse protocol under control conditions. The instantaneous current recorded upon stepping to the different test potentials following the initial pulse to +70 mV reversed closed to E_{Cl}. Bathing the cell in NaSCN shifted the reversal potential from a mean of 4±4 pA to −51±3 pA ($n=6$) consistent with previous studies (Greenwood *et al*., 2001; Piper *et al*., 2002). It can also be seen from Figure 3A that substitution of the external Cl^- for SCN^- prolonged the decay of the inward currents and this is consistent with the modulatory effect of external anions on this channel in smooth muscle cells

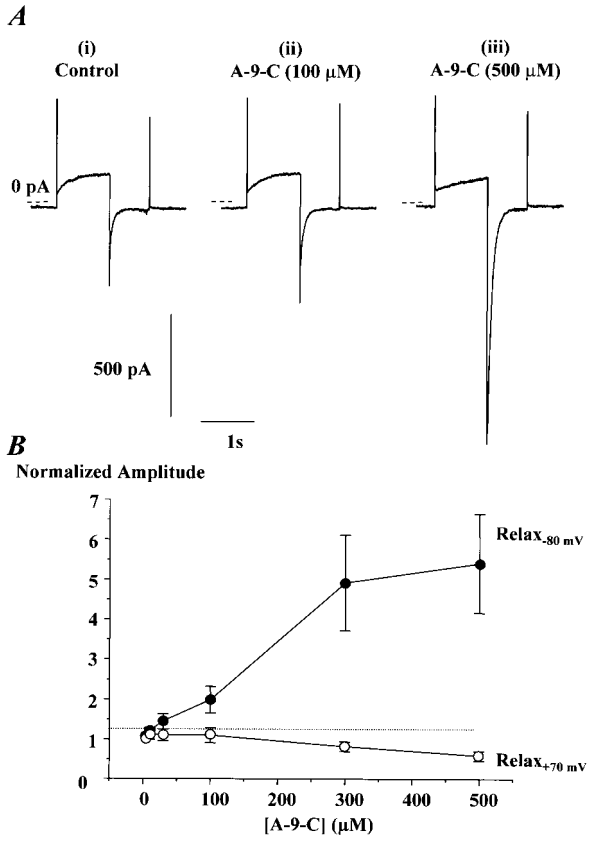

Figure 2 Concentration-effect curve for the effect of A-9-C (10–500 μM) on $I_{Cl(Ca)}$ stimulated by 500 nM $[Ca^{2+}]_i$. (A) shows examples of currents recorded from a single rabbit pulmonary artery myocyte in the absence and presence of increasing concentrations of A-9-C (100 μM and 500 μM). The holding potential was -50 mV, and the cell was stepped to $+70$ mV, then -80 mV at 20 s intervals. (B) current relaxations normalized to current in the absence of A-9-C at $+70$ mV and -80 mV plotted against concentration of A-9-C in μM. Points are mean$\pm$s.e.mean, $n=4$.

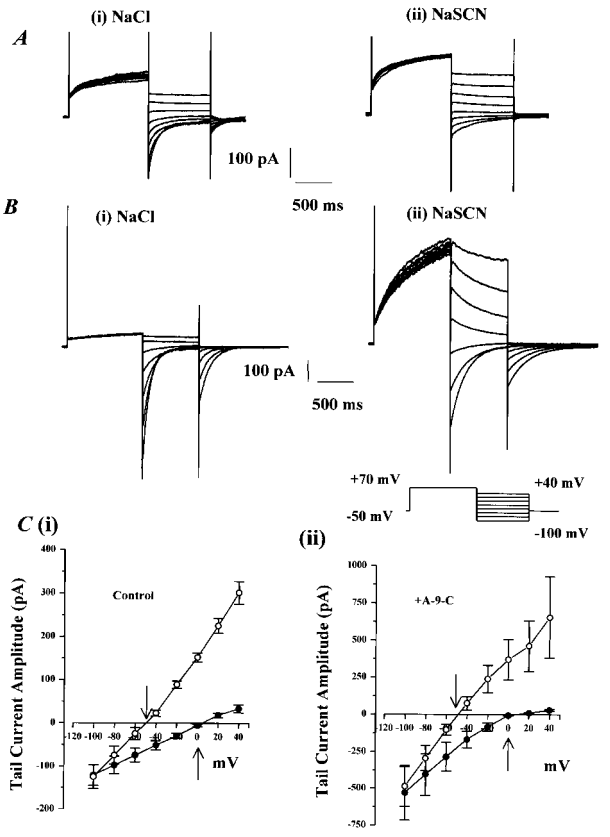

Figure 3 Reversal potential of $I_{Cl(Ca)}$ in the absence and presence of A-9-C. The reversal potential of currents evoked by 500 nM Ca^{2+} was determined using a two-step protocol. Cells were held at -50 mV and stepped $+70$ mV for 1.5 s followed by a 750 ms test step to potentials between -100 mV and $+40$ mV. Panels A and B show representative ensemble of currents elicited by this protocol from cells bathed in an external solution containing NaCl (i) or NaSCN (ii) in the absence (A) or presence (B) of 500 μM A-9-C. Panel C shows the mean current-voltage relationship of the current recorded immediately after stepping to the test step ($n=5-8$) in the absence (Ci) or presence (Cii) of 500 μM A-9-C. Filled symbols represent currents recorded in an external solution containing NaCl and filled symbols shows currents recorded with external NaSCN. Arrows show the reversal potential for each external solution.

(Greenwood & Large, 1999) and also in *Xenopus* oocytes (Qu & Hartzell, 2000). In the presence of 500 μM A-9-C the reversal potential with NaCl in the external solution was close to E_{Cl} (Figure 3B). Substitution of the external Cl^- with SCN^- produced a similar shift in reversal potential (from -2 ± 2 mV to -45 ± 2 mV, $n=5$) as the control current. The presence of SCN^- in the external solution also prolonged the decay of the augmented inward current recorded in the presence of A-9-C (Figure 3B). For example the time constant for the current decay at -80 mV was increased from 79 ± 6 ms to 203 ± 39 ms in SCN^- ($P<0.01$, paired t-test; $n=5$) which was similar to the increase in control values at this potential (68 ± 5 ms to 206 ± 17 ms; $P<0.01$, paired t-test; $n=6$). These data confirm that the current recorded in the presence of A-9-C is produced by activation of the same channels as the control currents.

Are the effects of A-9-C and niflumic acid through a common mechanism?

Experiments were conducted to determine if the effects of A-9-C on $I_{Cl(Ca)}$ activated by pipette solutions containing 500 nM Ca^{2+} were through a similar mechanism to niflumic acid (NFA). In these series of experiments application of 500 μM A-9-C increased the amplitude of the relaxation at -80 mV from -192 ± 22 pA to -516 ± 85 pA ($n=7$) and this was associated with a small inhibition of the outward relaxation at $+70$ mV (see Figure 4). When 100 μM NFA was applied in the continued presence of A-9-C the augmented inward current at -80 mV was rapidly inhibited (Figure 4A,B) and the mean amplitude of $I_{relax-80\ mV}$ was reduced to -62 ± 15 pA ($n=7$). These effects were similar to those produced by NFA alone on control currents where 100 μM NFA alone decreased the amplitude of $I_{relax-80\ mV}$ from 196 ± 46 pA to -68 ± 33 pA ($n=3$, see also Piper *et al.*, 2002) and markedly slowed the decay of this current. Washout of NFA in the continued presence of A-9-C caused the current to return to pre-application values, i.e. there was no further increase in current with NFA (washout phenomenon) as described by Piper *et al.* (2002). When the reverse experiment was performed i.e. 500 μM A-9-C was applied in the continued presence of 100 μM NFA the second agent

failed to significantly modify the effects of NFA that have been described previously (Piper *et al*., 2002). Thus, the amplitude of $I_{relax-80\ mV}$ in the presence of A-9-C and NFA was -72 ± 33 pA ($n=3$). These data show that A-9-C and NFA act through a similar mechanism and the effects of NFA appear to predominate. Interestingly close examination of the currents recorded in the continued presence of A-9-C with initial applications of NFA revealed the dominant effect of NFA. Figure 4C shows clearly that the normal mono-exponential decay of the augmented current at -80 mV recorded in the presence of A-9-C only (mean time constant was 55 ± 2 ms, $n=4$) became biphasic (Figure 4C) in the early phases of NFA blockade. Thus, the current recorded in the presence of A-9-C after 30 s application of NFA was well fitted by two exponential with mean values of τ_{fast} and τ_{slow} of 9 ± 0.6 ms and 255 ± 23 ms, respectively.

Investigation into the potentiatory effect of A-9-C at −80 mV

Experiments were conducted to determine if the marked augmentation of the current amplitude at -80 mV by A-9-C was due to a direct enhancement of the current at that potential or if prior block of the current at $+70$ mV was necessary. If the former were to be true then A-9-C should augment the conductance at -80 mV without prior depolarisation. It can be seen from Figure 5 that this is not the case. When pulmonary artery myocytes were stepped to -80 mV from -50 mV A-9-C had no effect on the amplitude of $I_{relax-80\ mV}$. Mean data are summarised in Figure 5B. However, upon stepping from -80 mV to $+70$ mV i.e. the reverse of the protocol used previously (see Figure 1) A-9-C produced a small inhibition of the current at $+70$ mV ($52\pm11\%$, $n=8$; Figure 5B). Moreover, the instantaneous inward current observed as the cell was repolarized from $+70$ mV to the holding potential of -50 mV was enhanced by A-9-C (Figure 5). These data suggest that for A-9-C to augment the current amplitude at negative potentials the cell must first be depolarised so that channel block can occur.

Effect of A-9-C on Cl- currents evoked by different $[Ca^{2+}]_i$

A-9-C may augment the Cl^- channel by increasing the sensitivity of the channel protein to Ca^{2+}. To investigate this

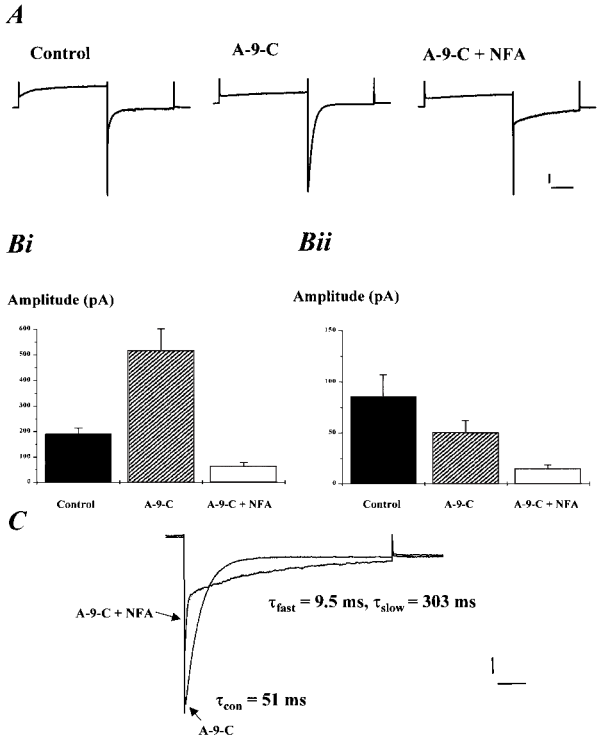

Figure 4 Effect of NFA applied in the continued presence of A-9-C. Panel A shows representative currents evoked by 500 nM Ca^{2+} using the standard voltage protocol. Currents were recorded in the absence of any Cl^- channel blocker (control), after 2 min application of 500 μM A-9-C and after the application of 100 μM NFA in the continued presence of A-9-C. Scale bars represent 100 pA and 250 ms. Panel B shows the mean data for the amplitude of the relaxation at -80 mV (Bi) and $+70$ mV (Bii) in the absence of any agent (filled box), in the presence of A-9-C alone (hatch) and in the presence of A-9-C plus NFA (open box). Panel C shows an expanded representative current recorded at -80 mV in the presence of A-9-C and after 30 s application of NFA in the continued presence of A-9-C. Mono- and bi-exponential fits to the current decay are shown as dotted lines overlaying the data. Scale bars represent 100 pA and 100 ms.

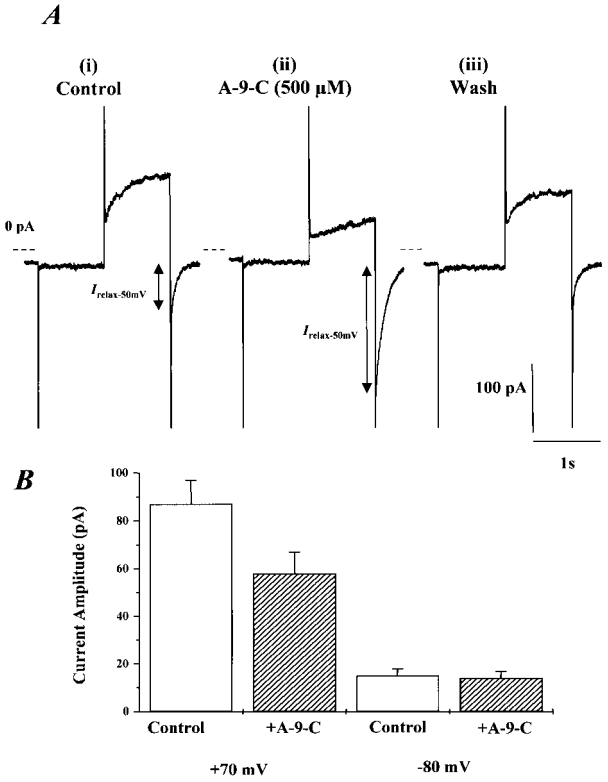

Figure 5 Augmentation of the current amplitude at -80 mV requires prior depolarization. Panel A shows representative currents evoked by stepping a cell held at -50 mV to -80 mV for 1.5 s followed by a step to $+70$ mV for 750 ms (the reverse protocol of Figures 1 and 2). Currents were recorded in the absence (Ai) and presence (Aii) of 500 μM A-9-C and following 3 min washout of A-9-C (Aiii). The inward relaxation recorded at -50 mV following the depolarization to $+70$ mV from -80 mV is highlighted. Panel B shows the mean data from five experiments showing the amplitude of current relaxation at $+70$ mV ($I_{relax+70\ mV}$) and -80 mV ($I_{relax-80\ mV}$) in the absence (open bars) and presence (hatched bars) of 500 μM A-9-C.

hypothesis we repeated the experiment shown in Figure 1 using pipette solutions containing 20 nM, 100 nM or 1 μM Ca^{2+}. When the pipette solution contained 20 nM no Cl^- current was recorded at the holding potential (mean amplitude at -50 mV was -15 ± 6 pA, $n=4$) and depolarization to potentials as positive as $+150$ mV did not elicit an outward current relaxation. 500 μM A-9-C had no effect in these cells. Pipette solutions containing 100 nM Ca^{2+} also did not activate a significant Cl^- current at -50 mV (mean amplitude $=-10\pm2$ pA, $n=9$) but upon stepping to $+90$ mV a small, yet distinct outward relaxation (mean amplitude was 23 ± 6 pA) was detected (see Figure 6i). Upon repolarization to -80 mV an inward, deactivating tail current was observed. This current had a mean amplitude of -72 ± 18 pA and could be fitted by a single exponential with a mean τ value of 36 ± 5 ms. Rupture of the cell membrane with 1 μM Ca^{2+} in the pipette solution produced a massive (>500 pA) initial increase in holding current that decayed rapidly to reach a sustained steady-state level of -50 ± 9 pA ($n=12$). This rundown phenomenon was not affected by bathing the cells in A-9-C before whole cell access had been achieved ($n=3$). Depolarization to $+90$ mV yielded a prominent outward relaxation (Figure 6ii, mean amplitude $=152\pm25$ pA) that was followed upon repolarization to -80 mV by a large inward current (mean amplitude $=-273\pm23$ pA). The control currents evoked by 1 μM Ca^{2+} were significantly ($P<0.01$) larger than those elicited by 100 nM Ca^{2+} at both the holding and test potentials. Nevertheless A-9-C enhanced the amplitude of $I_{relax-80\ mV}$ and inhibited the outward relaxation at $+90$ mV elicited by 100 nM Ca^{2+} and 1 μM Ca^{2+} to the same extent (Figure 6B). Furthermore, in cells dialysed with 1 μM Ca^{2+} the degree of current enhancement produced by A-9-C was identical whether applied immediately after whole cell access (i.e. when the current amplitude was very large) or after the current had rundown to smaller levels. Hence the potentiatory effects of A-9-C was not proportional to the degree of current activation. These observations suggest that A-9-C does not affecting the Ca^{2+}-sensitivity of the underlying channel.

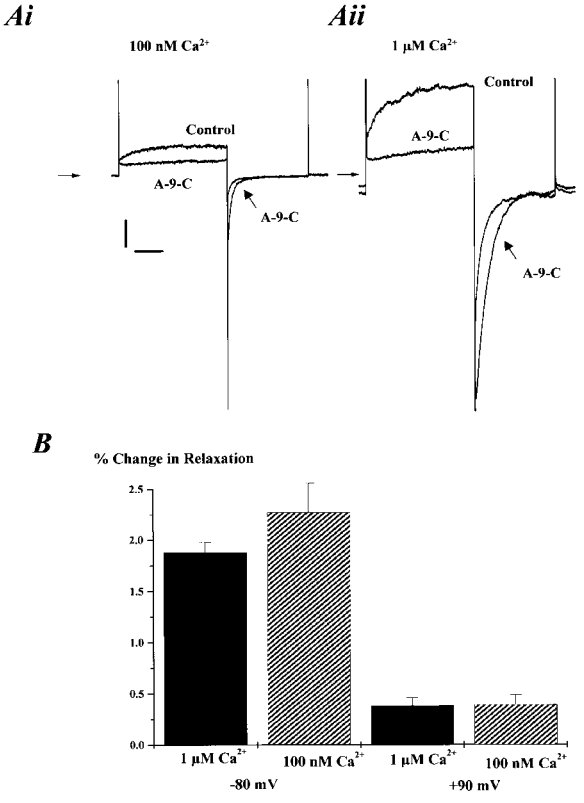

Figure 6 Effect of A-9-C on currents evoked by different $[Ca^{2+}]$. Panel A shows representative currents evoked by pipette solutions containing 100 nM Ca^{2+} (Ai) or 1 μM Ca^{2+} (Aii) in the absence (control) and in the presence of 500 μM-A-9-C (A-9-C). Voltage-dependent properties were studied by depolarizing from the holding potential of -50 mV to $+90$ mV for 750 ms followed by a repolarizing step to -80 mV. Scale bars represent 50 pA and 250 ms. Panel B shows the mean data for the effect of 500 μM A-9-C on the amplitude of the current relaxations at $+90$ mV and -80 mV. Each bar is the mean of 9–12 experiments with error bars representing the s.e.mean.

Discussion

The present study shows that A-9-C is a relatively ineffective blocker of $I_{Cl(Ca)}$ when activated by a sustained level of $[Ca^{2+}]_i$. A-9-C had no significant effect on the amplitude of the holding current at -50 mV but reduced the amplitude of the outward current at $+70$ mV and increased the amplitude of the subsequent $I_{Cl(Ca)}$ on stepping to -80 mV by approximately 300%. A-9-C had no significant effect on the decline of the current at -80 mV but slowed the decay significantly at less negative potentials. The effects of A-9-C were readily reversible upon washout of the agent and were not dependent on the concentration of Ca^{2+} used to generate the Cl^- current.

Voltage-dependence of Cl^- channel blockers

The inability of A-9-C to inhibit $I_{Cl(Ca)}$ activated by a sustained level of $[Ca^{2+}]_i$. was similar to a previous study on structurally dissimilar open channel blocking agents NFA and DCDPC (Piper *et al*., 2002). It has been proposed that these agents produce a simultaneous block and enhancement of Ca^{2+}-activated Cl^- channels (Piper *et al*., 2002). Thus, NFA and DCDPC reduced the amplitude of voltage-dependent current relaxations recorded on stepping to $+70$ mV and augmented $I_{Cl(Ca)}$ at the holding potential of -50 mV. In addition, washout of NFA and DCDPC was associated with a pronounced increase in instantaneous current amplitude at all potentials (Piper *et al*., 2002). The differences between A-9-C and NFA probably reflect the more marked voltage-dependent association of A-9-C with the channel protein highlighted in earlier studies on $I_{Cl(Ca)}$ (Hogg *et al*., 1993; 1994b; Qu & Hartzell, 2001). At the holding potential A-9-C binding to the channel is low but when the cell is depolarised then A-9-C associates with the channel probably within the pore resulting in an inhibition of outward Cl^- currents. Upon repolarization to -80 mV A-9-C unbinds relatively rapidly from the channel producing a short phase of current growth as A-9-C leaves the channel that is followed by channel closure resulting in a 'hooked' appearance of the inward current. The decay of the inward current at -80 mV is then comparable to control currents as

without A-9-C being bound to the channel this process is determined solely by the inherent channel kinetics. A-9-C also produced small changes in the decay of spontaneous $I_{Cl(Ca)}$ at −50 mV in rabbit portal vein myocytes (Hogg *et al*., 1993). In comparison, NFA and DCDPC are less voltage-dependent inhibitors of spontaneous $I_{Cl(Ca)}$ and at negative voltages the drugs associate freely with the channel and the net effect of these agents binding at −50 mV is a small increase in the resting current (see Piper *et al*., 2002). The slow decay of the inward tail current upon repolarization to −80 mV from +70 mV produced by these agents is due probably to rapid binding and unbinding consistent with agents that produce flickery blockade (Hille, 2001; Hogg *et al*., 1993; 1994b).

Cl^- channel blockers stimulate the channel

The accepted observation that NFA and A-9-C inhibit the Cl^- channel is supported by the reduction in the amplitude of outward currents by these agents in the present study and Piper *et al*. (2002). However, the data of the present study and Piper *et al*. (2002) suggest that there is also a contemporaneous stimulation of these channels. This stimulatory effect is revealed with this experimental model when the drug is washed out (in the case of NFA or DCDPC) or the drug dissociates from the channel within the time-scale of the protocol (in the case of A-9-C). As the potentiation of $I_{Cl(Ca)}$ by A-9-C required that the cell was first held at positive potentials where channel block occurred these results suggest that there may be a link between open channel block of $I_{Cl(Ca)}$ and the potentiation of current amplitude. Moreover, the effects of A-9-C and NFA appear to be mediated through the same binding site (or sites, see later) with the effects of NFA dominating when the drugs are presented together because of A-9-C's weaker affinity for the channel. The observation that DIDS, a non-open channel blocker of $I_{Cl(Ca)}$, did not augment currents evoked by 500 nM Ca^{2+} (Piper *et al*., 2002) supports a link between channel stimulation and an intra-pore inhibitory site. Moreover, NFA and DCDPC always inhibited outward current but not inward current flow (Piper *et al*., 2002). This situation may be analogous to the effects of internal tetraethyl ammonium on K^+ channels that are due to an interaction of the blocker with a binding site within the conduction pathway (Hille, 2001).

Whilst the effects of blockade and stimulation are linked implicitly there is no clear evidence to discern between a single or multiple binding sites being responsible for the two opposing effects. A single binding site would require that a perturbation of channel structure that leads to channel stimulation outlast the simple effect of blocking the pore. The observation that the effects of A-9-C and NFA are not additive supports a single site model with the NFA having a greater dwell time at the site. A two site model would require that the inhibitory site has a weaker affinity for NFA and A-9-C than the stimulatory site. Evidence for the latter hypothesis is provided by the observation in the present study that enhancement of the instantaneous current recorded on stepping to −80 mV occurred at lower concentrations of A-9-C than are required to produce block at +70 mV. There is a caveat to this observation in that the degree of inhibition at +70 mV produced by A-9-C may not be a true reflection of the extent of block if the effect of A-9-C at this potential is the product of simultaneous channel block and channel stimulation.

Mechanism underlying the modulatory effect of A-9-C

Until the channel that generates $I_{Cl(Ca)}$ is cloned and the structure is determined the mechanism by which Cl^- channel blockers stimulate $I_{Cl(Ca)}$ can only be speculated. However, the data in the present study suggest that Cl^- channel blockers stimulate directly the Cl^- channel protein and this effect is more obvious when the channel is activated by a sustained increase in $[Ca^{2+}]_i$. Previous studies have shown that blockers of $I_{Cl(Ca)}$ activate large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) at higher concentrations than required to inhibit $I_{Cl(Ca)}$ (Ottolia & Toro, 1994; Greenwood & Large, 1995; Toma *et al*., 1996). Moreover, the stimulatory effect of NFA type compounds on BK_{Ca} in portal vein myocytes was inversely proportional to the $[Ca^{2+}]_i$ (Greenwood & Large, 1995). The data of the present study suggests that Cl^- channel blockers increase the probability of chloride channel opening by a mechanism similar to that with $I_{K(Ca)}$ i.e. a direct stimulation of the channel protein. As the degree of current enhancement at −80 mV produced by A-9-C was independent of $[Ca^{2+}]$ between 100 nM and 1 μM then an increase in Ca^{2+} sensitivity can be dismissed. However, the Cl^- channel has to be activated for the stimulatory effect to be observed as A-9-C failed to have any effect on cells dialysed with 20 nM Ca^{2+}. Consequently, Cl^- blocking agents simultaneously stimulate $I_{Cl(Ca)}$ as well as inhibit the conductance by a channel blocking mechanism.

An alternative explanation for the stimulatory actions of A-9-C is that this agent impairs an underlying inactivation of the Ca^{2+}-activated Cl^- channel. It has been shown that agents that block K^+ channels such as tetraethyl ammonium also slow channel inactivation (Choi *et al*., 1991). Recent studies have shown that Ca^{2+}-activated Cl^- channels in tracheal and pulmonary artery myocytes are suppressed by Ca-calmodulin-dependent phosphorylation (Wang & Kotlikoff, 1997; Greenwood *et al*., 2001). However, this process did not seem to be involved in the stimulatory effects of NFA in a previous study (Piper *et al*., 2002). Alternatively, the inactivation process may be a direct consequence of Ca^{2+} binding to the channel. Interestingly currents activated by 500 nM or 1 μM Ca^{2+} undergo a period of rundown following the initial rupture of the cell membrane (Greenwood *et al*., 2001; Piper *et al*., 2002). However, this marked rundown of the current activated by 1 μM Ca^{2+} was not affected by pre-incubation with A-9-C in the present study.

The data from the present study confirm previous observations that the inhibitory effect of Cl^- channel blockers is modified by a sustained level of $[Ca^{2+}]_i$. These observations are not simply epi-phenomenal as there is a marked variation in effectiveness of Cl^- channel blockers on $I_{Cl(Ca)}$ in different smooth muscle preparations. Moreover, Cl^- channel blockers are used to probe for a functional role for Cl^- channels in smooth muscle cells excitability. In the light of our recent studies on the effect of Cl^- channel blockers on $I_{Cl(Ca)}$ activated by a sustained increase in $[Ca^{2+}]_i$ it is a necessary caveat that a lack of effect of Cl^- channel blockers does not preclude a functional role of Cl^- channels. A recent study by Hirst *et al*. (2002) on spontaneous

excitatory potentials caused by the activation of $I_{Cl(Ca)}$ in the interstitial cells of the guinea-pig gastric antrum revealed that NFA increased the frequency and amplitude of the spontaneous discharge of regenerative potentials. Moreover, while A-9-C did inhibit spontaneous excitatory potentials upon washing out A-9-C the frequency of discharge of potentials was increased (Hirst *et al*., 2002). These data show that in multicellular preparations, as in single cells, certain Cl^- channel blockers may not simply inhibit Cl^- currents but instead may also have a potentiatory effect.

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(Received August 19, 2002
Accepted September 23, 2002)