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RESEARCH PAPER

Inhibition of vascular calcium-gated chloride currents by blockers of $K_{Ca}1.1$, but not by modulators of $K_{Ca}2.1$ or $K_{Ca}2.3$ channels

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Background and purpose: Recent pharmacological studies have proposed there is a high degree of similarity between calcium-activated Cl^- channels (CaCCs) and large conductance, calcium-gated K^+ channels ($K_{Ca}1.1$). The goal of the present study was to ascertain whether blockers of $K_{Ca}1.1$ inhibited calcium-activated Cl^- currents (I_{ClCa}) and if the pharmacological overlap between $K_{Ca}1.1$ and CaCCs extends to intermediate and small conductance, calcium-activated K^+ channels.
Experimental approaches: Whole-cell Cl^- and K^+ currents were recorded from murine portal vein myocytes using the whole-cell variant of the patch clamp technique. CaCC currents were evoked by pipette solutions containing 500 nM free [Ca^{2+}].
Key results: The selective $K_{Ca}1.1$ blocker paxilline (1 μM) inhibited I_{ClCa} by ~90%, whereas penitrem A (1 μM) and iberiotoxin (100 and 300 nM) reduced the amplitude of I_{ClCa} by ~20%, as well as slowing channel deactivation. Paxilline also abolished the stimulatory effect of niflumic acid on the CaCC. In contrast, an antibody against the Ca^{2+}-binding domain of murine $K_{Ca}1.1$ had no effect on I_{ClCa} while inhibiting spontaneous $K_{Ca}1.1$ currents. Structurally different modulators of small and intermediate conductance calcium-activated K^+ channels ($K_{Ca}2.1$ and $K_{Ca}2.3$), namely 1-EBIO, (100 μM); NS309, (1 μM); TRAM-34, (10 μM); UCL 1684, (1 μM) had no effect on I_{ClCa}.
Conclusions and implications: These data show that the selective $K_{Ca}1.1$ blockers also reduce I_{ClCa} considerably. However, the pharmacological overlap that exists between CaCCs and $K_{Ca}1.1$ does not extend to the calcium-binding domain or to other calcium-gated K^+ channels.
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Abbreviations: CaCCs, calcium-activated Cl^- channels; I_{ClCa}, calcium-activated Cl^- currents; PV, portal vein; STOCs, spontaneous transient outward currents

Introduction

In the absence of a protein identified with this function, research into the physiological role of calcium-activated chloride channels (CaCCs) has relied upon pharmacological tools. However, as Greenwood and Leblanc (2007) have pointed out, agents shown to block CaCCs also modulate a number of different ion channels, but with a consistent pharmacological cross-over between CaCCs and large conductance calcium-activated potassium channels (BK_{Ca} or $K_{Ca}1.1$; nomenclature follows Alexander *et al.*, 2008). Not only do a wide range of structurally disparate chloride channel blockers such as niflumic acid, anthracene-9-carboxylate and ethacrynic acid enhance $K_{Ca}1.1$ (Ottolia and Toro, 1994; Greenwood and Large, 1995; Toma *et al.*, 1996), but two structurally disparate activators of $K_{Ca}1.1$ channels, namely the benzimidazole NS1619 and the pimarene isopimaric acid (Olesen *et al.*, 1994; Holland *et al.*, 1996; Huang *et al.*, 1997; Imaizumi *et al.*, 2002) augment calcium-activated chloride currents (I_{ClCa}) in vascular myocytes at concentrations that enhance $K_{Ca}1.1$ channels (Saleh *et al.*, 2007). Interestingly, chloride channel blockers such as niflumic acid or anthracene-9 carboxylic acid not only block I_{ClCa}, but paradoxically augment these currents when the underlying channels are stimulated persistently (Piper *et al.*, 2002; Piper and Greenwood, 2003; Ledoux *et al.*, 2005). Taken together, these findings led to the speculation that both types of channel contain a stimulatory site with a common

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structural motif; the $K_{Ca}1.1$ protein comprises some aspect of the native CaCC (Greenwood and Leblanc, 2007) or tentatively that the interaction between CaCCs and $K_{Ca}1.1$ is so tightly linked structurally and functionally that the pharmacology of one directly affects the other.

Although the study by Saleh *et al.* (2007) showed that agents known to stimulate $K_{Ca}1.1$ channels produce a similar effect on CaCCs, it still remains to be determined if specific blockers of $K_{Ca}1.1$ channels exert a parallel influence on CaCCs, as our hypothesis of a structure–function relationship would imply. To substantiate this conceptual framework, we first examined the effects of compounds recognized to interact specifically with $K_{Ca}1.1$ at different binding sites, namely paxilline, penitrem A and iberiotoxin, on I_{ClCa} elicited by elevated clamped Ca^{2+} concentrations in murine vascular myocytes. Secondly, we determined if an antibody raised against an epitope located in the putative Ca^{2+}-binding domain of murine $K_{Ca}1.1$ (*mSlo* encoded) affects I_{ClCa}. Thirdly, we assessed whether these interactions are unique to $K_{Ca}1.1$ by exploring the effects of specific inhibitors of the other two major subfamilies of calcium-activated K^+ channels encoding for the small ($K_{Ca}2.1$) and intermediate ($K_{Ca}2.3$) conductance K_{Ca} channels. These studies revealed that the pharmacological overlap is restricted to CaCCs and $K_{Ca}1.1$, and to agents that interact with the channel pore.

Methods

Preparation of cells and solutions

All animal care and experimental procedures complied with the United Kingdom Animals Act (1986). BALB/c mice (6–8 weeks old) were killed by cervical dislocation in accordance with schedule 1. After opening the abdomen, the portal vein (PV) was removed and immediately placed in chilled physiological salt solution composed of 125 mM NaCl, 5.4 mM KCl, 15.4 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, 0.34 mM KH_2PO_4, 10 mM glucose, 11 mM HEPES and 0.1 mM $CaCl_2$ (pH was adjusted to 7.2 with NaOH). The PV was freed of fat and connective tissue, and then cut into longitudinal strips and individual smooth muscle myocytes isolated using a sequential protease and collagenase digestion procedure as described by Saleh and Greenwood (2005).

I_{ClCa} and I_{BKCa} were recorded using the whole-cell voltage clamp technique from single smooth muscle cells isolated, as described earlier, from murine hepatic PV. A small aliquot of smooth muscle cells stored in 0.1 mM $CaCl_2$ physiological salt solution was placed in a glass chamber on the stage of a XPC-T30I trinocular inverted microscope (Pyser SGI, Edenbridge, UK) and allowed to adhere for 15–20 min. To record I_{ClCa}, cells were superfused at a rate of 2 mL·min^{-1}, with an external solution composed of 126 mM NaCl, 11 mM glucose, 10 mM HEPES, 10 mM TEA-Cl, 1.2 mM $MgCl_2$ and 1.5 mM $CaCl_2$ (pH was adjusted to 7.2 with NaOH). An external solution comprising 136 mM NaCl, 5 mM KCl, 11 mM glucose, 10 mM HEPES, 1.2 mM $MgCl_2$ and 1.5 mM $CaCl_2$ was superfused when recording spontaneous transient outward currents (STOCs).

Electrophysiology

Recordings were performed using the whole-cell configuration of the patch clamp technique with an Axopatch-200B (Molecular Devices, Sunnyvale, CA, USA) patch clamp amplifier. Voltage clamp protocols were computer driven using a D/A and A/D acquisition system (Digidata 1322A board; Molecular Devices) and pClamp 8.2 software (Molecular Devices). Patch pipettes were manufactured from borosilicate glass and fire polished, giving pipettes with resistance of between 5 and 7 MΩ. Macroscopic I_{ClCa} was recorded using pipette solutions containing 106 mM CsCl, 20 mM TEA, 3 mM Na_2ATP, 0.2 mM GTP-Na, 10 mM HEPES, 10 mM BAPTA, 1.1 mM $MgCl_2$ and a sustained Cl^- channel activation evoked by a fixed free [Ca^{2+}] of 500 nM obtained through the addition of 7.8 mM $CaCl_2$ as calculated by EqCal (Biosoft, Ferguson, MO, USA; Greenwood *et al.*, 2001, 2004; Britton *et al.*, 2002; Piper *et al.*, 2002; Angermann *et al.*, 2006). The pH was adjusted to 7.2 with CsOH. Under these conditions, contribution from K^+ channels was negligible, and the membrane conductance was dominated by Cl^- channel activity. A number of different voltage clamp protocols were employed. A step every 20 s from −50 to +70 mV for 1 s followed by repolarization to −80 mV for 500 ms was used to ascertain when I_{ClCa} had stabilized, and to investigate the time-course of different drug effects. Due to a rundown in channel activity (see Angermann *et al.*, 2006), biophysical properties and drug effects were measured only after a stable response had been established ~5 min after patching. A current–voltage (I–V) relationship was constructed by stepping membrane potential every 15 s from −50 mV to voltages ranging from −80 to +120 mV for 1 s (20 mV increments). To determine the reversal potential of elicited currents, a two-step protocol was employed. Cells were initially depolarized from −50 to +80 mV for 1.5 s to activate channels and then stepped to a range of test potentials between −100 and +40 mV for 750 ms at 20 s intervals.

STOCs were recorded, using the whole-cell configuration of the patch clamp technique, at a holding potential of 0 mV (theoretical E_{Cl}) using a pipette solution comprising 126 mM KCl, 10 mM HEPES, 0.1 mM BAPTA and 1.2 mM $MgCl_2$. The pH was adjusted to 7.2 with KOH. To compensate for the variation in both amplitude and width of STOCs, mean peak amplitude and total area under the curve were obtained over a period of 1 min prior to application of iberiotoxin, and 1 min upon stabilization in the presence of iberiotoxin.

Statistical Analysis

All data shown are means ± SEM taken from at least three animals. Statistical tests were performed using either paired Student's *t*-test or analysis of variance, and current–voltage graphs were fit using least square regression to either a linear or Boltzmann sigmoidal equation.

Materials

Drugs were purchased from Sigma-Aldrich (Poole, UK) with the exception of iberiotoxin and anti-K_{Ca} 1.1 antibody, which were purchased from Alomone Labs (Jerusalem, Israel);

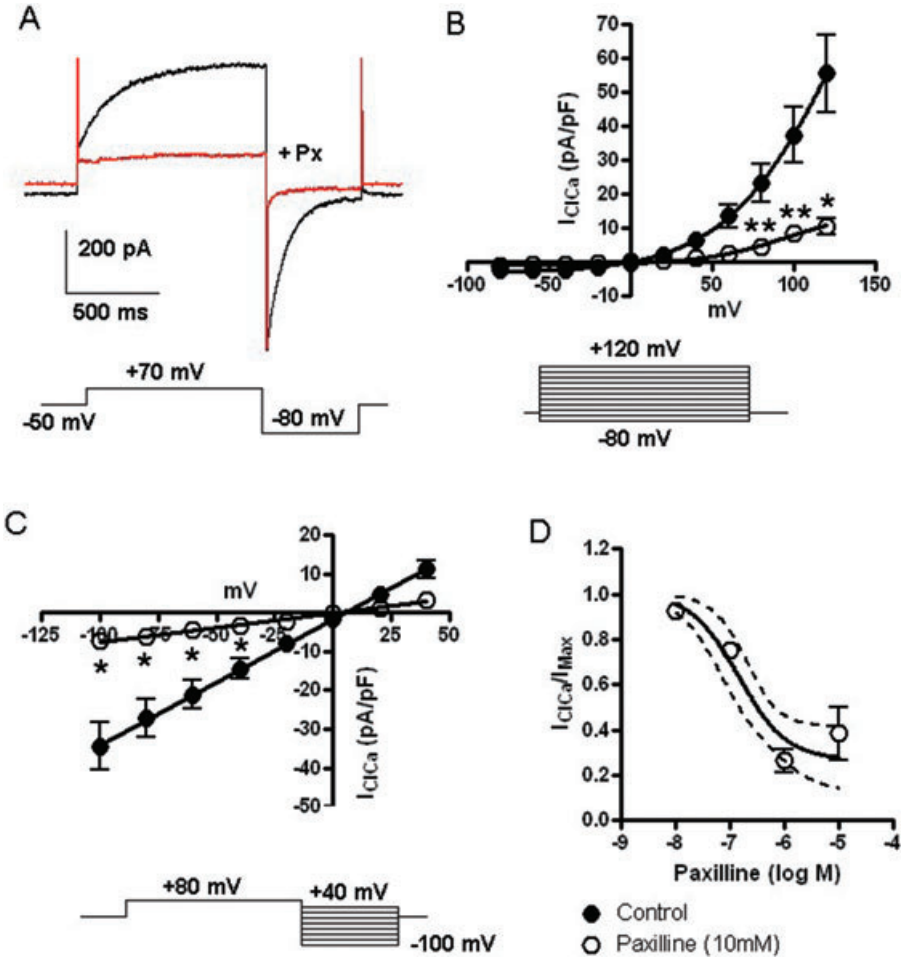

Figure 1 Effects of paxilline on I_{ClCa}. (A) A sample trace showing I_{ClCa} recorded in the absence and after 5 min application of 10 μM paxilline (Px). I_{ClCa} was evoked by step depolarization to +70 mV followed by repolarization to –80 mV. (B) The I–V relationship of the voltage and time-dependent current evoked by stepping to voltages ranging between –80 and +120 mV in the absence and presence of paxilline. (C) The reversal potential of the evoked current was not affected by paxilline; voltage protocol is shown below the graph. (D) The concentration–response relationship for paxilline inhibition of I_{ClCa} recorded at +70 mV. Each point is the mean data from at least four cells (bars showing SEM). *$P < 0.05$ and **$P < 0.01$ for paired Student's *t*-test comparisons between data acquired before and after application of paxilline.

UCL 1684 was purchased from Tocris (Bristol, UK), and 17β-oestradiol was purchased from Merck Chemicals Ltd. (Nottingham, UK).

Results

Native current in murine PV myocytes

Upon achieving the whole-cell configuration, I_{ClCa} evoked by 500 nM free Ca^{2+} exhibited rapid rundown until a stable level of current was achieved which remained constant for the duration of the experiment similar to I_{ClCa} in rabbit pulmonary artery myocytes (Angermann *et al.*, 2006; Saleh *et al.*, 2007). Under stable conditions, the mean inward whole-cell current evoked at –50 mV was –39 ± 7 pA ($n = 7$), and depolarization to +70 mV evoked an immediate outward current of 70 ± 9 pA, which increased over a period of 750 ms to 305 ± 50 pA (I_{Late}) with a mean time constant (τ_{act}) of 278 ± 31 ms. Repolarization to –80 mV evoked an inward current of 465 ± 80 pA (I_{-80mV}) with a τ_{close} of 75 ± 3 ms. These characteristics and the distinctive outward rectification were identical to that previously reported in the murine PV, rabbit pulmonary artery and rabbit coronary artery (Greenwood *et al.*, 2001; Britton *et al.*, 2002; Piper *et al.*, 2002; Saleh *et al.*, 2007).

Effects of $K_{Ca}1.1$ modulators on I_{ClCa}

Paxilline, penitrem A and iberiotoxin are all considered to be highly selective blockers of $K_{Ca}1.1$ channels. Experiments were undertaken to assess whether these agents could block I_{ClCa} in murine PV myocytes. As Figure 1 shows, application of 10 μM paxilline, a concentration in excess of that needed for total block of $K_{Ca}1.1$ channel (Sanchez and McManus, 1996; Li and Cheung, 1999), strongly inhibited a typical I_{ClCa} recorded at +70 mV and inward tail current at –80 mV (Figure 1A). The late outward current recorded at the holding potential of +70 mV decreased from +20.2 ± 4.1 pA·pF^{-1} to +4.1 ± 0.7 pA·pF^{-1} ($n = 4$, $P < 0.01$). Panel B shows mean I–V relationships for I_{ClCa} measured at the end of 1 s steps ranging from –80 to +120 mV that were obtained in the absence and presence of 10 μM paxilline. Paxilline blocked I_{ClCa} by more than 75% ($n = 5$) at all potentials tested. This pronounced inhibi-

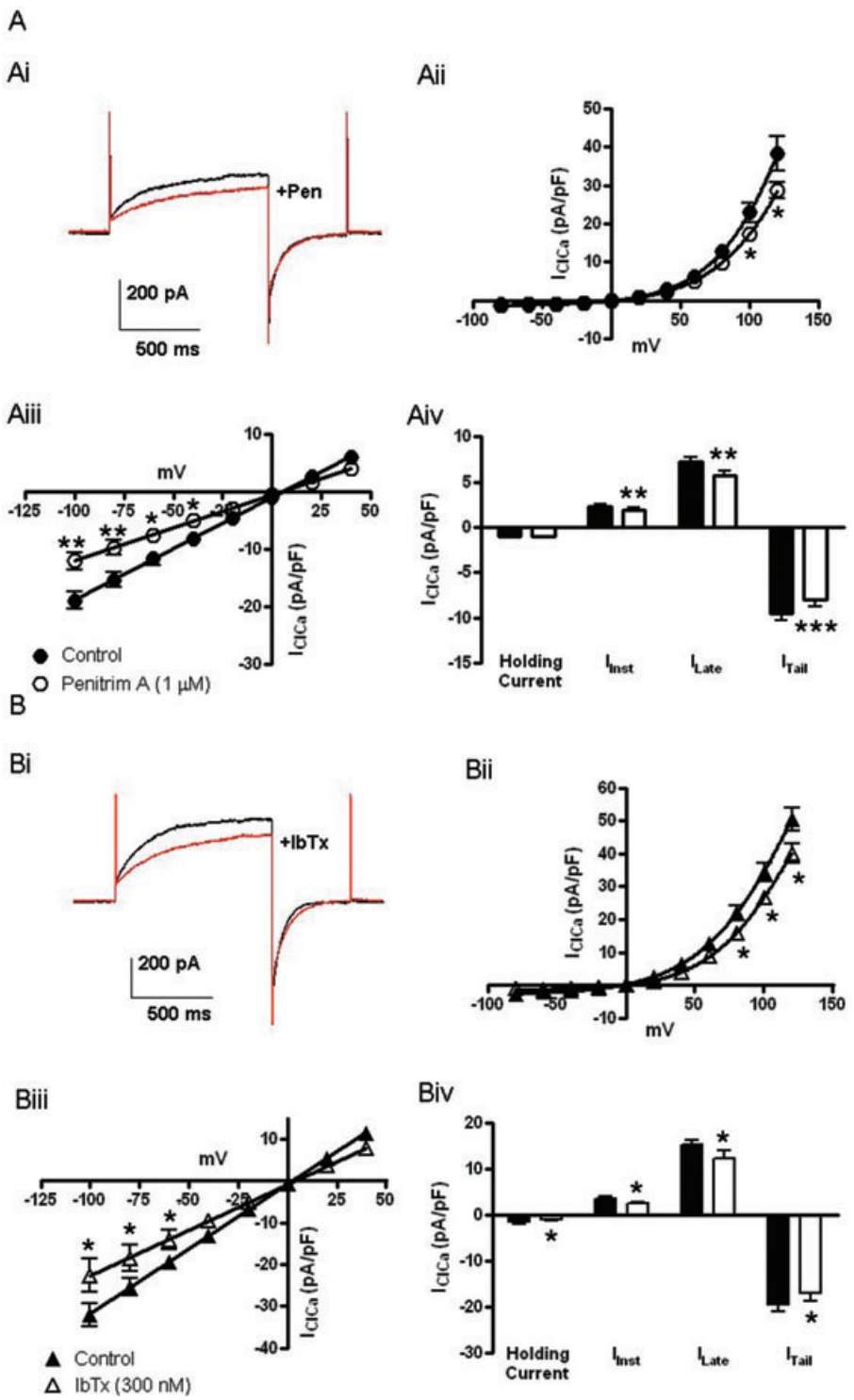

Figure 2 Effects of penitrem A and iberiotoxin on I_{ClCa}. (A) The effect of 10 μM penitrem A (Pen) on I_{ClCa}. (A)i shows representative currents recorded in the absence and presence of penitrem A (5 min, 1 μM). (A)ii and (A)iii show the effect of penitrem on I–V relationships and reversal potential of I_{ClCa}. (A)iv shows the effect of penitrem A at different voltages with the corresponding currents and amplitudes. (B) The same variables as in (A), but for 300 nM iberiotxin (IbTx). Each point or bar is the mean data from at least four cells (bars showing SEM). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for paired Student's *t*-test comparisons between data acquired before and after application of the channel modulator.

tion was not associated with a change in the reversal potential for the whole-cell current (Figure 1C). Construction of a paxilline concentration–response generated an IC_{50} of 147 nM with a 95% confidence interval ranging from 59 to 365 nM (Figure 1D). A second series of experiments were performed with a structurally dissimilar agent, penitrem A, and the potent peptide blocker iberiotoxin, which interacts with $K_{Ca}1.1$ channels at a site different from that binding paxilline

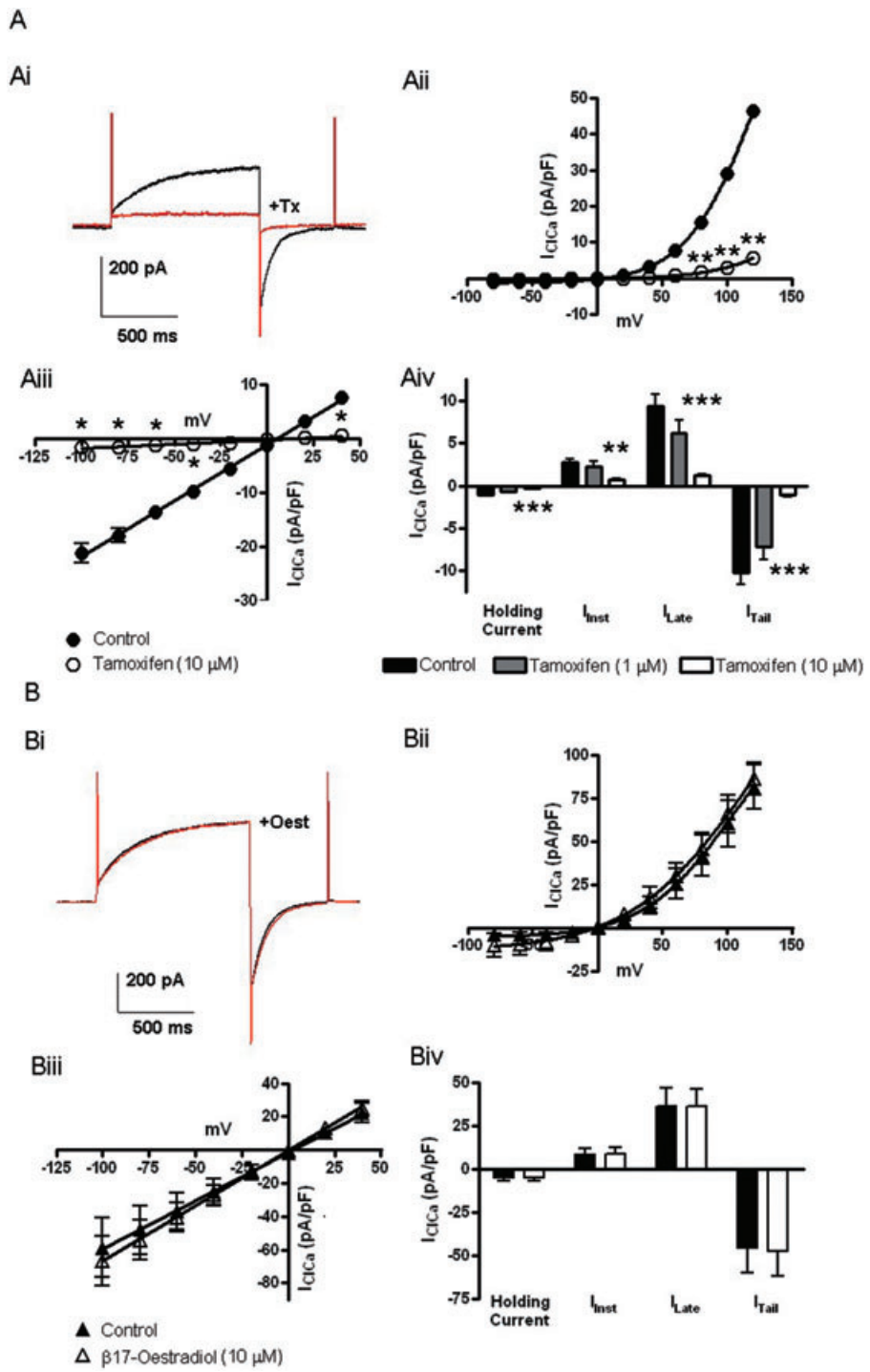

Figure 3 Effects of tamoxifen and 17β-oestradiol on I_{ClCa}. Modulation by tamoxifen (Tx, 10 μM; A) and 17β-oestradiol (Oest, 10 μM; B) of I_{ClCa}. (A)i and (B)i show sample traces, in the same cell, control I_{ClCa} and I_{ClCa} in the presence of the channel modulator, recorded at +70 mV followed by repolarizing to –80 mV. (A)ii and (B)ii show effects on the I–V relationships of the voltage and time-dependent current evoked by stepping to voltages ranging between –80 and +120 mV. In (A)iii and (B)iii, the current evoked immediately on repolarization to voltages ranging from –100 to +40 mV, is shown. The bar charts [(A)iv and (B)iv] detail different aspects of I_{ClCa} evoked by a depolarizing step to +70 mV followed by a repolarizing step to –80 mV; control currents and those evoked in the presence of modulator are shown. Each point or bar represents the mean data from at least four cells (bars showing SEM). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for paired Student's *t*-test comparisons between data acquired before and after application of the channel modulator.

(see Discussion). Penitrem A also inhibited I_{ClCa} (Figure 2A) when applied at a concentration greater than that required to produce maximal inhibition of the $K_{Ca}1.1$ current (Knaus *et al.*, 1994), but to a lesser degree than paxilline. Again, channel kinetics and reversal potential remained unchanged by the application of penitrem A. Application of 100 nM iberiotoxin produced a small inhibition of I_{ClCa} that was comparable to the effect of penitrem A (data not shown).

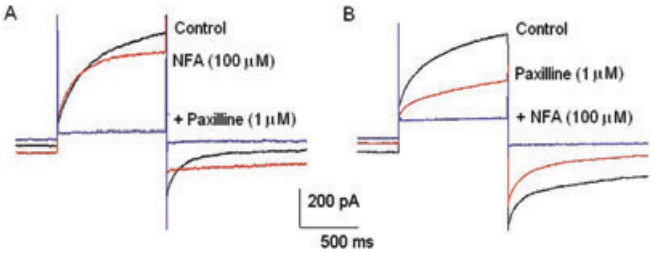

Figure 4 Co-application of niflumic acid and paxilline. (A) A sample trace showing in the same cell, inhibition of control I_{ClCa} by the initial application of niflumic acid (100 μM) followed by co-application of paxilline (1 μM). (B) Another sample trace demonstrating the reverse application; paxilline followed by co-application of niflumic acid. I_{ClCa} was evoked by step depolarization to +70 mV followed by repolarization to –80 mV.

However, iberiotoxin (100 nM) slowed the decay at –80 mV considerably (τ_{close} increased from 70.5 ± 6.8 to 97.5 ± 9.2 ms, $n = 6$, $P < 0.001$). Increasing the iberiotoxin to 300 nM produced a slightly greater inhibition (Figure 2B) and a similar slowing of channel closure at –80 mV (τ_{close} increased from 62 ± 6 ms to 85 ± 8 ms, $n = 6$, $P < 0.001$; Figure 2).

A final series of experiments were performed with tamoxifen, which blocks swelling-activated Cl^- currents (e.g. Greenwood & Large, 1998) but also modulates $K_{Ca}1.1$ channels. In the presence of auxillary β subunits of the $K_{Ca}1.1$ channel, tamoxifen augments channel activity, whereas this agent reduces the unitary conductance of the pore-forming subunit alone (Dick *et al.*, 2001). Application of 10 μM tamoxifen abolished I_{ClCa} (Figure 3A,i–iv). The inward current recorded at the holding potential of –50 mV was decreased ($n = 7$, $P < 0.001$; Figure 3A,iv), and the immediate outward current evoked by depolarization to +70 mV was also reduced ($P < 0.01$). Both the I time-dependent component of the outward current and inward tail current evoked by repolarization to –80 mV were markedly reduced ($P < 0.001$). The reversal potential did not change significantly (Figure 3A,iii). At a concentration of 1 μM, tamoxifen induced a slight reduction of evoked current, but this inhibition did not reach statistical significance (Figure 3A,iv). As tamoxifen is a partial agonist at oestrogen receptors, we studied the effect of the full agonist, 17β-oestradiol (10 μM), on I_{ClCa}. In contrast to the marked inhibitory effect of tamoxifen, 17β-oestradiol had no effect on I_{ClCa} in these cells ($n = 3$; Figure 3B,i). These data show that structurally dissimilar blockers of $K_{Ca}1.1$ channels also inhibit I_{ClCa}. It is worth emphasizing that none of the $K_{Ca}1.1$ blockers produced any stimulatory effects in contrast to the actions of chloride channel blockers such as niflumic acid on these currents (see Piper *et al.*, 2002; Piper and Greenwood, 2003; Ledoux *et al.*, 2005).

Combination of niflumic acid and paxilline produces marked inhibition of I_{ClCa}

Niflumic acid is a Cl^- channel blocker that produces a paradoxical stimulation of inward I_{ClCa} when the channel is activated persistently (Piper *et al.*, 2002; Ledoux *et al.*, 2005). As paxilline was an effective blocker of control I_{ClCa}, experiments were undertaken to determine whether the augmented currents produced by the application of 100 μM niflumic acid were also sensitive to this agent. As Figure 4 shows, 1 μM paxilline almost completely abolished I_{ClCa} recorded in the continued presence of niflumic acid with I_{late} reduced by 84 ± 4% ($n = 6$, $P < 0.001$). In contrast, application of 10 μM penitrem A in the continued presence of 100 μM niflumic acid induced no significant change on the stimulated current ($n = 4$; data not shown).

Effect of a $K_{Ca}1.1$ antibody

The experiments of the previous sections focused upon agents that block $K_{Ca}1.1$ channels through an interaction with the channel pore. The next series of experiments were undertaken to determine if an antibody targeted against the calcium-binding domain of the *mSlo*-encoded α subunit of the $K_{Ca}1.1$ channel could also affect I_{ClCa}. Initial experiments on STOCs, which are generated by the random activation of $K_{Ca}1.1$ channels by quanta of Ca^{2+} release from the sarcoplasmic reticulum (Benham and Bolton, 1986) were performed to confirm the inhibitory effect of the antibody on $K_{Ca}1.1$ activity. At a holding membrane potential of 0 mV, STOCs had a mean peak amplitude of 107 ± 5.8 pA ($n = 6$ cells), a mean area-under-curve of 7476 ± 531 pA.ms and showed stability over 10 min (Figure 5A). Application of iberiotoxin (10 nM) to cells not dialysed with a pipette solution containing antibody reduced mean peak amplitude by 57 ± 6% ($n = 3$, $P < 0.01$; Figure 5B,D) and area-under-curve over a 5 min period by 66 ± 11% ($P < 0.01$; Figure 5B,E). Intracellular dialysis of the anti-K_{Ca} 1.1 antibody (1:200) in the absence of iberiotoxin reduced STOC activity to the same degree as external application of iberiotoxin with the mean peak amplitude and area-under-curve decreasing by 55 ± 5 and 70 ± 2%, respectively ($P < 0.01$; $n = 3$, Figure 5C). Confirmation that the antibody was directed effectively against the $K_{Ca}1.1$ channel was provided by the lack of effect of iberiotoxin after intracellular dialysis of the $K_{Ca}1.1$ antibody in all cells dialysed with the antibody (data not shown). In contrast to these marked effects on STOCs, incorporation of the $K_{Ca}1.1$ antibody in the pipette solution had no effect on the I_{ClCa}. Figure 6A shows that the amplitude and kinetics of I_{ClCa} recorded from cells of similar capacitance in the presence and absence of the $K_{Ca}1.1$ antibody were identical (mean data is shown in Figure 6C). Figure 1B shows that the characteristic rundown of I_{ClCa} was not affected by intracellular dialysis with the $K_{Ca}1.1$ antibody. Consequently, the activation of the CaCC by calcium does not involve the same calcium-binding motif as $K_{Ca}1.1$ channels.

Effects of $K_{Ca}2.1$ and $K_{Ca}2.3$ modulators

Our experiments to date have highlighted a marked overlap in the pharmacology of $K_{Ca}1.1$ currents and I_{ClCa}, but it is not known whether this feature extends to other calcium-gated K^+ channels. We therefore embarked on a series of experiments to examine whether modulators of intermediate- and small-conductance calcium-activated potassium channels ($K_{Ca}2.3$ and $K_{Ca}2.1$, respectively) affected I_{ClCa}. We investigated the effects of the activators of $K_{Ca}2.3$ and $K_{Ca}2.1$, 1-EBIO and NS309 (Jensen *et al.*, 2001; Strøbaek *et al.*, 2004), at concen-

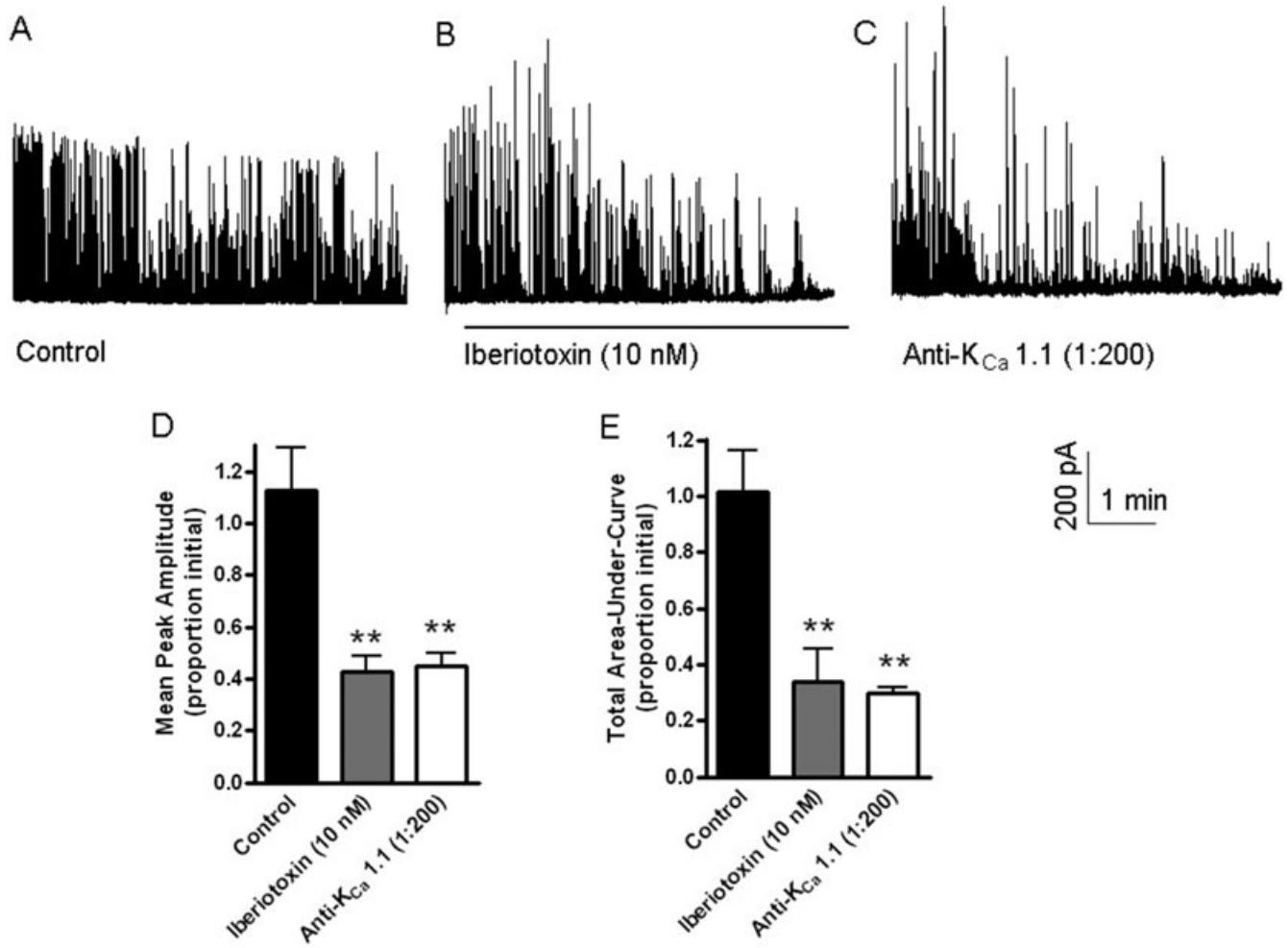

Figure 5 Modulation of spontaneous transient outward currents (STOCs) by anti-K_{Ca} 1.1 antibody. (A–C) Representative recordings of STOCs at 0 mV under control conditions, after 5 min application of 10 nM iberiotoxin (B) or after 10 min intracellular dialysis with an anti-K_{Ca} 1.1 antibody (1:200, C). Bar chart (D) demonstrates the absolute changes in mean peak STOC amplitude over a 1 min period before and 5 min after application of iberiotoxin or following dialysis with anti-K_{Ca} 1.1 antibody in the absence of iberiotoxin. (E) The absolute changes in total area encapsulated within STOC and baseline over a similar time period. Each bar is the mean data from at least three cells (bars showing SEM). $^{**}P < 0.01$ for paired Student's *t*-test; comparisons between data acquired before and after application of the channel modulator.

trations in excess of that required to stimulate these respective channels. Neither 100 μM 1-EBIO nor 1 μM NS309 had any effect on the amplitude or kinetics of I_{ClCa} in murine PV myocytes ($n = 5$ and 4, respectively, Figure 7A,B). Similarly, no effect on I_{ClCa} was observed with application of the specific $K_{Ca}2.3$ inhibitor TRAM-34 at a supramaximal blocking concentration of 10 μM ($n = 4$, Figure 7C; Wulff *et al.*, 2000) or application of 1 μM UCL 1684, a potent $K_{Ca}2.1$ blocker with an IC_{50} of 6 nM (Dunn, 1999; $n = 4$, Figure 7D). These experiments confirm that the pharmacological cross-over is restricted to CaCCs and $K_{Ca}1.1$, and does not extend to other calcium-dependent K^+ channels.

Discussion

This paper represents a detailed investigation of the degree of pharmacological overlap between CaCCs and subfamilies of calcium-activated K^+ channels. It shows clearly that three structurally unrelated blockers of ion channels encoded by the *mSlo* gene (KCNM1) all inhibit I_{ClCa} to some degree. In addition, tamoxifen, which can either augment or inhibit $K_{Ca}1.1$ currents depending upon the presence of the β1 subunit, almost abolished I_{ClCa} in PV myocytes. In contrast, an antibody raised against the C-terminal of $K_{Ca}1.1$ had no effect on I_{ClCa} nor did blockers and activators of intermediate and small conductance calcium-activated K^+ channels. These data consolidate the view postulated by Greenwood and Leblanc (2007) and Saleh *et al.* (2007) that CaCCs have a structural similarity to $K_{Ca}1.1$ or that the latter channel protein may somehow alter the function of the native Cl^- channel complex due to crosstalk in a microenvironment shared by both channels.

Blockade of CaCCs

The three classical $K_{Ca}1.1$ blockers used in this study are structurally very disparate and have different modes of action. Iberiotoxin is a 37 amino acid peptide extracted from the scorpion *Buthus tamulus* with a 70% homology to

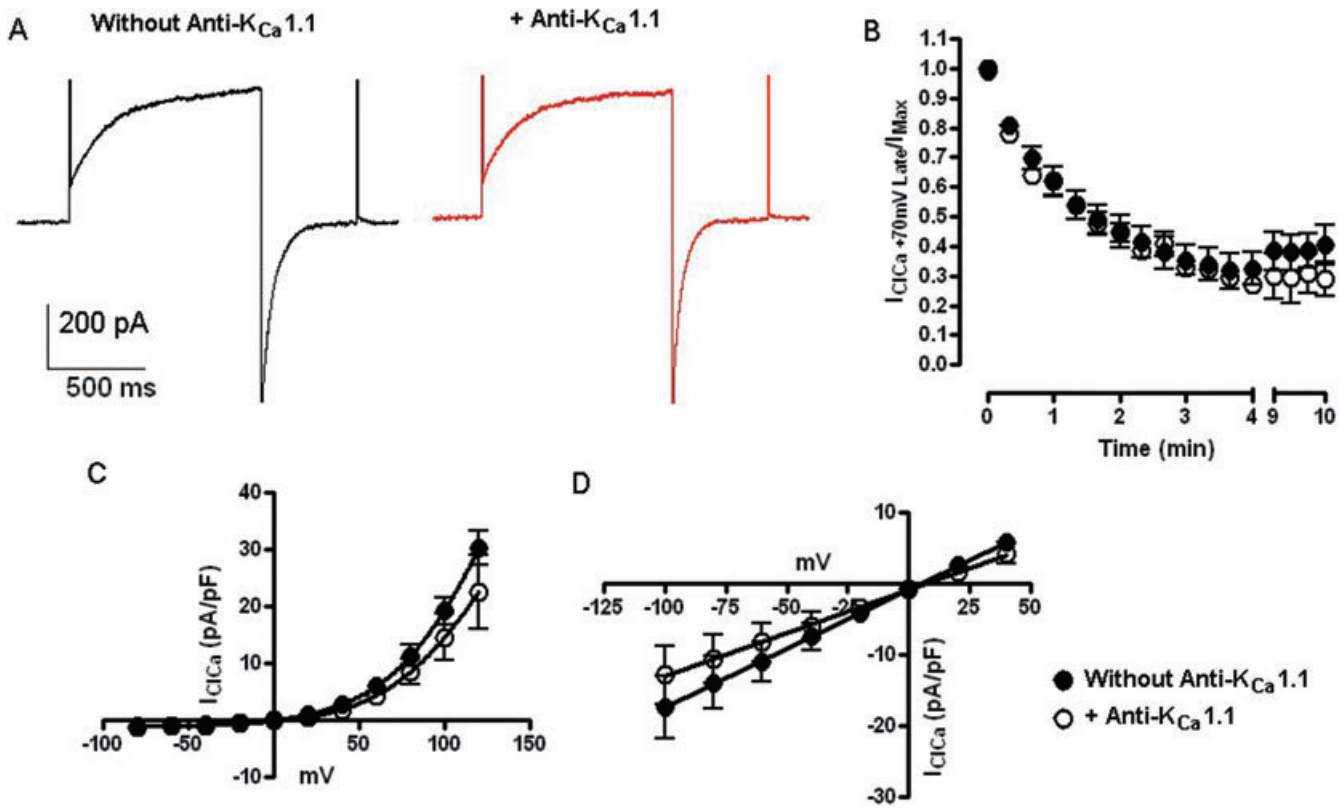

Figure 6 Lack of effect of anti-K_{Ca} 1.1 antibody on I_{ClCa}. (A) A comparison of I_{ClCa} recorded in a cell 10 min after achieving whole-cell access in a control cell (left-hand trace) or one dialysed with the anti-K_{Ca} 1.1 antibody (1:200: right-hand trace). (B) The lack of effect evoked by the anti-K_{Ca} 1.1 antibody (1:200) within the patch pipette when compared to control over a period of 10 min. Points represent late current evoked on stepping to +70 mV at a time period after acquiring whole-cell access, expressed as normalized current evoked immediately upon breaking the seal (relative to the initial current recorded at time = 0). (C) and (D) The lack of effect of the antibody on the I–V relationship of the voltage and time-dependent current evoked by stepping to voltages ranging between −80 and +120 mV, and the current evoked immediately on repolarization from +80 mV to voltages ranging from −100 to +40 mV respectively. Each point represents mean data from at least three cells (bars showing SEM).

charybdotoxin (Candia *et al.*, 1992; Giangiacomo *et al.*, 1992). Both peptides are less effective from the cytoplasmic side and are considered to bind to the external mouth of the pore (Candia *et al.*, 1992; Giangiacomo *et al.*, 1992). In contrast to charybdotoxin, which blocks other calcium-dependent and -independent K^+ channels, iberiotoxin is considered to be highly selective for K_{Ca}1.1 channels (Gao and Garcia, 2003). Penitrem A is a weaker but selective blocker of K_{Ca}1.1 that inhibits the binding of tritiated charybdotoxin (Knaus *et al.*, 1994), suggesting an interaction at a common binding site. Paxilline is a highly potent blocker of K_{Ca}1.1 (K_i in smooth muscle 10–38 nM, Knaus *et al.*, 1994; Sanchez and McManus, 1996; Li and Cheung, 1999) whose potency is related inversely to activating [Ca^{2+}] (Sanchez and McManus, 1996). Paxilline blocks K_{Ca}1.1 channels irrespective of the side of application and, in contrast to penitrem A, augments charybdotoxin binding (Knaus *et al.*, 1994), indicative of a different binding site. The experiments with these agents in the present study provide some startling revelations. First, the finding that iberiotoxin inhibits I_{ClCa} in PV myocytes and alters the rate of deactivation is the first observation of this peptide affecting any other ion channel other than K_{Ca}1.1 (Gao and Garcia, 2003). The change in I_{ClCa} kinetics, even with 100 nM iberiotoxin, suggests that this agent may be a gating modifier of the CaCC. Second, the ability of selective K_{Ca}1.1 blockers, which act at different sites to impede K^+ flux through K_{Ca}1.1 channels, to inhibit I_{ClCa} provides support for the proposition that the native Cl^- channel complex contains proteins resembling K_{Ca}1.1. Interestingly, an antibody that interacts with an epitope in the C-terminus calcium-binding domain of *mSlo* encoded proteins failed to affect I_{ClCa}. This suggests that Ca^{2+} sensing by the Cl^- channel involves a protein configuration different from the EF motif of K_{Ca}1.1.

In addition to an effect on I_{ClCa}, paxilline also ablated the stimulatory effect of niflumic acid on the Cl^- channel. This effect was not shared by penitrem A. Such a result leads to the tentative speculation that the stimulatory site on the Cl^- channel proposed by Ledoux *et al.* (2005) is contiguous with the paxilline blocking site. Logically, this suggests that the ability of niflumic acid and similar agents to augment K_{Ca}1.1 currents (see Greenwood and Large, 1995) is mediated by a similar binding site.

Lack of overlap with K_{Ca}2.1 and K_{Ca}2.3

Small conductance calcium-activated K^+ (K_{Ca}2.1) channels are encoded by KCNN1-3 genes (Stocker, 2004), have a unitary

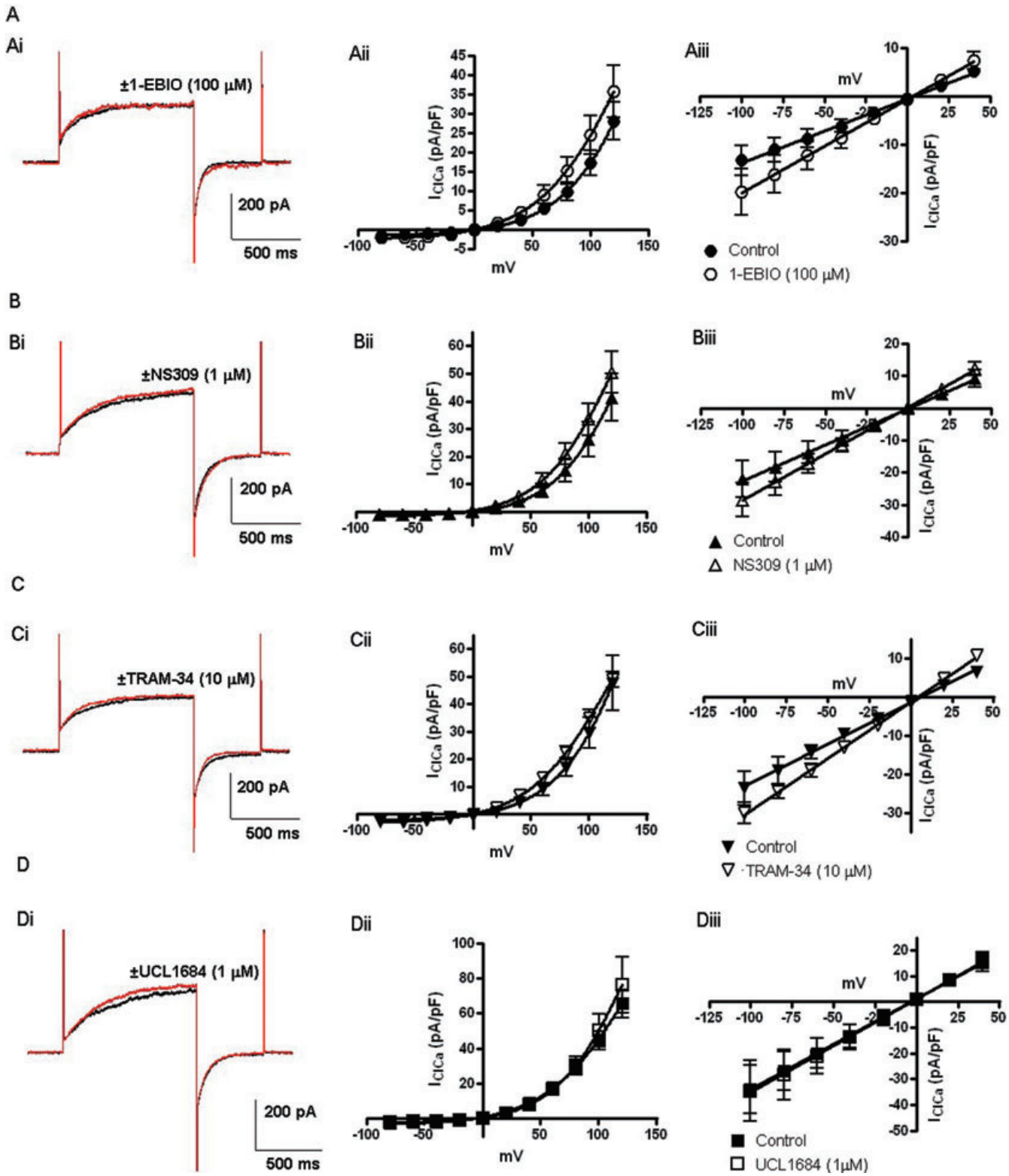

Figure 7 Effects of K_{Ca} 2.1 and K_{Ca} 2.3 modulators on I_{ClCa}. Modulation by 1-EBIO (100 μM; A), NS309 (1 μM; B), TRAM-34 (10 μM; C) and UCL 1684 (1 μM; D) is shown in this figure with a sample trace (left) showing in the same cell, control I_{ClCa} and I_{ClCa} evoked in the presence of a K_{Ca} 2.1 or K_{Ca} 2.3 modulator by depolarization to +70 mV followed by repolarizing to –80 mV. Middle and right graphs portray the I–V relationships of the voltage and time-dependent current evoked by stepping to voltages ranging between –80 and +120 mV, and the current evoked immediately on repolarization to voltages ranging from –100 to +40 mV, respectively; control currents and currents evoked in the presence of relevant modulator are shown. Each point represents the mean data from at least three cells (bars showing SEM).

conductance of about 3 pS, are voltage insensitive and are activated by low [Ca^{2+}] via an interaction with calmodulin (Stocker, 2004; Wei *et al*., 2005). Three isoforms of $K_{Ca}2.1$ exist that are about 65% homologous (Stocker, 2004). Intermediate conductance calcium-activated K^+ ($K_{Ca}2.3$) channels are encoded by KCNN4 and have about 40% homology with $K_{Ca}2.1$ channels (Stocker, 2004). Both channel subtypes are similar to $K_{Ca}1.1$ in the pore region and transmembrane domains, but are very different in the C and N termini (Faber and Sah, 2003). In contrast to the effect of $K_{Ca}1.1$ blockers (present study) and activators (Saleh *et al*., 2007), modulators of $K_{Ca}2.1$ or $K_{Ca}2.3$ had no effect on I_{ClCa}. Thus, 1-EBIO and NS309 at concentrations in excess of that required to maximally activate $K_{Ca}2.1$ and $K_{Ca}2.3$ (Strøbaek *et al*., 2004) had no effect on I_{ClCa} amplitude or kinetics. Moreover, application of the selective $K_{Ca}2.1$ or $K_{Ca}2.3$ blockers TRAM-34 and UCL 1684, again at supramaximal concentrations for blockade of the pertinent K^+ channel, had no effect on I_{ClCa}. Consequently, CaCCs in vascular smooth muscle would not include a contribution from KCNN gene products nor would they have any structural similarity to $K_{Ca}2.1$ or $K_{Ca}2.3$.

In conclusion, this study adds to the weight of evidence that CaCCs and $K_{Ca}1.1$ have a considerable pharmacological overlap. As we know nothing about the molecular identity of CaCCs (Leblanc *et al*., 2005), these findings, allied to previous experiments with Cl^- channel blockers (Greenwood and Large, 1995), may give a tantalizing glimpse into possible avenues of future investigation. Certainly, experiments on the ability of $K_{Ca}1.1$ modulators to affect ion currents generated by the over-expression of any bestrophin gene (see Hartzell *et al*., 2005) or the recently identified TMEM16 gene (Caputo *et al*., 2008; Schroeder *et al*., 2008; Yang *et al*., 2008), which are the main molecular candidates for CaCCs should be undertaken.

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Conflict of interest

None.

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