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A comparison of the effects of SCA40, NS 004 and NS 1619 on large conductance Ca^{2+}-activated K^+ channels in bovine tracheal smooth muscle cells in culture

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1 The effects of imidazopyrazine derivative, SCA40, on the activity of single large conductance, Ca^{2+}-activated K^+ (BK_{Ca}) channels in inside-out and outside-out patches from bovine tracheal smooth muscle (BTSM) cells in culture have been compared with those of two established BK_{Ca} channel openers, NS 004 and NS 1619.

2 The presence of BK_{Ca} channels on inside-out patches of BTSM membranes was confirmed by the single channel conductance (240 pS), selectivity for K^+, dependence of channel activity on $[Ca^{2+}]_i$, and sensitivity to the selective BK_{Ca} channel blocker, iberiotoxin.

3 NS 004 and ND 1619 (3-30 μM) induced concentration-related increases in open state probability of BK_{Ca} channels when applied to either inside-out or outside-out BTSM patches, thus confirming that these compounds are activators of the BK_{Ca} channel in this preparation.

4 SCA40 (0.1-10 μM) had no effect on the activity of BK_{Ca} channels when applied to either inside-out or outside-out patches which subsequently responded to the application of NS 004 (10-20 μM).

5 It is concluded that SCA40 does not have a direct effect on BK_{Ca} channel activity in BTSM patches and that the previously reported relaxant action of SCA40 on tracheal smooth muscle is unlikely to be mediated by this mechanism.

Keywords: BK_{Ca} channels; maxi-K^+ channels; bovine trachealis; potassium channel openers; patch-clamp; SCA40; NS 004; NS 1619; iberiotoxin

Introduction

The potential therapeutic application of openers of plasmalemmal K^+ channels has led to a considerable research effort over the past decade (for review see Longman & Hamilton, 1992). However, until recently the only known target for this class of compounds was the ATP-sensitive K^+ channel (K_{ATP}). With the discovery of agents like the substituted benzimidazolones, NS 004 and NS 1619 (Olesen & Wätjen, 1992; Olesen *et al.*, 1993; 1994a), and an extract from the medicinal herb, *Desmodium adscendens* (DHS-1; McManus *et al.*, 1993), attention has shifted towards openers of the large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}). NS 004 and NS 1619 have been shown to activate directly BK_{Ca} channels in a variety of preparations (Olesen & Wätjen, 1992; McKay *et al.*, 1994; Olesen *et al.*, 1994a,b; Sellers & Ashford, 1994). Direct opening of BK_{Ca} channels has also been described for DHS-1 (McManus *et al.*, 1993).

Recently, a series of imidazopyrazine derivatives have been synthesized which possess smooth muscle relaxant actions (Bonnet *et al.*, 1992). The most potent of these compounds, SCA40, has been reported to exert its relaxant effects by opening BK_{Ca} channels, a conclusion based on the sensitivity of the SCA40-induced relaxation to the BK_{Ca} channel blocker, charybdotoxin (ChTX), and on the observation that the relaxation was attenuated when 80 mM KCl was used as the spasmogen (Laurent *et al.*, 1993). However, the assertion that SCA40 acts via direct opening of BK_{Ca} channels has recently been disputed (Cook *et al.*, 1995).

In the present study we have attempted to resolve whether SCA40 directly activates BK_{Ca} channels by investigating its effects on single patch-clamped BK_{Ca} channels in bovine cultured tracheal smooth muscle cells, and have compared the actions of SCA40 with those of the established BK_{Ca} channel openers, NS 004 and NS 1619.

A preliminary account of the present work has been presented to the British Pharmacological Society (Macmillan *et al.*, 1994).

Methods

Isolation and culture of bovine tracheal smooth muscle cells

Strips of trachealis muscle from bovine tracheae were excised from animals at the local abattoir and transported to the laboratory in ice-cold Krebs solution. The tracheal muscle was dissected free from the surrounding connective tissue, chopped into 1 mm^3 pieces and digested in collagenase (Sigma Type IV) for 60 min to separate the smooth muscle cells. The resultant suspension was centrifuged for 6 min at 800 r.p.m. and the pellet resuspended in Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM) and antibiotics (2.5 $\mu g\ ml^{-1}$ amphotericin B and 50 $\mu g\ ml^{-1}$ gentamicin) and plated onto 175 cm^2 flasks. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere until they reached confluency. The cultures were passaged using trypsin-EDTA (Gibco-BRL) and grown on glass coverslips for electrophysiological recording. The presence of smooth muscle cells in the preparation was confirmed by positive immunocytochemical staining for actin and myosin (Twort & Van Breemen, 1988).

Electrophysiological recording solutions

The following solutions were used for recording BK_{Ca} channel activity: solution A had the following composition (in mM):

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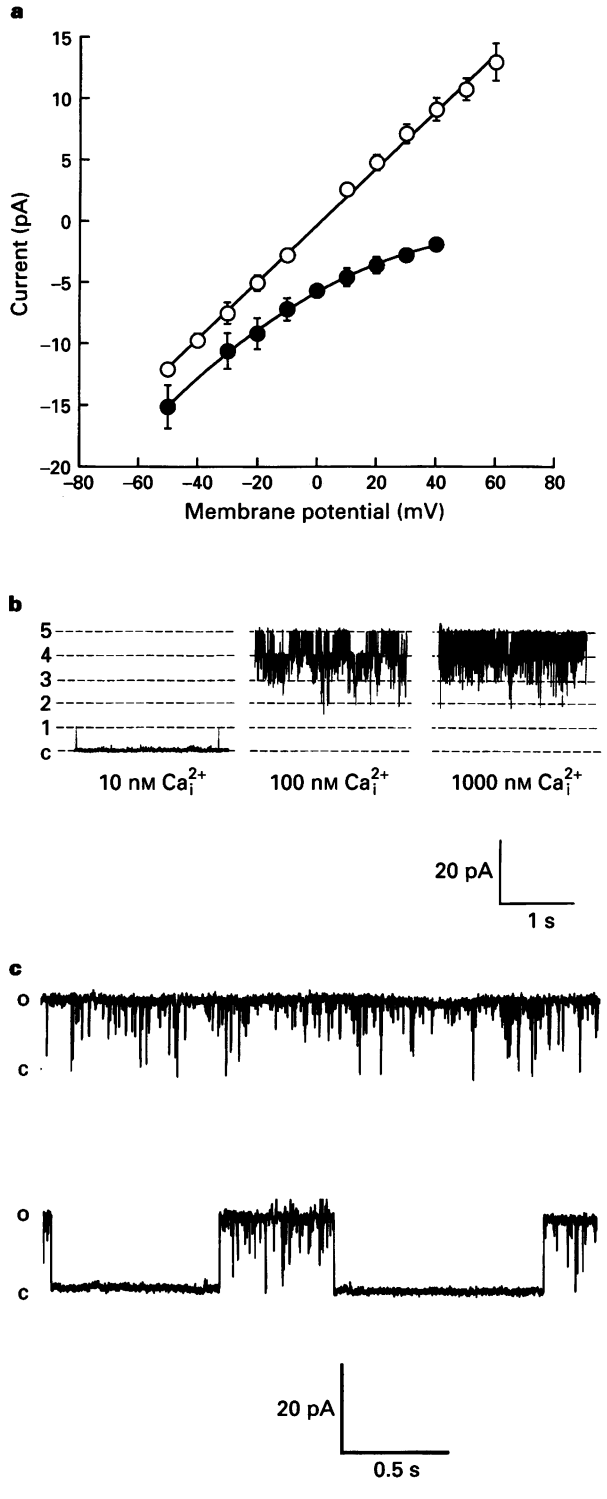


Figure 1 Characterization of the BK_{Ca} channel in cultured bovine tracheal smooth muscle cells (BTSM) cells. (a) Unitary current/voltage relationship for the BK_{Ca} channel in inside-out patches. (○) Solution B (K^+-rich) on both sides of the patch; (●) solution B (K^+-rich) in pipette, solution A (Na^+-rich) bathing the intracellular surface of the patch. Each point is the mean ± s.e.mean from at least 6 experiments. (b) Effect of Ca^{2+} on BK_{Ca} channel activity in an excised inside-out patch. Typical channel currents recorded from a patch bathed with solution B (K^+-rich) on both sides of the membrane. The solution bathing the intracellular face of the patch contained 10, 100 or 1000 nM Ca^{2+}. The membrane potential was held at +30 mV. In this and subsequent figures the closed state of the channel is denoted by 'c', and upward deflections represent outward current across the membrane. (c) Blocking effect of iberiotoxin on BK_{Ca} channel activity. *Upper trace*: example of channel activity in an inside-out patch held at +60 mV. *Lower trace*: channel activity recorded from another inside-out patch held at +60 mV with 100 nM iberiotoxin in the patch pipette (i.e. bathing the extracellular surface of the membrane). The concentration of Ca^{2+} bathing the intracellular face of the patch was 1 μM in both instances. Both sides of the patches were bathed in solution B.

NaCl 145, KCl 6, $CaCl_2$ 2, $MgCl_2$ 1, D-glucose 5, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10, adjusted to pH 7.4 with NaOH. Solution B contained (in mM): KCl 140, $MgCl_2$ 1, HEPES 10, 2 ethyleneglycol-*bis*-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA) 2, and an appropriate amount of $CaCl_2$ to make solutions of the desired free Ca^{2+} concentration (pH adjusted to 7.4 with KOH). The free Ca^{2+} concentration in these solutions was calculated using the EQUAL programme (Biosoft, Cambridge, U.K.) and was either 10, 100 or 1000 nM. For the two patch-clamp configurations used in these experiments (inside-out and outside-out) both the pipette and the superfusant contained solution B, unless otherwise stated.

Drugs

Stock solutions of SCA40 (10 mM), NS 004 (10 mM) and NS 1619 (10 mM) were prepared in absolute ethanol. In control experiments the highest concentration of ethanol (0.3% v/v) did not in itself influence BK_{Ca} channel activity. Stock solutions of iberiotoxin (10 μM) were made in distilled water. Iberiotoxin was obtained from Research Biochemicals Incorporated (Natick, MA, U.S.A.). SCA40 (6-bromo-8-methylaminoimidazo[1,2-a]pyrazine-2-carboritrile), NS 004 (1-(2-hydroxy-5-chlorophenyl)-5-trifluoromethyl-2-benzimidazolone) and NS 1619 (1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoro-methyl-2(3H) benzimidazolone)were synthesized by Syntex Discovery Research. Other chemicals were obtained from Sigma (U.K.) or from Fisons (U.K.). Final drug concentrations were obtained by adding to the superfusant appropriate volumes from concentrated stock solutions.

Patch-clamp recording

Single channel recording was carried out at room temperature (18–22°C) by the patch-clamp technique (Hamill *et al.*, 1981). Patch electrodes were pulled from 1.5 mm o.d. borosilicate glass capillaries (Clark Electromedical Instruments, U.K.) on a Flaming-Brown type micropipette puller (Sutter Instrument Company). Resistances of the patch electrodes when filled with solution B were between 5 and 12 MΩ and gave seal resistances of 2–10GΩ. Single channel recording was carried out on cells between passages 2–10 (2–5 days after passage). A commercial patch-clamp amplifier (RK 300, Biologic, France) was used to record channel activity. Currents were low-pass filtered at 2 kHz (eight-pole Bessel filter), digitized via a CED 1401 interface sampling at 10 kHz (Cambridge Electronic Design, Cambridge, U.K.) and stored on the hard disk of the computer for subsequent off-line analysis. Channel data were analysed for open-state probability (NP_o) with a two threshold transition algorithm which used a 50% threshold to detect events (Cambridge Electronic Design software). A minimum of 30 s of data were used to estimate NP_o. Solutions were introduced to the cells either by bath perfusion or with a commercial rapid solution changer (Biologic, France).

Data analysis

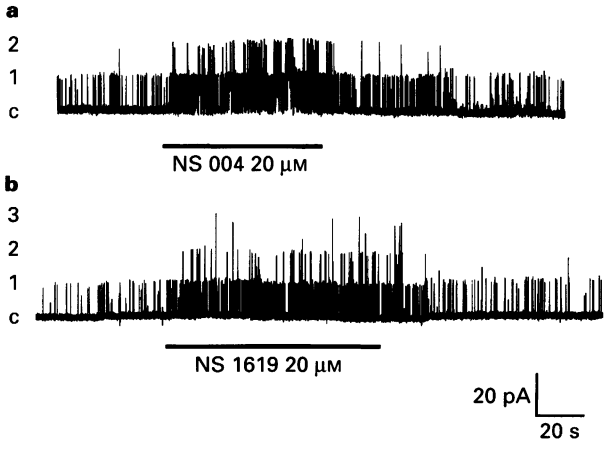
The responses to the test compounds were quantified by comparing the open-state probability under control conditions with that in the presence of drug (NP_o drug/NP_o control). Data are presented as means ± s.e.mean and, where appropriate, statistical comparisons have been made with Student's unpaired, two-tailed *t* tests.

Results

Characterization of BK_{Ca} channels on BTSM cells

Putative BK_{Ca} channel activity was observed in 51/69 excised patches and over 90% of the active patches contained more than one channel. The current-voltage (I/V) relationship with symmetrical 140 mM K^+ solution was linear in the membrane potential range −80 to +80 mV, and yielded a mean slope conductance of 240 ± 10 pS and a reversal potential of approximately 0 mV (Figure 1a, open symbols; $n = 16$ inside-out patches). When the concentration of K^+ bathing the intracellular face of the patch was changed to 6 mM the reversal potential was shifted to a value more positive than +50 mV (Figure 1a, solid symbols). Extrapolation of the I/V relationship under these asymmetrical K^+ conditions revealed a reversal potential approaching +80 mV, which is close to that expected for a K^+-selective channel.

The open-state probability of the putative BK_{Ca} channel at a membrane potential of +30 mV was increased by raising the concentration of Ca^{2+} bathing the intracellular face of the patch from 10 nM to 1000 nM ($P_o = 0.001 \pm 0.002$, 0.77 ± 0.12 and 0.92 ± 0.09 at 10, 100 and 1000 nM Ca^{2+}, respectively; $n = 5$ patches). An example of influence of Ca^{2+} on channel activity in a single inside-out patch is illustrated in Figure 1b.



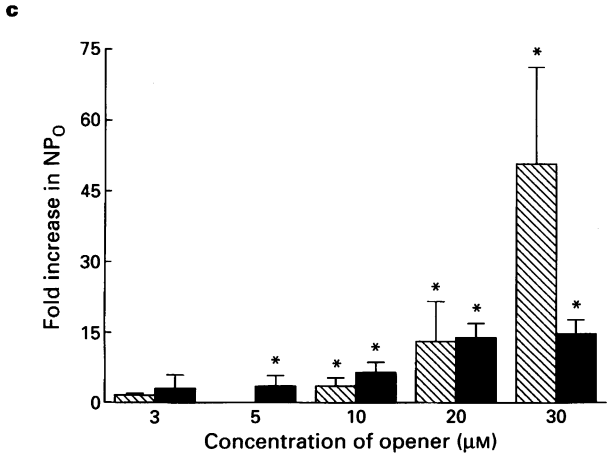


Figure 2 Application of 20 μM NS 004 (a) and 20 μM NS 1619 (b) to the intracellular face of two patches induced a reversible activation of BK_{Ca} channel activity (patches held at +70 mV). Drug application is indicated by the horizontal bars. The intracellular concentration of Ca^{2+} was 10 nM in both (a) and (b). (c) Concentration-dependence of the effects of NS 004 (solid columns) and NS 1619 (hatched columns) on BK_{Ca} channel activity. The columns show the fold increase (mean ± s.e.mean) in channel activity (NP_o drug/NP_o control) produced by NS 004 or NS 1619 in 3–6 inside-out patches. Asterisks denote significant difference from control value, $P < 0.05$.

The effect of iberiotoxin (100 nM) on channel activity was examined in 4 inside-out BTSM patches (toxin in the pipette). With this patch configuration, the channel activity at a membrane potential of +60 mV was blocked by iberiotoxin with a characteristic pattern of long closures interspersed with periods of normal activity ($P_o = 0.37 \pm 0.2$ in toxin-treated patches *versus* 0.98 ± 0.1 in 4 control patches; $P < 0.05$). This blocking action of iberiotoxin was also seen in outside-out patches ($n = 3$) where it was also shown to be reversible upon washout of the toxin. An example of the effect of iberiotoxin on BK_{Ca} channel activity in a single inside-out BTSM patch is shown in Figure 1c.

Effects of NS 004 and NS 1619 on BK_{Ca} channel activity

NS 004 (3–30 μM) markedly increased the activity of BK_{Ca} channels when applied to the intracellular surface of inside-out patches. An example of the effect of 20 μM NS 004 is shown in Figure 2a, where NS 004 induced a 16 fold increase in open-state probability. Activation of the channel was readily reversed by washout of NS 004 (Figure 2a). NS 004 induced a similar degree of channel activation irrespective of whether the concentration of Ca^{2+} bathing the cytosolic surface of inside-out patches was 10 nM ($n = 19$) or 100 nM ($n = 11$). However, NS 004 did not increase channel activity when the intracellular surface of the patch was bathed with a nominally Ca^{2+}-free solution ($n = 3$; data not shown). NS 1619 (3–30 μM) also dramatically increased the activity of BK_{Ca} channels when applied to the intracellular surface of inside-out patches held at +70 mV. An example of the effect of 20 μM NS 1619 in a single inside-out patch is shown in Figure 2b, where NS 1619 induced a 17 fold increase in open-state probability. As with NS 004, the action of NS 1619 was readily reversed by washout of the drug. A summary of the responses to NS 004 and NS 1619 (10 nM Ca^{2+} bathing the intracellular side of the patches) is shown in Figure 2c.

When applied to the extracellular face of outside-out patches held at a membrane potential of +60 mV, both NS 004 (20 μM; $n = 4$) and NS 1619 (20 μM; $n = 3$) induced increases in channel activity of 21.6 ± 3.1 and 15.8 ± 5.5 fold, respectively.

Effects of SCA40 on BK_{Ca} channel activity in BTSM cells

SCA40 (1, 5 and 10 μM), applied for 1–2 min to the cytosolic surface of inside-out patches of BTSM cells exposed to an intracellular Ca^{2+} concentration of 10 nM and held at +60 mV, did not induce statistically significant increases in BK_{Ca} channel activity (NP_o drug/NP_o control = 0.80 ± 0.01 ($n = 3$), 0.89 ± 0.27 ($n = 4$) and 0.98 ± 0.17 ($n = 4$) at 1, 5 and 10 μM, respectively). Nevertheless, each of these patches responded to the subsequent application of 10 μM NS 004 (NP_o drug/NP_o control = 5.41 ± 0.93; $P < 0.05$; data pooled from all 11 patches). An example of the action of 10 μM SCA40 on a cell which responded subsequently to NS 004 (10 μM) is shown in Figure 3a.

In 4 inside-out patch experiments in which the concentration of Ca^{2+} bathing the cytosolic surface was fixed to 100 nM, SCA40 (1, 5 and 10 μM, applied cumulatively for 1 min at each concentration) still failed to modify BK_{Ca} channel activity (data not shown).

To test whether SCA40 exerted an activator action when applied to the extracellular surface of patches, 3 outside-out patch experiments were performed. Under these conditions, SCA40 (1 and 10 μM, applied cumulatively for 1–2 min at each concentration) was still without effect on BK_{Ca} channel activity (NP_o drug/NP_o control = 1.21 ± 0.24 at 10 μM SCA40; $P > 0.05$; $n = 4$). In these same patches, channel activity was increased by the subsequent application of 20 μM NS 004 ($NP_o = 21.6 \pm 3.1$; $P < 0.05$). An example of the lack of effect of SCA40 (10 μM) in a single outside-out patch experiment is shown in Figure 3b.

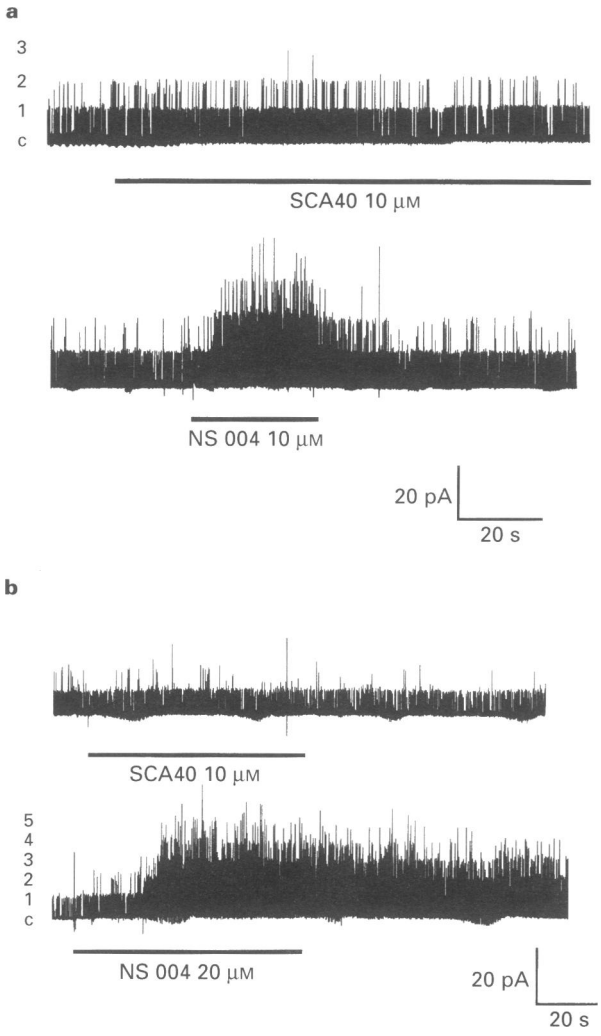


Figure 3 Actions of SCA40 and NS 004 on BK_{Ca} channel activity in inside-out (a) and outside-out (b) BTSM patches. (a) Application of 10 μM SCA40 to the intracellular surface of the patch is indicated by the horizontal bar. The lower trace shows the same cell after washout of SCA40 and during the application of 10 μM NS 004 to the cell (horizontal bar). The membrane potential of the patch was +60 mV and the intracellular concentration of Ca^{2+} was 10 nM. (b) The application of 10 μM SCA40 to the extracellular surface of the patch is indicated by the horizontal bar in the upper trace. The lower trace shows the effect of 20 μM NS 004 on the same patch after washout of SCA40. The membrane potential of the patch was +60 mV and the intracellular concentration of Ca^{2+} was 10 nM.

Discussion

BK_{Ca} channels in cultured BTSM cells

The channel most commonly encountered in patches from BTSM cells was a BK_{Ca} channel, as defined by its large conductance (240 pS), selectivity for K^+, dependence on intracellular Ca^{2+} concentration and sensitivity to the specific BK_{Ca} channel blocker, iberiotoxin (Galvez *et al.*, 1990). The presence of a 249 pS charybdotoxin-sensitive BK_{Ca} channel in acutely dissociated BTSM cells has been reported previously (Green *et al.*, 1991), and BK_{Ca} channels have also been identified in canine (McCann & Welsh, 1986; 266 pS), rabbit (Kume *et al.*, 1989; 120 pS), porcine (Huang *et al.*, 1987; 214 pS) and guinea-pig (Murray *et al.*, 1991; 130 pS) tracheal smooth muscle cells. Over 90% of the active patches in the present study contained more than one BK_{Ca} channel, which is in agreement with the findings of Green *et al.* (1991) in acutely isolated BTSM cells and demonstrates that the cultured BTSM cells retain a high density of BK_{Ca} channels. A similar high density of BK_{Ca} channels has also been reported in canine (McCann & Welsh, 1986) and porcine (Huang *et al.*, 1987) tracheal smooth muscle. An interesting contrast between the present study and that of Green *et al.* (1991) is that we failed to observe a sub-population of large conductance Ca^{2+}-independent K^+ channels. This may be due to the use of cultured BTSM cells in the present experiments instead of the acutely isolated cells used by Green *et al.* (1991).

Effect of NS 004 and NS 1619 on the activity of BK_{Ca} channels

Application of the BK_{Ca} channel activator, NS 004 (3–30 μM), to either inside-out or outside-out patches from BTSM cells consistently produced BK_{Ca} channel activation which was readily reversed by washout of the drug. The potency of NS 004 in the present study was similar both to that reported by Olesen *et al.* (1994b) to induce activation of BK_{Ca} channels in inside-out patches from mouse cerebellar granule cells and to that described by McKay *et al.* (1994) for activation of either BK_{Ca} channels in inside-out patches from GH3 cells or rat brain BK_{Ca} channels reconstituted into planar lipid bilayers.

NS 1619 (3–30 μM) produced an activation of BTSM BK_{Ca} channels that was qualitatively similar to that produced by NS 004, and similar to its previously described action on BK_{Ca} channels in aortic smooth muscle cells (Olesen *et al.*, 1994a) and rat hypothalamic neurones (Sellers & Ashford, 1994).

In the present study the ability of NS 004 to activate BK_{Ca} channels in inside-out patches was lost when the cytoplasmic face of the membrane was bathed in a Ca^{2+}-free solution. Abolition in Ca^{2+}-free medium of the activator action of NS 1619 on BK_{Ca} channels in aortic smooth muscle cells has been described previously (Olesen *et al.*, 1994a), and together these results demonstrate that neither compound is able to substitute for Ca^{2+} to activate the BK_{Ca} channel. A similar loss of activator potency in Ca^{2+}-free medium has been seen with the structurally unrelated BK_{Ca} channel opener, DHS-1 (McManus *et al.*, 1993).

Effect of SCA40 on BK_{Ca} channel activity on BTSM cells

The activity of BK_{Ca} channels in BTSM cells was not increased by SCA40 (1–10 μM) in either inside-out or outside-out patches. To ensure that the channels recorded in these experiments were capable of being activated, application of SCA40 was always followed by the application NS 004. The lack of effect of SCA40 persisted irrespective of whether the concentration of Ca^{2+} bathing the cytosolic side of the membrane was 10 nM or 100 nM.

The manifest lack of activity of SCA40 in the present experiments contrasts with the consistent activation induced by both NS 004 and NS 1619, and suggests that SCA40 does not directly activate BK_{Ca} channels in BTSM cells. The original claim that SCA40 is a BK_{Ca} channel activator was largely based on the observation that its relaxant action in guinea-pig isolated trachealis muscle was blocked by the BK_{Ca} channel blocker, ChTX, and markedly attenuated when high K^+ medium was used to contract the tissue (Laurent *et al.*, 1993). It is conceivable then that a species difference in the channel structure could explain the present negative findings, and that BK_{Ca} channels on guinea-pig trachealis cells possess a recognition site for SCA40 which is absent on BK_{Ca} channels in bovine trachealis. However, SCA40 is a potent relaxant of carbachol- and histamine-contracted bovine isolated trachealis muscle (R.C. Small, personal communication). In this latter study the maximum relaxant effect was achieved at a SCA40 concentration of 1 μM, which suggests that the concentration range used in the present study was appropriate to see an action if one existed.

If SCA40 does not directly open BK_{Ca} channels, how then is it able to relax tracheal smooth muscle? This question has recently been addressed by Cook *et al.* (1995)

who examined the pharmacology of SCA40 in guinea-pig isolated trachealis muscle and found that although its relaxant effects were attenuated in K^+-rich Krebs solution and by ChTx (confirming the original observations of Laurent *et al.*, 1993), this was due to the fact that the high K^+ and ChTX treatments in themselves promoted Ca^{2+} entry (and therefore contraction), and that this action produced a functional antagonism of the SCA40-induced relaxation. On the basis of these and other data, Cook *et al.* (1995) concluded that the smooth muscle relaxant action of SCA40 was most likely to arise from the potent inhibition by this compound of phosphodiesterase type III. The findings of the present study provide indirect support for this alternative mechanism of action.

In conclusion, our experiments confirm earlier reports that NS 004 and NS 1619 are able to activate BK_{Ca} channels by a direct action on the channel, and extend these observations to the BK_{Ca} channel in BTSM cells in culture. The failure of SCA40 to activate this channel in BTSM cells provides evidence at the single channel level that the relaxant action of this compound seen by others is not likely to be mediated by a *direct* interaction of the compound with the BK_{Ca} channel. This conclusion is in contrast to that of Laurent *et al.* (1993) but is entirely consistent with the findings of Cook *et al.* (1995).

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