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Effects of the BK_{Ca} channel activator, NS1619, on rat cerebral artery smooth muscle

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1 We have investigated the actions of NS1619, a putative activator of large conductance calcium-activated potassium channels (BK_{Ca}) by use of the patch-clamp technique on smooth muscle cells enzymatically isolated from the rat basilar artery.

2 Using whole cell current-clamp to measure membrane potential, addition of 30 μM NS1619 produced cellular hyperpolarization, moving the membrane potential towards the calculated equilibrium potential for potassium. This hyperpolarization was rapidly reversed by IbTX (100 nM), a selective inhibitor of BK_{Ca}.

3 In whole cell recordings made from cells voltage-clamped at 0 mV using the perforated-patch technique, addition of NS1619 (10–30 μM) activated an outward current, which reversed following washout of NS1619.

4 This outward current was unaffected by application of either glibenclamide (5 μM), an inhibitor of ATP-sensitive potassium channels, or apamin (100 nM), an inhibitor of small-conductance calcium-activated potassium channels. However, this current was almost completely abolished by iberiotoxin (IbTX; 50–100nM).

5 Depolarizing voltage steps activated small outward currents from cells held at −15 mV. Application of NS1619 (10–30 μM) increased the size of these currents, producing a shift to the left of the current-voltage (*I-V*) relationship. These currents were largely inhibited by IbTX (100 nM).

6 Measurements of the unitary amplitude of the single channels activated by NS1619 which could be resolved in whole cell recordings yielded a value of 5.6±0.14 pA at 0 mV.

7 NS1619 (10–30 μM) directly activated single channels contained in excised inside-out and outside-out membrane patches. In both configurations NS1619 (10–30 μM) rapidly increased the open probability of a large conductance calcium-dependent channel. The activation produced by NS1619 was calcium-dependent and inhibited by external IbTX (100 nM). The unitary current amplitude was unaffected by NS1619.

8 By use of conventional whole cell recording methods and conditions that suppressed BK_{Ca} openings, outward potassium currents were activated by depolarizing potentials positive to −35 mV from a holding potential of −65 mV. NS1619 (10–30 μM) inhibited this current in a concentration-dependent manner. This inhibition was reversed following washout of NS1619, recovering to 60–90% of control values within 2 min.

9 Ba^{2+} currents, measured by conventional whole cell recording, were activated by depolarizing voltage steps from negative holding potentials. NS1619 (1–30 μM) inhibited the evoked current in a concentration-dependent manner, yielding an IC_{50} value of 7 μM with a Hill coefficient approaching unity. This inhibition was reversible, with the currents recovering to 65–100% of control values after washout of NS1619 for 2 min.

10 NS1619 (0.3–100 μM) induced concentration-dependent relaxation of basilar artery segments contracted with histamine/5-HT (IC_{50} = 12.5±2.0 μM; n=4). This relaxation curve was shifted to the right, but not abolished, when the tissue was treated with a blocker of BK_{Ca} channels (IbTX; 100nM). Additionally, NS1619 produced concentration-dependent relaxation of basilar artery contracted with a depolarizing, isotonic salt solution containing 80 mM K^+.

11 Thus NS1619 produces hyperpolarization of basilar artery myocytes through direct activation of BK_{Ca} and also directly inhibits Ca^{2+} currents and voltage-activated K^+ channels. We discuss the implications of these results for its vasorelaxant actions.

Keywords: NS1619; large-conductance, calcium-activated K^+ channel; iberiotoxin; delayed rectifier K^+ channel; L-type calcium channel; vasorelaxation; basilar artery

Introduction

In recent years membrane hyperpolarization of vascular smooth muscle cells has been shown to be an important mechanism for producing vasodilatation. A number of drugs, collectively termed potassium channel openers, have recently been described. Exemplified by cromakalim and pinacidil, as well as several endogenous compounds, they produce hyperpolarization by activating ATP-sensitive K^+ channels (reviewed by Quayle & Standen, 1994; Nelson & Quayle, 1995). Activation of other types of K^+ channel could also produce hyperpolarization and ultimately relaxation. Large-conductance calcium-activated potassium channels (BK_{Ca}) have been reported in most types of smooth muscle, and their dual modulation by membrane potential and $[Ca^{2+}]_i$ fits well with one of their proposed roles as a regulator of myogenic tone in arterial smooth muscle (Brayden & Nelson, 1992). Because of the large unitary conductance of BK_{Ca} channels, BK_{Ca} openers are potentially potent vasorelaxing agents.

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Recently a number of chemically different compounds, the substituted benzimidazoles, the soyasaponins and the fenamates (McManus *et al.*, 1993; Olesen *et al.*, 1994a,b; Ottalia & Toro, 1994) have been described that activate BK_{Ca}. These compounds have been shown to hyperpolarize smooth muscle cells cultured from bovine aorta (Olesen *et al.*, 1994a). In smooth muscle cells isolated from the rat portal vein, NS1619 activated BK_{Ca} currents and also inhibited currents through Ca^{2+} channels (Edwards *et al.*, 1994).

Here, we have investigated the actions of NS1619 in cells freshly isolated from a cerebral artery, the basilar artery. We find activation of BK_{Ca} channels, in both whole cells and excised membrane patches. In addition, NS1619 inhibited voltage-activated K^+ channels, and was a potent inhibitor of L-type calcium channels.

Results of some of these experiments have been communicated to the British Pharmacological Society (Holland *et al.*, 1995).

Methods

Preparation and cell isolation

Male Wistar rats (200–400g) were killed by stunning followed by cervical dislocation. Basilar arteries were removed and placed in an ice cold buffer containing (mM): NaCl 136, HEPES 10, KCl 5.6, $NaHCO_3$ 4.17, $CaCl_2$ 2.6, $MgCl_2$ 1, NaH_2PO_4 0.44 and $NaHPO_4$ 0.42, adjusted to pH 7.4.

Isolated basilar artery myocytes were obtained by a method previously described for mesenteric artery (Quayle *et al.*, 1994). The basilar artery was transferred for a period of 10 min to a buffer identical in composition to the one described above, except that it contained a low concentration of calcium (0.1 mM). The artery was then digested for 30 min at 37°C in low calcium buffer that additionally contained (mg ml^{-1}): papain 1.5, bovine serum albumin (Sigma) 1.0 and dithiothreitol (Sigma) 1.0. The artery was then transferred to fresh low calcium buffer additionally containing (mg ml^{-1}): collagenase type F (Sigma) 1.5, hyaluronidase type I-S (Sigma) 1.0 and albumin 1.0 and incubated at 37°C for a further 10 min. Finally the digested artery was transferred to fresh low calcium buffer containing albumin (1.0 mg ml^{-1}). Single isolated myocytes were dispersed by gentle trituration with a wide bore pipette. Cells were stored at 4°C and used within 8 h.

Recording methods

For whole cell experiments patch pipettes were pulled from thin-walled borosilicate glass tubing (o.d. 1.5 mm, Clarke Electromedical). They were coated with dental sticky wax (Kemdent) and fire polished. Their resistance when filled with electrolyte was in the range 1–7 MΩ. Seals formed following the application of negative pressure were in the order of 10 GΩ. For experiments on excised patches pipettes were made as above, but of thick walled glass giving resistances of 7–12 MΩ.

Currents were recorded at a bandwidth of 10 kHz with a List EPC-7. Continuous recordings were stored on a modified digital audio tape (DAT) recorder, while those activated in response to voltage steps were digitised with an Axon instruments A-D interface and stored on a microcomputer. A suite of programmes developed using the AxoBASIC library (Davies, 1993) was used to apply command potentials and to analyse current records.

Whole cell potassium currents

Whole cell K^+ currents were measured by either the conventional or perforated-patch configuration of the patch-clamp technique (Hamill *et al.*, 1981; Rae *et al.*, 1991). The bathing (external) solution contained (mM): NaCl 136, glucose 10, HEPES 10, KCl 6, $MgCl_2$ 1, and $CaCl_2$ adjusted to pH 7.4. For perforated-patch recording the pipette solution contained (mM): KCl 136, HEPES 10, $MgCl_2$ 1, EGTA 0.5 and amphotericin B 200 μg ml^{-1}, adjusted to pH 7.2 with KOH such that final K^+ concentration was 140 mM. Amphotericin B was made as a stock (6 mg 100 μl^{-1}) in dimethylsulphoxide (DMSO, Sigma Tissue Culture Grade) and stored at −20°C; 20 μl of this stock was diluted in 5 ml of pipette solution prior to use. This was stored in darkness at 0°C and used within 4 h. The pipette (internal) solution for conventional whole cell recording of K^+ currents contained (mM): KCl 140, glucose 10, HEPES 10, EGTA 5 and $MgCl_2$ 0.5, adjusted to pH 7.2. Na_2ATP 2 mM and GTP 0.5 mM were added on the day of the experiment and the pH readjusted to 7.2.

For current-clamp recordings, the solution was the same as that used for conventional whole cell recording except EGTA was omitted.

Single potassium channel currents

Single channel recordings were made with excised inside-out or outside-out membrane patches. For inside-out patches, the cytoplasmic face of the patch was bathed with either a 500 nM Ca^{2+} solution or Ca^{2+}-free solution. The 500 nM Ca^{2+} solution contained (mM): KCl 140, glucose 10, HEPES 10, EGTA 5, $CaCl_2$ 3.74 and $MgCl_2$ 1, pH 7.2, while, for the Ca^{2+}-free solution $CaCl_2$ was omitted and EGTA 10 mM was added, giving a calculated free $[Ca^{2+}]$ of <1 nM. The pipette (external) solution contained the external solution described above. For outside-out patches the pipette (internal) solution used was the 500 nM Ca^{2+} described above and the bath solution was the external solution described above.

Whole cell Ca^{2+} currents

Whole cell currents through Ca^{2+} channels were measured by conventional whole cell recording with 10 mM Ba^{2+} as the charge carrier. The bathing (external) solution contained (mM): NaCl 130, $BaCl_2$ 10, glucose 10, HEPES 10, KCl 5.4, $MgCl_2$ 11 and EGTA 0.1, adjusted to pH 7.4. The pipette (internal) solution contained (mM): CsCl 130, EGTA 5, $MgCl_2$ 1 adjusted to pH 7.2 with TEA-OH. Glucose 10 mM, Na_2ATP 2 mM and GTP 0.5 mM were added on the day of the experiment and the pH readjusted to pH 7.2. We ensured that currents had reached a maximum amplitude by activating Ba^{2+} currents periodically (every 15 s) until currents reached a steady state. This was usually within 2 min.

The solution bathing cells under test could be rapidly changed via a seven-channel superfusion system that had an extremely small dead space (<20 μl). Experiments were conducted at room temperature, 18–22°C.

Solutions

NS1619 (1-(2'-hydroxy-5'-trifluoromethylphenyl) trifluoromethyl-2-(3H)benzimidazolone; a gift from Neurosearch Denmark) and glibenclamide (Sigma) were made up as a 10 mM stock solutions in DMSO (Sigma). Iberiotoxin (a gift from Zeneca Pharmaceuticals, Cheshire) was dissolved in distilled water to give a stock solution of 100 μM. Apamin (Sigma) was dissolved in dilute acetic acid giving a 1 mM stock solution. All drugs were diluted into flow solution immediately prior to use and the working concentrations are given throughout.

Recording mechanical tone

Segments of basilar artery were mounted in a wire myograph at 37°C for the isometric recording of tension. The bath solution used in these experiments was a modified Krebs-Henseleit solution containing (mM): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 1.8, KH_2PO_4 1.2, $NaHCO_3$ 25, HEPES 5, glucose 11. In order to prevent endothelial release of NO causing relaxation of the tissue 50 μM N^{ω}-nitro-L-arginine methyl ester

(L-NAME) was added to all bathing solutions. Basilar artery rings were stretched to an equivalent of a transmural pressure of 100 mmHg, the diameter calculated and then set to 90% of this value. The tissue was then allowed to equilibrate for 45 min. Active tone was established either by the addition of 1 μM histamine and 2 μM 5-HT or an isotonic depolarizing solution containing 80 mM K^+. After a stable level of tone was achieved NS1619 (0.3–100 μM) was added cumulatively to the bath. The effects of NS1619 were normalized by expressing the induced relaxation as a percentage of the maximum relaxant response to 1 μM nifedipine/1 mM EGTA. In a number of experiments 100 nM iberiotoxin was added 2 min prior to constructing the relaxant curve to NS1619.

Data analysis

Wherever possible concentration-effect data have been fitted to the equation:

$$Fx = \frac{(X/E)^n}{1 + (X/E)^n}$$

where X = concentration of effector compound, E = 50% of the maximum response, and n = slope factor, using a commercial fitting programme. The solution to this equation was then used to produce a best fit curve to the data. Graphs and text display the data as the mean ± s.e.mean. Where quoted, IC_{50} values were calculated for each data set and the mean ± s.e.mean calculated from these values. The significance of differences between means was assessed by Student's unpaired *t* test.

Results

Here we have investigated the effects of NS1619 on the ion channels contained in smooth muscle cells isolated from a small cerebral artery. Rat basilar myocytes selected for recording were relaxed, with dimensions of approximately 125 μm length by 10 μm diameter. Values for whole-cell capacitance and cellular input resistance measured at −60 mV, in response to a 10 mV depolarizing step, were 16.3 ± 3.5 pF and 6.3 ± 1.5 GΩ respectively (n = 7). All currents were measured as peak current and results are expressed as mean ± s.e.mean except where otherwise stated.

Effects of NS1619 on the membrane potential

When a physiological concentration gradient for potassium exists across the cell membrane, the opening of potassium channels will increase potassium efflux from the cell, resulting in membrane hyperpolarization provided the membrane potential is more positive than the potassium equilibrium potential (E_K). Therefore any agent that increases the open probability of potassium channels should produce membrane hyperpolarization. The effects of NS1619 on the membrane potential of rat isolated basilar myocytes were studied by use of the whole cell current-clamp.

Figure 1 illustrates the effect of NS1619 on the membrane potential of two cells measured under whole cell current clamp conditions. Their initial potentials were approximately −10 mV and −40 mV respectively. Superfusion with a solution containing 30 μM NS1619 caused both cells to hyperpolarize to around −70 mV (the calculated E_K is −79.5 mV). In each case the hyperpolarization was rapidly reversed, in the continued presence of NS1619, by the selective blocker of BK_{Ca} channels, iberiotoxin (IbTX, 100 nM). These experiments show that NS1619 can produce hyperpolarization of basilar myocytes and suggest that this effect results from the activation of BK_{Ca} channels.

Activation of a whole cell outward current by NS1619

Figure 2a illustrates the effect of NS1619 on the steady-state current recorded from a rat basilar myocyte using the perforated-patch technique. The cell was voltage-clamped at 0 mV throughout and was continuously superfused with a physiological solution containing 6 mM K^+, such that the opening of any potassium channels would produce outward currents. Prior to the addition of NS1619, the basal level of channel activity was very low, as expected because of the low intracellular calcium concentration of smooth muscle cells isolated from cerebral arterioles (approximately 500 nM at 0 mV, Kamishima & McCarron, 1995). When 20 μM NS1619 was added, a large outward current was activated, which reached a maximum value of around 500 pA after approximately 2 min. After removal of the NS1619, the outward current slowly declined, so that even after washing for 3 min the residual level of current was higher than the level that existed prior to exposure to NS1619. The ability of NS1619 to activate an outward current was confirmed in comparable experiments performed on seven other cells. On each occasion 1–4 min elapsed before the outward current reached a maximum. The mean NS1619-activated current was 210 ± 37 pA (n = 8).

Effects of potassium channel inhibitors on NS1619-activated current

To characterize the channel type activated by NS1619, we studied the effects of a number of specific K^+ channel inhibitors on the NS1619-activated current.

Iberiotoxin

Figure 2b illustrates the inhibition of the NS1619-activated current by the selective blocker of BK_{Ca}, iberiotoxin (IbTX). For the duration of this experiment the cell was continuously superfused with a physiological solution containing 6 mM K^+ and was clamped at 0 mV so that any potassium current would appear as outward current. Approximately 3 min after the addition of 20 μM NS1619 an outward current of approximately 180 pA was activated. When IbTX (100 nM) was ad-

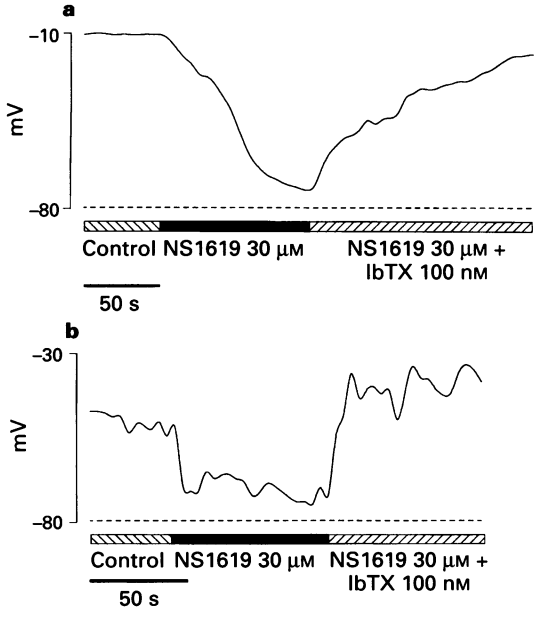

Figure 1 Effect of NS1619 on membrane potential. Basilar arterial myocytes recorded using current clamp conditions: (a) and (b) both show 30μM NS1619-induced hyperpolarization. The calculated E_K in these experiments is shown by the continuous dotted line. NS1619 and IbTX were applied where indicated.

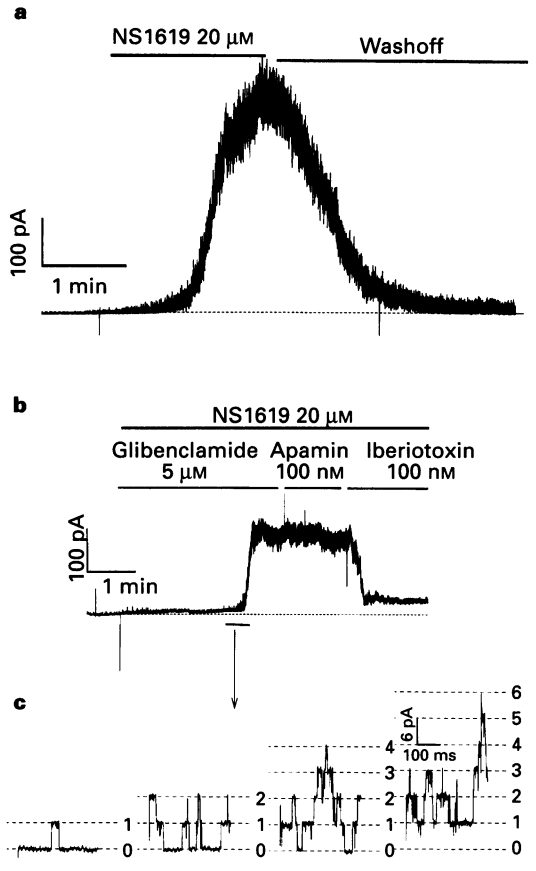

Figure 2 Selective activation of BK_{Ca} channels by NS1619 activated outward currents. Access was gained by use of the perforated patch technique with amphotericin. The cells were bathed in 6 mM K^+ and held at 0 mV throughout. The zero current levels are indicated by the continous dotted lines. The records were filtered at 1.0 kHz and sampled at 5 kHz. (a) Illustrates that following the addition of 20 μM NS1619, large outward currents were activated, which reversed following washout. (b) Shows that the current activated by NS1619 was selectively inhibited by iberiotoxin (IbTX). Where indicated glibenclamide, apamin and IbTX were added. (c) Activation of a large-conductance channel following the addition of 20 μM NS1619. The records show four segments of current recording expanded from record (b) during the beginning of the response to NS1619. The sections expanded are indicated by the bar under the record. The broken lines indicate the current levels corresponding to 0, 1, 2 etc. channels open.

ded, in the continued presence of NS1619, a rapid (<30s) and near complete inhibition of the NS1619-activated current was produced. This contrasted with the relatively slow reversal of the current observed following washout of NS1619 (see Figure 2a). The ability of IbTX to inhibit the NS1619-activated currents was confirmed in comparable experiments performed on a further four cells.

Glibenclamide and apamin

Glibenclamide is a relatively specific inhibitor of ATP-sensitive K^+ channels in smooth muscle at concentrations below 10 μM, (for review see Quayle & Standen, 1994). Figure 2b illustrates that NS1619 activated an outward current in the presence of 5 μM glibenclamide. Figure 2b additionally shows that apamin, a specific inhibitor of small conductance calcium-activated potassium channels (Hugues *et al.*, 1982), was also without effect on the NS1619-activated current. These findings were confirmed in three additional experiments.

Whole cell currents activated by step potentials from depolarized holding potentials are increased by NS1619 and inhibited by IbTX

The gating of BK_{Ca} channels is voltage-dependent, increasing when the membrane potential is depolarized (Latorre *et al.*, 1989). To characterize further the channels activated by NS1619, we studied the effects of NS1619 on whole cell currents activated by depolarizing command potentials. Whole cell access was gained using the perforated-patch technique with amphotericin B. This configuration was employed to ensure intracellular calcium concentrations and buffering were maintained as close to physiological levels as possible. Cells were superfused throughout with a physiological salt solution containing 6 mM K^+ and a holding potential of −15 mV was used to inactivate delayed rectifier (K_V) potassium channels (see Figure 6), ensuring that BK_{Ca} channels dominated the

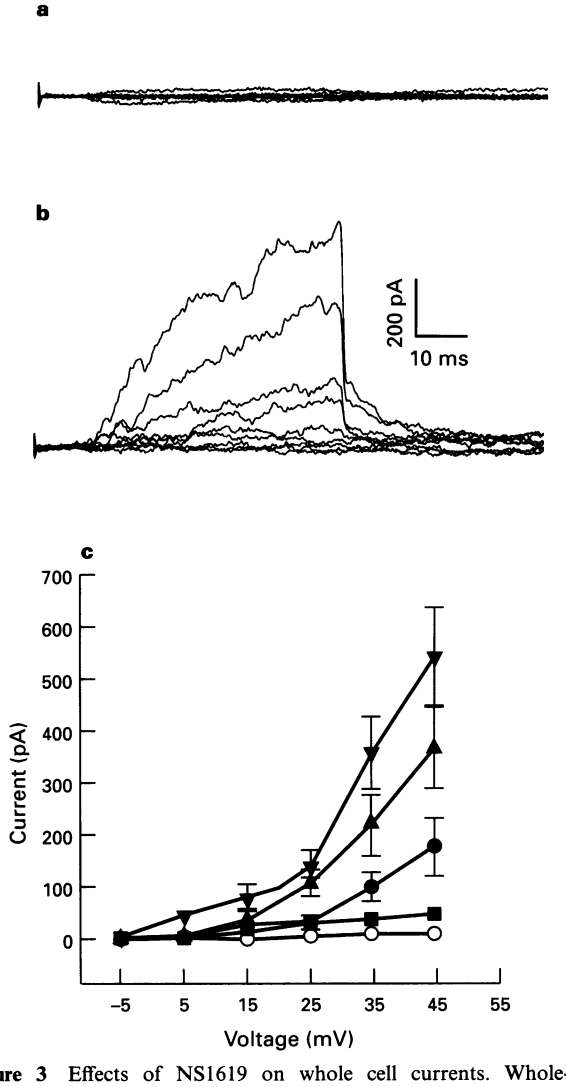

Figure 3 Effects of NS1619 on whole cell currents. Whole-cell currents were recorded, by use of the perforated-patch configuration (amphotericin B), in response to voltage steps (−35 to 45 mV) from a holding potential of −15 mV. Records shown were filtered at 1 kHz for display and have been leak subtracted. (a) Current records obtained in the absence of NS1619. (b) Current records from the same cell 1 min after the addition of 30 μM NS1619. (c) Effect of increasing concentrations of NS1619 on whole cell *I-V* curves. Mean current-voltage relations from 6 experiments using perforated-patch recording are illustrated. Voltage protocol −35 to 45 mV from a holding potential of −15 mV. Each cell was exposed to control external solution (○) and solutions containing 10 (●), 20 (▲), 30 μM (▼) NS1619 and 30 μM NS1619 and 100 nM IbTX (■). The points show mean peak outward current following leak subtraction ± s.e. mean. $n=6$.

outward currents. Figure 3a illustrates an experiment where currents were activated by a sequence of command potentials, from −35 mV increasing in 10 mV increments to a final level of +45 mV. The outward currents activated under control conditions were extremely small (Figure 3a). In the experiment shown, the outward current activated following a step to +45 mV was 11pA. Following the addition of 30 μM NS1619 to the same cell for 1 min, the peak outward currents increased substantially (Figure 3b). In this particular experiment an outward current of 730 pA was activated following a step to +45 mV.

Figure 3c illustrates the mean current-voltage relationship (*I-V*) measured from 6 cells following exposure to increasing concentrations of NS1619 (10, 20, and 30 μM) for 1 min. On stepping to +45 mV the mean control current was 11.3±3 pA, increasing to 177±54 pA, 367±79 pA and 540±92 pA following the addition of 10, 20 and 30 μM NS1619 respectively. In addition, Figure 3c shows that the current activated by NS1619 was inhibited by IbTX. When 100 nM IbTX was included together with 30 μM NS1619 the peak outward current activated by a step potential to +45 mV was reduced to 48±11 pA ($n=6$).

The current activated by NS1619 flows through large-conductance channels

In many of our whole cell recordings, single channel openings could be resolved early in the activation by NS1619. Figure 2c shows expanded segments of current taken from the recording of Figure 2b showing progressive channel activation. The channel activated by NS1619, measured by producing an amplitude histogram, has a unitary amplitude of 6.2 pA (at 0 mV) in this cell, while the mean value from 8 cells was 5.6±0.1 pA. Similar values were obtained when the traces were subjected to noise analysis (data not shown).

Rapid activation of BK_{Ca} in inside-out membrane patches

The substantial delay in activation of whole-cell current by NS1619 raises the possibility that NS1619 is causing channel activation by acting at an internal site, or perhaps by an indirect mechanism, for example by causing intracellular release of Ca^{2+}. We therefore investigated the activation of channels by NS1619 in excised inside-out membrane patches.

In these experiments, the cytoplasmic face of the membrane was continually superfused with either 'Ca^{2+}-free' or 500 nM Ca^{2+} solution (see Methods) and recordings were made at 0 mV. BK_{Ca} channels were identified on the basis of their Ca^{2+}-sensitivity and unitary current amplitude. Figure 4a shows the effect of NS1619 applied to the internal face of an inside-out patch. The patch was initially exposed to Ca^{2+}-free solution, in which channel openings were very infrequent (p_{open} measured over approximately 1 min was 0.001). Ca^{2+} 500 nM solution caused a slight increase in activity, doubling p_{open}, while NS1619 30 μM caused a rapid and significant ($P<0.05$) increase in activity ($P_{open}=0.081$). NS1619 did not however substitute as a ligand for Ca^{2+}, as changing to a Ca^{2+}-free solution in the continued presence of NS1619, led to an immediate decrease in activity to control level.

The unitary amplitude of the channel activated by NS1619 in the experiment illustrated in Figure 4a was 5.1 pA. The mean value of 5.9±0.2 pA obtained from 4 cells agrees well with the value for BK_{Ca} that we obtained from whole cell recordings (see Figure 2c), and with a previous report in which unitary currents were measured under the same conditions from rat mesenteric arterial myocytes (Langton *et al.*, 1991). In experiments on 4 further patches we also found that the p_{open} of BK_{Ca} channels was increased by NS1619 (10 and 30 μM) in the presence of 500 nM Ca^{2+}, an effect that was reversed by returning to Ca^{2+}-free solution. The results from these experiments are summarised in Figure 4b.

Activation of IbTX-sensitive channels in outside-out patches

We also examined the effects of NS1619 on channels in excised outside-out membrane patches. Figure 5a and b illustrates the effects of 30 μM NS1619 on the activity of channels in an outside-out patch held at 0 mV. Under control conditions with 500 nM calcium on the cytoplasmic face of the patch P_{open} was 0.07, and increased to 0.24 following the addition of 30 μM NS1619 to the external side of the patch, with 2 and occasionally 3 simultaneous channel openings. The activation in outside-out patches was also more rapid than the activation observed in the whole cell configuration. Externally applied IbTX (100 nM) in the continued presence of NS1619, led to a rapid reduction in channel activity ($n=2$). NS1619 did not significantly alter the mean unitary amplitude of BK_{Ca} channels as shown by the amplitude histograms in Figure 5c. In 2 patches, unitary current amplitudes were 5.9 and 5.8 pA in 500 nM Ca^{2+} and 5.8 and 6.0 pA following the addition of 30 μM NS1619.

Inhibition of voltage-activated (K_V) channels by NS1619

Edwards *et al.* (1994) have recently reported that NS1619 inhibits delayed rectifier K^+ (K_V) currents in cells from the rat portal vein. We sought to confirm this in cerebral myocytes. We used experimental conditions that minimized activation of BK_{Ca}. This was achieved by use of conventional whole cell configuration with 5 mM EGTA and no added Ca^{2+} in the patch pipette solution and by including 100 nM IbTX in the

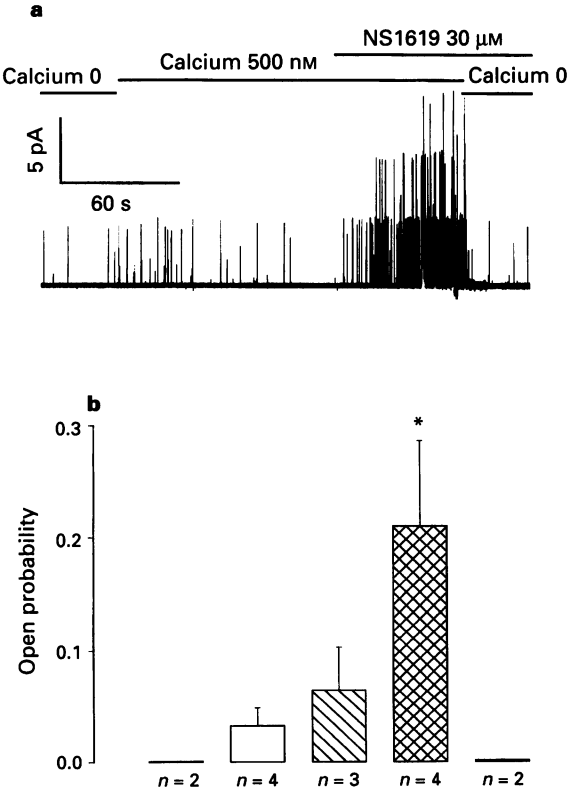

Figure 4 Calcium-dependence of activation of channels in an excised patch by NS1619. (a) Records obtained from an excised inside-out patch held at 0 mV in physiological K^+ gradient. (b) Panels from left to right show activity when the solution bathing the cytoplasmic face of the patch contained <1 nM Ca^{2+}; 500 nM Ca^{2+}; 500 nM Ca^{2+} + 10 μM NS1619; 500 nM Ca^{2+} + 30 μM NS1619 and 30 μM NS1619 with <1 nM Ca^{2+}. Mean (±s.e.mean) P_{open} values, measured over 50–70 s periods, from BK_{Ca} channels in excised patches exposed to the Ca^{2+} and NS1619 concentrations indicated, $^*P<0.05$.

extracellular bath solutions. Similar conditions have previously been reported to inhibit the activation of calcium-dependent potassium currents in rabbit coronary smooth muscle cells (Volk *et al.*, 1991). Under these conditions the dominant outward current is through voltage-activated K^+ channels. Figure 6a shows outward currents recorded from a cell using these conditions, in response to depolarizing voltage steps from a holding potential of −65 mV. Addition of 10 μM NS1619 reduced the outward currents, while 30 μM NS1619 led to further reduction. For comparison, Figure 6d shows the currents recorded on stepping to +35 mV under control conditions and in the presence of 10 and 30 μM NS1619. The peak outward

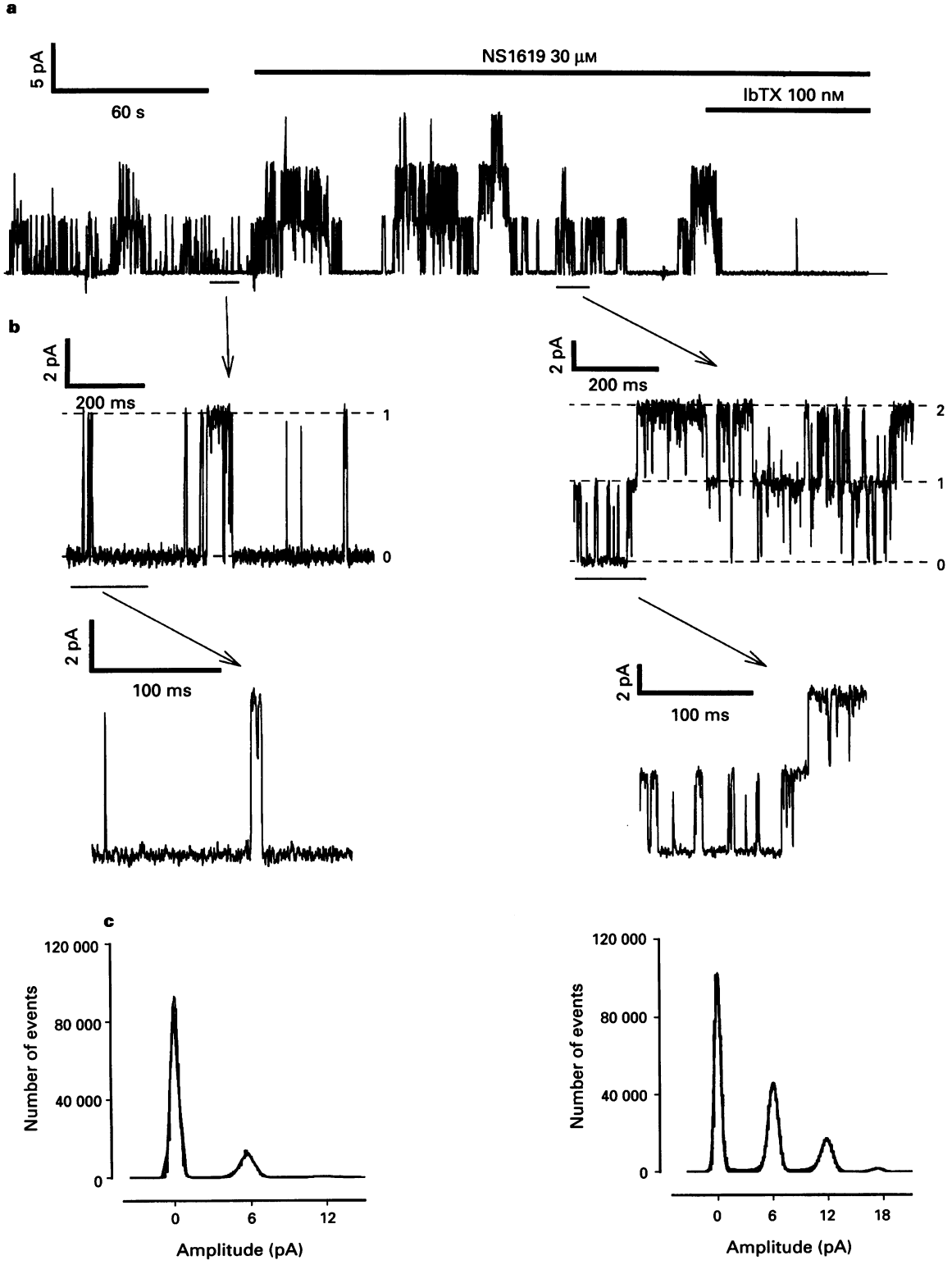

Figure 5 Channel activation by NS1619 in an outside-out patch. (a) Recording from an outside-out patch held at 0 mV. The pipette solution contained 140 mM K^+ and 500 nM calcium; bath solution contained 6 mM K^+. NS1619 and IbTX were applied externally as indicated. (b) Expanded segments from the recording in (a) before (left) and after (right) exposure to 30 μM NS1619. (c) Current amplitude histograms constructed from the traces shown in (a) before (left) and after (right) exposure to 30 μM NS1619. Note that single channel current amplitudes are not affected by exposure to NS1619 although channel P_{open} is increased.

currents were 90, 66 and 43 pA respectively. Figure 6e shows mean current-voltage relations from similar experiments performed on 4 cells. For each cell, currents were normalised by dividing by the maximum current recorded for that cell. It is clear that NS1619 caused a concentration-dependent reduction of outward current. We confirmed that the current observed

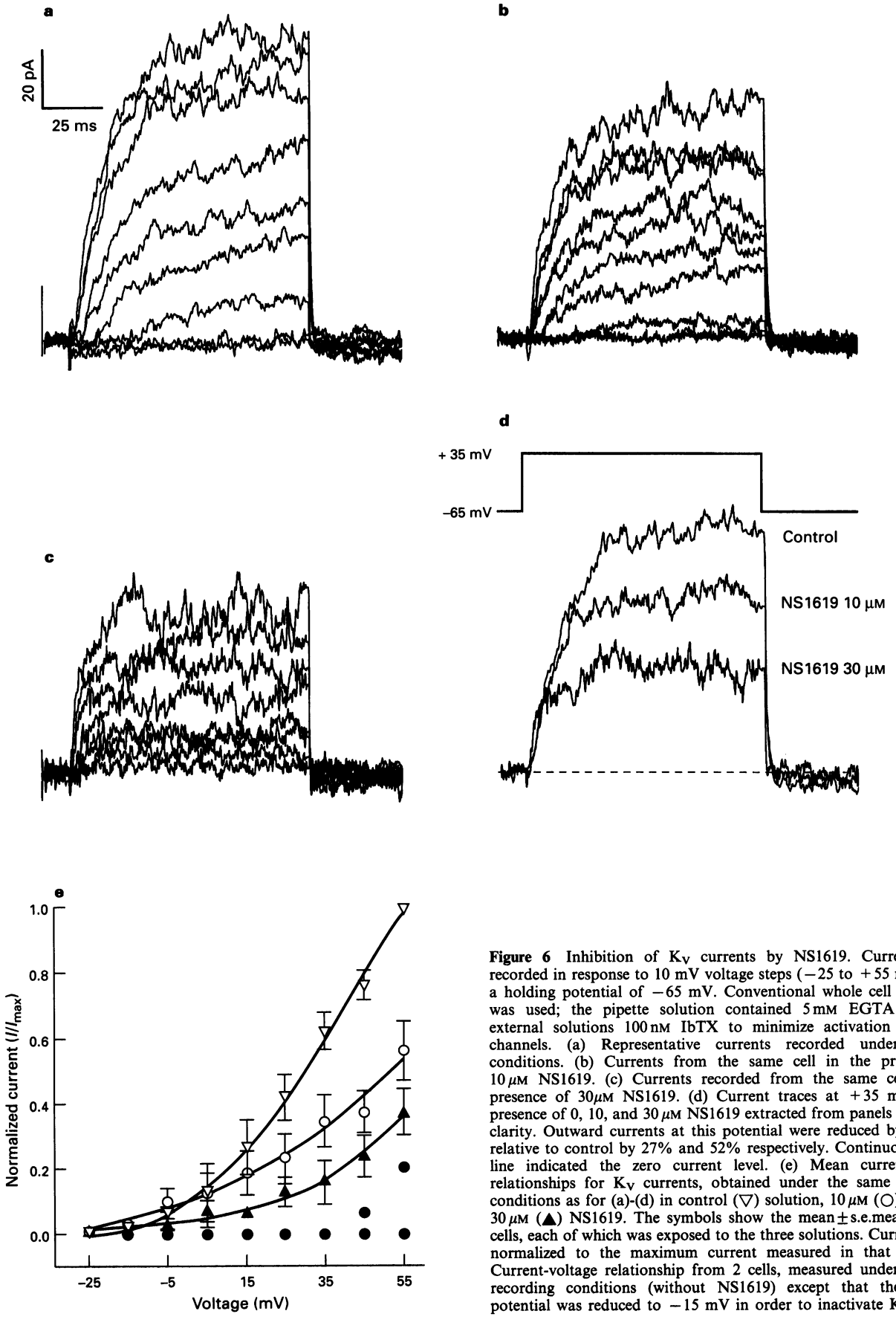

Figure 6 Inhibition of K_V currents by NS1619. Currents were recorded in response to 10 mV voltage steps (−25 to +55 mV) from a holding potential of −65 mV. Conventional whole cell recording was used; the pipette solution contained 5 mM EGTA and the external solutions 100 nM IbTX to minimize activation of BK_{Ca} channels. (a) Representative currents recorded under control conditions. (b) Currents from the same cell in the presence of 10 μM NS1619. (c) Currents recorded from the same cell in the presence of 30 μM NS1619. (d) Current traces at +35 mV in the presence of 0, 10, and 30 μM NS1619 extracted from panels (a)-(c) for clarity. Outward currents at this potential were reduced by NS1619 relative to control by 27% and 52% respectively. Continuous dotted line indicated the zero current level. (e) Mean current-voltage relationships for K_V currents, obtained under the same recording conditions as for (a)-(d) in control (▽) solution, 10 μM (○) NS1619, 30 μM (▲) NS1619. The symbols show the mean ± s.e.mean from 4 cells, each of which was exposed to the three solutions. Currents were normalized to the maximum current measured in that cell. (●) Current-voltage relationship from 2 cells, measured under identical recording conditions (without NS1619) except that the holding potential was reduced to −15 mV in order to inactivate K_V.

using these recording conditions was through voltage-activated K^+ channels by changing the holding potential to −15 mV to produce inactivation of K_V channels. This more depolarized holding potential was used because K_V channel availability is steeply voltage-dependent, with 50% inactivation produced in the range −25 to −30 mV for smooth muscle cells (Beech & Bolton, 1989; Volk *et al.*, 1991). In two experiments using this holding potential no outward current was detectable up to +35 mV (filled circles in Figure 6e). This provided strong evidence that the channel we were selectively activating and then blocking with NS1619 was K_V.

Inhibition of currents through Ca^{2+} channels by NS1619

The vasorelaxant effects of NS1619 were initially ascribed to its ability to open BK_{Ca} channels of vascular smooth muscle, resulting in hyperpolarization. Recently, however, Edwards *et al*, (1994) reported that 33 μM NS1619 completely inhibited Ca^{2+} currents in rat portal vein smooth muscle and suggested that this effect may underlie its functional effects. We investigated the concentration and voltage-dependence of Ca^{2+} channel inhibition in basilar arterial cells. In all experiments we used 10 mM external Ba^{2+} to give substantial currents through L-type Ca^{2+} channels in these cells (Langton & Standen, 1993). Figure 7a illustrates the effects of externally applied NS1619 (1, 3, 10, and 30 μM) on the inward Ba^{2+} currents elicited by a voltage step from −88 mV to +2 mV. The maximal inhibition by NS1619 occured within 1 min and Figure 7a shows a clear concentration-dependent decrease in Ba^{2+} current. In similar experiments on 4 cells the mean control current at +2 mV was

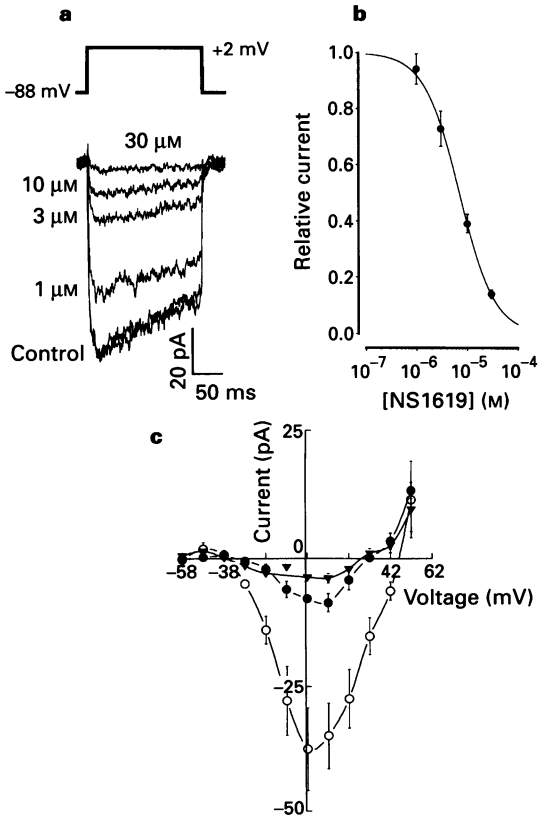

Figure 7 Inhibition of Ca^{2+} channel currents by NS1619. (a) Representative inward currents recorded in response to voltage steps from −88 to +2 mV. The external solution contained 10 mM Ba^{2+} to which NS1619 (1−30 μM) was added. (b) Concentration-effect relationship for the inhibition of Ba^{2+} currents by NS1619. The points show the mean ± s.e.mean from 4 cells and the best fit line has a Hill coefficient of 1.3 and an IC_{50} of 6.9 μM. (c) Current-voltage relationships for the Ba^{2+} current measured from 4 cells without (○), in 10 μM (●) and 30 μM (▼) NS1619.

−48 ± 12 pA, which was reduced to : −42 ± 12 pA, −30 ± 4 pA, −16 ± 3 pA and −6 ± 1.0 pA by 1, 3, 10, and 30 μM NS1619. The effect of NS1619 on Ba^{2+} currents was readily reversible, recovering to 85% of control values ($n=3$) after washing with NS1619-free solution for 2 min.

The concentration-effect curve for the inhibition of Ba^{2+} current by NS1619 measured from 4 cells is shown in Figure 7b. The curve is well fitted with a Hill coefficient of 1.3 and yields an IC_{50} value of 7 μM. Figure 7c illustrates whole cell Ba^{2+} currents activated following a series of depolarizing potentials, from an initial level of −58 mV rising in +10 mV increments to a final level of +52 mV. Cells were voltage-clamped at −68 mV throughout. NS1619 inhibited Ba^{2+} currents in a concentration-dependent manner, with 30 μM NS1619 producing almost complete inhibition of the current. The level of inhibition did not vary with changes in the step potential, indicating that the block of L-type Ca^{2+} channels by NS1619 was not voltage-dependent (data not shown).

Effects of NS1619 on mechanical tone

Active tone was established in segments of basilar artery either by the addition of an agonist mixture (1 μM histamine plus 2 μM 5-HT; neither agonist producing a stable contraction by themselves) or by replacing the bathing solution with an isotonic salt solution containing 80 mM K^+. The arterial segments contracted in response to either treatment but this increased tone was not maintained unless the bathing solution contained a NO synthase inhibitor such as L-NAME, suggesting that an intact endothelial layer was maintained in this preparation.

The cumulative addition of NS1619 (0.3−100 μM) to arterial rings contracted with the agonist mixture resulted in a concentration-dependent inhibition of tone ($IC_{50}=12.45\pm2.0$ μM; maximum relaxation 98 ± 2%; $n=4$) (Figure 8a). When agonist-contracted artery segments were treated with 100 nM iberiotoxin to block BK_{Ca} channels prior to the addition of NS1619, the relaxant curve was significantly shifted to the right, approximately 2 fold ($IC_{50}=21.1\pm0.3$ μM; maximum relaxation 89 ± 3%; $n=4$; $P<0.05$). The position of this curve lay between the relaxation curve against agonist-induced tone and the curve obtained against 80 mM K^+-induced tone which was also significantly shifted to the right (Figure 8b) ($IC_{50}=46.1\pm7.7$ μM; $n=8$; $P<0.01$). The possibility that IbTX was having some functional effect other than by K^+ channel blockade was addressed by repeating the relaxant curve against 80 mM K^+ but in the presence of 100 nM IbTX. The relaxation curve under these conditions superimposes on that of the curve against 80 mM K^+ alone, suggesting that the functional effects of IbTX on the response of the agonist contracture to NS1619 are being mediated via its ability to block an increase in K^+ conductance.

Discussion

NS1619-induced hyperpolarization results from the activation of BK_{Ca}

NS1619 hyperpolarized cells, moving the membrane potential towards E_K (Figure 1). This hyperpolarization was reversed by IbTX, a component isolated from the venom of the scorpion *Buthus tamalus*. IbTX is a potent and selective inhibitor of BK_{Ca} channels, binding to a specific site in the external 'mouth' of the channel and occluding the permeation of ions through the channel (Galvez *et al.*, 1989; Candia *et al.*, 1992).

Our experiments on the currents induced by NS1619 provide good evidence that the selective activation of BK_{Ca} channels was responsible for the production of this current. In intact cells using the perforated-patch technique NS1619 activated whole-cell currents which were unaffected by either the K_{ATP} channel blocker, glibenclamide, or apamin, a blocker of small conductance calcium-activated potassium channels. In

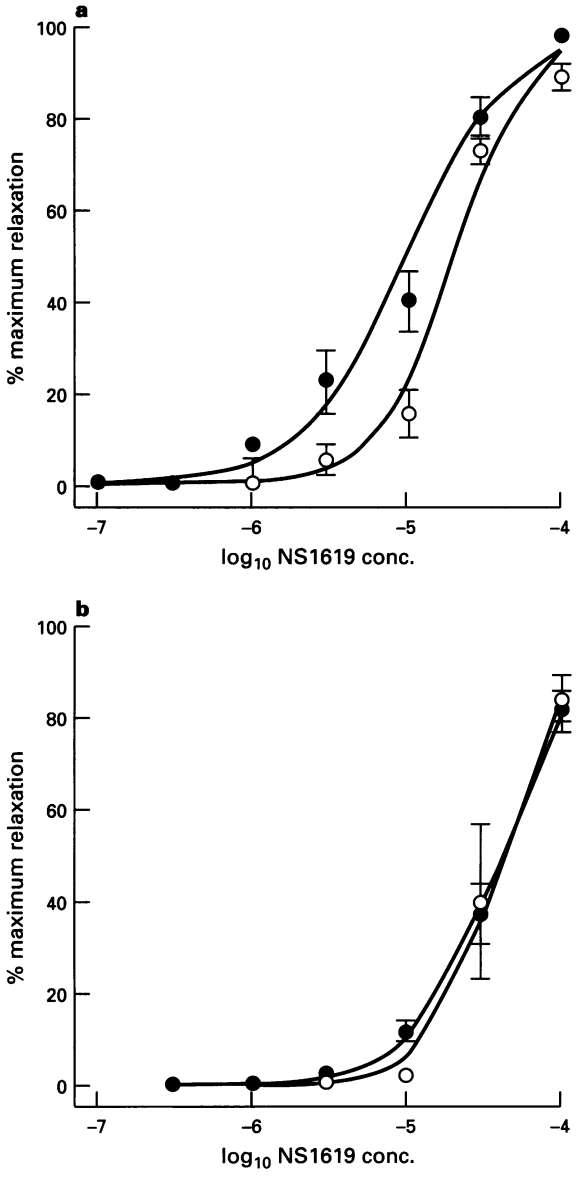

Figure 8 Relaxation of basilar artery rings by NS1619. (a) Concentration-effect curves to NS1619 against agonist- (1 μM histamine/2 μM 5-HT) supported tone (●) or agonist + 100 nM IbTX-supported tone (○). (b) Concentration-effect curves to NS1619 against 80 mM K^+-supported tone (●) or 80 mM K^+ + 100 nM IbTX-supported tone (○). Relaxant responses are expressed as a percentage of the maximum response to nifedipine and EGTA in the same arterial ring. Results expressed as mean ± s.e.mean; n of at least 4 for all results.

contrast, IbTX rapidly abolished the NS1619-activated current. Further evidence that the channel underlying these currents is BK_{Ca} comes from the amplitude of the unitary currents that could be resolved from whole cell recordings during the activation by NS1619. The mean unitary current amplitude was 5.6 pA at 0 mV with 6 mM K^+ externally, a value that agrees well with previous studies of BK_{Ca} channels in vascular smooth muscle (Langton *et al.*, 1991).

NS1619 increases IbTX-sensitive whole cell currents activated by depolarization

The open probability of BK_{Ca} channels is voltage-, as well as calcium-dependent, increasing with membrane depolarization. However, unlike other voltage-dependent K^+ channels they do not show voltage-dependent inactivation (Latorre *et al.*, 1989). Delayed rectifier (K_V) channels in our cells were strongly inactivated by depolarized holding potentials, and so we were able to isolate BK_{Ca} currents activated by step depolarization by using a holding potential of −15 mV. This leads to almost complete inactivation of K_V, such that BK_{Ca} channels dominate the outward currents. Under these conditions NS1619 greatly increased the outward currents (Figure 3b and c), this increase was blocked by IbTX. Although it is clear that NS1619 can shift the activation properties of BK_{Ca} channels, allowing channels to be activated at more negative potentials; the dual modulation of these channels makes it unclear whether NS1619 is affecting the calcium or voltage-sensitivity of the channel. However, a recent study of BK_{Ca} activation by the fenamates reported that the mechanism of action of these compounds was by increasing the calcium-sensitivity, rather than the voltage-sensitivity of the channel (Ottalia & Toro, 1994). Whether a similar mechanism explains our results will require further experimentation.

A possible site of action of NS1619

The activation of BK_{Ca} channels by NS1619 in intact cells could be explained in a number of ways. NS1619 could produce activation by a direct effect on the channel or a closely associated site, or indirectly by causing release of calcium from intracellular stores. We found that NS1619 applied directly to the cytoplasmic face of inside-out patches or the external surface of outside-out patches activated BK_{Ca} channels. This observation agrees well with previous results obtained in bovine cultured aortic smooth muscle cells (Olesen *et al.*, 1994a) and suggests that NS1619 acts directly, either on the channel protein itself or on a closely associated membrane-bound component.

Under whole cell recording conditions there was a substantial delay (1–4 min) between the addition of NS1619 and the activation of an outward current. This agrees with previous reports (Edwards *et al.*, 1994; Olesen *et al.*, 1994a) where contact times with NS1619 of 2–6 min were reported prior to full levels of activation being produced. NS004, a compound structurally related to NS1619, has also been reported to have a slow onset of action (McKay *et al.*, 1994). Additionally, the effects of NS1619 were slow to wash off, as described by Olesen *et al.* (1994a). These slow responses to the application or removal of drug cannot be attributed to our perfusion system, as the cell under study was continuously superfused and exchanges of solutions were rapid (<2 s). This delay in channel activation in the whole cell (see Figure 2), an effect also observed in on cell recordings from coronary artery myocytes (Holland unpublished observation), contrasts with the very rapid activation of BK_{Ca} channels in excised patches (see Figure 4 and 5). A possible explanation for these results is that the site of action of NS1619 is intracellular, explaining the almost instantaneous activation seen using inside-out patches. The delay in activation in whole cells might therefore be explained by the requirement for NS1619 to cross the cell membrane. However, the reported high lipophilicity of NS1619 (Olesen, 1994) should mean that this compound would cross the membrane rapidly leading to channel activation. This predicted rapid activation was observed in experiments using outside-out patches (Figure 5). In this respect NS1619 differs from the recently described soyasaponin analogues which have been reported to activate BK_{Ca} only from the cytoplasmic membrane surface (McManus *et al.*, 1993). We suspect that the difference in activation between intact cells and outside-out patches is due both to an effective concentration of NS1619 being more rapidly achieved at the cytoplasmic face of the patch and to the higher calcium concentration present at the cytoplasmic face of the patches. Therefore we feel that an internal site of action of NS1619 best accounts for the results of our studies.

[Ca^{2+}] is required for the activation of BK_{Ca} by NS1619

A previous report (Edwards *et al.*, 1994), based on conventional whole cell recordings from rat portal vein smooth muscle cells using a calcium-free pipette solution buffered with EGTA, suggested that the activation of BK_{Ca} channels by NS1619 was independent of the presence of cytosolic calcium. This indicates that NS1619 could act as a substitute for calcium, in contrast to a previous report using cultured bovine aortic smooth muscle cells (Olesen *et al.*, 1994a). In our experiments using inside-out patches, calcium-free solutions always reversed channel activation even in the presence of high concentrations of NS1619. This difference can probably be explained by incomplete buffering of intracellular calcium in the region of the cell membrane when using the conventional whole cell configuration.

Inhibition of delayed rectifier potassium (K_V) channels by NS1619

In the present study NS1619 was noted to inhibit delayed rectifier potassium channels in a concentration-dependent manner. This inhibition of K_V by NS1619 was fast in onset readily reversible, and independent of voltage. This observation partially agrees with a previous report (Edwards *et al.*, 1994) that 10 μM NS1619 inhibited K_V currents in rat portal vein smooth muscle cells. However, in contrast to Edwards *et al.* (1994), who reported that an increase in the concentration of NS1619 above 10 μM reduced the inhibition of K_V currents, we found that 30 μM NS1619 further depressed K_V currents. The reasons for this discrepancy are not immediately obvious but, in the present study, we noted that, in spite of the presence of high extracellular concentrations of IbTX and dialysing the cell with 5 mM EGTA, at depolarized potentials in the presence of 30 mM NS1619 some BK_{Ca} activation can be seen (see Figure 6c). If, as seems likely, this also occurred in the experiments performed by Edwards *et al.* where only an extracellular toxin blocker was used to prevent BK_{Ca} activation, this might appear in the whole cell record as an apparent reduction in the level of inhibition of K_V currents as the concentration of NS1619 was increased.

Inhibition of Ba^{2+} currents by NS1619

Our results confirmed that NS1619 inhibits L-type Ca^{2+} currents, as first demonstrated by Edwards *et al.* (1994) in rat portal vein smooth muscle. NS004, a structurally related compound also described as an activator of BK_{Ca} channels (Olesen *et al.*, 1994b; McKay *et al.*, 1994), has also been reported to inhibit Ca^{2+} currents (Sargent *et al.*, 1993). This action of NS1619 was fast in onset, (maximum inhibition <1 min) and readily reversible. NS1619 blocked in a 1:1 fashion with an IC_{50} of 7 μM. This block did not show voltage-dependence.

NS1619-induced relaxation of basilar artery

A previous study (Edwards *et al.*, 1994) has reported that block of BK_{Ca} by specific inhibitors did not inhibit NS1619-induced relaxation and that the relaxation-induced by NS1619 does not involve the opeing of K^+ channels. Our results, obtained in a cerebrovascular artery, suggest that while K^+ channel opening is not necessary for NS1619-induced relaxation it may act to increase the relaxant potency of the compound. This result fits well with the electrophysiological results obtained in cells from this tissue where NS1619 appears to be slightly more potent as a blocker of $I_{Ca^{2+}}$ than as an opener of BK_{Ca}. The ability of the compound to cause relaxation of tone induced by 80 mM K^+, where the opening of K^+ channels could not lead to hyperpolarization, indicated that BK_{Ca} channel opening is not required for relaxation. However, under conditions where hyperpolarization can occur NS1619 appears to be a more potent relaxant. The sensitivity of this effect to IBTX indicates that this was due to activation of BK_{Ca} channels. This also indicates that of the mixed effects on K^+ channels observed in this tissue (BK_{Ca} activation/K_V inhibition) the opening of BK_{Ca} channels predominates in terms of the effect of this compound on outward current and hence membrane potential. This is also shown by the current clamp experiments where NS1619 always shifted membrane potentials towards E_K which would provide a further drive to reduce calcium influx through voltage-sensitive Ca^{2+} channels. This result differs from the reported effects of NS1619 in rat portal vein, a spontaneously active blood vessel, where it appears that the blockade of I_{KV} is the predominant effect leading to a net inhibitory effect of this compound on outward current (Edwards *et al.*, 1994). This may reflect a difference between these sites in the vascular system in their K^+ channel density or K^+ channel populations or to similar differences betwen phasic and tonic smooth muscles. These intriguing questions will only be answered by a more detailed study of ion channel populations throughout the vascular system.

In conclusion, in intact cells NS1619 could reduce the activity of Ca^{2+} channels by two routes; directly, by its Ca^{2+} channel blocking action, and indirectly by activating BK_{Ca} channels producing hyperpolarization, so decreasing the P_{open} of Ca^{2+} channels through their voltage-dependence. Functionally, both of these effects could contribute to its vasorelaxant properties. In cerebral arteries, as in rat portal vein, NS1619 appeared slightly more potent as a Ca^{2+} channel inhibitor than as a BK_{Ca} channel activator; such that this action may dominate its functional effects. Nevertheless, NS1619 is an effective BK_{Ca} channel activator and this accounts for its hyperpolarizing action in cerebral arterial smooth muscle cells. Activators of BK_{Ca} that do not have the additional inhibitory effects of NS1619 on K_V and Ca^{2+} channels would be of considerable experimental and potentially therapeutic interest.

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