Br. J. Pharmacol. (1994), **113**, 1538–1547

Ion channel modulation by NS 1619, the putative BK_{Ca} channel opener, in vascular smooth muscle

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1 The effects of NS 1619, the putative BK_{Ca} channel opener, were investigated on rat intact portal veins and on single smooth muscle cells enzymatically separated from the same tissue.

2 Under whole-cell patch clamp conditions with K-rich pipettes, exposure of single cells held at −10 mV to NS 1619 (10–33 μM) induced a noisy, outward current which reached a maximum (33 μM NS 1619; mean 35.8 ± 17 pA, $n = 8$) within about 6 min.

3 On stepping to test potentials (range −50 to +50 mV) from a holding potential of −10 mV, the NS 1619-induced noisy current exhibited time-dependent activation and marked outward rectification.

4 The stimulation of outward currents by NS 1619 at −10 mV was independent of the presence of Ca^{2+} in the bath or pipette solutions but was antagonized by either charybdotoxin (250 nM) or penitrem A (100 nM) in the bath solution.

5 Stationary fluctuation analysis of the noisy current induced by NS 1619 at −10 mV yielded a value of 70 ± 8 pS ($n = 4$) (under the quasi-physiological conditions of the experiment) for the unitary conductance of the channel involved.

6 At −10 mV, NS 1619 (10–33 μM) rapidly inhibited spontaneous transient outward currents.

7 With a holding potential of −90 mV, NS 1619 (10–33 μM) produced a reduction of outward currents evoked by depolarizing steps to +50 mV, an effect associated with marked inhibition of the delayed rectifier current, $I_{K(V)}$.

8 NS 1619 (3–100 μM) produced a concentration-dependent inhibition of spontaneous activity in rat portal vein characterized by a reduction in the amplitude and duration of the tension waves. This inhibition was slightly potentiated in the presence of either charybdotoxin (250 nM) or penitrem A (1 μM). NS 1619 also totally inhibited contractions of rat aorta induced by KCl (both 20 mM and 80 mM).

9 Under whole-cell recording conditions and using Cs-rich pipettes, Ca-currents evoked in portal vein cells by stepping from a holding potential of −90 mV to test potentials in the range −30 to +50 mV were totally inhibited in the presence of 33 μM NS 1619.

10 NS 1619 (33 μM) inhibited the induction of $I_{K(ATP)}$ by levcromakalim (10 μM).

11 It is concluded that NS 1619 activates the large conductance, Ca^{2+}-sensitive channel, BK_{Ca} and over the same concentration range it inhibits both K_V and L-type Ca-channels. The observed NS 1619-induced mechanical inhibition in rat portal vein and aorta seems most likely to be due to the observed inhibition of Ca-currents.

Keywords: NS 1619; charybdotoxin; penitrem A; levcromakalim; B_{KCa}; K_V; L-type Ca-channels; K_{ATP}; rat blood vessels; whole-cell patch clamp; relaxation

Introduction

Over the past few years, many agents have been described which can open plasmalemmal potassium (K) channels. The best known of these constitute the diverse chemical entities known as the *K-channel openers*, typified by levcromakalim, pinacidil and aprikalim. The target for these agents is the ATP- and glibenclamide-sensitive K-channel, K_{ATP} (see Edwards & Weston, 1993).

More recently, however, openers of the large conductance Ca^{2+}-sensitive K-channel (BK_{Ca}) have been described. These include the dehydrosaponins, the prototypes of which were extracted from the herb *Desmodium adscendens* (McManus *et al.*, 1993) and the benzimidazoles, initially exemplified by NS004. This agent hyperpolarizes neuroblastoma cells and increases the open probability of charybdotoxin-sensitive BK_{Ca} channels in inside-out membrane patches (Olesen & Wätjen, 1992). NS 1619, a close chemical relative of NS004, was recently shown to hyperpolarize bovine aortic smooth muscle cells and to shift the voltage activation characteristics of charybdotoxin-sensitive whole cell currents in a hyperpolarizing direction (Olesen *et al.*, 1994b).

The objective of the present study was to investigate further the profile of action of NS 1619 in rat vascular smooth muscle. Using both whole portal veins and freshly isolated single cells from this tissue together with segments of rat aorta, it was hoped to clarify the effects of this agent on both mechanical activity and on whole-cell K-currents. In further experiments, the action of NS 1619 on whole-cell Ca currents was also evaluated since inhibition of such currents by the related molecule, NS004, has been reported (Sargent *et al.*, 1993). A preliminary account of our observations has been presented to the British Pharmacological Society (Schneider *et al.*, 1994).

Methods

All experiments were performed on tissues removed from male Sprague-Dawley rats (100–125 g body weight), previously killed by stunning and bleeding. The whole-cell

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voltage-clamp experiments were performed on single smooth muscle cells isolated from portal veins while mechanical studies were carried out on both whole portal veins and segments of aorta.

Cell separation

After dissection, the portal veins were placed in a physiological salt solution (PSS) containing 0.5 mM Ca^{2+} and carefully cleaned of fat and connective tissue with fine scissors in conjunction with a dissecting microscope. Each vein was then opened along its longitudinal axis and transferred to an identical PSS to which collagenase and pronase had been added. The tissue was agitated for 21 min at 37°C and then washed in Kraftbrühe (Klöckner & Isenberg, 1985). The veins were then cut into approximately six segments and triturated in Kraftbrühe with a wide bore, smooth-tipped pipette. The dispersed cells were used for experiments within 8 h of separation, during which time they were stored at 6°C in Kraftbrühe.

Single-cell electrophysiology

Experiments were performed at 23°C using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). Patch pipettes were pulled from Pyrex glass (H 15/10, Jencons, UK) and had resistances of 3–4MΩ when filled with the internal (intracellular) solution. Voltage commands and data acquisition were performed as described by Noack *et al.* (1992). For cell stimulation and for recording and analysing data, the pCLAMP 5.5 programme was used (Axon Instruments, U.S.A.). Data acquisition and storage were as described by Ibbotson *et al.* (1993b).

As in previous studies, we used a double holding potential protocol (see Noack *et al.*, 1992; Edwards *et al.*, 1993) to distinguish between the time-dependent currents, $I_{K(A)}$ and $I_{K(V)}$, and the time-independent current, $I_{BK(Ca)}$. With Ca-PSS in the bath, $I_{K(A)}$, $I_{K(V)}$ and $I_{BK(Ca)}$ were all available on stepping from a holding potential of −90 mV to more positive potentials up to +50 mV. However, when cells were clamped at a holding potential of −10 mV, $I_{K(A)}$ and $I_{K(V)}$ became inactivated allowing the characteristics of $I_{BK(Ca)}$ alone to be examined by stepping to a series of potentials in the range −50 mV to +50 mV. In all experiments, the currents evoked by 500 ms voltage steps from the stated holding potential were measured at their peak level.

The effects of NS 1619, levcromakalim, charybdotoxin and penitrem A were each investigated by adding the appropriate amount(s) of these agents to the main reservoir containing the external solution to ensure that responses were obtained under steady-state conditions. The bath (volume: 1 ml) was continuously perfused (0.7 ml min^{-1}) with fresh solution using a pump (Microperpex, Pharmacia LKB); a second identical pump was used to remove excess solution from the recording chamber.

Whole-tissue mechanical studies

Whole portal veins were mounted in a Krebs PSS at 37°C under 10 mN tension for isometric recording. Whole dorsal aortae were cut into 4 segments which were each opened longitudinally and similarly mounted for isometric tension recording.

The tissues were allowed to equilibrate for approximately 45 min before they were exposed at 15 min intervals to increasing concentrations of NS 1619 added cumulatively. Modification of the relaxant effect of NS 1619 was determined by preincubating portal veins with charybdotoxin or penitrem A for 20 min before exposure to NS 1619 in the continuing presence of the modifying agent. Aortic contractions to 20 mM or 80 mM KCl (which were substituted for equimolar concentrations of NaCl in the Krebs PSS) were allowed to plateau before further addition of NS 1619.

Mechanical responses were recorded and this activity was integrated with respect to time by use of an Apple Macintosh computer in conjunction with MacLab hardware (MacLab 8) and software (Chart, version 2.5) (Analog Digital Instruments).

Drugs and solutions

Electrophysiological studies The low-Ca^{2+} PSS used during cleaning of the veins comprised (mM): KCl 130, $CaCl_2$ 0.05, taurine 20, pyruvate 5, creatine 5, HEPES 10, fatty acid-free albumin 1 mg ml^{-1}, buffered with methanesulphonic acid to pH 7.4. Collagenase (Type VIII, Sigma) 1 mg ml^{-1} and pronase (Calbiochem) 0.2 mg ml^{-1} were added to this solution for cell separation. Kraftbrühe comprised (mM): KCl 85, KH_2PO_4 30, $MgSO_4$ 5, Na_2ATP 5, K-pyruvate 5, creatine 5, taurine 20, β-OH-butyrate 5, fatty acid-free albumin 1 mg ml^{-1}, pH adjusted to 7.2 with KOH.

Two bath (external) solutions were used for the measurement of K-currents. One of these was a calcium-containing PSS (Ca^{2+}-PSS) comprising (mM): NaCl 125, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11, HEPES 10, buffered with NaOH to pH 7.30; aerated with O_2. The composition of the Ca^{2+}-free bath solution (Ca^{2+}-free PSS) was similar except that the $CaCl_2$ was omitted. The $MgCl_2$ concentration was increased to 3.7 mM to compensate for the loss of divalent cations and 1.0 mM EGTA (ethylene glycol-*bis* β-aminoethyl ether tetraacetic acid) was also added. Ca-PSS was used during the measurement of Ca-currents.

Two pipette (internal) solutions differing in their respective Ca^{2+} concentrations were employed in the study of K-currents. The Ca-containing pipette solution contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, HEPES 10, glucose 11, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, buffered to pH 7.30 with KOH. Using fura-2 and atomic absorbtion spectrophotometry, the estimated free Ca^{2+} concentration of this solution was 1 μM derived from the Ca^{2+} contamination of the constituents. The Ca^{2+}-free pipette solution was prepared similarly but the addition of 1.2 mM EGTA which yielded a free Ca^{2+} concentration of <1 nM (fura-2). A third, caesium-rich pipette solution was used for measurement of Ca^{2+} currents. This was similar to the Ca^{2+}-free pipette solution, except that the KCl and K_2HPO_4 were each replaced by equimolar concentrations of CsCl and the pH of the solution was adjusted to pH 7.30 with CsOH.

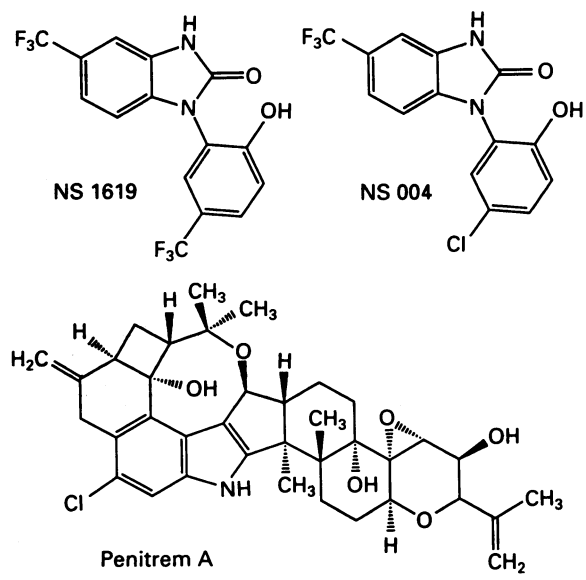

Figure 1 Structural formulae of some modulators of BK_{Ca} described in the text.

Organ bath studies The physiological salt solution used for the whole-tissue mechanical studies comprised (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1. This solution was gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C.

NS 1619 (Figure 1, NeuroSearch), levcromakalim (Pfizer Central Research) and penitrem A (Figure 1) were each first dissolved in dimethyl sulphoxide (DMSO) to produce a concentrated stock solution (20 mM) from which dilutions were prepared with bath solution immediately before they were required. Synthetic charybdotoxin (Latoxan) was dissolved in glucose-free bath solution (pH 7.30) and used immediately. Unless otherwise stated, all reagents and compounds were obtained from Sigma.

Data analysis

Treatment effects were analysed by 2-way within subject (repeated measures) ANOVA (Statistica v.3.0a:Statsoft) or Student's two-tailed paired t test as appropriate. P values less than 0.05 were considered to be significant.

Results

Characteristics of NS 1619-induced changes at a holding potential of − 10 mV

General effects When the whole-cell recording mode was achieved with a pipette solution containing 1 μM Ca^{2+} and a Ca^{2+}-free PSS in the bath, the dominant currents observed on stepping from a holding potential of − 90 mV to a series of more positive test potentials were $I_{K(A)}$ and $I_{K(V)}$ (see methods). $I_{K(A)}$ activated and inactivated rapidly (within 200 ms at − 20 mV) and was only visible at test potentials within the range − 30 mV to 0 mV. At more positive voltages this current was masked by the larger current component, $I_{K(V)}$, which activated more slowly than $I_{K(A)}$ and which inactivated only slightly over the period of the test pulse (Figure 2a). At a holding potential of − 10 mV $I_{K(A)}$ and $I_{K(V)}$ inactivated, and under this condition these time-dependent currents were essentially abolished (Figure 2b). On changing the bath solution to Ca^{2+}-PSS, a larger, noisy, time-dependent, outwardly rectifying current was generated with an identical test protocol and a holding potential of − 10 mV (compare mean currents, Figures 2b and 3a). This current showed no signs of inactivation even after holding for several minutes at positive potentials (data not shown). Under these conditions, spontaneous transient outward currents (STOCs) were observed in approximately 20% of cells (Figure 3c).

Exposure to NS 1619 (10–33 μM) immediately abolished any STOCs which were present and there was a gradual increase in the level of outward current. This reached a maximum (33 μM NS 1619; mean 35.8 ± 17 pA, $n = 8$) within about 6 min and was associated with a marked increase in current noise (Figure 3c). On stepping to test potentials in the range − 40 mV to + 50 mV, the large, noisy, time-dependent, non-inactivating, outwardly rectifying current (Figure 3a) was markedly enhanced by NS 1619 in a concentration-dependent manner (mean currents in 33 μM NS 1619, Figure 3b; $I-V$ relationships for 10 and 33 μM NS 1619, Figure 4a). In the presence of 33 μM NS 1619, the zero current potential was shifted from − 35.5 ± 6.6 mV to − 38.4 ± 4.9 mV ($P < 0.05$, $n = 8$).

Since the noisy, outwardly-rectifying current was non-inactivating, the effects of NS 1619 cannot be attributed to modification of inactivation characteristics. Thus, to determine whether there was an NS 1619-induced shift in the *activation* threshold of the currents available on stepping from − 10 mV, the net effects of two concentrations of NS 1619 (10 μM and 33 μM) were calculated by subtracting the control currents (Figure 3a and open circles, Figure 4a) from the currents available in the presence of these two concentrations of NS 1619 (closed symbols, Figure 4a). It can be clearly seen that NS 1619 did indeed shift the activation threshold of the non-inactivating current to more negative values ($P < 0.05$, Figure 4b).

In the absence of Ca^{2+}, i.e. with Ca^{2+}-free PSS in the bath and a Ca^{2+}-free pipette solution, STOCs were never detected but the ability of NS 1619 to enhance outward currents was

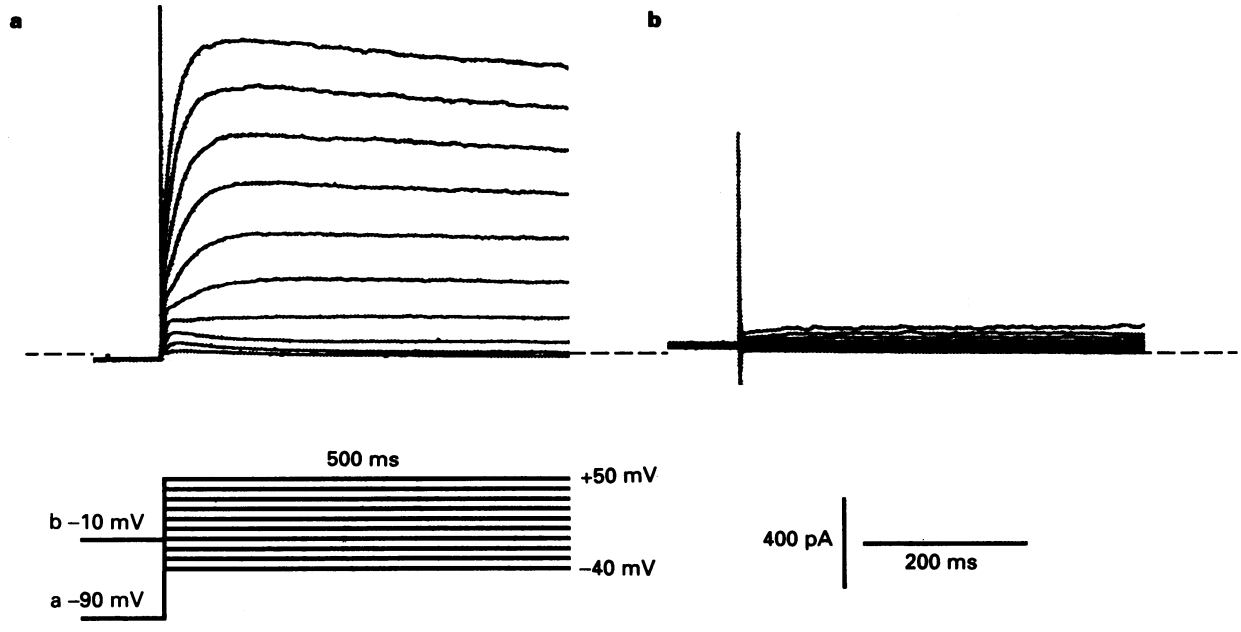

Figure 2 Effect of holding potential on whole-cell currents available in rat portal vein cells bathed in Ca-free PSS. The pipette solution contained approximately 1 μM calcium and no EGTA. (a) Cells were held at − 90 mV and stepped to test potentials from − 40 to + 50 mV in 10 mV increments. $I_{K(A)}$ activated and inactivated rapidly and was visible in the range − 30 mV to 0 mV. At more positive potentials, $I_{K(V)}$ masked all other currents and inactivated only slowly during the test pulse. (b) At a holding potential of − 10 mV, $I_{K(A)}$ and $I_{K(V)}$ inactivated leaving only low-amplitude, non-inactivating currents. Each trace represents the computer-derived mean from the same 5 cells under the two conditions. In all traces, zero current is indicated by a dashed line.

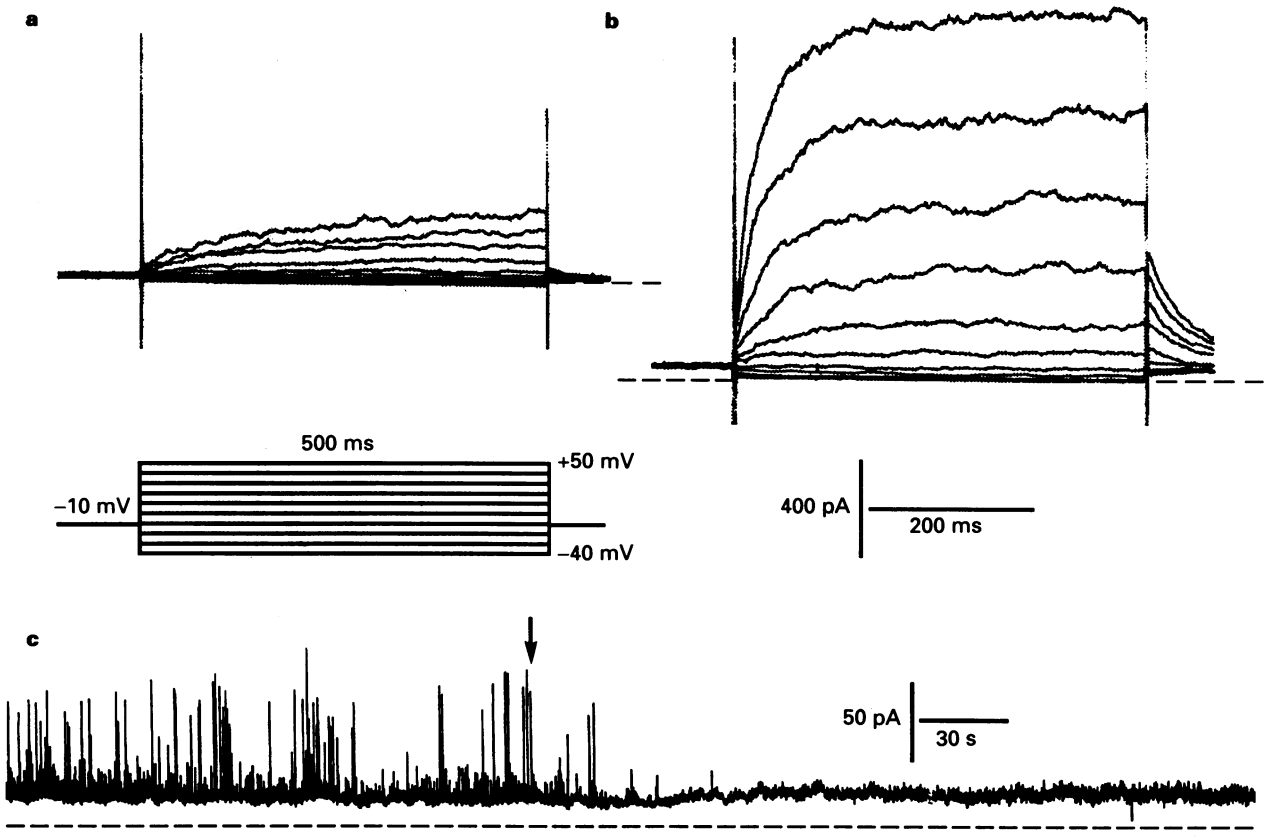

Figure 3 Effect of calcium and NS 1619 on whole-cell currents available from − 10 mV in rat portal vein cells. The pipette solution contained approximately 1 μM calcium and no EGTA. (a) When cells were bathed in Ca^{2+}- PSS, a noisy, outwardly-rectifying, time-dependent current was obtained on stepping from a holding potential of − 10 mV to test potentials from − 40 to + 50 mV in 10 mV increments. (b) Subsequent exposure to 33 μM NS 1619 produced a further increase in this current, an effect which was particularly striking at test potentials more depolarized than − 10 mV. Each trace represents the computer-derived mean from the same 5 cells under the various conditions. (c) In the presence of 2.5 mM calcium, spontaneous transient outward currents (STOCs) were evident in some cells under whole-cell recording conditions at a holding potential of − 10 mV. In the single example shown, exposure to 20 μM NS 1619 (arrow) rapidly inhibited the STOCs. The onset of the gradual development of a noisy, outward current is also visible. In all traces, zero current is indicated by a dashed line.

still observed. Thus, under these conditions, 33 μM NS 1619 produced an increase in the noisy, time-dependent, outwardly-rectifying current identical (ANOVA, $P>0.05$) to that seen in the presence of Ca^{2+} (compare the $I-V$ relationships, open and closed inverted triangles in Figure 4b).

Effects of charybdotoxin and penitrem A

With Ca^{2+}-PSS in the bath, exposure to 250 nM charybdotoxin abolished any STOCs which were present (data not shown) and totally inhibited the stimulation of the noisy, outwardly rectifying current by 10–33 μM NS 1619 ($I-V$ relationships for 20 μM NS 1619, Figure 4c). With Ca^{2+}-free PSS in the bath, the current induced by 33 μM NS 1619 was also abolished by 250 nM charybdotoxin (data not shown).

These results, together with the electrophysiological characteristics of the changes induced by NS 1619 strongly indicated that the current involved was $I_{BK(Ca)}$. To confirm this view, experiments were also carried out with 100 nM penitrem A, a recently-described, selective inhibitor of BK_{Ca} (Knaus *et al.*, 1994). In the presence of this agent, the currents induced by NS 1619 (33 μM) were totally inhibited ($I-V$ relationships, Figure 4d).

Effects of NS 1619 on currents available from a holding potential of − 90 mV

On stepping from a holding potential of − 90 mV, the evoked currents comprised $I_{K(A)} + I_{K(V)} + I_{BK(Ca)}$ but because of the very rapid inactivation of $I_{K(A)}$ (see Figure 2), this current did not contribute to the measured peak current which comprised approximately 74% $I_{K(V)}$ and 26% $I_{BK(Ca)}$. With Ca^{2+}-PSS as the bath solution and 1 μM Ca^{2+}-containing PSS in the pipette, NS 1619 (10–33 μM) *inhibited* the total currents evoked on stepping from − 90 mV to the range of test potentials ($I-V$ relationships, Figure 5a). Since NS 1619 increases $I_{BK(Ca)}$ (Figures 3 and 4), these inhibitory effects strongly indicate an inhibition of $I_{K(V)}$. This action was confirmed by exposing cells to 20 μM NS 1619 in the absence of Ca^{2+} from the bath and pipette together with the presence of charybdotoxin to eliminate any effects of NS 1619 on $I_{BK(Ca)}$ ($I-V$ relationships, Figure 5b). To clarify the *resultant* effect of NS 1619-induced inhibition of $I_{K(V)}$ and activation of $I_{BK(Ca)}$, difference curves were constructed from the data presented in Figure 5a (Figure 5c). These clearly show that 10 μM and 33 μM NS 1619 each produced an overall *inhibitory* effect on K-currents, the degree of which depended not only on the extent to which $I_{K(V)}$ was compromised but also on the degree of stimulation of $I_{BK(Ca)}$.

Noise analysis of current stimulated by NS 1619

Using stationary fluctuation analysis of the basal level of current noise and of that in the presence of 33 μM NS 1619, we estimated the unitary current and unitary conductance of those ionic channels which carried the NS 1619-induced outward current. This procedure was performed for a membrane potential of − 10 mV to allow a sufficient number of the

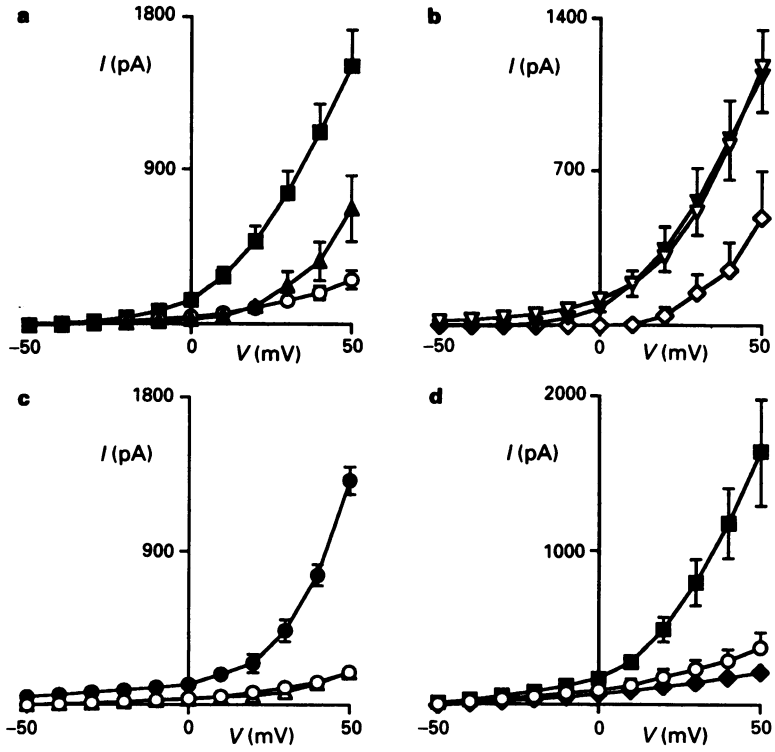

Figure 4 Effect of NS 1619 on currents available on stepping from − 10 mV. (a) with Ca-PSS in the bath a small outwardly-rectifying current was evident under control conditions (○; $n = 16$). This current was increased in a concentration-dependent manner by 10 μM (▲, $n = 5$) and 33 μM (■, $n = 8$) NS 1619. (b) Subtraction of the control current at each test potential from the corresponding current in the presence of NS 1619 (10 μM, ◇; 33 μM, ▼) shows the current, $I_{BK(CA)}$ induced by the drug. Note that increasing the concentration of NS 1619 shifts the activation potential of $I_{BK(Ca)}$ in a hyperpolarizing direction. The magnitude of $I_{BK(Ca)}$ in the presence of 33 μM NS 1619 but under calcium-free conditions (EGTA in bath and pipette solutions; ▽, $n = 5$) is shown for comparison. (c) With calcium in the bath and pipette solutions (control, ○), the effect of 20 μM NS 1619 (●) was completely inhibited when 250 nM charybdotoxin was present in the bath (△) ($n = 5$). (d) The effect of 33 μM NS 1619 was totally inhibited by 100 nM penitrem A (control, ○; 33 μM NS 1619, ■; NS 1619 + penitrem A, ◆; $n = 5$).

target channels to be opened by NS 1619 but to keep the total open probability relatively low (<0.1; see Figure 4 for the voltage-dependency). Interference between the NS 1619-induced outward current and the other currents modified by this drug ($I_{K(V)}$ and the calcium current, I_{Ca}; see Figure 5 and Figure 7, respectively) can be excluded since both $I_{K(V)}$ and I_{Ca} inactivate at − 10 mV and neither a shift of the mean current (μ) nor a change of the current variance (σ^2) would result from inhibition of either $I_{K(V)}$ or I_{Ca} or both of these currents.

Under control conditions, the background noise ranged from 2.5–3.5 pA, giving a current variance of 6.25–12.25 $(pA)^2$, respectively. In the presence of NS 1619, background current increased with an accompanying increase in noise and the mean quotient $(\Delta\sigma^2)/\Delta\mu)$ was 2.7 pA ($n = 4$). This value represents the mean current carried by the K-channels opened by NS 1619 under a normal K-gradient and at a potential of − 10 mV. Application of the Goldman-Hodgkin-Katz equation yielded a value for the single channel conductance of 70 ± 8 pS ($n = 4$).

Effects of NS 1619 on mechanical activity in rat blood vessels

The characteristic spontaneous mechanical activity of rat portal vein was inhibited by NS 1619 (3 to 100 μM) in a concentration-dependent manner. This was characterized by a gradual reduction in the amplitude and duration of the tension waves with little effect on their frequency. Attempts were made to antagonize the effects of NS 1619 with either charybdotoxin (250 nM) or penitrem A (1 μM). Both inhibitors increased the integrated spontaneous mechanical activity of the portal vein (by 140 ± 24%, charybdotoxin, $n = 4$ and by 82 ± 25%, penitrem A, $n = 4$). However, the relaxant effects of NS 1619 were not inhibited but were slightly potentiated by both inhibitors (Figure 6a,b), suggesting that NS 1619-induced activation of BK_{Ca} was not solely responsible for the observed mechano-inhibitory effects of this agent. We therefore investigated the possibility that NS 1619 might relax smooth muscle by a mechanism independent of K-channel opening using segments of rat aorta contracted with either 20 mM or 80 mM KCl. Under each of these conditions, NS 1619 produced full inhibition of the contractions induced by both 20 mM and 80 mM KCl (Figure 6c).

Effects of NS 1619 on calcium currents

The ability of NS 1619 to relax an 80 mM KCl-induced contraction together with the failure of either charybdotoxin or penitrem A to antagonize the mechano-inhibitory effects of this agent suggested that NS 1619 might inhibit calcium currents. This possibility was reinforced by the report that the closely-related molecule NS 004 (Figure 1) inhibits L-type calcium currents in cardiac myocytes (Sargent *et al.*, 1993).

With Ca^{2+}PSS in the bath and a Cs-rich pipette solution, Ca-currents (Figure 7) were evoked in portal vein cells by stepping from a holding potential of − 90 mV to test potential in the range − 30 mV to + 40 mV. These currents were totally inhibited by 1 μM nifedipine (data not shown) and thus almost certainly represent Ca^{2+} flux through L-type calcium channels. In the presence of 33 μM NS 1619 these currents were essentially totally inhibited by NS 1619 (33 μM) within a period of 10 min ($I-V$ relationship, Figure 7b).

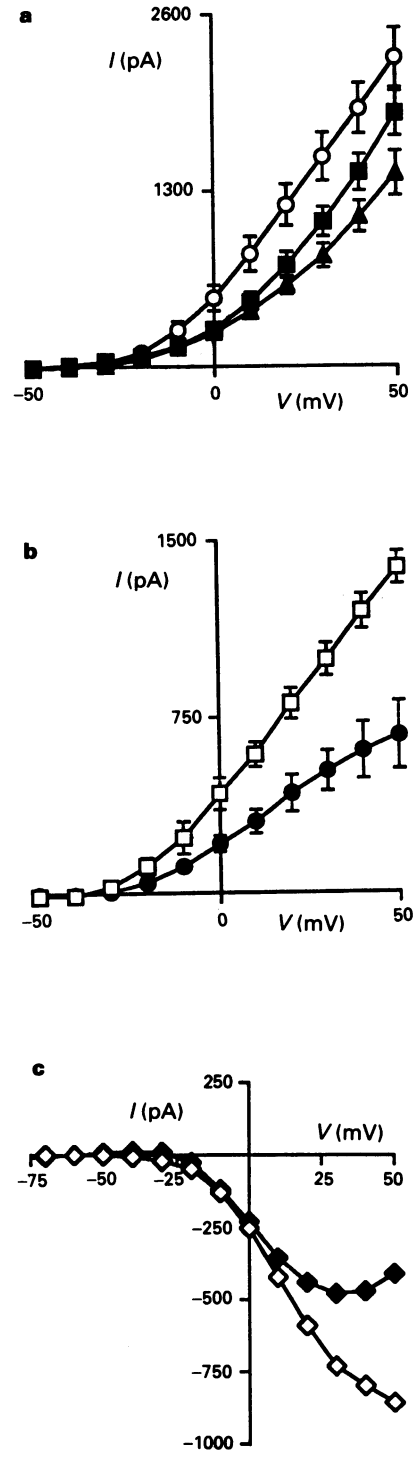

Figure 5 Effect of NS 1619 on currents available from − 90 mV with Ca-PSS in the bath. (a) An outwardly-rectifying current which activated at − 35 mV was evident under control conditions (○; $n = 16$). This total current was inhibited by 10 μM NS 1619 (▲, $n = 5$) although 33 μM NS 1619 (■, $n = 8$) produced a smaller inhibition. (b) In the presence of charybdotoxin (to eliminate $I_{BK(Ca)}$), the inhibitory effect of NS 1619 on $I_{K(V)}$ was evident (control, □; 20 μM NS 1619, ●, $n = 4$). (c) Transformation of data presented in (a) to illustrate more clearly the effect of NS 1619 on total currents. Subtraction of the control current at each test potential from the corresponding current in the presence of NS 1619 (10 μM, ◇; 33 μM, ◆) shows that the drug inhibited a current which activated at approximately − 35 mV. There was little difference in the magnitude of inhibition of current by 10 μM or 33 μM NS 1619 over the range of test potentials from − 40 mV to 0 mV. At more positive test potentials the inhibitory effect of 33 μM NS 1619 was reduced because of its comparatively larger stimulatory effect on $I_{BK(Ca)}$ (see Figure 4).

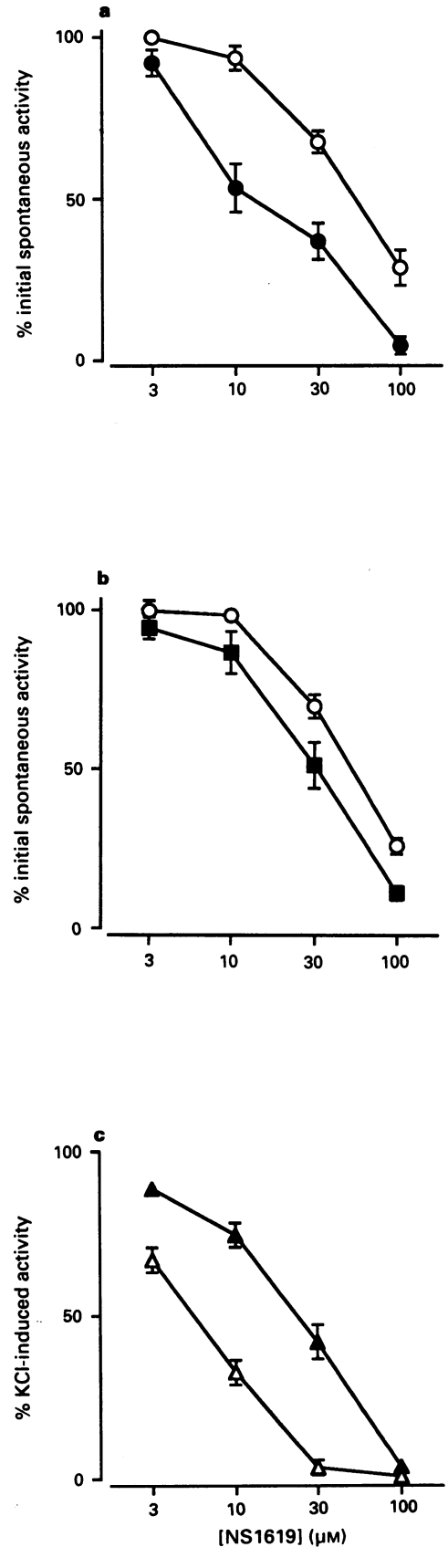

Figure 6 Mechano-inhibitory effect of NS 1619 in rat portal vein and aorta. NS 1619 produced a concentration-dependent inhibition of spontaneous activity in rat portal veins (○; a,b). This effect was not antagonized in the presence of either 250 nM charybdotoxin (●; a) or 1 μM penitrem A (■; b). (c) In rat aorta, NS 1619 fully inhibited contractions induced by either 20 mM (△) or 80 mM (▲) KCl ($n = 4$).

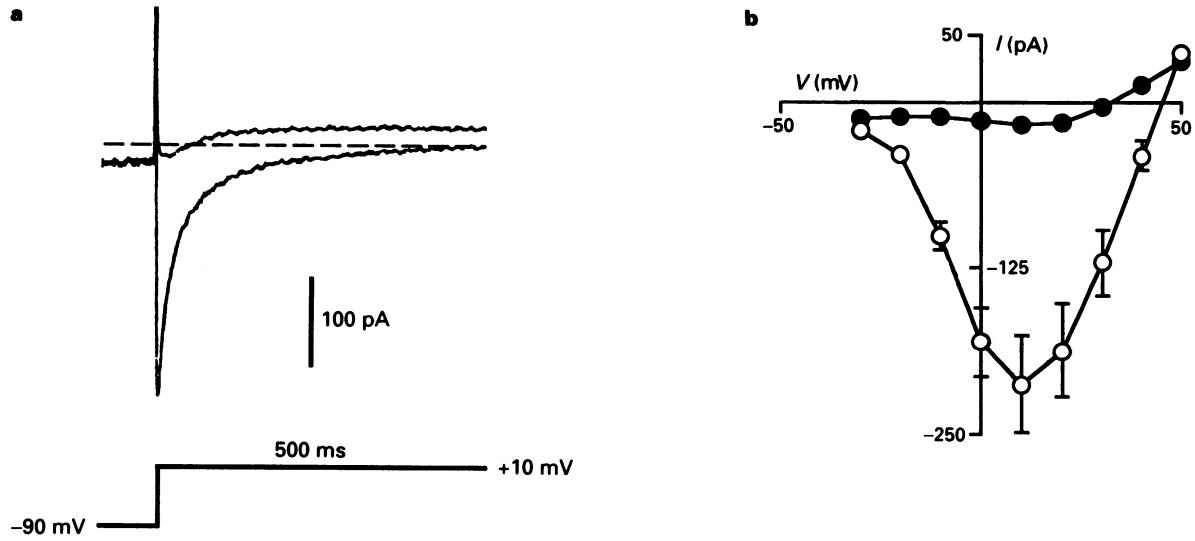

Figure 7 Effect of NS 1619 on calcium currents in rat portal vein cells. (a) When cells were held at − 90 mV then stepped to + 10 mV for 500 ms a rapidly-activating and -inactivating calcium current was observed (○). This current was abolished by exposure to 33 μM NS 1619 (●). (b) Current-voltage relationship for peak calcium currents evoked on stepping (at 8 s intervals) from a holding potential of − 90 mV to a series of test potentials in the absence (○) or presence (●) of 33 μM NS 1619 ($n = 3$).

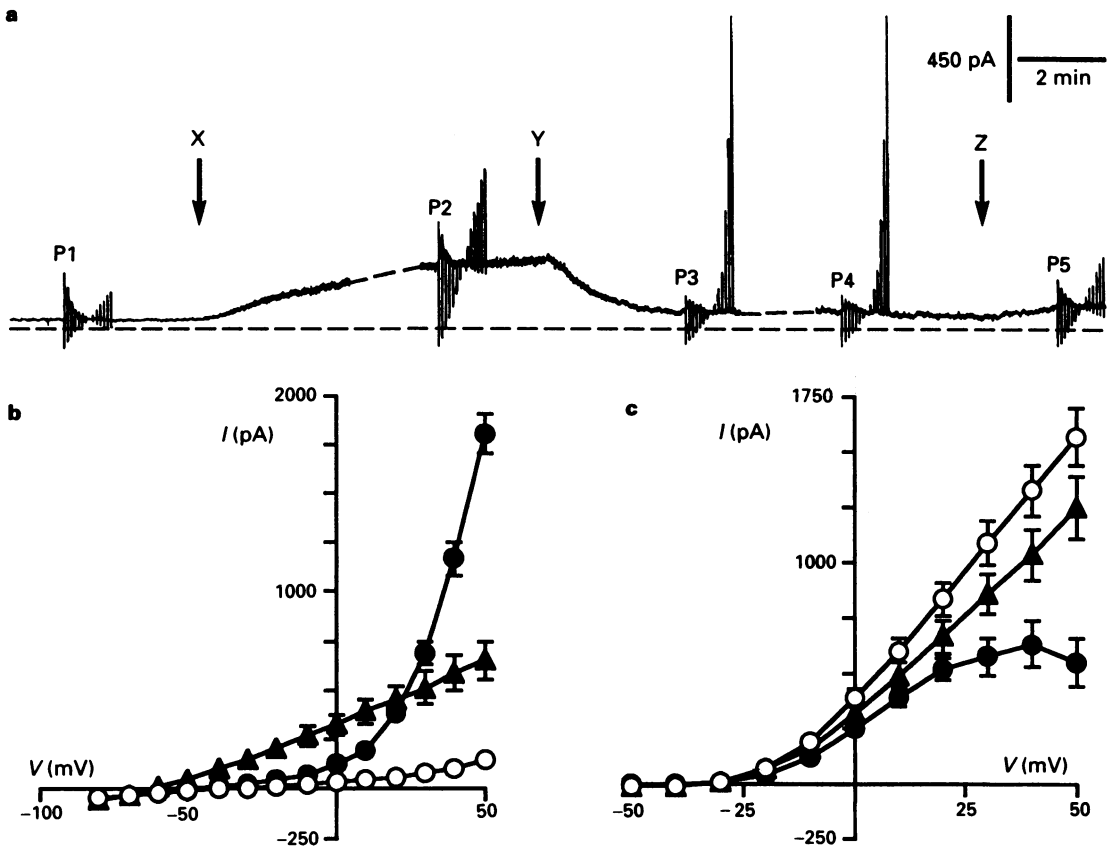

Figure 8 Inhibition of effects of levcromakalim by NS 1619. (a) Recording from a single cell clamped at a holding potential of − 10 mV under calcium-free whole-cell recording conditions. On stepping to a series of test potentials ranging from − 90 mV to + 50 mV in 10 mV increments (P1), a non-inactivating, voltage-insensitive current with a reversal potential of − 40 mV was obtained. On exposure to 10 μM levcromakalim (X) there was a marked increase in the holding current and application of the test potentials (P2) yielded larger, but still voltage-insensitive, currents with a reversal potential of approximately − 60 mV. In the continued presence of levcromakalim, 33 μM NS 1619 (Y) produced a rapid inhibition of the levcromakalim-induced increase in the holding current. The reversal potential became less negative and there was a marked increase in the voltage-dependent outward current component, $I_{BK(Ca)}$ (P3, P4). On washout (Z), the effects of NS 1619 on $I_{BK(Ca)}$ were rapidly reversed (P5). (b) Currents obtained at test potentials in 4 such experiments when cells were stepped from a holding potential of − 10 mV; (○) control, P1; (▲) levcromakalim, P2; (●) levcromakalim + NS1619, P4. (c) Shows inhibition of $I_{K(V)}$ (calculated by subtracting currents at each test potential after stepping from − 10 mV from those obtained on stepping from − 90 mV) by levcromakalim (▲) and further inhibition in the presence of levcromakalim + NS 1619 (●) ($n = 4$).

Interaction between NS 1619 and levcromakalim

Drugs like levcromakalim, which open the so-called ATP-sensitive K-channel (K_{ATP}) are inhibited by agents which inhibit $I_{K(V)}$ (Edwards & Weston, 1993, Ibbotson *et al.*, 1993a,b). Thus, the unexpected finding in the present study that NS 1619 was an inhibitor of $I_{K(V)}$ prompted an examination of the interaction between NS 1619 and levcromakalim.

As shown in Figure 8, cells were held at − 10 mV and first exposed to levcromakalim (10 μM) which produced a characteristic increase in current noise associated with the development of an outward current (Figure 8a). Stepping from the holding potential to a range of test potentials (P2, Figure 8a) confirmed that the levcromakalim-induced current was voltage- and time-independent with the previously-described characteristics of $I_{K(ATP)}$ ($I-V$ relationship, Figure 8b, see also Noack *et al.*, 1992; Edwards *et al.*, 1993). When this current had reached a peak, cells were additionally exposed to NS 1619 (33 μM) and after a short delay, $I_{K(ATP)}$ declined. Subsequent application of stepping voltage protocols (P3, P4, Figure 8a) confirmed that a current with the voltage-dependency and outwardly-rectifying characteristics characteristic of $I_{BK(Ca)}$ had indeed been activated $I-V$ relationship, Figure 8b). On wash-out of NS 1619, but in the continued presence of levcromakalim, the stimulation of $I_{BK(Ca)}$ was rapidly lost (within 2 to 3 min) whereas inhibition of the levcromakalim-induced current was maintained for up to 10 min (P5, Figure 8a). Consistent with our previously-described findings with levcromakalim (Noack *et al.*, 1992; Edwards *et al.*, 1993) and with NS 1619 (present study) use of depolarizing pulse protocols from a holding potential of − 90 mV (applied at the broken lines on the current trace in Figure 8a but removed for clarity) confirmed that both agents inhibited $I_{K(V)}$ ($I-V$ relationship, Figure 8c).

Discussion

Induction of $I_{BK(Ca)}$ by NS 1619

Exposure to NS 1619 of freshly-isolated portal vein cells, induced a noisy, time- and voltage-dependent, outwardly-rectifying current and stationary fluctuation analysis of the associated current noise was used to estimate the unitary conductance of the channel opened by NS 1619. This method may yield erroneous values unless the channel open probability is low and if other currents such as $I_{K(V)}$ and I_{Ca} are still present. We thus analysed current noise after holding at − 10 mV for several minutes under which conditions $I_{K(V)}$ and I_{Ca} are largely inactivated (see Figures 2 and 7). In addition, since − 10 mV was close to the activation threshold for BK_{Ca} in the present study, we estimate that the open probability of this channel would have been much less than 0.1 (see Figure 4 and Olesen *et al.*, 1994a). With this method an estimate for the unitary conductance underlying the NS 1619-induced current of 70 ± 8 pS were obtained (at − 10 mV and under the quasi-physiological conditions of the experiment, $n = 4$). When corrected for symmetrical (150 mM) K^+ conditions, this yielded a value of approximately 140 pS which lies within the range of values previously reported for BK_{Ca} in smooth muscle (Bolton & Beech, 1992). On the basis of this relatively large unitary conductance, together with its charybdotoxin and penitrem A sensitivity, we conclude that the NS 1619-induced current can be identified as $I_{BK(Ca)}$. This confirms the recent findings of Olesen *et al.* (1994a) obtained using cultured bovine aortic smooth muscle. As in these cultured cells, NS 1619 produced a concentration-dependent increase in $I_{BK(Ca)}$ in rat portal vein cells, apparently by shifting the $I_{BK(Ca)}$ − voltage relationship to more negative levels. This shift did not appear to be primarily due to a change in the calcium-sensitivity of BK_{Ca} since, in the present study, 33 μM NS 1619 stimulated similar increases in the magnitude of $I_{BK(Ca)}$ in the presence or absence of calcium.

Inhibition of $I_{K(V)}$ by NS 1619

In both the present investigation and in that of Olesen *et al.* (1994a), the effects of NS 1619 on $I_{BK(Ca)}$ were measured after optimising the conditions for the detection of this current. However, an important finding of the present study was that over a similar range of concentrations, NS 1619 not only enhanced $I_{BK(Ca)}$ but also inhibited $I_{K(V)}$. Thus, despite the magnitude of the stimulatory effect of NS 1619 on $I_{BK(Ca)}$ (measured from − 10 mV), a comparison of the total evoked currents before and after exposure to NS 1619 showed that the compound had a net *inhibitory* effect on the total outward current (measured after stepping from − 90 mV). Since BK_{Ca} is clearly opened by NS 1619, the inhibition of the total current strongly suggests a very substantial inhibition of $I_{K(V)}$ which was confirmed by experiments performed in the presence of charybdotoxin (in which $I_{BK(Ca)}$ was abolished), thus simplifying the determination of $I_{K(V)}$.

This inhibition contrasts with the reported lack of effect of NS 1619 on $I_{K(V)}$ in neuronal cells (Olesen *et al.*, 1994a) and the reasons for this discrepancy are not clear. However, numerous gene products have been identified which associate into homo- or heteromultimers to produce voltage-sensitive K-channels with differing gating properties (see Pongs, 1993). Thus, the marked difference between the very rapid activation of neuronal delayed reactifier currents (Olesen *et al.*, 1994a) and the slower time-course of activation of $I_{K(V)}$ in smooth muscle (present study) may reflect differences in the multimeric composition of neuronal and smooth muscle delayed rectifier channels. If so, it would not be surprising if the underlying channels exhibited different drug sensitivities.

Relaxant effects of NS 1619 in whole tissues

The rat portal vein exhibits spontaneous mechanical activity. This is generated by the discharge of electrical multi-spike complexes which at their peak shift the membrane potential into the range − 10 to 0 mV (Southerton *et al.*, 1988). Since inhibitors of both $I_{K(V)}$ (phentolamine; Ibbotson *et al.*, 1993b) and $I_{BK(Ca)}$ (charybdotoxin and penitrem A; present study) increase spontaneous activity in portal vein, both K_V and BK_{Ca} are probably involved in the repolarization phase of such complexes. Thus, it was of interest to study the effects of NS 1619 on spontaneous mechanical activity in whole portal veins.

NS 1619 produced a concentration-dependent inhibition of spontaneous mechanical activity characterized by a reduction in tension wave amplitude and duration with little effect on wave frequency. Despite the findings from the whole-cell clamp experiments which indicated a net inhibitory action on outward currents in single cells, we initially assumed that the mechanical inhibition of a whole vein must somehow reflect a dominant opening effect of NS 1619 on BK_{Ca} (since inhibition of K_V should depolarize cell membranes and produce conditions favourable for an increase in the frequency and magnitude of the spontaneous contractions). However, the relaxant effect of NS 1619 was not reduced following preincubation of the vessels with either charybdotoxin or penitrem A at concentrations identical to, or even greater than, those which totally inhibited $I_{BK(Ca)}$ in the presence of NS 1619 (whole-cell recording, present study.)

A further indication that the relaxant effect of NS 1619 was not due to the opening of K-channels was provided by the finding that NS 1619 produced a full relaxation of rat aorta precontracted with 80 mM KCl (see Hamilton *et al.*, 1986). Based on these observations, and the finding that NS 004 (a compound structually very similar to NS 1619; see Figure 1) inhibited L-type calcium channels (Sargent *et al.*, 1993), we considered the possibility that NS 1619 might also inhibit plasmalemmal calcium channels.

Inhibition of calcium-currents and STOCs by NS 1619

Using the whole-cell recording configuration, we found that NS 1619 (33 μM) was an effective inhibitor of nifedipine-sensitive calcium currents (I_{Ca}) in portal vein. This effect was not readily reversible, in contrast to the actions of NS 1619 on $I_{BK(Ca)}$ and $I_{K(V)}$ which were rapidly reversed on washout of the drug. In tissue-bath experiments, the inhibition of portal vein spontaneous mechanical activity by NS 1619 was also only slowly reversible and it thus seems likely that the observed inhibition of I_{Ca} rather than activation of $I_{BK(Ca)}$ plays the major role in the spasmolytic effect of NS 1619.

One interesting and unexpected observation was the rapid inhibition of STOCs by NS 1619. These currents are thought to be carried by BK_{Ca} channels which open in response to transient increases in $[Ca^{2+}]_i$ released spontaneously from inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular Ca-stores close to the membrane (Benham & Bolton, 1986; Ohya *et al.*, 1988; Bolton & Lim, 1989). STOCs are only observed when Ca^{2+} is present extracellularly (Hume & Leblanc, 1989) and they may represent a pathophysiological phenomenon associated with Ca^{2+} leak from the extracellular medium into the cell through the gigaseal between cell and patch pipette (Bolton, personal communication). In favour of this is the finding in intact single jejunal cells that the $[Ca^{2+}]_i$ measured by use of the dye Indo-1 was significantly lower than that in the same cells held under whole-cell clamp conditions (Pacaud & Bolton, 1991).

Irrespective of the mechanism involved in the induction of STOCs, the importance of Ca^{2+} release from stores in their production (Benham & Bolton, 1986; Ohya *et al.*, 1988; Bolton & Lim, 1989) makes these currents sensitive indicators of Ca^{2+} release from stores. Their inhibition by NS 1619 could indicate that this compound modifies intracellular Ca^{2+} release from, or sequestration into IP_3 sensitive Ca-stores. Alternatively, if Ca^{2+} entry *via* L-type Ca-channels is involved, inhibition of these by NS 1619 (see above) would inhibit STOC production. These alternatives and their possible contribution to the mechano-inhibitory action of NS 1619 are the subject of further study.

Interaction between NS 1619 and levcromakalim

In the present study, NS 1619 inhibited $I_{K(ATP)}$ induced by levcromakalim, a property shared by chemically-diverse inhibitors of the smooth muscle $I_{K(V)}$ such as phentolamine, guanethidine and ciclazindol (see Edwards & Weston, 1993). To account for this and for the finding that procedures designed to reduce intracellular ATP simultaneously inhibit $I_{K(V)}$ (rat portal vein, Noack *et al.*, 1992; rabbit atria, Ogbaghebriel & Shrier, 1994; frog skeletal muscle, Fink & Wettwer, 1978), we proposed that K_{ATP} and K_V might be different phosphorylated states of the same channel (Edwards *et al.*, 1993). Recently, however, an inwardly-rectifying K-channel inhibited by micromolar concentrations of intracellular ATP and opened by (50 μM) pinacidil has been cloned from rat heart (Ashford *et al.*, 1994). This channel, which seems likely to be the cardiac K_{ATP}, comprises α-subunits consisting of only two membrane-spanning segments linked by a pore region, a structure which shares homology with the S5-pore-S6 region of K_V (J. Adelman, personal communication). Thus the pharmacological features which led us to propose that K_{ATP} and K_V might be two states of a single channel (Edwards *et al.*, 1993) may rather indicate that modulators of K_{ATP} and K_V directly or indirectly interact with sites commmon to both channel types (and which probably lie in the vicinity of their respective pore-forming regions). In favour of this is the recent finding that deletion of four transmembrane segments (S1 to S4) of the *delayed rectifier*, K_V (leaving the S5-pore-S6 structure), produces subunits which associate to form K-selective, *inwardly-rectifying* channels (Tytgat *et al.*, 1994).

Conclusions

The present study has shown that the pharmacology of NS 1619 is more complex than originally described by Olesen *et al.* (1994a). Our observation that NS 1619 can inhibit calcium currents is perhaps not surprising in view of the fact that the closely-related benzimidazole NS 004 (Figure 1) exhibits this property (Sargent *et al.*, 1993). However, an unexpected finding was the NS 1619-induced inhibition of $I_{K(V)}$ and $I_{K(ATP)}$, a property shared by many structurally-diverse molecules (see Edwards & Weston, 1993). The ability of NS 1619 simultaneously to open BK_{Ca} and to *inhibit* L-type calcium channels is not unique but is also shared by niguldipine (Klöckner *et al.*, 1989). Furthermore, BK_{Ca} is activated by the chloride channel inhibitor, niflumic acid (Toro *et al.*, 1993). Other agents also exhibit similarly diverse properties. Thus, openers of K_{ATP} also inhibit K_V (levcromakalim, aprikalim, P1060: Edwards *et al.*, 1993; Ibbotson *et al.*, 1993b) and chloride channels (levcromakalim, P1075, minoxidil sulphate and diazoxide: Sheppard & Welsh, 1993; Holevinsky *et al.*, 1994). It is not yet clear whether the multiple excitatory and inhibitory effects of these agents result from their direct interaction with channel structural components or involve modulation of biochemical pathways which regulate channel gating.

Perhaps the most striking feature of NS 1619 was its ability to stimulate $I_{BK(Ca)}$ in smooth muscle although this action was almost certainly not responsible for a major component of the observed relaxant effects of this molecule in the intact vessels studied. Nevertheless, in those tissues such as bladder or trachea in which BK_{Ca} is perhaps more important (Suarez-Kurtz *et al.*, 1991; Small *et al.*, 1993) and in those experimental situations in which $I_{BK(Ca)}$ can be studied in isolation, NS 1619 would seem to be a useful tool for the investigation of the pharmacology of BK_{Ca}.

NS 1619 was a generous gift from Dr S.P. Olesen (NeuroSearch). Helpful discussions with Dr D.J. Beech, Professor T.B. Bolton, Dr G. Kaczorowski and Dr O.B. McManus are gratefully acknowledged. G.E. was supported by Pfizer Central Research.

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(Received July 15, 1994
Revised August 30, 1994
Accepted August 31, 1994)