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Investigation of the effects of 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) on membrane currents in rat portal vein

A.J. Kirkup, G. Edwards & [1]A.H. Weston

School of Biological Sciences, G38 Stopford Building, University of Manchester, Manchester M13 9PT

1 The effects of 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) were investigated on evoked and spontaneous currents in freshly-isolated cells from the rat portal vein by use of conventional whole-cell recording and perforated-patch techniques.

2 At a holding potential of −60 mV in potassium-free, caesium-containing solutions, NPPB (10 μM) inhibited calcium (Ca)-sensitive chloride currents ($I_{Cl(Ca)}$) evoked by caffeine (10 mM) and by noradrenaline (10 μM) by 58% and 96%, respectively.

3 At a holding potential of −2 mV in potassium (K)-containing solutions, NPPB (10 μM) inhibited charybdotoxin-sensitive K-currents ($I_{BK(Ca)}$) induced by noradrenaline (10 μM) and acetylcholine (10 μM) by approximately 90%. In contrast, $I_{BK(Ca)}$ induced by caffeine (10 mM) was unaffected in the presence of NPPB (10 μM). Conversely, $I_{BK(Ca)}$ elicited by caffeine (2 mM) was reduced by approximately 50% whereas $I_{BK(Ca)}$ evoked by noradrenaline (50 μM) was not significantly inhibited by NPPB.

4 In K-containing solutions, NPPB (10 μM) abolished spontaneous transient outward currents (STOCs) and induced a slowly-developing outward K-current. Bath application of glibenclamide (10 μM) abolished the outward current but did not antagonize the inhibitory effects of NPPB on STOCs or on $I_{BK(Ca)}$ evoked by noradrenaline.

5 In caesium-containing solutions, NPPB (30 μM) inhibited voltage-sensitive Ca-currents.

6 In Ca-free, K-containing solutions and in the presence of glibenclamide (5 μM), $I_{BK(Ca)}$ induced by 20 μM NS1619 was enhanced by NPPB (10 μM).

7 It is concluded that NPPB inhibits agonist-induced $I_{Cl(Ca)}$ in rat portal vein smooth muscle. However, this agent also inhibits agonist-evoked $I_{BK(Ca)}$ and STOCs. Moreover, NPPB inhibits voltage-sensitive Ca-currents and stimulates a glibenclamide-sensitive K-current and $I_{BK(Ca)}$. The effects of this agent on evoked $I_{Cl(Ca)}$ and $I_{BK(Ca)}$ and on STOCs probably involves an inhibitory action on intracellular Ca-stores.

Keywords: Chloride currents; potassium currents; calcium currents; spontaneous transient outward currents; perforated-patch technique; NPPB; noradrenaline; caffeine; glibenclamide; NS1619

Introduction

The ability of noradrenaline to increase chloride (Cl) conductance in smooth muscle was first demonstrated by Byrne & Large (1987) in freshly-isolated rat anococcygeus cells. Subsequent investigations on vascular smooth muscle revealed similar effects of noradrenaline in rabbit and rat portal veins (Byrne & Large, 1988; Pacaud *et al.*, 1989) and in rabbit ear and pulmonary arteries (Amédée *et al.*, 1990a,b; Wang & Large, 1993). Evidence from these and other studies suggested that agonist-induced release of calcium (Ca) from an intracellular store was responsible for the activation of a Ca-sensitive Cl-current, which was termed $I_{Cl(Ca)}$ (Large, 1991). Since the Cl-equilibrium potential (E_{Cl}) of smooth muscle is approximately −25 mV (Aickin & Brading, 1982; Aickin & Vermue, 1983), induction of $I_{Cl(Ca)}$ may represent an important mechanism by which agonists produce smooth muscle depolarization and trigger the activation of voltage-sensitive Ca-channels (Amédée & Large, 1989; Pacaud *et al.*, 1991).

To date, however, the true contribution of agonist-induced Cl-currents to smooth muscle excitation-contraction coupling in whole tissues is uncertain due to the lack of selective Cl-channel inhibitors. For example, Amédée *et al.* (1990b) demonstrated that the noradrenaline-activated $I_{Cl(Ca)}$ in rabbit ear artery was sensitive to 4, 4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS; 0.1–0.5 mM) and to frusemide (0.5–1.0 mM). However, these agents were more potent at inhibiting ATP-induced cation currents in the same cell type. More recent studies have shown that Cl-currents in rabbit portal vein can be reduced by compounds such as 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS), DIDS, anthracene-9-carboxylate (A-9-C) and niflumic acid. However, these drugs also increased the magnitude of agonist-evoked Ca-sensitive potassium (K) currents (Hogg *et al.*, 1994a,b).

NPPB (5-nitro-2-(3-phenylpropylamino)-benzoic acid) is a potent inhibitor of an epithelial Cl-conductance (Wangemann *et al.*, 1986). Furthermore, this agent completely blocks outwardly-rectifying Cl-channels present in canine tracheal epithelium (Li *et al.*, 1990) and rat colonic enterocytes (Singh *et al.*, 1991; Venglarick *et al.*, 1994). The objective of the present study was to obtain a profile of the action of NPPB by use of agonist-evoked and spontaneously-generated currents from smooth muscle cells freshly-dispersed from rat portal vein. Using this approach it was hoped to determine the suitability of this agent as a Cl-channel modulator for the investigation of the role of such channels in smooth muscle excitability. A preliminary account of some of these observations has been presented to the British Pharmacological Society (Kirkup & Weston, 1995).

Methods

All the experiments were conducted on portal veins from male Sprague-Dawley rats (100–250 g), previously killed by stunning and cervical dislocation.

[1] Author for correspondence.

Patch-clamp studies

Isolation of cells Portal vein cells were prepared as previously described (Ibbotson *et al.*, 1993) and used within 9 h of separation. All experiments were performed at room temperature (22–26°C) with patch pipettes made from Pyrex glass (Cat. No. 687–055, Jencons, UK) and of resistance 3–10 MΩ when filled with internal solution.

Single-cell electrophysiology To reduce the 'run-down' of agonist-induced currents and voltage-sensitive Ca-currents which occurs when using conventional whole-cell recording (Amédée *et al.*, 1990b; Zhang & Bolton, 1995), amphotericin B was used in most experiments to produce 'perforated' patches (Horn & Marty, 1988; Rae *et al.*, 1991). A fresh amphotericin B stock solution (30 mg ml^{-1}) was made each day by dissolving the drug in dimethyl sulphoxide. Immediately before experimentation an amphotericin B-containing pipette solution (300 μg ml^{-1}) was prepared and used for up to 90 min. Pipette tips were briefly dipped into filtered pipette solution and then back-filled with the amphotericin B-containing solution. After formation of giga-seals, 15–25 min were allowed for amphotericin B to diffuse into the cell membrane. During this period, the access resistance dropped to 15–40 MΩ thus facilitating the recording of whole-cell currents. All other voltage-clamp recordings were obtained from single cells by conventional whole-cell recording methods. Voltage-clamp protocols were delivered and membrane current responses were recorded by means of an Axopatch-1D amplifier (Axon instruments) interfaced to a 12-bit analogue-to-digital converter (Axon Instruments, TL-1) and an AT-compatible computer equipped with pClamp 5.5 software. Membrane current data were stored on digital audio tape (Sony) and monitored continuously on a Gould Windograf chart recorder. Data were not corrected for leak or capacitance currents. The liquid junction potentials were corrected with an offset circuit before each experiment.

The isolated cells were superfused with a filtered (Millipore 0.22 μm filter unit) Ca^{2+}-containing physiological salt solution (PSS; see solutions below). A perfusion rate of approximately 0.7 ml min^{-1} was achieved with a peristaltic pump (LKB Microperpex) and a second identical pump was used to remove the solution from the recording chamber. Cells were left to equilibrate in the perfusing external solution for 15–20 min before experimentation commenced. Noradrenaline (10–50 μM) and acetylcholine (10 μM) were each applied to the cells by pressure ejection from borosilicate pipettes (1B100F-4, World Precision Instruments, UK) of tip diameter 2–4 μm for a period of 500 ms. Caffeine (2–10 mM) was applied by pressure ejection for 1 s (Baron *et al.*, 1991). The tips of application pipettes were positioned 40–80 μm from the cell surface and pneumatic pressures of 10–14 psi, generated with a PV820 Pneumatic Picopump (World Precision Instruments, UK), were applied. Electrophysiological responses were examined only in those cells which appeared relaxed, were phase-dense and which contracted in response to application of the agonist in use.

Treatment of data

Where applicable, results are expressed as the mean ± s.e.mean with the number of observations in parentheses. Analysis of variance (ANOVA), Student's paired or unpaired *t* test were used as necessary to test for statistical differences between data and *P* values of less than 0.05 were taken to be significant.

Solutions

Patch-clamp studies The normal external solution (K-PSS) was of the following composition (mM): NaCl 126, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 10, glucose 11. The normal pipette solution comprised (mM): KCl 126, $MgCl_2$ 1.2, HEPES 10, glucose 11, EGTA 1. In order to inhibit K-currents, KCl was sometimes isosmotically replaced with CsCl in both external and pipette solutions (Cs-PSS and Cs-pipette solution, respectively). To determine the ionic nature of the caffeine-induced inward currents, NaCl in the external solution was sometimes isosmotically replaced with either 79.2 mM sodium glutamate, sodium isethionate (ICN Pharmaceuticals) or sodium thiocyanate (low Cl-PSS) or with 70.3 mM Tris hydrochloride (low Na-PSS). Furthermore, tetraethylammonium chloride (TEA; 10 mM) was also added to the Cs-PSS and the Cs-pipette solution and the NaCl and CsCl were reduced accordingly. All of these solutions were adjusted to pH 7.20 with 3 M NaOH.

The composition of the Ca-free bath solution (Ca-free PSS) was (mM): NaCl 125, KCl 4.8, $MgCl_2$ 3.7, KH_2PO_4 1.2, EGTA (ethylene glycol-*bis* β-aminoethyl ether tetraacetic acid) 1.0, glucose 11, HEPES 10, buffered with 3M NaOH to pH 7.20. The Ca-free pipette solution contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, HEPES 10, EGTA 1.2, glucose 11, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, buffered to pH 7.20 with 3M KOH. The estimated free Ca concentration of this solution was < 1 nM. For the measurement of Ca-currents a different caesium-rich pipette solution (Ca-free, Cs-pipette solution) was used. This was similar to the Ca-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 7.20 with CsOH. All the external solutions were continuously bubbled with O_2 and, when appropriate, contained 1 μM propranolol to inhibit any β-adrenoceptor-mediated responses.

The enzyme solution for dispersing cells from portal vein comprised (mM): KOH 130, $CaCl_2$ 0.05, taurine 20, pyruvate 5, creatine 5, HEPES 10, collagenase (1 mg ml^{-1}; Sigma Type VIII), pronase (0.2 mg ml^{-1}; Calbiochem), fatty acid-free albumin (1 mg ml^{-1}). The pH was titrated to 7.40 with methanesulphonic acid.

Drugs

The following drugs were used: acetylcholine, amphotericin B, caffeine, charybdotoxin (ChTX; Latoxan), glibenclamide, NPPB (Cookson Chemicals, Southampton, UK), noradrenaline bitartrate, NS1619 (1-(2′-hydroxy-5′-trifluoromethyl-phenyl)-5-trifluoromethyl-2(3*H*)benzimidazolone; NeuroSearch) and propranolol (Zeneca). Unless otherwise stated, all drugs and reagents were obtained from Sigma. Stock solutions of glibenclamide and NPPB were each made up in ethanol and diluted in the appropriate PSS. Propranolol was dissolved in twice-distilled water before addition to PSS. Caffeine was dissolved in the appropriate PSS to give the required concentration for filling pressure-ejection pipettes. ChTX was prepared as a stock solution in a Ca-free PSS and diluted in the superfusate immediately before use. A stock solution of NS1619 was made up in dimethyl sulphoxide before subsequent dilution in the external solution. Noradrenaline bitartrate and acetylcholine were dissolved in 0.1 N HCl or twice-distilled water, respectively, before dilution in PSS to give the required concentration for filling pressure-ejection pipettes.

Results

Ionic nature of caffeine- and noradrenaline-evoked inward currents

In K-PSS and at a holding potential of −77 mV which is the calculated potassium equilibrium potential (E_K) for the solutions used, caffeine (10 mM) generated inward currents (−147 ± 46 pA; n = 14; Figure 1a(i)) which were of significantly greater magnitude than those induced by 10 μM noradrenaline (−38 ± 6 pA; n = 12; Figure 1b(i); $P < 0.05$). In the case of noradrenaline, this inward current has been previously identified as a Ca-sensitive Cl-current ($I_{Cl(Ca)}$; Pacaud *et al.*, 1989; Kirkup *et al.*, 1994). The caffeine-induced inward current also required the presence of Ca in the PSS (data not shown) and was further characterized by determining its reversal potential

(E_{rev}) by use of bath solutions of varying composition. To prevent contamination from K^+ conductances, these measurements were conducted in Cs- and TEA-containing solutions (*see* Methods). To determine the E_{rev} of caffeine-induced currents, voltage-ramps (holding potential of 0 mV; +50 mV to −50 mV or +100 mV to −100 mV for 500 ms) were applied to cells before and during exposure to caffeine (10 mM). The intersection of the voltage-ramp pulse in the absence with that in the presence of caffeine yielded an estimate of the E_{rev} of the induced current.

In Cs-PSS, the E_{rev} value of the caffeine-induced current was −0.3 ±1.2 mV (n = 15), a value close to and not significantly different from the calculated chloride equilibrium potential (E_{Cl}; −2 mV). In isethionate- and glutamate-containing low Cl-PSS, the E_{rev} of the caffeine-induced current was shifted to the significantly different values of +17±2.4 mV (n = 6; $P<0.005$) and +20±2.1 mV (n = 5, $P<0.001$), respectively, which were close to the value of the altered E_{Cl} (+20 mV). In contrast, in thiocyanate-containing low Cl-PSS, the E_{rev} of the caffeine-induced current moved to a value of −38±4.8 mV (n = 5, $P<0.001$), a direction opposite to that of the altered E_{Cl}. This negative shift in the estimated E_{rev} value indicates that thiocyanate is more permeant than chloride through this conductance pathway. In low Na-PSS, the E_{rev} value was unchanged (+0.7±4.1 mV; n = 6; $P>0.05$).

Collectively, these data suggest that the inward currents activated by caffeine in rat portal vein are (like those induced by noradrenaline; Pacaud *et al.*, 1989; Kirkup *et al.*, 1994) carried by Cl with little contribution from cations.

Effects of NPPB on noradrenaline- and caffeine-evoked $I_{Cl(Ca)}$

In Cs-containing solutions and at a holding potential of −60 mV, NPPB produced a concentration-dependent inhibition (1 μM, 6±21%: 10 μM, 58±9%: 100 μM, 84±8%: n = 5) of $I_{Cl(Ca)}$ evoked by caffeine (10 mM). From these experiments, a concentration of 10 μM NPPB (the approximate EC_{50} value) was selected for use in the remainder of the study. Thus, in the presence of 10 μM NPPB, $I_{Cl(Ca)}$ evoked by noradrenaline (10 μM) was reduced by 94±3% (n = 7), a degree of inhibition which was significantly greater ($P<0.05$) than when $I_{Cl(Ca)}$ was induced by caffeine (Figure 2a,b). After 5 min washout of NPPB, partial recovery of the caffeine- and noradrenaline-induced inward currents was observed (Figure 2a,b). Responses following successive exposure to either caffeine or noradrenaline were reproducible (Figure 2c). Glibenclamide (10 μM) had no significant effect on caffeine-induced $I_{Cl(Ca)}$ (control:- 100±23 pA; 10 μM glibenclamide:- 101±17 pA; n=4; $P>0.05$).

Ionic nature of caffeine- and noradrenaline-elicited outward currents

In K-PSS but at a holding potential of −2 mV (the calculated E_{Cl} under these conditions), caffeine (10 mM) elicited large (760±55 pA; n=23; Figure 1a(ii)) outward currents. Noradrenaline (10 μM) produced outward currents which were significantly smaller (505±69 pA; n=14: Figure 1b(ii), $P<0.01$). Neither caffeine- nor noradrenaline-induced currents were observed in K-free solutions and they were reversibly attenuated by 48±11% (n=4; $P<0.05$) and by 35±4% (n=3; $P<0.05$), respectively, in the presence of 100 nM char-

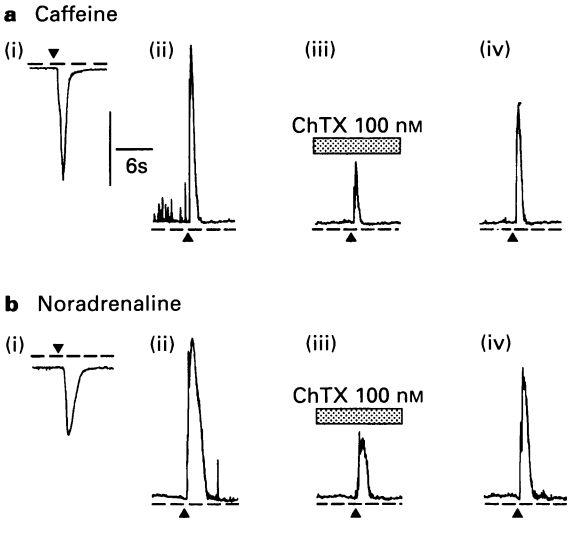

Figure 1 Effect of holding potential and of charybdotoxin (ChTX) on the currents generated by caffeine and noradrenaline in K-PSS. At a holding potential of −77 mV (the potassium equilibrium potential) pressure-ejection-application of (a)(i) caffeine (▼, 10 mM, 14 psi for 1 s) or (b)(i) noradrenaline (▼, 10 μM, 10 psi for 500 ms) induced inward currents. In contrast, at a holding potential of −2 mV (the chloride equilibrium potential), pressure-ejection-application of (a)(ii) caffeine (▲, 10 mM, 14 psi for 2 s) or (b)(ii) noradrenaline (▼, 10 μM, 10 psi for 400 ms) elicited large outward potassium currents which were attenuated (a)(iii), (b)(iii) after 5 min exposure to charybdotoxin (100 nM, stippled horizontal bar). This reduction was partially reversed after a 5 min washout (a)(iv), (b)(iv). The records (a,b) were obtained from four different cells and the dashed lines refer to the zero current potential. The vertical calibration bar represents 100 pA in (a)(i) and (b)(i), 200 pA in (a)(ii)–(a)(iv) and 400 pA in (b)(ii)–(b)(iv)

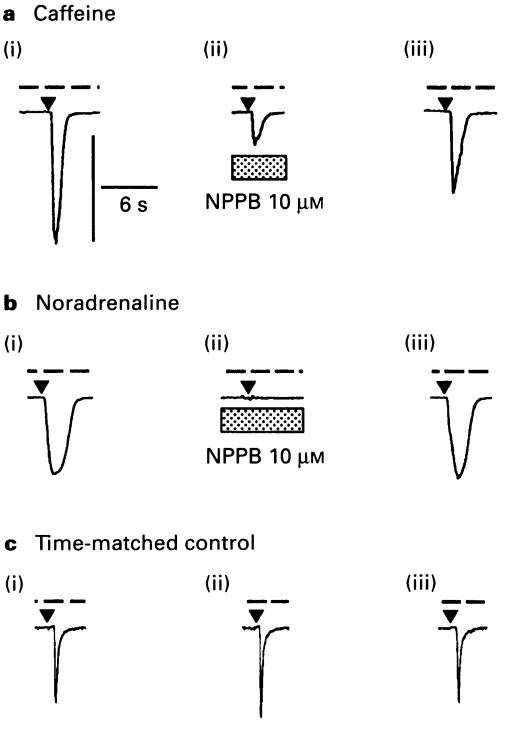

Figure 2 Effect of NPPB on caffeine- and noradrenaline-induced chloride currents at a holding potential of −60 mV in Cs-PSS. Pressure-ejection application of (a)(i) caffeine (▼, 10 mM, 14 psi for 1 s) or (b)(i) noradrenaline (▼, 10 μM, 14 psi for 500 ms), induced inward chloride currents. After 5 min exposure to 10 μM NPPB (a)(ii), (b)(ii), (stippled horizontal bar) the magnitude of the elicited chloride current was reduced, an effect that was partially reversed after a 5 min washout period (a)(iii), (b)(iii). (c)(i) Recordings from a cell to which caffeine (▼, 10 mM, 14 psi for 1 s) was applied at 5 min intervals (c)(ii)–(c)(iii) thus demonstrating that consistent responses could be obtained from the single portal vein cells. The records (a, b and c) were obtained from three different cells and the dashed lines refer to the zero current potential. The vertical calibration bar represents 50 pA in (a), 100 pA in (b) and 20 pA in (c).

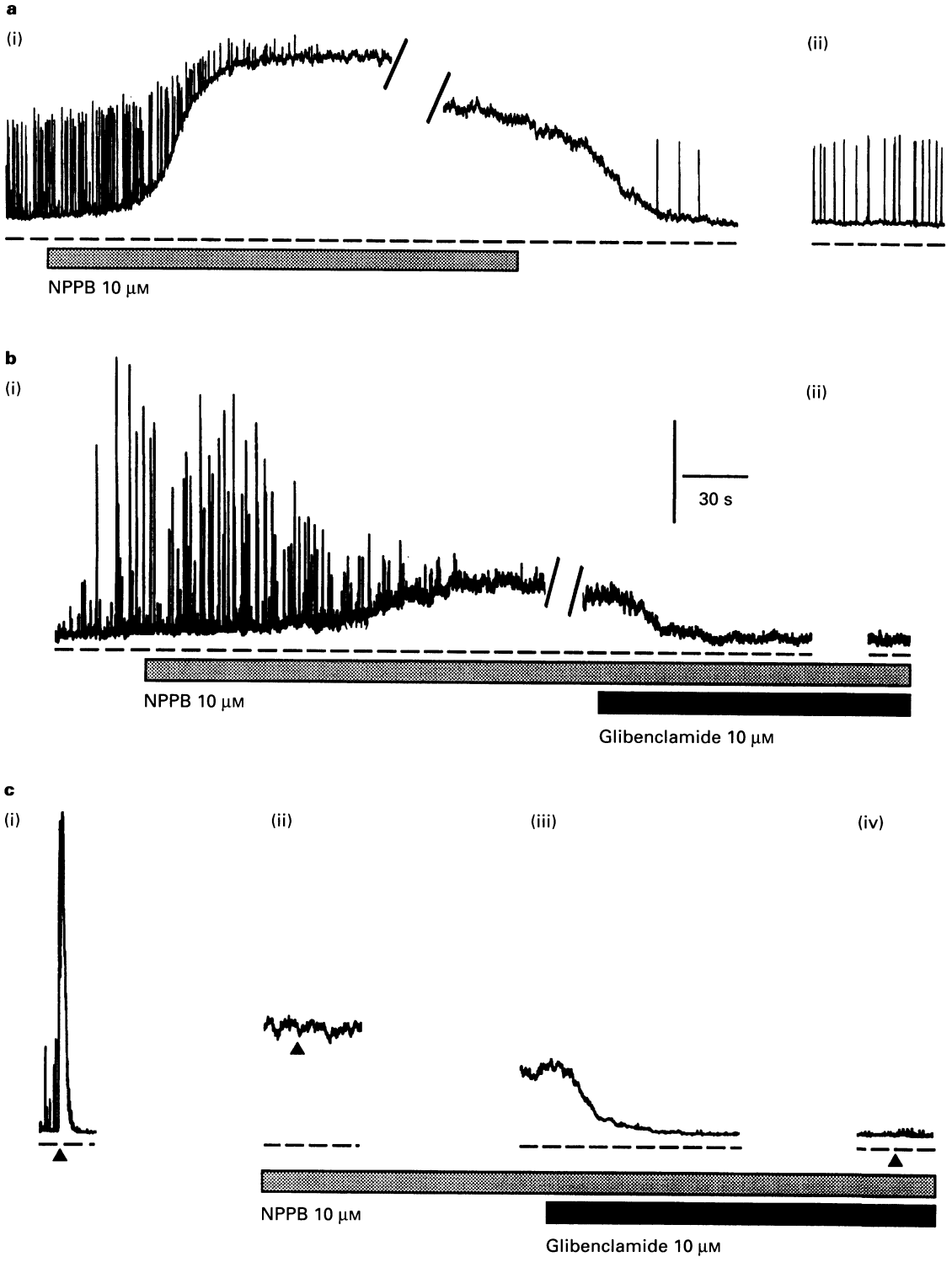

Figure 3 Effects of NPPB on the holding current, spontaneous transient outward currents (STOCs) and noradrenaline-induced potassium currents at a holding potential of −2 mV. (a)(i) Bath application of 10 μM NPPB (stippled horizontal bar) induced a slowly-developing outward current (which exhibited run-down) and inhibited STOCs, effects which were reversed after 5 min washout (a)(ii). The gap in (a)(i) was 2 min. (b) In another cell (b)(i), bath-applied glibenclamide (10 μM, solid horizontal bar) inhibited the outward current induced by 10 μM NPPB (stippled horizontal bar). However, after 5 min in the continued presence of both NPPB and glibenclamide (b)(ii), STOCs had not reappeared. The gap in (b)(i) was 2 min. (c)(i) Application of noradrenaline (▲, 10 μM, 14 psi for 500 ms), elicited an outward potassium current. After 5 min in the presence of 10 μM NPPB which itself induced an outward current (c)(ii), application of noradrenaline (▲, 10 μM, 14 psi for 500 ms) failed to elicit a response. Bath application of 10 μM glibenclamide (c)(iii), (solid horizontal bar) abolished the current induced by NPPB but, after 5 min had elapsed, failed to reverse the inhibitory effects of NPPB on noradrenaline-induced currents (c)(iv). The records (a,b and c) were obtained from three different cells and the dashed lines refer to the zero current potential. The vertical calibration bar represents 200 pA in (a) and (c) and 100 pA in (b). The holding potential in all records was −2 mV.

ybdotoxin (ChTX; Figures 1a(iii–iv) and 1b(iii–iv)), an inhibitor of BK_{Ca}, the large-conductance Ca-sensitive potassium channel. Spontaneous transient outward currents (STOCs) were also sometimes observed at a holding potential of −2 mV. These were reversibly abolished by application of either caffeine (2–10 mM), noradrenaline (10–50 μM) or acetylcholine (10 μM) and inhibited by exposure to 100 nM ChTX (data not shown).

Effects of NPPB on evoked, spontaneous and non-inactivating K-currents

In K-containing solutions and at a holding potential of −2 mV, the effects of NPPB (10 μM) were complex. After a latent period of 2–3 min, this agent induced a noisy outward current (93 ± 13 pA, 42 out of 56 cells tested; Figure 3a-c) which reached a maximum over a further period of about 5 min. The characteristics of this current were investigated by use of a voltage-stepping protocol in which cells were held at −10 mV and stepped to a series of test potentials, in 10 mV increments, for 500 ms. Prior to exposure to NPPB, these voltage-steps gave rise to a series of time-independent, outwardly-rectifying currents which became noisier at potentials positive to +10 mV (Figure 4a(i)). In the presence of 10 μM NPPB, a time-independent, outwardly-rectifying current was induced which was associated with a *further* increase in current noise (Figure 4a(ii)). In seven cells tested, the E_{rev} of the current induced by NPPB was −77 ± 4.9 mV a value close to and not significantly different from the E_K (−77 mV) under these conditions. Furthermore, the zero current potential of these cells was shifted from −24 ± 3 mV to a hyperpolarized value of −45 ± 5 mV. The K-current induced by NPPB was inhibited by glibenclamide (Figure 3b).

In addition to these effects, application of 10 μM NPPB also inhibited STOCs (Figure 3a,b) and $I_{BK(Ca)}$ evoked by noradrenaline (10 μM) and by acetylcholine (10 μM) was reduced by 88 ± 5% (n = 8; $P<0.01$) and by 87 ± 8% (n = 4; $P<0.05$), respectively (Figure 5b-c). Surprisingly, however, NPPB was without significant effect on the 10 mM caffeine-induced $I_{BK(Ca)}$ (control:- 823 ± 48 pA; 10 μM NPPB:- 836 ± 55 pA; n = 16; $P > 0.05$; Figure 5a).

These differential inhibitory effects of NPPB could indicate a specific effect of this agent on noradrenaline-induced Ca^{2+} release. However, the magnitude of K-currents produced by application of caffeine (10 mM) was significantly larger than that induced by noradrenaline (10 μM). Thus, the differential inhibitory effects of NPPB on these evoked currents could have resulted from different degrees of functional antagonism if the amount of Ca^{2+} released by 10 mM caffeine were greater than that liberated by 10 μM noradrenaline. To investigate this postulate, the effects of NPPB (10 μM) on the responses to application of 2 mM caffeine and 50 μM noradrenaline were examined. $I_{BK(Ca)}$ elicited by caffeine (2 mM) was inhibited by 47 ± 12% (control:- 388.8 ± 73.6 pA; 10 μM NPPB:- 167.6 ± 43.6; n = 8; $P<0.005$) whereas NPPB (10 μM) had no significant effect on $I_{BK(Ca)}$ induced by application of 50 μM noradrenaline (control:- 482 ± 73 pA; 10 μM NPPB:- 329 ± 72 pA; n = 5; $P > 0.05$).

Exposure to 10 μM glibenclamide in the continuing presence of NPPB did not reverse the attenuation of STOCs (n = 5; Figure 3b) or the inhibition of noradrenaline- (n = 6; Figure 3c) or acetylcholine-evoked $I_{BK(Ca)}$ (data not shown). In the absence of NPPB, glibenclamide (10 μM) had no effect on either STOCs or on noradrenaline-evoked $I_{BK(Ca)}$ (data not shown).

Effects of NPPB on calcium-currents in Cs-PSS

At a holding potential of −60 mV in caesium-containing solutions (Cs-PSS and Ca-free, Cs-pipette solution), stepping to a series of depolarizing test potentials resulted in the activation of calcium currents (I_{Ca}) with a typical bell-shaped current-

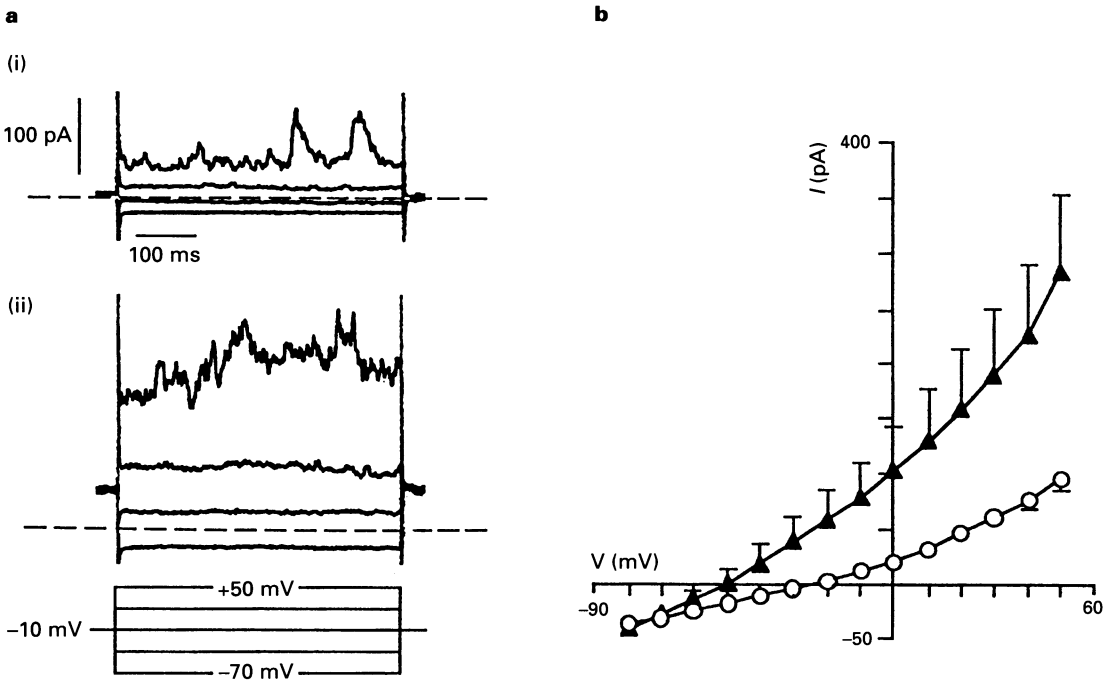

Figure 4 Effect of NPPB on whole-cell currents in potassium-containing solutions. (a)(i) On stepping cells from a holding potential of −10 mV to a series of test potentials for 500 ms, a series of time-independent, outwardly-rectifying currents resulted. At positive potentials, these currents were sometimes accompanied by spontaneous transient outward currents (+50 mV pulse). In the presence of 10 μM NPPB (a)(ii), an additional time-independent, outwardly-rectifying current was stimulated which was associated with a further increase in current noise. (b) Current-voltage relationships obtained on stepping to a range of test potentials from the holding potential of −10 mV in the absence (○) and presence of 10 μM NPPB (▲). The dashed lines in (a)(i) and (a)(ii) indicate the zero current potential. Each point in (b) represents the mean ± s.e.mean, n = 7.

voltage relationship and an activation threshold of around −20 mV (Figure 6). These currents were inhibited by nifedipine (1 μM; data not shown) suggesting they were carried by L-type Ca-channels. The magnitude of these calcium-currents at +10 mV was −207 ± 43 pA (n = 6). After 5 min in the presence of 10 μM NPPB, there was no significant effect on the magnitude of these inward currents (−186 ± 50 pA; n = 5; P > 0.05). However, exposure to 30 μM NPPB for 5 min produced a significant reduction of I_{Ca} (−72 ± 23 pA; n = 6; P<0.01) an effect which was only slowly reversible on washout (data not shown). Glibenclamide (10 μM), which itself had no effect on I_{Ca} (data not shown), failed to antagonize the inhibitory effects of NPPB (30 μM) on I_{Ca} (data not shown).

The effect of NPPB on the kinetics of inactivation of I_{Ca} was also investigated. Under control conditions, the inactivation of I_{Ca} (over a 200 ms period after the inward current peak) was best explained by a single exponential function with a time constant (τ) value of 34 ± 4 ms (n = 5). In the presence of 30 μM NPPB, the inactivation of I_{Ca} could still be described by a single exponential function, but τ was significantly increased to 62 ± 6 ms (n = 5; P<0.005).

Effects of NPPB on $I_{BK(Ca)}$ induced by NS1619 in Ca-free conditions

Under essentially calcium-free conditions (Ca-free pipette solution and Ca-free PSS), and using the conventional whole-cell recording configuration, only a very small outward current was elicited by stepping (for 500 ms) to a test potential of +50 mV from a holding potential of −10 mV, since at these depolarizing potentials, delayed rectifier channels were inactivated. After 10 min pre-exposure to, and in the continued presence of, 5 μM glibenclamide (to inhibit $I_{K(ATP)}$), 20 μM NS1619 (an opener of $BK_{(Ca)}$; Edwards *et al.*, 1994; Olesen *et al.*, 1994) had little effect on the current at the holding potential but produced a marked increase in the outward current at the test potential (Figure 7a). In 8 cells this increase reached a plateau within 10 min (data not shown). After 15 min exposure to 20 μM NS1619, NPPB (10 μM) produced a further increase in $I_{BK(Ca)}$ (P<0.05) which appeared to be due to a small but significant shift (ANOVA, P<0.05) in the voltage-sensitivity of activation to more hyperpolarized potentials (Figure 7b). In the presence of glibenclamide (5 μM), NPPB did not induce an outward current at the holding potential (Figure 6a), indicating that $I_{K(ATP)}$ did not contribute to the observed increase in outward current at the +50 mV test potential.

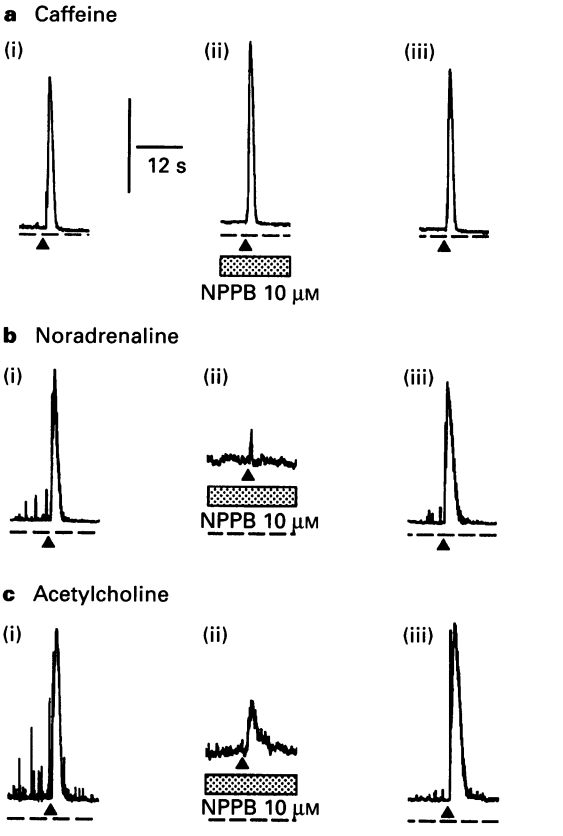

Figure 5 Effect of NPPB on caffeine-, noradrenaline- and acetylcholine-evoked potassium currents at a holding potential of −2 mV. Application of (a)(i) caffeine (▲, 10 mM, 14 psi for 1 s), (b)(i) noradrenaline (▲, 10 μM, 14 psi for 500 ms) and (c)(i) acetylcholine (▲, 10 μM, 14 psi for 500 ms) by pressure-ejection, elicited outward potassium currents. After 5 min exposure to 10 μM NPPB (stippled horizontal bar) which induced a slowly-developing, noisy outward current (see Figure 3), the magnitude of (b)(ii)−(b)(iii) noradrenaline- and (c)(ii)−(c)(iii) acetylcholine-activated potassium currents was reversibly attenuated. In contrast, caffeine-induced potassium currents (a)(ii)−(a)(iii) were unaffected in the presence of NPPB. Experimental traces (a, b and c) were obtained from three different cells and the dashed lines refer to the zero current potential. The vertical calibration bar represents 500 pA in (a) and 200 pA in (b) and (c).

Discussion

Nature of caffeine- and noradrenaline-induced currents

In the present study, a Ca-sensitive Cl-current, $I_{Cl(Ca)}$, was evoked by noradrenaline (as previously described by Kirkup *et al.*, 1994) and by caffeine. In addition, these agents and acetylcholine also stimulated a Ca- and ChTX-sensitive potassium current ($I_{BK(Ca)}$). These observations are consistent with earlier findings in rat portal vein which were obtained by conventional whole-cell recording techniques (Pacaud *et al.*, 1989; Baron *et al.*, 1991; Loirand *et al.*, 1992). Interestingly, ChTX was less potent against evoked than spontaneous $I_{BK(Ca)}$. This could be explained by the fact that agonist-evoked currents were consistently of greater magnitude than STOCs, a possible reflection of different amounts of Ca^{2+} release associated with the two events. Furthermore, $I_{BK(Ca)}$ in rat portal vein smooth muscle does not appear to be as sensitive to ChTX as previously described in studies on rabbit portal vein or human mesenteric artery (*see* Beech & Bolton, 1989; Smirnov & Aaronson, 1992). This may be a species difference or may reflect the use of less pure toxin in the earlier studies.

The effects of agonists on $I_{Cl(Ca)}$ and $I_{BK(Ca)}$ could be consistently evoked several times in individual cells provided that sufficient time was allowed between successive exposures to these agents. Such reproducible responses were a critical feature of the present investigation and allowed the effects of NPPB on these currents to be characterized with some degree of confidence.

NPPB-mediated inhibition of agonist-induced $I_{Cl(Ca)}$ and $I_{BK(Ca)}$

$I_{Cl(Ca)}$ evoked by caffeine and by noradrenaline was inhibited by NPPB with a potency similar to that of niflumic acid in rabbit portal vein (Hogg *et al.*, 1994b) and the extent of this inhibition was inversely related to the magnitude of the induced Cl-current. This action could have been exerted directly on $Cl_{(Ca)}$ since NPPB apparently exerts inhibitory actions on such a channel in *Xenopus* oocytes (Wu & Hamill, 1992). Furthermore, evidence has been obtained that the inhibition of $I_{Cl(Ca)}$ in smooth muscle by both niflumic acid (which is structurally-related to NPPB) and by A-9-C is also exerted on Cl_{Ca} itself (Hogg *et al.*, 1993b; 1994a,b).

However, a common feature which links the agonist-induced, NPPB-sensitive conductances measured in the present study is their sensitivity to the intracellular Ca-concentration ($[Ca^{2+}]_i$). Thus in smooth muscle, caffeine releases Ca from intracellular stores *via* the 100 pS, ryanodine-sensitive Ca-

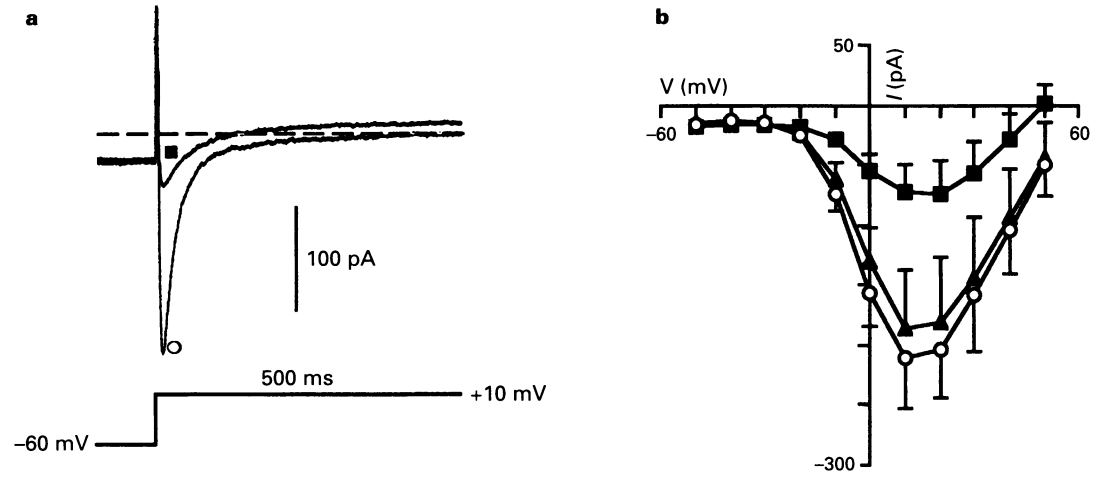

Figure 6 Effect of NPPB on Ca-currents in rat portal vein cells in caesium-containing solutions. (a) When cells were held at −60 mV then stepped to +10 mV for 500 ms a rapidly-activating and -inactivating Ca-current was observed (○). This current was attenuated in the presence of 30 μM NPPB (■). (b) Current-voltage relations for peak Ca-currents evoked on stepping for 500 ms (at 4s intervals) from a holding potential of −60 mV to a series of test potentials in the absence (○) or presence of 10 μM (▲) or 30 μM NPPB (■). Note that the magnitude of the Ca-current was reduced in a concentration-dependent manner. The dashed line in (a) denotes the zero current potential. Each point represents the mean ± s.e.mean, n = 5–6.

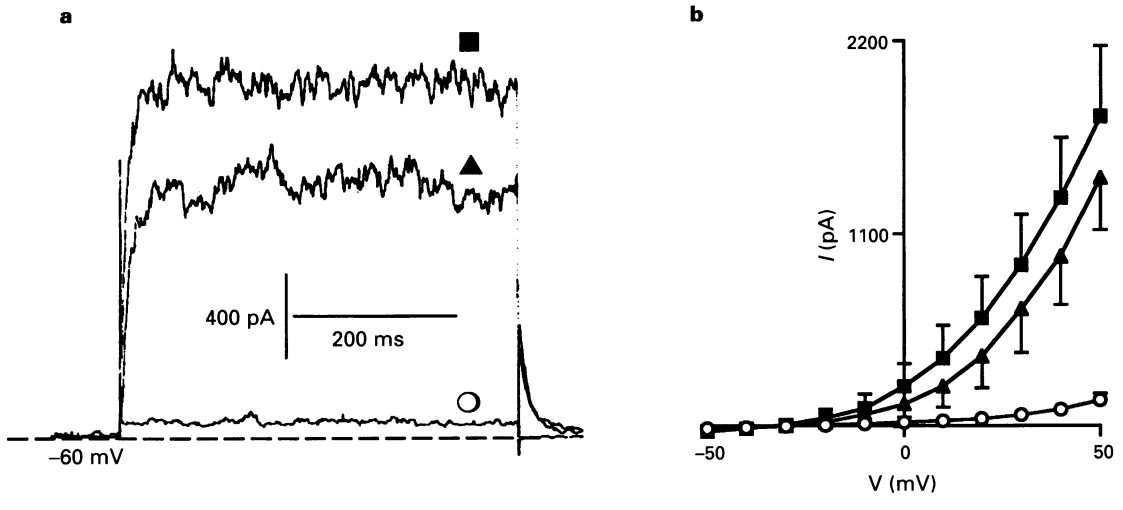

Figure 7 Effect of NPPB on $I_{BK(Ca)}$ induced by NS 1619. (a) Using whole-cell recording techniques, and under essentially calcium-free conditions in the presence of 5 μM glibenclamide, only a small outward current was observed on stepping from a holding potential of −10 mV to a test potential of +50 mV for 500 ms (○). NS 1619 (20 μM, ▲) induced a noisy outward current, $I_{BK(Ca)}$, which was evident at the test potential but not the holding potential. This current was enhanced by the additional presence of NPPB (10 μM, ■). (b) Current-voltage relationships obtained on stepping to a range of test potentials from the −10 mV holding potential (symbols as above). The dashed line in (a) refers to the zero current potential. Each point represents the mean ± s.e.mean, n = 4.

channel (Meissner, 1994). There is also indirect evidence that noradrenaline and acetylcholine initiate Ca^{2+} release from intracellular stores *via* an inositol-1,4,5-trisphosphate ($InsP_3$)-sensitive Ca-channel (Loirand *et al.*, 1992; Pacaud *et al.*, 1993; Ehrlich & Bezprozvanny, 1994). The resulting increase in $[Ca^{2+}]_i$ then plays a major role in activating Ca-sensitive ion channels (Pacaud *et al.*, 1989; Amédée *et al.*, 1990a, b; Large, 1991). Thus, the inhibitory effects of NPPB on $I_{Cl(Ca)}$ and on $I_{BK(Ca)}$ could have been exerted indirectly by reducing the amount of stored Ca available for ion channel activation. Support for this conclusion can be derived from the finding that the selected concentration of NPPB was a more effective inhibitor of caffeine-induced $I_{Cl(Ca)}$ than $I_{BK(Ca)}$. Since $I_{BK(Ca)}$ requires a lower $[Ca^{2+}]_i$ for full activation than does $I_{Cl(Ca)}$ (Marty *et al.*, 1984; Hogg *et al.*, 1993a,b), any net reduction in $[Ca^{2+}]_i$ by NPPB should indeed preferentially inhibit $I_{Cl(Ca)}$.

Initial experiments showed that NPPB inhibited $I_{BK(Ca)}$ induced by 10 μM noradrenaline whereas the same current elicited by 10 mM caffeine was unaffected. However, $I_{BK(Ca)}$ produced by 2 mM caffeine was significantly attenuated by 10 μM NPPB whereas that stimulated by 50 μM noradrenaline was unchanged. Such results clearly suggest that the ability of NPPB to inhibit agonist-induced $I_{BK(Ca)}$ depends on the amount of calcium liberated by the stimulating agonist.

Inhibition of STOCs by NPPB

NPPB inhibited STOCs, an action which contrasts with the effects of niflumic acid and other Cl-channel inhibitors (Hogg *et al.*, 1994a, b). STOCs are thought to be carried by BK_{Ca} channels which open in response to transient increases in $[Ca^{2+}]_i$ following spontaneously liberation from intracellular stores close to the membrane (Benham & Bolton, 1986; Ohya *et al.*, 1988; Bolton & Lim, 1989). Wang and coworkers (1992)

proposed that background Ca^{2+} influx may maintain an adequate $[Ca^{2+}]_i$ with which the quantal release of Ca^{2+} from stores summates to produce a concentration that is sufficient to stimulate the opening of Ca-sensitive ion channels. Since there are no reports which suggest that smooth muscle spontaneously hyperpolarizes (see Wang *et al.*, 1992), STOCs might represent a pathophysiological phenomenon (T.B. Bolton, personal communication). However, spontaneous *depolarizations* have been recorded from whole segments of guinea-pig mesenteric vein (van Helden, 1991). Since these are probably the result of the activation of Ca-sensitive Cl-currents, the spontaneous release of intracellular Ca^{2+} might indeed occur under physiological conditions.

Irrespective of the mechanism involved, the importance of Ca^{2+} release from stores in the production of STOCs (Benham & Bolton, 1986; Ohya *et al.*, 1988; Bolton & Lim, 1989) makes these currents sensitive indicators of store release. Thus their inhibition by NPPB, together with the reduction of agonist-evoked, Ca-sensitive currents ($I_{Cl(Ca)}$ and $I_{BK(Ca)}$) strongly suggests that this compound modifies intracellular Ca^{2+} release from, or sequestration into, intracellular stores. Such a possibility is supported by the finding that a chloro-derivative of NPPB inhibits vasopressin-induced Ca-release from intracellular stores in a clonal (A7r5) cell line (Pon *et al.*, 1993).

Potentiation of $I_{BK(Ca)}$ *by NPPB*

These experiments were originally designed to determine whether the marked inhibitory action of NPPB on agonist-induced $I_{BK(Ca)}$ was exerted by a direct effect on the channel. They were thus conducted in Ca-free conditions and using NS1619, the benzimidazolone derivative which is capable of opening BK_{Ca} in the absence of Ca^{2+} (Edwards *et al.*, 1994). In the absence of caffeine or noradrenaline, NPPB paradoxically produced a modest potentiation of current flow.

Potentiation of $I_{BK(Ca)}$ is a feature common to many Cl-channel inhibitors, such as A-9-C, DIDS, SITS and a variety of fenamates (Hogg *et al.*, 1994a,b; Ottolia & Toro, 1994; Teramoto & Brading, 1994). The mechanism involved in this action of NPPB was not investigated. However, it seems unlikely that it involved changes in $[Ca^{2+}]_i$ since the experiments were conducted in Ca-free conditions in which Ca^{2+} availability from either intracellular stores or from the extracellular fluid would have been severely limited. Interestingly, the ability of NPPB to enhance $I_{BK(Ca)}$ and to inhibit STOCs is a property shared by the benzimidazolone derivative, NS1619 (Edwards *et al.*, 1994).

Inhibition of calcium-currents by NPPB

NPPB produced a concentration-dependent inhibition of nifedipine-sensitive Ca-currents in the rat portal vein and significantly increased their time constant of inactivation. This action is consistent with the report that NPPB can inhibit I_{Ca} in guinea-pig ventricular myocytes (Walsh, 1994). Little inhibition of I_{Ca} was observed in the presence of 10 μM NPPB whereas exposure to 30 μM NPPB produced a marked diminution of this current. It thus seems unlikely that inhibition of Ca-currents could have made a significant contribution to the reduction in agonist-induced $I_{Cl(Ca)}$ and $I_{BK(Ca)}$ or STOCs observed in the present study.

Induction of $I_{K(ATP)}$ *by NPPB*

The activation of a small conductance, $[Ca^{2+}]_i$ and TEA-sensitive K-channel in pig urethra by the Cl-channel inhibitor, niflumic acid, was briefly reported by Teramoto & Brading (1994). In the present study, exposure to NPPB resulted in the slow development of an outward K-current which resembled that evoked by the K-channel openers levcromakalim, P1060, and aprikalim in rat portal vein (Noack *et al.*, 1992; Ibbotson *et al.*, 1993). It suggested that NPPB might open ATP-sensitive K-channels (K_{ATP}), a possibility which was confirmed by the observation that the induced K-current was sensitive to glibenclamide. To our knowledge, this is the first time that an inhibitor of $I_{Cl(Ca)}$ has been shown to induce $I_{K(ATP)}$, a Ca-insensitive current carried by a small conductance K-channel (see Edwards & Weston, 1993; 1994).

Conclusions

The present investigation has demonstrated the complex pharmacology of NPPB in rat portal vein. In addition to its inhibitory effects on agonist-induced $I_{Cl(Ca)}$, NPPB also attenuated evoked $I_{BK(Ca)}$ and STOCs. Furthermore, NPPB inhibited nifedipine-sensitive Ca-channels and under certain conditions, it stimulated both $I_{K(ATP)}$ and $I_{BK(Ca)}$. The effects of this agent on evoked Cl-and K-currents are probably exerted indirectly by an initial inhibitory action on intracellular Ca-stores.

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