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The effects of nifedipine and other calcium antagonists on the glibenclamide-sensitive K^+ currents in smooth muscle cells from pig urethra

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1 The effects of nifedipine on both levcromakalim-induced membrane currents and unitary currents in pig proximal urethra were investigated by use of patch-clamp techniques (conventional whole-cell configuration and cell-attached patches).

2 Nifedipine had a voltage-dependent inhibitory effect on voltage-dependent Ba^{2+} currents at -50 mV ($K_i = 30.6$ nM).

3 In current-clamp mode, subsequent application of higher concentrations of nifedipine ($\geqslant 30$ μM) caused a significant depolarization even after the membrane potential had been hyperpolarized to approximately -82 mV by application of 100 μM levcromakalim.

4 The 100 μM levcromakalim-induced inward current (symmetrical 140 mM K^+ conditions, -50 mV) was inhibited by additional application of three different types of Ca antagonists (nifedipine, verapamil and diltiazem, all at 100 μM). In contrast, Bay K 8644 (1 μM) possessed no activating effect on the amplitude of this glibenclamide-sensitive current.

5 When 100 μM nifedipine was included in the pipette solution during conventional whole-cell recording at -50 mV, application of levcromakalim (100 μM) caused a significant inward membrane current which was suppressed by 5 μM glibenclamide. On the other hand, inclusion of 5 μM glibenclamide in the pipette solution prevented levcromakalim from inducing an inward membrane current.

6 The levcromakalim-induced K^+ channel openings in cell-attached configuration were suppressed by subsequent application of 5 μM glibenclamide but not of 100 μM nifedipine.

7 These results suggest that in pig proximal urethra, nifedipine inhibits the glibenclamide-sensitive 43 pS K^+ channel activity mainly through extracellular blocking actions on the K^+ channel itself.

Keywords: Glibenclamide; levcromakalim; glibenclamide-sensitive 43 pS K^+ channel; Ca antagonists; voltage-dependent Ba^{2+} currents

Introduction

Ca antagonists (Ca^{2+} entry blockers or Ca^{2+} channel blockers) at low concentrations (<1 μM) inhibit the amplitude of voltage-dependent Ca^{2+} currents in a use-, frequency- and voltage-dependent manner due to profound and selective inhibitory effects on voltage-dependent Ca^{2+} channels. In contrast, higher concentrations of Ca antagonists ($\geqslant 30$ μM) also block voltage-dependent Na^+ channels (human cardiacmyocytes, Bustamante, 1985; rat cultured ventricular cells, Yatani & Brown, 1986; Yatani *et al*., 1988) and voltage-dependent K^+ channels (*Helix* neurones, Gola & Ducreux, 1985; rabbit intestine, Terada *et al*., 1987a, b; rat alveolar epithelial cells, Jacobs & DeCoursey, 1990; rabbit cardiacmyocytes, Gotoh *et al*., 1991; *shaker* K^+ channels, Avdonin *et al*., 1997), with a much higher IC_{50} value than that for voltage-dependent Ca^{2+} channels.

Relaxation of smooth muscle can be brought about by administration of Ca antagonists, and also by drugs which activate K^+ channels. The ability of K^+ channel activating drugs to relax smooth muscle is normally considered due to their hyperpolarizing effect on the membrane, which will switch off L-type Ca^{2+} channels and thus reduce extracellular Ca^{2+} entry. One of the target channels for K^+ channel opening drugs is the ATP-sensitive K^+ channel (K_{ATP}). Much effort has been made to elucidate the vasodilating mechanisms of K_{ATP} openers and Ca antagonists in vascular smooth muscle (Hamilton *et al*., 1986; Greenwood & Weston, 1993). However, the precise interaction between these two types of drugs still remains elusive. The K^+ channel openers in current use may have more than one mechanism of action; for example cromakalim also has a Ca antagonistic action (30 μM, rat portal vein, Okabe *et al*., 1990; 20 μM, canine proximal colon, Post *et al*., 1991) as well as the K^+ channel opening effect.

In the urological field, both Ca antagonists and K^+ channel openers have been shown to be clinically effective for the treatment fo motor urge incontinence (reviewed by Andersson, 1988; 1992; Langtry & McTavish, 1990), and it is thus of interest to investigate the mechanisms of action of these drugs, and the interaction between the two types of drugs. No date are currently available concerning the effects of Ca antagonists on glibenclamide-sensitive K^+ currents or K^+ channels.

We have previously demonstrated the presence of a glibenclamide-sensitive 43 pS K^+ channel in pig proximal urethra, concluding that K_{ATP} opener-induced urethral relaxation would be an undesirable side effect for the treatment of bladder instability and urge incontinence (Teramoto & Brading, 1996; 1997; Teramoto *et al*., 1997a, b). In the present experiments, we have investigated the effects of Ca antagonists on the Ba^{2+} currents and the glibenclamide-sensitive K^+ currents (mainly activated by the (−)-enantiomer of cromakalim, namely levcromakalim) in pig proximal urethra. We

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discuss whether or not nifedipine may modulate the sulphonylurea receptor associated with the glibenclamide-sensitive channels.

Methods

Fresh urethra from female pigs, was collected from a local abattoir still attached to its bladder and transported to the laboratory in a cold solution (at 4–6°C) of composition (mM): Na^+ 137, K^+ 5.9, Mg^{2+} 0.5, Ca^{2+} 0.5, Cl^- 128.3, HCO_3^- 15.4, $H_2PO_4^-$ 1.2 and glucose 11.5, which had previously been bubbled with 97% O_2 and 3% CO_2 (pH 7.25–7.3). The proximal region of the pig urethra (1–2 cm from the bladder neck) was excised and the connective tissue and mucosa removed under a dissection microscope.

Cell dispersion and recording procedure

Pig urethral myocytes were freshly isolated by the gentle tapping method (Teramoto & Brading, 1997; Teramoto *et al*., 1997a,b). Relaxed spindle-shaped cells, with length varying between 400 μm and 500 μm, were isolated and stored at 4°C. The dispersed cells were normally used within 2 h for experiments. Patch-clamp experiments were performed at room temperature (21–23°C) as described previously (Hamill *et al*., 1981; Teramoto & Brading, 1996; 1997; Teramoto *et al*., 1997a,b). Junction potentials between bath and pipette solutions were measured with a 3 M KCl reference electrode and were <2 mV, so that correction for these potentials was not made. Capacitance noise was kept to a minimum by maintaining the test solution in the electrode as low as possible. At the beginning of each experiment, the series resistance was compensated.

Drugs and solutions

For recoring glibenclamide-sensitive membrane currents, the following solutions were used: physiological salt solution (PSS) containing (mM): Na^+ 140, K^+ 5, Mg^{2+} 1.2, Ca^{2+} 2, glucose 5, Cl^- 151.4, HEPES 10, titrated to pH 7.35–7.40 with Tris base (sometimes 140 mM K^+ PSS was obtained by replacing 135 mM Na^+ with equimolar K^+); high potassium pipette solution containing (mM): K^+ 140, Cl^- 140, glucose 5, ethylene glycol-*bis* (β-aminoethylether) *N,N,N′,N′*-tetraacetic acid (EGTA) 5, and HEPES 10/Tris (pH 7.35–7.40). For recording voltage-dependent Ba^{2+} currents, high caesium pipette solution contained (mM): Cs^+ 130, tetraethylammonium (TEA^+) 10, Mg^{2+} 2, Cl^- 144, glucose 5, EGTA 5, ATP 5, HEPES 10/Tris (pH 7.35–7.40). Ba^{2+} 10 mM bath solution (mM): Ba^{2+} 10, TEA^+, 135, Cl^- 155, glucose 10, HEPES 10/Tris (pH 7.35–7.40). For cell-attached recordings, the pipette and bath solution were high potassium solution (mM): K^+ 140, Cl^- 140, EGTA 5, glucose 5, HEPES 10/Tris (pH 7.35–7.40) producing symmetrical 140 mM K^+ conditions. Cells were allowed to settle in the small experimental chamber (80 μl in volume). The bath solution was superfused by gravity throughout the experiments at a rate of 2 ml min^{-1}. The following chemicals were used: ATP, collagenase (type I), dimethysulphoxide (DMSO), EGTA, glibenclamide, HEPES, papain, TEAC1 and (±)-verapamil (Sigma, Dorset, U.K.), Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) and pinacidil (RBI Chemicals Ltd., St. Albans, U.K.), diltiazem (Tanabe Pharmaceutical Ltd., Osaka, Japan) and Tris (BDH Chemicals Ltd., Dorset, U.K.). Levcromakalim was kindly provided by SmithKline Beecham Pharmaceuticals (Harlow, U.K.). K_{ATP} openers (levcromakalim and pinacidil), glibenclamide and Ca antagonists were prepared daily as 100 mM stock solutions in DMSO. Bay K 8644 was dissolved to 1 mM in DMSO. The final concentration of DMSO was less than 0.3%, and this concentration was shown not to affect potassium channels in pig urethra.

Data analysis

The whole-cell current data were low-pass filtered at 500 Hz by an 8 pole Bessel filter, sampled at 20 ms intervals and analysed on a computer (Macintosh PowerBook 1400c, Apple Computer UK Limited, Uxbridge, U.K.) by the commercial software 'MacLab 3.4.2' (ADInstruments Pty Ltd., Castle Hill, Australia). For single-channel recordings, the stored data were low-pass filtered at 2 kHz (−3 dB) and sampled into the computer with a digitalized interval of 80 μs with 'PAT' program (kindly provided by Dr J. Dempster, the University of Strathclyde, U.K.); events briefer than 80 μs were not included in the evaluation. Continuous traces in the figures were obtained from records filtered at 500 Hz for presentation (digital sampling interval, 25 ms). Values for the channel open state probability (P_{open}) were measured at −50 mV for 2 min.

$$NP_o = (\sum_{j=1}^{N} t_j.j)/T$$

where t_j is the time spent at each current level corresponding to $j = 0,1,2, \ldots N$, T is the duration of the recording, and N is taken as the maximum number of channels observed in the patch membrane where P_{open} was relatively high. Data points were fitted by use of least-squares fitting.

Statistics

Statistical analyses were performed with either Student's t test for paired values or analysis of variance (ANOVA) test (two-factor with replication). Changes were considered significant at $P<0.01$. Data are expressed as mean with the s.d.

Results

Effects of nifedipine on the voltage-dependent Ba^{2+} currents in pig proximal urethra

When the pipette was filled with a Cs^+-TEA^+ solution containing 5 mM EGTA and the bath was superfused by 10 mM Ba^{2+} solution containing 135 mM TEA^+, application of depolarizing step pulses (10 mV increment from −40 mV to +40 mV for 1 s duration, every 20 s) from a holding potential of −50 mV produced an inward Ba^{2+} current at potentials more positive than −40 mV. The maximum current amplitude was obtained at +10 mV. The inward currents were reduced by nifedipine as shown in Figure 1a, which compares the current-voltage (*I*-V) relationships in control solution and 4 min after 20 nM nifedipine was applied to the bath. The inhibition by nifedipine was voltage-dependent, the relative amplitude of the inhibition increasing linearly by approximately 6.8% per 10 mV shift in the depolarizing direction (in the range −10 mV to +40 mV, results not shown). Figure 1b shows the concentration-response relationship for the effect of nifedipine on the relative amplitude of the inward Ba^{2+} current evoked by a pulse depolarization to +10 mV from −50 mV ($K_i = 30.6$ nM).

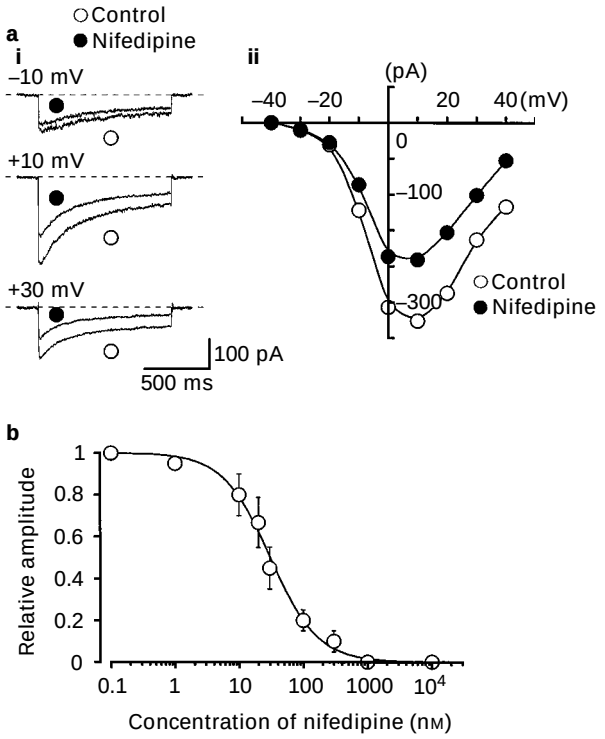

Figure 1 Effects of nifedipine on voltage-dependent Ba^{2+} inward currents at a holding membrane potential of -50 mV in single cells of pig proximal urethra. The pipette solution was Cs^+-TEA^+ solution containing 5 mM EGTA and the bath solution was 10 mM Ba^{2+} containing 135 mM TEA^+. (a) (i) Original current traces before (control) and after application of 20 nM nifedipine at the indicated pulse potentials. (ii) Current-voltage relationships obtained in the absence (control) or presence of 20 nM nifedipine. The current amplitude was measured as the peak amplitude of the Ba^{2+} inward current in each condition. The lines were drawn by eye. (b) Relationship between relative inhibition of the peak amplitude of Ba^{2+} current and the concentration of nifedipine. The peak amplitude of the Ba^{2+} current elicited by a step pulse to $+10$ mV from the holding potential of -50 mV just before application of nifedipine was normalized as 1.0. The curve was drawn by fitting the equation by the least-squares method: relative amplitude of voltage-dependent Ba^{2+} current $= 1/\{1+K_i/D)^{n}{}_H$ where K_i, D and n_H are the inhibitory dissociation constant, concentration of nifedipine (nM) and Hill coefficient, respectively. The following values were used for the curve fitting: $K_i = 30.6$ nM, $n_H = 1.1$. Each symbol indicates the mean of 4–6 observation with s.d. shown by vertical lines.

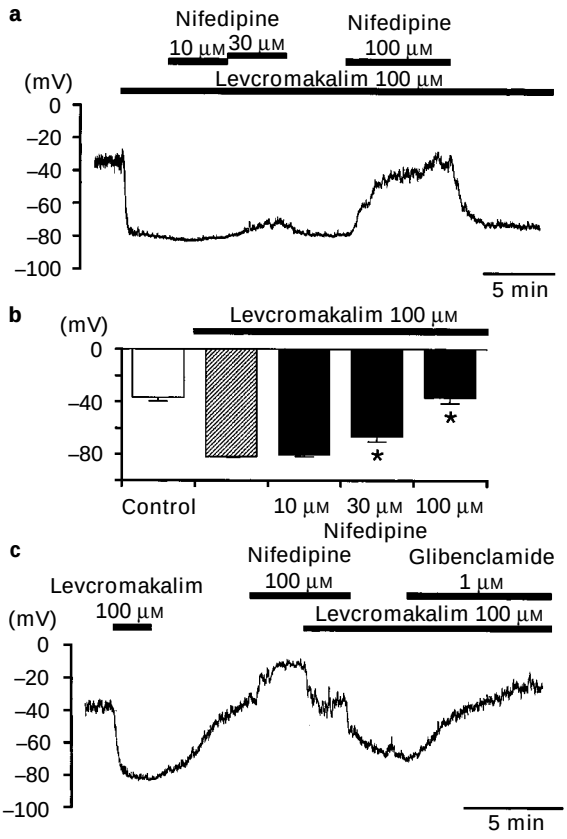

Figure 2 Concentration-dependent inhibitory effects of nifedipine on the 100 μM levcromakalim-induced hyperpolarization in dispersed urethral smooth muscle cells by use of current-clamp mode. The bath solution was PSS and the pipette solution was 140 mM KCl containing 5 mM EGTA. (a) Effects of nifedipine on the 100 μM levcromakalim-induced membrane hyperpolarization. (b) Concentration-response relationships for the inhibitory effects of nifedipine. Control indicates the membrane potential just before the application of 100 μM levcromakalim. Each column shows the mean of 4–8 observations with s.d. *Significantly different from the control ($P<0.01$). (c) Effects of application of either 100 μM nifedipine or 1 μM glibenclamide on the membrane potential.

Effects of nifedipine on the levcromakalim-induced hyperpolarization

In current-clamp mode, application of 100 μM levcromakalim immediately caused a stable hyperpolarization (to -81.9 ± 0.4 mV, $n=8$) which was very close to the theoretical potassium equilibrium potential ($E_K = -84.2$ mV) under the present experimental conditions (Figure 2a). When nifedipine was additionally applied in a cumulative manner, nifedipine ($\geqslant 30$ μM) caused a significant reversal of the hyperpolarization, although lower concentrations ($\leqslant 10$ μM) had no significant effect (Figure 2a, b, $n=5$). When 100 μM nifedipine was applied before additional application of 100 μM levcromakalim (Figure 2c), nifedipine itself significantly depolarized the cell membrane to approximately -15 mV (-17 ± 4 mV, $n=5$) and the peak amplitude of the 100 μM levcromakalim-induced hyperpolarization was dramatically inhibited (-36.6 mV±4.6 mV, $n=8$). On removal of nifedipine, the levcromakalim-induced hyperpolarization gradually returned, although its amplitude was smaller than that of the previous application. Additional application of glibenclamide (1 μM) depolarized the cell membrane slightly beyond the control level (-27.2 ± 2 mV, $n=4$). Extracellular application of 0.3% DMSO had no effect (data not shown).

Effects of Ca antagonists on the glibenclamide-sensitive membrane currents at -50 mV

To investigate further the inhibitory effects of nifedipine on the levcromakalim-induced stable hyperpolarization, whole-cell voltage-clamp experiments were performed in symmetrical 140 mM K^+ conditions (bath solution 140 mM K^+ PSS, pipette solution 140 mM KCl containing 5 mM EGTA, $E_K = 0$ mV) at a holding potential of -50 mV. As shown in Figure 3a, application of levcromakalim (100 μM) rapidly caused an inward current which, after reaching a peak value, gradually decreased in amplitude at constant potential (-50 mV). When 100 μM nifedipine was additionally applied, the remaining levcromakalim-induced inward current was immediately inhibited. On removal of nifedipine, the amplitude of the levcromakalim-induced current showed a gradual recovery towards the steady state value it would have reached without the intervention of nifedipine. Subsequent application of 5 μM glibenclamide suppressed the inward

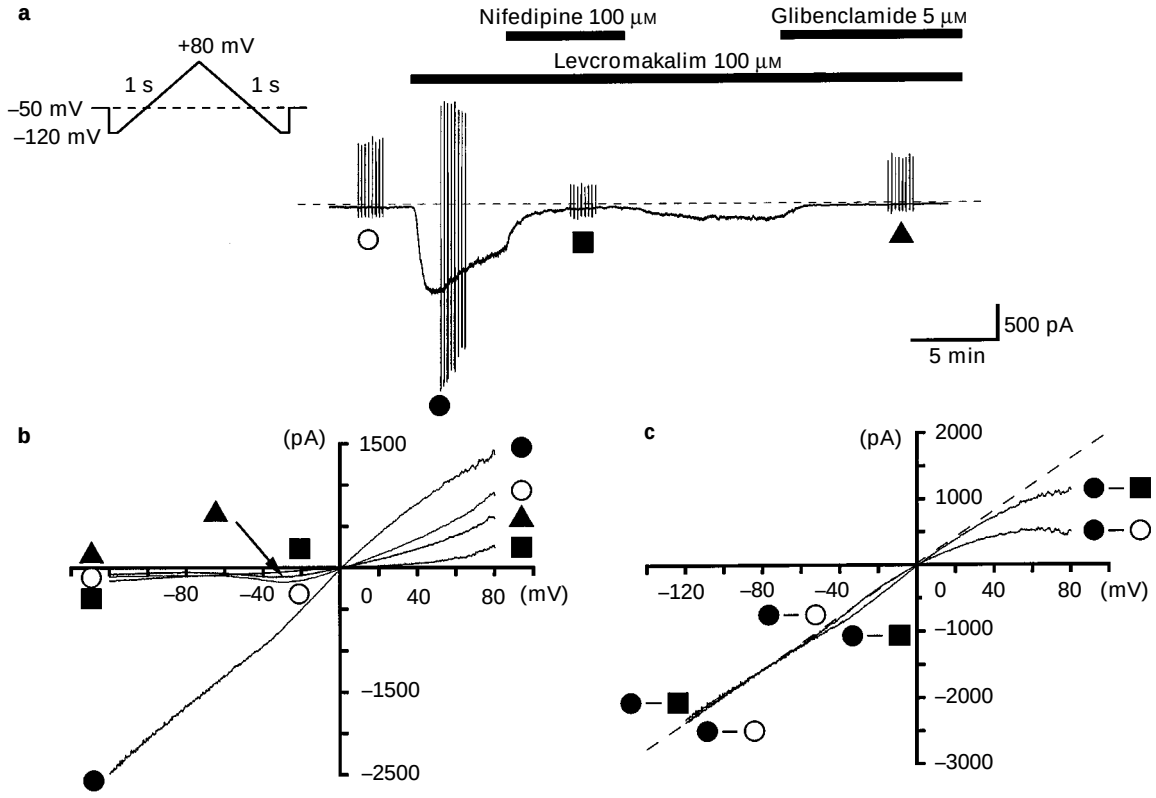

Figure 3 Inhibitory effects of nifedipine on the K_{ATP} opener-induced glibenclamide-sensitive inward membrane current at -50 mV. Whole-cell recording, bath solution 140 mM K^+ PSS, pipette solution 140 mM K^+containing 5 mM EGTA. (a) Current trace. The vertical lines are responses to triangular ramp potential pulses of 200 mV s^{-1} from -120 mV to $+80$ mV, applied after an initial 100 ms conditioning pulse to -120 mV (see inset). Levcromakalim (100 μM) caused an inward membrane current (peak amplitude about 1.3 nA) which gradually decayed. The current was suppressed by additional application of 100 μM nifedipine, recovered to a steady state amplitude after nifedipine was removed, and was then suppressed to the same level by 5 μM glibenclamide. The dashed line indicates zero current line. (b) *I*-V curves measured from the negative-going limb (the falling phase) of the ramp pulse. Each symbol is the same as in the current trace. The lines are mean membrane currents from the 8 ramps in each condition. (c) Net membrane currents. The levcromakalim-induced membrane current was obtained by subtraction of the two mean ramp currents recorded before and during application of 100 μM levcromakalim. The nifedipine-sensitive membrane current was obtained by subtraction of the membrane currents in the absence and presence of 100 μM nifedipine when levcromakalim was present in the bath solution.

current to a value close to that seen in the presence of nifedipine. In the same experiment, eight triangular ramp potential pulses (see inset in Figure 3a) were applied in order to obtain current-voltage relationships under each condition. Figure 3b shows the averaged membrane currents during the falling phase of the ramp pulses under the various experimental conditions. The levcromakalim-induced membrane current was obtained by subtracting the averaged control current from the membrane current in the presence of 100 μM levcromakalim, demonstrating not only a selective K^+ permeability (the reversal potential was -0.9 ± 0.4 mV, $n=6$; $E_K = 0$ mV) but also an inward rectification at positive potentials (Figure 3c). The 100 μM nifedipine-sensitive membrane current in the presence of levcromakalim was obtained by subtracting the mean membrane current in the presence of 100 μM nifedipine from the membrane current in its absence. The nifedipine-sensitive current overlapped the levcromakalim-induced current from -120 mV to 0 mV. A similar inhibitory effect of nifedipine was observed on the inward current induced by 100 μM pinacidil at -50 mV (Figure 4a). Figure 4b shows that the amplitude of the 100 μM levcromakalim-induced inward current was also suppressed to a level close to that caused by glibenclamide (5 μM), by subsequent application of other Ca antagonists at 100 μM (verapamil, (i); diltiazem (ii)). On removal of the Ca antagonists, the amplitude of the 100 μM levcromakalim-induced inward current at -50 mV gradually increased to a steady value that was less than the original level, but probably similar to the level that would have occurred without application of the Ca antagonists, due to run down of the channels under these conditions (Teramoto *et al.*, 1997a, b). Applying 1 μM Bay K8644, a selective voltage-dependent Ca^{2+} channel agonist, did not augment the levcromakalim-induced current (Figure 4d, 4 min application $n=5$).

Experiments to elucidate the route of access of glibenclamide or nifedipine

In the experiments described so far, the drugs were applied in the bathing solution, and were not included in the pipette solution. However, it is not clear whether their site of action is extracellular or intracellular. In order to clarify the matter, 5 μM glibenclamide or 100 μM nifedipine was applied intracellularly by including each drug in the pipette solution to determine if they had a similar inhibitory potency on the 100 μM levcromakalim-induced membrane current at -50 mV when administered by this route. Approximately 10 min after the establishment of conventional whole-cell configuration,

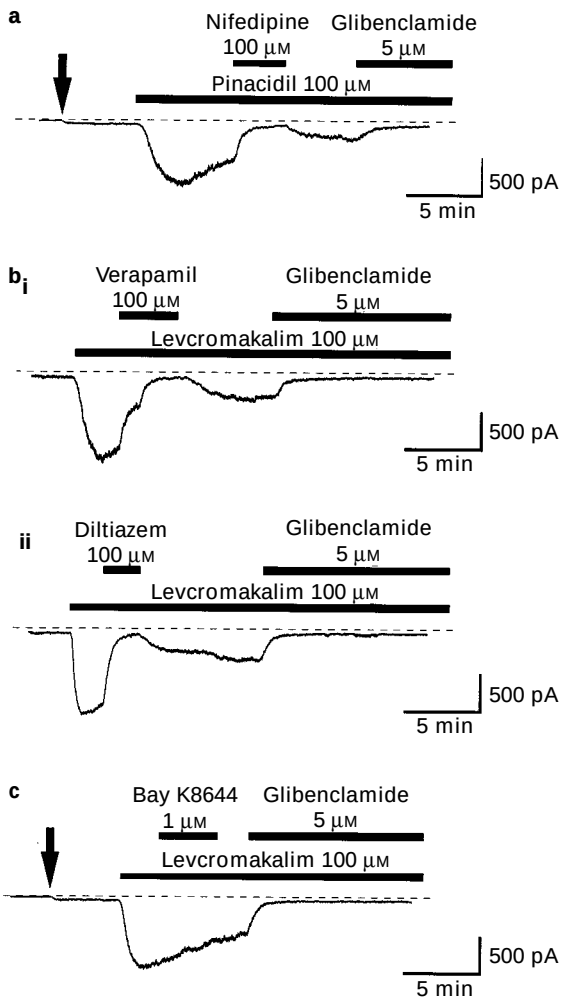

Figure 4 Effects of Ca antagonists and Bay K8644 on K_{ATP} opener-induced glibenclamide-sensitive inward membrane currents. Whole-cell recording at -50 mV. Bath solution 140 mM K^+ PSS (introduced replacing PSS at the arrows), pipette solution 140 mM K^+ containing 5 mM EGTA. The dashed lines indicate the zero current level. (a) Application of nifedipine 100 μM suppressed the 100 μM pinacidil-induced glibenclamide-sensitive inward current. (b) The 100 μM levcromakalim-induced membrane current was suppressed by subsequent application of 100 μM verapamil (i) or diltiazem (ii). (c) Additional application of 1 μM Bay K 8644 (4 min duration) did not enhance the amplitude of the 100 μM levcromakalim-induced membrane current.

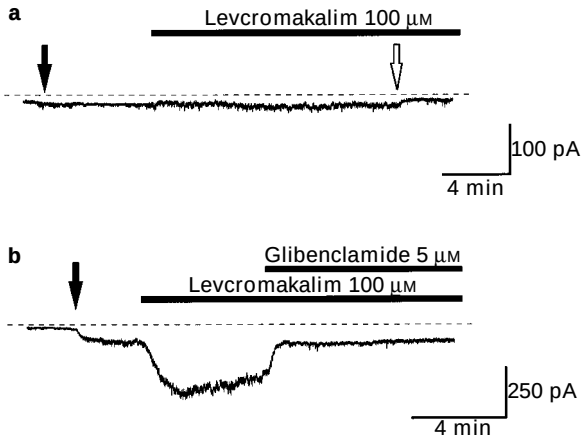

Figure 5 Effects of levcromakalim on the membrane current at -50 mV when either 5 μM glibenclamide or 100 μM nifedipine was included in the pipette solution. Whole-cell configuration. Levcromakalim (100 μM) was applied approximately 15 min after the conventional whole-cell configuration had been established. The bath solution was initially 5 mM K^+ PSS and changed to 140 mM K^+ PSS between the solid and open arrows. Pipette solution 140 mM K^+ containing 5 mM EGTA. The dashed lines indicate the zero current line. (a) The pipette solution contained 5 μM glibenclamide, which prevented activation of the 100 μM levcromakalim-induced membrane current. (b) The pipette solution contained 100 μM nifedipine. Under this condition, 100 μM levcromakalim induced an inward current, but the amplitude was only about a quarter of that seen without nifedipine in the pipette (see text). The levcromakalim-induced inward current was suppressed by subsequent application of 5 μM glibenclamide.

and thus allowing intracellular perfusion with the drug, the bath solution was changed from 5 mM K^+ PSS to 140 mM K^+ PSS and, 5 min later, 100 μM levcromakalim was applied in the bathing solution. When 5 μM glibenclamide was included in the pipette solution, application of 100 μM levcromakalim did not cause any detectable inward current (Figure 5a, $n=5$). On the other hand, with 100 μM nifedipine in the pipette solution, 100 μM levcromakalim caused an inward current, but only about a quarter of that induced without nifedipine in the pipette (4.5 ± 0.6 pA pF^{-1} $n=5$ (nifedipine), vs 19.7 ± 4.5 pA pF^{-1}, $n=10$ (control), $P<0.01$). This stable levcromakalim-induced inward current was further suppressed by additional application of 5 μM glibenclamide (Figure 5b). To minimize cell-to-cell variation for these experiments, the effect of the drugs was examined on cells isolated from the same animal under the same experimental conditions (such as a top size of the glass pipette and a flow rate of levcromakalim). The effects of glibenclamide and nifedipine on the K^+ channels activated by 100 μM levcromakalim were also studied by use of single-channel recording in the cell-attached configuration under symmetrical 140 mM K^+ conditions at -50 mV. In approximately 25% of the membrane patches (25 patches in 96 patches), glibenclamide-sensitive 43 pS K^+ channel openings were observed in the presence of 100 μM levcromakalim, demonstrating the relatively low probability of recording from these channels in pig urethra. In Figure 6a, a 2.14 pA channel was activated in the patch by bath application of 100 μM levcromakalim. Additional bath application of 100 μM nifedipine caused a small, reversible inhibition of the channel activity to an NP_o of 0.76 ± 0.13 when the control 100 μM levcromakalim-induced NP_o value was normalized as 1.0 ($n=4$, Figure 6b (i)). On removal of nifedipine, the channel activity completely recovered to the control level (0.93 ± 0.07, $n=4$). Approximately 6 min later, bath application of 5 μM glibenclamide completely but reversibly abolished the channel activity (NP_o value 0, Figure 6b (ii)). On the other hand, if 100 μM nifedipine was included in the pipette solution with the cell attached configuration, extracellular application of levcromakalim never appeared to activate glibenclamide-sensitive 43 pS K^+ channels ($n=18$), although we would have expected to see them on at least four occasions.

Discussion

Non-selective blocking effects of Ca antagonists on K^+ currents

In this paper we have been able to demonstrate that Ca antagonists, at a concentration an order of magnitude higher than required to block voltage-sensitive Ca^{2+} channels, have an inhibitory effect on K_{ATP} openers-induced membrane currents.

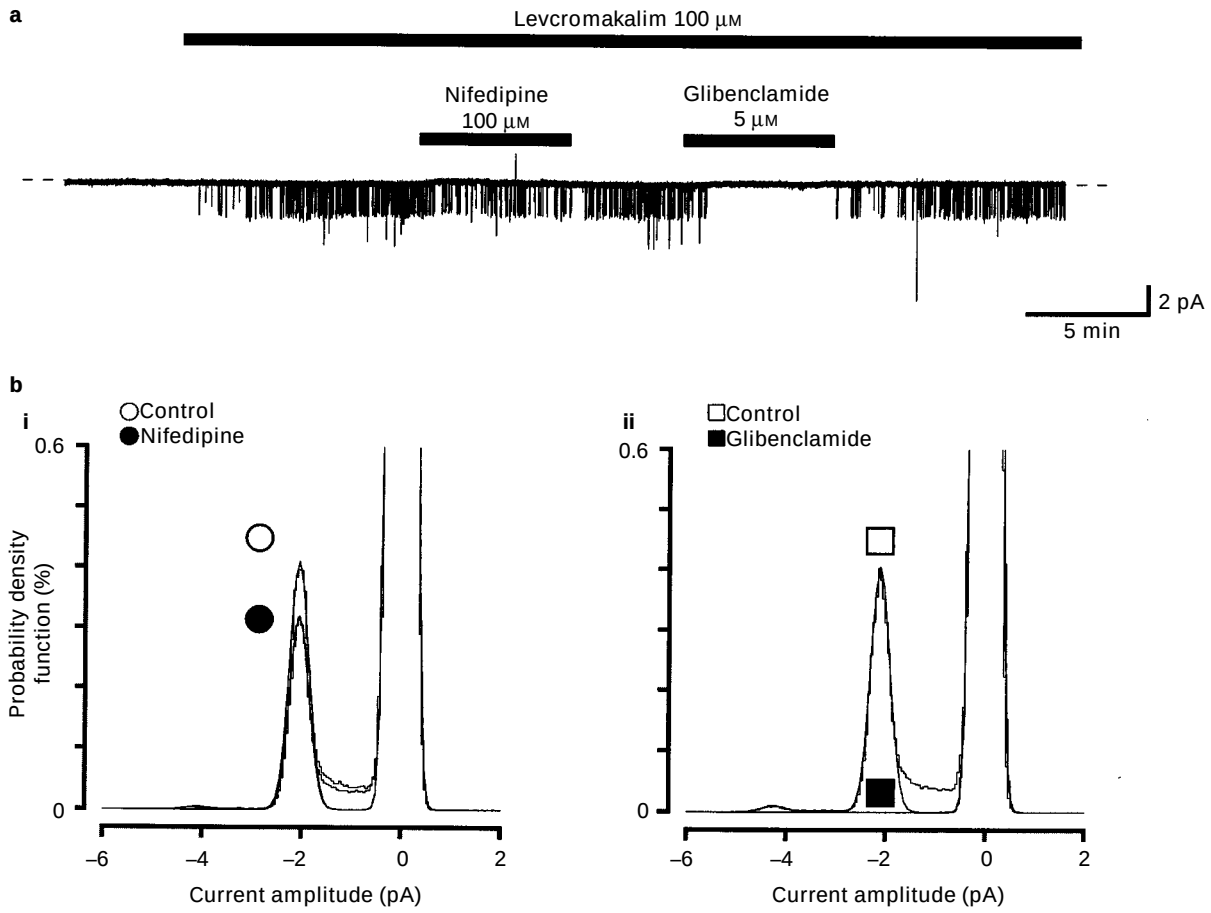

Figure 6 Effects of either 100 μM nifedipine or 5 μM glibenclamide on the 100 μM levcromakalim-induced K^+ channel. Cell-attached patch at -50 mV. Levcromakalim 100 μM was added to the bath solution (symmetrical 140 mM K^+ conditions). (a) Additional application of 100 μM nifedipine (6 min duration) reversibly reduced the activity of the 2.14 pA K^+ channel. After removal of nifedipine, 6 min later, application of glibenclamide (6 min duration) abolished channel activity. The dashed line indicates the current when the channel was not open. (b) All-point amplitude histograms in the presence of either 100 μM nifedipine or 5 μM glibenclamide (obtained during the last 2 min of a 6 min application. Histograms in the absence (control; just before the application of each blocker) or presence of each blocker (100 μM nifedipine or 5 μM glibenclamide) were superimposed. Continuous lines in the histograms are theoretical curves fitted with the Gaussian distribution, by the least-squares method. The abscissa scales show the amplitude of the current (pA) and the ordinate scales show the percentage value of the probability density function (%) for the recording period (2 min). The relative NP_o values ($\pm$s.d.) in the presence of 100 μM nifedipine were 0.76 ± 0.13, after washing-out nifedipine 0.94 ± 0.07, and in the presence of 5 μM glibenclamide 0 ($n=4$). The values in the presence of the drugs were significantly different from the control ($P<0.01$, ANOVA).

Several studies have shown that the target K^+ channels for K_{ATP} openers in some smooth muscle possess intracellular Ca^{2+} sensitivity (rat azygous vein, pinacidil, Hermsmeyer, 1988; rabbit aorta, cromakalim, Gelband *et al.*, 1989; Gelband & McCullough, 1993; rat portal vein, nicroandil, Kajioka *et al.*, 1990; canine colon, levcromakalim, Carl *et al.*, 1992). It is therefore possible that the inhibitory effects of the Ca antagonists that we have shown on the K^+ currents in the pig urethral myocytes are indirect, and due to their effects on the L-type Ca^{2+} channels. Blocking these channels might lead to a reduction of Ca^{2+} influx followed by a decrease of intracellular Ca^{2+} concentration, which directly or indirectly could switch off the levcromakalim-activated K^+ channels. It is equally possible that the Ca antagonists might directly block K^+ channels in a non-selective manner. The results from the present experiments suggest that the latter possibility is probably correct. It seems neither an interaction with voltage-dependent Ca^{2+} channels nor extracellular Ca^{2+} influx through them is related to the activity of the glibenclamide-sensitive K^+ currents in pig urethra, and that the effects of Ca antagonists are due to a non-selective K^+ channel blockade. We have used levcromakalim in order to avoid the non-selective effects of cromakalim, and although the inward levcromakalim-activated K^+ current in symmetrical high K^+ conditions at -50 mV was inhibited by nifedipine, it was not augmented by 1 μM Bay K8644, a concentration known to activate L-type Ca^{2+} channels (Hille, 1992) and result in Ca^{2+} influx. Under more physiological K^+ conditions, 100 μM levcromakalim generated a prolonged hyperpolarization, stabilizing the membrane potential at about -82 mV, a potential at which virtually all of the L-type Ca^{2+} channels will be closed, excluding entry of Ca^{2+} through these channels. Nevertheless, under these conditions 100 μM nifedipine depolarized the membrane and reduced the membrane conductance in a manner similar to 5 μM glibenclamide. These pharmacological observations, which suggest that changes in intracellular Ca^{2+} concentration are not involved in this action of nifedipine, are strongly supported by our previous observations that neither the levcromakalim-induced glibenclamide-sensitive K^+ currents nor the levcromakalim-induced glibenclamide-sensitive 43 pS K^+ channel exhibit intracellular Ca^{2+} sensitivity in pig proximal urethra (Teramoto & Brading, 1996; Teramoto *et*

al., 1997a,b). Thus the inhibitory effects of Ca antagonists on both the levcromakalim-induced hyperpolarization and the levcromakalim-induced membrane currents in pig urethra are not due to a decreased Ca^{2+} entry but to a block of the glibenclamide-sensitive mechanisms.

It is interesting that the glibenclamide-sensitive current is inhibited by three different structural types of Ca antagonists (dihydropyridine derivatives, nifedipine; phenylalkylamine derivatives, verapamil; benzothiazepine derivatives, diltiazem). This is in contrast to the blocking effect of the Ca antagonists on voltage-dependent K^+ currents (Terada *et al*., 1987a) in which nicardipine, diltiazem and verapamil inhibit not only with different potencies but also exhibit different mechanisms of action. Moreover, a similar inhibitory effect of Ca antagonists on the 100 μM pinacidil-induced glibenclamide-sensitive inward membrane currents is observed in pig urethra. These results suggest that inhibitory actions of Ca antagonists on the K_{ATP} opener-induced glibenclamide-sensitive K^+ currents may be a common property of Ca antagonists at high concentrations.

Mechanisms by which Ca antagonists block glibenclamide-sensitive currents

Recently, it has been found that in the cloned K_{ATP} of pancreatic β-cells, K_{ATP} comprises a complex of (at least) two different proteins: the sulphonylurea receptor (SUR1) and an inwardly rectifying K^+ channel subunit (Kir6.2) (Inagaki *et al*., 1995). It is important to determine the binding sites of Ca antagonists for blocking effects on the channel activity of the glibenclamide-sensitive K_{ATP}. It has been shown that in rat pancreatic β-cells application of high concentrations (100 μM) of verapamil or nifedipine do not inhibit [^{3}H]-glibenclamide binding, suggesting that Ca antagonists do not interact with the sulphonylurea receptors (Kramer *et al*., 1988). In contrast, in cat ventricular myocytes the activity of K_{ATP} was clearly blocked by intracellular but not by extracellular application of verapamil (3 μM), leading to the conclusion that verapamil had access to its binding site from within the cell (Kimura *et al*., 1992). In the present experiments, extracellular application of either 100 μM Ca antagonists or 5 μM glibenclamide completely suppressed the 100 μM levcromakalim-induced membrane current at -50 mV. However, intracellular application of 5 μM glibenclamide also completely blocked the levcromakalim-induced inward current in symmetrical 140 mM K^+ conditions. Under similar conditions, intracellular application of 100 μM nifedipine only reduced, but did not completely abolish the levcromakalim-activated current. In the cell-attached configuration, 100 μM levcromakalim in the bathing solution activated the 43 pS K^+ channel, and this was completely suppressed by additional bath application of glibenclamide (5 μM) but only partially suppressed by nifedipine (100 μM), even in the same membrane patches. These results suggest that glibenclamide and levcromakalim can access their respective binding sites equally well when applied extracellularly or intracellularly, presumably by diffusion through the membrane. On the other hand, nifedipine is far less effective when applied intracellularly. These results suggest that high concentrations of Ca antagonists are likely to inhibit the glibenclamide-sensitive 43 pS K^+ channel via a different inhibitory pathway from that of glibenclamide. It is probable that the binding site for nifedipine is readily accessible from the extracellular solution, and that its minor inhibitory effects on the levcromakalim-induced current when applied intracellularly, and its ability to decrease of the NP_o value of K_{ATP} in cell-attached patches when applied extracellularly is due to its diffusion through the membrane generating a low concentration for binding to an extracellular site. In confirmation of this hypothesis is the apparent ability of Ca antagonists, when included in the pipette solution (100 μM) in cell-attached configuration, to prevent extracellular application of levcromakalim (or pinacidil) from activating the glibenclamide-sensitive 43 pS K^+ channel.

Although we cannot be certain as to the exact binding sites for nifedipine on the glibenclamide-sensitive K^+ channels, it does not appear to be acting through the sulphonylurea receptor in pig urethra. Further studies may cast light upon the exact nature of the binding sites of Ca antagonists to the glibenclamide-sensitive 43 pS K^+ channel in pig urethra, and thus identify a potentially useful site for producing more potent blockers for glibenclamide-sensitive K^+ channels.

In conclusion, we demonstrated that extracellular application of different types of Ca antagonists inhibit the levcromakalim-induced glibenclamide-sensitive K^+ currents in smooth muscle myocytes from pig urethra, through a blockade of the glibenclamide-sensitive 43 pS K^+ channel.

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