British Journal of Pharmacology (1996) 119, 772-778

Inhibition by P1075 and pinacidil of a calcium-independent chloride conductance in conditionally-immortal renal glomerular mesangial cells

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1 Depolarization of mesangial cells has been shown to occur following an outward movement of chloride ions from the cell. We have shown previously that mesangial cells from the *H*-2*K*^{b}-tsA58 transgenic mouse possess a significant whole-cell chloride conductance and consequently are a suitable preparation for the study of potential chloride channel inhibitors.

2 The effects on the whole-cell chloride conductance of the chloride channel inhibitor, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and the potassium channel openers, (KCOs) P1075 and pinacidil were investigated in mesangial cells from the *H*-2*K*^{b}-tsA58 transgenic mouse cultured in permissive conditions (at 33°C in the presence of 50 u ml^{-1} murine γ-interferon).

3 In symmetrical solutions of 140 mM tetramethylammonium chloride (TMACl) the whole-cell chloride conductance was 1.08 ± 0.05 nS ($n = 63$) and this could be reversibly inhibited by 5×10^{-5} M NPPB.

4 Both P1075 and pinacidil inhibited the whole-cell chloride conductance. This inhibition was not reversible after drug washout and was demonstrated only when drugs were applied to the extracellular surface of the cells. Very low concentrations of the drugs were found to reduce the chloride conductance after 16 h incubation but under no circumstances studied was the conductance totally inhibited, leaving a mean residual current of 0.33 ± 0.03 nS ($n = 12$).

5 The effects of different peptide calcium concentrations on the magnitude of the residual current in the presence of the drugs were investigated. The residual current was reduced with 10^{-8} M calcium in the pipette and increased with 10^{-3} M pipette calcium. Therefore, these data suggest that P1075 and pinacidil selectively inhibit a calcium-independent chloride conductance in mesangial cells from the *H*-2*K*^{b}-tsA58 transgenic mouse.

Keywords: *H*-2*K*^{b}-tsA58; P1075; pinacidil; chloride conductance

Introduction

Recently, several drugs have been shown to have effects on both chloride and potassium channels. Niflumic acid is an inhibitor of chloride currents in the AtT-20 mouse anterior pituitary cell line (Korn *et al.*, 1991) and in *H*-2*K*^{b}-tsA58 transgenic mouse mesangial cells (Barber *et al.*, 1995) and has been found to potentiate openings in large conductance, calcium-activated potassium channels found in vesicles prepared from pig coronary arteries (Ottolia & Toro, 1994) as well as to increase a potassium current in rabbit corneal epithelium (Rae & Farrugia, 1992). Similarly, it has been shown that the chloride channel blockers SITS (4-acetamido-4-isothiocyanostilbene-2,2'-disulphonic acid) and DIDS (4,4'-diisothiocyanostilbene-2,2'-disulphonic acid), (Hogg *et al.*, 1994) block ATP-sensitive potassium channels in guinea-pig ventricular myocytes (Furukawa *et al.*, 1993). Correspondingly, the potassium channel openers (KCOs) diazoxide and levcromakalim, and the sulphonylureas, tolbutamide and glibenclamide, which normally inhibit ATP-sensitive potassium channels, have been shown to inhibit the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) (Sheppard & Welsh, 1992). Furthermore, glibenclamide has been shown to inhibit an outwardly rectifying chloride channel found in mouse cortical collecting tubule cells (Volk *et al.*, 1995) and a cyclic AMP-activated chloride channel in guinea-pig ventricular myocytes (Tominaga *et al.*, 1995).

Another KCO P1075 (*N*-cyano-*N'*-(1,1-dimethylpropyl)-*N''*-3-pyridylguanidine) has been shown to cause the activation of a K^+ conductance in A10 smooth muscle cells (Holevinsky *et al.*, 1994). However, Holevinsky *et al.* (1994) have also demonstrated that P1075 inhibited a chloride conductance in the same cells. In other, earlier experiments, diphenylamine-2-carboxylate,5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and niflumic acid have been used as chloride channel inhibitors in tissues including the shark rectal gland (Di Stefano *et al.*, 1985), rabbit kidney collecting tubule (Wangemann *et al.*, 1986) and mouse pituitary cells (Korn *et al.*, 1991) but they were used at high concentrations (50-100 μM). Holevinsky *et al.* (1994) obtained a 50% inhibition with 300 nM P1075 and this suggests that P1075 may have a much higher potency.

The primary role of renal glomerular mesangial cells is thought to be the maintenance of glomerular structure (Mené *et al.*, 1989). Mesangial cells are similar to smooth muscle cells, containing actin and myosin (Kreisberg *et al.*, 1985) and cultured rat mesangial cells have been shown to depolarize in response to application of various vasoactive hormones (Okuda *et al.*, 1986). Depolarization is brought about by the activation of an inward chloride current in both calcium-dependent and calcium-independent mechanisms (Kremer *et al.*, 1989; 1992) and leads to cell contraction (Venkatachalam & Kreisberg, 1985).

Mesangial cells isolated from the *H*-2*K*^{b}-tsA58 transgenic mouse (Jat *et al.*, 1991) have a significant chloride permeability (Barber *et al.*, 1995) and therefore, as such, are useful for study of potential chloride channel inhibitors. We have used mesangial cells from this mouse, cultured in permissive conditions, to investigate the inhibition of the chloride conductance by P1075 and pinacidil.

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Methods

Cell culture

Mesangial cells were prepared as described previously (Barber *et al.*, 1995). Briefly, mice were killed by cervical dislocation and their kidneys were removed. Thin slices of the kidney cortex were subjected to successive sieving, centrifugation and further sieving steps before the glomeruli were caught on a 300 mesh sieve. These, and the subsequent cellular outgrowth (Kreisberg & Karnovsky, 1983) were cultured in permissive conditions at 33°C in a humidified atmosphere containing 7% CO_2. The culture medium was made up from Dulbecco's Minimum Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 u ml^{-1} penicillin and streptomycin (PS, all from Gibco BRL, Paisley) and 50 u ml^{-1} recombinant murine γ-interferon (Genzyme, Cambridge, U.S.A.). Cells were passaged weekly and fed twice weekly. Cells were plated out into 35 mm Petri dishes (from R. & L. Slaughter, Upminster, Essex) and grown in permissive conditions. Experiments were performed between 24 h and 7 days after passage on cells between passages 8–15.

Electrophysiology

Whole-cell recordings were obtained according to the technique of Hamill *et al.* (1981). Cells were viewed at 400× magnification with a Nikon TMS microscope supported on an air table (Wentworth Engineering Ltd., Sandy, Bedfordshire). Pipette electrodes were pulled from filamented, borosilicate glass (GC120-TF10, Clark Electromedical Instruments, Reading) on a Narishige PB-7 two-stage electrode puller (Narishige, Tokyo, Japan) and were fire-polished. Pipettes were positioned onto the cell-surface by use of Narishige MN153 coarse and MO103 hydraulic 3-dimensional manipulators. Filled pipettes had resistances of between 4–8 MΩ and all seal resistances were greater than 1 GΩ with cell access resistances of less than 10 MΩ. Experiments were performed at room temperature (21–24°C). To improve seal formation, cells were pretreated with 0.5 g l^{-1} trypsin (Worthington Biochemicals, Bury St. Edmunds, Suffolk) in a solution of 135 mM NaCl and 0.6 mM ethylenediaminetetraacetic acid (EDTA) for 15–120 s. Seal formation in the absence of trypsin pretreatment was negligible. This is believed to be a result of the secretory nature of these cells (Mené *et al.*, 1989).

Drugs were applied from a gravity-driven perfusion system and were present for 20 min before starting recording. The membrane stability of these cells is low and so all data are recorded from separate cells. Where control traces or data are shown, these refer to experiments conducted in cells from the same passage as those treated with drug. In experiments to investigate the effects of drug washout, cells were pretreated with the relevant drug. In the experiments, drugs were applied for 20 min and were removed at least 10 min prior to attempting seal formation.

Data acquisition

Whole-cell currents were obtained with a List EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). The voltage clamp protocols, data acquisition and analysis was performed with pCLAMP Version 5.5 software (Axon Instruments, Foster City, CA, U.S.A.) installed on an Elonex PC 333 microcomputer equipped with a Tecmar Labmaster A/D D/A board (Axon Instruments). Cells were held at 0 mV and a series of pulses (Figure 1a), each of 80 ms duration were applied to the cells. Pulses were stepped from −100 mV to +100 mV in 12.5 mV intervals and, between each pulse, cells were held at 0 mV for 25 ms. This protocol was repeated twice and was used in all experiments. Current measurements were obtained by averaging a 70 ms period following the end of the capacitative transient in each recording. Whole-cell conductances were determined as the gradient of the current/voltage relationship at 0 mV in symmetrical solutions obtained from these pulse protocols.

Solutions

Experiments were performed in symmetrical solutions containing 140 mM tetramethylammonium chloride (TMACl), 1 mM ethyleneglycol-*bis*-(β-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA), 2 mM free Mg^{2+} (2.005 mM $MgCl_2$) and 1 μM free Ca^{2+} (0.8995 mM $CaCl_2$) where the free Mg^{2+} and Ca^{2+} concentrations were determined using binding constants calculated by Fabiato & Fabiato (1979). Solutions were buffered to pH 7.4 with 10 mM Na^{2+}-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and all chemicals were of analytical grade from Fisons (Loughborough) with the following exceptions: TMACl, from Fluka Chemicals Ltd. (Gillingham, Dorset), NPPB, from Sigma (Poole) and P1075 and pinacidil which were a generous gift from Leo Pharmaceuticals Ltd., Ballerup, Denmark. Both P1075 and pinacidil were dissolved in dimethylsulphoxide (DMSO, 25 mg ml^{-1}) and were diluted as appropriate so that the final concentration of DMSO was less than 0.1%.

All data are presented as the mean ± standard error of the mean with the number of experiments given in parentheses. Statistics were performed using Student's *t* test for unpaired data and the data are described as significant when $P<0.05$.

Results

In symmetrical solutions of 140 mM TMACl, the mean whole-cell conductance in mesangial cells from the *H*-2*K*^{b}-tsA58 transgenic mouse cultured in permissive conditions, was 1.08 ± 0.05 nS ($n=63$). Application of 5×10^{-5} M NPPB to the extracellular bathing solution inhibited the conductance (g = 0.27 ± 0.03 ns, $n=4$) and these effects were readily reversible following exchange of the bathing solution with drug-free solution. After the removal of NPPB, the whole-cell conductance was 0.96 ± 0.06 nS ($n=4$, $P<0.7$ vs controls, Student's *t* test for unpaired data). Original current recordings obtained from single cells and a current/voltage plot showing the effects of NPPB are shown in Figure 1.

To investigate the effects of P1075 it was applied to the bath solution at concentrations of 10^{-11} M, 10^{-9} M or 10^{-5} M. Each population of cells was exposed to a single drug concentration and it was found that with each concentration of drug, P1075 inhibited the whole-cell chloride conductance. This is shown in Figure 2 for cells in the presence of 10^{-5} M P1075 when the conductance is 0.33 ± 0.04 nS ($n=6$) and is significantly different from controls in the absence of drugs ($P<0.001$, Student's *t* test for unpaired data). In the presence of 10^{-9} or 10^{-11} M P1075, the conductances were 0.33 ± 0.10 nS ($n=6$) and 0.35 ± 0.06 nS ($n=7$) respectively and these are also significantly different from the conductance measured in the absence of drug ($P<0.001$, Student's *t* test for unpaired data). When P1075 (10^{-7} M) was dissolved in the pipette solution and applied to the intracellular surface of the membrane, the whole-cell conductance was 1.05 ± 0.08 nS ($n=6$, Figure 2). This is not significantly different from the conductance in the absence of drug ($P>0.9$, Student's *t* test for unpaired data).

The effects of pinacidil (10^{-3} M) were similar to those of P1075 and caused an inhibition of the whole-cell chloride conductance, (Figure 3). Furthermore, pinacidil had no effect when applied to the intracellular surface of the cells. In the presence of pinacidil (10^{-3} M) in the pipette, the conductance was 0.99 ± 0.07 nS ($n=4$) and this is not significantly different from controls ($P>0.7$, Student's *t* test for unpaired data).

Neither the effects of P1075 nor those of pinacidil were reversible after washout, when the same procedure, shown previously to be effective in removing the effects of NPPB, was repeated twice and the cells allowed to recover for 30 min. When either P1075 (10^{-5} M) or pinacidil (10^{-3} M) were added to the medium bathing the external surface of the membrane,

the whole-cell conductances were 0.33 ± 0.04 nS ($n=6$) and 0.58 ± 0.12 nS ($n=6$) respectively. Following washout, the conductances were unchanged at 0.37 ± 0.06 nS ($n=5$, Figure 2) and 0.50 ± 0.11 nS respectively ($n=6$, Figure 3). This suggests that the effects of P1075 and pinacidil on the chloride conductance are irreversible.

However, when the concentrations of P1075 or pinacidil were reduced 10^{-15} M and 10^{-7} M respectively, no inhibitory effect was observed. In the presence of P1075 (10^{-15} M) the whole-cell conductance was 0.94 ± 0.14 nS ($n=6$) and was 1.17 ± 0.14 ($n=6$) in the presence of pinacidil (10^{-7} M). This could be explained if the drugs were highly-potent reversible

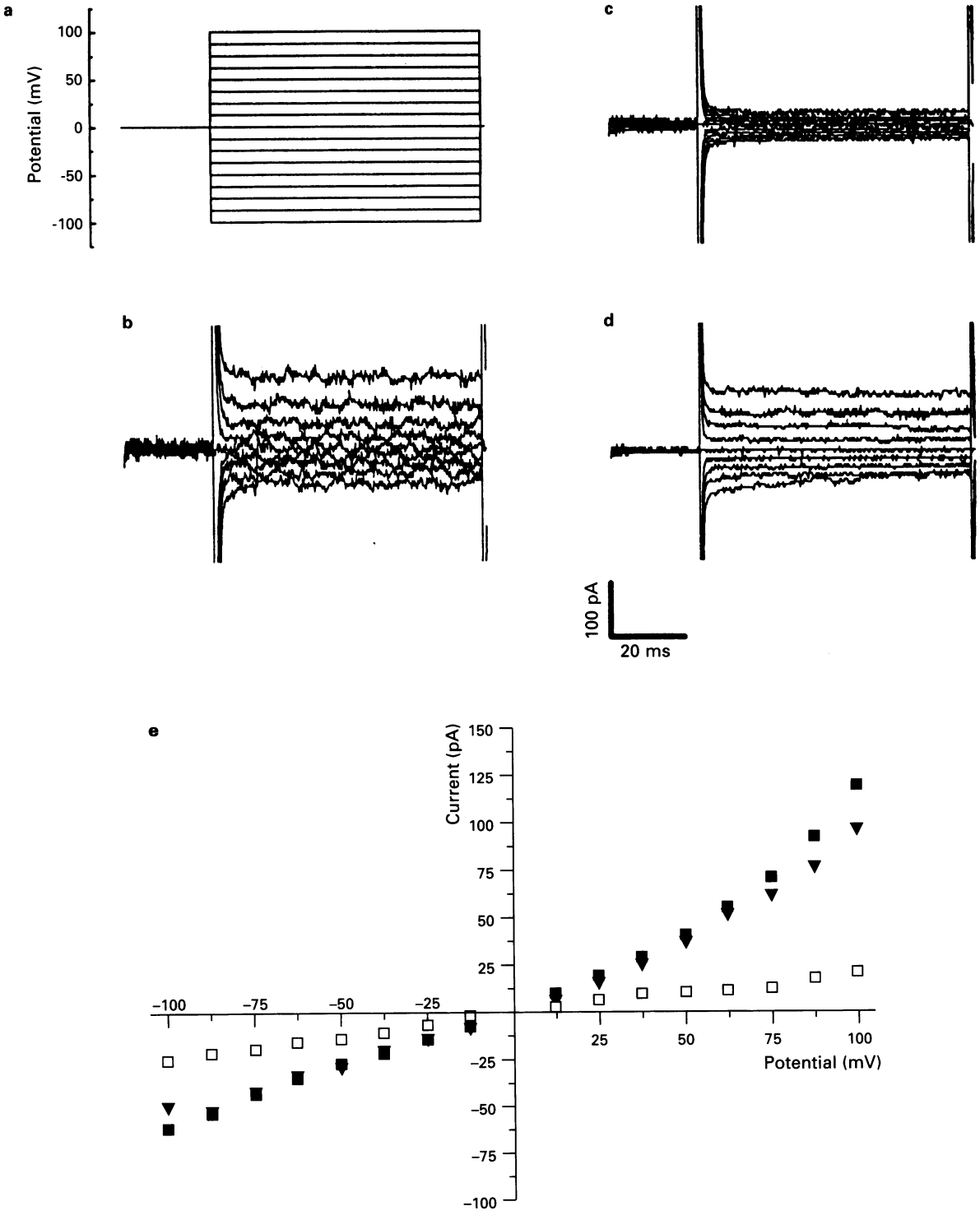
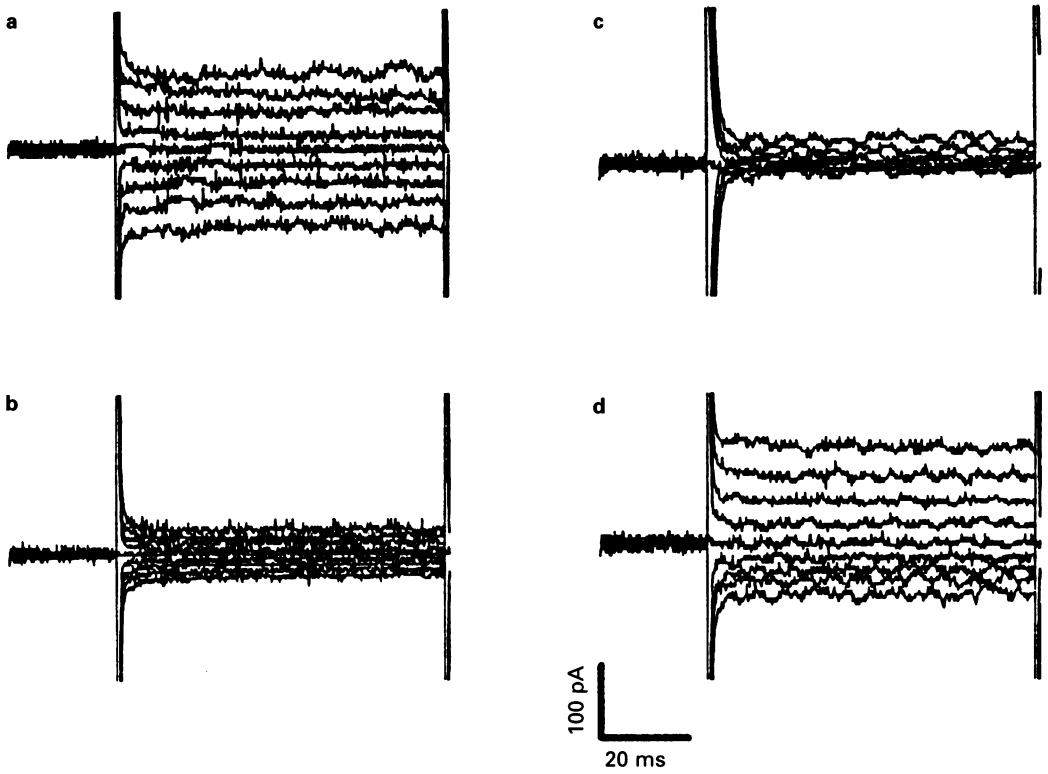


Figure 1 The effects of NPPB (5×10^{-5}M) on the chloride currents in mesangial cells from the *H-2K*^{b}-tsA58 transgenic mouse cultured in permissive conditions. In (a) the voltage pulse protocol is shown. Panels (b)–(d) show original current traces from three separate cells, recorded in control conditions (b), in the presence of 5×10^{-5} M NPPB (c) and following washout of NPPB (d). The current-voltage relationships, calculated from these recordings, are shown in (e). For the data shown, the conductances were 0.84 nS (control, ■, mean $= 1.07 \pm 0.09$ nS, $n=6$), 0.25 nS (NPPB, □, mean $= 0.27 \pm 0.03$ nS, $n=4$) and 0.76 nS (after washout, ▼, mean $= 0.96 \pm 0.06$ nS, $n=4$) respectively. Points were obtained from the recordings shown in (b)–(d) and are the mean of the current measured over a period of 70 ms. For each point, the s.e.mean was <0.5 pA. All experiments were performed in symmetrical solutions containing 140 mM TMACl and 1 μM free Ca^{2+} and, for clarity, only currents recorded at 25 mV intervals are shown in (b)–(d).

inhibitors but, the absence of any effect of pinacidil at 10^{-7} M makes this hypothesis unlikely, assuming that both drugs act in the same manner. A second explanation for these results is that drug binding may have been limited by the duration of the experiment.

To investigate this, cells were incubated for 16 h in supplemented DMEM containing either 10^{-15} M P1075 or 10^{-11} M pinacidil. The cells were washed twice to remove the drugs and experiments were performed in symmetrical solutions of TMACl in the absence of drugs. In these experiments the whole-cell conductances were 0.32 ± 0.04 nS ($n=6$) and 0.35 ± 0.05 nS ($n=6$) for P1075 and pinacidil respectively. This experiment indicates that the previous experimental conditions with low drug concentrations limited drug binding and supports the hypothesis of an irreversible inhibition of the whole-cell chloride conductance by P1075 and pinacidil in these cells.

It is known that mesangial cells have both calcium-dependent and -independent chloride conductances (Kremer *et al.*, 1992) and we have shown that mesangial cells from the *H*-2K^b-tsA58 transgenic mouse also possess calcium-dependent and



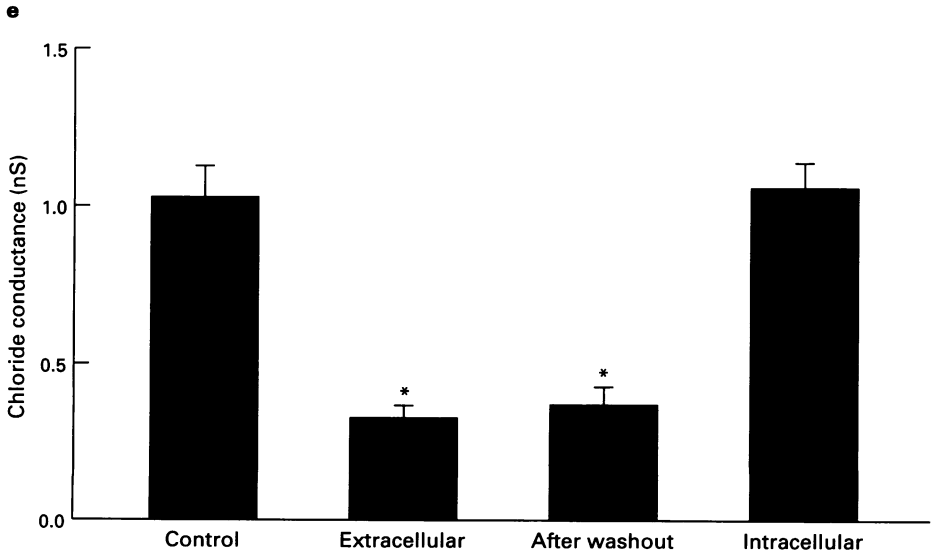


Figure 2 The effects of P1075 on the whole-cell chloride conductance. Original current recordings are shown for single cells in the absence of P1075 (a), in the presence of 10^{-5} M P1075 (b), following drug removal (c) and after intracellular application of 10^{-7} M P1075 (d). The control conductance was 1.03 ± 0.09 nS ($n=6$) and was inhibited by application of P1075 to the extracellular surface of the cell ($g=0.33 \pm 0.04$ nS, $n=6$). This inhibition was not reversible following washout ($g=0.37 \pm 0.06$ nS, $n=5$) and could not be obtained with intracellular application of P1075 ($g=1.05 \pm 0.08$, $n=6$). A summary of these data is shown in (e) where * indicates that the data are significantly different from the controls ($P<0.001$). All experiments were performed in symmetrical solutions containing 140 mM TMACl using the pulse protocol shown in Figure 1 (a).

calcium-independent chloride conductances (Barber *et al.*, 1995). In the presence of P1075 or pinacidil a residual conductance of 0.40 ± 0.03 nS ($n=41$) is observed. This is similar in magnitude (0.37 nS) to the calcium-dependent conductance in the presence of 10^{-6} M calcium (calculated from the total chloride conductance of 1.08 ± 0.05 nS ($n=63$) less the nominally calcium-independent chloride conductance, 0.71 ± 0.06 nS ($n=6$), from Barber *et al.*, 1995). Therefore, we investigated whether the drugs in this study were inhibiting the calcium-independent component of the chloride conductance. In the following experiments the pipette solution contained 140 mM TMACl at pH 7.4 with 1 mM EGTA and 2 mM free Mg^{2+} as before, and either 10^{-3} M, 10^{-6} M or 10^{-8}M free calcium. Cells were bathed in the original TMACl solution containing either P1075 (10^{-5} M), pinacidil (10^{-5} M) or NPPB (5×10^{-5} M) and the effects of changes of pipette calcium concentration on the inhibition of the chloride conductance by these drugs are shown in Figure 4. The results show that increasing pipette calcium significantly increases the residual component of the conductance (the

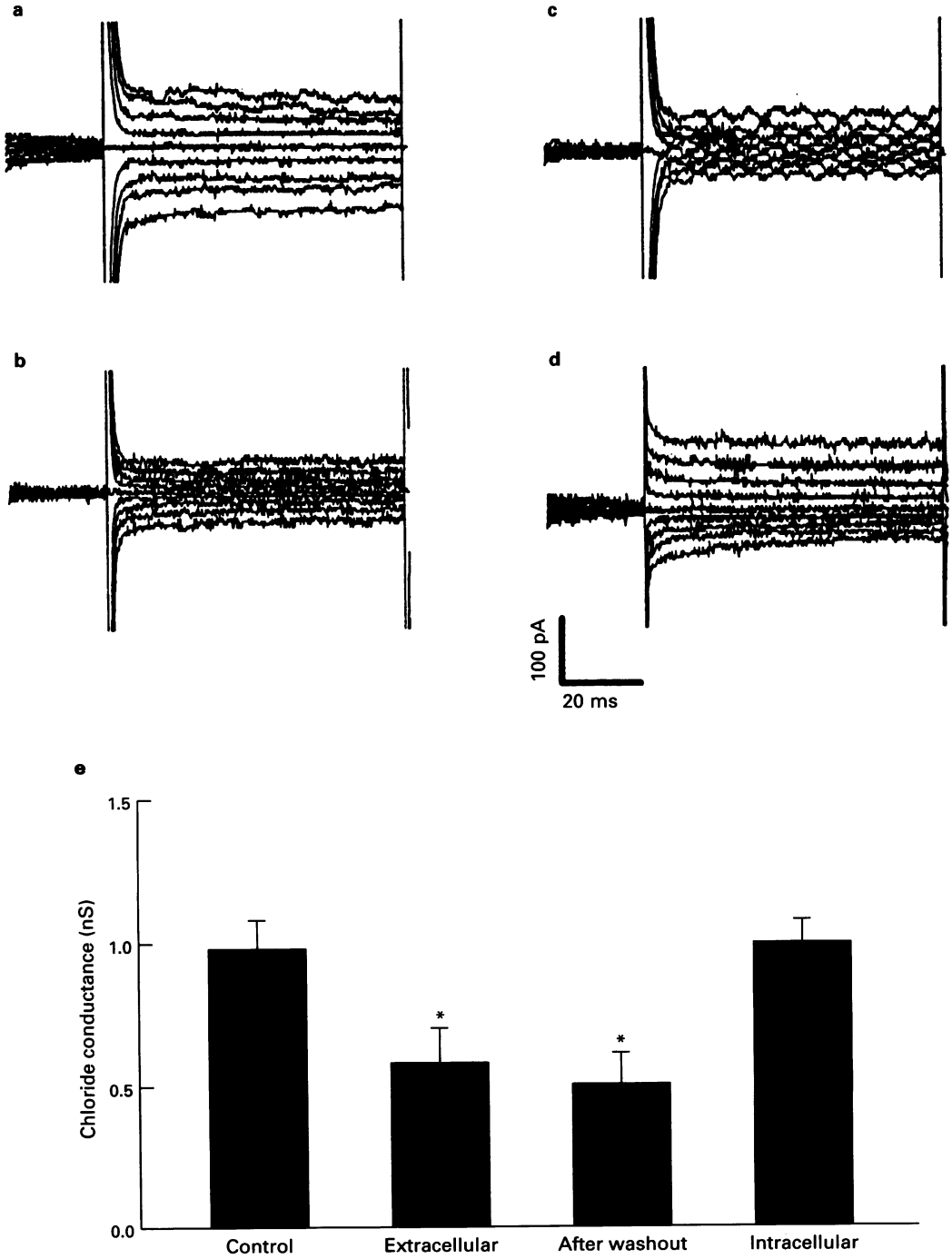


Figure 3 The effects of pinacidil (10^{-3} M) on the whole-cell chloride conductance. Original current recordings are shown for single cells in the absence of pinacidil (a), in the presence of pinacidil (b), following drug removal (c) and after intracellular application of pinacidil (d). The control conductance was 0.98 ± 0.09 nS ($n=6$) and was inhibited by application of pinacidil to the extracellular surface of the cell ($g=0.58 \pm 0.12$ nS, $n=6$). This inhibition was not reversible following washout ($g=0.50 \pm 0.12$ nS, $n=6$) and could not be obtained with intracellular application of pinacidil ($g=0.99 \pm 0.07$, $n=5$). A summary of these data is shown in (e) where *indicates that the data are significantly different from the controls ($P<0.001$). All experiments were performed in symmetrical solutions containing 140 mM TMACl using the pulse protocol shown in Figure 1 (a).

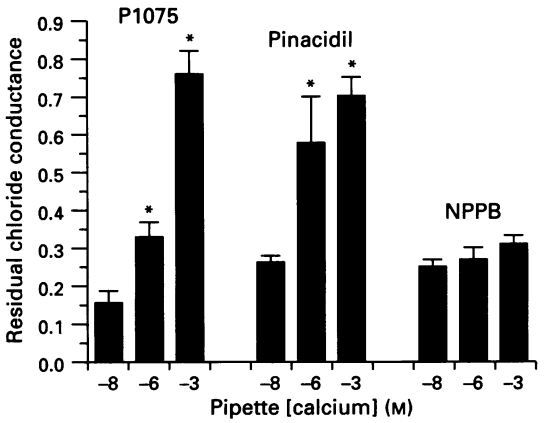


Figure 4 The mean effects of changing pipette free calcium concentrations on the residual whole-cell conductance. Increases in pipette calcium concentration increased the residual conductances in the presence of both P1075 (10^{-5} M) and pinacidil (10^{-3} M), ($P<0.05$) but not NPPB (5×10^{-5} M), ($P>0.2$). *Indicates that the data are significantly different from the conductance recorded in the presence of 10^{-8} M calcium ($P<0.05$).

calcium-dependent component) in the presence of both P1075 or pinacidil ($P<0.05$, Student's t test for unpaired data) but not NPPB ($P>0.2$, Student's t test for unpaired data). Interestingly, the residual current in the presence of 10^{-3} M calcium in the pipette is 0.72 ± 0.03 nS ($n=19$) and is similar to the magnitude (0.64 nS) of the calcium-dependent component of the chloride conductance with 10^{-4} M pipette calcium (from Barber *et al.*, 1995). This indicates that the calcium-dependent component of the conductance is unaffected by either P1075 or pinacidil. Taken together, these data suggest that P1075 and pinacidil selectively and irreversibly inhibit the calcium-independent component of the chloride conductance, acting from the external surface of the membrane. The residual conductance in the presence of NPPB does not change and this is consistent with the non-selective chloride channel blocking activity of NPPB.

Discussion

This study shows that P1075 and pinacidil are irreversible inhibitors of the calcium-independent component of the whole-cell chloride conductance in mesangial cells from the *H*-2K^b-tsA58 transgenic mouse. Furthermore, they are active only from the external surface of the membrane.

In 1986, Wangemann *et al.* reported a study in which they had tested 219 different potential chloride channel blockers in the thick ascending limb of the loop of Henle of the rabbit and the shark rectal gland. In an analysis of the structures of the compounds that possessed potent inhibitory properties, they found several features in common. These were the presence of negatively charged carboxyl group at one end of the molecule and that potent inhibitors were predominantly secondary amines. They argued that there was a requirement for an apolar residue and that inhibitory potency could be increased by the presence of a nucleophilic substituent into a benzoate ring and the introduction of alkyl spacers (CH_2) between the apolar residue and the secondary amine. The structures of P1075 and pinacidil meet some of these predictions for a chloride channel inhibitor and are shown in Figure 5 with the structure of NPPB for comparison.

Mesangial cells from the *H*-2K^b-tsA58 transgenic mouse possess a significant chloride conductance as is expected of mesangial cells (Barber *et al.*, 1995). Cells from this mouse proliferate at 33°C in the presence of γ-interferon but cease cell division at 37°C in the absence of the γ-interferon (Barber & Henderson, 1995). There is no difference in the absolute

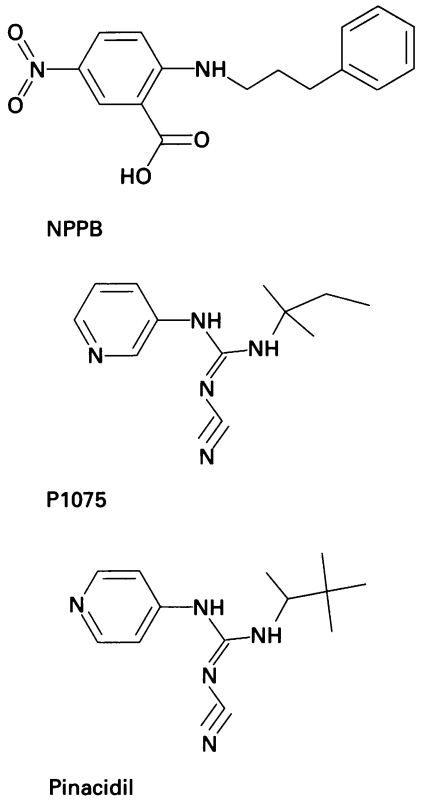


Figure 5 The structures of P1075, pinacidil and NPPB.

magnitude of the chloride conductance between cells grown in the different conditions, although differences have been found with respect to the effects of the nephrotoxic mycotoxin ochratoxin A (Barber *et al.*, 1995) which inhibits anion conductances in the renal epithelial MDCK cell line (Gekle *et al.*, 1993). On account of the significant chloride permeability of mesengial cells and their shared characteristics with smooth muscle cells, these cells provide a suitable preparation to study potential chloride channel inhibitors. The inhibition by P1075 has been demonstrated in cells grown in non-permissive culture conditions (data not shown) but due to the similarity of the whole-cell chloride conductance in cells grown in permissive and non-permissive culture conditions, this paper describes only the data obtained from cells grown in permissive culture conditions.

The absence of a dose-dependent relationship for concentrations of up to 10^{-11} M P1075 and 10^{-7} M, pinacidil, together with the continued inhibition following drug removal, suggested that these drugs might be irreversible inhibitors of the chloride conductance. This was confirmed by the 16 h incubations with low concentrations of the drugs. This contrasts with the inhibition by NPPB which could be removed with drug washout. NPPB acts by altering of the rate of transition between different channel kinetic states and effectively reduces chloride channel open probability (Dreinhöfer *et al.*, 1988; Alton & Williams, 1992). The mechanism for the irreversible inhibition by P1075 and pinacidil is not known, but whether P1075 and pinacidil work by a similar mechanism, reducing transition rate constants, can only be determined by single channel recordings. The data presented in this study also indicate that P1075 and pinacidil are effective only from the external surface of the channel and this indicates that the binding site must be accessible only from the outside of the channel.

In experiments with P1075 and pinacidil, the residual conductance could be correlated with the nominally calcium-dependent component of the chloride conductance in these cells that has been described previously (Barber *et al.*, 1995). This shows that the calcium-dependent component of the con-

ductance is unaffected by these drugs and from which, it is concluded that these drugs act specifically on the calcium-insensitive component of the conductance.

Therefore, it is concluded that these potassium channel openers, P1075 and pinacidil, are irreversible antagonists of the whole-cell chloride conductance in mesangial cells from the *H-2K*^{b}-tsA58 transgenic mouse. They act at the external surface of the cell membrane and are selective for the calcium-independent component of the conductance.

R.D.B. was an M.R.C. Research Student. Mouse tissue was kindly donated by Dr M.D. Noble of the Ludwig Institute for Cancer Research, London and glomeruli were isolated by Dr S.J. White of the University of Sheffield. P1075 and pinacidil were generous gifts of Leo Pharmaceuticals, Ballerup, Denmark.

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(Received December 15, 1995
Revised July 12, 1996
Accepted July 16, 1996)