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Activation of potassium currents by inhibitors of calcium-activated chloride conductance in rabbit portal vein smooth muscle cells

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1 The conventional whole-cell recording technique was used to study the effects of the chloride channel inhibitors ethacrynic acid, anthracene-9-carboxylic acid (A-9-C) and indanyloxyacetic acid (IAA) on membrane currents in rabbit portal vein smooth muscle cells at a holding potential of 0 mV.

2 Using a pipette solution that contained 1×10^{-4} M 1,2-*bis* (2-aminophenoxy)-ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA) and a normal bathing solution the addition of ethacrynic acid (2×10^{-4} M to 1×10^{-3} M) inhibited spontaneous transient outward currents (STOCs) and evoked a concentration-dependent current at a holding potential of 0 mV. A similar current was activated by IAA (5×10^{-4} M to 1×10^{-3} M) but not by A-9-C ($1-5 \times 10^{-3}$ M) at a holding potential of 0 mV.

3 The amplitude of the current evoked by ethacrynic acid and IAA was linearly related to potential between -30 and 0 mV and displayed outward rectification at positive potentials. The current induced by A-9-C was evident only at potentials positive to +20 mV.

4 Glibenclamide (1×10^{-5} M) abolished the current evoked by ethacrynic acid and IAA at potentials negative to +10 mV and partially inhibited the current positive to +10 mV. The glibenclamide-insensitive current at positive potentials was completely inhibited by 1×10^{-3} M TEA. The A-9-C-evoked current was insensitive to glibenclamide and abolished by 1×10^{-3} M TEA.

5 The glibenclamide-sensitive current activated by ethacrynic acid was not sustained and declined to control levels in the continued presence of ethacrynic acid. However, the outwardly rectifying current recorded at +50 mV was well maintained over the same period.

6 Outwardly rectifying currents evoked by ethacrynic acid and A-9-C were observed with a pipette solution containing 1×10^{-2} M BAPTA in cells bathed in Ca-free extracellular solution containing 5×10^{-4} M BAPTA and 1×10^{-5} M cyclopiazonic acid.

7 It is concluded that all three chloride-channel blockers activated an outwardly rectifying, TEA-sensitive current. Moreover, ethacrynic acid and IAA evoked an additional glibenclamide-sensitive current which was present at all potentials between -30 and +50 mV.

Keywords: Potassium currents; vascular smooth muscle; chloride channel blockers, ethacrynic acid, anthracene-9-carboxylic acid, indanyloxyacetic acid

Introduction

A wide range of compounds, such as levcromakalim and pinacidil, have been shown to activate a small conductance, glibenclamide-sensitive potassium channel in various types of smooth muscle cell (*e.g.* Noack *et al.*, 1992a; Beech *et al.*, 1993). However, recently an extract from the plant *Desmodium adscendens* (dehydrosoyasoponin) has been shown to activate large conductance, calcium-activated potassium channels (BK_{Ca}) in membranes of bovine trachea inserted into lipid bilayers (McManus *et al.*, 1993). This compound and two new synthetic benzimidazolones NS-004 and NS1619, which have been shown to activate BK_{Ca} in bovine aortic smooth muscle membranes (Olesen *et al.*, 1994), canine coronary artery myocytes (Xu *et al.*, 1994), rat portal vein smooth muscle cells (Edwards *et al.*, 1994) and bovine tracheal cells (Macmillan *et al.*, 1995), represent the first generation of compounds which activate BK_{Ca} in smooth muscle cells. Subsequently, it was shown that the fenamates niflumic, flufenamic and mefenamic acid, which have chemical structures similar to the benzimidazolones blocked smooth muscle calcium-activated chloride currents ($I_{Cl(Ca)}$; Hogg *et al.*, 1994a; Greenwood & Large, 1995) and also, at higher concentrations, activated BK_{Ca} in porcine coronary artery membranes (Ottolia & Toro, 1994) and in whole-cell recordings from canine coronary artery and rabbit portal vein (Xu *et al.*, 1994; Greenwood & Large, 1995). In the latter paper it was established that the outward current evoked by the fenamates was sensitive to 1×10^{-3} M tetraethyl ammonium (TEA) and had a current-voltage relationship with pronounced outward rectification and was comparable to the current evoked by the structurally similar compound NS-004 (Xu *et al.*, 1994). The aim of the present study was to investigate whether other chloride channel blockers, which have different chemical structures from the putative BK_{Ca} openers, NS-004 and NS 1619, could evoke similar outward currents to those reported for the fenamates in rabbit portal vein smooth muscle cells. The three agents studied, anthracene-9-carboxylic acid (A-9-C), ethacrynic acid and indanyloxyacetic acid (IAA) in the range 1×10^{-4} M to 1×10^{-3} M, inhibited chloride currents in rabbit portal vein smooth muscle cells (Hogg *et al.*, 1994b; Greenwood *et al.*, 1995). However, ethacrynic acid also induced an outward current in rabbit portal vein cells held at 0 mV (Greenwood *et al.*, 1995) and A-9-C increased noradrenaline-evoked potassium currents in the same cell type (Hogg *et al.*, 1994b). The present study shows that all three chloride channel blockers activated an outwardly rectifying current in concentrations between 2×10^{-4} M and 5×10^{-3} M

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which was inhibited by TEA. In addition, ethacrynic acid and IAA also evoked a glibenclamide-sensitive current which could be recorded at all potentials between −50 and +50 mV.

Methods

Experiments were carried out on smooth muscle cells freshly dispersed from rabbit portal vein. Female New Zealand White rabbits were killed by i.v. injection of sodium pentobarbitone and single cells obtained by enzymatic dissociation. After removal of adipose and connective tissue, strips of portal vein were incubated in nominally Ca-free physiological salt solution (PSS) for 10 min at 37°C. Strips were then exposed to Ca-free PSS containing protease (Sigma type I crude; 0.2–0.3 mg ml^{-1}) for 5 min followed by Ca-free PSS containing collagenase (Sigma type XI; 0.5–1 mg ml^{-1}) for 10 min. Cells were dissociated by gently passing the muscle strips through the mouth of a wide-bore glass pipette. Cells were stored on cover slips in PSS containing 0.75 mM Ca^{2+} at 4°C and used within 8 h of dispersion. Whole-cell currents were measured from cells constantly superfused with PSS using a patch clamp amplifier (List EPC 7; List-Electronic; Darmstadt, Germany). The normal extracellular solution (PSS) contained, (mM): NaCl (126), KCl (6), $MgCl_2$ (1.2), $CaCl_2$ (1.5), HEPES (10) and glucose (11) and was adjusted to pH 7.2 with NaOH. The normal pipette solution contained (mM): KCl 126, $MgCl_2$ (1.2), HEPES (10), glucose (11), 1,2-*bis* (2-aminophenoxy)-ethane-N,N, N',N'-tetraacetic acid (BAPTA) either 1×10^{-4} M or 1×10^{-2} M and the pH was adjusted to 7.2 with KOH.

All voltage protocols were generated and membrane currents recorded using Cambridge Electronic Design hardware and voltage clamp software. For analysis of voltage ramps, currents were low-pass filtered at 1 kHz prior to digitization at 2.5 kHz. Cells were held at a holding potential of 0 mV to inactivate the delayed rectifier and transient outward currents and current-voltage relationships were constructed by stepping the membrane potential to −50 mV for 30 ms and then changing the voltage steadily (voltage ramp) to +50 mV at a rate of 0.06 mV ms^{-1}. It should be noted that in rabbit portal vein smooth muscle cells the delayed rectifier and the transient outward current would not recover from inactivation during the voltage protocol used in the present study (see Beech & Bolton, 1989a) and would not therefore contribute to the ramp-evoked current. Voltage ramps in the absence of any drug were repeated 2–3 times to obtain an averaged current-voltage relationship. The same protocol was then executed in the presence of the drug. The effect of a drug on spontaneous transient outward currents (STOCs; calcium-activated potassium currents) was determined by measuring individual STOCs over a 3 min period before and after the addition of any drug and the mean amplitude and frequency calculated. In the text, *n* represents the number of cells required to obtain the mean value which is presented ± s.e. mean. Statistical comparisons were made by Student's unpaired *t* test where significance was taken at *P* value < 0.05. All drugs were dissolved in the PSS superfusing the cells and introduced to the recording chamber downstream of the main PSS reservoir. Drugs were applied for between 2–6 min and reached the cells within 50 s. Anthracene-9-carboxylic acid (A-9-C) and etha-

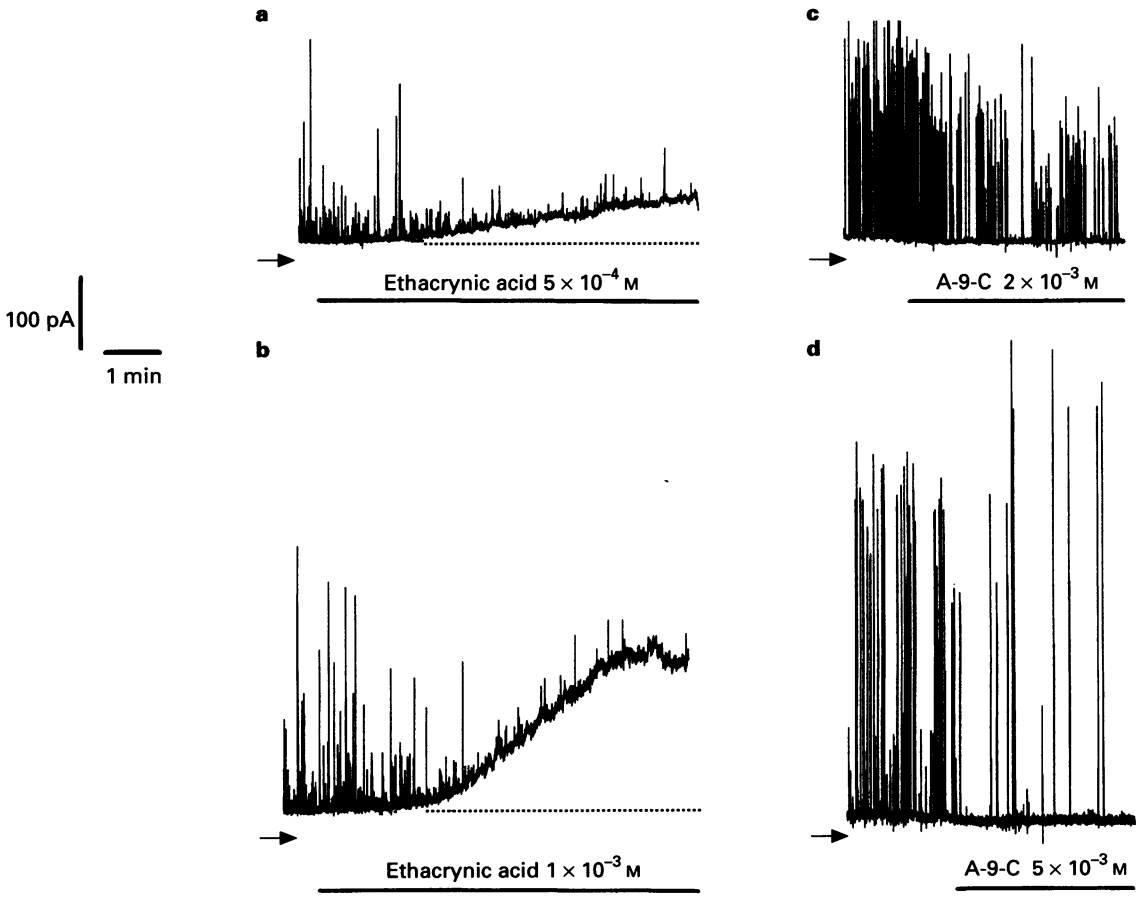

Figure 1 Effect of ethacrynic acid and A-9-C on potassium-currents recorded from smooth muscle cells at a holding potential of 0 mV. In all panels the rapid upward deflections represent STOCs and the dotted line shows the resting current level. (a and b) Show the effect of increasing concentrations of ethacrynic acid (5×10^{-4} M and 1×10^{-3} M) on STOCs and holding current; (c and d) show the effect of A-9-C (2×10^{-3} M and 5×10^{-3} M) on potassium currents at the holding potential of 0 mV. Note in (d) that 5×10^{-3} M A-9-C decreased STOC frequency but increased STOC amplitude. In each panel the horizontal arrow denotes the zero current level.

crynic acid were obtained from Sigma (Poole, Dorset) and were prepared as stock solutions in dimethylsulphoxide (DMSO) each day and diluted to the required concentrations in the extracellular solution. Addition of A-9-C to PSS to obtain a final concentration of 5×10^{-3}M reduced the pH to approximately 5.5. The change in pH was subsequently readjusted to pH 7.2 with NaOH which increased the solubility of A-9-C. Indanyloxyacetic acid (IAA; Research Biochemicals International, Natick, U.S.A.) was prepared as a stock solution in ethanol. In all experiments the solvent concentration was normally below 0.1% except for the highest concentration of A-9-C (5×10^{-3} M) where the DMSO concentration was 0.4%. No concentration of solvent had any effect on membrane currents. Cyclopiazonic acid (Calbiochem, La Jolla, USA) and glibenclamide (Sigma) were dissolved in DMSO. Caffeine was dissolved in the extracellular solution and tetraethyl ammonium (TEA) was dissolved in distilled water.

Results

Effect of ethacrynic acid and A-9-C on currents studied in cells held at 0 mV

Membrane currents were studied in cells in K-containing conditions at a holding potential of 0 mV where it has been shown that the delayed rectifier and the transient outward current would be largely inactivated (Beech & Bolton, 1989b; Bolton & Beech, 1992; Noack *et al.*, 1992a). With a K-rich pipette solution containing 1×10^{-4} M BAPTA, smooth muscle cells exhibited STOCs which were relatively constant in amplitude and frequency for the duration of the experiment. Figure 1a and b shows that ethacrynic acid (5×10^{-4}–1×10^{-3} M) induced a slowly developing outward current which was readily reversible upon wash out. The current produced by 1×10^{-3} M ethacrynic acid at 0 mV had a time to peak of 256 ± 38 s and a mean amplitude of 175 ± 25 pA ($n = 13$) whereas 5×10^{-4} M ethacrynic acid elicited a smaller current (55 ± 12 pA; $n = 8$). Lower concentrations of ethacrynic acid and concentrations of A-9-C up to 5×10^{-3} M failed to activate an outward current at 0 mV (Figure 1c and d). Interestingly, 5×10^{-3} M A-9-C reduced the resting current level in some cells (see Figure 3b; mean reduction $= 18 \pm 3$ pA; $n = 10$) but we have no explanation for this observation. In addition to evoking an outward current in most cells, ethacrynic acid also inhibited the amplitude and frequency of STOCs. Thus, 2×10^{-4} M ethacrynic acid, which did not evoke a current at 0 mV, reduced the STOC amplitude and frequency to respectively 75 ± 10 and 72 ± 10% of control values ($n = 6$) and in the presence of 1×10^{-3} M ethacrynic acid, STOC amplitude and frequency were reduced to 19 ± 5 and 32 ± 5% respectively of control values ($n = 6$).

A-9-C had more varied effects on spontaneous currents at 0 mV. Thus, 1, 2 and 5×10^{-3} M A-9-C reduced STOC frequency to 75 ± 9, 54 ± 8 and 51 ± 8% ($n = 6$–10) of control values, respectively. A-9-C 2×10^{-3} M reduced STOC amplitude to 48 ± 7% ($n = 8$) of control values but 5×10^{-3} M A-9-C had less of an effect on STOC amplitude and markedly increased current amplitude in some cells (for example see Figure 1d). The mean STOC amplitude recorded in the presence of 5×10^{-3} M A-9-C was 104 ± 5% of control values ($n = 10$). It is evident that whereas ethacrynic acid simply decreased STOC activity A-9-C had a more complex effect on the spontaneous currents.

Properties of the current evoked by ethacrynic acid at 0 mV

As Figure 1 shows, the current evoked by ethacrynic acid at 0 mV developed steadily with little increase in the variance of the current. The current-voltage relationship of this conductance was established by measuring currents in response to a ramp change in voltage from −50 to +50 mV in the absence and presence of ethacrynic acid (5×10^{-4} and 1×10^{-3} M). In the absence of any drug the ramp-evoked current was negligible at potentials negative to 0 mV and then rectified in an outward direction positive to this potential (Figure 2a). STOCs were often superimposed on the depolarization-induced current and therefore the mean current was calculated at 10 mV increments from −50 mV to +50 mV. In the majority of cells (15 out of 21), 1×10^{-3} M ethacrynic acid evoked a current at all potentials between −30 mV and +50 mV (Figure 2a). The overall current-voltage relationship was approximately linear negative to 0 mV but at more positive potentials the current induced by ethacrynic acid exhibited outward rectification (Figure 2a). In a few cells (6 out of 21) 1×10^{-3} M ethacrynic acid produced negligible current at 0 mV although there were drug-induced currents at positive potentials similar to that seen with A-9-C (see later).

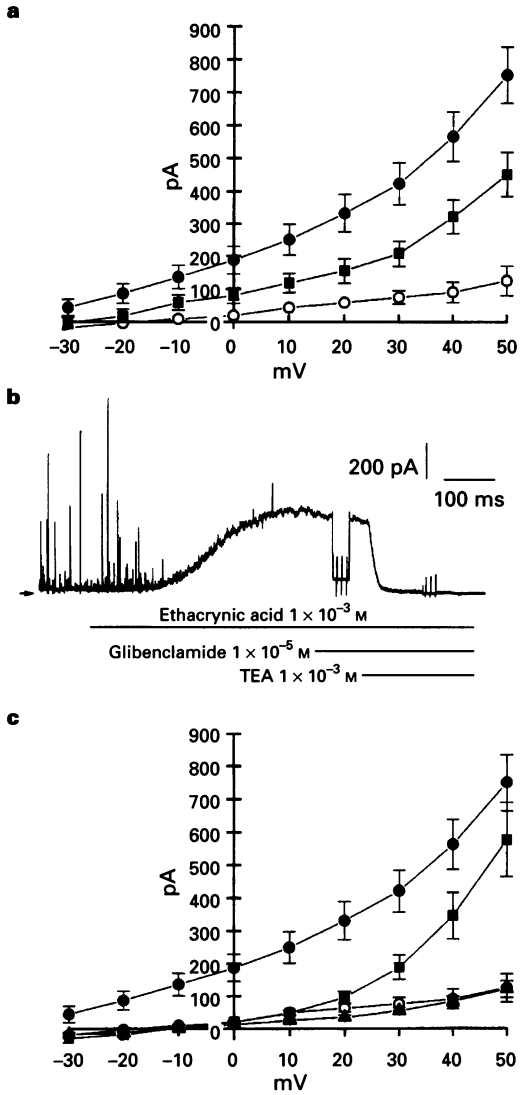

Figure 2 Properties of the current activated by ethacrynic acid. (a) The current evoked by the ramp change in potential is illustrated from −30 mV to +50 mV in control conditions (○) and in the presence of 5×10^{-4} M ethacrynic acid (■) and 1×10^{-3} M ethacrynic acid (●). (b) Shows a representative current evoked by 1×10^{-3} M ethacrynic acid at a potential of 0 mV and the reduction of this current by 1×10^{-5} M glibenclamide and 1×10^{-3} M TEA. Note that when the ramps were applied (vertical deflections) the gain was decreased. The horizontal arrow shows the zero current level at this holding potential. (c) Effect of 1×10^{-5} M glibenclamide (■) and glibenclamide plus 1×10^{-3} M TEA (▲) on the current evoked by a ramp change in potential in the presence of 1×10^{-3} M ethacrynic acid (●); (○) represents the control current-voltage plot in the absence of ethacrynic acid and potassium-channel blockers. Each point represents the mean of between 5–7 cells ± s.e. mean.

The pharmacology of the ethacrynic acid-induced current was investigated using the K-channel blockers TEA and glibenclamide. Glibenclamide (1×10^{-5} M), which does not affect BK_{Ca} in rabbit mesenteric arterial cells (Langton *et al.*, 1991), produced a marked reduction of the current elicited by ethacrynic acid at 0 mV (Figure 2b; $n=6$). The current-voltage relationship showed that glibenclamide completely blocked the ethacrynic acid-induced currents at potentials between −50 and +10 mV but some activated current remained at +20 to +50 mV in the presence of glibenclamide (Figure 2c). TEA at 1×10^{-3} M is considered to be a relatively selective blocker of large conductance K-channels in rabbit portal vein smooth muscle cells (Bolton & Beech, 1992) and completely abolished the glibenclamide-insensitive current (Figure 2c). When TEA (1×10^{-3} M) was added before glibenclamide the outwardly rectifying properties of the ethacrynic acid-evoked current were abolished at positive potentials leaving the almost linear current-voltage relationship of the glibenclamide-sensitive response ($n=5$). These results suggest that ethacrynic acid generates two separate potassium currents in rabbit portal vein cells, one which has a linear current-voltage relationship and is glibenclamide-sensitive and another which is outwardly rectifying and is TEA-sensitive.

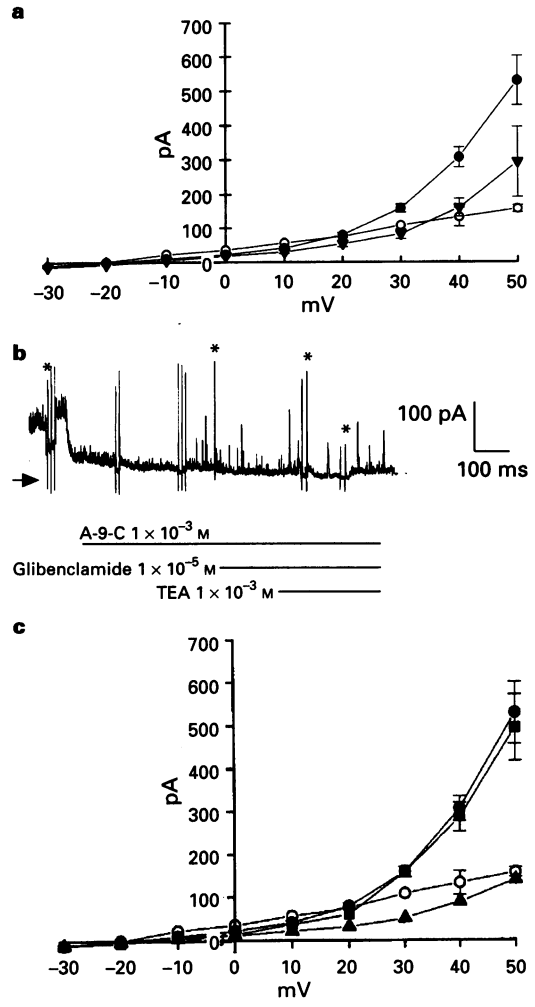

Figure 3 Characteristics of the current induced by A-9-C. (a) The current evoked by the ramp change in potential is illustrated from −30 mV to +50 mV in control conditions (○) and in the presence of 2×10^{-3} M A-9-C (▼) and 5×10^{-3} M A-9-C (●). (b) shows a representative trace illustrating the effects of glibenclamide and TEA on the current induced by 5×10^{-3} M A-9-C. When the ramps were applied (at the point denoted by the *) the gain was halved so that the vertical calibration represents 200 pA under these conditions. The horizontal arrow shows the zero current level at this holding potential. (c) Effect of 1×10^{-5} M glibenclamide (■) and glibenclamide plus 1×10^{-3} M TEA (▲) on the current evoked by a ramp change in potential in the presence of 5×10^{-3} M A-9-C (●); (○) denotes the control current in the absence of A-9-C and the potassium-channel blockers. Each point represents the mean of 7 cells ± s.e. mean.

Characterization of the A-9-C-induced current

In comparison to ethacrynic acid, A-9-C did not evoke a current at the holding potential of 0 mV (Figure 1 and 3b). However, when a ramp change in voltage was produced in the presence and absence of A-9-C it could be seen that this agent increased the current at potentials positive to 0 mV in a concentration-dependent manner (Figure 3a). Comparing Figures 2a and 3a it can be seen that A-9-C was a less potent activator of outward currents than ethacrynic acid. Moreover, the currents elicited by A-9-C were not inhibited by 1×10^{-5} M glibenclamide but were completely abolished by 1×10^{-3} M TEA (Figure 3b and 3c; $n=7$). Therefore, A-9-C appears to activate only BK_{Ca} in rabbit portal vein smooth muscle cells.

Time course of the two potassium currents activated by ethacrynic acid

Since ethacrynic acid evoked two types of K-current it was of interest to ascertain whether the time course of activation and

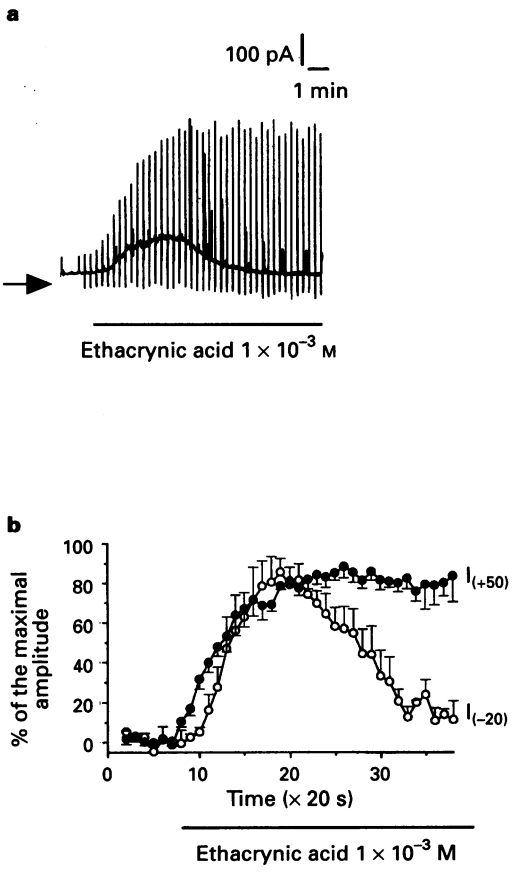

Figure 4 Time course of the two potassium currents evoked by ethacrynic acid. (a) Shows a representative trace of the ethacrynic acid-evoked currents recorded at the holding potential of 0 mV. Ethacrynic acid (1×10^{-3} M) was applied for the period denoted by the horizontal bar and voltage ramps were applied every 20 s. The zero current level is represented by the horizontal arrow. (b) Shows a plot of the ethacrynic acid-evoked currents recorded at −20 mV ($I_{(-20)}$) and +50 mV ($I_{(+50)}$). The abscissa scale shows the current recorded at each time point represented as a % of the maximum current obtained at both test potentials in each cell. The ordinate scale shows the time of recording. Each symbol denotes the mean of 5 cells ± s.e. mean where (○) represents the current recorded at −20 mV and (●) represents the current recorded at +50 mV (see text for further details).

inactivation of these currents was similar. Experiments on the time course of both currents were performed using a pipette solution containing 1×10^{-4} M BAPTA and cells were bathed in 'nominally' Ca-free PSS to abolish STOCs which allowed small changes of current to be observed. Figure 4a shows the effect of 1×10^{-3} M ethacrynic acid where voltage ramps were applied every 20 s. It can be seen from Figure 4a that the current evoked at 0 mV reached a peak in approximately 3 min and then steadily declined in the continued presence of the drug. Analysis of the voltage ramp-evoked currents show that in comparison to the current at 0 mV the current at +50 mV remained at a sustained level during exposure to ethacrynic acid (Figure 4b). In Figure 4b the current recorded at a membrane potential of −20 mV ($I_{(-20)}$) represents the glibenclamide-sensitive current while the current at +50 mV ($I_{(+50)}$) was mostly TEA-sensitive. Figure 4b shows that the current at −20 mV starts to develop more slowly than the current at +50 mV where the time to reach 10% of the maximal current was 100±12 s at −20 mV and 28±4 s at +50 mV. This difference was statistically significant ($P<0.01$, $n=5$) and these data indicate that the TEA-sensitive is activated prior to the glibenclamide-sensitive current. However, there was no significant difference in the total time to peak current (256±38 s at −20 mV compared to 432±78 s at +50 mV). The most marked effect highlighted by Figure 4b was the gradual run-down of the current recorded at −20 mV to a level approximately 10% of the maximum current whereas the current at +50 mV did not fade over the same time period. Thus, the glibenclamide-sensitive current activated by ethacrynic acid exhibited marked run-down in the presence of the drug and was activated more slowly than the sustained, outwardly rectifying TEA-sensitive current.

Effect of ethacrynic acid and A-9-C in conditions of 'strong' intracellular Ca-buffering

It has been shown previously that the ability of NS-004 and fenamates to evoke $I_{K(Ca)}$ was not reduced by increasing the Ca-buffering of the pipette solution (Xu *et al.*, 1994; Greenwood & Large, 1995). Experiments were therefore performed in the present study to determine whether ethacrynic acid and A-9-C could activate an outwardly rectifying current in conditions where intracellular and extracellular calcium were buffered to low levels. In order to achieve this, a pipette solution containing 1×10^{-2} M BAPTA was used and cells were bathed in Ca-free PSS containing 5×10^{-4} M BAPTA and 1×10^{-5} M cyclopiazonic acid, an inhibitor of the Ca-ATPase in smooth muscle sarcoplasmic reticulum (Uyama *et al.*, 1993). The latter agent was used following a brief exposure to 1×10^{-2} M caffeine in order to deplete the intracellular calcium stores. With the above conditions, 5×10^{-3} M A-9-C increased current 'noise' at the holding potential of 0 mV (Figure 5a) and substantially increased the current at potentials positive to 0 mV (Figure 5b). Thus, at +50 mV the amplitude of the A-9-C-evoked current was 558±159 pA (Figure 5b). At the holding potential of 0 mV 1×10^{-3} M ethacrynic acid evoked a mean current of 95±12 pA which was glibenclamide-sensitive in 5 out of 7 cells. This value was not significantly smaller than the current recorded with a pipette solution containing 1×10^{-4} M BAPTA in cells bathed in Ca-free PSS used in the time course experiments (136±26 pA, $n=8$). In comparison, the ethacrynic acid-evoked current recorded at +50 mV, which is predominantly a TEA-sensitive current, was significantly larger in cells where the calcium was buffered to a lower level (1091±71 pA, $n=7$) compared to 'weakly' buffered cells where the mean evoked current was 615±100 pA (compare Figures 2a and 5c).

Effects of indanyloxyacetic acid (IAA) on membrane currents at a holding potential of 0 mV

In the final series of experiments we examined whether IAA, which is an inhibitor of $I_{Cl(Ca)}$ in rabbit portal vein (Greenwood *et al.*, 1995) and for which there is no evidence as a K-channel opener, could activate an outward current at concentrations higher than those required to block $I_{Cl(Ca)}$. Figure 6a shows

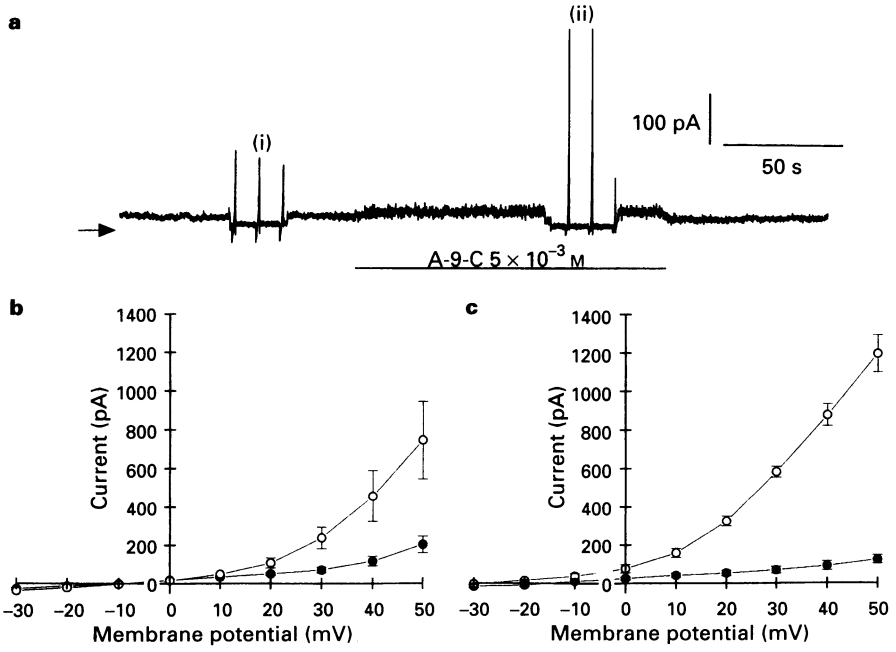

Figure 5 The effect of ethacrynic acid and A-9-C on membrane currents recorded in conditions of 'strong' intracellular calcium buffering. Cells were bathed in calcium-free PSS ($+5 \times 10^{-4}$ M BAPTA) containing 1×10^{-5} M cyclopiazonic acid and membrane currents were recorded with a pipette solution containing 1×10^{-2} M BAPTA. (a) Shows a representative trace of the effect of 5×10^{-3} M A-9-C on currents recorded at 0 mV and in response to ramp changes in potential. Ramps were applied in the absence of any drug (control, (i)) and in the presence of A-9-C (ii). The horizontal arrow denotes the zero current level in this cell. (b) Shows the currents recorded in response to a ramp change in potential in the absence (control, ●) and presence of 5×10^{-3} M A-9-C (○). Each point is the mean of 5 cells±s.e. mean. (c) Shows the currents recorded in response to a ramp change in potential in the absence (control, ●) and presence of 1×10^{-3} M ethacrynic acid (○). Each point is the mean of 7 cells±s.e. mean.

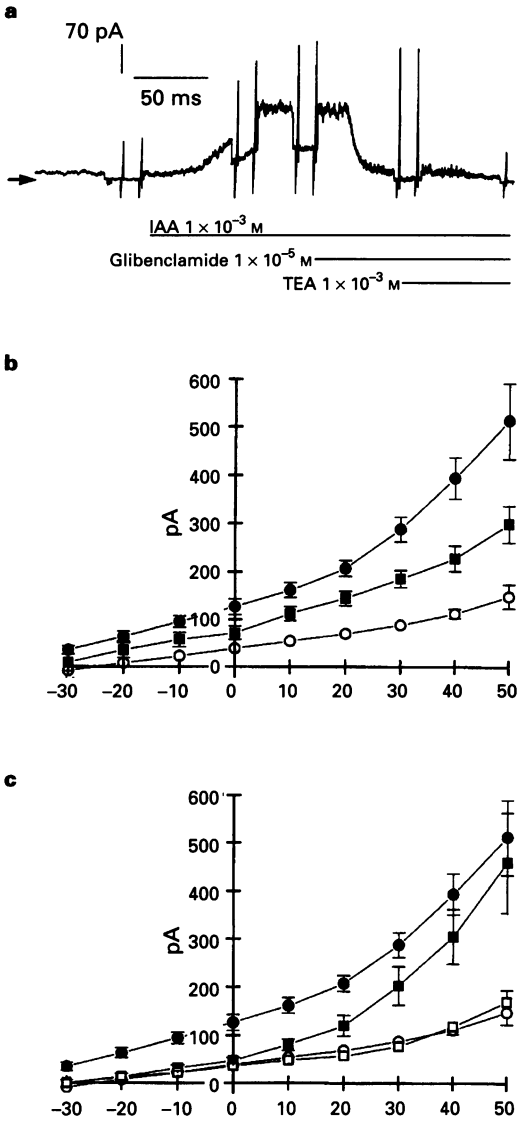

Figure 6 The properties of currents activated by IAA. (a) Shows a representative trace of the effect of 1×10^{-3} M IAA at a holding potential of 0 mV. These experiments were carried out in zero external calcium to remove STOCs. The gain was halved when voltage ramps (vertical deflections) were applied in both the absence and presence of IAA. The horizontal arrow denotes the zero current level in this cell. (b) Shows the concentration-dependence of the effect of IAA on ramp-evoked currents. Currents induced by 5×10^{-4} M and 1×10^{-3} M IAA are represented by (■) and (●) respectively and each point denotes the mean of 7–8 cells. Control currents are shown as (○). (c) Shows the effect of glibenclamide and TEA on the currents evoked by 1×10^{-3} M IAA. Currents were evoked by a voltage ramp in control conditions (○), in 1×10^{-3} M IAA alone (●) and in the presence of 1×10^{-5} M glibenclamide (■) and glibenclamide plus 1×10^{-3} M TEA (□). Each point denotes the mean of 6–8 cells ± s.e. mean.

that IAA (1×10^{-3} M) evoked a large outward current at 0 mV in a cell bathed in nominally Ca-free PSS (to reduce STOC discharge so that currents produced by IAA could be recorded accurately). The time to peak was 91 ± 13 s and the amplitude of the mean evoked current to 5×10^{-4} M and 1×10^{-3} M IAA was 59 ± 11 pA ($n = 5$) and 110 ± 12 pA ($n = 8$) respectively. It can be observed from Figure 6b that the current evoked by a ramp change in voltage was increased significantly from control values at all potentials between −10 and +50 mV by 5×10^{-4} M and 1×10^{-3} M IAA. The addition of 1×10^{-5} M glibenclamide in the presence of 1×10^{-3} M IAA rapidly and markedly reduced the evoked current at 0 mV (mean reduction = 94 ± 2%, $n = 5$; Figure 6a and 6c). Glibenclamide inhibited all evoked currents at potentials between −50 and 0 mV but appeared to have less effect at +50 mV (Figure 6c). Application of 1×10^{-3} M TEA in the presence of IAA completely inhibited the glibenclamide-resistant current (Figure 6c; $n = 5$). Thus, IAA had a similar electrophysiological and pharmacological profile to ethacrynic acid.

Discussion

The experiments of the present study show that ethacrynic acid, A-9-C and IAA activated potassium currents in rabbit portal vein smooth muscle cells. Ethacrynic acid and IAA evoked two potassium currents with distinctive characteristics. One conductance had an approximately linear current-voltage relationship between −30 and +50 mV and was blocked by glibenclamide. The second conductance was glibenclamide-resistant, displayed pronounced outward rectification at positive potentials and was blocked by 1×10^{-3} M TEA. Only this latter current was activated by A-9-C. These latter results suggest that ethacrynic acid, A-9-C and IAA activated a current with characteristics similar to BK_{Ca} and therefore these agents can be added to NS 1619, NS-004, dehydrosoyasoponin and the fenamates as activators of BK_{Ca} in smooth muscle cells (McManus *et al.*, 1993; Edwards *et al.*, 1994., Olesen *et al.*, 1994; Xu *et al.*, 1994; Greenwood & Large, 1995; Macmillan *et al.*, 1995). However it should be stressed that the chloride channel blockers are less efficacious agonists of BK_{Ca} than either NS 1619 or NS-004 which activate BK_{Ca} in the concentration range 5×10^{-6} M to 3×10^{-5} M (Olesen *et al.*, 1994; Xu *et al.*, 1994). The glibenclamide-sensitive current evoked by ethacrynic acid and IAA was similar to the current evoked by the 'classical' K-channel openers, levcromakalim and pinacidil in a variety of smooth muscle cells including rat portal vein (Noack *et al.*, 1992a; Edwards *et al.*, 1994), rabbit portal vein (Beech *et al.*, 1993) and rat mesenteric artery (Zhang & Bolton, 1995). Interestingly the glibenclamide-sensitive current evoked by ethacrynic acid exhibited marked run-down in the continued presence of the drug and which is also similar to the levcromakalim- and pinacidil-induced currents recorded from rabbit portal vein and rat mesenteric artery smooth muscle cells (Beech *et al.*, 1993; Zhang & Bolton, 1995) and the current termed I_{met} recorded in ATP-depleted rat portal vein cells (Noack *et al.*, 1992b). Beech *et al.* (1993) observed that the run-down of the levcromakalim-induced current was reduced when the perforated patch recording technique was used and preliminary experiments with ethacrynic acid and perforated patch recording supports this observation (data not shown). This indicates that an intracellular mediator is responsible for optimal activation of the glibenclamide-sensitive current and is washed out with whole-cell dialysis. Beech *et al.* (1993) proposed also that the target channel for levcromakalim was regulated by intracellular nucleotide diphosphates, although previous reports suggested that the ATP-sensitive K-channel was activated by levcromakalim (*e.g.* Noack *et al.*, 1992b). The data from the present study does not elucidate the nature of the potassium-channel activated by ethacrynic acid and IAA, but establishes that in addition to activating BK_{Ca} these two agents also induced a current very similar to that generated by compounds classified as typical K-channel openers. However, levcromakalim and pinacidil are not considered to activate BK_{Ca} and NS 1619 or NS-004 do not generate a glibenclamide-sensitive current (Olesen *et al.*, 1994; Xu *et al.*, 1994) and therefore ethacrynic acid and IAA appear to be unusual in activating two distinct potassium currents.

Previously it was shown that the fenamates decreased the amplitude and frequency of STOCs at approximately the same concentrations which activated BK_{Ca} (Greenwood & Large, 1995). Essentially similar data were found with ethacrynic acid and A-9-C (the effect of IAA was not studied) although the actions of A-9-C appeared to be more complex. It is possible

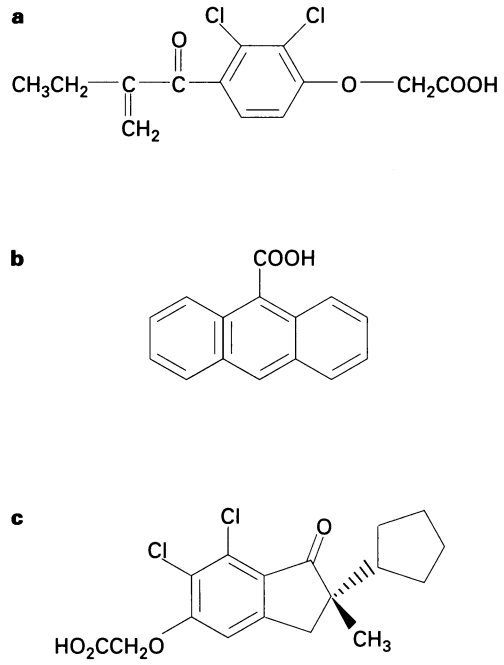

Figure 7 Chemical structure of (a) ethacrynic acid, (b) anthracene-9-carboxylic acid (A-9-C) and (c) indanyloxy acetic acid (IAA) used in the present study.

that the effect of these agents on STOC activity is related to their ability to stimulate BK_{Ca}. This might be mediated by an effect on the intracellular calcium store (sarcoplasmic reticulum, SR) to reduce the release of calcium which generates STOCs, e.g. by stimulating BK_{Ca} in the sarcoplasmic reticulum, as it has been shown that calcium movements into the SR can be modulated by potassium channel activation (Fink & Stephenson, 1987). However, it is also possible that there is an interaction between these compounds and calcium ions at the calcium-activated potassium channel in the sarcolemma (see later).

Niflumic, flufenamic and mefenamic acid block $I_{Cl(Ca)}$ and activate BK_{Ca} in rabbit portal vein cells (Hogg *et al.*, 1994b; Greenwood & Large, 1995). Ethacrynic acid, A-9-C and IAA, which are structurally dissimilar from each other and the fenamates (cf. Figure 7 and Figure 1 of Greenwood & Large, 1995), also block $I_{Cl(Ca)}$ in rabbit portal vein smooth muscle cells (Hogg *et al.*, 1994a; Greenwood *et al.*, 1995) and in the present work have been shown to activate potassium currents. Thus, a general property of chloride-channel blockers in smooth muscle may be the ability to activate BK_{Ca}, albeit at higher concentrations than are necessary to inhibit $I_{Cl(Ca)}$. There are marked differences in the concentration required to inhibit $I_{Cl(Ca)}$ and evoke $I_{K(Ca)}$ for each compound. Thus niflumic acid inhibited spontaneous transient inward currents (STICs, calcium-activated chloride-currents) with an IC_{50} of approximately 2×10^{-6} M but evoked a potassium current only at concentrations greater than 1×10^{-4} M (Greenwood & Large, 1995) indicating a high degree of selectivity for $I_{Cl(Ca)}$. With ethacrynic acid the concentration difference between inhibition of $I_{Cl(Ca)}$ and activation of BK_{Ca} was only approximately two fold and thus exhibits a lower degree of selectivity for the two calcium-activated currents. Evidence suggests that the fenamates and A-9-C alter the kinetics of the chloride channel and the data indicate that these agents inhibit $I_{Cl(Ca)}$ by blocking the open channel (Hogg *et al.*, 1994a,b; Greenwood & Large, 1995). Interestingly these agents activated BK_{Ca} only. In comparison, ethacrynic acid and IAA which appear to inhibit $I_{Cl(Ca)}$ without greatly altering channel kinetics (Greenwood *et al.*, 1995) evoked a glibenclamide-sensitive current in addition to BK_{Ca}. Thus, there may be a correlation between the mechanism of chloride channel blockade and the identity of potassium channels opened by these compounds. Nevertheless the data suggest that there are common binding sites on calcium-activated chloride and potassium channels.

It has been proposed by Ottolia & Toro (1993) that fenamates increase the activity of BK_{Ca} in porcine coronary artery membranes by increasing the sensitivity of the channel protein to calcium ions. In agreement with this proposed mechanism, NS-004 and NS 1619 do not activate BK_{Ca} in isolated patches bathed in calcium-free solution (Olesen *et al.*, 1994; Macmillan *et al.*, 1995). Moreover, Vandier *et al.* (1995) showed that the stimulatory action of NS-004 on pulmonary artery potassium currents was increased by raising the intracellular calcium concentration. Also NS 1619 and NS-004 did not evoke outward currents in smooth muscle cells bathed in zero calcium PSS and recorded with pipettes containing high concentrations of calcium chelator (Edwards *et al.*, 1994; Xu *et al.*, 1994; Macmillan *et al.*, 1995). The present study shows that the activation of BK_{Ca} by ethacrynic acid and A-9-C was increased when the calcium concentration in the pipette and bathing solution was reduced. In these experiments the pipette solution contained 1×10^{-2} M BAPTA and cells were bathed in zero calcium PSS ($+5 \times 10^{-4}$ M BAPTA) and 1×10^{-5} M CPA which depletes the intracellular store (Suzuki *et al.*, 1992). In these conditions it would be expected that the intracellular calcium concentration would be very low and although intracellular calcium was not measured directly STOCs were not observed and caffeine (1×10^{-2} M) failed to activate a potassium-current in cells at 0 mV. Nevertheless in these conditions ethacrynic acid and A-9-C evoked TEA-sensitive outward currents at +50 mV that were larger than in normal conditions when calcium was present in the bathing solution and with a lower concentration of BAPTA (1×10^{-4} M) in the pipette. The TEA-sensitive current evoked by flufenamic acid and niflumic acid at 0 mV was also significantly larger when calcium buffering was higher (Greenwood & Large, 1995). The apparent antagonism by calcium ions of the activation of BK_{Ca} by the fenamates led to the proposal that these compounds may behave as partial agonists at the calcium binding site (Greenwood & Large, 1995). The present data indicate that ethacrynic acid and A-9-C may also behave in this manner. However, Ottolia & Toro (1993) suggested that the binding site for the fenamates was at the extracellular surface of the cell membrane while calcium binds to the channel protein at the cytoplasmic side to activate BK_{Ca}. Further experiments are required to elucidate whether the antagonism between the activators and calcium ions on BK_{Ca} activity results from direct competition at a single site or whether an allosteric interaction is involved.

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