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Effect of frusemide, ethacrynic acid and indanyloxyacetic acid on spontaneous Ca-activated currents in rabbit portal vein smooth muscle cells

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1 The effect of frusemide, ethacrynic acid and indanyloxyacetic acid was investigated on spontaneous calcium-activated chloride ($I_{Cl(Ca)}$) and potassium currents ($I_{K(Ca)}$) in rabbit portal vein cells with the perforated patch technique.

2 Frusemide ($0.3-1.0\times10^{-3}$ M) reduced the amplitude of spontaneous transient inward chloride currents (STICs) in a concentration-dependent manner. The degree of inhibition on STIC amplitude was similar between -50 and $+30$ mV and frusemide did not alter the STIC reversal potential (E_{rev}).

3 The voltage-dependent exponential decay of STICs, which is thought to represent closure of chloride channels, was not altered by frusemide.

4 The amplitude and frequency of spontaneous potassium outward currents (STOCs) were not altered by frusemide. Since both STICs and STOCs are activated by calcium released from intracellular stores these data indicate that frusemide may block directly $I_{Cl(Ca)}$.

5 Ethacrynic acid ($2-5\times10^{-4}$ M) decreased the amplitude of STICs in a concentration-dependent manner by a similar amount at potentials of -50 to $+30$ mV but did not alter the STIC E_{rev}. However, these concentrations of ethacrynic acid also reduced STOC amplitude and 5×10^{-4} M ethacrynic acid evoked a sustained outward current in most cells at 0 mV; thus ethacrynic acid has a more complex action than simple block of $I_{Cl(Ca)}$.

6 Indanyloxyacetic acid reduced both STIC amplitude and decay time without affecting STOCs and thus also seems to inhibit directly $I_{Cl(Ca)}$. It is discussed whether block of $I_{Cl(Ca)}$ mediates the vasodilator effect of these agents.

Keywords: Loop diuretics; calcium-activated currents; vascular smooth muscle; frusemide; ethacrynic acid, indanyloxyacetic acid

Introduction

Loop diuretics are used clinically in the treatment of congestive heart failure and essential hypertension. Whereas there is little doubt that much of their beneficial effect is due to their diuretic action there is also evidence to suggest that these agents may also relax vascular smooth muscle to produce vasodilatation. Thus, frusemide administered intravenously to patients with congestive heart failure increased venous capacitance prior to diuresis (Dikshit *et al.*, 1973) and reduced blood pressure in hypertensive patients by an action not directly correlated with a decrease in plasma volume (Gerkens, 1987). Furthermore, *in vitro* experiments have demonstrated that frusemide inhibited both the spontaneous and the evoked contractions to noradrenaline and angiotensin II in the rat isolated portal vein (Blair-West *et al.*, 1972). Some evidence has been put forward to suggest that the effect of frusemide on the vasculature is mediated by prostaglandin production (see Gerber, 1983; Gerkens, 1987). However, recent experiments have shown that frusemide relaxed vascular smooth muscle when cyclo-oxygenase was inhibited which indicates that at least part of the inhibitory effect is independent of prostaglandin release (Stevens *et al.*, 1992; Barthelmebs *et al.*, 1994). Moreover, since the vasorelaxant effect of frusemide was not altered by removal of the endothelium it seems likely that frusemide directly reduces smooth muscle contractility (Stevens *et al.*, 1992; Greenberg *et al.*, 1994).

It has been shown that noradrenaline stimulates a calcium-activated chloride current ($I_{Cl(Ca)}$) in rabbit portal vein (Byrne & Large, 1988) and it was suggested that this conductance, in addition to a non-selective cation current, may mediate the noradrenaline-induced depolarization and at least part of the subsequent contraction in vascular smooth muscle (Amédée & Large, 1989). Later it was shown that frusemide inhibited the noradrenaline-induced $I_{Cl(Ca)}$ in rabbit ear artery cells (Amédée *et al.*, 1990) which indicates that this action may contribute to the vasodilator effect of frusemide. However, in this latter paper the mechanism by which frusemide inhibits $I_{Cl(Ca)}$ was not studied. For example, it is possible that frusemide inhibited $I_{Cl(Ca)}$ by inhibiting the release of calcium from the sarcoplasmic reticulum which is responsible for the noradenaline-induced $I_{Cl(Ca)}$ (Amédée *et al.*, 1990) or even by blocking the α-adrenoceptor which mediates the noradrenaline-evoked current. In the present work we have re-investigated the action of frusemide on spontaneous calcium-activated chloride and potassium currents which are both activated by pulses of calcium released from the sarcoplasmic reticulum and these spontaneous currents occur independently of α-adrenoceptor activation (Wang *et al.*, 1992). Recently, it has been suggested that the decay of spontaneous transient inward currents ($I_{Cl(Ca)}$, STICs) in rabbit portal vein represents the closure of chloride channels (Hogg *et al.*, 1993a, b) and consequently agents that block open chloride channels may produce a characteristic change in the decay rate of STICs. Indeed, some established chloride channel blocking compounds have been shown to shorten or lengthen the decay rate of STICs (Hogg *et al.*, 1993b; 1994a) and niflumic acid converts the normal exponential decay into a bi-exponential form at some membrane potentials (Hogg *et al.*, 1994b). These data suggest that these agents interact with the chloride channels after they have

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opened and the quantitative results provide some information on the rate with which the blockers interact with the channel (Hogg *et al.*, 1994b). Thus it would seem that the STIC may represent a good model to investigate the mechanisms by which drugs inhibit $I_{Cl(Ca)}$ in smooth muscle. In this paper we describe the effects of frusemide and another loop diuretic, ethacrynic acid, on STICs and compare the results on spontaneous transient outward currents (STOCs, calcium-activated potassium currents). In addition, we have studied the action of indanyloxyacetic acid (IAA-94) which has also been shown to possess vasodilator activity. IAA-94 has been demonstrated to be a potent inhibitor of endothelin-induced depolarization and contraction in renal blood vessels and mesangial cells and rat aorta which are considered to involve the opening of $I_{Cl(Ca)}$ (Ijima *et al.*, 1991; Takenaka *et al.*, 1992).

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 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 and were then 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 with the perforated patch method using a patch clamp amplifier (List EPC 7; List-Electronic; Darmstadt, Germany). To obtain a perforated patch the antibiotic nystatin (ICN; 200 μg ml^{-1}) was dissolved in dimethylsulphoxide (DMSO) and included in the pipette solution which was prepared every 3 h. Cells were constantly superfused at a rate of 2–3 ml min^{-1} with an external PSS containing (mM): NaCl 126, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 10 and glucose 11 which was adjusted to pH 7.2 with NaOH. The pipette solution contained (mM): KCl 126, $MgCl_2$ 1.2, HEPES 10, glucose 11, EGTA 0.2 and the pH was adjusted to 7.2 with KOH. In K-free experiments, 126 mM KCl was replaced by an equimolar amount of NaCl in the pipette solution and was omitted from the external solution.

All currents were recorded on magnetic video tape and played back onto a Gould brush recorder to allow amplitudes to be calculated. Analysis of the time course of the spontaneous currents was performed using the SIGAVG signal-averaging programme via a CED 1401 interface (both systems Cambridge Electronic Design, Science Park, Cambridge). Signals were low-pass filtered at 1 kHz prior to digitization and sampled at 2.5 kHz by the software. Ten to twenty individual STICs were used to produce an averaged current and decay time constants were obtained by fitting an exponential with a least squares fitting routine. In the text, n, represents the number of cells required to obtain the mean value which is presented ± s.e.mean. All drugs were dissolved in the PSS perfusing the cells from stock solutions and introduced to the recording chamber downstream of the main PSS reservoir. All drugs were applied for between 2–4 min at which times the inhibitory effects of the drugs appeared to have reached equilibrium. Ethacrynic acid (Sigma, Poole, Dorset) and indanyloxyacetic acid (IAA-94; Research Biochemicals Incorporated, Natick, U.S.A.) were prepared as stock solutions in DMSO. The final concentration of DMSO did not exceed 0.1% in the bathing solution and this concentration had no effect on any of the electrical signals studied. Frusemide was obtained as a pre-injection format dissolved in water (Antigen Pharmaceuticals, Roscrea, Ireland). Statistical significance was assessed by Student's t test.

Results

Effect of frusemide and ethacrynic acid on the amplitude of spontaneous calcium-activated chloride currents

The effect of loop diuretics on STICs was investigated in rabbit portal vein cells initially with potassium-containing pipette and bathing solutions which approximate to physiological conditions. Figure 1a is a control record of STICs from a cell continuously superfused with PSS and shows that the amplitude and frequency of STICs remained stable over the period during which the inhibitors were studied. Figure 1b and c illustrate the effect of frusemide and ethacrynic acid (both at 5×10^{-4} M) on STICs in two different cells at a holding potential (V_h) of −77 mV. This potential was selected because it is the theoretical value of the potassium equilibrium potential so that the chloride currents could be studied without contamination from STOCs. The amplitude of STICs was rapidly attenuated by the addition of 5×10^{-4} M frusemide to the bathing solution (Figure 1b) and the mean reduction in STIC amplitude after 3 min exposure to 5×10^{-4} M frusemide was 50 ± 6% (n=7 cells). Frusemide, 10^{-3} M, inhibited STIC amplitude by 60 ± 5% (n=4). The threshold concentration of frusemide for decreasing STIC amplitude in the time course of the present experiments (about 3 min) was between $1-3 \times 10^{-4}$ M. In 4 cells the mean reduction in STIC amplitude by 3×10^{-4} M frusemide was 27 ± 4% whereas 10^{-4} M frusemide was without effect. Frusemide did not alter the holding current in any concentration used. In 7 cells the effect of frusemide was reversible while in 8 cells even prolonged washing produced only partial recovery of STIC amplitude (to 0.5–0.8 of the control amplitude). In Figure 1b it seems that

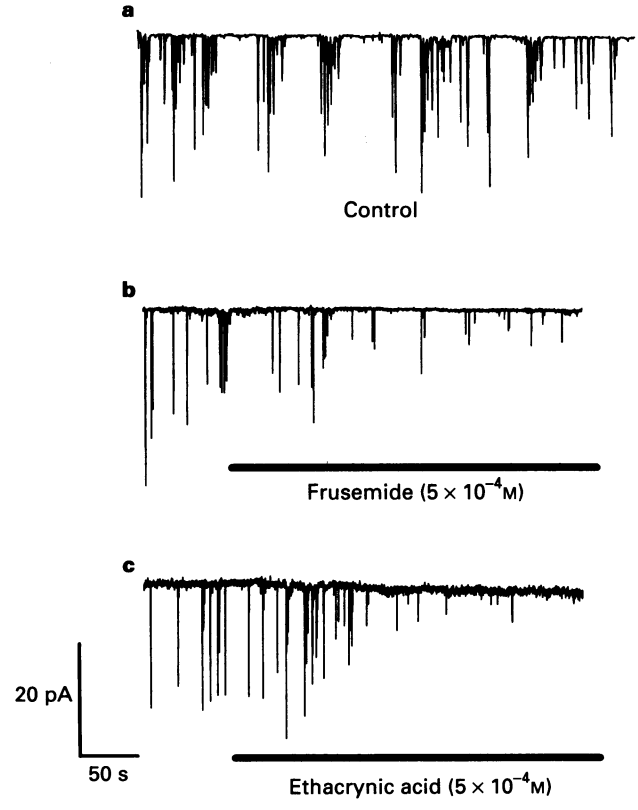

Figure 1 The effect of frusemide and ethacrynic acid on STICs. The holding potential, V_h, was −77 mV in potassium-containing solutions. (a) Shows control STICs and in (b) and (c) the drugs were added to the bathing solution as indicated by the horizontal bars.

frusemide may have inhibited STIC frequency but this apparent effect is likely to be due to the fact that some of the smaller STICs had been reduced to below the noise level.

Previously, we have demonstrated that some chloride channel blockers are more potent as the membrane is depolarized (Hogg *et al.*, 1993b; 1994a, b). In order to test the voltage-dependence of these agents it is necessary to carry out the experiments in potassium-free pipette and external solutions to remove spontaneous transient outward potassium currents (STOCs) which become prominent as the membrane potential is depolarized. Figure 2a illustrates the influence of membrane potential on STIC amplitude in the absence or presence of 5×10^{-4} M frusemide. It can be seen that the reversal potential (E_{rev}) was not changed by the inhibitor. Thus in 9 cells the control E_{rev} was -3 ± 2 mV and in 5×10^{-4} M frusemide E_{rev} was -2 ± 4 mV ($n=3$). Figure 2b demonstrates that the inhibitory effect of frusemide was similar at holding potentials of -50 to $+30$ mV and therefore is voltage-independent.

The addition of ethacrynic acid (5×10^{-4} M) to the bathing solution also produced a rapid decrease in STIC amplitude (Figure 1c) without changing the holding current. At -77 mV in K-containing solutions, 2×10^{-4} M and 5×10^{-4} M ethacrynic acid reduced STIC amplitude by $56 \pm 6\%$ ($n=4$) and $71 \pm 6\%$ ($n=8$), respectively. The effect of ethacrynic acid was also poorly reversible. Further experiments were carried out with ethacrynic acid in K-free conditions at -50 mV where the degree of inhibition was $45 \pm 8\%$ ($n=4$) and $76 \pm 9\%$ ($n=3$) for 2×10^{-4} M and 5×10^{-4} M ethacrynic acid, respectively. Also E_{rev} measured in K-free conditions was not altered by 2×10^{-4} M ethacrynic acid ($E_{rev}=2 \pm 3$ mV, $n=3$) compared to control ($E_{rev}=-3 \pm 2$ mV). Figure 2b demonstrates that the degree of inhibition by 2×10^{-4} M ethacrynic acid was similar at membrane potentials of -50 to $+30$ mV and therefore its effects are not voltage-dependent.

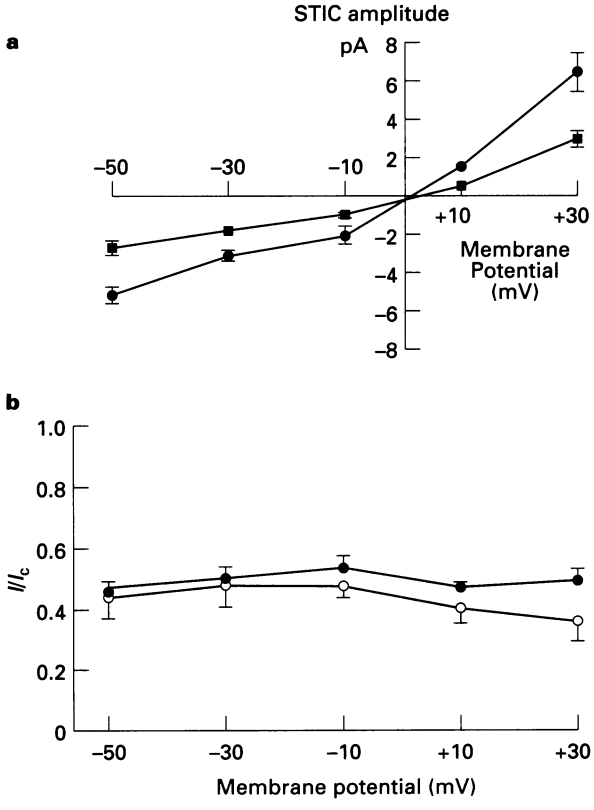

Figure 2 Effect of membrane potential on the inhibitory effect of frusemide and ethacrynic acid on STIC amplitude. (a) Shows the influence of membrane potential on STIC amplitude in the absence (●) or presence of 5×10^{-4}M frusemide (■); (b) illustrates the inhibitory effect of frusemide (●, 5×10^{-4}M) and ethacrynic acid (○, 2×10^{-4}M) at different holding potentials. I and I_C are the current amplitudes in the presence and absence of the inhibitors.

Effect of frusemide and ethacrynic acid on the decay of STICs

Earlier studies have shown that the STIC decays exponentially (Hogg *et al.*, 1993a,b) and that some chloride channel blockers produce a marked alteration in the time constant of the STIC decay (Hogg *et al.*, 1993b; 1994a,b). Figure 3a illustrates the effect of 5×10^{-4} M frusemide on the STIC time course at -50 mV in K-free conditions. In the presence of frusemide the decay remained exponential but the reduction in amplitude was not accompanied by a change in decay time constant (τ, 111 ms in frusemide compared to a control value of 106 ms). In 7 cells with 5×10^{-4} M frusemide the τ value was 101 ± 11 ms compared to the control τ value of 93 ± 7 ms. Frusemide, 10^{-3} M, also did not affect τ at -50 mV (in frusemide, $\tau=98 \pm 5$ ms compared to a control value of 101 ± 5 ms, $n=4$). It has been shown that some chloride channel antagonists produce a voltage-dependent effect on τ (Hogg *et al.*, 1993b; 1994a,b) but this did not occur with frusemide as is shown in Figure 3b. The control time constant at $+50$ mV, which was prolonged compared to the value at -50 mV (see Hogg *et al.*, 1993b), was not different from the value in the presence of frusemide 251 ms vs 231 ms, respectively. In 7 cells at $+50$ mV, the τ values in the absence and presence of 5×10^{-4} M frusemide were 195 ± 19 ms and 177 ± 22 ms, respectively. In 10^{-3} M frusemide at $+50$ mV, τ was 207 ± 16 ms compared to a control τ of 212 ± 10 ms ($n=7$).

The reduction in STIC amplitude by ethacrynic acid, also, was not accompanied by a change in the decay time at either -50 mV or $+50$ mV (Figure 4). The control τ values at -50 mV and $+50$ mV were 87 ± 6 ms and 265 ± 17 ms and in 5×10^{-4} M ethacrynic acid τ was 93 ± 6 ms and 278 ± 17 ms ($n=4$). The STIC decay time constants at -50 mV and $+50$ mV also were not altered by 2×10^{-4} M ethacrynic acid ($n=4$).

Effect of frusemide and ethacrynic acid on spontaneous potassium currents

We have shown that STICs and STOCs often occur as biphasic events (Wang *et al.*, 1992; Hogg *et al.*, 1993a) and that both currents are blocked by agents (e.g. caffeine) that deplete calcium from the sarcoplasmic reticulum. Consequently it seemed that both STICs and STOCs are triggered by the same pulses of calcium released randomly from the sarcoplasmic reticulum (Wang *et al.*, 1992; Hogg *et al.*, 1993a). It is possible that agents that decrease STIC

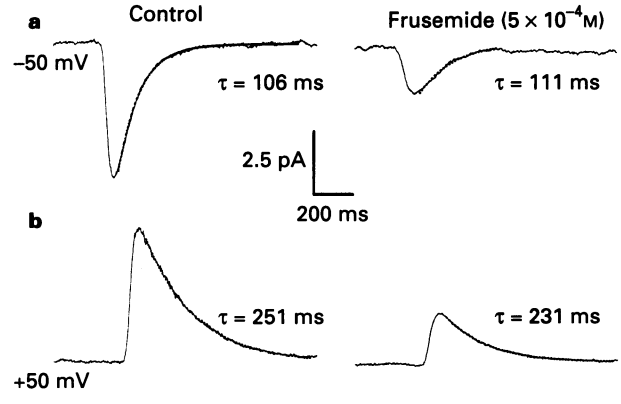

Figure 3 The effect of frusemide on the time course of STICs at different membrane potentials. The holding potential was -50 mV in (a) and $+50$ mV in (b). The time constant (τ) was estimated from an averaged current derived from 10–20 individual currents. K-free bathing and pipette solutions were used. The dotted curves show the exponential fits.

amplitude may produce this effect by depleting or inhibiting the release of calcium from the intracellular store. This possibility was examined by studying the effect of the loop diuretics on STOCs in K-containing solutions at 0 mV, i.e. close to the chloride equilibrium potential. Figure 5a shows that frusemide (10^{-3} M) has no inhibitory effect on the frequency and amplitude of STOCs and this result was found in 6 cells. Exposure of cells to 2×10^{-4} M ethacrynic acid (Figure 5b) had no effect on STOC frequency (0.4 ± 0.08 Hz compared to a control value of 0.5 ± 0.05 Hz) but resulted in a small but significant reduction ($P < 0.05$) in STOC amplitude (204 ± 17 pA compared to a control mean of 262 ± 44 pA, $n = 4$). Addition of 5×10^{-4} M ethacrynic acid produced a pronounced reduction of STOC amplitude (Figure 5c) and evoked an outward current in 6 out of 10 cells tested. The mean outward current was 59 ± 13 pA at 0 mV which peaked after 4–5 min and reversed after washout of ethacrynic acid. In the four other cells, ethacrynic acid (5×10^{-4} M) did not produce any change in holding current. In the presence of 5×10^{-4} M ethacrynic acid the STOC amplitude decreased by $69 \pm 9\%$ ($n = 10$). Consequently the inhibitory effect of frusemide on STIC amplitude does not appear to be mediated by an effect on the intracellular calcium store but in contrast, it seems that ethacrynic acid has an action on the intracellular store.

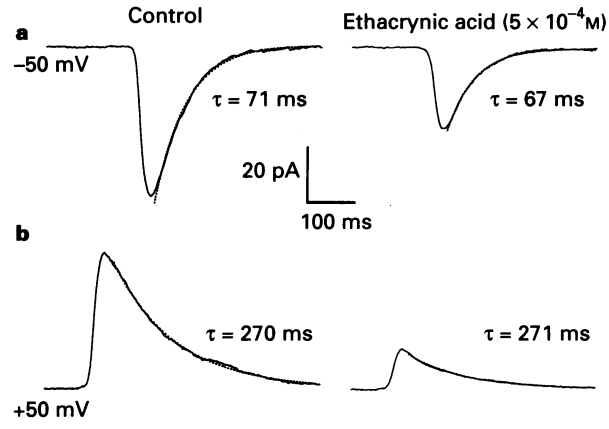

Figure 4 The effect of ethacrynic acid on the time course of STICs. The holding potential was −50 mV in (a) and +50 mV in (b). K-free conditions were used. The dotted curves show the exponential fits.

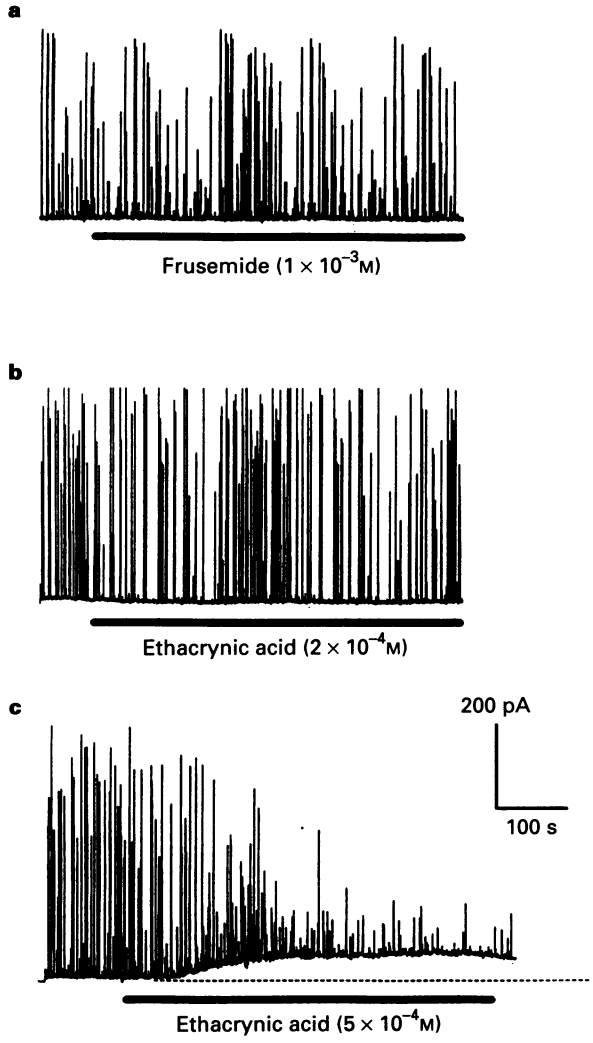

Figure 5 The effect of frusemide and ethacrynic acid on STOCs. The holding potential was 0 mV with K-containing solutions. In (c) the horizontal dashed line indicates the control holding current value.

Effect of IAA-94 on STICs and STOCs

Figure 6a shows that addition of 10^{-4} M IAA-94 to the bathing solution produced a pronounced reduction in STIC amplitude without altering the holding current In 4 cells, 10^{-4} M IAA-94 reduced STIC amplitude by $75 \pm 7\%$ and STICs were blocked totally by 5×10^{-4} M IAA-94 ($n = 3$). In contrast to the loop diuretics, the decrease in STIC amplitude caused by IAA-94 was accompanied by an increase in the STIC decay rate. Figure 6b illustrates that the STIC decay remained exponential in the

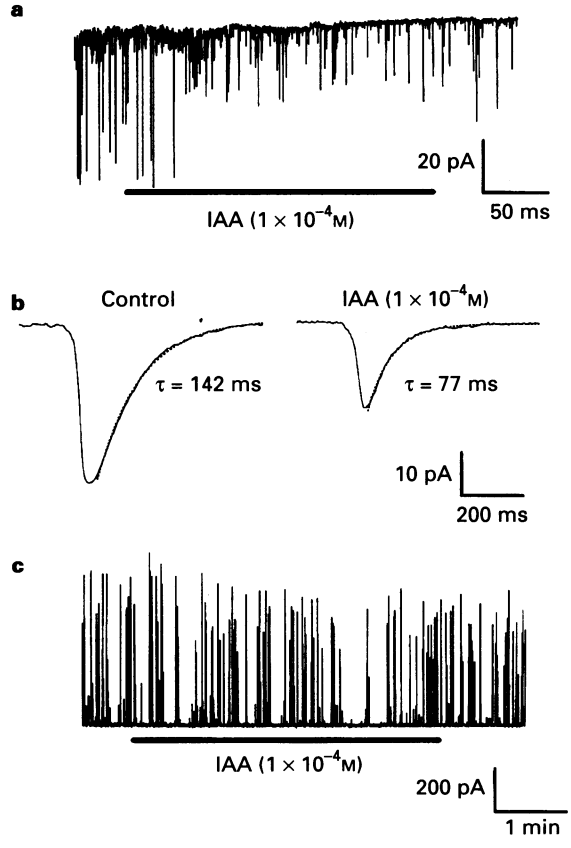

Figure 6 The effect of indanyloxyacetic acid (IAA-94) on STICs and STOCs. K-containing solutions were used and the holding potential in (a) and (b) was −77 mV and in (c) was 0 mV. (a) Illustrates an experimental trace while in (b) the effect of IAA-94 on averaged STICs from (a) is shown. The dotted curves show the exponential fits.

presence of 10^{-4} M IAA-94 but τ was reduced from a control value of 142 ms to 77 ms. In 4 cells the mean control τ was 106 ± 10 ms and in 10^{-4} M IAA-94 τ was 72 ± 2 ms ($P < 0.05$).

In contrast, IAA-94 had no effect on either the amplitude or the frequency of STOCs (Figure 6c). Thus, in 3 cells the ratio of STOC amplitude in the presence of IAA-94 compared to control values was 1.03 ± 0.09 and the ratio of STOC frequency was 1.0 ± 0.13. Therefore it seems unlikely that the reduction in STIC amplitude by IAA-94 was due to an effect on the intracellular calcium store but rather was due to direct inhibition of the chloride conductance.

Discussion

The data from this study indicate that all three compounds, frusemide, ethacrynic acid and IAA-94 inhibited $I_{Cl(Ca)}$ in rabbit portal vein cells but with different characteristics. Since frusemide and IAA-94 did not decrease STOC amplitude it seems unlikely that the reduction in STIC amplitude produced by these agents was due to inhibition of the calcium release mechanism of the sarcoplasmic reticulum which is the source of the calcium signal that triggers both STOCs and STICs. Rather, it is possible that frusemide and IAA-94 block directly the calcium-activated chloride conductance (see below). In contrast concentrations of ethacrynic acid that reduced STIC amplitude also decreased STOC amplitude. This could be due to an effect on the intracellular calcium store or block of the Ca-activated potassium channels that underlie STOCs. The present experiments do not differentiate between these two explanations but the ability of 5×10^{-4} M ethacrynic acid to evoke an outward current (potassium) in some cells at 0 mV indicates that ethacrynic acid can activate a potassium current rather than block potassium channels. Consequently the reduction in STOC amplitude may indicate a depletion of intracellular calcium stores which would also lead to a reduction in STIC amplitude. However it should be noted that 2×10^{-4} M ethacrynic acid produced a greater decrease in STIC amplitude than in STOC amplitude and therefore it is possible that this loop diuretic also inhibits $I_{Cl(Ca)}$ directly in vascular smooth muscle. This would be in agreement with the finding that ethacrynic acid inhibits both chloride transport and tritiated IAA-94 binding in epithelial cells (Landry *et al.*, 1987). Nevertheless ethacrynic acid has apparently complex effects on rabbit portal vein cells and is not simply a direct inhibitor of $I_{Cl(Ca)}$.

The present results with spontaneous chloride currents in portal vein cells confirm the blocking effect of frusemide on $I_{Cl(Ca)}$ reported for noradrenaline-evoked currents in rabbit ear artery cells (Amédée *et al.*, 1990). The latter study did not present any information on the mechanism of action of frusemide which was one of the major aims of the present work but it seems likely that reduction of the noradrenaline-evoked $I_{Cl(Ca)}$ by frusemide is not due to an inhibitory action on the intracellular calcium store (see above). Also since spontaneous chloride currents were inhibited by frusemide in the present study, reduction of noradrenaline-evoked $I_{Cl(Ca)}$ probably occurs as a direct block of the chloride conductance rather than the α-adrenoceptor. Marty and his coworkers (Evans *et al.*, 1986) observed a similar degree of inhibition of $I_{Cl(Ca)}$ by frusemide in the same concentration-range in rat lacrimal gland cells. In their experiments, frusemide displayed a weak degree of voltage-dependence and also appeared to be more effective against large rather than small currents. It was concluded therefore that frusemide bound to the open state of the channel (Evans *et al.*, 1986). In portal vein cells the reduction of STIC amplitude by frusemide was similar at potentials between -50 and $+30$ mV and consequently there appears to be no voltage-dependence in smooth muscle. Also it appears that frusemide did not affect the STIC τ value, which represents the mean channel open time if the STIC decay represents channel closure (Hogg *et al.*, 1993a,b). In the light of these comments it has been shown that the inhibitory action of anthracene-9-carboxylic acid (A-9-C) and niflumic acid on STIC amplitude is increased by depolarization (Hogg *et al.*, 1993b; 1994a,b) which would be expected if the negatively charged form of the molecule blocks the channel. Moreover the reduction in STIC amplitude by both these compounds was accompanied by a concentration-dependent alteration in the STIC decay time, consistent with rapid block of the open channel (for fuller explanation see Hogg *et al.*, 1993b; 1994a,b). By analogy the present data show that frusemide does not alter the STIC decay time constant which suggests that either the chloride conductance is inhibited before channel opening or that $I_{Cl(Ca)}$ is inhibited by a 'slow' channel blocking mechanism in which the apparent mean open time is little affected. In contrast, the reduction of STIC amplitude by IAA-94 was associated with a significant decrease in the STIC decay τ value which suggests that this compound may interact with the open calcium-activated chloride channel and dissociate slowly relative to the mean channel open time. Also IAA-94 was more potent than frusemide and did not affect intracellular calcium stores and therefore might serve as a useful probe to investigate the role of $I_{Cl(Ca)}$ in smooth muscle. However, it should be pointed out that IAA-94 is considerably less potent than niflumic acid which inhibits STICs in the micromolar concentration range and appears to be selective against $I_{Cl(Ca)}$ (Hogg *et al.*, 1994b). However, the observation that IAA-94 inhibited $I_{Cl(Ca)}$ in rabbit portal vein cells supports the previous hypothesis that the attenuation of the endothelin-induced depolarization and vasoconstriction by IAA-94 in the renal microcirculation was due to block of chloride channels (Ijima *et al.*, 1991; Takenaka *et al.*, 1992).

Finally it is worth considering whether inhibition of $I_{Cl(Ca)}$ might contribute to the vasodilator action of frusemide which has been observed in many studies (see Introduction). Chloride ions are transported into smooth muscle cells primarily by the Cl^-/HCO_3^- exchanger with possible involvement of the Na^+, K^+, $2Cl^-$ cotransporter with the result that the chloride equilibrium potential is normally considered to be between -20 mV and -30 mV (Aickin, 1990). The resting chloride conductance is low in smooth muscle but any stimulus (e.g. a pharmacological agent) that increases intracellular calcium might activate $I_{Cl(Ca)}$ and thereby produce membrane depolarization and contraction. There is evidence that frusemide inhibits the cotransporter in some tissues in concentrations greater than 10^{-4} M (Aickin & Brading, 1990; Cabantchik & Greger, 1992) which may convey a vasorelaxant effect (see O'Donnell & Owen, 1994). The concentrations of frusemide required to inhibit $I_{Cl(Ca)}$ in our experiments were rather high ($\geqslant 10^{-4}$ M) but this is the case also in some functional studies. Indeed there was a good degree of similarity in the quantitative data in our electrophysiological study and some contraction experiments. For example, in rat portal vein about 3×10^{-4} M frusemide inhibited spontaneous contraction by approximately 40% after 10 min exposure and inhibited maximum noradrenaline and angiotensin-induced contractions by respectively 25% and 52% (Blair-West *et al.*, 1972). In guinea-pig pulmonary artery, 3×10^{-4} M frusemide (10 min contact time) decreased the noradrenaline-evoked contraction by 40% (Stevens *et al.*, 1992). Also several types of canine veins pre-contracted with noradrenaline or a stable thromboxane-mimetic were relaxed by 30–70% with 3×10^{-4} M frusemide (Greenberg *et al.*, 1994). In our study 3×10^{-4} M frusemide reduced $I_{Cl(Ca)}$ by 27% after a 3 min exposure. Consequently it is possible that blockade of $I_{Cl(Ca)}$ may contribute to the vasodilator action of frusemide when used in relatively high concentrations.

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