British Journal of Pharmacology (1995) 116, 2939-2948 © 1995 Stockton Press All rights reserved 0007-1188/95 $12.00

Comparison of the effects of fenamates on Ca-activated chloride and potassium currents in rabbit portal vein smooth muscle cells

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1 The perforated patch and conventional whole-cell recording techniques were used to study the action of flufenamic, mefenamic and niflumic acid on calcium-activated chloride and potassium currents in rabbit portal vein smooth muscle cells.

2 In K-conditions at a holding potential of -77 mV flufenamic acid and mefenamic acid decreased the amplitude of spontaneous transient inward currents (STICs, calcium-activated chloride currents, $I_{Cl(Ca)}$) in a concentration-dependent manner. The potency sequence was niflumic > flufenamic > mefenamic acid.

3 At -77 mV 1×10^{-5} M flufenamic acid increased the STIC exponential decay time constant (τ). At higher concentrations the STIC decay was described by 2 exponentials with an initial decay (τ_f) faster than the control τ value and a second exponential (τ_S) which had a time constant slower than the control τ value. Low concentrations of mefenamic acid had no effect or decreased the τ value whereas in higher concentrations biphasic currents were recorded.

4 In K-free conditions the inhibitory effect of both flufenamic and mefenamic acid on STIC amplitude was greater at $+50$ mV compared to -50 mV, showing that the effect of these agents was voltage-dependent.

5 In cells held at 0 mV in K-containing conditions the fenamates reduced both the frequency and amplitude of spontaneous transient outward currents (STOCs, calcium-activated potassium currents, $I_{K(Ca)}$). The concentration range to produce these effects was higher than that to decrease STIC amplitude and the potency sequence was flufenamic > niflumic $\geqslant$ mefenamic acid.

6 All these compounds in concentrations greater than 5×10^{-5} M evoked a 'noisy' potassium current at 0 mV which reached a maximum after approximately 3 min. This current was readily reversible on washout of the drug and could be elicited several times in the same cell. The current-voltage relationship of the fenamate-evoked current exhibited pronounced outward rectification characteristic of $I_{K(Ca)}$.

7 The current evoked by 2×10^{-4} M flufenamic acid and 5×10^{-4} M niflumic acid was not affected by 1×10^{-5} M glibenclamide but was markedly inhibited by 1×10^{-3} M tetraethylammonium. Furthermore, large currents were activated by flufenamic and niflumic acid in the presence of caffeine and cyclopiazonic acid (an inhibitor of the sarcoplasmic reticulum Ca-ATPase) to deplete intracellular Ca-stores.

8 Conventional whole-cell recording was performed with pipette solutions in which the ability to buffer changes in intracellular calcium was varied by altering the concentration of the calcium chelator (2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA). Flufenamic acid (2×10^{-4} M) and niflumic acid (5×10^{-4} M) both evoked large outward currents when recordings were made with either 1×10^{-4} M or 1×10^{-2} M BAPTA. Furthermore, bathing the cells in nominally calcium-free extracellular solution did not reduce the amplitude of the evoked currents.

9 It is concluded that both flufenamic and mefenamic acid inhibit $I_{Cl(Ca)}$ by a mechanism similar to niflumic acid, possibly open channel blockade. Furthermore, at concentrations greater than 5×10^{-5} M all three fenamates inhibited STOC activity and evoked directly an outward current which resembled $I_{K(Ca)}$.

Keywords: Fenamates; calcium-activated currents; vascular smooth muscle; flufenamic acid; mefenamic acid; niflumic acid

Introduction

In recent studies we have been investigating the pharmacology of calcium-activated chloride currents ($I_{Cl(Ca)}$) in vascular smooth muscle. Experiments on spontaneous transient inward currents (STICs), which are randomly occurring calcium-activated chloride currents triggered by calcium ions released sporadically from the sarcoplasmic reticulum (SR, Wang *et al.*, 1992), showed that concentrations of $>1 \times 10^{-4}$ M of most classical chloride channel blockers are needed to inhibit $I_{Cl(Ca)}$ in rabbit portal vein (Hogg *et al.*, 1994a). However, the non-steroidal anti-inflammatory drug, niflumic acid, reduced the amplitude of $I_{Cl(Ca)}$ in the micromolar range (Pacaud *et al.*, 1989; Janssen & Sims, 1992; Akbarali & Giles, 1993; Hogg *et al.*, 1994b; Lamb *et al.*, 1994). Moreover, analysis of the action of niflumic acid on the STIC decay time course, which is thought to represent the kinetics of chloride channel closure (Hogg *et al.*, 1993), indicated that niflumic acid binds to a site within the channel after it has opened to inhibit the chloride current (open channel blockade, Hogg *et al.*, 1994b). A subsequent and unexpected observation was that niflumic acid and two structurally-related compounds, flufenamic and mefenamic acid, potentiated large conductance calcium-activated potassium channels (BK_{Ca}) of coronary artery membranes re-

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constituted into lipid bilayers (Ottolia & Toro, 1994). This represents a novel action of the fenamates but it is possible that this effect may be restricted to BK_{Ca} channels in lipid bilayers since some properties of these channels differ from those of native BK_{Ca} channels. For example the sensitivity to Ca^{2+} ions of BK_{Ca} channels in bilayers is 10–100 times less than that of BK_{Ca} channels in their natural environment (see Bolton & Beech, 1992). Consequently we have compared the effects of flufenamic acid, mefenamic and niflumic acid (see structures in Figure 1) on calcium-activated potassium currents ($I_{K(Ca)}$) and $I_{Cl(Ca)}$ with whole cell and perforated patch recording in freshly dispersed rabbit portal vein smooth muscle cells. The results show that all three compounds appear to block open calcium-activated chloride-channels and also activate BK_{Ca} but with different potencies and with a different potency ratio.

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 then dissociated by gently passing the muscle strips through the mouth of a wide-bore glass pipette. Cells 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 constantly superfused with PSS by 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) dissolved in dimethyl sulphoxide (DMSO) was included in the pipette solution to give a final concentration of 100–200 μg ml^{-1}. The nystatin-containing pipette solution was prepared every 3 h. The normal extracellular solution 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, EGTA 0.2 and the pH was adjusted to 7.2 with KOH. In K-free experiments 126 mM KCl in the pipette solution was replaced by an equimolar amount of CsCl and KCl was omitted from the external solution. For experiments using the conventional whole-cell technique pipette solutions were prepared which contained either 1×10^{-4} M or 1×10^{-2} M 1,2-bis (2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA).

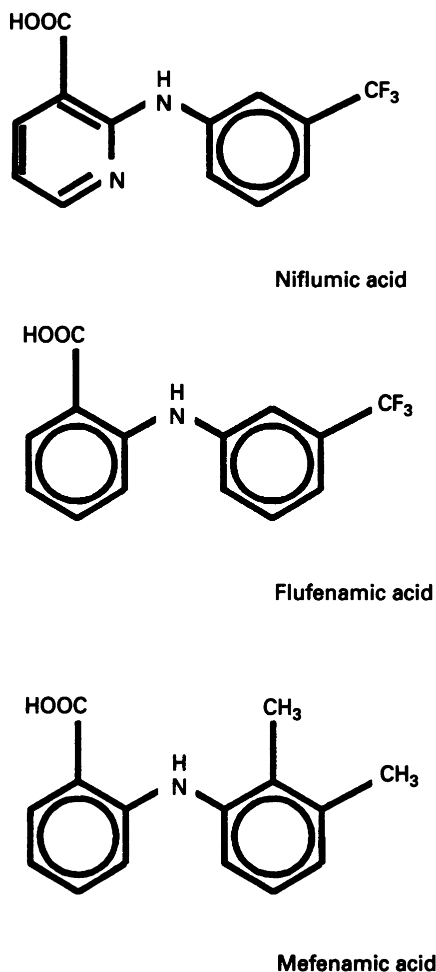

Figure 1 Chemical structure of niflumic acid, flufenamic acid and mefenamic acid used in the present study.

All membrane 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 spontaneous currents was performed by use of 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 before digitization and sampled at 2.5 KHz by the software. STICs recorded over a 1–3 min period were averaged and the mean current of 10–20 events was fitted by an exponential described by the equation $A^t = Ae^{-(t/\tau)}$ using a least squares fitting routine. Individual STOCs were also recorded over a 2 min period and the mean 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 use of an unpaired Student's *t* test at the 0.05 probability level and any significance is indicated in the text. 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. Flufenamic acid, mefenamic acid and niflumic acid (all Sigma; Poole, Dorset) were prepared as stock solutions in DMSO each day and diluted to the required concentrations in the extracellular solution. For experiments on the effect of high concentrations of fenamates on K-currents the DMSO concentration did not exceed 0.25% and the solvent had no effect on membrane currents at this concentration. Cyclopiazonic acid (CPA) was purchased from Calbiochem (La Jolla, U.S.A.) and dissolved in DMSO as was glibenclamide (Sigma). Caffeine was dissolved in the extracellular solution and tetraethylammonium (TEA) was dissolved in distilled water.

Results

Effect of flufenamic and mefenamic acid on STICs recorded in K-conditions

In the first series of experiments we investigated the action of flufenamic acid and mefenamic acid on STICs to estimate the potency of these compounds in inhibiting $I_{Cl(Ca)}$ and to see if they acted by blocking open channels. At a holding potential of −77 mV, the theoretical potassium equilibrium potential, the amplitude of control currents was stable for over 10 min and ranged from 13 to 77 pA with a mean amplitude of 26±4 pA (n=20). Inclusion of flufenamic acid in the superfusing PSS reduced the amplitude of STICs rapidly and produced a maximal effect within 1–2 min (Figure 2a and b). The inhibitory effect of flufenamic acid on STIC amplitude was concentration-dependent and completely reversible within 2 min of perfusing the cells with normal PSS (Figure 2). Thus, 1, 2 and 5×10^{-5} M flufenamic acid reduced the amplitude of the STICs to respectively 71±6%, 52±11% and 9±4% of the control value. In general low concentrations of flufenamic acid

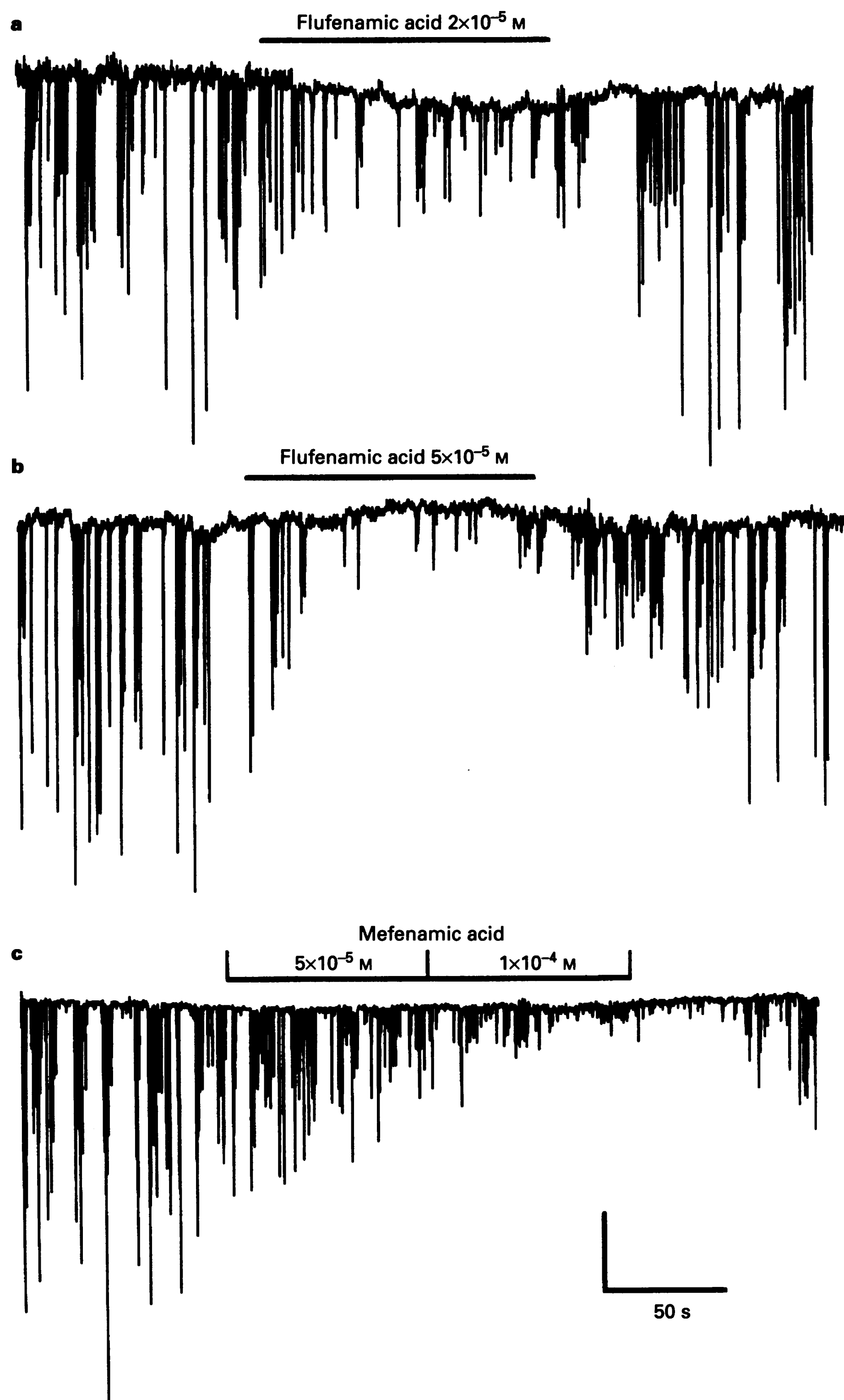

Figure 2 Effect of flufenamic acid and mefenamic acid on STICs recorded at a holding potential of −77 mV. Traces are continuous records with STICs shown as downward deflections. Cells were exposed to flufenamic acid and mefenamic acid for the periods denoted by the horizontal bars. In (a) and (b) the traces are from the same cell which was treated with flufenamic acid (a) 2×10^{-5} M and (b) 5×10^{-5} M separated by a washout period of 6 min. In (c) the cell was superfused with a solution containing 5×10^{-5} M and 1×10^{-4} M mefenamic acid sequentially. The vertical scale bar represents 20 pA for (a) and (b) and 10 pA for (c).

did not affect the STIC frequency, although higher concentrations produced an apparent reduction. Whilst this may be due to the abolition of small amplitude currents we cannot rule out a direct effect on STIC frequency by an unknown mechanism.

In comparison to the effects of flufenamic acid, mefenamic acid was a less potent inhibitor of STIC amplitude. Figure 2c shows that 5×10^{-5} M mefenamic acid produced a smaller attenuation of STIC amplitude compared to flufenamic acid (compare Figure 2b). Figure 6a shows that for any concentration flufenamic acid produced a greater inhibition of STIC amplitude than mefenamic acid. Thus, the concentration required to produce a 50% inhibition (IC_{50}) at −77 mV was approximately 1.9×10^{-5} M and 7×10^{-5} M for, respectively,

flufenamic and mefenamic acid. The inhibitory action of mefenamic acid was also readily reversible and control amplitudes were attained within 2–5 min after washout. The concentration-effect curves for flufenamic and mefenamic acid were compared with the data for niflumic acid (taken from Hogg *et al.*, 1994b) in Figure 6a. The potency sequence for inhibition of STICs was niflumic > flufenamic > mefenamic acid.

Effect of flufenamic and mefenamic acid on the time constant of STIC decay in K^+-conditions

Control STICs recorded at −77 mV decayed in a manner which could be fitted well by a single exponential which had a mean time constant (τ) of 89 ± 6 ms ($n=20$; e.g. Figures 3 and 4). Exposure of smooth muscle cells to flufenamic acid resulted in a change of the decay characteristics which was concentration-dependent. Thus, in the presence of 1×10^{-5} M flufenamic acid the STIC decay remained mono-exponential but the τ value was increased by a factor of 1.4 ± 0.06 ($n=5$). When the cells were exposed to 2×10^{-5} M fluefenamic acid the decay process became bi-exponential (Figure 3) with an initial decay which could be described by a time constant (τ_f) faster than the control τ value and a second exponential which was slower (τ_s) than the control τ. In 2×10^{-5} M flufenamic acid the absolute value of τ_f was between 16 and 22 ms with a mean normalized value of 0.3 ± 0.04 of control. In comparison τ_s was between 100 and 132 ms in the presence of 2×10^{-5} M flufenamic acid (mean normalized value $= 1.85 \pm 0.11$). Increasing the concentration of flufenamic acid to 5×10^{-5} M prolonged τ_s to between 120 to 250 ms (mean normalized value $= 2.7 \pm 0.8$, $n=5$) although in 5×10^{-5} M flufenamic acid τ_f was not reduced significantly ($P<0.05$) compared to the effects of 2×10^{-5} M flufenamic acid. Figure 3b shows that the decay of STICs was well fitted by 2 exponentials in the presence of flufenamic acid, which is similar to the effect found for niflumic acid in these cells and was interpreted to represent open-channel block (Hogg *et al.*, 1994b).

The reduction in STIC amplitude by 1×10^{-5} M mefenamic acid was not accompanied by a change in τ value, but 2×10^{-5} M mefenamic acid accelerated the STIC decay (Figure 4) and τ was reduced to 0.85 ± 0.03 of the control value. A further speeding up of the STIC decay was observed in 4 out of 7 cells in the presence of 5×10^{-5} M mefenamic acid (τ-value in drug compared to control value $= 0.76 \pm 0.1$). The reduction in τ value by 2 and 5×10^{-5} M was statistically significant ($P<0.05$). In the other 3 cells exposed to 5×10^{-5} M mefenamic acid and all cells bathed in 1×10^{-4} M mefenamic acid the STIC decay was fitted by 2 exponentials (see Figure 4). With 5×10^{-5} M and 1×10^{-4} M mefenamic acid the fast component of biphasic currents had similar values (0.27 ± 0.05 and 0.26 ± 0.06 of control values respectively) but the effect on τ_s was concentration-dependent (2.0 ± 0.2 and 3.1 ± 0.3 times the control τ value for 5×10^{-5} M and 1×10^{-4} M, respectively).

Influence of membrane potential on the effects of flufenamic and mefenamic acid

It has been shown previously that the inhibitory effect of some chloride-channel antagonists is increased by depolarization in rabbit portal vein cells (Hogg *et al.*, 1994a,b). Therefore, we compared the effects of flufenamic and mefenamic acid at two holding potentials, −50 mV and +50 mV, where the driving force for chloride ions is roughly equal (calculated $E_{Cl} = -2$ mV) but in the opposite direction. These experiments were carried out in K-free conditions with Cs^+ as the main intracellular cation (see Methods) to eliminate unwanted potassium currents. At +50 mV, 1×10^{-5} M flufenamic acid inhibited STICs to 0.31 ± 0.07 ($n=5$) of control values which was significantly different ($P<0.05$) than the effect at −50 mV (0.49 ± 0.07 of control, $n=8$). Mefenamic acid (5×10^{-5} M) was

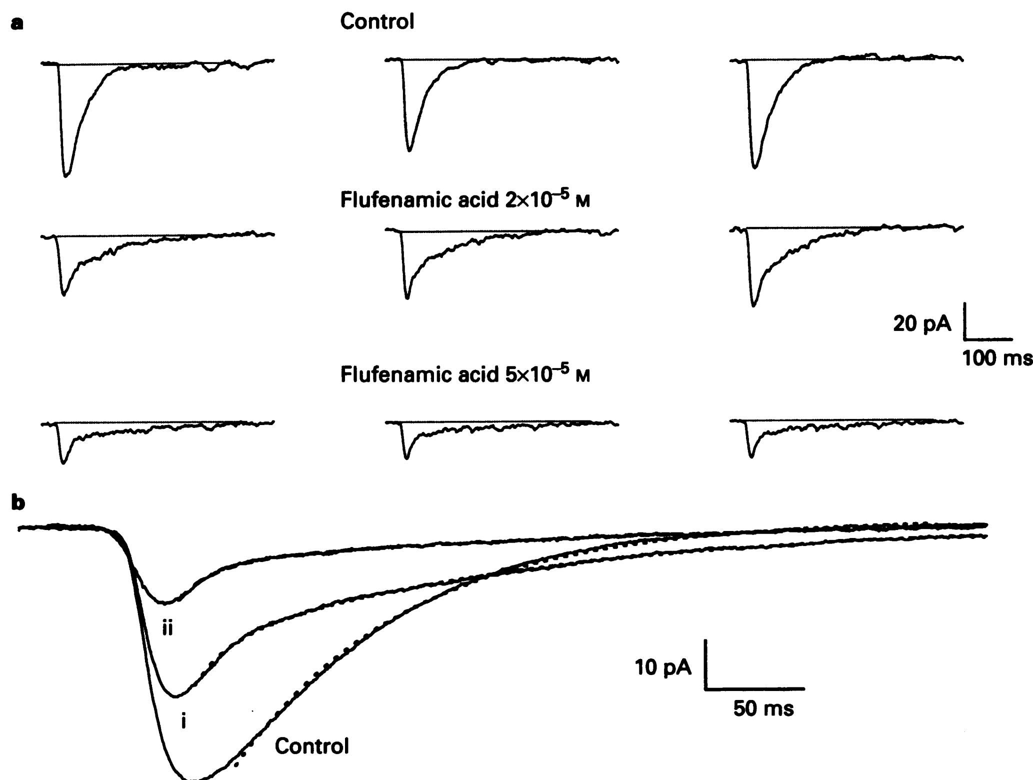

Figure 3 Effect of flufenamic acid on the decay of STICs recorded with a K-containing pipette solution at a holding potential of −77 mV. In (a), single STICs are shown in the control conditions (upper row) and in the presence of 2 and 5×10^{-5} M flufenamic acid (middle and lower rows). The horizontal line shows the baseline current level. (b) The averaged STICs in the absence (control) and presence of 2×10^{-5} M flufenamic acid (i) and 5×10^{-5} M flufenamic acid (ii). The control current decayed exponentially and had a τ value of 93 ms whereas in the presence of 2×10^{-5} M flufenamic acid the decay was biphasic ($\tau_f = 22$ ms, $\tau_s = 171$ ms). In 5×10^{-5} M flufenamic acid τ_f was 18 ms and τ_s was 222 ms. The dotted line shows the exponential fit of the 3 averaged currents.

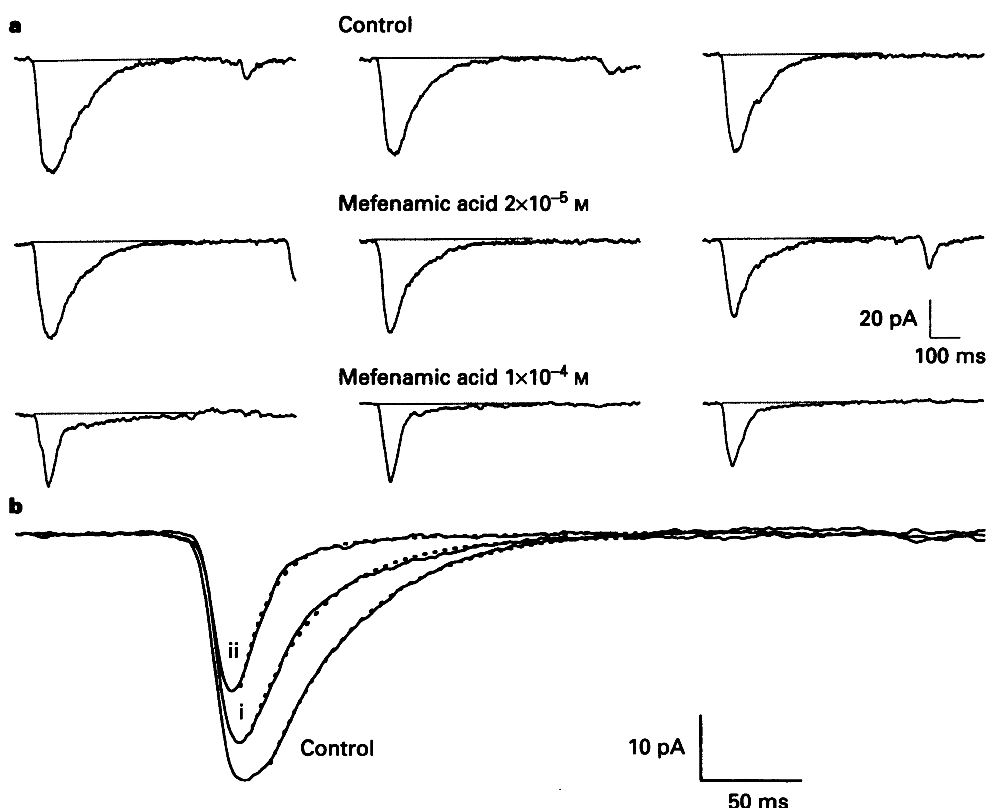

Figure 4 Effect of mefenamic acid on the decay of STICs recorded with a K-containing pipette solution at a holding potential of −77 mV. (a) Single STICs are shown in the control conditions (upper row) and in the presence of 2×10^{-5} M and 1×10^{-4} M mefenamic acid (middle and lower rows). The horizontal dotted line shows the baseline current level. (b) Averaged STICs in the absence (control) and presence of 2×10^{-5} (i) and 1×10^{-4} M mefenamic acid (ii). The control current decayed exponentially and had a τ value of 86 ms which was decreased to 72 ms in 2×10^{-5} M mefenamic acid. In the presence of 5×10^{-5} M mefenamic acid the decay was biphasic ($\tau_f=37$ ms, $\tau_s=145$ ms). In 1×10^{-4} M mefenamic acid τ_f was 29 ms and τ_s was 210 ms. The dotted line shows the exponential fit of the currents.

also more effective at +50 mV compared to −50 mV (STICs reduced to 0.39 ± 0.04 of control values at +50 mV compared to 0.48 ± 0.05 at −50 mV, $n=6$ for each potential). Consequently there is a small but significant ($P<0.05$) increase in potency of flufenamic and mefenamic acid with membrane depolarization.

Effect of flufenamic and mefenamic acid on K-currents recorded at 0 mV

Recent reports have shown that in lipid bilayers of porcine coronary artery (Ottolia & Toro, 1994) niflumic acid, flufenamic acid and mefenamic acid activated large conductance Ca-activated K-currents. Thus, we assessed whether flufenamic and mefenamic acid as well as niflumic acid could evoke a K-current in smooth muscle cells of the rabbit portal vein. Using the perforated patch technique, cells superfused with K-containing PSS at potentials between −50 mV and 0 mV exhibited spontaneous transient outward K-currents (STOCs) which became larger as the holding potential was made less negative (see Benham & Bolton, 1986). The amplitude of STOCs recorded at 0 mV was stable for over 10 min (range, 100 pA to, 800 pA) and was unaffected by 1×10^{-5} M flufenamic acid ($n=4$) applied for 4 min. In comparison, 2×10^{-5} M, 5×10^{-5} M and 1×10^{-4} M flufenamic acid inhibited STOC amplitude to 0.87 ± 0.05, 0.49 ± 0.09 and 0.20 ± 0.06 of control values respectively (Figures 5a–c and 6c). Furthermore, STOC frequency was reduced by 2×10^{-5} M, 5×10^{-5} M and 1×10^{-4} M flufenamic acid to 0.81 ± 0.08, 0.57 ± 0.15 and 0.37 ± 0.06 of the control value ($n=6-7$, Figures 5a–c and 6d). Exposure of cells to 2×10^{-4} M flufenamic acid completely abolished STOCs in 18 out of 20 cells studied. Mefenamic acid and niflumic also had an inhibitory effect on STOCs (Figures 5d–f and 6). Thus, exposure of cells to 2×10^{-4} M mefenamic acid decreased STOC amplitude to 0.64 ± 0.11 of control values and the STOC frequency was reduced to 0.77 ± 0.15 of control values ($n=7$). In comparison, 2 and 5×10^{-4} M niflumic acid decreased STOC amplitude to respectively 0.41 ± 0.15 and 0.11 ± 0.09 of control values and reduced STOC frequency to 0.57 ± 0.18 and 0.15 ± 0.1 of control values ($n=6$). The concentration-effect curves of the three fenamates on STIC amplitude, STOC amplitude and frequency are shown in Figure 6. The potency sequence for inhibiting STOC activity was flufenamic > niflumic ≥ mefenamic acid.

Figure 5 shows that all the fenamates at a concentration greater than 5×10^{-5} M evoked an outward current at 0 mV. This current developed gradually with a steady increase in current 'noise' as the current approached a maximum and readily reversed upon washout of the drug. The time to peak was dependent on the concentration of fenamate e.g. 3.3 ± 0.3 min for 2×10^{-4} M flufenamic acid and 2.4 ± 0.41 min for 5×10^{-4} M flufenamic acid. The evoked current did not inactivate in the continued presence of the applied fenamate and after washout of the drug additional currents could be elicited by further addition of the fenamate to the cell. Outward currents were seen in 3 out of 6 cells superfused with 5×10^{-5} M flufenamic acid (mean = 13 ± 8 pA) and 1×10^{-4} M flufenamic acid (mean = 18 ± 5 pA). In the other 3 cells 1×10^{-4} M flufenamic acid increased current noise without a change in holding current. Higher concentrations of flufenamic acid consistently evoked an outward current with a mean increase produced by 2×10^{-4} M and 5×10^{-4} M flufenamic acid of 32 ± 5 pA (18 out of 20 cells, Figure 6b) and 191 ± 3 pA (3 out of 3 cells) respectively. Mefenamic acid and niflumic acid at concentrations greater than 1×10^{-4} M also evoked outward

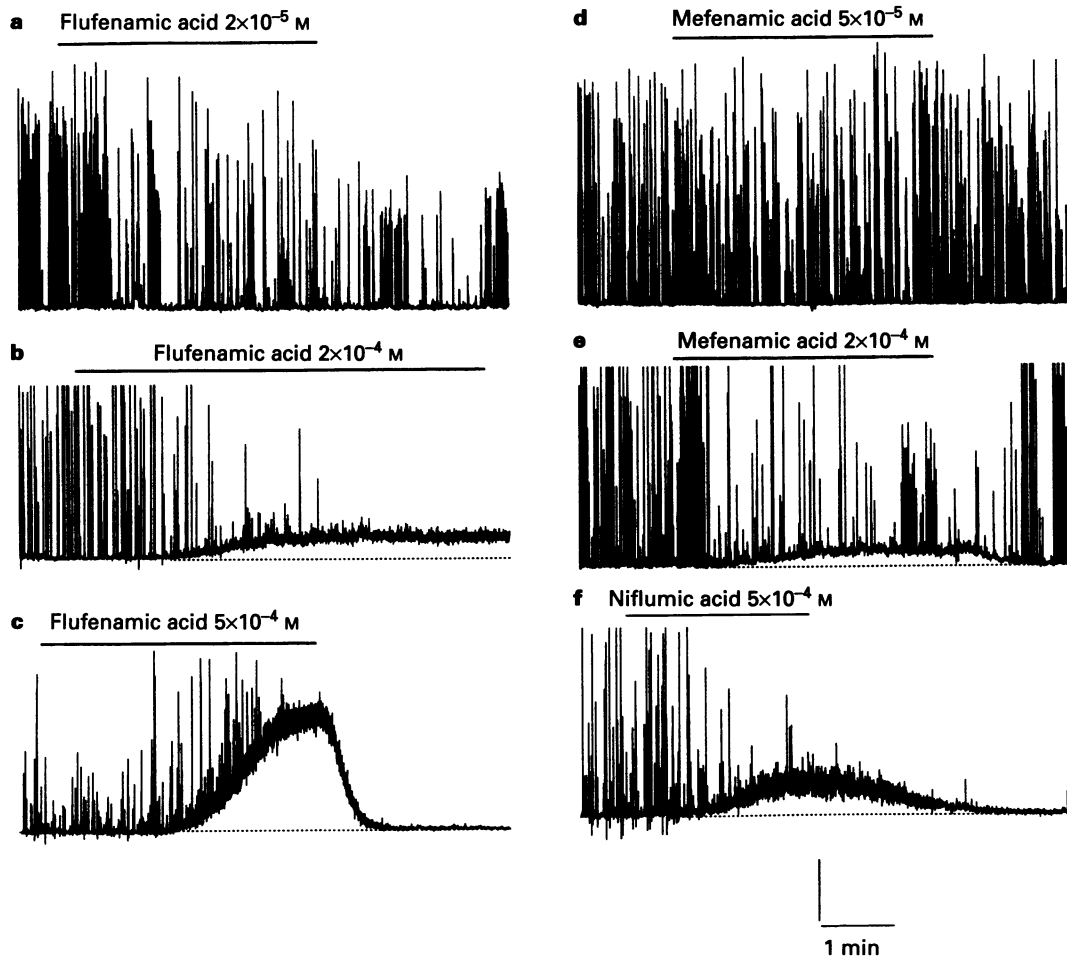

Figure 5 Effect of flufenamic acid, mefenamic acid and niflumic acid on K-currents recorded with the perforated patch technique at a holding potential of 0 mV. In all panels the upward deflections represent STOCs and the dotted line shows the resting current level. Drugs were applied for the period denoted by the horizontal bar. Vertical scale bar represents 100 pA in (a), (c), (d) and (f) and 50 pA in (b) and (e). (a–c) Show the effect of increasing concentrations of flufenamic acid (2×10^{-5} M, 2×10^{-4} M and 5×10^{-4} M) on STOCs and holding current. (d) Shows the lack of effect of 5×10^{-5} M mefenamic acid on STOCs and (e) shows the developed outward current produced by 2×10^{-4} M mefenamic acid. (f) Shows the sustained current produced by exposure of the cell to 5×10^{-4} M niflumic acid.

currents (Figures 5d–e and 6b). Niflumic acid, 2 and 5×10^{-4} M produced mean currents of 14 ± 5 and 39 ± 4 pA respectively ($n=6-8$). In comparison, 2×10^{-4} M mefenamic acid evoked a mean current of 19 ± 4 pA (8 out of 8 cells).

Characterization of the outward current evoked by cells exposed to the fenamates

Smooth muscle cells exhibit several types of K-channels which could underlie the fenamate-evoked outward current and therefore we attempted to characterize the fenamate-induced conductance. The 'noisy' current evoked by flufenamic acid at 0 mV was markedly quieter when the holding potential was changed to -20 mV and increased substantially at $+20$ mV ($n=3$ cells, data not shown). This suggested that the current activated by flufenamic acid was outwardly rectifying with channel activity increasing with potentials positive to 0 mV and indicated that the fenamate-induced current was a calcium-activated K-current ($I_{K(Ca)}$). To determine the current-voltage relationship of the fenamate-evoked conductance the potential was changed continuously from -50 mV to $+50$ mV over 1.5 s (voltage ramp) in the absence and presence of various concentrations of fenamates. As Figure 7b shows, the current in response to a ramp change in potential in the absence of drug (control trace), and the outward rectification became apparent at approximately -5 mV. At potentials positive to -20 mV STOCs were superimposed on the ramp current. The mean current recorded at $+10$ and $+30$ mV was 34 ± 3 and 65 ± 6 pA respectively ($n=17$). Figure 7 (a and b) show that exposure of a cell held at 0 mV to 2×10^{-4} M flufenamic acid induced a sustained current and altered the ramp-evoked current especially at more positive potentials (see Figure 7b and Table 1). Thus, the increase in current evoked by 2×10^{-4} M flufenamic acid was greater at $+50$ mV compared to $+10$ mV (Table 1, $n=10$). Figure 7b also shows the effect of 2 and 5×10^{-4} M niflumic acid on the ramp-evoked current. Qualitatively similar effects on the ramp-evoked current to those for flufenamic acid were observed but the absolute increase in current produced by niflumic acid was markedly less at each potential studied (Table 1). Mefenamic acid (2×10^{-4} M) also had a greater effect on the current recorded at $+50$ mV compared to $+10$ mV (Table 1). The current-voltage relationship supported the hypothesis that the fenamate-evoked current was $I_{K(Ca)}$.

To confirm that the current evoked by flufenamic acid was $I_{K(Ca)}$ experiments were performed to investigate the pharmacological properties of the evoked conductance. TEA is considered to be a relatively selective blocker of B_{KCa} channels in the concentration range $0.1-1\times10^{-3}$ M in rabbit portal vein cells (Bolton & Beech, 1992) and glibenclamide blocks ATP-sensitive K-currents evoked by the K-channel opener levcromakalim in various smooth muscle cells (Noack *et al.*, 1992; Beech *et al.*, 1993). Figure 8 shows that the current evoked by 2×10^{-4} M flufenamic acid and 5×10^{-4} M niflumic acid was not affected by 1×10^{-5} M glibenclamide (mean reduction in 4–6 cells after 4 min was 2 ± 4 and 4 ± 4%, respectively). In

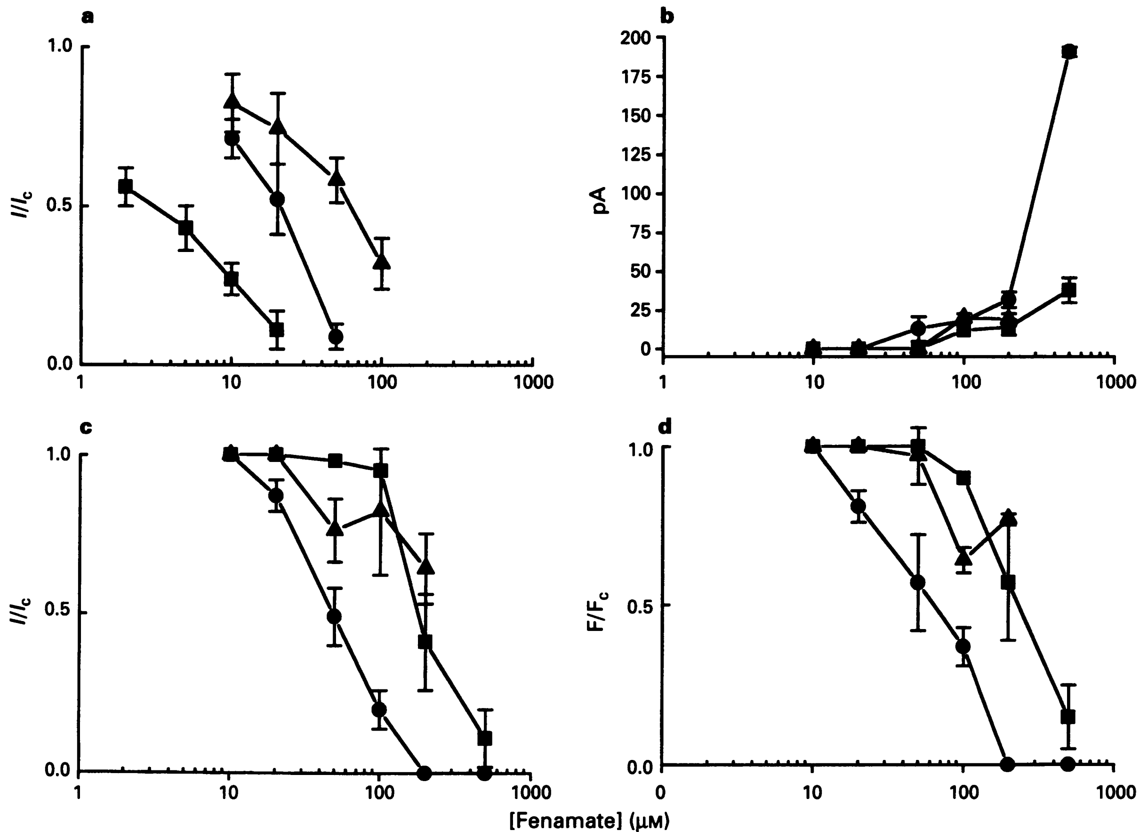

Figure 6 Comparison of the effects of flufenamic, mefenamic and niflumic acid to inhibit STOCs and to evoke a potassium current with their ability to inhibit STICs. In all panels the currents were recorded using the perforated patch technique in K-containing conditions at either −77 mV (a) or 0 mV (b, c and d). (a) Inhibitory effects of the fenamates on STICs. Data for flufenamic and mefenamic acid were from the present study whereas the niflumic acid data were taken from Hogg *et al.* (1994b) for comparison. The abscissa scale represents the current amplitude in the presence of each concentration of fenamate normalized to the individual control levels in the absence of any drug. (b) Ability of the fenamates to evoke a sustained current at 0 mV. The abscissa scale represents the current amplitude and each point represents the mean current evoked at each concentration. (c) Effect of the fenamates on STOC amplitude which is shown on the abscissa scale as normalised to the control value in the absence of the drug. (d) Effect of the fenamates on STOC frequency which is shown on the abscissa scale as normalised to the control value in the absence of the drug. In all panels the ordinate scale represents the fenamate concentration and the symbols denote (●) flufenamic, (■) niflumic and (▲) mefenamic acid respectively. Each point is the mean of between 5 and 22 cells ± s.e.mean.

contrast the currents evoked by 2×10^{-4} M flufenamic acid and 5×10^{-4} M niflumic acid were markedly and rapidly reduced by 1×10^{-3} M TEA (Figure 8). The mean reduction in the fenamate-induced current at 0 mV by 1×10^{-3} M TEA was 93±2% and 91±4% for 2×10^{-4} M flufenamic acid and 5×10^{-4} M niflumic acid respectively (n=4–6). Thus, the pharmacological studies indicate that the current evoked by the fenamates was $I_{K(Ca)}$.

The effects of the fenamates on STOCs suggested that these agents may activate $I_{K(Ca)}$ as a consequence of the release of calcium from intracellular stores. Therefore experiments were carried out to investigate the effects of fenamates on cells in which the intracellular Ca-stores had been depleted. Cells were treated with 1×10^{-5} M cyclopiazonic acid (CPA), which has been shown to be an inhibitor of the sarcoplasmic reticulum Ca-ATPase in smooth muscle (Uyama *et al.*, 1993). Moreover, 1×10^{-2} M caffeine was also added to the bathing solution to deplete the Ca-stores. In these conditions STOCs were abolished which suggests the stores had been depleted. In the continued presence of CPA and caffeine 5×10^{-4} M flufenamic acid and 5×10^{-4} M niflumic acid produced mean currents at 0 mV of 140±31 and 21±3 pA respectively (n=4 for each drug). These values are similar to the control values obtained earlier in the absence of CPA and caffeine (see Figure 6b). Furthermore, the current-voltage relationship of the fenamate-evoked currents in the presence of CPA and caffeine were similar to the control current-voltage curves. Consequently, $I_{K(Ca)}$ activated by the fenamates is not mediated by Ca released from the internal Ca-store.

Finally, experiments were performed with conventional whole-cell recording in which the pipette solution contained different concentrations of BAPTA to vary the degree of Ca-buffering in the cell. With 1×10^{-2} M BAPTA in the pipette, no STOCs were observed (see Figure 9a and b) and caffeine did not evoke $I_{K(Ca)}$ suggesting that the internal Ca was buffered at a low level. Under these conditions 2×10^{-4} M flufenamic acid and 5×10^{-4} M niflumic acid evoked large K-currents (Figure 9a) which were 131±16 pA (n=8) and 105±30 pA (n=3), respectively. Moreover, removal of Ca from the bathing solution had no effect on the flufenamic acid-induced current recorded with a pipette solution containing 1×10^{-2} M BAPTA (mean current=147±26 pA, n=3, Figure 9b). With 1×10^{-4} M BAPTA in the pipette solution the degree of Ca-buffering was less than with 1×10^{-2} M BAPTA as STOCs were always observed with this solution (Figure 9c). With 1×10^{-4} M BAPTA in the pipette the mean amplitude of the current evoked by 2×10^{-4} M flufenamic acid was 88±13 pA (n=4).

Discussion

The results from this study show that niflumic, flufenamic and mefenamic acid block $I_{Cl(Ca)}$ and activate $I_{K(Ca)}$ in freshly dispersed rabbit portal vein smooth muscle cells. In addition the fenamates reduced both the frequency and amplitude of STOCs. The concentration to inhibit by 50% (IC_{50}) STOC amplitude and frequency was about $5-7\times10^{-5}$ M and

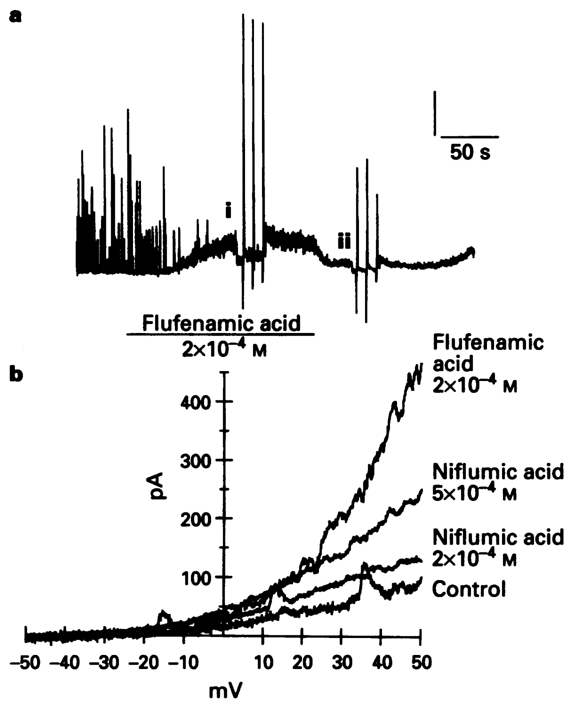

Figure 7 Effect of flufenamic acid and niflumic acid on the current evoked by a ramp change in potential from −50 mV to +50 mV over 1.5 s. (a) Shows a representative current evoked by 2×10^{-5} M flufenamic acid at a potential of 0 mV. Ramps were performed during (i) and after (ii) exposure to the drug. When the ramps were applied the gain was halved and the vertical calibration bar represents 100 pA under these conditions and 50 pA otherwise. (b) The current evoked by the ramp change in potential from −50 mV to +50 mV under control conditions and in the presence of niflumic acid 2 and 5×10^{-4} M and flufenamic acid 2×10^{-4}. The ordinate scale shows the membrane potential (mV) and the abscissa scale shows the current in pA.

Table 1 Effect of flufenamic acid, mefenamic acid and niflumic acid on ramp-evoked currents at various potentials

	Membrane potential (mV)			
	+10	+30	+50	n
Flufenamic acid 2×10^{-4} M	21±7 pA	131±30 pA	344±35 pA	10
Mefenamic acid 2×10^{-4} M	20±8 pA	43±15 pA	71±5 pA	5
Niflumic acid 2×10^{-4} M	16±9 pA	24±4 pA	58±18 pA	5
Niflumic acid 5×10^{-4} M	19±9 pA	48±20 pA	255±38 pA	5

Each column denotes the mean increase in current produced by each fenamate measured at three potentials (+10, +30 and +50 mV). Currents were evoked by changes in the membrane potential using a ramp from -50 to +50 mV over 1.5 s. Currents in the absence of a drug were then subtracted at each potential from the current recorded in the presence of the fenamate.

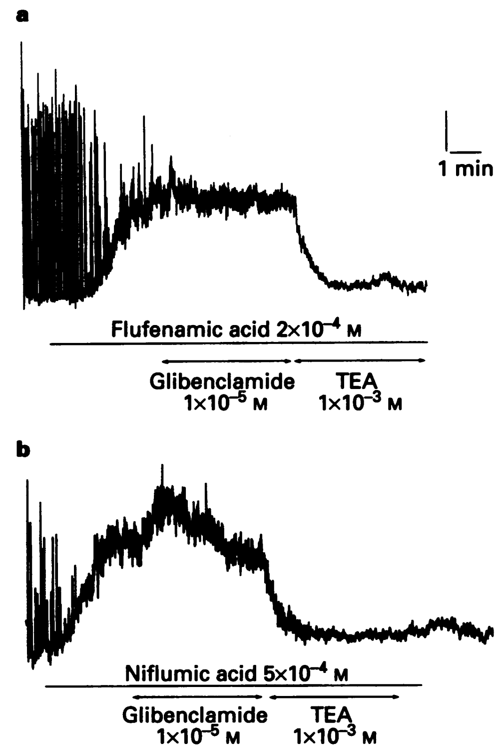

Figure 8 Effect of tetraethylammonium (TEA) and glibenclamide on sustained currents evoked by flufenamic and niflumic acid. Both panels show a representative trace for cells held at 0 mV which exhibit STOCs. Flufenamic acid 2×10^{-4} M (a) and niflumic acid 5×10^{-4} M (b) were applied for the period denoted by the horizontal bar and both agents abolished STOCs and elicited a sustained outward current. Exposure of the cells to 1×10^{-5} M glibenclamide in the continued presence of flufenamic acid or niflumic acid did not affect the evoked current. TEA (1×10^{-3} M) in the continued presence of either flufenamic acid or niflumic acid produced a rapid and complete inhibition of the current. Vertical calibration bar represents 25 pA in the upper panel and 15 pA in the lower panel.

2×10^{-4} M for flufenamic and niflumic acid respectively. The IC_{50} for mefenamic acid was not estimated because of solubility limitations but was in excess of 2×10^{-4} M. Consequently the potency ratio against STOC frequency and amplitude was flufenamic > niflumic ≥ mefenamic acid. The present work does not reveal the mechanism for inhibiting STOC activity but the most obvious explanation is that the fenamates either reduce the spontaneous release of Ca^{2+} from the intracellular calcium-store responsible for triggering STOCs or block the calcium-activated potassium-channels that underlie STOCs (Benham & Bolton, 1986). Since these agents appear to activate the calcium-activated potassium-channels it seems unlikely that simple channel block of K-channels is involved. However, it is possible that the fenamates reduce STOC activity by a more elaborate interaction with the potassium channel (see later).

Inhibition of $I_{Cl(Ca)}$

The IC_{50} values for the inhibition of STICs were approximately 2×10^{-6} M, 2×10^{-5} M and 7×10^{-5} M for niflumic, flufenamic and mefenamic acid respectively (Hogg *et al.*, 1994b and the present study). In the concentration range that flufenamic and mefenamic acid decreased STIC amplitude these compounds also reduced STOC amplitude and frequency. If the action on STOCs is due to an inhibitory effect on the intracellular Ca-store, which is also the source of Ca^{2+} ions responsible for triggering STICs (Wang *et al.*, 1992), then part of the reduction in STIC amplitude by flufenamic and mefenamic acid might be mediated by an effect on the Ca-store. Nevertheless low concentrations of these two compounds decreased STIC amplitude without an effect on STOCs and all concentrations of these agents reduced STIC more than STOC amplitude. Thus, flufenamic and mefenamic acid probably inhibit $I_{Cl(Ca)}$ directly. Niflumic acid had no effect on STOCs in the concentration range required to inhibit STICs and therefore the potency for blocking $I_{Cl(Ca)}$ is niflumic > flufenamic > mefenamic acid which is different from the potency sequence for inhibiting STOCs or activating $I_{K(Ca)}$ (see later). The characteristics of the flufenamic acid- and mefenamic acid-induced block on the

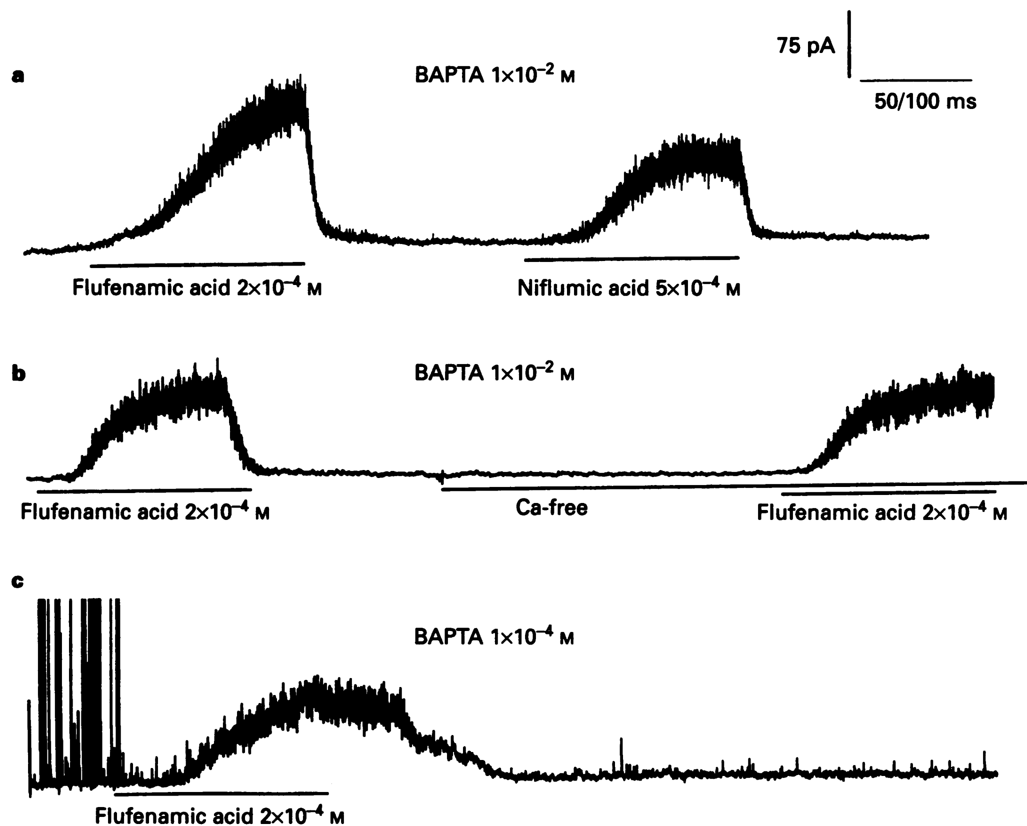

Figure 9 Flufenamic acid- and niflumic acid-evoked currents recorded with conventional whole-cell techniques using pipette solutions in which the Ca-buffering is varied. All three panels show representative traces of cells held at 0 mV and recorded with pipette solutions containing either BAPTA 1×10^{-2} M ('strong' Ca-buffering; (a) and (b)) or BAPTA 1×10^{-4} M ('weak' Ca-buffering; (c)). (a) Currents were evoked in the same cell by flufenamic acid 2×10^{-4} M and niflumic acid 5×10^{-4} applied during the period denoted by the horizontal bar. (b) Shows the effect of superfusing the cells with nominally Ca-free extracellular solution on currents elicited by flufenamic acid 2×10^{-4} recorded with a pipette solution containing BAPTA 1×10^{-2} M. The current shown on the left of the trace was induced by flufenamic acid in cells bathed in extracellular solution containing 1.5 mM Ca^{2+}. (c) Currents in response to flufenamic acid 2×10^{-4} M recorded with a pipette solution in which the Ca was 'weakly' buffered (BAPTA 1×10^{-4} M). The upward deflections at the beginning of the trace are STOCs which are rapidly abolished by flufenamic acid.

STIC time course were similar qualitatively to that of niflumic acid. The potency of flufenamic and mefenamic acid was increased similarly by membrane depolarization (see Hogg *et al.*, 1994b). Moreover, in the presence of both flufenamic and mefenamic acid the normal exponential STIC decay was converted into a bi-exponential time course. With niflumic acid the data were consistent with a mechanism of the drug blocking the open chloride channel (Hogg *et al.*, 1994b). The analysis used by Hogg *et al.* (1994b) could not be applied in the present study because the fast exponential of STIC decay (τ_f) did not appear to be dependent on the blocker concentration. However, it is possible that this occurred because τ_f had reached a lower limit. We have not recorded STICs with a time constant faster than 15–30 ms and therefore processes other than simple channel closure may determine the decay when STICs become so rapid. However, τ_s was increased in a concentration-dependent manner and overall the data suggest that flufenamic and mefenamic acid are also channel blocking agents. In conclusion we feel that all three fenamates bind to a common site within the conducting pore of the calcium-activated chloride channel after it has opened to inhibit $I_{Cl(Ca)}$ and dissociate quickly relative to the mean channel open time.

Activation of $I_{K(Ca)}$

The results of the present study show that the three fenamates activate a potassium current with perforated patch and conventional whole-cell recording. In the present experiments the holding potential of 0 mV would be expected to inactivate the delayed rectifier as well as the transient outward current (Bolton & Beech, 1992; Noack *et al.*, 1992) but would not affect BK_{Ca} channels. The current-voltage characteristics of the control potassium current in the absence of fenamates indicated that a calcium-activated potassium current was the predominant current in our experimental conditions (as demonstrated by Beech & Bolton, 1989). The current activated at 0 mV by the fenamates was 'noisy', non-inactivating and the membrane currents at positive potentials were progressively increased. These results suggest that the fenamates potentiate $I_{K(Ca)}$ and this conclusion was supported by the pharmacological data which showed that a relatively low concentration of TEA (1×10^{-3} M) decreased the current by more than 90% at 0 mV. Moreover, since 1×10^{-5} M glibenclamide had a negligible effect on the fenamate-evoked current it is unlikely that the fenamates activate the same potassium current described for the typical potassium channel opener, levcromakalim in both rat and rabbit portal vein smooth muscle cells (Noack *et al.*, 1992; Beech *et al.*, 1993).

Our data in freshly dispersed rabbit portal vein cells support the observation of Ottolia & Toro (1994), who showed that the fenamates activate $I_{K(Ca)}$ in lipid bilayers. However, the potency-ratio presented by these authors (flufenamic$\simeq$niflumic>mefenamic acid) differed slightly from the present study which may reflect intrinsic differences between potassium currents in bilayers and freshly dispersed cells. The effects of the fenamates in the present study were also similar to those for the putative BK_{Ca} channel opener NS 1619 in rat portal vein smooth muscle cells (Edwards *et al.*, 1994) which inhibited STOCs and evoked a noisy, slowly developing current at −10 mV. NS 1619 and the fenamates along with the soyasa-

ponins, which potentiate maxi-potassium channels in bovine tracheal smooth muscle (McManus *et al.*, 1993), represent a novel range of agents which activate large conductance potassium channels.

The activation of $I_{K(Ca)}$ by the fenamates does not seem to result from the release of Ca^{2+} from intracellular stores. Currents of similar amplitude to control values were obtained in cells treated with CPA and caffeine. As no STOCs were recorded under these conditions it seems that the Ca-stores had been greatly depleted. Moreover, with whole-cell recording, flufenamic and niflumic acid activated $I_{K(Ca)}$ in cells which had been dialyzed with 1×10^{-2} M BAPTA. In these conditions no STOCs were observed and caffeine did not evoke $I_{K(Ca)}$ which is evidence that the internal calcium levels were buffered to very low levels. The amplitude of fenamate-evoked $I_{K(Ca)}$ with 1×10^{-2} M BAPTA was significantly larger than the currents recorded with 1×10^{-4} M BAPTA in the pipette solution and was markedly enhanced compared to perforated patch recording. In the latter two conditions the buffering of calcium would be relatively weak and the present data support the work of Ottolia & Toro (1994), who proposed that the fenamates interact with the channel protein directly. The present data also suggest that the fenamates do not increase the sensitivity of the channel protein to Ca^{2+} as proposed by Ottolia & Toro (1994) and for the soyasaponins (McManus *et al.*, 1993). Thus, the evoked current amplitude was not reduced when the cells were dialyzed by a pipette solution which strongly buffered calcium levels. Furthermore, bathing the cells in nominally calcium-free solution did not affect the amplitude of the fenamate-elicited current in the above conditions.

It is interesting that inhibition of STOC activity and stimulation of $I_{K(Ca)}$ occurs in a similar concentration range for each of the drugs although the effect on STOCs does occur at slightly lower concentrations. These observations suggest that a common mechanism might be responsible for reducing spontaneous calcium-activated potassium currents and activating $I_{K(Ca)}$. It is possible that the fenamates might bind to a single site which would prevent the binding of Ca^{2+} to the channel leading to the inhibition of STOCs. This interaction would lead to a reduction of STOC amplitude and could reduce STOC frequency although other mechanisms may possibly be involved in the latter effect. At higher concentrations of drug the interaction at the calcium-binding site leads to channel activation and development of $I_{K(Ca)}$. In this manner the fenamates may act as partial agonists at the calcium binding site.

In conclusion, the evidence indicates that the fenamates have at least two distinct sites of action in the rabbit portal vein. First, these compounds bind to a site within the conducting pore of calcium-activated chloride channels to inhibit Cl^- permeation. Secondly, the fenamates seem to interact directly with calcium-activated potassium channels to activate $I_{K(Ca)}$. It is possible that the latter action might modify the binding of Ca^{2+} to the channel protein. Therefore the fenamates act as antagonists of calcium-activated chloride-channels and openers of calcium-activated potassium-channels.

This work was supported by the Wellcome Trust.

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(Received August 8, 1995
Revised August 11, 1995
Accepted August 16, 1995)