Action of niflumic acid on evoked and spontaneous calcium-activated chloride and potassium currents in smooth muscle cells from rabbit portal vein

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1 The action of niflumic acid was studied on spontaneous and evoked calcium-activated chloride ($I_{CaCl}$) and potassium ($I_{KCl}$) currents in rabbit isolated portal vein cells.

2 With the nystatin perforated patch technique in potassium-containing solutions at a holding potential of $-77$ mV (the potassium equilibrium potential), niflumic acid produced a concentration-dependent inhibition of spontaneous transient inward current (STIC, calcium-activated chloride current) amplitude. The concentration to reduce the STIC amplitude by 50% ($IC_{50}$) was $3.6 \times 10^{-8}$ M.

3 At $-77$ mV holding potential, niflumic acid converted the STIC decay from a single exponential to 2 exponential components. In niflumic acid the fast component of decay was faster, and the slow component was slower than the control decay time constant. Increasing the concentration of niflumic acid enhanced the decay rate of the fast component and reduced the decay rate of the slow component.

4 The effect of niflumic acid on STIC amplitude was voltage-dependent and at $-50$ and $+50$ mV the $IC_{50}$ values were $2.3 \times 10^{-8}$ M and $1.1 \times 10^{-8}$ M respectively (cf. $3.6 \times 10^{-8}$ M at $-77$ mV).

5 In K-free solutions at potentials of $-50$ mV and $+50$ mV, niflumic acid did not induce a dual exponential STIC decay but just increased the decay time constant at both potentials in a concentration-dependent manner.

6 Niflumic acid, in concentrations up to $5 \times 10^{-5}$ M, had no effect on spontaneous calcium-activated potassium currents.

7 Niflumic acid inhibited noradrenaline- and caffeine-evoked $I_{CaCl}$ with an $IC_{50}$ of $6.6 \times 10^{-6}$ M, i.e. was less potent against evoked currents compared to spontaneous currents. In contrast niflumic acid ($2 \times 10^{-5}$ M–$5 \times 10^{-5}$ M) increased noradrenaline- and caffeine-induced $I_{KCl}$.

8 The results are discussed with respect to the mechanism of block of $I_{CaCl}$ by niflumic acid and its suitability as a pharmacological tool for assessing the role of $I_{CaCl}$ in physiological mechanisms.

Keywords: Niflumic acid; vascular smooth muscle; calcium-activated chloride current; calcium-activated potassium current

Introduction

Experiments with patch pipette techniques have revealed that noradrenaline acts on $\alpha_1$-adrenoceptors to stimulate simultaneously a calcium-activated chloride current ($I_{CaCl}$), calcium-activated potassium current ($I_{KCl}$) and a calcium-permeable cation current ($I_{Ca}$) in the rabbit portal vein (Byrne & Large, 1988; Wang & Large, 1991) and in the rabbit ear artery (Amédée et al., 1990). Since it has been shown that an anion and a cation conductance increase is responsible for the noradrenaline-induced depolarization recorded with micro-electrodes in isolated cells of the rabbit portal vein (Amédée & Large, 1989), it is possible that $I_{CaCl}$ and $I_{Ca}$ may have important roles in producing depolarization and contraction in vascular smooth muscle. Also it is relevant that $I_{CaCl}$ has now been observed in several types of smooth muscle and can be activated by various pharmacological agents (see Introduction of Hogg et al., 1994). Therefore it would be interesting to assess the contribution of $I_{CaCl}$ and $I_{Ca}$ to agonist-induced depolarization in smooth muscle. In order to do this it would be necessary to have selective blocking drugs to dissect out the roles of these conductance mechanisms.

Recently we have embarked on a series of experiments to investigate the characteristics of established chloride channel antagonists in blocking $I_{CaCl}$ in vascular smooth muscle cells. It has been demonstrated that 4-acetamido-4'-isothiocyanato-2,2'-dithiobimane-2,2'-dithiobimane (SITS), 4,4'-disothiocyanato-2,2'-dithiobimane (DIDS) and anthracene-9-carboxylic acid (A-9-C) inhibit evoked $I_{CaCl}$ in rat portal vein (Baron et al., 1991). Also it was shown in rabbit portal vein that these compounds were more potent against spontaneous transient inward currents (STICs, chloride currents activated by spontaneous release of calcium from caffeine-sensitive intracellular stores) than against $I_{CaCl}$ elicited by noradrenaline and caffeine (Hogg et al., 1994). It was found that all 3 compounds had low potency as the concentration to inhibit $I_{CaCl}$ by 50% was greater than $10^{-4}$ M i.e. the potency against $I_{CaCl}$ is less than the action of DIDS and SITS against their well-established effects on Cl⁻–HCO₃⁻ exchange in smooth muscle (e.g. see Aickin & Braden, 1983). Consequently it seems unlikely that these channel blockers would be of use as pharmacological tools for assessing the physiological role of $I_{CaCl}$. A more promising candidate might be niflumic acid, a non-steroidal anti-inflammatory agent, which at a concentration of 10 $\mu$M produced marked attenuation of $I_{CaCl}$ in rat portal vein (Pacaud et al., 1989).

The relatively high potency of niflumic acid has been confirmed in canine and guinea-pig tracheal cells (Janssen & Sims, 1992) and in rabbit oesophageal smooth muscle (Akbarali & Giles, 1993).

In the present work we have undertaken a quantitative study of the action of niflumic acid against spontaneous and evoked $I_{CaCl}$ in the rabbit portal vein. Evidence will be presented to substantiate the relatively high potency of niflumic acid and experiments will be described which indicate that niflumic acid inhibits $I_{CaCl}$ at least partly, by an open channel blocking mechanism.

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Methods

Experiments were carried out on freshly dispersed smooth muscle cells from the rabbit portal vein. Rabbis (2.5-2.5 kg) of either sex were killed by exsanguination and rapidly dissected. The portal vein was removed and used en bloc. It was defatted and cut into thin strips. The strips were then kept in a physiological salt solution with 0.75 mM Ca2+ at 4°C and were used on the same day. Whole-cell currents were measured with the perforated patch method with a patch clamp amplifier (List EPC-7; List Electronic, Darmstadt, Germany) at room temperature. In order to obtain a perforated patch nystatin (75-200 µg ml-1) was contained in the patch pipette solution. The external salt solution contained (mM): NaCl 126, KCl 6, MgCl2 1.2, CaCl2 1.5, HEPES 10 and glucose 11 and the pH was adjusted to 7.2 with NaOH. In potassium-free conditions, 6 mM KCl was omitted and in some experiments 5 mM tetraethylammonium chloride (TEA-Cl) was added to the patch-pipette solution. The pipette solution contained (mM): KCl 126, MgCl2 1.2, HEPES 10, glucose 11 and EGTA 0.2. In potassium-free conditions 126 mM KCl was replaced by an equimolar amount of NaCl. In some experiments the effect of niflumic acid on voltage-gated divalent cation currents was studied. For these studies 10 mM BaCl2 was added to the bathing solution instead of calcium so that Ba2+ was the charge carrier. Also in these latter studies the pipette solution contained 126 mM CsCl and 10 mM EGTA.

In experiments where noradrenaline was used, 10-8 M propranolol was included in the bathing solution to remove any β-adrenoceptor-mediated response. The recordings were made in a static bathing solution and the external solution was changed with a push-pull arrangement of the recording chamber.

Some records were illustrated by playback from videotape onto a Gould brush recorder. Analysis of the time course of spontaneous transient currents was carried out on a personal computer. Signals were filtered at 400 Hz prior to digitisation and currents were sampled at 800 Hz using a CED 1401 laboratory interface and captured on the hard disk of the computer. Capture and averaging of signals were performed with the SIGAVG signal-averaging programme. The pipette solution was done with a voltage clamp analysis programme (both Cambridge Electronic Design, Science Park, Cambridge).

Exponential fits were obtained using a least squares fitting routine and the fitting procedure was weighted towards large current values. From each cell 10-20 spontaneous chloride or potassium currents were averaged to obtain amplitude and time course data. An exponential curve fit to the current decay (in Figure 1b) was drawn using an IC50 value (concentration of niflumic acid to inhibit STIC amplitude to 50% of the control value) obtained by linear regression of the experimental data points. In the text, n values refer to the number of cells used to obtain the mean determinations. The values given in the text are means ± s.e.m. and statistical significance was estimated with either Student's t test or paired t test. Chemicals used were bovine serum albumin (fatty acid free), caffeine, dithiothreitol, noradrenaline bitartrate, nystatin, papain (type IV), TEA-Cl (all Sigma, Poole, Dorset); niflumic acid (Aldrich, Gillingham, Dorset); collagenase (CLS2 247 U mg-1, Worthington, Reading, Berkshire).

Results

Effect of niflumic acid on STICs

In the first series of experiments we investigated the action of niflumic acid on STICs in potassium-containing solutions. Figure 1a shows a record from a cell which was held under voltage clamp at a command potential of −77 mV (the potassium equilibrium potential, Ek) and niflumic acid was added to the bathing solution in a cumulative manner. It can be seen quite clearly that there was progressive diminution of STIC amplitude and in the presence of 10-4 M niflumic acid the STICs were blocked. The onset of inhibition was rapid and was apparent within about 10 s of adding niflumic acid to the external solution. Also the antagonism was readily reversible and usually complete within 1-3 min after removing niflumic acid from the cells (Figure 1a). The concentration-effect of niflumic acid on STIC amplitude is illustrated graphically in Figure 1b. The continuous curve is drawn according to the Langmuir isotherm and the estimated concentration to inhibit STIC amplitude to 50% of the control value (IC50) at −77 mV with potassium containing solutions was 3.6 ± 10-4 M. The potency of niflumic acid against STIC amplitude at any given membrane potential did not differ in potassium-free conditions which were used in some experiments to investigate the voltage-dependence of the action of niflumic acid.

It was apparent in the majority of cells at −77 mV that the reduction in STIC amplitude by niflumic acid was accompanied by a marked alteration of the STIC time course. Figure 2a illustrates averaged STICs in the absence and presence of various concentrations of niflumic acid taken from the same cell as shown in Figure 1. In control conditions the STIC decay time course can be described by a single exponential with a time constant (τc) of 77 ms (control curve in Figure 2b). In the presence of niflumic acid the STIC decay time course became more complex and appeared to consist of two distinct phases (Figure 2a). The semi-logarithmic plots show that the initial decay was faster than the control while the slow component was slower than the control decay (Figure 2b). In Figure 2b the straight lines with niflumic acid represent the slow exponential component from which the slow time constant (τs) was calculated. The fast time constant (τf) was obtained by subtracting the slow component from the total current. In the presence of 5 × 10-4 M niflumic acid, τf was 28 ms and τs was 196 ms and in 5 × 10-5 M niflumic acid τf was 18 ms and τs was 711 ms. Thus an increase in the concentration of niflumic acid accelerated the fast component of the STIC decay but reduced the rate of decay of the slow component but both components remained exponential in niflumic acid. It should be emphasised that the fast phase is not a spontaneous transient outward current (calcium-activated potassium current, STOC) which theoretically might be observed if inadequate cell dialysis with the pipette solution had occurred. First, because of their voltage-dependence STOCs are very rarely observed at potentials negative to −50 mV. Secondly and most convincingly, the rapid component of the STIC decay was very insensitive to niflumic acid and therefore STICs insensitive to niflumic acid (see Figure 6). Evidence has been put forward to suggest that the STIC decay represents closure of the calcium-activated chloride channels (Hogg et al., 1993) and the alteration of the STIC decay by niflumic acid could therefore be explained by blockade of open ion channels. The usual scheme (see Colquhoun & Sheridan, 1981) to describe open channel block is:

\[ \beta^o \left( \frac{1}{a} \right) \left( \frac{1}{a_k} \right) \left( \frac{1}{k_b} \right) \]

(1) closed channel → open channel → blocked channel

Assuming the normal opening rate (β) is faster than the closure rate (α) of calcium-activated chloride channels then STIC decay in control conditions is determined by α = 1/τc (see Hogg et al., 1993). In the presence of an open channel blocking agent (with association and dissociation rate constants of respectively k_b and k_a) it is expected that the STIC decay should consist of two components as was seen experimentally. Moreover it is predicted with certain assumptions (see Colquhoun & Sheridan, 1981) that

\[ \frac{1}{\tau_c} + \frac{1}{\tau_s} - \frac{1}{\tau_{c0}} = k_a C_b + k_b \]

where C_b is the antagonist concentration. If open channel block was responsible for the reduction in STIC amplitude
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Figure 1  The effect of niflumic acid on spontaneous transient inward current (STIC) amplitude. (a) Shows experimental records in a single cell where niflumic acid was added cumulatively. Inward currents are represented as downward deflections and the records in (a) are continuous. Note the rapid reversal on washout. Potassium-containing solutions and the holding potential was −77 mV. (b) Illustrates the concentration-effect relationship of niflumic acid on STIC amplitude. The amplitude is normalised to the individual control values as 1 and the mean control amplitudes were between 43 and 125 pA. Each point is the mean from 6 cells.

Figure 2  The effect of niflumic acid on spontaneous transient inward current (STIC) time course at −77 mV. (a) Shows averaged STICs in increasing concentrations of niflumic acid. Note the long tail in the presence of 5 × 10⁻⁵ M niflumic acid; (b) illustrates the decay of STICs plotted on a semi-logarithmic scale. The amplitude of the currents are plotted on a logarithmic scale and the straight lines were drawn by eye. In niflumic acid the lines represent the slow exponential components which were used to calculate the fast components by subtraction from the total current. (●), Control; (▲) 5 × 10⁻⁴ M; (▼) 5 × 10⁻⁵ M niflumic acid.
then it is expected that a plot of \((1/\tau_r + 1/\tau_s - 1/\tau_{on})\) against niflumic acid concentration would be linear. The results from the cell in Figure 2 are plotted according to equation (2) in Figure 3. It can be seen that a linear relationship fits the data points well and the intercept \(k_{-a}\) is 25 s\(^{-1}\) and the slope \(k_{+b}\) was \(3.8 \times 10^5\) M\(^{-1}\) s\(^{-1}\). In 5 out of 6 cells in potassium-containing solutions at \(-77\) mV, niflumic acid produced similar results to those illustrated in Figures 2 and 3 and the mean \(k_{+b}\) was \(5.8 \pm 1.1 \times 10^5\) M\(^{-1}\) s\(^{-1}\) and \(k_{-a}\) was \(26 \pm 5.1\) s\(^{-1}\). In the sixth cell the fast component was not present and niflumic acid appeared only to increase the \(\tau\) value (i.e. corresponding to \(\tau_s\)). It seems unlikely that the fast component was not seen in this cell because of the voltage-dependent action of niflumic acid and the fast component was not observed at more depolarized potentials (e.g. see Figure 5 and see discussion). Nevertheless, overall the data are consistent with open channel block by niflumic acid.

**Voltage-dependent effect of niflumic acid**

Previously we had demonstrated that A-9-C reduced STICs in a voltage-dependent manner whereas the inhibitory action of DIDS and SITS was not influenced by membrane potential (Hogg et al., 1994). We have carried out similar experiments with niflumic acid in potassium-free bathing and pipette solutions to eliminate STOCs which become prominent at potentials positive to \(-50\) mV. Figure 4a shows the effect of \(10^{-6}\) M niflumic acid at \(-50\) mV and \(+50\) mV in the same cell. At \(-50\) mV this concentration of the blocker reduced the STIC amplitude by about 10% whereas at \(+50\) mV the attenuation was more marked (about 30%). The concentration-effect curves for several cells are shown in Figure 4b at \(-50\) and \(+50\) mV and the curve at \(-77\) mV from Figure 1b is also added for comparison. The calculated IC\(50\) values at \(-50\) and \(+50\) mV were respectively \(2.3 \times 10^{-4}\) M and \(1.1 \times 10^{-4}\) M at \(-77\) mV. Therefore it is concluded that the potency of niflumic acid was increased by depolarization by about two fold between \(-50\) and \(+50\) mV.

We also investigated the effect of niflumic acid on the STIC \(\tau\) at various membrane potentials and these experiments were also carried out in potassium-free conditions to remove any interference from STOCs. Figure 5a shows averaged STICs in the absence (control) and in the presence of \(5 \times 10^{-4}\) M niflumic acid at holding potentials of \(-50\) mV and \(+50\) mV. It was apparent that the fast component of decay was no longer observed in the presence of niflumic acid at these potentials (to be discussed later) and that only the slow component was present (cf. Figure 2a). At \(-50\) and \(+50\) mV, niflumic acid greatly prolonged the STIC decay in addition to reducing the STIC amplitude (i.e. qualitatively similar to the effect of niflumic acid on the slow component of decay at \(-77\) mV, Figure 2). The decays of the averaged currents in Figure 5a are plotted on a semi-logarithmic scale in Figure 5b. In both the absence and presence of niflumic acid.
acid the STICs decayed exponentially and the control time constants at \(-50\) and \(+50\) mV were respectively 96 ms and 213 ms. In the presence of \(5 \times 10^{-6} \text{M}\) niflumic acid the \(\tau\) values were 263 ms (\(-50\) mV) and 485 ms (\(+50\) mV). The effect of two concentrations of niflumic acid at \(-50\) mV and \(+50\) mV are shown in Table 1 and there are several conclusions. First, the increase in the STIC \(\tau\) value was concentration-dependent as \(5 \times 10^{-6} \text{M}\) niflumic acid produced a greater effect on \(\tau\) than \(10^{-5} \text{M}\) niflumic acid. Secondly, although the absolute \(\tau\) values are larger at \(+50\) mV than \(-50\) mV (see Hogg et al., 1993), the ratios of the \(\tau\) values (drug:control) are no greater at \(+50\) mV than at \(-50\). Therefore the increase in \(\tau\) does not appear to be voltage-dependent.

**Effect of niflumic acid on STOCs**

In order to ensure that the effect of niflumic acid was not mediated by an action on the intracellular calcium store which is the primary source of calcium for triggering STICs we investigated the action of niflumic acid on spontaneous transient outward currents (STOCs). These are spontaneous calcium-activated potassium currents which are triggered by the same calcium store responsible for STICs (Wang et al., 1992). Figure 6a shows a continuous record of STOCs in a cell held at 0 mV (i.e. close to \(E_c\)) before and after the addition of \(5 \times 10^{-5} \text{M}\) niflumic acid to the bathing solution. From this trace and averaged STOCs illustrated in Figure 6b it is apparent that niflumic acid did not affect STOCs. With \(5 \times 10^{-6} \text{M}\), \(10^{-5} \text{M}\) and \(5 \times 10^{-5} \text{M}\), \(n = 3\) at each concentration) niflumic acid did not alter the amplitude, time to peak, half-decay time and frequency of STOCs. It can be concluded that niflumic acid does not modify the intracellular calcium store or the calcium-activated potassium channels responsible for STOCs.

**Effect of niflumic acid on evoked calcium-activated chloride and potassium currents**

It has been demonstrated previously that DIDS, SITS and A-9-C were less potent in inhibiting evoked chloride currents compared to STICs (Hogg et al., 1994). Consequently we investigated the effect of niflumic acid on noradrenaline- and caffeine-evoked \(I_{\text{CACHL}}\) in potassium-free conditions at a holding potential of \(-50\) mV. Figure 7 shows the effect of two concentrations of niflumic acid on noradrenaline-induced \(I_{\text{CACHL}}\) (Figure 7a) and caffeine-evoked \(I_{\text{CACHL}}\) (Figure 7b) and it can be seen that niflumic acid produces a concentration-

**Table 1** Effect of niflumic acid on the spontaneous transient inward current (STIC) decay time constant

| Niflumic acid concentration | Control \(\tau\) (ms) | In niflumic acid \(\tau\) (ms) | \(c/a\) | \(d/b\) |
|-----------------------------|-----------------------|-----------------------------|--------|--------|
| \(10^{-6} \text{M}\), \(n = 8\) | \(-50\) mV (a) 99 ± 12 | \(-50\) mV (c) 133 ± 39 | 1.34  | 1.51   |
|                             | \(+50\) mV (b) 159 ± 19 | \(+50\) mV (d) 295 ± 39 |       |        |
| \(5 \times 10^{-6} \text{M}\), \(n = 5\) | \(-50\) mV (a) 115 ± 12 | \(-50\) mV (c) 303 ± 38 | 2.63  | 2.32   |
dependent inhibition of $I_{\text{Ca}}$ which is rapidly reversible. It is interesting that in Figure 7b niflumic acid appears to prolong the duration of the induced current which persisted after washout of the drug. There is no obvious explanation for this observation. Niflumic acid produced similar effects on both noradrenaline- and caffeine-evoked currents and consequently the data were pooled. Figure 8 illustrates the concentration-effect relationship of niflumic acid on evoked $I_{\text{Ca}}$ at $-50 \text{mV}$ obtained from several cells and the estimated $IC_{50}$ was $6.6 \times 10^{-4} \text{M}$. Also included on this graph are the data on STICs at $-50 \text{mV}$ (appropriate curve from Figure 4b) and it can be seen quite clearly that the potency of niflumic acid against STICs ($IC_{50} = 2.3 \times 10^{-4} \text{M}$) is greater than against evoked chloride currents.

We also studied the effect of niflumic acid on $I_{\text{K(Ca)}}$ stimulated by noradrenaline in potassium-containing solutions at 0 mV (which is close to the chloride equilibrium potential, $-2 \text{mV}$). A typical experiment is shown in Figure 7c where it can be seen that $5 \times 10^{-5} \text{M}$ niflumic acid potentiates the noradrenaline-evoked $I_{\text{K(Ca)}}$ and this enhancement is sustained in the continued presence of niflumic acid. The potentiating effect of niflumic acid was concentration-dependent as in the presence of $2 \times 10^{-4} \text{M}$ and $5 \times 10^{-5} \text{M}$ niflumic acid the noradrenaline-evoked $I_{\text{K(Ca)}}$ was increased respectively 1.7 ± 0.4 fold ($n = 6$) and 2.5 ± 0.3 ($n = 8$) fold. Therefore niflumic acid increases the amplitude of evoked $I_{\text{K(Ca)}}$ without affecting spontaneous calcium-activated potassium currents.

**Effect of niflumic acid on voltage-activated divalent cation currents**

The relative high potency (see Discussion) of niflumic acid against $I_{\text{Ca}}$ suggests that this agent might be used to appraise the role of calcium-activated chloride currents in producing membrane depolarization and contraction evoked by noradrenaline (or other excitants) in smooth muscle. Consequently it seemed worthwhile to see if niflumic acid inhibited voltage-gated calcium currents which might be expected to be the essential link between depolarization which is generated by niflumic acid and muscle contraction. In these experiments we used pipettes filled with 126 mM CsCl and 10 mM EGTA (see Methods) and the external solution contained 10 mM BaCl$_2$ (no added calcium) so that Ba$^{2+}$ was the charge carrier. Full current-voltage experiments were not carried out but in experiments where the holding potential was $-70 \text{mV}$ the amplitude of inward currents evoked by command steps duration of (1500 ms) to $0 \text{mV}$ was not altered by $2 \times 10^{-4} \text{M}$ $-5 \times 10^{-5} \text{M}$ niflumic acid. For example, in one series of experiments the amplitude of the control barium current was 88 ± 12 pA and in the presence of $2 \times 10^{-5} \text{M}$ niflumic acid the current $59 \pm 9 \text{pA}$ ($n = 4$). In another series of experiments in $5 \times 10^{-5} \text{M}$ niflumic acid the inward current was $79 \pm 13 \text{pA}$ compared to a control value of $73 \pm 12 \text{pA}$ ($n = 3$). Therefore it can be concluded that niflumic acid in concentrations up to $5 \times 10^{-5} \text{M}$ does not inhibit the influx of divalent cations through voltage-gated calcium channels.

**Discussion**

This paper demonstrates that niflumic acid is a relatively potent blocker of calcium-activated chloride currents in rabbit portal vein and inhibits $I_{\text{Ca}}$ in the micromolar range. It will be suggested later that niflumic acid inhibits $I_{\text{Ca}}$ by blocking open chloride channels and therefore comparison of quantitative data in different preparations might give an indication whether the calcium-activated chloride conductance differs from tissue to tissue. Dose-response curves with niflumic acid have not been constructed in other smooth muscle tissues but it has been reported that in the presence of $10^{-5} \text{M}$ niflumic acid a tail current, presumed that many chloride currents activated by the influx of calcium, was greatly reduced but a small component still persisted in the presence of $10^{-4} \text{M}$ niflumic acid in rabbit oesophageal smooth muscle (Akbarali & Giles, 1993) whereas this concentration of niflumic acid blocked STICs in the present work. This suggests that there might be a difference in $I_{\text{Ca}}$ in portal vein and oesophagus or that another channel can contribute to the tail current in rabbit oesophageal smooth muscle cells. The $IC_{50}$ of niflumic acid against $I_{\text{Ca}}$ in *Xenopus* oocytes was estimated to be 17 μM (White & Aylwin, 1990) which is almost an order less potent than against STICs at $-50 \text{mV}$ ($IC_{50}$ of $2.2 \times 10^{-4} \text{M}$) in the present study. This difference is sufficiently large to suggest that there might be a difference between $I_{\text{Ca}}$ in rabbit portal vein and oocytes. It should be noted that niflumic acid is not specific for $I_{\text{Ca}}$ because this agent inhibits cyclic AMP-activated chloride currents in amphibian retinal pigment epithelial cells with an $IC_{50}$ of about $2.5 \times 10^{-4} \text{M}$ (Hughes & Segawa, 1993). Also niflumic acid is a potent inhibitor ($IC_{50}$ of $6.3 \times 10^{-5} \text{M}$) of anion exchange in human red cells where it has been suggested that niflumic acid interacts with the band 3 protein (Cousin & Motais, 1979). It is interesting to speculate that this compound could block chloride channels and further inhibit anion transport in red blood cells (also see Cousin & Motais, 1982a,b).

A striking result was that niflumic acid inhibited STICs more potently than evoked currents. Since niflumic acid increased the noradrenaline-evoked $I_{\text{K(Ca)}}$ without altering STOCs (spontaneous calcium-activated potassium currents) it is possible that this compound increases the evoked release of calcium from the intracellular store without altering the spontaneous release of calcium. It was observed previously that DIDS, SITS and A-9-C increased the evoked $I_{\text{K(Ca)}}$ (Hogg et al., 1994) and it may be a general property of chloride channel blocking agents to enhance the amount of calcium released from the intracellular store.

There is some evidence to suggest that niflumic acid inhibits $I_{\text{K(Ca)}}$ by blocking open chloride channels. First, the action of niflumic acid is voltage-dependent which suggests the blocking site is within the membrane electrical field. The rapid onset and reversibility indicates the site with which niflumic acid binds is readily accessible from the external solution and therefore taking these two points together it is tempting to speculate that the site of action is the chloride channel. Secondly, at $-77 \text{mV}$ in the presence of niflumic acid the STIC decay consisted of two exponentials rather than one and the time constants of both exponentials were depen-
dent on concentration of niflumic acid in a manner consistent with open channel block according to scheme (1). Moreover the data represented in Figure 3 give quantitative support for an open channel blocking mechanism over a large concentration range. However, it is necessary to explain why in the presence of niflumic acid the STIC decay was described by two exponentials at \(-77\) mV but only a single component was observed at more positive potentials. It should be emphasized that the fast component at \(-77\) mV was not a STOC (see Results for arguments) but was a chloride current. The STIC decay at \(-50\) mV and \(+50\) mV (e.g. see Figure 5a) resemble in some respects the slow component recorded at \(-77\) mV (e.g. Figure 2a). At \(-50\) and \(+50\) mV in the presence of niflumic acid the STIC decay was exponential and had a similar absolute value, for any given concentration of niflumic acid, to \(\tau_c\) at \(-77\) mV. Therefore it can be concluded that the fast component is not observed at potentials more positive than \(-77\) mV. A common observation is that STOCs and STICs often occur as biphasic events which suggests that the same calcium pulse activates both potassium and chloride currents (see Hogg et al., 1993). Therefore since the STOC time course is not altered by niflumic acid it is unlikely that the slow decay of STICs in the presence of the blocking agents can be attributed to a prolonged time course of the calcium signal that triggers the STIC. From scheme (1) two components of STIC decay would be recorded only if chloride channel opening was rapid compared to the blocking acid time constant. We found that the rate of opening of calcium-activated chloride channels in smooth muscle but the STIC rise time is 40–60 ms which is likely to be slow relative to chloride channel opening. There are no data on the factors that determine the STIC rise time although the rate of rise of calcium at the internal surface of the cell membrane which contains calcium-activated chloride channels remains a distinct possibility. Nevertheless since the STIC rise time is relatively slow it is probable that significant block of the chloride channels will occur during the STIC rise time. If the rate of association \((k_b)\) of niflumic acid with the chloride channel was rapid compared to the STIC rise time it might not be expected to record all (or any) of the rapid phase of block and only the component associated with unblocking of the channel might be observed. In the context of the model for the effects of niflumic acid this fast component of block is resolved at \(-77\) mV but not at \(-50\) mV or \(+50\) mV because the rate of association of niflumic acid with the channel is slow compared to the STIC rise time at \(-77\) mV but not at more positive potentials. For this to occur it would be necessary for \(k_{ub}\) to be voltage-dependent and at more depolarized potentials block of channels might occur during the STIC rise time and only the unblocking slow component is observed. The experimental data confirm that membrane depolarization increases the potency of niflumic acid which indicates that as the inside of membrane is made more positive the rate of association of the negatively charged molecules of niflumic acid with binding sites inside the open chloride channel is increased. Previously it has been demonstrated at the frog motor end-plate in the presence of ligandocaine derivatives, which block channels opened by acetylcholine, the decay of the endplate current (e.p.c.) is prolonged but remains mono-exponential at \(-30\) mV whereas at more negative potentials the e.p.c. decay is obviously bi-exponential (Beam, 1976). These data are qualitatively similar to the results of the present experiments. In conclusion, it seems likely that at least part of the blocking action of niflumic acid \((K_0)\) can be attributed to block of open chloride channels.

The equilibrium constant of niflumic acid for the open chloride channel \((K_0)\) can be estimated from \((k_{ub})/k_{bu}\) which is calculated to be \(4.5 \times 10^{2} \text{ M}^{-1}\) at \(-77\) mV. This is about an order greater than the \(I_{Ca}^{\text{Ca}}\) \((3.6 \times 10^{4} \text{ M})\) calculated from inhibition of STIC amplitude at \(-77\) mV which suggests that niflumic acid might have an additional action (other than blocking open channels) to inhibit \(I_{Ca}^{\text{Ca}}\). Alternatively the value of \(K_0\) might be an overestimate resulting from the imperfections of using STICs as a model as outlined in the previous paragraph.

Finally, it is worth commenting on the potential usefulness of niflumic acid as a pharmacological tool. It is evident that niflumic acid does not inhibit either the \(\alpha_2\)-adrenoceptor recognition site (as the noradrenaline-induced \(I_{Ca}^{\text{Ca}}\) was not blocked) or the voltage-dependent calcium channel. Also in two cells, noradrenaline-evoked \(I_m\) (unpublished observation) was not reduced and thus it seems that niflumic acid might be useful in defining the role of \(I_{Ca}^{\text{Ca}}\) in producing noradrenaline-evoked depolarization and contraction. However it seems that niflumic acid appears to increase the amount of calcium released from the intracellular store in response to stimulation with noradrenaline. If this increased release of calcium can activate contractile proteins niflumic acid will tend to increase contractility of smooth muscle to excitants such as noradrenaline in addition to blocking calcium-activated chloride channels which are also stimulated by noradrenaline. Therefore the effect of niflumic acid to inhibit \(I_{Ca}^{\text{Ca}}\) might be used to assess the role of calcium released from the intracellular store in the contractility of vascular smooth muscle. In these circumstances it might be difficult to assess the role of \(I_{Ca}^{\text{Ca}}\) in smooth muscle contraction with niflumic acid.

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References

AICKIN, C.C. & BRADING, A.F. (1983). Towards an estimate of chloride permeability in the smooth muscle of guinea-pig vas deferens. J. Physiol., 336, 179–197.

AKBARALI, H.I. & GILES, W.R. (1993). Ca\(^{2+}\) and Ca\(^{2+}\)-activated Cl\(^{-}\) currents in rabbit oesophageal smooth muscle. J. Physiol., 460, 117–133.

AMÉDÉE, T., BENHAM, C.D., BOLTON, T.B., BYRNE, N.G. & LARGE, W.A. (1990). Potassium, chloride and non-selective cation conductances opened by noradrenaline in rabbit ear arteries. J. Physiol., 428, 551–568.

AMÉDÉE, T. & LARGE, W.A. (1989). Microelectrode study on the ionic mechanisms which contribute to the noradrenaline-induced depolarization in isolated cells of the rabbit portal vein. Br. J. Pharmacol., 97, 1331–1337.

BARON, A., PACAUD, P., LOIRAND, G., MIRONNEAU, C. & MIRONNEAU, J. (1991). Pharmacological block of Ca\(^{2+}\)-activated Cl\(^{-}\) current in rat vascular smooth muscle cells in short term primary culture. Pflügers Archiv., 419, 553–558.

BEAM, K.G. (1976). A voltage-clamp study of the effect of two lidocaine derivatives on the time course of end-plate currents. J. Physiol., 258, 279–300.

BYRNE, N.G. & LARGE, W.A. (1988). Membrane ionic mechanisms activated by noradrenaline in cells isolated from the rabbit portal vein. J. Physiol., 404, 557–573.

COLOQUHOUN, D. & SHERIDAN, R.E. (1981). The modes of action of gallamine. Proc. R. Soc. B., 211, 181–203.

COUSIN, J.L. & MOTAIAS, R. (1979). Inhibition of anion permeability by amphiphilic compounds in human red cell: evidence for an interaction of niflumic acid with the band 3 protein. J. Membr. Biol., 46, 125–153.

COUSIN, J.L. & MOTAIAS, R. (1982a). Inhibition of anion transport in the red blood cell by anionic amphiphilic compounds. I. Chemical properties of the flufenamate-binding site on the band 3 protein. Biochim. Biophys. Acta, 687, 147–155.
COUSIN, J.L. & MOTAIS, R. (1982b). Inhibition of anion transport in the red blood cell by anionic amphiphilic compounds II. Chemical properties of the flufenamate-binding site on the band 3 protein. Biochim. Biophys. Acta., 687, 156–164.

HOGG, R.C., WANG, Q. & LARGE, W.A. (1993). Time course of spontaneous calcium-activated chloride currents in smooth muscle cells from the rabbit portal vein. J. Physiol., 464, 15–31.

HOOG, R.C., WANG, Q. & LARGE, W.A. (1994). Effects of Cl channel blockers on Ca-activated chloride and potassium currents in smooth muscle cells from rabbit portal vein. Br. J. Pharmacol., 111, 1333–1341.

HUGHES, B.A. & SEGAWA, Y. (1993). cAMP-activated chloride currents in amphibian retinal pigment epithelial cells. J. Physiol., 466, 749–766.

JANSSEN, L.J. & SIMS, S.M. (1992). Acetylcholine activates non-selective cation and chloride conductances in canine and guinea-pig tracheal myocytes. J. Physiol., 453, 197–218.

PACAUD, P., LOIRAND, G., LAVIE, J.L., MIRONNEAU, C. & MIRONNEAU, J. (1989). Calcium-activated chloride current in rat vascular smooth muscle cells in short-term primary culture. Pflügers Arch., 413, 629–636.

WANG, Q., HOOG, R.C. & LARGE, W.A. (1992). Properties of spontaneous inward currents in smooth muscle cells isolated from the rabbit portal vein. J. Physiol., 451, 525–537.

WANG, Q. & LARGE, W.A. (1991). Noradrenaline-evoked cation conductance recorded with the nystatin whole-cell method in rabbit portal vein cells. J. Physiol., 435, 21–39.

WHITE, M.M. & AYLWIN, M. (1990). Niflumic acid and flufenamic acids, are potent, reversible blockers of Ca2+-activated Cl− channels in Xenopus oocytes. Mol. Pharmacol., 37, 720–724.

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