British Journal of Pharmacology (1999) 127, 1470–1478
http://www.stockton-press.co.uk/bjp

Calcium antagonistic properties of the cyclooxygenase-2 inhibitor nimesulide in human myometrial myocytes

[1]Gregory A. Knock & *,[1]Philip I. Aaronson

[1]London Myometrial Group and Division of Pharmacology and Therapeutics, King's College London, St. Thomas' Hospital Campus, Lambeth Palace Road, London SE1 7EH

1 The non-steroidal anti-inflammatory drug nimesulide is a selective inhibitor of cyclooxygenase-2 which relaxes spontaneously contracting human myometrium *in vivo* and is potentially a useful tocolytic drug. Part of the relaxant action of nimesulide may be *via* block of myometrial Ca^{2+} channels. Here, we describe the Ca^{2+} channel blocking properties of nimesulide in freshly dispersed human term-pregnant myometrial smooth muscle cells (HMSMCs).

2 Both L- and T-components of the whole cell Ca^{2+} channel current were inhibited by 100 μM nimesulide (38 ± 3 and 35 ± 1% block, respectively). At physiological pH inside and outside the cell ($pH_o/pH_i = 7.4/7.2$), this block did not depend on the holding or test potential, although a degree of use-dependence was observed during high frequency stimulation at a higher concentration of drug (300 μM).

3 At $pH_o/pH_i = 6.8$, under which condition the concentration of the non-ionized form of the drug is increased 3 fold compared to pH 7.4, nimesulide blocked the L-type current more potently (58 ± 3% inhibition at 100 μM, $P < 0.01$) compared to physiological pH. Nimesulide caused a 7 mV leftward shift in the availability curve of the current at pH 6.8, suggesting that the affinity of the drug for the inactivated channel is approximately 4 fold higher than its affinity for the closed channel. We speculate that acidification and depolarization of the myometrium during the intense and prolonged contractions of labour might increase the potency of nimesulide as a Ca^{2+} channel antagonist, promoting its action as a tocolytic agent.

Keywords: Nimesulide; tocolysis; human; myometrium; calcium current; calcium antagonists; labour

Abbreviations: ANOVA, analysis of variance; COX, cyclooxygenase; DMSO, dimethylsulphoxide; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; HMSMCs, human myometrial smooth muscle cells; HP, holding potential; *I-V*, current voltage relationship; NIM, nimesulide; pH_o/pH_i, pH outside and inside the cell; PSS, physiological salt solution; TEA-Cl, tetraethylammonium chloride

Introduction

Nimesulide (N-(4-nitro-2-phenoxyphenyl)-methane-sulphonilamide, NIM), is a non-steroidal anti-inflammatory drug which inhibits prostaglandin synthesis by cyclooxygenase (COX) with a 30–100 fold selectivity for the inducible isoform of enzyme, COX-2 (Taniguchi *et al*., 1995; Miralpeix *et al*., 1997). COX-2 is the predominant isoform in foeto-placental membranes and myometrium (Slater *et al*., 1995; 1997), whereas the constitutive isoform, COX-1, is expressed in foetal tissues. Nimesulide is therefore a potentially useful tocolytic agent (Sawdy, 1997), enabling the targeting of foeto-placental and uterine COX and avoiding the harmful foetal side effects that preclude the use of non-selective COX-inhibitors such as indomethacin (Norton *et al*., 1993).

At concentrations close to those expected to exist in the maternal reproductive organs during acute tocolysis therapy (Davis & Brogden, 1994), it was recently shown that nimesulide inhibited *in vitro* spontaneous contractions of myometrial strips taken from pregnant women at term, and that it also significantly inhibited the voltage-gated Ca^{2+} current in isolated human myometrial smooth muscle cells (HMSMCs) (Sawdy *et al*., 1998) at a holding potential close the resting membrane potential in human myometrium (−50 mV), as reported by Inoue *et al*. (1990). However, current amplitude was less affected by the drug than was contraction.

*Author for correspondence; E-mail: p.aaronson@umds.sc.uk

The myometrium demonstrates complex electrical activity, characterized by action potentials arising from periodic slow waves (Kawarabayashi *et al*., 1986). In addition, HMSMCs have been reported to exhibit both T- and L-type Ca^{2+} currents (Inoue *et al*., 1990; Young *et al*., 1993). Since Ca^{2+} channel blockade by most drugs appears to be state-dependent (Lee & Tsien, 1983; Sanguietti & Kass, 1984), it seemed possible that the effect of nimesulide might be influenced by the conditions used to characterize channel block. Moreover, the effect of most Ca^{2+} antagonists is pH-dependent. Although the pH of the myometrium seems never to have been measured during labour, reports indicating that plasma lactate levels rise progressively during labour and sharply at delivery, and indeed measurably during individual contractions (Marx & Greene, 1964), suggest strongly that a fall in myometrial pH occurs during parturition. In the present study, therefore, we have investigated the state- and pH-dependency of nimesulide's effect on both T- and L-type calcium currents in isolated human myometrial smooth muscle cells from pregnant women at term.

Methods

Cell isolation

Myometrial biopsies were taken from the middle of the upper edge of the lower segment incision in women undergoing routine elective caesarean section at term (following informed

consent) and placed immediately into cold physiological salt solution (PSS). Small pieces of myometrium ($2-3$ mm^3) were incubated for 45 min at 37°C in low-Ca^{2+} PSS containing collagenase (mixture of Sigma types I and XI, total 2.5 mg ml^{-1}). Cells were dispersed by transferring the digested tissue pieces to fresh low-Ca^{2+} PSS and triturating through a wide bore glass pipette. Cells were stored in low-Ca^{2+} PSS at 4°C.

Solutions and chemicals

The conventional whole-cell patch clamp technique was used to record calcium channel currents. Current amplitudes were magnified by using 10 mM Ba^{2+} as the charge carrier. Low-Ca^{2+} PSS contained (in mM): NaCl 130, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 0.015, HEPES 10, and glucose 10, pH 7.4 with NaOH. The pipette solution contained (in mM): CsCl 135, $MgCl_2$ 2.5, MgATP 5, HEPES 10, and ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) 10 (adjusted to pH 7.2 or 6.8 with CsOH). The bath solution contained (in mM): NaCl 120, tetraethylammonium chloride (TEA-Cl) 4, CsCl 1, $MgCl_2$ 1.2, $BaCl_2$ 10 (or in some experiments $CaCl_2$ 1.5), HEPES 10 and glucose 10 (adjusted to pH 7.4 or 6.8 with NaOH). In Na^+-free PSS, NaCl was replaced by TEA-Cl. Nimesulide was prepared from a 100 mM stock solution in dimethylsulphoxide (DMSO). Final DMSO concentrations were too low to affect currents, as described previously (Sawdy *et al.*, 1998).

Statistics

Statistical comparisons between two sample means were made using Student's paired or unpaired *t*-test, where appropriate and between three sample means using analysis of variance (ANOVA). Differences were considered significant when $P<0.05$. Curve fitting was by non-linear regression using SigmaPlot 4.0.

Results

Characteristics of Ca^{2+} channel currents in HMSMCs

The voltage-dependence of activation and inactivation of the Ca^{2+} current using 10 mM Ba as charge carrier (I_{Ba}) was investigated. Figure 1 shows the current/voltage relationships ($I-V$) of I_{Ba}, from holding potentials (HP) of -80 and -50 mV (Figure 1A). The current activated at a relatively negative potential (-60 mV) from holding potential -80 mV, and demonstrated an initial rapid component of decay which was not apparent when the holding potential was -50 mV, in which case the apparent threshold of

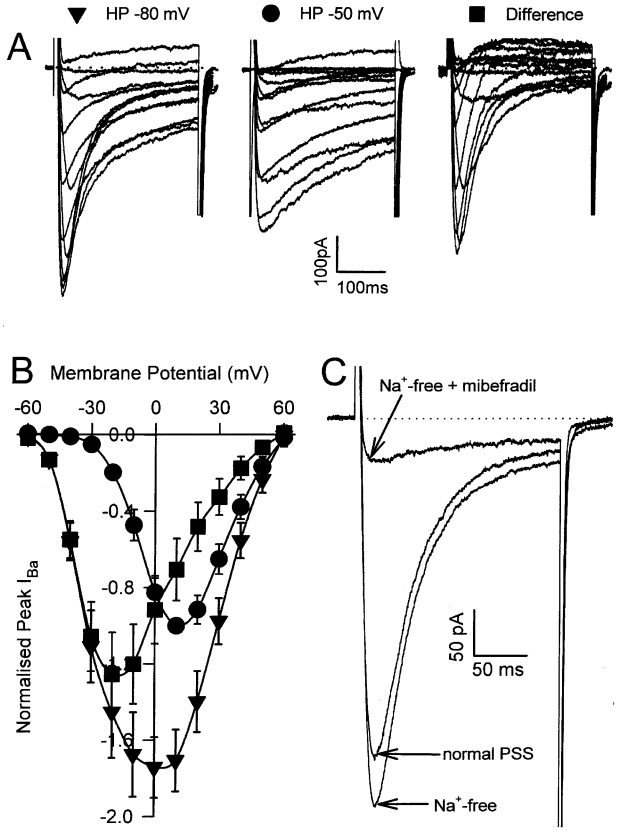

Figure 1 Ca^{2+} channel currents in HMSMCs. (A) Current-voltage relationships of currents elicited by 300 ms depolarizations to between -60 and $+60$ mV in a representative cell, from a holding potential (HP) of either -80 or -50 mV, and the difference (subtracting currents at HP -50 mV from currents at HP -80 mV). (B) Peak amplitude of currents from HP -80, -50 mV and the difference, normalized to the $+10$ mV pulse from HP -50 mV (mean and s.e.mean in the same 6 cells). (C) Example of T-type Ca^{2+} current, isolated by stepping to -20 mV from HP -80 mV. The current is not inhibited by replacing external Na^+ with TEA-Cl, but is blocked by 1 μM mibefradil, revealing a small L-type component.

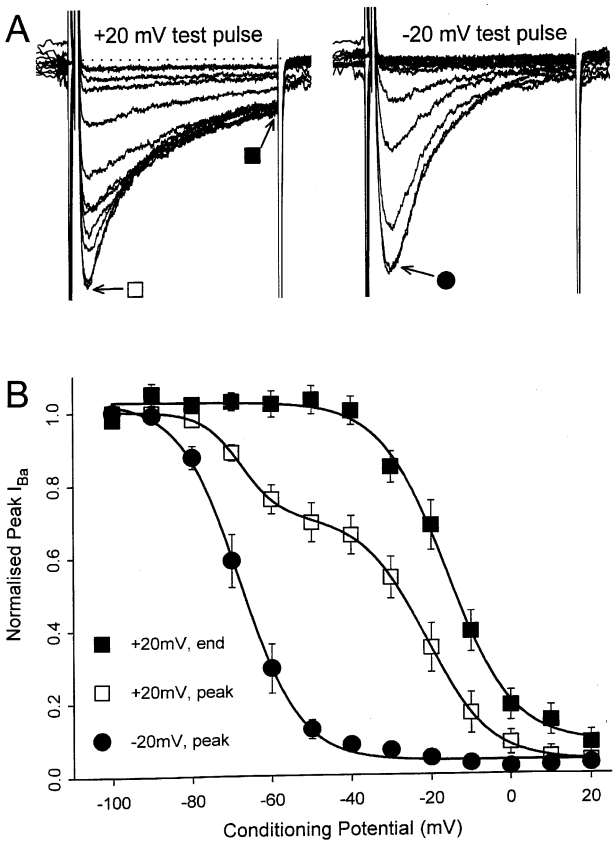

Figure 2 Availability of I_{Ba} in HMSMCs. (A) Currents in a representative cell elicited by 300 ms depolarization to $+20$ or -20 mV following 5 s preconditioning pulses between -100 and $+20$ mV. (B) Points are the mean and s.e.mean of peak current at -20 mV ($n=6$), peak current at $+20$ mV ($n=9$) and currents at the end of the 300 ms $+20$ mV depolarization ($n=9$). Data are normalized to the current elicited following the -100 mV pre-conditioning pulse and fitted to single or double component Boltzmann distributions.

activation was around −40 mV. When the currents elicited from HP −50 mV were subtracted from the currents evoked from HP −80 mV, a rapidly inactivating current with negative threshold of activation was observed. This rapidly-inactivating component of current reached a maximal amplitude at −20 mV, while the slowly-inactivating current evoked from HP −50 mV reached its peak at +10 or +20 mV (Figure 1B). The rapidly- and slowly-inactivating components of the current are suggestive of T-type and L-type currents respectively. The terms T-component and L-component are hereafter used to describe the two current components. The identity of T current was also confirmed by its resistance to Na^+-removal and its inhibition by the T-selective Ca^{2+} antagonist, mibefradil (Mishra & Hermsmeyer, 1994) (effective inhibition with 0.1–1 μM, $n=3$) (Figure 1C).

Figure 2A shows the availability of I_{Ba} at a +20 mV test potential, observed using a range of 5 s conditioning potentials from −100 and +20 mV. The current, measured at its peak (shown in Figure 2B, open squares), demonstrated a biphasic inactivation curve which was fitted with a double component Boltzmann function:

$$y = A/[1 + (\exp((V_m - V_{Half1})/K_1] + B/[1 + \exp((V_m - V_{Half2})/K_2)] + C \quad (1)$$

where y is the normalized current at any potential, V_m is the membrane potential, V_{Half1} and V_{Half2} are the half-inactivation potentials, K_1 and K_2 are the slope factors of the low (A) and high threshold (B) inactivating fractions of the current, respectively; and C is the fraction of current not inactivated. V_{Half1} was −68.4±4 mV and V_{Half2} was −19.4±2 mV. The fraction of A was 36.8±6.8% and the fraction of B 56.5±7.3% in nine cells.

The T-component should be most obvious at the peak of the current, and also at more negative test potentials, where it is prominent and the L-component is only slightly activated. Conversely, the L-component should predominate at more positive test potentials, and at the end of the pulse (300 ms), since the T-component will have almost completely inactivated. The availability of end-of-pulse current at a test potential of +20 mV (L-component, filled squares) was therefore compared with that of the peak current at a test potential of −20 mV (T-component, filled circles), elicited following 5 s conditioning potentials, using a single component Boltzmann function:

$$y = (1 - A)/[1 + \exp((V_m - V_{Half})/K) + A] \quad (2)$$

The end of pulse currents at the +20 mV test pulse had a high voltage-range of inactivation ($V_{Half}=-16.4\pm7.6$ mV, $n=8$; Figure 2B, solid squares). By contrast, the peak currents at the −20 mV test pulse had a low threshold of inactivation ($V_{Half}=-65.3\pm1.8$ mV, $n=6$; Figure 2B, solid circles). These values were not significantly different than those derived from the two-component fitting of the overall current peak.

Taken together, the results of Figures 1 and 2 indicate that HMSMCs exhibit two distinct components of Ca^{2+} channel current; a T-type component with negative ranges of activation and inactivation and a rapid decay, and an L-type component with more positive ranges of activation and inactivation, and slower inactivation kinetics.

Voltage-dependence of current blockade by nimesulide

In these experiments a concentration of nimesulide (100 μM) that causes 40–50% inhibition of Ca^{2+} current was used (Sawdy *et al.*, 1998). Figure 3 illustrates the effects of 100 μM nimesulide on the current-voltage curves in six cells resulting when HP was either −80 (Figure 3A and C) or −50 mV (Figure 3B and D). Currents were measured at both peak (Figure 3A and B) and end of pulse (Figure 3C and D) (300 ms). Regardless of the holding potential, both peak and end-of-pulse current amplitudes (i.e. both T- and L-components) were diminished by nimesulide over the whole range of potentials (Figure 3). Apparent thresholds of activation and potentials of maximum current amplitude at either holding potential, however, were unaffected by nimesulide. A complete dose response for the L-component at +10 mV is presented in Figure 9.

The effect of nimesulide on the availability of the T- and L-components was assessed using the protocol described for Figure 2. We did not use specific inhibitors to isolate the L- or T-components of the current in these experiments because of the pronounced voltage-dependence of block demonstrated by dihydropyridines (Sanguinetti & Kass, 1984 and others), wherein the resultant leftward shift in the availability would influence the interpretation of the remaining current as T-type and would interfere with potential nimesulide-mediated changes in current availability. For the availability of T-component, peak current at −20 mV was measured, and for the L-component, the current was measured at the end of a test pulse to +20 mV. 100 μM nimesulide produced no significant change in V_{Half}

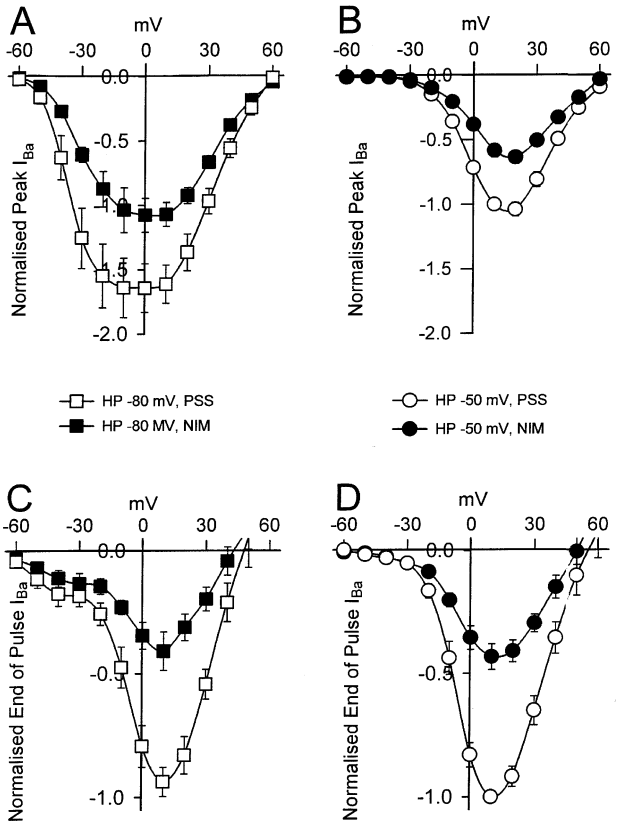

Figure 3 Influence of 100 μM nimesulide on current/voltage relationships in HMSMCs. (A, B) Peak current from holding potentials (HP) of −80 mV (A) and −50 mV (B) in PSS and 100 μM nimesulide (NIM), with data normalized to the current at +10 mV from HP −50 mV. (C, D) End of pulse current from HP −80 mV (C) and −50 mV (D) in PSS and 100 μM nimesulide (NIM), with data normalized to the current at +10 mV from HP −50 mV. All data points are mean and s.e.mean of normalized currents from the same six cells.

for either L-component (-16.5 ± 1.7 mV, $n=8$) or T-component (-66.6 ± 1.7 mV, $n=6$), indicating that no shift occurred in the potential-dependency of availability for either type of current (Figure 4).

In order to examine possible effects of more prolonged conditioning potentials on the response to nimesulide, the effect of 100 μM drug on currents elicited by pulses to +10 mV were compared when membrane potential was held for several minutes at −40 and −50 mV. Using measurements of the current integral as an overall estimate of current amplitude, there was no significant difference in percent inhibition (resting block) at the two holding potentials (see Figure 5).

Effect of nimesulide on current kinetics

Figure 3 illustrates that nimesulide apparently caused a greater inhibition of L-type current at the end of the pulse than at the peak of the current, suggesting that it may have enhanced the apparent rate of inactivation. Currents elicited by depolarizations to +10 mV from HP −50 mV, in the presence and absence of nimesulide, were therefore fitted with a single component exponential function:

$$y = [(1 - B)^{*}\exp(-t/tau) + B] \quad (3)$$

where y is current amplitude, t is time, *tau* is the time constant (ms), and B is a constant representing non-inactivating current. Because Ba^{2+} changes current kinetics, these experiments were also repeated with 1.5 mM Ca^{2+} as the charge carrier. *tau* was significantly reduced by 100 μM nimesulide (NIM) with both 10 mM Ba^{2+} (PSS, 211 ± 29 ms vs NIM, 105 ± 8 ms, $P<0.01$, paired test, a 41 ± 6% change, $n=12$) and 1.5 mM Ca^{2+} (PSS, 116 ± 23 ms vs NIM, 69 ± 6 ms, $P<0.05$, paired test, a 31 ± 6% change, $n=9$) as charge carrier. The kinetics of T-type currents were unaffected by nimesulide (PSS, 37 ± 4 ms vs NIM 33 ± 3 ms, $n=7$).

Use-dependency and recovery from inactivation of L-type current

The increased decay rate of the current in nimesulide suggested that the drug might have been causing a degree of use-dependent block. This possibility was examined using the following protocol and was adopted: following a control burst of short (50 ms), high frequency (4 Hz) steps to +10 mV from a −50 mV holding potential, 300 μM nimesulide was applied during a 3 min resting period at −50 mV. This was immediately followed by an identical burst of short rapid steps to +10 mV in the continued presence of nimesulide.

Under control conditions, current amplitude fell progressively during the rapid short depolarizations, but recovered during the three minute rest (Figure 6A), and then fell in a similar manner during a second train of pulses. When nimesulide was added during the rest period, current amplitude was immediately decreased during the first of the subsequent train of test pulses (Figure 6B), and then fell further during the train. Results of a number of experiments of this type are shown in Figure 6C. Normalization of the data revealed that the fall in current amplitude during the pulse train applied in the presence of drug was exaggerated compared to that observed during the control period (Figure 6D).

These results suggested that the effect of nimesulide on the current was use-dependent, possibly because drug unbinding from the channel was incomplete during high-frequency stimulation. In order to investigate this more directly, the effect of nimesulide (300 μM) on recovery of the L-type current from inactivation was also investigated. Cells were stepped for 500 ms to +10 mV from a holding potential of −50 mV in the presence or absence of drugs, and then a second depolarization to +10 mV was imposed, with the interval between the two pulses increased incrementally to determine the time-depe-

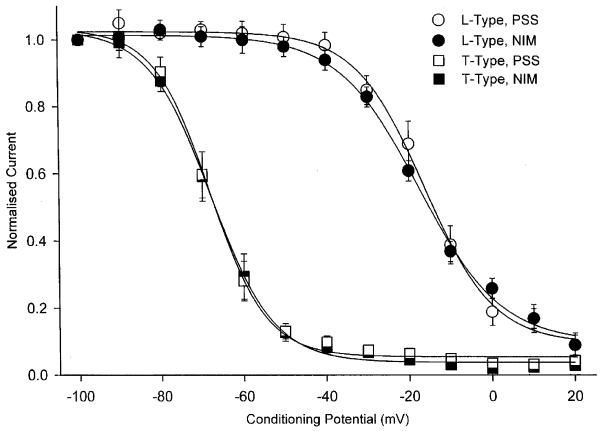

Figure 4 Influence of nimesulide on availability of T-type and L-type Ca^{2+} currents in HMSMCs. 5 s pre-conditioning pulses to between −100 mV and +20 mV followed by 300 ms depolarizations to either −20 mV (T-type current, $n=6$, peak current measured) or to +20 mV (L-type current, $n=8$, end of pulse current measured) in the absence (PSS) and presence (NIM) of 100 μM nimesulide.

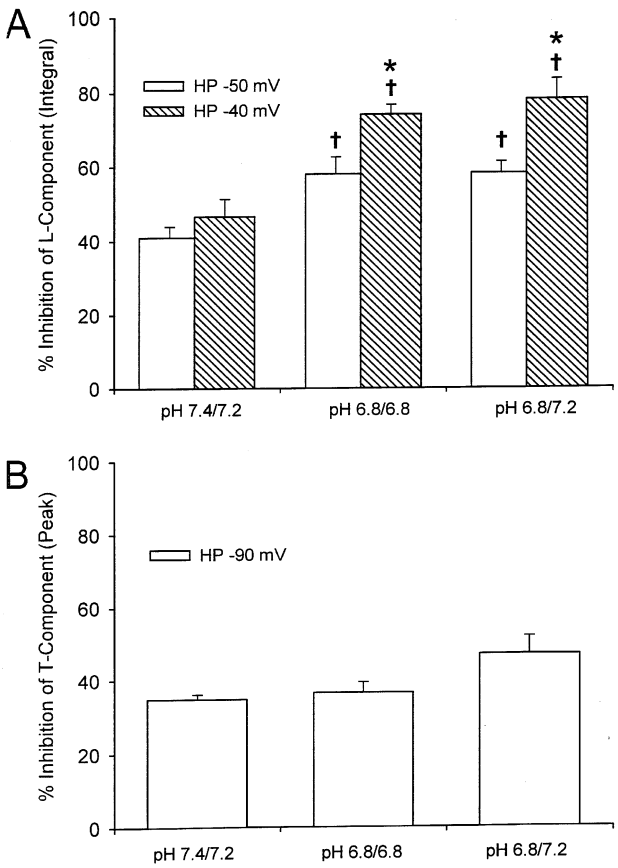

Figure 5 Summary of the percent inhibition of L-type (A: integrated current) and T-type (B: Peak current) components of I_{Ba} in conditions of differing pH_o/pH_i and different holding potentials. *Inhibition of L-type current was significantly greater at HP −40 mV than at HP −50 mV ($P<0.01$, $n=6-12$ cells). †Inhibition of L-type current was significantly greater at pH 6.8/6.8 and pH 6.8/7.2 than at pH 7.4/7.2 for both HP −50 mV ($P<0.05$ by ANOVA) and HP −40 mV ($P<0.001$ by ANOVA).

nency of recovery. Figure 7 shows that the recovery of these currents from inactivation was significantly slowed in the presence of 300 μM nimesulide.

Effect of lowering pH on block of Ca^{2+} channel currents by nimesulide

The modulated receptor hypothesis (see discussion) states that Na^+ and Ca^{2+} channel antagonists in their ionized forms bind preferentially to the open state of the channel, and in their neutral forms interact predominantly with the inactivated and resting states. Nimesulide is a weak acid with a pK_A of 6.5 (Magni, 1991). According to the Henderson-Hassalbach equation, nimesulide will be 88.8% ionized at pH 7.4 (pH outside the cell or pH_o) and 83.4% ionized at pH 7.2 (pH inside the cell and pipette or pH_i), and will become less ionized as the pH is decreased. We therefore investigated whether the degree of resting block by nimesulide and the effect of nimesulide on channel inactivation were altered when both pH_o and pH_i were lowered to 6.8 (i.e. $pH_o/pH_i = 6.8/6.8$) (66.6% ionization; a trebling of the neutral drug concentration).

In addition, in order to evaluate whether the ionized form of the drug played any role in channel blockade, the effect of 100 μM nimesulide on resting block and steady-state inactivation was also evaluated in cells where the pH_o was 6.8 and pH_i was 7.2 (i.e. $pH_o/pH_i = 6.8/7.2$). Due to ion trapping, these conditions result in a similar steady-state intracellular concentration of neutral drug as with pH 6.8 on both sides of the membrane, but increase the steady-state intracellular concentration of ionized drug by 2.5 fold.

Estimates of the degree of resting block of the T- and L-components of I_{Ba} produced by 100 μM nimesulide at the pH_o/pH_i of 7.4/7.2, 6.8/6.8 and 6.8/7.2 are described in Figure 5. The T-component was isolated using 300 ms test depolarizations to -20 mV from HP -90 mV. Using this protocol, and due to the unusually large T-current in these cells, peak current was approximately 90% T-type. L-type current was isolated by applying 300 ms steps to either $+10$ or $+20$ mV from HP -50 mV and measuring the integral of the current (At HP -50 mV, T-current is more than 90% inactivated). Percent inhibition of the L-component but not the T-component of I_{Ba} by 100 μM nimesulide was significantly greater under both sets of lower pH conditions than at 7.4/7.2 (L-component: $P<0.05$ by ANOVA, $n=9-12$ cells; T-component: $P=$NS by ANOVA, $n=6-7$ cells).

As shown in Figure 8, inactivation curves when $pH_o/pH_i = 6.8/6.8$ for L-component (isolated using a $+20$ mV test pulse and measuring current at the end of the pulse) and T-component (isolated using a -20 mV test pulse and measuring peak current) were both significantly shifted to the left by 100 μM nimesulide, giving a mean shift in the V_{half} of -7.3 ± 1.3 mV for L-component (from -17.7 ± 1.0 mV in PSS to -22.0 ± 1.5 mV in NIM, $n=6$, $P<0.01$, paired t-test) and -4.6 ± 0.8 mV for T-component (from -63.4 ± 2.1 mV in PSS to -68.0 ± 2.7 mV in NIM, $n=6$, $P<0.01$, paired t-test).

Nimesulide also produced a similar hyperpolarizing shift in the inactivation of both L- and T-components of I_{Ba} when $pH_o/pH_i = 6.8/7.2$. The mean shift in V_{half} was -9.6 ± 2.2 mV for L-component (from -16.2 ± 2.1 mV in PSS to -25.8 ± 1.1 mV in nimesulide, $n=6$, $P<0.01$, paired t-test) and -5.9 ± 0.9 mV for T-component (from -61.7 ± 3.3 mV in PSS to -67.6 ± 4.1 mV in nimesulide, $n=7$, $P<0.01$, paired t-test).

To confirm this apparent voltage-dependence of block at the lower pH_o/pH_i (either 6.8/6.8 or 6.8/7.2) the apparent

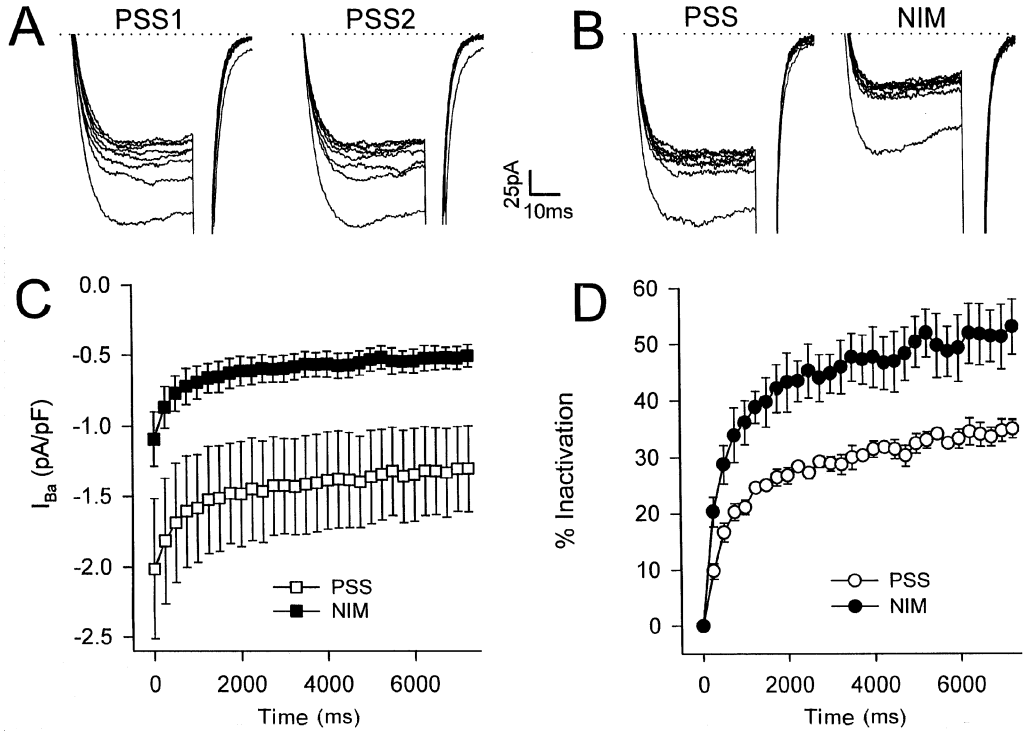

Figure 6 Use-dependence of I_{Ba} current block by nimesulide in HMSMCs. Currents elicited by a 7 min burst of short and rapid (50 ms, 4 Hz) depolarizations to $+10$ mV from a holding potential of -50 mV, followed by a 3 min rest period at -50 mV and then a further 7 min burst of short rapid pulses to $+10$ mV. Nimesulide was applied at the start of the rest period and was present throughout the second burst of rapid pulses. (A) Representative control experiment showing traces before (PSS1) and after (PSS2) a 3 min rest period, in the absence of nimesulide. (B) Representative current traces before (PSS) and after (NIM, 300 μM nimesulide) the 3 min rest period, respectively. Every fourth current trace is shown. (C) Effect of nimesulide on peak current in four cells (mean and s.e.mean). (D) Same data as in (C) normalized to the zero time-point, showing per cent inactivation of current.

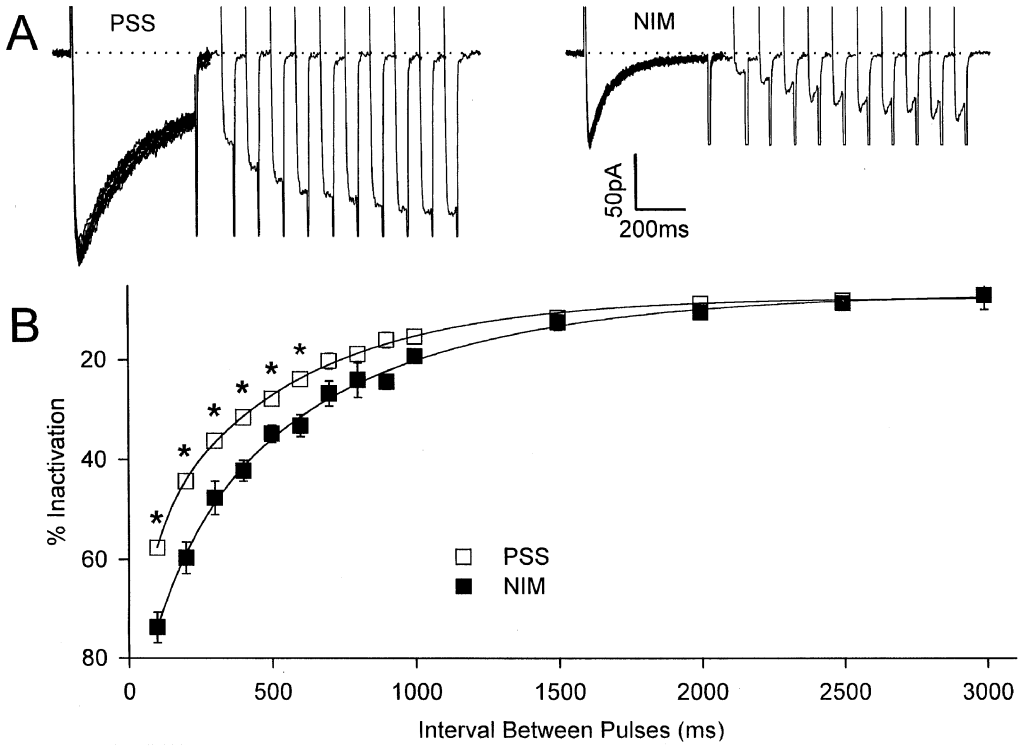

Figure 7 Influence of nimesulide on I_{Ba} recovery from inactivation in HMSMCs. 500 ms depolarization pulses from −50 mV to +10 mV followed at increasing intervals by a further 50 ms pulse to +10 mV. (A) Representative traces showing currents in the presence of PSS and 300 μM nimesulide (NIM) in the same cell. (B) Per cent inactivation at each time interval, in the absence (PSS) and presence of 300 μM nimesulide (NIM), calculated by taking the peak current amplitude at each 50 ms pulse as a percentage of the corresponding 500 ms pre-pulse (mean and s.e.mean, $n=5$ cells). *$P<0.05$ by student t-test.

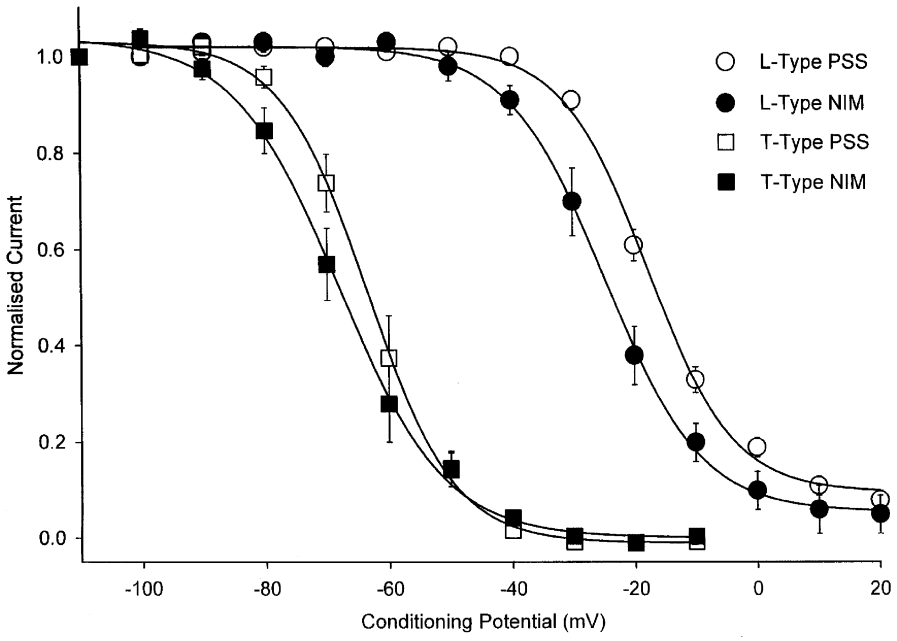

Figure 8 Influence of nimesulide on availability of T- and L-components of I_{Ba} in HMSMCs when pH_o/pH_i is 6.8/6.8. 5 s pre-conditioning pulses to between −110 and +20 mV followed by 300 ms depolarizations to either −20 mV (T-type, $n=6$, peak current measured) or to +20 mV (L-type, $n=6$, end of pulse current measured) in the absence (PSS) and presence (NIM) of 100 μM nimesulide.

inhibition of the L-component of I_{Ba} by 100 μM nimesulide was further estimated using a slightly depolarized HP of −40 mV. This blockade was significantly greater than from HP −50 mV, both at $pH_o/pH_i=6.8/6.8$ and 6.8/7.2 ($P<0.05$) but not at $pH_o/pH_i=7.4/7.2$ (Figure 5). At HP −40 mV, there was a similar significant variation in the degree of inhibition between the three pH conditions at HP −50 mV ($P<0.001$ by ANOVA, $n=6-9$ cells).

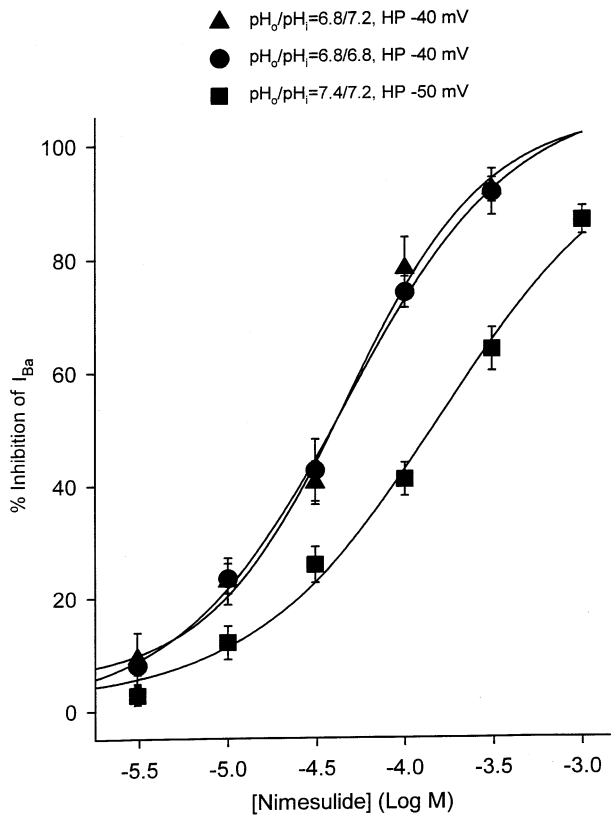

Figure 9 Effect of extracellular (pH_o) and intracellular pH (pH_i) on the concentration-dependent inhibition of L-type Ca^{2+} current by nimesulide. Points represent the mean and s.e.mean of integrated current at +10 mV in 9–12 cells at each concentration. Steady state inhibition at each concentration was reached within 3 min.

A full dose-response for inhibition of L-component by nimesulide at $pH_o/pH_i = 7.4/7.2$ is presented in Figure 9 (squares). This mean data was fitted to a sigmoidal curve and an EC_{50} of 155 μM for nimesulide was estimated. Further dose-responses were constructed using $pH_o/pH_i = 6.8/6.8$ (circles) or 6.8/7.2 (triangles) from an HP of −40 mV. Sigmoidal curves were also fitted to these two sets of mean data and estimates of EC_{50} values of 46 and 47 μM, respectively were obtained. The similar sensitivity of the current to nimesulide under these two sets of conditions, together with the similar shift in the availability (see above), suggest that the concentration of ionized drug inside the cell (which is 2.5 times greater when $pH_o/pH_i = 6.8/7.2$ than when it is 6.8/6.8 or 7.4/7.2) does not influence the degree of inhibition.

As at normal physiological pH (i.e. $pH_o/pH_i = 7.4/7.2$, see above), current decay of the L-component was accelerated by nimesulide when pH_o/pH_i was 6.8/6.8 (*tau*: PSS, 142±19 ms vs 100 μM NIM, 75±2 ms, $n=9$, $P<0.01$ paired *t*-test) and when it was 6.8/7.2 (*tau*: PSS, 161±21 ms vs 100 μM NIM, 90±7 ms, $n=9$, $P<0.01$ paired *t*-test). The percentage change in *tau* was similar under all three sets of pH conditions (7.4/7.2: 41±6%, 6.8/6.8: 41±6% and 6.8/7.2: 42±3%).

Discussion

L- and T-type Ca^{2+} currents in HMSMCs

We have provided electrophysiological and pharmacological evidence for both L-type and T-type Ca^{2+} currents in freshly dispersed HMSMCs. The apparent absence of a Na^+ current contrasts with several studies in pregnant and non-pregnant rat myometrium, where co-expression of L-type Ca^{2+} current with fast TTX-sensitive Na^+ current was evident, but no T-type current was identified (Ohya & Sperelakis, 1989; Miyoshi *et al*., 1991; Inoue & Sperelakis, 1991; Yoshino *et al*., 1997). mNa$_v$2.3 mRNA expression was also detected in mouse uterus (Knittle *et al*., 1996). In human myometrium a Na^+ current is clearly present in cultured pregnant human myometrial cells and a leiomyosarcoma cell line (Young & Herndon Smith, 1991; Kusaka & Sperelakis, 1996), but has not been reported in freshly dispersed pregnant myometrial cells (Inoue *et al*., 1990; Young *et al*., 1991; 1993), in which a low threshold T-type Ca^{2+} current was observed. We have confirmed the presence of a T-type current in our HMSMCs with inhibition by the selective T-type channel blocker mibefradil, while the lack of effect of Na^+ removal on the transient inward current confirms the lack of a Na^+ current.

Resting block of L- and T-type currents by nimesulide

In a recent study (Sawdy *et al*., 1998) we reported that nimesulide, a selective COX-2 inhibitor (Taniguchi *et al*., 1995; Miralpeix *et al*., 1997) and potential tocolytic agent (Sawdy *et al*., 1997), relaxes spontaneously contracting human myometrium *in vitro*, and suggested that, at least in part, this occurs *via* inhibition of the myometrial calcium current. We have now further characterized the calcium antagonist properties of nimesulide.

The block of I_{Ba} by 100 μM nimesulide at physiological pH ($pH_o = 7.4$, $pH_i = 7.2$) did not appear to demonstrate any dependency on the holding potential. No shift of the inactivation curve of either L-type currents or T-type currents was observed when 5 s conditioning pulses were used, and block of the current was similar at HP −50 and −40 mV. Furthermore, nimesulide did not alter the shape of the $I-V$ curve at either holding potential (−80 or −50 mV), either when peak current was measured or when current was measured at the end of the pulse, and there was no discernible shift in the apparent threshold of activation or potential of maximum activation.

An interesting aspect of block by nimesulide is its lack of selectivity for either L-type or T-type currents. The T-type calcium current is quite pronounced in myometrial smooth muscle cells, but its relevance to myometrial contractility is unknown. However, the current may be large enough that despite the very negative range of inactivation, a small percentage of channels available at the resting membrane potential may be enough to contribute to initiating slow wave depolarization, which then causes the firing of action potentials. It is possible therefore that block of T-type channels may contribute to nimesulide's inhibitory effect on myometrial contractility.

State-dependent channel block by nimesulide

The observation that considerable block of current was observed during the first depolarization following a 3 min incubation with nimesulide (Figure 6C), indicated that the greatest part of the block developed under these conditions was *via* binding of nimesulide to the channel in its closed (resting) state. However, nimesulide also blocked the L-type current significantly more when measured at the end of the 300 ms pulse than at the current peak, suggesting that it might accelerate current decay. This was confirmed by

calculating time constants of current decay in the absence and presence of nimesulide. This effect was similar and significant either with 10 mM Ba^{2+} or with 1.5 mM Ca^{2+} as charge carrier, and was apparently independent of pH. In addition to accelerating current decay, nimesulide significantly enhanced the decline of the current which developed during a train of brief high frequency depolarizations, and also significantly slowed recovery of the current following a 500 ms voltage step. These enhanced current decay and use-dependent properties suggest that block of I_{Ba} by nimesulide may also be promoted by channel opening (Yeh *et al*., 1982; Lee & Tsien, 1983).

The phenylalkylamines, including verapamil and its derivative D600, and the benzothiazepine diltiazem, are predominantly frequency- and use-dependent and appear to bind preferentially to the open state of the channel (Lee & Tsien, 1983; McDonald *et al*., 1993). Furthermore, the degree of use-dependence of a drug may be predicted by the degree of ionization of the drug at physiological pH. This is evident with D600 (pKA 8.5, 93% ionized at pH 7.4) and verapamil (pKA 9.7, 96% ionized at pH 7.3) which are both wholly use-dependent at physiological pH (Sanguinetti & Kass, 1984; Uehara & Hume, 1985). Diltiazem is intermediate (pKA 7.7, 67% ionized at pH 7.4) so that lowering the pH to 6.5 increases the per cent of ionized drug to 95% and increases the degree of use-dependence (Uehara & Hume, 1985). On the other hand, the dihydropridines such as nifedipine (pKA 1.0), nicardipine, nisoldipine and nitrendipine are wholly neutral and cause large hyperpolarizing shifts in the steady-state inactivation, indicative of preferential binding to the inactivated state of the channel and demonstrate little or no use-dependence at physiological pH (Sanguinetti & Kass, 1984; Uehara & Hume, 1985; Bean *et al*., 1986; Terada *et al*., 1987).

Nimesulide differs from these antagonists, in that due to its sulfonilamide group it is a weak acid (Magni, 1991). Thus lowering the pH from 7.4 to 6.8 decreases the amount of ionized drug and increases the amount of the neutral form of the drug from 11.2 to 33.4%. This 3 fold increase in concentratioan of neutral drug paralleled the increased block by nimesulide when pH_o/pH_i was 6.8/6.8 (Figure 5). Furthermore, if the ionized form of the drug was contributing to resting block, when pH_o/pH_i was 6.8/7.2, where the concentration of ionized drug inside the cell would be increased 2.5 fold by an ion trapping effect, and neutral drug concentration would be the same as at 6.8/6.8, the potency of nimesulide would be even greater. Since this was not the case, we concluded that the neutral form of the drug is predominantly responsible for block of the resting channel.

In addition, nimesulide caused a 7.3 mV leftward shift of the inactivation curve of the L-component when the pH was 6.8 both in the bath and pipette solutions. According to Bean *et al*. (1984), this shift can be used to calculate the affinity of the drug for the inactivated state (K_I), as follows:

$$\Delta V_{half} = k\ln[(1 + N/K_R)/(1 + N/K_I)] \quad (5)$$

Where ΔV_{half} is the shift in the inactivation, k is the slope factor, and N is the drug concentration. Assuming that only the neutral form of the drug binds to the resting state of the channel, the affinity for the resting state, K_R, is estimated from an approximate EC_{50} of 155 μM at pH 7.4 of which 11.2% is in the neutral form and, assuming that changing the pH does not change the affinity of the drug for the channel, giving a K_R of 18 μM for the neutral form of the drug. With a ΔV_{half} of -7.3 mV at pH 6.8, K_I is estimated as 4.7 μM for the neutral form of the drug.

The possibility that nimesulide binds with a higher affinity to the inactivated state of the channel is entirely consistent with the observation that, at pH_o 6.8, when the holding potential was set at -40 mV there was significantly more block than when the holding potential was -50 mV. The fact that this reduction in holding potential did not cause additional block of the L-component at pH_o 7.4 or shift the current availability, was most likely because the concentration of neutral drug was very low under this condition.

The absence of a significantly greater hyperpolarizing shift in current availability when pH_o/pH_i was 6.8/7.2 compared to when it was 6.8/6.8, suggests that the ionized form of the drug does not contribute to inactivated channel blockade.

Taken together these observations are consistent with the concept that nimesulide blocks the Ca^{2+} channel in resting, inactivated and open states. The block of resting and inactivated channels is largely caused by the neutral form of the drug. The effect of nimesulide on current decay was similar under all three sets of pH conditions and was not apparently influenced by the concentrations and relative proportions of neutral and ionized drug in the bath and in the intracellular medium. However, the underlying rate of current decay was also accelerated at the lower pH, making interpretation of these data difficult.

Nimesulide also caused a small but significant leftward shift in the availability of the T-component when pH_o/pH_i was either 6.8/6.8 or 6.8/7.2. The extent to which this effect might contribute to nimesulide's potency as a myometrial relaxant is unclear, given our lack of knowledge concerning the role of the T-current in myometrial contractility.

Channel block, pH, and tocolysis

The increased potency of nimesulide at lower pH is also potentially of relevance to the use of nimesulide as a tocolytic agent. Although the pH of the myometrium appears not to have been measured during labour, there is substantial circumstantial evidence for a significant myometrial acidification at this time. It is thought that during labour there is progressive ischemia of the myometrium which is only relieved after delivery. The lactate concentration in the maternal venous circulation increases to 4–5 mM during the final stages of labour (Piquard *et al*., 1991). This lactate almost certainly originates in the myometrium, since peaks in blood lactate have been observed to occur in association with labour contractions (Marx & Greene, 1964). Precisely how far the intracellular pH falls during labour is not known; however small transient intracellular acidifications were associated with spontaneous contractions of very thin strips of rat myometrium even in oxygenated solution (Taggart & Wray, 1993). An *in vivo* study of rats showed that artificially induced ischemia reduced pH from 7.3 to 7.0 (Harrison *et al*., 1994). Acidification of the myometrium during labour in women should be even more pronounced, since the muscle is thicker and contractions are much more prolonged; more prolonged agonist-induced contractions in rat myometrial strips caused a much greater acidification than spontaneous contractions (Taggart & Wray, 1994). In addition, lactic acid is likely to accumulate in the myometrium, since at delivery there is a reduction in maternal arterial pH which is attributed to a flooding of lactic acid into the circulation (Cohen *et al*., 1970). Both acidification of the myometrium during the contractions of labour, and the increased level of Ca^{2+} channel inactivation associated with prolonged slow wave depolarizations, are predicted by our results to greatly enhance the *in vivo* potency of nimesulide as a myometrial relaxant. Nimesulide,

which is otherwise a rather weak Ca^{2+} antagonist, may therefore be targeted to myometrial Ca^{2+} channels during labour, in addition to its COX-2 inhibitory action in foeto-placental membranes.

We would like to thank the Tommy's Campaign for supporting this study.

References

BEAN, B.P., STUREK, M., PUGA, A. & HERMSMEYER, K. (1986). Calcium channels in muscle cells isolated from rat mesenteric arteries. *Circ. Res.,* **59,** 229–235.

COHEN, A.V., SCHULMAN, H. & ROMNEY, S.L. (1970). Maternal acid-base metabolism in normal human parturition. *Am. J. Obstet. Gynecol.,* **107,** 934–938.

DAVIS, R. & BROGDEN, R.N. (1994). Nimesulide. An update of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy. *Drugs,* **48,** 432–454.

HARRISON, N., LARCOMBE-MCDOUALL, J.B., EARLEY, L. & WRAY, S. (1994). An in vivo study of the effects of ischaemia on uterine contraction, intracellular pH and metabolites in the rat. *J. Physiol.,* **476,** 349–354.

INOUE, Y., NAKAO, K., OKABE, K., IZUMI, H., KANDA, S., KITAMURA, K. & KURIYAMA, H. (1990). Some electrical properties of human pregnant myometrium. *Am. J. Obstet. & Gynecol.,* **162,** 1090–1098.

INOUE, Y. & SPERELAKIS, N. (1991). Gestational change in Na^+ and Ca^{2+} channel current densities in rat myometrial smooth muscle cells. *Am. J. Physiol.,* **260,** C658–C663.

KAWARABAYSHI, T., IKEDA, M., SUGIMORI, H. & NAKANO, H. (1986). Spontaneous electrical activity and effects of noradrenaline on pregnant human myometrium recorded by the single sucrose gap method. *Acta Physiologica Hungaria,* **67,** 71–82.

KNITTLE, T.J., DOYLE, K.L. & TAMKUN, M.M. (1996). Immunolocalisation of the $mNa_v2.3$ Na^+ channel in mouse heart: upregulation in myometrium during pregnancy. *Am. J. Physiol.,* **270,** C688–C696.

KUSAKA, M. & SPERELAKIS, N. (1996). Genistein inhibition of fast Na^+ current in uterine leiomyosarcoma cells is independent of tyrosine kinase inhibition. *Biochimica. et Biophysica. Acta.,* **1278,** 1–4.

LEE, K.S. & TSIEN, R.W. (1983). Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature,* **302,** 790–794.

MAGNI, E. (1991). Nimesulide: an overview. *Drug Investigation,* **3** (Suppl 2), 1–3.

MARX, G.F. & GREENE, N.M. (1964). Maternal lactate, pyruvate, and excess lactate production during labor and delivery. *Am. J. Obstet. Gynecol.,* **90,** 786–793.

MCDONALD, T.F., PELZER, D. & TRAUTWEIN, W. (1993). Cat ventricular muscle treated with D600: characteristics of calcium channel block and unblock. *J. Physiol. London,* **414,** 569–586.

MIRALPEIX, M., CAMACHO, M., LOPEZ-BELMONTE, J., CANALIAS, F., BELETA, J., PALACIOS, J.M. & VILA, L. (1997). Selective induction of cyclo-oxygenase-2 activity in the permanent human endothelial cell line HUV-EC-C: biochemical and pharmacological characterization. *Br. J. Pharmacol.,* **121,** 171–180.

MIYOSHI, H., URABE, T. & FUJIWARA, A. (1991). Electrophysiological properties of membrane currents in single myometrial cells isolated from pregnant rats. *Pflugers Arch.,* **419,** 386–393.

MISHRA, S.K. & HERMSMEYER, K. (1994). Selective inhibition of T-type Ca^{2+} channels by Ro 40-5967. *Circ. Res.,* **75,** 144–148.

NORTON, M.E., MERRILL, J. & COOPER, B.A. (1993). Neonatal complications after administration of indomethacin for preterm labour. *N. Engl. J. Med.,* **329,** 1602–1607.

OHYA, Y. & SPERELAKIS, N. (1989). Fast Na^+ and slow Ca^{2+} channels in single uterine muscle cells from pregnant rats. *Am. J. Physiol.,* **257,** C408–C412.

PIQUARD, F., SCHAEFER, A., DELLENBACH, P. & HABEREY, P. (1991). Is fetal acidosis in the human fetus maternogenic during labor? A reanalysis. *Am. J. Physiol.,* **261,** R1294–R1299.

SANGUINETTI, M.C. & KASS, R.S. (1984). Voltage-dependent block of calcium channel current in the calf cardiac purkinje fiber by dihydropyridine calcium channel antagonists. *Circ. Res.,* **55,** 336–348.

SAWDY, R., KNOCK, G.A., BENNETT, P.R., POSTON, L. & AARONSON, P.I. (1998). Nimesulide and indomethacin inhibit contractility and the Ca^{2+} channel current in myometrial smooth muscle from pregnant women. *Br. J. Pharmacol.,* **125,** 1212–1217.

SAWDY, R., SLATER, D., JONES, G., POSTON, L. & BENNETT, P.R. (1997). Potential of the cyclo-oxygenase type-2 selective inhibitor nimesulide in prevention of preterm labour. *J. Soc. Gynecol. Investig.,* **4** (suppl 1), 337.

SLATER, D.M., BERGER, L.C., NEWTON, R., MOORE, G.E. & BENNETT, P.R. (1995). Expression of cyclooxygenase types 1 and 2 in human fetal membranes at term. *Am. J. Obstet. Gynecol.,* **172,** 77–82.

SLATER, D.M., DENNIS, W., JONES, G.D., POSTON, L. & BENNETT, P.R. (1997). Expression of cyclooxygenase types 1 and 2 in human myometrium; changes in relation to gestational age and labour onset. *J. Soc. Gynecol. Investig.,* **4** (Supp), 146.

TAGGART, M.J. & WRAY, S. (1993). Occurrence of intracellular pH transients during spontaneous contractions in rat uterine smooth muscle. *J. Physiol.,* **472,** 23–31.

TANIGUCHI, Y., IKESUE, A., YOKOYAMA, K., NODA, K., DEGUCHI, H., NAKAMURA, T., TODA, A. & SHIMENO, H. (1995). Selective inhibition by nimesulide, a novel non-steroidal anti-inflammatory drug, with prostaglandin endoperoxide synthase-2 activity in vitro. *Pharmacol. Sci.,* **1,** 173–175.

TERADA, K., KITAMURA, K. & KURIYAMA, H. (1987). Blocking actions of Ca2+ antagonists on the Ca^{2+} channels in the smooth muscle cell membrane of rabbit small intestine. *Pflugers Arch.,* **408,** 552–557.

UEHARA, A. & HUME, J.R. (1985). Interaction of organic calcium channel antagonists with calcium channels in single frog atrial cells. *J. Gen. Physiol.,* **85,** 621–648.

YEH, J.Z. (1982). A pharmacological approach to the structure of the Na channel. In: *Squid Axon in Proteins in the Nervous System: Structure and Function.* New York, Alan R Liss, Inc, pp. 17–49.

YOSHINO, M., WANG, S.Y. & KAO, C.Y. (1997). Sodium and calcium inward currents in freshly dissociated smooth myocytes of rat uterus. *J. Gen. Physiol.,* **110,** 565–577.

YOUNG, R.C. & HERNDON-SMITH, L. (1991). Characterization of sodium channels in cultured human uterine smooth muscle cells. *Am. J. Obstet. Gynecol.,* **164,** 175–181.

YOUNG, R.C., HERNDON-SMITH, L. & ANDERSON, N.C. (1991). Passive membrane properties and inward calcium current of human uterine smooth muscle cells. *Am. J. Obstet. Gynecol.,* **164,** 1132–1139.

YOUNG, R.C., SMITH, L.H. & MCLAREN, M.D. (1993). T-type and L-type calcium currents in freshly dispersed human uterine smooth muscle cells. *Am. J. Obstet. & Gynaecol.,* **169,** 785–792.

(Received February 8, 1999
Revised March 3, 1999
Accepted April 26, 1999)