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Effect of nimesulide and indomethacin on contractility and the Ca^{2+} channel current in myometrial smooth muscle from pregnant women

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1 The non-steroidal anti-inflammatory drug (NSAID) indomethacin inhibits both constitutive and inducible forms of cyclo-oxygenase (COX-1 and COX-2, respectively), while nimesulide is a selective COX-2 inhibitor. Uterine COX-2 is upregulated before and during term and pre-term labour, and prostaglandins play a crucial role in parturition. We therefore evaluated the effects of these drugs on myometrial contractility and the voltage-gated Ca^{2+} channel current in tissue strips and isolated human myometrial smooth muscle cells (HMSMC) from myometrial biopsies taken with informed consent from women undergoing caesarean section at term (not in labour).

2 Nimesulide and indomethacin caused almost complete inhibition of spontaneous myometrial contractions at concentrations of 100 and 300 μM, respectively. The Ca^{2+} channel current was inhibited in a concentration-dependent manner by both drugs, with a 40% reduction of the current at 100 μM nimesulide and 300 μM indomethacin. Nimesulide also accelerated the decay of the Ca^{2+} channel current.

3 The inhibition of the Ca^{2+} channel current by 100 μM nimesulide and 300 μM indomethacin was unaffected by the presence of either $PGF_{2\alpha}$ or PGE_2 (30 μM), and was of similar magnitude whether 10 mM Ba^{2+} or 1.5 mM Ca^{2+} was used as the charge carrier.

4 The concentrations of indomethacin and nimesulide required to suppress spontaneous contractility in human pregnant myometrium were much higher than those necessary to inhibit prostaglandin production. The results suggest that both nimesulide and indomethacin inhibit myometrial contractility *via* mechanisms independent of cyclo-oxygenase inhibition. Blockade of the Ca^{2+} current may contribute to this effect.

Keywords: Cyclo-oxygenase-2; non steroidal anti-inflammatory drugs; nimesulide; indomethacin; tocolysis; human; myometrium; Ca^{2+} current; premature labour

Introduction

Preterm labour occurs in 5–10% of pregnancies and is the principle cause of perinatal mortality (Cooper *et al*., 1993; Carroll *et al*., 1996). Beta-sympathomimetics, the most commonly used tocolytics, exhibit tachyphylaxis and cause potentially life threatening maternal side effects (Morrison, 1996; Carroll *et al*., 1996). Both term and preterm labour are associated with increased prostaglandin synthesis within the uterus (Skinner & Challis, 1985; Romero *et al*., 1988). Although they are highly effective in inhibition of preterm contractions and delay of delivery, the use of cyclo-oxygenase (COX) inhibitors such as indomethacin is limited by foetal side effects including constriction of the ductus arteriosis, renal tubular dysfunction and oligohydramnios, and neonatal complications including intracranial haemorrhage and necrotising entercolitis (Norton *et al*., 1993).

COX exists in two isoforms. COX-1 is constitutively expressed where prostaglandin synthesis is constant. COX-2 is inducible and associated with inflammation. COX 2, and not COX-1, is upregulated with labour in foetal membranes and in myometrium (Slater *et al*., 1995, 1997). Since prostaglandin synthesis in the foetus is constant it is probably mainly mediated by COX-1, and in this case a COX-2 selective inhibitor might therefore have the efficacy of indomethacin without side effects.

Nimesulide (N-(4-nitro-2-phenoxyphenyl)-methane-sulphonilamide), is a non-steroidal anti-inflammatory drug, which has been shown not to affect prostaglandin synthesis in tissues in which COX-1 is the predominant isoform (Bianco *et al*., 1991) but does inhibit COX-2 mediated prostaglandin synthesis (Taniguchi *et al*., 1995; Miralpeix *et al*., 1997). *In vitro*, nimesulide displays 30–100 fold selectivity for COX-2 compared with COX-1 (Miralpeix *et al*., 1997; Yamada *et al*., 1997). Nimesulide is in widespread clinical use in Europe as an anti-inflammatory agent and there have been no reports of complications of its use in pregnancy. Because of its potential use as a tocolytic agent we have undertaken experiments to assess the direct effect of nimesulide upon contractility of isolated human myometrium *in vitro* (Sawdy *et al*., 1997a). The effects of indomethacin upon contractility were also evaluated. The rapid action of both drugs in abolishing myometrial contractions suggested a mode of action other than through inhibition of prostaglandin synthesis and we therefore went on to investigate the hypothesis that nimesulide and indomethacin might block myometrial calcium channels.

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Methods

Contractility studies

Myometrium biopsies were taken from the middle of the upper margin of the lower segment incision in women undergoing routine elective caesarean section at term and placed immediately into cold physiological salt solution (PSS). Specimens were dissected to achieve muscle bundles of $10 \times 2 \times 2$ mm size within 2 h of collection. For each experiment four (or occasionally three) strips were mounted under 3 g resting tension, connected to an isometric transducer in a water bath containing PSS at 37°C and perfused steadily with 95% O_2 and 5% CO_2. There was a mean equilibration period of 90 min before the onset of spontaneous contractile activity. A baseline untreated period of 60 min was then observed before addition of nimesulide or indomethacin. In each experiment, two strips were treated with ascending concentrations of drug, and two (or occasionally one) strips were treated with appropriate increasing concentrations of the drug solvent, DMSO (maximum 0.1%). Each treatment period was 60 min after which the strips were flushed with fresh PSS and the drug or vehicle added. Strip viability was confirmed at the end of each experiment by observing response to 1 nM oxytocin.

Myometrial contractions were recorded and analysed using MacLab hardware with Chart 3.3 software. In each experiment a normalization procedure was used to compare contractions in the drug-treated strips to those in the vehicle-treated strips in order to control for spontaneous run-up or run-down which might occur over the course of the experiment. First, data were analysed to show the effect of drug or vehicle upon (1) mean contraction amplitude during a treatment (or control) interval, (2) mean area under each contraction during an interval (equivalent to mean work done per contraction), and (3) total work, equivalent to the summed area under all of the contractions occurring during a treatment interval. Then, each of these three parameters during each treatment interval in the control strips was expressed as a percentage of the initial, vehicle-free control period. The mean of the percentages for each successive vehicle-treatment period from the two control strips gave a measure of the effects of vehicle and also spontaneous run-up or run-down. This normalization procedure was carried out because regression analysis suggested that changes in activity (both spontaneous run-up and run-down were observed) in the two control strips from a biopsy were correlated ($r = 0.56$, $t = 2.12$, $P < 0.05$ one-tailed t-test). The measured parameters of contractile activity from each treatment interval in the drug-treated strips were then divided by the mean percentage for the same time interval in the two control strips in order to give the effect of the drug, corrected for spontaneous changes in contractility. The statistical significance of the effect of a concentration of drug was assessed using Student's paired t-test to compare the normalized total work during equivalent treatment intervals in the vehicle-treated and drug-treated strips. Rejection of the null hypothesis at $P < 0.05$ was used to indicate a significant effect of the drug.

Electrophysiological studies

HMSMC were isolated using a 45 min incubation at 37°C in low Ca^{2+} physiological saline solution (PSS) containing collagenase (Sigma type XI, 3 mg ml^{-1}). The conventional whole-cell patch clamp technique was used to record the Ca^{2+} channel current, which was magnified by using 10 mM Ba^{2+} as the charge carrier (Smirnov & Aaronson, 1992). Low Ca^{2+} PSS contained (in mM): NaCl 130, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 0.015, HEPES 10, and glucose 10. The pipette solution contained: CsCl 135, $MgCl_2$ 2.5, MgATP 5, HEPES 10, and EGTA 10. Currents were recorded in Ba^{2+}-PSS, containing: NaCl 120, TEA-Cl 4, CsCl 1, $MgCl_2$ 1.2, $BaCl_2$ 10, HEPES 10, and glucose 10. All solutions were buffered to pH 7.2 with NaOH. Effects of drugs were assessed following a 2 min control period during which the current amplitude was stable. The integral of currents (elicited by 200 ms depolarizations to +10 mV from a −50 mV holding potential) were calculated for each concentration of COX inhibitor. Two to three min were allowed for each dose of inhibitor to have its effect. Details of data handling are described in Smirnov & Aaronson (1992). Student's t-test was used to determine whether differences between sample means were significant at the $P < 0.05$ level.

In control experiments the highest concentration of the vehicle dimethyl sulphoxide (DMSO, 0.1%) did not significantly affect the amplitude of the Ca^{2+} current (10 mM Ba, capacitance-corrected integral current 407 ± 64 pA ms pF^{-1} vs 446 ± 79 in PSS, $n = 9$). Additional experiments were performed to determine whether current inhibition by 100 μM nimesulide and 0.1% DMSO was affected by the presence or absence of DMSO in the control period before nimesulide in DMSO was added, in order to separate the effect of nimesulide *per se* from the effect of DMSO. The inhibition was similar in both cases ($39 \pm 3\%$, $n = 9$ vs $41 \pm 3\%$ $n = 11$, respectively).

Time control experiments indicated that spontaneous run-down (or run-up) over the period of a typical dose response experiment varied from cell to cell but, on average, there was a small degree of run-up (in barium $12 \pm 20\%$ at 5 min, $18 \pm 31\%$ at 10 min and $17 \pm 32\%$ at 15 min, $n = 6$, and in calcium $12 \pm 13\%$ at 5 min and $11 \pm 11\%$ at 10 min, $n = 4$).

Results

Effects of nimesulide and indomethacin on myometrial contractility

Figure 1 illustrates an example of an experiment in which the effect on myometrial contractility of several concentrations of nimesulide was recorded. The lower trace shows the effect of vehicle (DMSO) alone, while the upper trace illustrates the effects of 1, 10, and 100 μM nimesulide. Nimesulide caused an inhibition of contractility which was complete in most strips at a drug concentration of 100 μM. Figure 2 shows the concentration-dependency of the inhibitory effect of nimesulide on total work done during a treatment period (A), mean work done per contraction (B) and mean contraction amplitude (C). The effect of nimesulide on each of these parameters was similar, and in all cases was significant at 100 μM drug.

In similar experiments, indomethacin also suppressed myometrial contractions, although over a somewhat higher concentration range. Indomethacin caused a block of the spontaneous contractions which was significant at 100 μM, and essentially complete at 300 μM. The concentration-dependency of the effect of indomethacin on the three indices of spontaneous activity is shown in Figure 2.

Inhibition of Ca^{2+} channel current by nimesulide and indomethacin

The Ba^{2+} current through the Ca^{2+} channel (I_{Ba}) was elicited in HMSMCs by 200 ms depolarizations to +10 mV applied at

0.1 Hz, from a holding potential of −50 mV, which is similar to the resting potential in human myometrium (Nakajima, 1971). Nimesulide (10–300 μM) reduced I_{Ba} (measured as the integral of the current) as shown in Figure 3. Figure 4 illustrates the concentration-dependency of the effect of nimesulide. Nimesulide also consistently accelerated the decay of I_{Ba}, so that the extent of current blockade measured at the end of the test pulse was greater than that measured at its peak. For example, with 100 μM nimesulide, the peak current was inhibited by 37±4%, while the end-of-pulse current was inhibited by 50±3%. ($n=11$, $P<0.05$).

Indomethacin also reduced I_{Ba} over this concentration range, but to a smaller extent (Figure 5). Unlike nimesulide, indomethacin had no consistent effect on the rate of decay of I_{Ba}. The mean concentration-dependency of the effect of indomethacin on the integrated current is shown in Figure 4.

In order to determine whether the response to nimesulide was due to an attenuation of intrinsic basal prostaglandin synthesis, the effect of 100 μM nimesulide was recorded when the drug was applied after a 3 min pre-incubation in (and in the continuing presence of) either 30 μM prostaglandin E_2 (PGE_2) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). As illustrated in Figure

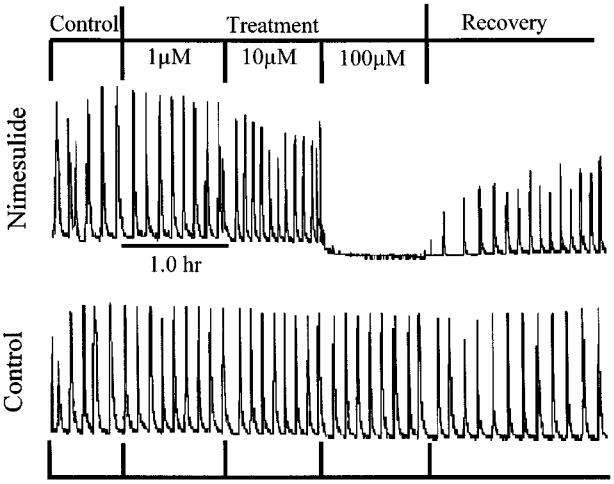

Figure 1 Effect of nimesulide (1, 10, and 100 μM, upper trace) on spontaneous isometric contractions in a strip of human myometrium. DMSO, applied to another strip from the same biopsy at the concentrations used to dissolve each concentration of nimesulide, had no effect (lower trace).

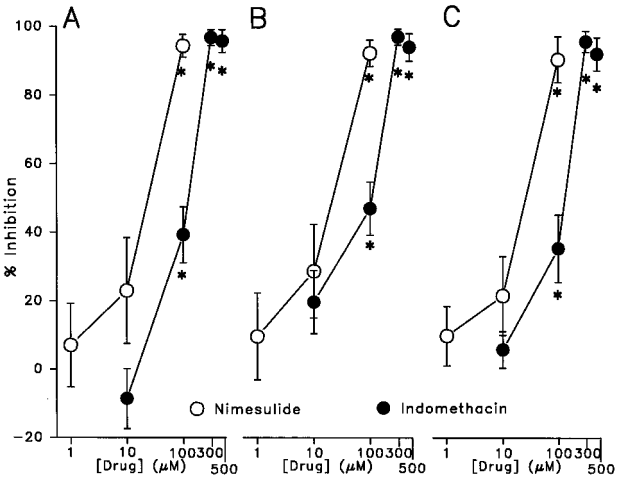

Figure 2 Concentration-dependency of the inhibition of myometrial contractility by nimesulide and indomethacin. (A) total work done per treatment interval, (B) work done per contraction, (C) peak contraction amplitude. Values are shown as mean and s.e.m. Each concentration of nimesulide was applied to 14 strips of myometrium (from seven biopsies). 10 μM indomethacin was applied to 23 strips from 11 biopsies, 100 μM indomethacin was applied to 25 strips from 13 biopsies, 300 μM indomethacin was applied to seven strips from three biopsies, and 500 μM indomethacin was applied to 12 strips from six biopsies. Asterisks indicate significant inhibition by drug ($P<0.05$).

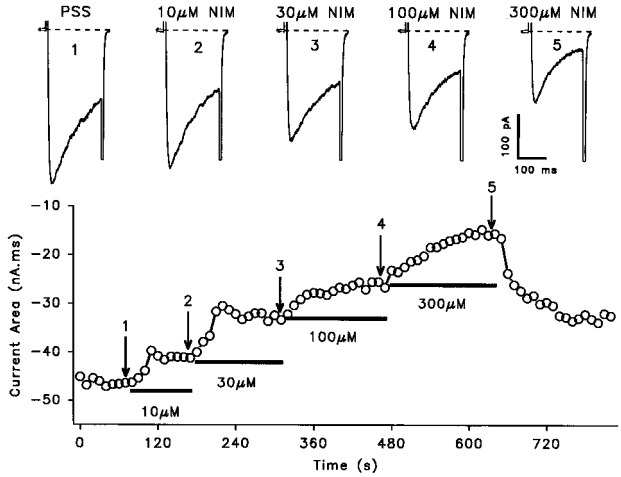

Figure 3 Effect of several concentrations of nimesulide on the Ca^{2+} channel current (I_{Ba}), elicited by 200 ms depolarizations to +10 mV from a holding potential of −50 mV, in a representative cell. The time course of the effect of nimesulide on the integrated current is illustrated below. Numbered traces correspond to the respective numbered points in the time course.

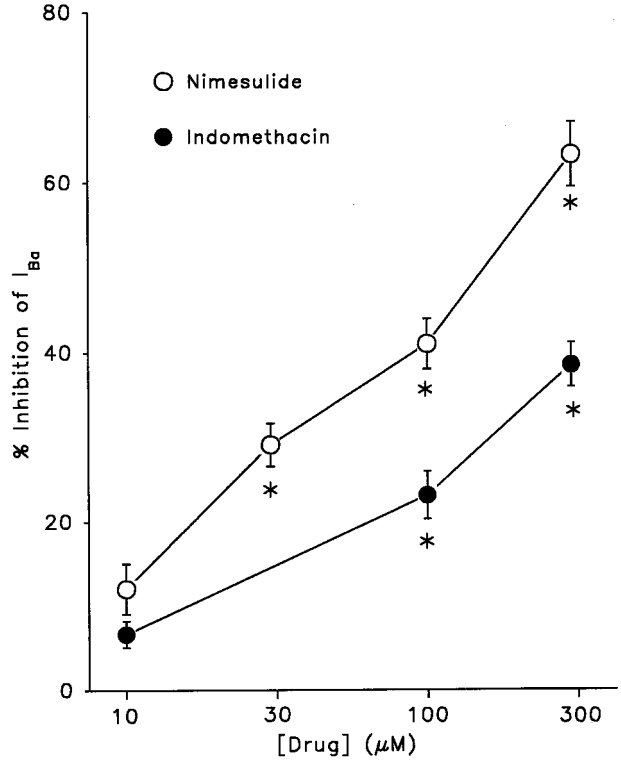

Figure 4 Concentration-dependency of the effect of nimesulide and indomethacin on the Ca^{2+} channel current. The current was integrated over the 200 ms step to +10 mV. Each value represents the mean and s.e.m. of the inhibition observed in 7–11 (nimesulide) or 5–7 (indomethacin) cells. Asterisks indicate significant inhibition by drug, $P<0.05$.

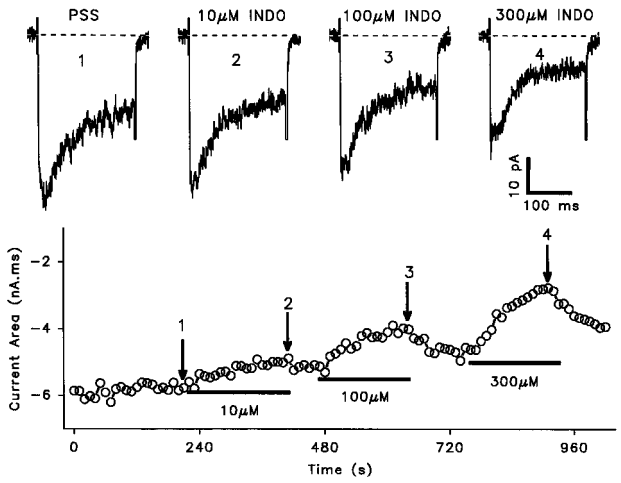

Figure 5 Effect of several concentrations of indomethacin on the Ca^{2+} channel current (I_{Ba}), elicited by 200 ms depolarizations to +10 mV from a holding potential of −50 mV, in a representative cell. The time course of the effect of indomethacin on integrated current is illustrated below. Numbered traces correspond to the respective numbered points in the time course.

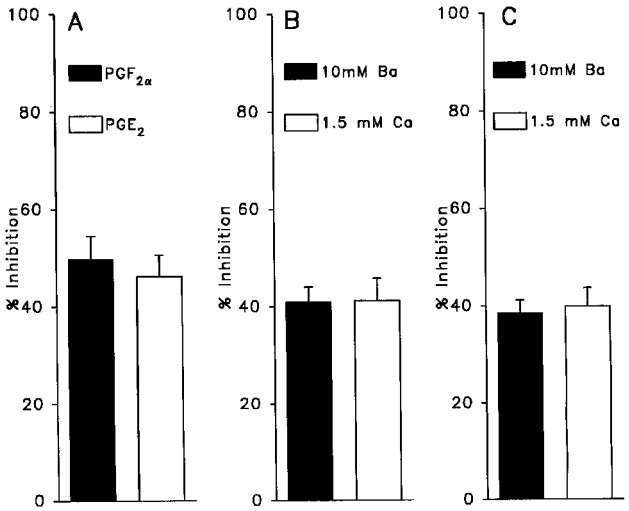

Figure 6 Lack of effects of prostaglandins and charge carrier on block of the Ca^{2+} channel current by nimesulide and indomethacin. (A) percent inhibition of the Ca^{2+} channel current by nimesulide in the presence of 30 μM $PGF_{2\alpha}$ or 30 μM PGE_2. 10 mM Ba^{2+} was used as the charge carrier, and these values are not significantly different than the control inhibition of the current, which is represented by the left column in panel (B). (B) similar inhibition by 100 μM nimesulide of the Ca^{2+} channel current with 10 mM Ba^{2+} or 1.5 mM Ca^{2+} as the charge carrier. (C) Similar inhibition by 300 μM indomethacin of the Ca^{2+} channel current with 10 μM Ba^{2+} or 1.5 mM Ca^{2+} as the charge carrier.

6A, the extent of I_{Ba} inhibition in the presence of either prostaglandin was similar to that observed in the absence of prostaglandin (shown by the left column in Figure 6B).

The response of the Ca^{2+} channel current to 100 μM nimesulide was also evaluated in nine cells using 1.5 mM Ca^{2+}, rather than 10 mM Ba^{2+}, as the charge carrier. The reduction of current amplitude induced by nimesulide when Ca^{2+} was used as the charge carrier was very similar to that observed with Ba^{2+} (Figure 6B). Moreover, the inhibition of the Ca^{2+} channel current by 300 μM indomethacin was similar whether 10 mM Ba^{2+} or 1.5 mM Ca^{2+} was used as the charge carrier (Figure 6C).

Discussion

Both nimesulide and indomethacin suppressed spontaneous myometrial contractility *in vitro*, with almost complete inhibition of activity at concentrations of 100 and 300 μM, respectively. The effect of both drugs on each index of spontaneous contractility was similar. At these concentrations, both drugs also reduced the Ca^{2+} channel current by approximately 40%. The inhibition of the Ca^{2+} channel current was unlikely to be secondary to an effect on prostaglandin production by cells, since the presence of either PGE_2 or $PGF_{2\alpha}$ in the solution did not significantly alter the extent of current blockade by nimesulide, and since the current amplitude declined rapidly after the application of both drugs.

A suppression by indomethacin of *in vitro* myometrial contractility was first described by Vane & Williams (1973), who found that 11 μM indomethacin gradually abolished both spontaneous myometrial contractions and uterine prostaglandin production in pregnant rat uterus. They concluded that intrinsic prostaglandin production maintained the spontaneous contractions in this preparation.

A similar effect of indomethacin and also fenoprofen on human myometrial strips was subsequently demonstrated by Johnson *et al.* (1975). In this study, however, the concentrations of COX inhibitors required to inhibit spontaneous contractions were very high, with less than 50% inhibition of contractility observed at 278 μM indomethacin and 191 μM fenoprofen. Crankshaw & Dyal (1994) similarly found that indomethacin concentrations up to 100 μM had no effect on spontaneous contractions of pregnant human myometrium *in vitro*. On the other hand, Garrioch (1978) observed that 50 μM indomethacin gradually abolished spontaneous contractions in non-pregnant and pregnant human myometrial strips, and Quaas *et al.* (1987) stated that human pregnant myometrial strips demonstrated little contractile activity when pre-treated with concentrations of indomethacin as low as 1 $\mu g\ ml^{-1}$ (2.8 μM). However, neither Garrioch (1978) nor Quaas *et al.* (1987) described the response to indomethacin of spontaneous contractions in any detail, and neither observation was supported by statistical analysis or any consideration of the effects of the drug vehicle. In our hands, indomethacin inhibited spontaneous myometrial activity over a concentration range somewhat lower than that found by Johnson *et al.* (1975).

In contrast, we have recently shown that nimesulide reduces PGE_2 production by human foetal membranes by 75 and 93% at 1 and 10 μM, while indomethacin reduces PGE_2 production by 82 and 90% at these concentrations. (Sawdy *et al.*, 1997b). Therefore the concentrations of both drugs required to suppress spontaneous *in vitro* contractions of human myometrium were approximately two orders of magnitude higher than those needed to inhibit uterine COX. These data agree with several previous reports which show that indomethacin, although more potent against COX-1, also inhibits COX-2 with micromolar (Mitchell *et al.*, 1993) or even submicromolar IC_{50} values (Miralpeix *et al.*, 1997; Yamada *et al.*, 1997). Similarly, nimesulide inhibits COX-2 at micromolar or submicromolar concentrations (Casolaro *et al.*, 1993; Miralpeix *et al.*, 1997; Yamada *et al.*, 1997). The foetal membranes and decidua are thought to be the principle sites of synthesis of oxytocic prostaglandins within the human uterus

(Skinner & Challis 1985), and the importance of the myometrium itself as a source of oxytocic prostaglandins is uncertain. The principle prostaglandin product of myometrium is prostacyclin (Bamford *et al*., 1980), which has no effect upon contractility (Crankshaw & Dyal, 1994).

These observations suggest that neither drug was likely to be suppressing *in vitro* contractility in pregnant human myometrium *via* an inhibition of prostaglandin production. However, both nimesulide and indomethacin reduced the Ca^{2+} channel current in human myometrial myocytes at concentrations which inhibited contractility. Nimesulide, which is currently being investigated as a tocolytic drug which may be free of adverse effects caused by COX-1 inhibition (Sawdy *et al*., 1997a), was slightly more potent as an antagonist of both the Ca^{2+} channel current and contractility. These results are therefore consistent with the possibility that, at least in part, both drugs may act as myometrial relaxants *via* Ca^{2+} channel blockade.

It is clear, however, that both drugs were less effective in inhibiting the Ca^{2+} channel current than in inhibiting contractility. The smaller effect on the current may imply that Ca^{2+} antagonism is only one of several mechanisms by which these drugs suppress contractions. It may also, however, stem from the methodological differences involved in studying contractility and ion channel currents. For example, the duration of the exposure of single cells to nimesulide or indomethacin was effectively limited by the durability of the cells under voltage clamp conditions and the possibility of slow run-down of the Ca^{2+} current. In addition, it might be that the drugs have some use-dependent effects on the Ca^{2+} channel which would favour blockade during the prolonged depolarizations and trains of action potentials which occur during spontaneous myometrial contractions. In agreement with this possibility, nimesulide blocked the current measured after 200 ms depolarization to a significantly larger extent than the current measured at its peak. Detailed analysis of the mechanisms of calcium antagonism by nimesulide will be the subject of future study. Finally, no data exist as to the relationship between Ca^{2+} channel blockade and the inhibition of spontaneous activity in this tissue, and it may be that partial reduction of the current has a disproportionate inhibitory effect on action potential activity and contractions.

Whether or not the effects of these drugs on the Ca^{2+} current bear any relevance to their *in vivo* tocolytic effect is unclear. However, total therapeutic plasma concentrations of indomethacin are approximately 1–2 μM, of which 90% is bound to plasma proteins (Berman *et al*., 1980). Both total and free plasma concentrations of indomethacin are therefore much lower during therapy than those which inhibited myometrial contractility and the Ca^{2+} current *in vitro*.

On the other hand, therapeutic total plasma concentrations of nimesulide are relatively high, ranging between 6 and 32 μM (Davis & Brogden, 1994). Nimesulide is 99% bound in plasma (Bree *et al*., 1993), indicating that its free plasma concentrations range is submicromolar. Nimesulide, however, is lipophilic, and its membrane concentration is therefore likely to be much higher than its free plasma concentration. It may be for this reason that nimesulide reaches a concentration in female reproductive organs which is 33–50% of the total plasma concentration (Davis & Brogden, 1994). In addition, many of the diverse effects of nimesulide which are thought to contribute to its *in vivo* anti-inflammatory action have relatively high (>10 μM) EC_{50} or IC_{50} values when these effects are assayed *in vitro* (Casolaro *et al*., 1993; Facino *et al*., 1993; Ottonello *et al*., 1995). In particular, it has been proposed that many of these actions are related to its ability to inhibit phosphodiesterase (PDE) type IV (Bevilacqua & Magni, 1993), and in neutrophils the concentration-dependency of the inhibition of PDE IV by nimesulide has an IC_{50} value of 30 μM (Bevilacqua *et al*., 1994).

These data suggest that nimesulide's total plasma concentration may better reflect its concentration at its sites of action than its free concentration. It is therefore not inconceivable that the effect of nimesulide on the Ca^{2+} channel current may play some role in reducing myometrial motility *in vivo*. On the other hand, the total plasma concentration of indomethacin is much too low for this drug to exert any Ca^{2+} channel blocking action *in vivo*, whereas its COX-antagonistic activity occurs at very low concentrations, so that these probably explain its tocolytic action.

In conclusion, our findings suggest that nimesulide, a potential tocolytic agent, may have a dual mechanism of action, inhibiting uterine contractility through a Ca^{2+} channel blocking mechanism and foetal membrane prostaglandin synthesis through inhibition of COX-2. Whether nimesulide ultimately enters widespread clinical practice as a useful tocolytic agent will depend upon the results of studies of its foetal side effect profile.

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