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Disturbance of peristalsis in the guinea-pig isolated small intestine by indomethacin, but not cyclo-oxygenase isoform-selective inhibitors

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1 Since the cyclo-oxygenase (COX) isoform-nonselective inhibitor indomethacin is known to modify intestinal motility, we analysed the effects of COX-1 and COX-2 inhibition on intestinal peristalsis.

2 Peristalsis in isolated segments of the guinea-pig small intestine was triggered by a rise of the intraluminal pressure and recorded *via* the pressure changes associated with peristalsis.

3 The COX-1 inhibitor SC-560, the COX-2 inhibitor NS-398 (both at 0.1 – 1 μM) and the isoform-nonselective inhibitors flurbiprofen (0.01 – 10 μM) and piroxicam (0.1 – 50 μM) were without major influence on peristalsis, whereas indomethacin and etodolac (0.1 – 10 μM) disturbed the regularity of peristalsis by causing nonpropulsive circular muscle contractions.

4 Radioimmunoassay measurements showed that SC-560, NS-398, indomethacin and etodolac (each at 1 μM) suppressed the release of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) from the intestinal segments.

5 Reverse transcription – polymerase chain reaction tests revealed that, relative to glyceraldehyde-3 phosphate dehydrogenase ribonucleic acid, the expression of COX-1 mRNA increased by a factor of 2.0 whereas that of COX-2 mRNA rose by a factor of 7.9 during the 2 h experimental period.

6 Pharmacological experiments indicated that the action of indomethacin to disturb intestinal peristalsis was unrelated to inhibition of L-type calcium channels, adenosine triphosphate-sensitive potassium channels or phosphodiesterase type IV.

7 These results show that selective inhibition of COX-1 and COX-2 does not grossly alter peristaltic motor activity in the guinea-pig isolated small intestine and that the effect of indomethacin to disturb the regular pattern of propulsive motility in this species is unrelated to COX inhibition.

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Abbreviations: ANOVA, one-way analysis of variance; ATP, adenosine triphosphate; cyclic AMP, cyclic adenosine monophosphate; COX, cyclo-oxygenase; DNA, deoxyribonucleic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; PPT, peristaltic pressure threshold; RNA, ribonucleic acid; RT – PCR, reverse transcription – polymerase chain reaction; TXA_2, thromboxane A_2

Introduction

A common mechanism of action of nonsteroidal anti-inflammatory drugs (NSAIDs) is inhibition of cyclo-oxygenase (COX) which exists in two isoforms, COX-1 and COX-2 (Vane *et al*., 1998; Mitchell & Warner, 1999; Warner *et al*., 1999). The gastrointestinal tract expresses high levels of COX-1 which functions as a house-keeping enzyme (Vane *et al*., 1998) but, under physiological conditions, contains only low levels of COX-2 (Kargman *et al*., 1996; Ferraz *et al*., 1997; Maricic *et al*., 1999). Most studies of the gastrointestinal function of COX isoforms relate to mucosal homeostasis, given that both COX-1 and COX-2 inhibition carries a risk of ulceration (Warner *et al*., 1999; Wallace *et al*., 2000), whereas little is known about the roles which COX-1 and COX-2 may play in the regulation of gastrointestinal motility.

Several investigations have shown that the NSAID indomethacin influences intestinal peristalsis, the clinically most relevant motor pattern of the gut. Thus, indomethacin alters the slow wave pattern in the canine colon (Franck *et al*., 1999), weakens peristaltic performance in the guinea-pig isolated ileum and colon (Bennett *et al*., 1976; Fontaine *et al*., 1977) and impairs peristalsis in the human colon (Bruch *et al*., 1978). These antiperistaltic actions are contrasted by the ability of indomethacin to evoke phasic contractions of the circular muscle in the guinea-pig small intestine (Maggi *et al*., 1994). It has not yet been examined, though, whether or not the intestinal motor effects of indomethacin are due to inhibition of COX-1 and/or COX-2. The first aim of this study, therefore, was to test a range of COX-1/COX-2

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isoform-selective and -nonselective inhibitors (SC-560, NS-398, indomethacin, flurbiprofen, piroxicam and etodolac) for their effects on propulsive motor activity in the guinea-pig isolated small intestine. The effects of sodium salicylate, the 5-lipoxygenase inhibitor Bay X 1005 and the thromboxane A_2 (TXA_2) receptor antagonist SQ-29,548 were also examined.

The second aim of our investigation was to check whether indomethacin and some of the other NSAIDs under study (SC-560, NS-398 and etodolac) inhibit the biosynthetic release of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), a stable metabolite of PGI_2, from the peristaltically active segments of intestine. A separate trial addressed the question whether the formation of 6-keto-$PGF_{1\alpha}$ changes when the intestinal segments are kept at rest or stimulated to perform peristaltic motor activity. Since injury and inflammation cause upregulation of COX-2 in the gastrointestinal tract (Feraz *et al*., 1997; Maricic *et al*., 1999), we also investigated the possibility that excision of the intestinal segments and their maintenance in organ baths alters the expression of COX-2 and/or COX-1.

The inhibitory effect of indomethacin on intestinal peristalsis is prevented by PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ (Bennett *et al*., 1976), which indirectly suggests that deficient PG synthesis accounts for the motor response to indomethacin. Although the reversal of indomethacin's adverse peristaltic motor action by PGE_1 was confirmed, we also addressed the possibility that indomethacin may disturb peristalsis by blocking L-type calcium channels in intestinal muscle (Northover, 1977; Burch *et al*., 1983; Franck *et al*., 1999), by preventing the effect of endogenous PGs to activate adenosine triphosphate (ATP)-sensitive potassium channels (Hide *et al*., 1995) or by inhibiting cyclic adenosine monophosphate (cyclic AMP)-specific phosophodiesterase of type IV (Flower & Vane, 1974; Newcombe *et al*., 1974; Beatty *et al*., 1976).

Methods

Propulsive peristalsis

Recording of peristalsis The small intestine of adult guinea-pigs (TRIK strain, either sex, 350–450 g body weight) was isolated, flushed of luminal contents and placed, for up to 4 h, in Tyrode solution kept at room temperature and oxygenated with a mixture of 95% O_2 and 5% CO_2 (Heinemann *et al*., 1999). The composition of the Tyrode solution was (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.6. The jejunum and ileum were divided into eight segments, each being approximately 10 cm long. Four intestinal segments were set up in parallel and secured horizontally in organ baths containing 30 ml of Tyrode solution at 37°C. In order to elicit propulsive peristalsis, prewarmed Tyrode solution was continuously infused into the lumen of the segments at a rate of 0.5 ml min^{-1} (Heinemann *et al*., 1999). The intraluminal pressure at the aboral end of the segments was measured with a pressure transducer whose signal was, *via* an analogue/digital converter, fed into a personal computer and recorded and analysed with the software 'Peristal 1.0' (Heinemann *et al*., 1999).

The fluid passing through the gut lumen was directed into a vertical outlet tubing which ended 4 cm above the fluid level in the organ bath. When fluid was infused, the intraluminal pressure rose slowly until it reached a threshold at which peristalsis was triggered (Figure 1). The aborally moving wave of peristaltic contraction resulted in a spike-like increase in the intraluminal pressure (the 'peristaltic wave'), which caused emptying of the segment if the maximal pressure of the peristaltic wave exceeded the level of 400 Pa as set by the position of the outlet tubing.

Experimental protocol The preparations were allowed to equilibrate in the organ bath for a period of 30 min during which they were kept in a quiescent state. Thereafter the bath fluid was renewed and peristaltic motility initiated by intraluminal perfusion of the segments. After basal peristaltic activity had been recorded for a 30 min period, the drugs to be tested were added to the bath, i.e., to the serosal surface of the intestinal segments, at volumes not exceeding 1% of the bath volume. The corresponding vehicle solutions were devoid of any effect.

Four sets of experiments were performed. Firstly, the peristaltic motor effects of SC-560, NS-398 (0.1–1 μM), Bay X 1005, indomethacin, etodolac (0.1–10 μM), flurbiprofen (0.01–10 μM), piroxicam (0.1–50 μM), sodium salicylate (1–100 μM) and SQ-29,548 (10 μM) were studied. Except SQ-29,548, the drugs were added to the bath in a cumulative manner at 15 min intervals. Secondly, the peristaltic motor effects of SC-560 (1 μM), NS-398 (1 μM) and the combination of SC-560 (1 μM) plus NS-398 (1 μM) were compared with each other during two consecutive 15 min periods. Thirdly, the ability of PGE_1 or U-46,619 to cancel out the peristaltic motor disturbance caused by indomethacin (3 μM) or S(−)-Bay K 8644 (1 μM) was explored. To this end, the segments were first exposed to indomethacin (3 μM) or S(−)-Bay K 8644 (1 μM) for two 15 min periods, after which vehicle, PGE_1 (0.1 μM) or U-46,619 (0.1 μM) was added to the bath and peristalsis recorded for two further 15 min periods. Fourthly, the peristaltic motor effects of S(−)-Bay K 8644 (0.1–3 μM), R(+)-Bay K 8644 (0.1–10 μM), cromakalim, glibenclamide (1–100 μM), forskolin (0.01–1 μM) and rolipram (0.1–30 μM) were examined, these drugs being added to the bath in a cumulative manner at 15 min intervals. Each protocol was carried out with at least six segments from six different guinea-pigs.

Evaluation of peristalsis The recordings of peristalsis were analysed with the software 'Peristal 1.0' with regard to four different parameters: the peristaltic pressure threshold (PPT), the residual baseline pressure, the amplitude (maximal pressure) of the peristaltic waves and the maximal acceleration of the peristaltic waves. PPT (Pa) is the intraluminal pressure at which a peristaltic wave is triggered. Inhibition of peristalsis was associated with an increase in PPT, and abolition of peristalsis manifested itself in a lack of propulsive motility in spite of an intraluminal pressure of 400 Pa. Although in this case PPT exceeded 400 Pa, abolition of peristalsis was expressed quantitatively by assigning PPT a value of 400 Pa in order to obtain numerical results suitable for further statistical evaluation. The residual baseline pressure (Pa) equals the minimal intraluminal pressure that is achieved after completion of each peristaltic wave and thus reflects a sensitive measure of the emptying capacity of the peristaltic waves (Heinemann *et al*., 1999). Further indices of

peristaltic effectiveness are the amplitude of the peristaltic waves (Pa) and the maximal acceleration of the peristaltic waves (Pa s^{-2}), which is determined not only by the speed with which the muscle contracts but also by the speed with which the contraction moves aborally to empty the segments.

The effects of drugs on peristalsis were quantified such that all peristaltic waves occurring during the 15 min observation periods were analysed and the peristalsis parameters averaged for each observation period. In addition, the time during which peristalsis was replaced by irregular nonperistaltic contractions was also determined for each 15 min observation period. In order to allow for a better comparison of the data obtained in different preparations, the average baseline parameters recorded in each experimental group were set as 100% and the parameters recorded in the presence of the drugs and substances expressed as a percentage of the baseline parameters.

Biosynthetic release of 6-keto-$PGF_{1\alpha}$

Experimental protocol Segments of the small intestine were set up in the organ baths and equilibrated for 30 min while they were kept at peristaltic rest. After a change of the bath fluid the segments were incubated for three periods of 30 min, the bath fluid being changed every 30 min. During this incubation period of 90 min the preparations were subjected to two different treatment protocols. In the first protocol, the segments were induced to perform continuous peristalsis for 90 min. Concomitantly with initiation of peristalsis the segments were exposed to NS-398 (1 μM), SC-560 (1 μM), indomethacin (1 μM), etodolac (1 μM) or their vehicle, the drugs being present throughout the 90 min incubation period. These experiments were carried out to assess the effect of COX inhibitors on the biosynthetic release of 6-keto-$PGF_{1\alpha}$ from peristaltically active gut segments.

The second protocol addressed the question as to whether peristaltic activity modulates the biosynthetic release of 6-keto-$PGF_{1\alpha}$. To this end, two experiments were carried out. In one experiment the segments were kept peristaltically quiescent during the first and second incubation periods and peristalsis elicited in the third incubation period only. In the other experiment the segments were induced to perform peristalsis during the first and second incubation period but kept at rest during the third incubation period.

In all experiments, aliquots of 1.5 ml bath fluid were collected at the end of the second and third incubation periods, frozen in liquid nitrogen and stored at -80°C until assay.

Radioimmunoassay Immunoreactive 6-keto-$PGF_{1\alpha}$ in the bath fluid was determined by a specific radioimmunoassay as described previously (Peskar *et al*., 1979; Schuligoi *et al*., 1997). The assay used synthetic 6-keto-$PGF_{1\alpha}$ as standard and 6-keto[5,8,9,11,12,14,15(n)-^{3}H]-$PGF_{1\alpha}$ (specific activity 4.8–7.4 TBq $mmol^{-1}$) as tracer. The assay detection limit, defined as 10% displacement of tracer binding to the antibody, amounted to 20 ± 2.3 pg 6-keto-$PGF_{1\alpha}$ per ml bath fluid ($n=4$).

COX-1 and COX-2 expression determined by reverse transcription–polymerase chain reaction (RT–PCR)

After excision of the small intestine, three full thickness rings (about 3 mm wide) were cut from intestinal segments before they were set up in the organ baths. Following a 30 min period of rest the segments were induced to perform continuous peristalsis for 90 min. After this time another set of three full thickness rings was collected from the segments. The tissue samples were immediately frozen and kept at -80°C until RT–PCR which was performed as described previously (Amann *et al*., 1999). Total ribonucleic acid (RNA) was extracted with Trizol (Life Technologies, Gaithersburg, MD, U.S.A.) and, after treatment with RNase-free DNase I (Roche, Vienna, Austria) to remove contaminating deoxyribonucleic acid (DNA), purified through a Nucleo-Spin Kit (Machery & Nagel, Düren, Germany).

RT of 0.8 μg RNA was performed with avian myoblastosis virus reverse transcriptase and an oligo $(dT)_{15}$ primer (Promega, Mannheim, Germany). Three- and 1-μl volumes of the RT products were used to amplify the COX-1/COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals, respectively. PCR was carried out in 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates (Sigma-RBI, Vienna, Austria), 50 pmol specific primers and 0.25 U Taq polymerase (Promega). After denaturation of the samples for 2 min at 95°C, specific cDNA was amplified in a RoboCycler (Stratagene, La Jolla, CA, U.S.A.) according to the cycling programme: 95°C for 1 min, 59°C for 1 min, and 72°C for 2 min. The final extension time was 4 min at 72°C. Thirty-six cycles were performed for COX-1 and COX-2, and 25 cycles for GAPDH, amplification.

Specific primers for guinea-pig COX-2 cDNA (sense: 5′-GGTGCATAGCGTAATGTCCATGT-3′ and antisense: 5′-ATGTAGGGTGGGTACATCATCTCT-3′) were chosen according to the published sequence of guinea-pig COX-2 DNA (Bracken *et al*., 1997). Since the nucleotide sequence of the guinea-pig COX-1 gene awaits disclosure, the COX-1 primers (sense: 5′-GTGTGACCTGCTGAAGGCTGAGCAC-3′ and antisense: 5′-CTTGCGGTACTCATTGAAGGGCTGC-3′) were chosen to match regions that are highly conserved among mammalian species (Bracken *et al*., 1997). The GAPDH primers obtained from Clontech (Palo Alto, CA, U.S.A.) also span a highly conserved region of the gene.

The PCR products were separated by electrophoresis in an agarose gel stained with ethidium bromide. The specific primers for COX-2, COX-1 and GAPDH yielded amplification products of 334, 468 and 450 base pairs, respectively. The ethidium bromide-stained bands were visualized under ultraviolet light with a Gel Doc 2000 system (Bio-Rad, Hercules, CA, U.S.A.) and quantified with the Quantity One software (Bio-Rad). GAPDH was used as an internal standard and changes in COX expression presented *via* the ratio of COX/GAPDH PCR products.

Drugs and solutions

The sources of the drugs used here were as follows. R(+)-Bay K 8644 and S(−)-Bay K 8644, cromakalim, etodolac, forskolin, (±)-flurbiprofen, glibenclamide, indomethacin, N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS-398), PGE_1, piroxicam, rolipram, sodium salicylate and [1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptaonic acid (SQ-29,548) were obtained from Sigma-RBI. 9,11-Dideoxy-9α, 11α-methanoepoxy-$PGF_{2\alpha}$ (U-46,619) and 6-keto-$PGF_{1\alpha}$ were bought from Cayman (Ann Arbor, MI, U.S.A.) and 6-

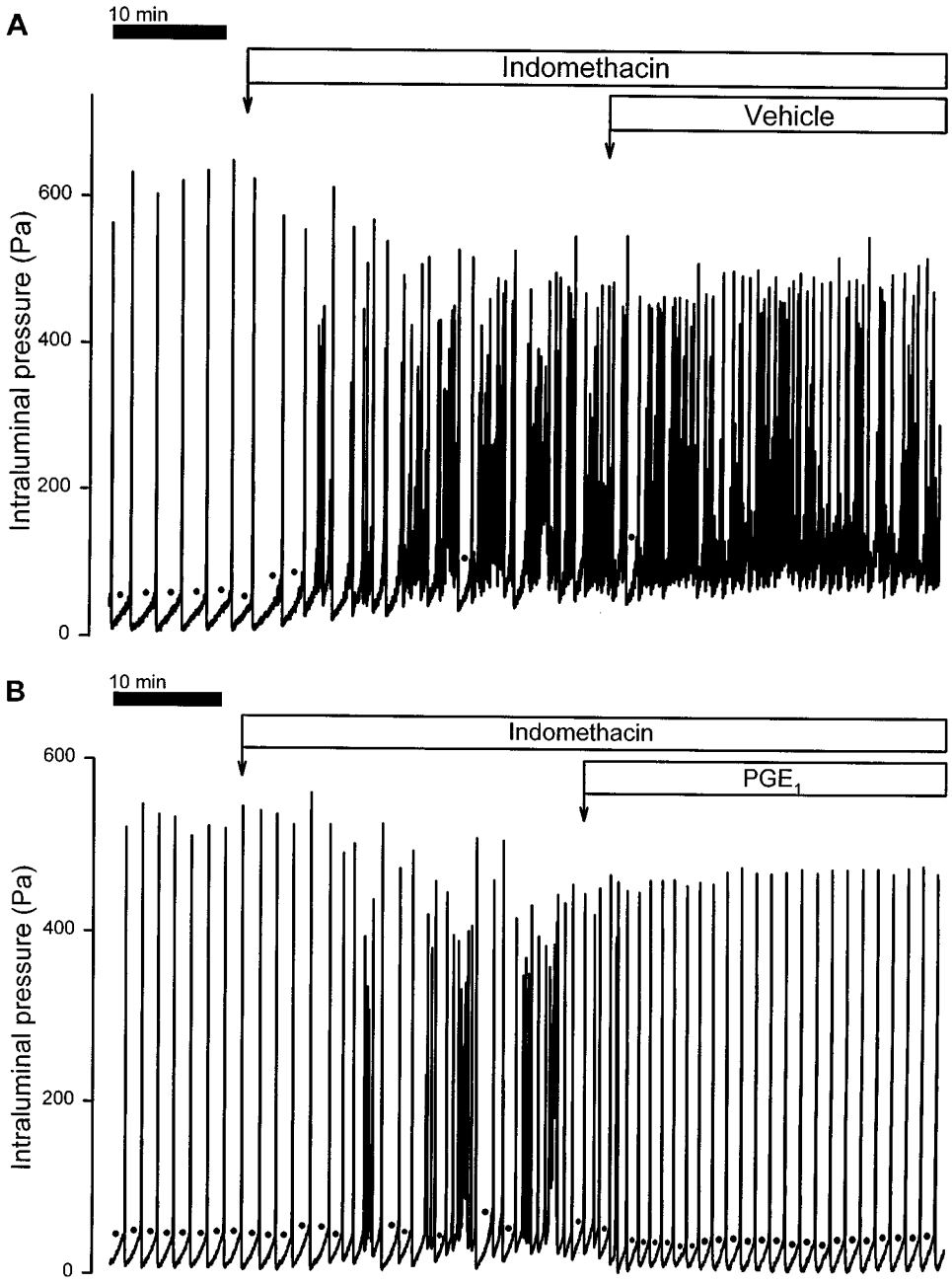

Figure 1 Recording of the peristaltic motor disturbance caused by indomethacin (3 μM) and the subsequent action of vehicle (A) and PGE_1 (0.1 μM; B) to stop the response to indomethacin. All drugs were added to the organ bath. The dots indicate the pressure threshold of the peristaltic waves.

keto[5,8,9,11,12,14,15(n)-^{3}H]-$PGF_{1\alpha}$ from Amersham (Vienna, Austria). Bay X 1005 was a gift of Bayer (Wuppertal, Germany) and 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC-560) was a gift of Searle (Skokie, IL, U.S.A.). The drugs were dissolved with appropriate media, the concentrations given hereafter in parenthesis referring to the stock solutions. R(+)-Bay K 8644, S(−)-Bay K 8644, etodolac, flurbiprofen, forskolin, rolipram, SQ-29,548 (10 mM) and SC-560 (2.5 mM) were dissolved in ethanol. Cromakalim, glibenclamide (100 mM), piroxicam (50 mM), Bay X 1005, NS-398 and U-46,619 (10 mM) were dissolved in dimethyl sulphoxide, PGE_1 (1 mM) in methanol, indomethacin (1 mM) in 0.5 M phosphate buffer of pH 7.4 and sodium salicylate (100 mM) in Tyrode solution. These stock solutions were diluted with Tyrode solution as required, except that of glibenclamide which was diluted with dimethyl sulphoxide. Care was taken that none of the organic solvents reached concentrations higher than 0.1% in the bathing solution.

Statistics

Quantitative data are presented as means ± s.e.mean (unless stated otherwise) of n experiments, n referring to the number

of guinea-pigs used in the test. The results were evaluated with Student's two sample *t*-test or with one-way analysis of variance (ANOVA) for repeated measures followed by Dunnett's test, as appropriate. Probability values of $P<0.05$ were regarded as significant.

Results

Peristaltic motor effects of COX-1, COX-2 and 5-lipoxygenase inhibitors and a TXA_2 receptor antagonist

Quantitative estimates of the peristalsis parameters at baseline were: PPT, 85 ± 5.4 Pa; residual baseline pressure, 18 ± 1.6 Pa; maximal pressure of peristaltic waves, 572 ± 9.0 Pa; and maximal acceleration of peristaltic waves, 283 ± 9.3 Pa s^{-2} ($n=59$). Administration of the COX isoform-nonselective inhibitor indomethacin to the organ bath disturbed the regularity of peristaltic motor waves by inducing circular muscle spasms of a stationary nature (Figure 1). The time during which peristalsis was replaced by these irregular nonperistaltic contractions was closely related to the concentration of indomethacin (Figure 2). Since the motor action of indomethacin became maximal within 10–15 min and was sustained (Figure 1), it was possible to record its concentration-response relationship in a cumulative manner at 15 min intervals (Figures 2 and 3). When the peristaltic waves remaining in between the nonpropulsive contractions were evaluated it was seen that indomethacin led to a concentration-dependent rise of PPT and the residual baseline pressure (Figure 3A,B) and to a quantitatively minor reduction of the amplitude (maximal pressure) and maximal acceleration of the peristaltic waves (Figure 3C,D).

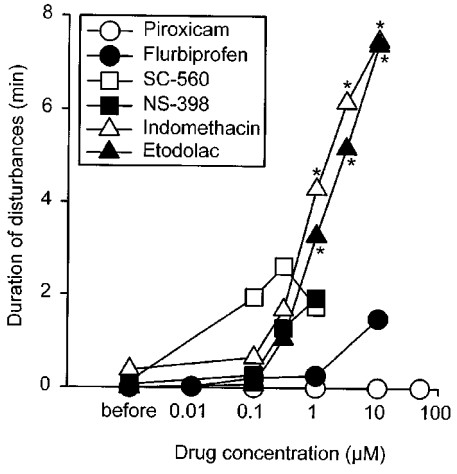

Figure 2 Concentration-response relationship for the effects of etodolac, flurbiprofen, indomethacin, piroxicam, SC-560 and NS-398 to disturb regular peristalsis. The concentration-response curves were recorded in a cumulative manner at 15-min intervals, and the ordinate gives the time during which peristalsis was replaced by irregular nonperistaltic contractions for each 15 min observation period. The values represent means; $n=8$ for piroxicam, $n=13-18$ for etodolac, flurbiprofen, indomethacin, NS-398 and SC-560. Standard errors of the mean are not shown because some of them overlap. * $P<0.05$ versus observation period immediately before drug administration (ANOVA for repeated measures followed by Dunnett's test).

Etodolac (0.1–10 μM), a COX isoform-nonselective inhibitor with some preference for COX-2 (Warner *et al*., 1999), disrupted the regularity of peristalsis in a manner similar to that of indomethacin (Figure 2) and attenuated the amplitude of the peristaltic waves (Figure 3C). In contrast, the COX isoform-nonselective inhibitors flurbiprofen (0.01–10 μM) and piroxicam (0.1–50 μM) did not significantly disturb the regularity of peristalsis (Figure 2). Of the four peristalsis parameters under study (Figure 3), only the maximal pressure of the peristaltic waves was decreased by both flurbiprofen and piroxicam, whereas PPT and the residual baseline pressure was enhanced by piroxicam only. The COX-1 selective inhibitor SC-560 (Smith *et al*., 1998) and the COX-2 selective inhibitor NS-398 (Futaki *et al*., 1994) were also without major influence on peristalsis (Figures 2 and 3). Both SC-560 (0.1–1 μM) and NS-398 (0.1–1 μM) failed to disturb the regularity of peristalsis to any significant extent (Figure 2). While SC-560 increased the residual baseline pressure and decreased the amplitude and maximal acceleration of the peristaltic waves to a small extent, NS-398 failed to alter the peristalsis parameters except for a slight attenuation of the peristaltic wave amplitude (Figure 3).

In further experiments it was tested whether combined administration of SC-560 plus NS-398 would disturb peristalsis to a larger extent than either drug alone. For this reason, the peristaltic motor effects of SC-560 (1 μM), NS-398 (1 μM) and SC-560 (1 μM) plus NS-398 (1 μM) were compared with each other. As shown in Table 1, neither treatment caused any significant disturbance of peristalsis, and combined exposure of segments to SC-560 plus NS-398

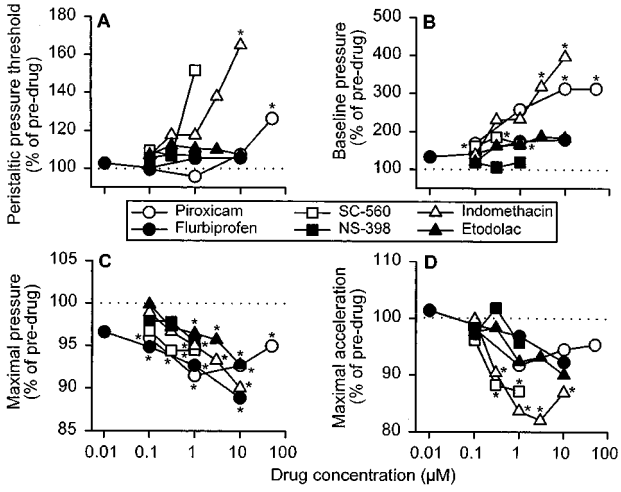

Figure 3 Concentration-response relationship for the effects of the COX isoform-nonselective inhibitors etodolac, flurbiprofen, indomethacin and piroxicam, the COX-1 isoform-selective inhibitor SC-560 and the COX-2 isoform-selective inhibitor NS-398 to alter peristaltic pressure threshold (PPT; A), residual baseline pressure (B), amplitude (maximal pressure; C) and maximal acceleration (D) of the peristaltic waves. The concentration-response curves were recorded in a cumulative manner at 15-min intervals, and the ordinate gives the average peristalsis parameters for each 15-min observation. The values represent means; $n=8$ for piroxicam, $n=13-18$ for etodolac, flurbiprofen, indomethacin, NS-398 and SC-560. Standard errors of the mean are not shown because some of them overlap. *$P<0.05$ versus observation period immediately before drug administration (pre-drug value = 100%; ANOVA for repeated measures followed by Dunnett's test).

Table 1 Failure of SC-560, NS-398 and SC-560 plus NS-398 to disturb the regularity of peristaltic waves

Drug	n	*Duration of disturbances in the 15-min period pre-drug* (min)	*Duration of disturbances in the first 15-min period post-drug* (min)	*Duration of disturbances in the second 15-min period post drug* (min)
SC-560 (1 μM)	9	0	1.17±0.77	0
NS-398 (1 μM)	10	0	1.59±1.22	0.51±0.51
SC-560 (1 μM) plus NS-398 (1 μM)	10	0	1.53±0.93	0.93±0.93

Intestinal segments were incubated with the drugs for two 15-min periods, and the time during which peristalsis was replaced by irregular nonperistaltic contractions in the 15-min period before, and two 15-min observation periods after, drug exposure evaluated. The values represent means±s.e.mean. No significant differences between the three observation periods and the three drug treatment groups were noted.

failed to disturb peristalsis to a larger degree than either drug alone.

The 5-lipoxygenase inhibitor Bay X 1005 (0.1–10 μM, $n=6$), the TXA_2 receptor antagonist SQ-29,548 (10 μM, $n=8$) and sodium salicylate (1–100 μM, $n=8$), a NSAID devoid of significant anti-COX activity (Warner *et al*., 1999), did not significantly alter any of the peristalsis parameters (data not shown).

Peristaltic motor effects of drugs acting on calcium or potassium channels or on cyclic AMP metabolism

The L-type calcium channel activator S(−)-Bay K 8644 and blocker R(+)-Bay K 8644, the ATP-sensitive potassium channel activator cromakalim and blocker glibenclamide, the adenylate cyclase stimulant forskolin and the inhibitor of phosphodiesterase type IV rolipram were tested for their effects on peristalsis. The drugs were added to the bath in a cumulative manner at 15 min intervals. Like indomethacin (Figure 1), S(−)-Bay K 8644 (0.1–3 μM) disturbed peristalsis by inducing nonperistaltic contractions, an effect that was concentration-dependent (Figure 4). At low concentrations (0.1–0.3 μM) which did not completely disrupt the regularity of peristaltic waves, S(−)-Bay K 8644 lowered PPT, the maximal pressure and the maximal acceleration of the peristaltic waves (Figure 5A,C,D). R(+)-Bay K 8644 (0.1–10 μM) did not disturb the regular pattern of peristalsis (Figure 4) but inhibited the effectiveness of peristaltic waves, and, at 10 μM, abolished peristaltic activity. At lower concentrations of R(+)-Bay K 8644, PPT and the residual baseline pressure were enhanced and the maximal pressure and the maximal acceleration of the peristaltic waves reduced (Figure 5).

Cromakalim (1–100 μM, $n=6$), glibenclamide (1–100 μM, $n=7$), forskolin (0.01–1 μM, $n=7$) and rolipram (0.1–30 μM, $n=6$) failed to disrupt the regularity of peristalsis (data not shown). The only effect seen with glibenclamide was a small rise of PPT at 100 μM (Figure 5A), while forskolin caused a minor reduction of the maximal pressure and the maximal acceleration of peristaltic waves (Figure 5C,D). In contrast, cromakalim and rolipram led to a concentration-related inhibition of peristalsis, which became manifest by a rise of PPT and the residual baseline pressure and a fall of the maximal pressure and the maximal acceleration of the peristaltic waves (Figure 5).

Effects of PGE_1 or U-46,619 to stop the peristaltic disturbances due to indomethacin or S(−)-Bay K 8644

The preparations were first exposed to indomethacin (3 μM) or S(−)-Bay K 8644 (1 μM) for two 15-min periods, after which vehicle, PGE_1 (0.1 μM) or the TXA_2 receptor agonist U-46,619 (0.1 μM) was added to the bath and peristalsis recorded for two further 15-min periods. In contrast to vehicle and U-46,619, PGE_1 promptly terminated the indomethacin-evoked irregularities of peristalsis (Figures 1 and 6). The peristaltic disturbances caused by S(−)-Bay K 8644 were also counteracted by PGE_1 (Figure 6).

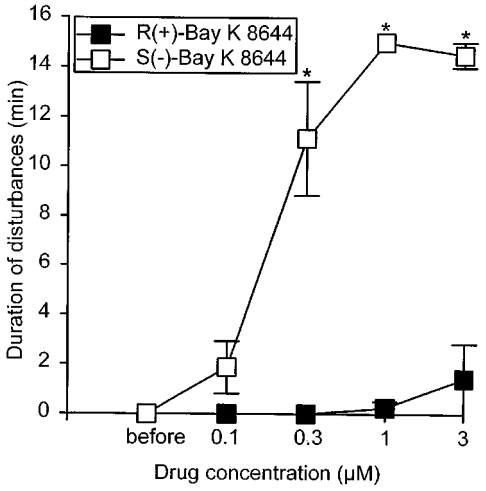

Figure 4 Concentration-response relationship for the effects of R(+)-Bay K 8644 and S(−)-Bay K 8644 to disturb regular peristalsis. The concentration-response curves were recorded in a cumulative manner at 15-min intervals, and the ordinate gives the time during which peristalsis was replaced by irregular nonperistaltic contractions for each 15 min observation period. The values represent means±s.e.mean, $n=6$. *$P<0.05$ versus observation period immediately before drug administration (ANOVA for repeated measures followed by Dunnett's test).

Effect of COX inhibitors on the biosynthetic release of 6-keto-$PGF_{1\alpha}$ from peristaltically active gut segments

Intestinal segments were incubated with COX inhibitors for 3 periods of 30 min, during which they were induced to perform continuous peristalsis. In the presence of vehicle there was a constant biosynthetic release of 6-keto-$PGF_{1\alpha}$ into the organ bath as assessed during the second and third incubation period. Since the prostanoid levels in the two incubation periods were similar, only those of the third period are presented (Figure 7). At the concentration of 1 μM, indomethacin, etodolac, SC-560 and NS-398 depressed the release of 6-keto-$PGF_{1\alpha}$ to a significant extent, the depression of 6-keto-$PGF_{1\alpha}$ being 93% for SC-560, 86% for indomethacin, 72% for NS-398 and 38% for etodolac versus the respective vehicle controls (Figure 7). In contrast, sodium

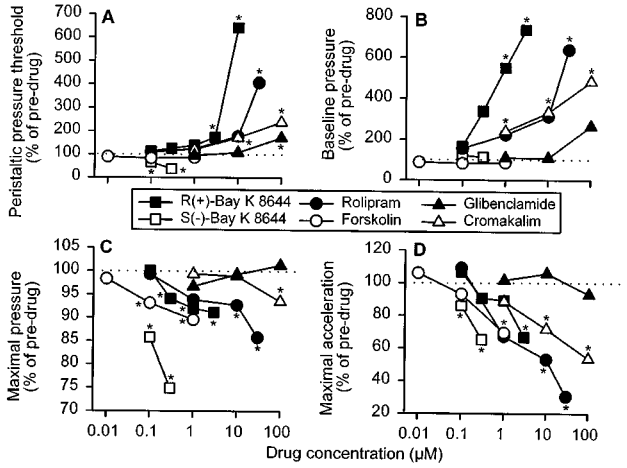

Figure 5 Concentration-response relationship for the effects of R(+)-Bay K 8644, S(−)-Bay K 8644, cromakalim, glibenclamide, forskolin and rolipram to alter peristaltic pressure threshold (PPT; A), residual baseline pressure (B), amplitude (maximal pressure; C) and maximal acceleration (D) of the peristaltic waves. The concentration-response curves were recorded in a cumulative manner at 15-min intervals, and the ordinate gives the average peristalsis parameters for each 15-min observation period. The values represent means; $n=6-7$. Standard errors of the mean are not shown because some of them overlap. $^*P<0.05$ versus observation period immediately before drug administration (pre-drug value = 100%; ANOVA for repeated measures followed by Dunnett's test).

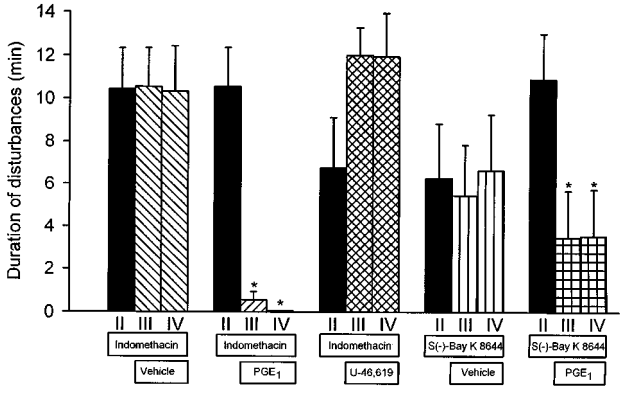

Figure 6 Effects of PGE_1 (0.1 μM), U-46,619 (0.1 μM) or vehicle to stop the peristaltic motor disturbance caused by indomethacin (3 μM) or S(−)-Bay K 8644 (1 μM). The segments were preincubated with indomethacin or S(−)-Bay K 8644 for two 15-min periods, after which the preparations were exposed to indomethacin plus vehicle, indomethacin plus PGE_1, indomethacin plus U-46,619, S(−)-Bay K 8644 plus vehicle or S(−)-Bay K 8644 plus PGE_1 for two further 15-min periods. The graph shows the time during which peristalsis was replaced by irregular nonperistaltic contractions in observation periods II–IV. The values represent means + s.e.mean, $n=8-9$. $^*P<0.05$ versus respective value recorded in period II (ANOVA for repeated measures followed by Dunnett's test).

salicylate (100 μM) failed to reduce the release of 6-keto-$PGF_{1\alpha}$ (Figure 7).

Relationship between peristaltic motility and biosynthetic release of 6-keto-$PGF_{1\alpha}$

The experiments revealed that peristaltic motor activity does not modulate the biosynthetic release of 6-keto-$PGF_{1\alpha}$ into the organ bath. Thus, the release of 6-keto-$PGF_{1\alpha}$ did not significantly change when the segments were first kept peristaltically quiescent and then induced to perform peristaltic motility (524 ± 118 and 550 ± 100 pg ml^{-1} 6-keto-$PGF_{1\alpha}$ in bath fluid, $n=12$) or when this order of activity was reversed to an initial period of peristalsis followed by a rest period (655 ± 153 and 619 ± 122 6-keto-$PGF_{1\alpha}$ pg ml^{-1}, $n=11$).

COX-1 and COX-2 mRNA expression during the in vitro *peristalsis experiments*

After set up in the organ baths, intestinal segments were allowed a 30 min period of rest which was followed by a 90 min period of peristaltic motor activity. RT–PCR showed that, relative to GAPDH, COX-1 and COX-2 mRNA expression significantly increased during the course of the 2 h *in vitro* peristalsis experiment (Figure 8). The expression of COX-1 mRNA rose by a factor of 2.0, whereas the expression of COX-2 mRNA, which was very low at the beginning, increased by a factor of 7.9 (Figure 8).

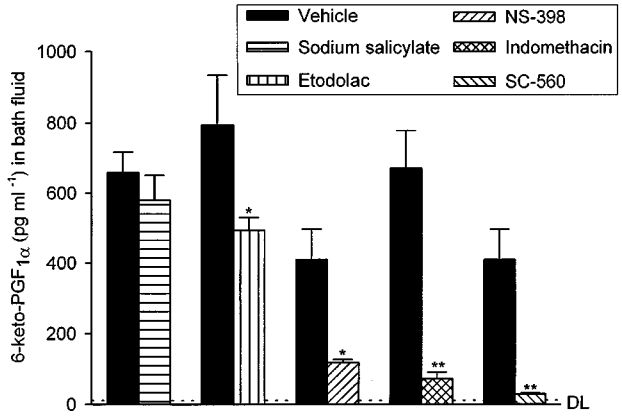

Figure 7 Effects of sodium salicylate, etodolac, indomethacin, SC-560 and NS-398 (all at 1 μM), relative to their vehicle, to inhibit the biosynthetic release of 6-keto-$PGF_{1\alpha}$ from intestinal segments into the organ bath. Intestinal segments performing peristalsis were incubated with vehicle or one of the drugs for three 30 min periods. The values which show the release of 6-keto-$PGF_{1\alpha}$ during the third incubation period represent means + s.e.mean, $n=5$. $^*P<0.05$, $^{**}P<0.01$ versus respective vehicle controls (two sample t-test). DL = detection limit.

Discussion

The major observation of this study was that, among the various test compounds with an inhibitory action on COX-1 and/or COX-2 (Table 2), only indomethacin and etodolac disrupted propulsive motility in the guinea-pig isolated small intestine, whereas other COX inhibitors were without major influence on peristalsis. Indomethacin and etodolac impaired peristaltic motor performance through the induction of nonpropulsive, segmental contractions which replaced the aborally moving peristaltic waves and thus disturbed the regularity of peristaltic motility. The parameters of the propulsive movements which remained in the presence of indomethacin and etodolac were not altered to any major extent, although the decrease in the amplitude and maximal acceleration of the peristaltic waves points to a minor

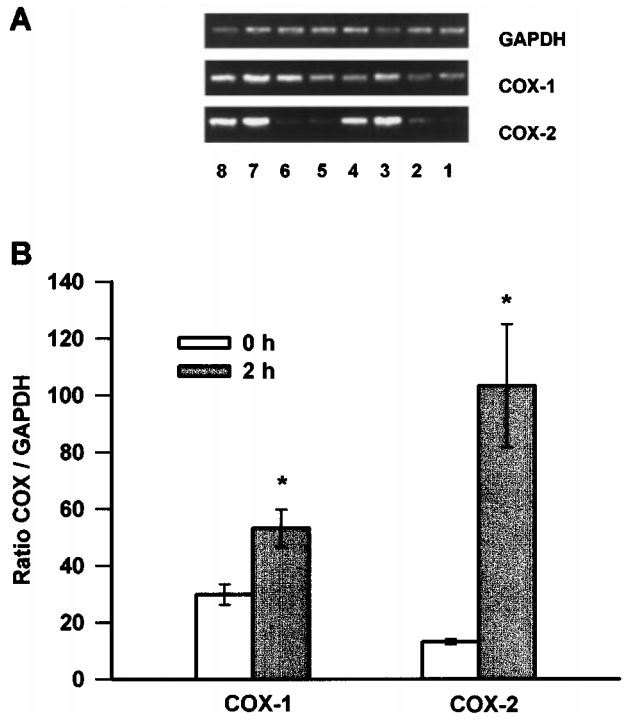

Figure 8 *In vitro* changes in the expression of COX-1 and COX-2 mRNA, relative to GAPDH mRNA, in peristaltically active gut segments as determined by RT–PCR. Tissues were collected immediately before the segments were set up in the organ baths (0 h) and after an experimental period of 2 h. (A) Agarose gel electrophoresis of RT–PCR products showing GAPDH, COX-1 and COX-2 mRNA expression in isolated gut segments at 0 h (lanes 1, 2, 5 and 6) and 2 h (lanes 3, 4, 7 and 8). (B) Quantitative results of COX-1 and COX-2 mRNA expression at 0 h and 2 h. Means ± s.e. mean, $n=6$. $^*P<0.05$ versus 0 h (two sample *t*-test).

Table 2 Potencies of the test compounds used in this study to inhibit COX-1 and COX-2

Compound	*IC_{50} for COX-1* (μM)	*IC_{50} for COX-2* (μM)	*References*
Etodolac	12	0.94	Warner *et al*., 1999
Flurbiprofen	0.075	0.77	Warner *et al*., 1999
Indomethacin	0.013	0.13	Warner *et al*., 1999
NS-398	6.9	0.042	Warner *et al*., 1999
Piroxicam	2.4	0.17	Warner *et al*., 1999
Sodium salicylate	4956	482	Warner *et al*., 1999
SC-560	0.009	6.3	Smith *et al*., 1998

The potency of etodolac, flurbiprofen, indomethacin, NS-398, piroxicam and sodium salicylate to inhibit COX-1 and COX-2 was estimated in the William Harvey human whole blood assay (Warner *et al*., 1999) while that of SC-560 was determined on the enzymatic activity of recombinant human COX-1 and COX-2 (Smith *et al*., 1998).

attenuation of peristaltic effectiveness. The type of indomethacin- and etodolac-induced peristaltic disturbance is profoundly different from the peristaltic motor inhibition caused by endogenous substances such as neuropeptide Y (Holzer *et al*., 1987) or ATP (Heinemann *et al*., 1999), which do not alter the regularity of peristalsis but gradually increase PPT to 400 Pa and thus cause peristaltic motility to cease in the absence of any contractile activity. The peristaltic motor effect of indomethacin and etodolac, though, is similar to the peristaltic motor disruption caused by the L-type calcium channel activator S(−)-Bay K 8644 (this study) and by the combination of apamin plus N-nitro-L-arginine methylester, which inhibit small conductance Ca^{2+}-dependent K^+ channels and nitric oxide synthase, respectively, and thus suppress ATP- and nitric oxide-mediated transmission from inhibitory motor neurons to the circular muscle (Waterman & Costa, 1994; Holzer *et al*., 1997).

The present finding that indomethacin and etodolac impaired peristalsis in the guinea-pig small intestine is in keeping with previous observations that NSAIDs weaken peristaltic performance in the guinea-pig (Bennett *et al*., 1976; Fontaine *et al*., 1977) and human (Bruch *et al*., 1978) small and large intestine. The induction of phasic stationary contractions is probably related to the ability of indomethacin to alter the slow wave pattern in the canine colon (Franck *et al*., 1999) and to evoke phasic contractions of the circular muscle in the guinea-pig small intestine (Maggi *et al*., 1994; Johnson *et al*., 1998). These phasic contractions are induced at the muscle level because they are insensitive to tetrodotoxin, a blocker of voltage-gated sodium channels involved in nerve conduction (Maggi *et al*., 1994). Whether a similar site of action accounts for the ability of indomethacin to disrupt propulsive motility was not possible to sort out, because any drug blocking nerve or muscle activity will eliminate the motor pattern of peristalsis (Johnson *et al*., 1996; Holzer *et al*., 1998). The present work addressed the question as to whether the peristaltic motor disturbance caused by indomethacin is due to inhibition of COX, in particular COX-1 or COX-2.

To this end, we compared a number of COX-1/COX-2 isoform-nonselective and -selective inhibitors (Table 2) in their peristaltic motor actions. Indomethacin, flurbiprofen, piroxicam and etodolac are considered to be COX-1/COX-2 nonselective, whereas SC-560 is a selective inhibitor of COX-1 and NS-398 a selective inhibitor of COX-2 (Table 2). Inhibition of COX by etodolac, indomethacin, SC-560 and NS-398 was proved by their ability to inhibit the biosynthetic release of 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2. The degree of inhibition caused by these compounds at a concentration of 1 μM resulted in a rank order of effectiveness (SC-560 ≃ indomethacin > NS-398 > etodolac) that fits roughly with their activity at COX-1 (see Table 2). This relationship suggests that 6-keto-$PGF_{1\alpha}$ release from peristaltically active segments of the guinea-pig small intestine arises predominantly from COX-1 activity. It remains to be elucidated whether the effect of NS-398 (1 μM) to depress the release of 6-keto-$PGF_{1\alpha}$ by 72% was due to a nonselective action on COX-1 in our preparation.

Sodium salicylate, a NSAID with very weak activity at COX (Table 2), failed to inhibit the biosynthetic release of 6-keto-$PGF_{1\alpha}$ and to alter peristaltic performance. Since effective concentrations of the TXA_2 receptor antagonist SQ-29,548 (10 μM; Ogletree *et al*., 1985) and the 5-lipoxygenase inhibitor Bay X 1005 (0.1–10 μM; Müller-Peddinghaus *et al*., 1993) were also without influence on peristalsis, it is inferred that thromboxanes and leukotrienes do not participate in the physiological control of intestinal peristalsis. In addition, the lack of effect of Bay X 1005 suggests that leukotrienes do not account for the peristaltic motor changes elicited by indomethacin and etodolac, a

possibility that may be envisaged from the ability of COX inhibitors to enhance leukotriene biosynthesis (Kuehl *et al*., 1984; Elliott *et al*., 1989).

The effect of indomethacin to disturb the regularity of peristalsis at concentrations of $\geqslant 1\ \mu$M, which inhibit both COX-1 and COX-2 in the human whole blood assay (Table 2), contrasted with the lack of peristaltic motor effect of COX-1 and COX-2 isoform-selective concentrations of SC-560 and NS-398 ($\leqslant 1\ \mu$M; Smith *et al*., 1998; Warner *et al*., 1999), respectively. It thus appeared as if peristaltic motility is impaired only if COX-1 and COX-2 are inhibited simultaneously. This inference, though, was refuted by the finding that combined blockade of COX-1 and COX-2 by fully effective concentrations of SC-560 and NS-398 failed to reproduce the peristaltic disturbance caused by combined COX-1 and COX-2 inhibition with indomethacin ($1-10\ \mu$M). From the mismatch between the test compounds' activity to block COX-1 and/or COX-2, their activity to suppress the biosynthetic release of 6-keto-$PGF_{1\alpha}$ from the intestine and their activity to impair intestinal peristalsis it need be deduced that the action of indomethacin and etodolac to disrupt the regularity of peristalsis is not related to blockade of COX-1 or COX-2.

The conjecture that NSAID-induced impairment of peristalsis arises from PG depletion through inhibition of COX has long been based on the observation that the antiperistaltic action of indomethacin can be terminated by PGE_1 and other PGs (Bennett *et al*., 1976). Although this activity of PGE_1 was confirmed, while the TXA_2 receptor agonist U-46,619 (0.1 μM; Tymkewycz *et al*., 1991) proved ineffective, the current study revealed that the action of PGE_1 was not NSAID-specific because the peristaltic irregularities elicited by the L-type calcium channel activator S(−)-Bay K 8644 were also counteracted by PGE_1. The data of the current study thus provide multiple evidence that indomethacin, and most probably etodolac, disturb peristalsis by an action other than inhibition of COX.

In further analysing indomethacin's antiperistaltic effect we addressed the possibility that this NSAID may act by blocking L-type calcium channels (Northover, 1977; Burch *et al*., 1983; Franck *et al*., 1999), by preventing the effect of endogenous PGs to activate ATP-sensitive potassium channels (Hide *et al*., 1995) or by inhibiting phosophodiesterase of type IV (Flower & Vane, 1974; Newcombe *et al*., 1974; Beatty *et al*., 1976). Of the drugs used to sort out these options, only the L-type calcium channel activator S(−)-Bay K 8644 ($0.1-3\ \mu$M; Ravens & Schöpper, 1990) was able to mimick the indomethacin-induced disturbance of peristalsis. However, it is very unlikely that the indomethacin-induced irregularities of peristalsis result from opening of L-type calcium channels because this NSAID does not activate, but blocks, L-type calcium channels in intestinal muscle (Northover, 1977; Burch *et al*., 1983; Franck *et al*., 1999) and because the L-type calcium channel blocker R(+)-Bay K 8644 ($0.1-10\ \mu$M; Ravens & Schöpper, 1990) inhibited peristalsis without disrupting the regularity of peristaltic waves.

A PG-dependent modulation of ATP-sensitive potassium channels (Hide *et al*., 1995) is likewise improbable to account for the indomethacin-induced irregularities of peristalsis, since activation of these channels with cromakalim ($1-100\ \mu$M; Buchheit & Bertholet, 1988) and their blockade with glibenclamide ($1-100\ \mu$M; Maggi *et al*., 1996) failed to disturb the regularity of peristalsis but raised PPT and reduced the effectiveness of peristaltic waves. Finally, we have also obtained two sets of data to rule out the possibility that indomethacin disrupts peristalsis *via* inhibition of type IV phosophodiesterase and subsequent accumulation of intracellular cyclic AMP (Flower & Vane, 1974; Newcombe *et al*., 1974; Beatty *et al*., 1976). Thus, the adenylate cyclase activator forskolin ($0.01-1\ \mu$M; Zafirov *et al*., 1985; Heinemann & Holzer, 1999) did not influence peristalsis, while selective inhibition of the cyclic AMP-specific phosphodiesterase by rolipram ($0.1-30\ \mu$M; Izzo *et al*., 1998) depressed peristaltic performance without causing irregularities. The latter effect is most probably a sequel of rolipram's ability to block enteric neurotransmission *via* noradrenaline-mediated activation of α_2-adrenoceptors (Izzo *et al*., 1998). Taken all current data together, it would appear that indomethacin induces nonperistaltic circular muscle contractions that disturb the regularity of peristalsis in the guinea-pig small intestine by an unknown mode of action that does not involve COX, L-type calcium channels, ATP-sensitive potassium channels or phosphodiesterase of type IV.

The finding that the expression of COX-2 mRNA markedly increased during the 2 h course of the *in vitro* peristalsis experiments, while that of COX-1 mRNA rose only moderately, is a discovery with potentially important implications. While it has previously been shown that trauma and inflammation increase the formation of COX-2 mRNA in the gastrointestinal tract *in vivo* (Ferraz *et al*., 1997; Maricic *et al*., 1999), the present data demonstrate that similar changes take place even in segments excised from the guinea-pig small intestine. Although the increased expression of COX-2 mRNA does not seem to have an impact on peristaltic motor regulation in the isolated gut, it needs to be considered that the enhanced production of COX-2 mRNA may influence the outcome of studies in which COX-dependent processes are investigated in isolated bowel segments. At present it is not clear in which cells of the guinea-pig isolated small intestine COX-2 mRNA expression increases during the course of the peristalsis experiments. Immunocytochemical studies in other species have shown that COX-2 is constitutively expressed in epithelial, neuroendocrine and lamina propria cells (Iseki, 1995; Ferraz *et al*., 1997; Nakajima *et al*., 1997).

Unlike other investigations which have suggested that PG release may be related to gastrointestinal motility (Singh, 1980; Yagasaki *et al*., 1980), our results negate a significant relationship between 6-keto-$PGF_{1\alpha}$ release and peristaltic motor activity. This is in keeping with the inability of selective COX-1 and COX-2 inhibitors to grossly modify the pattern of propulsive motility. The complete lack of effect of a COX-2 inhibitor indicates that in particular PGs generated by COX-2 are without significance for intestinal peristalsis, perhaps because they are formed by cellular systems that are remote from the effector cells of peristalsis. In contrast, PGs generated by COX-1 may play a permissive role in supporting peristaltic effectiveness, given that selective blockade of COX-1 and nonselective inhibition of COX-1 and COX-2 consistently lowered the amplitude of peristaltic waves.

In summary, we have found that, among various COX inhibitors, only indomethacin and etodolac disturb peristalsis

in the guinea-pig isolated small intestine, whereas other COX isoform-nonselective NSAIDs (flurbiprofen and piroxicam) as well as COX-1 and COX-2 isoform-selective inhibitors (SC-560 and NS-398) are without major influence on peristalsis. The antiperistaltic effects of indomethacin and etodolac appear to be brought about by an unkown mode of action that does not involve COX, L-type calcium channels, ATP-sensitive potassium channels or phosophodiesterase of type IV. Thus, the peristaltic irregularities caused by indomethacin and etodolac seem to represent a peculiar property of indole acetic acid-derived NSAIDs. Although it is not known whether these COX-unrelated effects apply to species other than the guinea-pig, the current observations advocate critical caution when indomethacin and etodolac are used as pharmacological tools for probing the involvement of PGs in functional tests.

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