

THE EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON CHOLINERGIC AND HISTAMINE-INDUCED CONTRACTIONS OF GUINEA-PIG ISOLATED ILEUM

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1 Eleven non-steroidal anti-inflammatory drugs (NSAID) reversibly inhibited contractions of the longitudinal muscle of the guinea-pig isolated ileum induced by acetylcholine, histamine, electrical transmural stimulation and nicotine in this order of increasing potency.

2 After the addition of prostaglandins E_1 , E_2 or $F_{2\alpha}$, with partially effective concentrations of NSAID (but not with higher concentrations which almost totally prevented the responses) the inhibitory effects of NSAID were reversibly lost, except for electrically induced contractions and prostaglandin $F_{2\alpha}$.

3 The effects of NSAID may be due to actions on biological membranes or on distribution of ions in addition to their inhibitory effect on prostaglandin synthesis. Prostaglandins may reverse the inhibition by non-selective sensitization of smooth muscle to various agonists.

Introduction

Prostaglandins seem to play a role in contractions of intestinal smooth muscle (Bergström, Eliasson, Von Euler & Sjöval, 1959; Bennett, Eley & Scholes, 1968; Fleshler & Bennett, 1969; Eckenfels & Vane, 1972; Ferreira, Herman & Vane, 1976). Since the demonstration that indomethacin inhibits prostaglandin synthesis in cell-free homogenates of guinea-pig lung (Vane, 1971), many authors have confirmed the inhibitory action of non-steroidal anti-inflammatory drugs (NSAID) on prostaglandin synthesis in various tissues (Flower, 1974).

Ehrenpreis, Greenberg & Belman (1973) and Bennett, Eley & Stockley (1975) found that indomethacin (40 $\mu\text{g/ml}$) substantially reduced contractions of the longitudinal muscle of guinea-pig isolated ileum in Krebs solution to nerve stimulation with transmural electrical pulses. Kadlec, Mašek & Šeferna (1974) obtained similar results with indomethacin (0.36 $\mu\text{g/ml}$) in a modified Krebs solution. The difference in the concentration of indomethacin needed depends on the composition of the bathing solution (Bennett *et al.*, 1975). All these authors reversed the inhibitory action of indomethacin with low concentrations of prostaglandin E .

Ehrenpreis *et al.* (1973) concluded that prostaglandins couple cholinergic nerve terminal excitation with acetylcholine release while Bennett *et al.* (1975) thought that prostaglandins modulate the response to cholinergic activity. Kadlec *et al.* (1974) postulated

that this prostaglandin-modulated action on cholinergic activity is mediated through inhibition of noradrenaline release.

Bennett *et al.* (1975) showed that indomethacin 2 $\mu\text{g/ml}$ also greatly depressed nicotine-induced contractions of guinea-pig isolated ileum whereas 5–20 $\mu\text{g/ml}$ caused little reduction in the contractions to exogenous acetylcholine and histamine (Chong & Downing, 1973; Bennett *et al.*, 1975). These inhibitory effects are reduced by small amounts of prostaglandins in the bath (Bennett *et al.*, 1975).

We have extended these observations to several other NSAID known to inhibit prostaglandin synthesis (Flower, 1974) in an attempt to understand better their mechanisms of action on intestinal smooth muscle.

Methods

Adult guinea-pigs of either sex were stunned and bled. Segments of ileum (4 cm) at least 10 cm from the caecum were set up under an initial load of 1 g in Krebs-Henseleit solution (composition g/l: KCl 0.35, NaCl 6.92, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.37, NaHCO_3 2.1, KH_2PO_4 0.16, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.29 and glucose 1.0) at 37°C gassed with a mixture of 5% CO_2 and 95% O_2 . Transmural stimulation was carried out as described

by Paton (1955) with rectangular pulses of 0.5 ms duration, 0.1 Hz, 5 to 25 V delivered and measured by a Grass stimulator. Muscle contraction was registered isometrically by a transducer.

The inhibitory effect of 11 NSAID (indomethacin, flufenamic, mefenamic and niflumic acids, bufexamac, alclofenac, clopirac, ibuprofen, phenylbutazone, oxyphenbutazone and amidopyrine) were assessed on these electrically-induced contractions. Five different concentrations (2.5, 10, 40, 100 and 200 $\mu\text{g/ml}$) were chosen to be tested, but only 3 or 4 of them were used for each drug according to the degree of inhibition observed. At least 4 experiments were performed at each drug concentration. The results are expressed as a percentage of the contractions obtained before addition of the drug. After an inhibition was observed, small amounts of prostaglandins E_1 (2.5 ng/ml), E_2 (2.5 ng/ml) or F_{2a} (tromethamine salt, 25 ng/ml) were added to the bath 12 min after the NSAID addition, but reversal of inhibition was not measured.

The same 11 NSAID were tested in parallel (12 min contact time) for inhibitory effects on acetylcholine, histamine and nicotine-induced contractions, using the lowest concentration tested which reduced the electrically-induced contractions by at least 50%. Segments of ileum were set up in identical conditions except that contractions were recorded on a kymograph with an isotonic lever (five-fold magnification). The submaximal contractions induced in the presence of the NSAID by 30 ng/ml histamine and 0.5 $\mu\text{g/ml}$ nicotine were recorded every 3 min and 6 min respectively. The results are expressed as a percentage of the average of at least 3 control responses obtained before addition of the NSAID. In 4 of the 8 experiments with each NSAID, prostaglandin E_1 (2.5 ng/ml) was added to the bath immediately after the first (nicotine) or second (histamine) evoked contraction following the NSAID addition (Figure 3). The height of the contractions in the presence of both prostaglandin E_1 and NSAID is expressed as a percentage of the average of at least 3 control contractions.

In each experiment the ileum was finally challenged with the agonist, after washing out the NSAID (and prostaglandin E_1 if present). The height of three consecutive contractions is expressed as a percentage of the control contractions.

In other similar experiments prostaglandins E_2 (2.5 ng/ml) and F_{2a} (tromethamine salt, 25 ng/ml) were tested in the presence of each NSAID but their effect was estimated quantitatively only for indomethacin, phenylbutazone (Figure 3) and flufenamic acid. The effects of prostaglandins E_1 , E_2 and F_{2a} were similarly tested in 4 experiments for each prostaglandin after the second evoked contraction induced by acetylcholine (20 ng/ml). The NSAID inhibition of contractions to acetylcholine has been described previously (Famaey, Fontaine & Reuse, 1977).

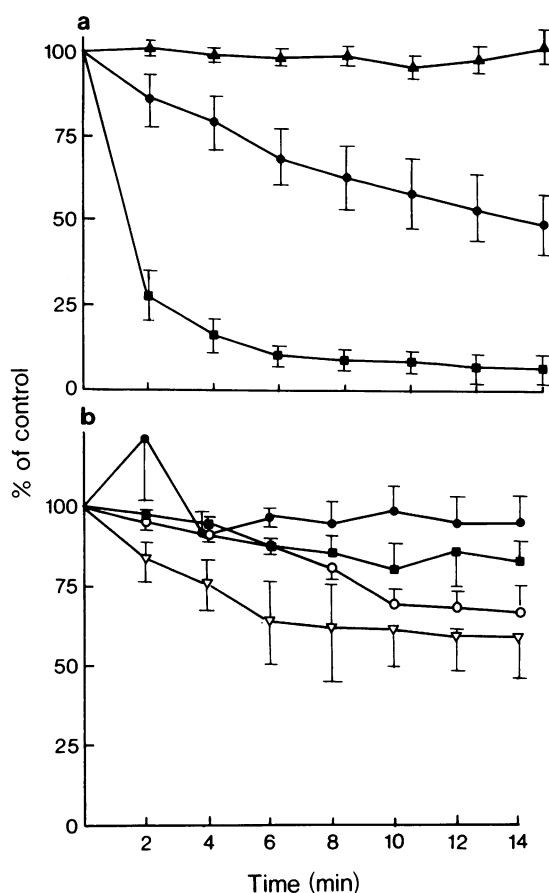


Figure 1 The influence of drug contact time on the inhibition by various concentrations of (a) flufenamic acid and (b) alclofenac on the responses of guinea-pig ileum to electrical stimulation: (▲) 2.5 $\mu\text{g/ml}$; (●) 10 $\mu\text{g/ml}$; (■) 40 $\mu\text{g/ml}$; (○) 100 $\mu\text{g/ml}$ and (▽) 200 $\mu\text{g/ml}$.

Results were analyzed by Student's *t* tests, except when differently quoted.

Drugs

Gifts of the following drugs were obtained: prostaglandins E_1 , E_2 and the tromethamine salt of prostaglandin F_{2a} (Upjohn Co, Kalamazoo, Michigan, U.S.A.), acetylcholine (Roche, Basel, Switzerland), phenylbutazone, oxyphenbutazone and amidopyrine (Ciba-Geigy, Basel, Switzerland), clopirac, bufexamac and alclofenac (Continental Pharma, Brussels, Belgium), ibuprofen (Boots Drug Co, Nottingham, Great Britain), indomethacin (Merck Sharp and Dohme, Rahway, New Jersey, U.S.A.), niflumic acid (Upsa, Agens, France), flufenamic and mefenamic

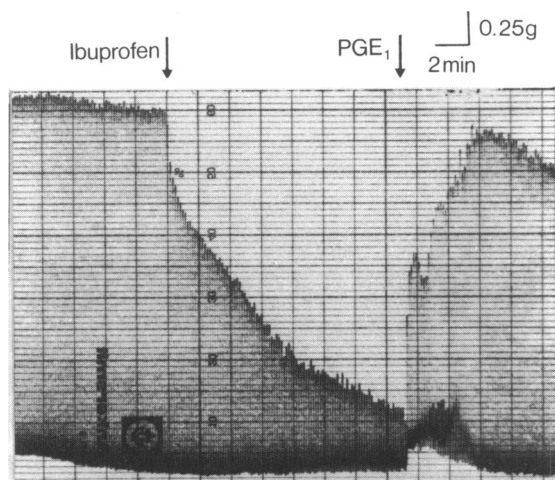


Figure 2 Almost total reversal by prostaglandin E_1 (PGE_1) 2.5 ng/ml of the inhibition induced by ibuprofen 40 μ g/ml on responses of guinea-pig ileum to electrical stimulation.

acids (Parke Davis & Co, Detroit, Michigan, U.S.A.). Histamine was purchased from Fluka A.G. (Buchs, Switzerland) and nicotine sulphate from BDH. All other chemicals were of the purest grade commercially available.

Results

All 11 NSAID tested caused a dose-related inhibition of the electrically-induced contractions of the guinea-pig ileum. The concentrations of NSAID used were 2.5–40 μ g/ml for all drugs except phenylbutazone, oxyphenbutazone and alclofenac which required 10–200 μ g/ml (Table 1, Figure 1). The effect increased progressively with the duration of contact and was maximum at about 12 min (Figure 1).

Prostaglandin E_1 or E_2 (2.5 ng/ml) reversed this submaximal inhibition, prostaglandin E_1 being somewhat more potent than prostaglandin E_2 (Figure 2), while prostaglandin $F_{2\alpha}$ (25 ng/ml) had little effect.

Prostaglandin E compounds clearly reversed a partial inhibition by NSAID but only slightly restored responses that had been almost abolished. The NSAID, at concentrations up to 200 μ g/ml which induced at least 50% inhibition (with the exception of alclofenac) of the electrically-induced contractions after a 12 min contact time, also inhibited contractions induced by histamine or nicotine ($P < 0.001$) (Figure 3). With every drug the inhibition of electrically-induced or nicotine-induced contractions was significantly higher than that of contractions to histamine (except for bufexamac, 10 μ g/ml where

inhibition of electrically-induced contraction was not significantly greater than that of histamine).

Indomethacin, flufenamic, mefenamic and niflumic acids, ibuprofen (all at 40 μ g/ml) and bufexamac (40 and 10 μ g/ml) inhibited nicotine-induced contractions significantly more than those to electrical stimulation.

Prostaglandin E_1 (2.5 ng/ml) completely reversed the NSAID-induced inhibition of contractions to histamine, nicotine and also to acetylcholine ($P < 0.05$ to $P < 0.001$ according to the drug and agonist—see Table 1) and even increased most of the contractions to above the control levels ($P < 0.001$ for nicotine, $P < 0.01$ for acetylcholine and $P < 0.05$ for histamine). There were no significant differences between the contractions obtained after washing out the NSAID (and prostaglandin E_1 if present) and the average of the control contractions, demonstrating the reversibility of the NSAID and the prostaglandin E_1 effects.

Prostaglandin E_2 (2.5 ng/ml) and prostaglandin $F_{2\alpha}$ (25 ng/ml) gave similar results for all the NSAID and for each of the 3 agonists, to those obtained with prostaglandin E_1 and their statistical analysis for indomethacin, flufenamic acid and phenylbutazone also gave the same P values.

As with electrically-induced contractions, prostaglandins markedly reversed submaximally inhibited contractions but had little effect on responses that had been totally inhibited (Table 1).

Discussion

The inhibitory effect of indomethacin on electrically-induced contractions of the guinea-pig ileum described by Ehrenpreis *et al.* (1973), Kadlec *et al.* (1974) and Bennett *et al.* (1975) was confirmed by us and extended to 10 other acidic NSAID which inhibit prostaglandin synthesis (Flower, 1974) but have different chemical structures.

Inhibition of acetylcholine-induced contractions of guinea-pig ileum by indomethacin (Bennett *et al.*, 1975) and by the other NSAID (Famaey *et al.*, 1977) has been described and inhibition by these agents is now extended to histamine- and nicotine-induced contractions. With every NSAID the inhibition of electrically-induced or nicotine-induced contractions was significantly greater than the inhibition of contractions to acetylcholine. Only indomethacin, flufenamic and niflumic acids, clopirac (all at 40 μ g/ml) and bufexamac (10 μ g/ml) inhibited histamine-induced contractions significantly more than those to acetylcholine. The comparison between the averages of the mean inhibition obtained with each drug for each of the four agonists shows that NSAID inhibit the contractions to histamine and to acetylcholine significantly less than those to nicotine or to electrical stimulations ($P < 0.001$, Mann Whitney U

Table 1 A statistical analysis of the inhibitory effects of 11 non-steroidal anti-inflammatory drugs (NSAID) on guinea-pig ileum contractions induced by 4 different agonists and of the reversal by prostaglandin E_1 of these inhibitions

NSAID	$\mu\text{g/ml}$	Stim	ACh (Famaeey Fontaine & Reuse, 1977)	ACh PGE ₁	Hist	Hist + PGE ₁	Nic	Nic + PGE ₁	Results of Student's <i>t</i> tests
Indomethacin	40	40.2 ± 10.3	77.8 ± 7.5	122.4 ± 10.4 <0.01	55.2 ± 4.7	109.8 ± 23.7 <0.05	1.9 ± 1.4	no increase	(1) < 0.05 (2) < 0.05 (3) < 0.001 (4) < 0.05 (5) < 0.01 (6) < 0.01
	10	76.8 ± 11.9	—	—	—	—	40.7 ± 5.7	174.9 ± 27.8 <0.001	(1) < 0.001 (2) < 0.02 (3) < 0.001 (4) < 0.05 (5) < 0.01 (6) < 0.01
Flufenamic acid	40	8.1 ± 2.8	64.9 ± 8.5	82.3 ± 14.0 <0.05	33.2 ± 7.4	78.2 ± 12.9 <0.02	0	no increase	(1) < 0.001 (2) < 0.02 (3) < 0.001 (4) < 0.05 (5) < 0.01 (6) < 0.01
	10	54.0 ± 10.1	—	—	—	—	18.2 ± 2.8	178.2 ± 29.3 <0.001	(1) < 0.01 (2) NS (3) < 0.001 (4) < 0.02 (5) < 0.01 (6) < 0.02
Mefenamic acid	40	33.0 ± 11.4	79.6 ± 5.3	110.0 ± 3.0 <0.02	70.7 ± 7.7	103.0 ± 10.7 <0.05	1.0 ± 0.5	no increase	(1) < 0.01 (2) NS (3) < 0.001 (4) < 0.02 (5) < 0.01 (6) < 0.02
	10	59.6 ± 8.0	—	—	—	—	33.8 ± 7.0	191.6 ± 16.2 <0.001	(1) < 0.001 (2) < 0.01 (3) < 0.001 (4) < 0.01 (5) < 0.001 (6) < 0.01
Niflumic acid	40	29.6 ± 7.2	93.3 ± 3.3	133.2 ± 15.7 <0.01	71.2 ± 5.4	173.2 ± 44.9 <0.01	10.1 ± 4.1	no increase	(1) < 0.001 (2) < 0.01 (3) < 0.001 (4) < 0.01 (5) < 0.001 (6) < 0.01
	10	92.5 ± 6.8	—	—	—	—	70.1 ± 12.4	205.7 ± 28.6 <0.01	(1) < 0.02 (2) NS (3) < 0.01 (4) < 0.05 (5) < 0.01 (6) < 0.001
Bufexamac	40	4.6 ± 1.1	43.2 ± 9.4	83.5 ± 8.0 <0.05	36.4 ± 10.1	71.4 ± 5.5 <0.01	0	no increase	(1) < 0.01 (2) < 0.01 (3) < 0.01 (4) NS (5) < 0.05 (6) < 0.05
	10	43.0 ± 7.3	66.0 ± 5.7	142.5 ± 5.7 <0.05	46.7 ± 12.9	82.9 ± 6.8 <0.01	15.7 ± 3.8	106.3 ± 10.8 <0.01	(1) < 0.01 (2) < 0.05 (3) < 0.01 (4) < 0.02 (5) < 0.001 (6) < 0.01
	2.5	88.3 ± 6.5	—	—	—	—	44.5 ± 7.2	161.6 ± 12.7 <0.001	(1) < 0.01 (2) NS (3) < 0.01 (4) < 0.01 (5) < 0.01 (6) NS
Alclofenac	200	60.2 ± 14.4	97.0 ± 2.5	150.7 ± 11.0 <0.001	96.4 ± 2.4	163.3 ± 18.7 <0.001	74.9 ± 5.7	249.7 ± 36.1 <0.001	(1) < 0.01 (2) NS (3) < 0.01 (4) < 0.01 (5) < 0.01 (6) NS
Clopirac	40	41.5 ± 11.5	79.5 ± 5.4	204.4 ± 33.3 <0.001	66.9 ± 3.3	163.6 ± 39.4 <0.001	37.1 ± 4.2	191.5 ± 29.8 <0.001	(1) < 0.01 (2) < 0.05 (3) < 0.001 (4) < 0.02 (5) < 0.001 (6) NS
Ibuprofen	40	39.5 ± 7.0	76.6 ± 5.5	191.3 ± 9.2 <0.001	72.8 ± 7.8	151.4 ± 4.2 <0.01	22.0 ± 2.6	132.4 ± 3.1 <0.001	(1) < 0.01 (2) NS (3) < 0.001 (4) < 0.02 (5) < 0.001 (6) < 0.02
Phenylbutazone	200	44.7 ± 10.0	74.3 ± 6.8	121.4 ± 14.0 <0.05	66.0 ± 2.7	116.3 ± 6.4 <0.02	32.9 ± 3.0	167.2 ± 23.5 <0.001	(1) < 0.05 (2) NS (3) < 0.01 (4) < 0.01 (5) < 0.01 (6) NS
Oxyphenbu- tazone	200	36.3 ± 4.5	74.5 ± 8.8	120.9 ± 18.6 <0.01	79.3 ± 5.1	151.8 ± 8.6 <0.01	31.9 ± 6.0	140.1 ± 11.8 <0.001	(1) < 0.05 (2) NS (3) < 0.02 (4) < 0.01 (5) < 0.001 (6) NS
Amidopyrine	40	36.0 ± 14.5	86.4 ± 8.1	126.7 ± 10.8 <0.05	76.8 ± 6.8	109.7 ± 3.0 <0.05	45.1 ± 4.6	159.5 ± 24.0 <0.001	(1) < 0.01 (2) NS (3) < 0.001 (4) < 0.01 (5) < 0.001 (6) NS

Stim = electrical stimulations; ACh = acetylcholine; Hist = histamine; Nic = nicotine
 Prostaglandin E_1 (PGE₁) was used at the concentrations given in the Methods section. The results are expressed as percentages of control con-
 tractions ± s.e. mean.
 The statistical significance of the reversing effects of prostaglandins was established by comparison with the results obtained from the same agonist
 in the presence of the same NSAID alone. Student's *t* tests refer to (1) acetylcholine compared with electrical stimulation; (2) acetylcholine
 compared with histamine; (3) acetylcholine compared with nicotine; (4) histamine compared with electrical stimulation; (5) histamine compared
 with nicotine; (6) nicotine compared with electrical stimulation. NS = not significant.

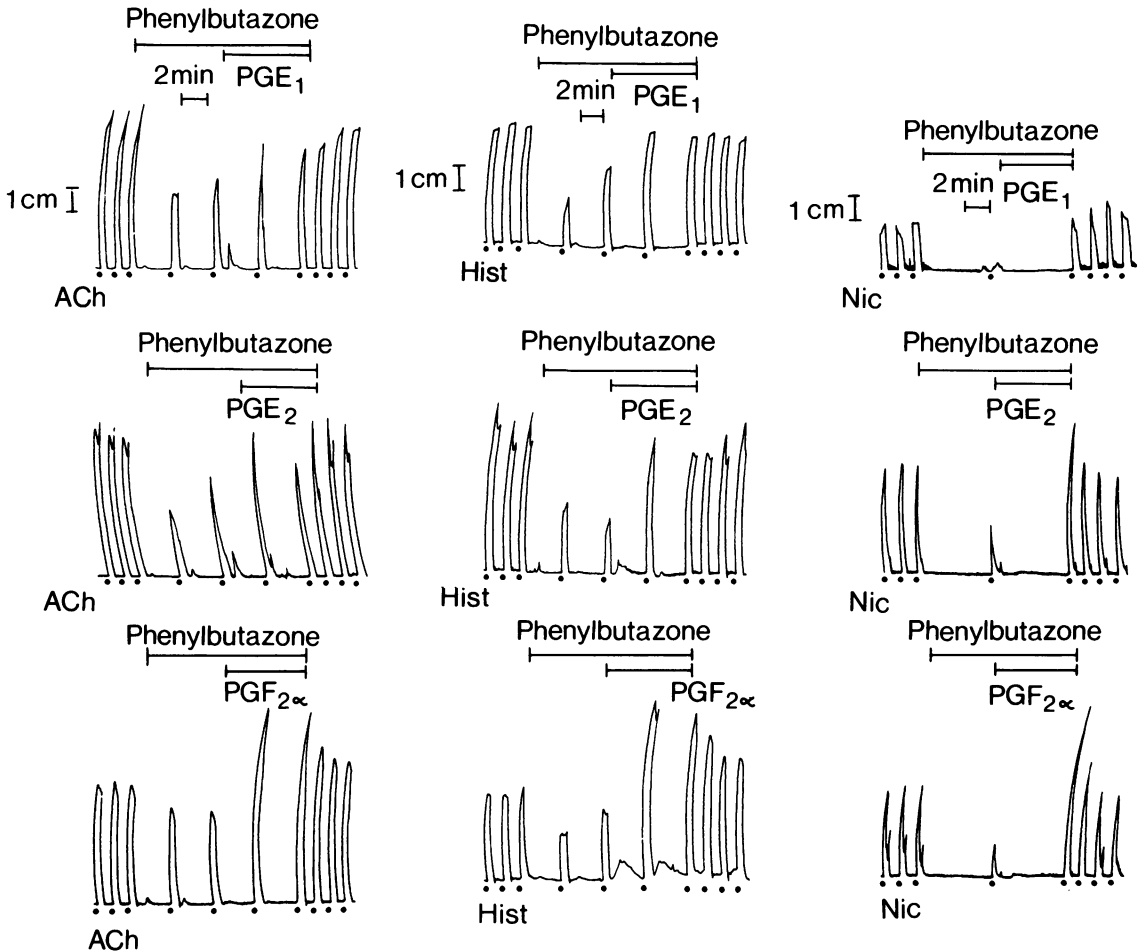


Figure 3 The effect of phenylbutazone 200 µg/ml on guinea-pig ileum contractions induced at ● by acetylcholine (ACh), histamine (Hist) and nicotine (Nic) and the reversing effects of prostaglandin E_1 (PGE_1 , 2.5 ng/ml) PGE_2 (2.5 ng/ml) and $PGF_{2\alpha}$ (25 ng/ml).

test) and that there is no significant difference between the inhibitory effects of NSAID on contractions to acetylcholine or to histamine and between those on contractions to nicotine or electrical stimulations.

As suggested by Ehrenpreis *et al.* (1973) the greater inhibition of contractions to nicotine or electrical stimulation (indirect cholinergic effects) than to acetylcholine or histamine (direct agonists) could be explained by interference with neuronal acetylcholine release. However, nicotine which acts on ganglia (Day & Vane, 1963) was significantly more affected by NSAID than was the response to electrical stimulation which involves post-ganglionic cholinergic nerves (Paton, 1955). This suggests that the NSAID could be exerting some ganglion blocking effect on the guinea-pig isolated ileum. Ganglion blocking effects have not been clearly demonstrated for NSAID in other *in vitro* or *in vivo* models. Although Bennett *et al.* (1975) have

obtained with indomethacin complete inhibition of nicotine-induced contractions and only partial inhibition of electrically-induced contractions, they did not point out any statistical difference between these two activities.

Our results show that all NSAID tested possess some spasmolytic activity as they all slightly inhibit contractions to histamine and to a lesser extent to acetylcholine. Others using similar concentrations, have demonstrated spasmolytic properties of several NSAID on gastrointestinal and vascular smooth muscles (Wilhelmi, 1949; Northover, 1967; Yamatake, Kato & Takagi, 1975).

We conclude that, in addition to effects which may be mediated by reduced acetylcholine release, similar to those previously described for indomethacin by Ehrenpreis *et al.* (1973), Kadlec *et al.* (1974) and Bennett *et al.* (1975), the NSAID exhibit on the

guinea-pig isolated ileum slight spasmolytic, moderate antihistaminic and perhaps some ganglion blocking properties.

Several NSAID possess some antihistaminic properties (Domenjoz, 1960; Parrot, Ruff & Saindelle, 1966). Histamine is thought to be an important mediator in the inflammatory process (Schayer, 1962; Spector & Willoughby, 1964). Most of the NSAID are able to reduce oedema induced experimentally by histamine or histamine-releasers although to a lesser degree than true antihistaminic drugs (Winter, 1966). This is in accordance with our results which indicate that NSAID have only moderate antihistaminic properties.

We found that prostaglandins E_1 , E_2 and F_{2a} completely reversed the submaximal inhibitory effect of NSAID on contractions to acetylcholine, histamine, nicotine and electrical stimulation (except for prostaglandin F_{2a} and electrical stimulation). Since NSAID inhibit prostaglandin synthesis at even lower concentrations than those used in our experiments (Vane, 1971), prostaglandins might be important for (1) the activation of ganglia by nicotine, (2) the release of acetylcholine by nerve stimulation, and (3) the contraction of smooth muscle by acetylcholine or histamine. Ehrenpreis *et al.* (1973) suggested this hypothesis for acetylcholine release by electrical nerve stimulation and extended it to the concept that morphine affects gastrointestinal motility by inhibiting prostaglandin-synthetase.

Another explanation was proposed by Bennett *et al.* (1975) who thought that prostaglandins might be involved in ileal contractions mediated by cholinergic nerves by increasing the response to released acetylcholine and possibly its amount. Prostaglandins might modulate responsiveness to other stimuli since they also reverse the inhibition of ileal histamine-induced contractions by NSAID. Eckenfels & Vane (1972) observed that following pretreatment of the guinea-pig colon with indomethacin, the initial contractor effect of histamine was unaffected but the contraction was not maintained unless prostaglandin E_2 was added.

Prostaglandins therefore seem to sensitize guinea-pig ileum non-selectively to all stimuli. This would

explain why prostaglandins reverse NSAID inhibition and even induce contractions above control levels.

These NSAID effects on guinea-pig isolated ileum probably involve actions apart from inhibition of prostaglandin synthesis. At concentrations similar to those used in our experiments these drugs also inhibit various proteolytic enzymes, uncouple mitochondrial oxidative phosphorylation and interfere with various biological membranes (lysosomes, mitochondria, erythrocytes and lymphocytes) as well as non-biological membranes (Famaey, Brooks & Dick, 1975). These membrane properties are related to movements of various ions including cellular uptake or release of sodium, potassium or calcium (Northover, 1971; Famaey & Whitehouse, 1976).

Preliminary data indicate that most of the anti-inflammatory steroids and chloroquine which also affect membranes (Weissman, 1964) have very similar effects on contractions of guinea-pig ileum (Famaey, Fontaine, & Reuse, 1975; Famaey *et al.*, 1977). However anti-inflammatory steroids with no known inhibitory effects on prostaglandins synthesis in crude homogenates (Flower, 1974) might affect the release (Gryglewski, Panczenko, Korbut, Grodzinska & Ocetkiewicz, 1975; Lewis & Piper, 1975) as well as the production of prostaglandins by entire cell preparations (Kantrowitz, Robinson, MacGuire & Levine, 1975; Tashjian, Voelkel, MacDonough & Levine, 1975).

In conclusion it seems that NSAID are able to inhibit, in a dose-dependent manner, the contractions induced by various agonists, probably by inhibiting ileal prostaglandin synthesis and by affecting membrane properties and altering their permeability to various ions. The reversal effect observed in the presence of prostaglandins must be related to this inhibition of synthesis but may also be related to a non-specific ileal sensitization by prostaglandins to the various agonists tested.

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