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Mechanism of the inhibitory action of indomethacin on smooth muscle

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Summary

1. Strips of muscle from the wall of the guinea-pig stomach contracted in response to electrical field stimulation (100 ms pulses, 0·2 Hz) or to histamine (1 μg/ml), and these responses were inhibited by indomethacin (2–20 mg/100 ml).

2. Glycerol-extracted strips of stomach muscle developed tension when exposed to a mixture of ATP, CP, magnesium chloride and calcium chloride. The tension response was not altered by indomethacin (50 mg/100 ml).

3. Indomethacin failed to alter the content of ATP or of CP in strips of stomach muscle.

4. The calcium content of strips of stomach muscle increased in response to 30 min of electrical stimulation (100 ms pulses, 0·2 Hz). The uptake of calcium and the contraction of the strips were inhibited by indomethacin (2–20 mg/100 ml) to a similar extent.

5. Calcium uptake by electrically stimulated guinea-pig aorta was inhibited by lower concentrations of indomethacin than were required by stomach muscle.

Introduction

Anti-inflammatory drugs of the analgesic-antipyretic type inhibit contraction of smooth muscle produced in response to a variety of stimulant drugs (Jaques & Domenjoz, 1950). Moreover, the inhibitory action on smooth muscle appears to be pharmacologically related to the protective action of these compounds on the inflamed peritoneum of the mouse (Hogan & Northover, 1970). An understanding of the mechanism of the inhibitory action of these compounds on smooth muscle may also afford, therefore, a better understanding of their anti-inflammatory action.

Methods

Albino guinea-pigs of either sex weighing 600–800 g were killed by a blow on the head and the stomach or descending thoracic aorta was removed and rinsed with Krebs solution. Four mucosa-free longitudinal strips were prepared from each stomach according to the method of Bailey (1970). Each strip was lightly blotted and weighed (100–200 mg) before mounting in an isolated organ bath. The aorta was opened longitudinally and all loose connective tissue removed from the adventitial side.

Glycerol-extracted muscle

Strips of smooth muscle were tied to glass rods at approximately their *in vivo* length, rinsed for 1 h in 2 mM magnesium chloride solution at room temperature and then extracted for 2–10 days in 35% glycerol solution at 4° C. The extracted strips were bathed at 37° C with a solution containing (mM): KCl, 80 ; tetrasodium ethylene diamine tetraacetate, 0·001 ; L-histidine monohydrochloride, 40, adjusted to pH 7·4 with KOH. Tension changes in the strips were recorded isometrically with a Grass force transducer (FT-03) after adjusting the baseline tension to 50 mg.

Experiments with living muscle

Except where otherwise stated, the muscle was immersed at 37° C in a solution having the following composition (mM): NaCl, 118 ; KCl, 4·7 ; KH_2PO_4, 1·2 ; $MgSO_4$, 1·2 ; $NaHCO_3$, 25 ; glucose, 11 ; $CaCl_4$, 0·4 ; and gassed with 95% oxygen and 5% carbon dioxide. The concentration of calcium ions in this fluid was checked at frequent intervals with a calcium ion electrode (Orion Research Inc., Cambridge, Massachusetts). The addition of indomethacin (20 mg/100 ml) had no significant effect on the measured concentration of calcium ions.

Some strips were stimulated electrically via a pair of platinum wires (4 cm long, 4 mm apart). Responses of the muscle were recorded either on a smoked paper kymograph at a tension of 200 mg by means of a side-writing isotonic lever giving a magnification of $\times 10$, or alternatively, by means of a force transducer, as described above.

Measurement of creatine (C) and creatine phosphate (CP)

Strips of muscle were removed from the organ bath and plunged into liquid nitrogen whilst still connected to their tissue holders. However, warm tissues do not freeze immediately when placed in liquid nitrogen due to the Leydenfrost phenomenon (Mommaerts, 1969). The possible error incurred by the slightly delayed freezing was estimated by rapidly freezing some tissues by placing them in contact with a block of aluminium cooled to the temperature of liquid nitrogen. The C, CP and adenosine triphosphate (ATP) contents of the tissues frozen by the two methods were not significantly different, indicating that the Leydenfrost phenomenon caused no appreciable error.

In a cold room at 4° C each strip of frozen muscle was ground in a Potter all-glass homogenizer with 10% trichloracetic acid solution (TCA), and the TCA extract centrifuged at 1000 g for 5 minutes. The clear supernatant was decanted and then made alkaline by adding 5 N NaOH solution. The recovery of added creatine by this procedure was 90–95%, which is similar to the findings of Ennor & Rosenberg (1952). The C content of the extract from each strip was estimated by the method of Ennor (1957).

Alternate TCA extracts were hydrolysed, thereby converting the CP present to C. The difference between the C content of hydrolysed and unhydrolysed extracts was taken as the content of CP. Ennor (1957) has shown that this method underestimates the content of CP by 11%.

Measurement of ATP

Frozen muscle was extracted with TCA in the way described above for C, and the TCA was then removed by shaking the extract in the cold room with 20 ml of diethyl ether (Analar grade, B.D.H.), and repeating the extraction four times. Residual ether in the aqueous phase was eliminated by warming to 50° C and bubbling air through it for 2 minutes.

The ATP content of an extract was measured by the firefly luminescence method of Strehler & McElroy (1957), using a freeze-dried extract of firefly lanterns (Sigma, FLE-50).

Measurement of calcium

Each piece of muscle was blotted, placed in a porcelain crucible and ashed in a muffle furnace at 600° C for 1 hour. The cooled ash was dissolved in 1 ml of 1 N HCl solution containing 100 μg $LnCl_3.7H_2O$, as suggested by Bianchi (1968). The concentration of calcium in the dissolved ash was measured in an atomic absorption spectrophotometer (Unicam SP 90A).

Chemicals

Doses of histamine hydrogen tartrate refer to the free base. Indomethacin (kindly donated by Dr. R. Hodgkinson, Merck Sharp and Dohme Ltd.), phenylacetic acid and 2:4-dinitrophenol were dissolved in a slight excess of sodium carbonate solution and quickly adjusted to pH 7·4 with HCl. Potassium cyanide solutions were adjusted to pH8 with HCl and then kept in closed containers until used. Potassium palmitate (0·1% solution) was prepared by dissolving palmitic acid in KOH solution, adjusting to pH 7·4 with HCl and then adding bovine serum albumin (Armour) in a final concentration of 3·5%. Papaverine was dissolved in HCl solution and adjusted to pH 6·5 with NaOH.

Results

Glycerol-extracted muscle

Strips of glycerol-extracted muscle developed no tension in response to the application of PC (4 mM), even in the presence of calcium chloride (10^{-5} M) plus magnesium chloride (10^{-2} M). Nevertheless, PC (4 mM) potentiated the response to a mixture of ATP, calcium chloride and magnesium chloride (Figs. 1 and 2). Tension increments could be reobtained at 30 min intervals for several hours provided the strips were replaced in the original bathing fluid after each response. Bozler (1968) reported similar findings with glycerol-extracted frog stomach muscle. Indomethacin (50 mg/100 ml) failed to alter the response to a mixture of ATP, CP, calcium chloride and magnesium chloride, whereas mersalyl (0·1–1·0 mM) inhibited the tension increments (Fig. 2). Mersalyl inhibits many ATP-ases, including those of the contractile proteins (Weber & Portzehl, 1954 ; Webb, 1966b).

Experiments with living muscle

Histamine (0·1–1·0 μg/ml) caused brisk isotonic shortening of guinea-pig stomach muscle which could be reobtained at 6 min intervals without evidence of tachy-

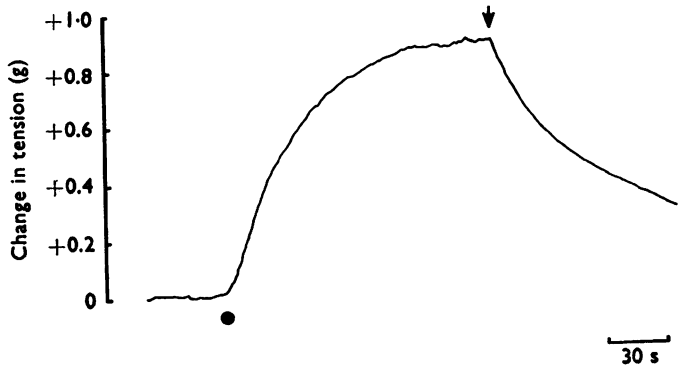

FIG. 1. Effect of ATP on tension in a glycerol-extracted strip of stomach muscle. At ● the bathing fluid was supplemented with ATP (2×10^{-3}M), PC (4×10^{-3}M), magnesium chloride (10^{-2}M), and calcium chloride (10^{-5}M). At ↓ the tissue was returned to its original bathing fluid.

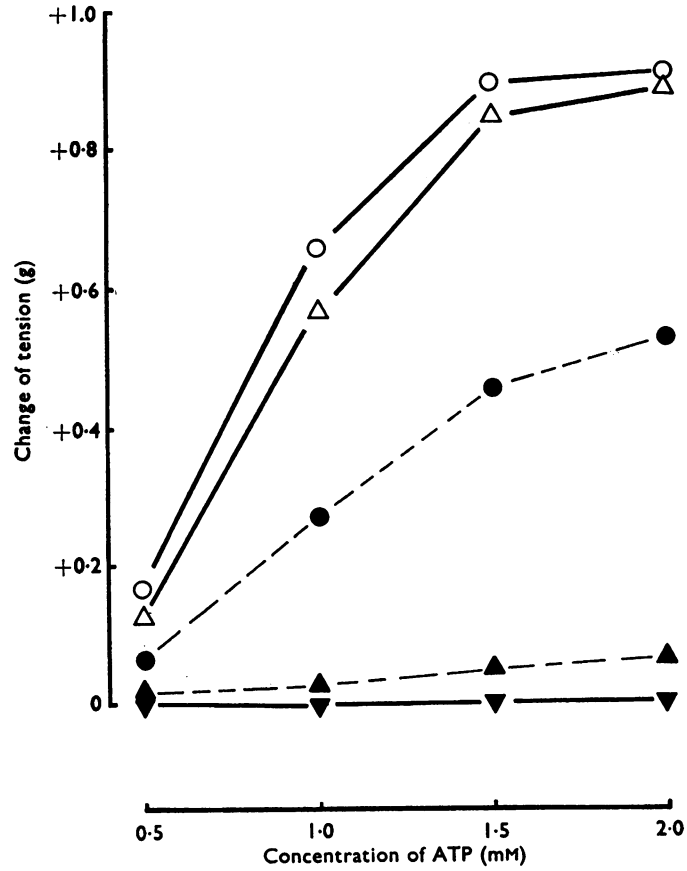

FIG. 2. Changes of tension in glycerol-extracted strips of stomach muscle recorded 2 min after adding ATP. Solid lines connect responses obtained with PC (4×10^{-3}M), magnesium chloride (10^{-2}M), and calcium chloride (10^{-5}M) in the bathing fluid. Responses ○——○ are controls; △——△, with indomethacin (0·5 mg/ml); ▼——▼, with mersalyl (1 mM); ●- - -●, with magnesium chloride (10^{-2}M), and calcium chloride (10^{-5}M) but without PC; ▲- - -▲, without PC, magnesium chloride or calcium chloride.

phylaxis for up to 10 hours. Similar doses of histamine also caused brisk increases in isometric tension. Addition of indomethacin (1–20 mg/100 ml) inhibited the responses to histamine.

Muscle dependent upon aerobic metabolism

Strips of stomach muscle bathed with glucose-free solution showed diminishing responses to histamine, particularly after the addition of 2-deoxy-D-glucose (0·1 mg/ml), as shown in Fig. 5. Deoxyglucose is an inhibitor of glycolysis (Webb, 1966a) and probably prevents the utilization of stored glycogen. The ability of the muscle to remain contracted in response to histamine was affected more than its ability to contract initially (Fig. 5). The residual supply of energy is probably derived from a non-carbohydrate substrate. Table 1 shows that exposure to glucose-free bathing fluid containing deoxyglucose slightly reduces the content of CP but not of ATP. Complete and rapid restoration of the contractile response to histamine was seen after adding glucose (1 mg/ml), sodium pyruvate (0·1 mg/ml) or potassium palmitate (50 μg/ml), whereas bovine serum albumin (1·8 mg/ml) was inactive (Fig. 5).

The addition of indomethacin (1–20 mg/100 ml) to the glucose-free fluid bathing stomach muscle maintained on pyruvate or palmitate as an energy source, caused inhibition of the response to histamine (Figs. 3 and 4), whereas the CP and ATP contents were unchanged (Table 1). 2:4-Dinitrophenol (0·018–18 mg/100 ml), on the other hand, reduced the content of both CP and ATP.

Muscle dependent upon glycolysis

Strips of stomach muscle were deprived of oxygen by gassing the bathing fluid with nitrogen (British Oxygen, White Spot grade) and aerobic metabolism further reduced by adding potassium cyanide (0·1 mg/ml) to the bathing fluid. Provided that the bathing fluid contained the usual concentration of glucose the tissue continued to respond to histamine with a brisk contraction and the content of ATP remained unchanged whilst the content of CP actually increased (Table 1). Removal of the glucose from the bathing fluid, however, caused a rapid decline in the response to histamine of the anaerobically maintained muscle. This deterioration in response is accounted for by the depletion of the stores of CP and ATP which occur under these circumstances, and which is not prevented by adding sodium pyruvate (Table 1).

Indomethacin (1–20 mg/100 ml) reduced the response to histamine of anaerobically maintained stomach muscle (Fig. 3) although the content of CP and ATP did not change significantly (Table 1). The inhibitory effect of indomethacin on glucose-maintained stomach muscle gassed with nitrogen or with oxygen plus carbon dioxide was significantly less than on aerobic muscle deprived of glucose but supplied with pyruvate or palmitate (Fig. 4).

Response of stomach muscle to electrical stimulation

Strips of stomach muscle contracted with a partially fused tetanus when stimulated electrically (100 ms pulses, 0·2 Hz), as shown in Fig. 6. Contractions were unaffected by tetrodotoxin (0·1 μg/ml). Since this concentration of tetrodotoxin blocks neuronally mediated responses of the guinea-pig stomach (Gershon, 1967)

TABLE 1. *Concentration of creatine (C), creatine phosphate (CP) and adenosine triphosphate (ATP) in stomach muscle under various conditions*

Substrate added to bathing fluid	Gas	Drug		Mean substrate µmol/g ± S.E.			CP+ATP µmol/g	$\frac{CP}{CP+C}$ %
		Name	Concentration mg/100 ml	C	CP	ATP		
Glucose 1 mg/ml	O_2/CO_2	Nil		0·25±0·06§	0·61±0·11‡	1·96±0·14†	2·57	71
,,	,,	Indomethacin	20	0·31±0·09	0·49±0·12	2·18±0·18	2·67	61
,,	,,	Dinitrophenol	0·018	0·32±0·04	0·70±0·08	1·80±0·10	2·50	69
,,	,,	,,	0·18	0·63±0·06¶	0·15±0·03*	1·81±0·20	1·96	19
,,	,,	,,	1·8	0·51±0·05¶	0·16±0·05*	2·05±0·25	2·21	24
,,	,,	,,	18	0·50±0·03¶	0·27±0·07*	1·44±0·12‖	1·71	35
,,	N_2	Nil		1·50±0·11¶	2·23±0·44*	2·24±0·19	4·47	60
,,	,,	Indomethacin	20	1·36±0·13¶	2·19±0·30*	2·09±0·13	4·28	62
Sodium pyruvate 0·1 mg/ml	O_2/CO_2	Nil		0·69±0·05¶	0·82±0·09	1·98±0·15	2·80	54
,,	,,	Indomethacin	20	0·83±0·12¶	0·78±0·06	1·78±0·21	2·56	48
,,	N_2	Nil		1·32±0·18¶	1·18±0·02*	0·77±0·09‖	0·95	12
Nil	O_2/CO_2	,,		0·45±0·04¶	0·31±0·04*	1·76±0·11	2·07	41
,,	,,	Deoxyglucose	10	0·47±0·06¶	0·26±0·04*	2·19±0·23	2·45	36
,,	,,	Dinitrophenol	18	0·55±0·08¶	0·14±0·05*	1·34±0·10‖	1·48	20
,,	N_2	Nil		1·38±0·13¶	0·38±0·12*	0·70±0·04‖	1·08	22

A significant difference exists (Student's t test, $P<0·05$) between the value marked § and values marked ¶, between the value marked ‡ and values marked *, and between the value marked † and values marked ‖.

it is concluded that the muscle was being stimulated directly in the present experiments. Indomethacin (1–20 mg/100 ml) inhibited the contractile response to electrical stimulation (Fig. 6).

Figure 7 shows that the calcium content of stomach muscle increased progressively during 30 min of electrical stimulation (100 ms pulses, 0·2 Hz). Lüllmann & Mohns (1969) reported similar findings using guinea-pig small intestinal muscle. Both indomethacin (7·20 mg/100 ml) and cinchocaine (0·5–5·0 mg/100 ml) caused a similar proportional reduction in the contractor response and in the uptake of calcium (Table 2). Phenylacetic acid is related chemically to indomethacin but is devoid of anti-inflammatory activity (Northover, 1964; Durant, Smith, Spickett & Szarvasi, 1965). Phenylacetic acid (1 mg/ml) failed to prevent either the contraction or the uptake of calcium in response to electrical stimulation (Table 2). Papaverine (5 mg/100 ml), on the other hand, abolished contraction of stomach muscle but failed to prevent the uptake of calcium (Table 2).

Like stomach muscle, the aorta showed an uptake of calcium in response to 30 min of electrical stimulation (Fig. 7). Table 2 shows that both indomethacin and cinchocaine inhibited the uptake of calcium by the aorta, but whereas lower concentrations of indomethacin were required by the aorta than by the stomach, similar concentrations of cinchocaine were required by the two tissues.

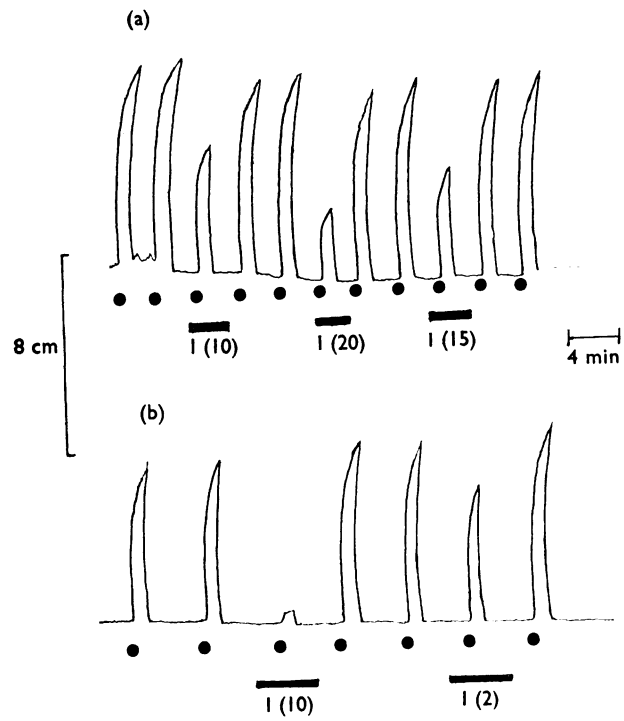

FIG. 3. Effect of indomethacin on responses of stomach muscle. Histamine (1 μg/ml) was applied at ●. Between each response the kymograph was stopped and the organ bath refilled with fresh bathing fluid. Indomethacin was present at the concentration (mg/100 ml) indicated during the periods marked I. In (a) the bathing fluid contained glucose (1 mg/ml) and potassium cyanide (0·1 mg/ml), and was gassed with nitrogen. In (b) the glucose-free bathing fluid contained deoxyglucose (0·1 mg/ml) and potassium palmitate (50 μg/ml) and was gassed with 95% oxygen plus 5% carbon dioxide.

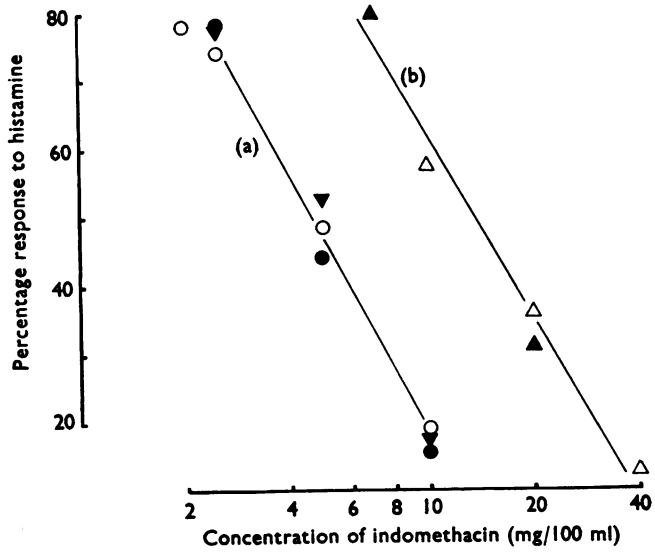

FIG. 4. Effect of indomethacin on the response of stomach muscle to histamine (1 μg/ml). Responses (●) were recorded isometrically and all other responses were recorded isotonically. △, Bathing fluid containing glucose (1 mg/ml) plus potassium cyanide (0·1 mg/ml) and gassed with nitrogen. All other responses were obtained with 95% oxygen plus 5% carbon dioxide. ▲, Responses in a bathing fluid containing glucose (1 mg/ml); ▼ and ○, responses in glucose-free bathing fluid with deoxyglucose (0·1 mg/ml). Sodium pyruvate (0·1 mg/ml) was added to the bathing fluid in the case of ▼, and potassium palmitate (50 μg/ml) was added in the case of ○. Responses to histamine in the presence of indomethacin were expressed as a percentage of the preceding response to histamine in the absence of indomethacin. Line (b) is the best fitting straight line through points △ and ▲, and line (a) is the best fitting straight line through the other points.

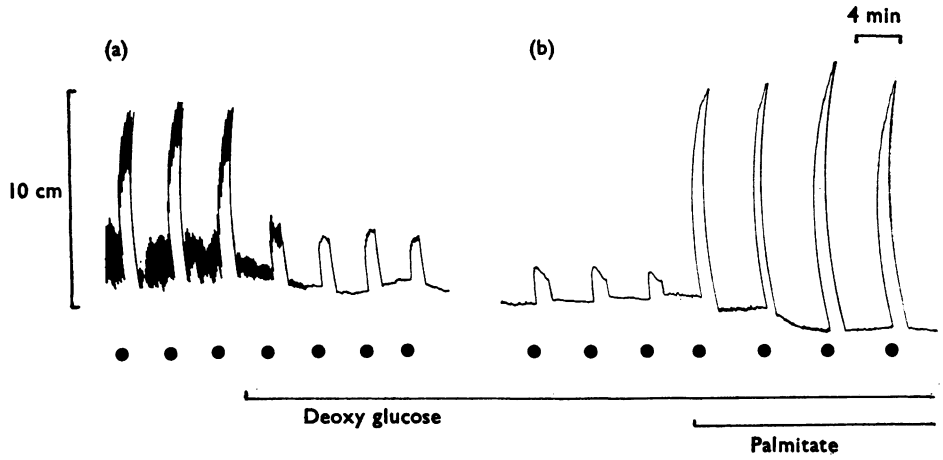

FIG. 5. Effect of deoxyglucose (0·1 mg/ml) and potassium palmitate (50 μg/ml) on the responses of stomach muscle to histamine in glucose-free bathing fluid gassed with 95% oxygen plus 5% carbon dioxide. Histamine (1 μg/ml) was applied at ●. Between each response the kymograph was stopped and the organ bath refilled with fresh bathing fluid. At the start of (a) the tissue had been in glucose-free bathing fluid for 2 hours. During the 70 min which elapsed between (a) and (b) the tissue was re-exposed to histamine at 6 min intervals.

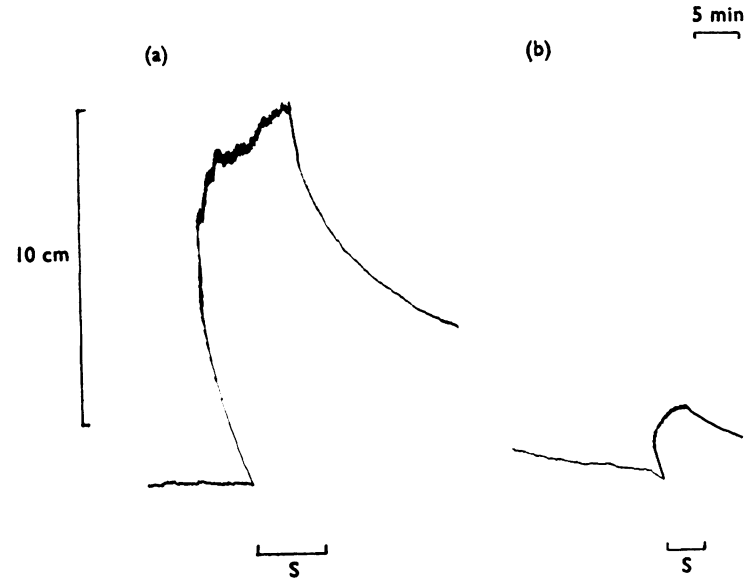

FIG. 6. Contraction of stomach muscle in response to electrical stimulation (100 ms pulses, 0·2 Hz) during the periods marked S. In (b) the bathing fluid contained indomethacin (20 mg/100 ml).

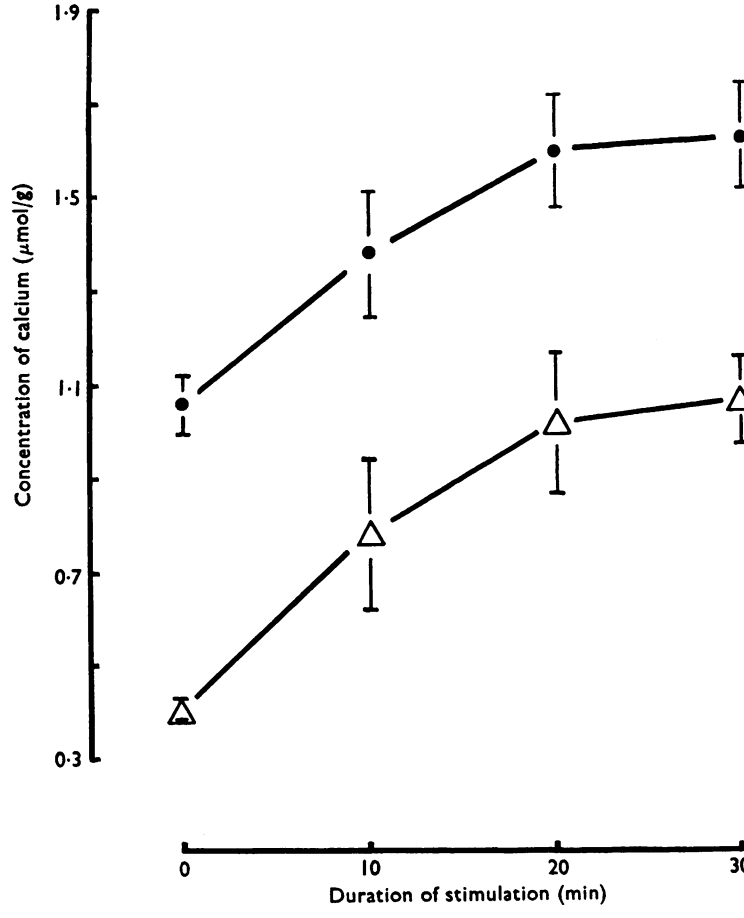

FIG. 7. Effect of electrical stimulation (100 ms pulses, 0·2 Hz) on the calcium content of stomach muscle (△) and aorta (●). The vertical bars represent mean ± standard error.

TABLE 2. *Responses of smooth muscle to electrical stimulation*

Drug: Name	Concentration mg/100 ml	Calcium content μmol/g ± S.E.: Unstimulated	Calcium content μmol/g ± S.E.: Stimulated ¶	Calcium uptake: μmol/g	Calcium uptake: as % of control	Height of contraction as % of control §
		(a) Stomach				
Control		0·41 ± 0·02	1·07 ± 0·09†	0·66	100	100
Indomethacin	7	0·40 ± 0·07	0·95 ± 0·17	0·57	86	83
Indomethacin	20	0·39 ± 0·05	0·65 ± 0·13*	0·25	38	22
Papaverine	5	0·41 ± 0·03	1·00 ± 0·08	0·59	89	2
Phenylacetic acid	100	0·38 ± 0·07	1·02 ± 0·20	0·64	97	94
Cinchocaine hydrochloride	0·5	0·42 ± 0·06	0·98 ± 0·16	0·56	85	83
Cinchocaine hydrochloride	1·0	0·44 ± 0·04	0·76 ± 0·09*	0·32	49	40
Cinchocaine hydrochloride	5·0	0·38 ± 0·06	0·49 ± 0·08*	0·11	17	11
		(b) Aorta				
Control		1·06 ± 0·06	1·63 ± 0·11‡	0·57	100	
Indomethacin	2	1·02 ± 0·04	1·41 ± 0·12	0·39	68	
Indomethacin	7	1·11 ± 0·08	1·23 ± 0·07‖	0·12	21	
Indomethacin	20	0·98 ± 0·05	1·06 ± 0·10‖	0·08	14	
Cinchocaine hydrochloride	0·5	0·93 ± 0·10	1·53 ± 0·13	0·60	105	
Cinchocaine hydrochloride	1·0	1·04 ± 0·07	1·30 ± 0·06‖	0·26	46	
Cinchocaine hydrochloride	5·0	1·17 ± 0·12	1·29 ± 0·08‖	0·12	21	

¶ Field stimulation with 100 ms pulses, 0·2 Hz for 30 minutes. § Field stimulation with 100 ms pulses, 0·2 Hz for 3 minutes. A significant difference exists (Student's *t* test, $P<0·05$) between value marked † and values marked *, and also between value marked ‡ and values marked ‖.

Discussion

Indomethacin appears to lack an effect on the contractile proteins of smooth muscle since it failed to reduce the contraction of glycerol-extracted strips of muscle in the present experiments. Görög & Kovacs (1970) reported that a number of analgesic-antipyretic drugs, including indomethacin, inhibited the ATP-ase activity of skeletal muscle actomyosin. The maximal inhibition produced by even very high concentrations of indomethacin was, however, less than 50%.

Indomethacin inhibited contraction of smooth muscle in the present experiments in concentrations which caused no significant depletion of ATP or of CP. A decline in the content of ATP and of CP was reported by Smith & Jeffrey (1956) in striated muscle under the influence of sodium salicylate. Kalbhen & Domenjoz (1967) reported, however, that anti-inflammatory doses of sodium salicylate and phenylbutazone failed to reduce the ATP content of the inflamed rat foot. Using pieces of bovine cartilage *in vitro*, Bröhr & Kalbhen (1968) found that various anti-inflammatory drugs inhibited the incorporation of sulphate in mucopolysaccharides, but whereas some of these drugs decreased the content of ATP, others actually increased it. There was no correlation between the change in sulphate incorporation and the change in ATP content.

The inhibitory effect of indomethacin on smooth muscle appears to be associated with, and probably caused by, an inhibition of the influx of calcium into the muscle cell. There was a good correlation between the prevention of contraction of the stomach in response to electrical stimulation by both indomethacin and cinchocaine, and their respective inhibitory effects on the uptake of calcium (Table 2). Several drugs, mostly local anaesthetics, reduce the movement of ions, including calcium ions, through cell membranes (Shanes, 1958 ; Ritchie & Greengard, 1966 ; Cuthbert, 1967 ; Kuperman, Altura & Chezar, 1968). This appears to be the first time, however, that a similar mechanism has been invoked to account for the actions of indomethacin.

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