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CHEMICAL FACTORS AFFECTING SPONTANEOUS MOTILITY OF THE SMALL INTESTINE IN THE RAT—1. SULFHYDRYL REACTANTS*

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Abstract—To determine whether the sulfhydryl group is involved in the mechanism of intestinal smooth muscle contractility, relative reactivities of a variety of sulfhydryl reactants with the cysteine sulfhydryl group were evaluated by a modification of the indophenol method (Basford and Huennekens[10]) along with parallel studies of the effects of the same sulfhydryl reactants on spontaneous motility and response to acetylcholine of isolated segments of rat ileum in Ringer's solution. Characteristics of intestinal contractility of normal and treated segments were continuously recorded on a photometric apparatus devised to measure frequency and amplitude of isotonic contractions. Compounds highly reactive toward the sulfhydryl group included the following: lead acetate, zinc chloride, cadmium chloride, *p*-hydroxymercuribenzoic acid, 2-iodoacetamide, 2,4-dinitrofluorobenzene, hydrogen peroxide, iodine in potassium iodide, alloxan, N-ethylmaleimide, patulin, N-phenylmaleimide. These were compared with a number of compounds which do not react with the SH group including: urea, sorbitol hydrate, inosine, sucrose, lecithin, and glycyl-L-serine. Results of this study demonstrate that reagents of widely varied structures but with high reactivity toward the SH group profoundly affect the spontaneous motility of the isolated small intestine of the rat. Curves are given to illustrate the decrease in energy of acetylcholine-induced contraction with increased concentrations of sulfhydryl reactants. Representative traces of individual experiments demonstrating normal motility and the inhibitory effects of sulfhydryl reactants are given. In all cases studied compounds showing high reactivity toward the SH group, at appropriate concentrations (10^{-2} to 10^{-5} M) can completely inhibit spontaneous motility of the small intestine; all can block the acetylcholine-induced contraction. Many at appropriate concentrations (10^{-4} to 10^{-7} M) can induce contraction.

IN THEIR now classic studies, Engelhardt and Ljubimova[1, 2] described the enzymic activity as well as the elastic properties of myosin isolated from striated muscle. The elasticity and the ATPase activity of myosin fibres were greatly reduced when treated with compounds known to react with sulfhydryl groups, such as copper or silver salts, and iodoacetic acid.

The sulfhydryl group has been repeatedly implicated in the mechanism of muscle contraction in the extensive studies of Bacq,[3] Weber and Portzehl,[4] Szent-Gyorgyi,[5] Koshtoyanz and Turpaev,[6] Mendez,[7] Bailey and Perry,[8] and others.

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Bacq[3] has demonstrated that a wide variety of compounds highly reactive towards the sulfhydryl group (the 'thioloprives') produce the 'Lundsgaard effect' with loss of activity in striated muscle of the frog.

Although the story is by no means complete there is general agreement that at least in the contraction of striated muscle, sulfhydryl-containing proteins play a vital role.

Most of the literature on chemical mechanisms of muscle contraction has been concerned with striated muscle, but it seems reasonable that, in general, smooth muscle would have similar properties. This view is expressed by Csapo[9] as a consequence of his extensive studies of uterine muscle.

The present work was undertaken in an effort to determine whether and by what mechanism the sulfhydryl group is similarly involved in the regulation or control of intestinal smooth muscle contractility. Relative reactivities of a variety of chemical agents toward the cysteine sulfhydryl group were evaluated along with parallel

TABLE 1. EFFECTS OF VARIOUS REAGENTS ON INTESTINAL CONTRACTION AND THE CYSTEINE SULFHYDRYL GROUP

Reagents	A Minimal concn.* to produce 10% of maximal contraction (M × 5·7)	B Mean concn. to prevent ACh contraction (M × 5·7)	C Per cent SH reacted† (1 min at 25°)
Group I			
Cadmium chloride	10^{-7}	10^{-4}	100
p-Hydroxymercuribenzoic acid	10^{-6}	10^{-3}	100
Lead acetate	10^{-7}	10^{-4}	100
Zinc chloride	10^{-7}	10^{-3}	100
Group II			
2,4-Dinitrofluorobenzene	No contr.	10^{-4}	67
Iodoacetamide	No contr.	10^{-3}	75
Group III			
Hydrogen peroxide	10^{-7}	10^{-5}	67
Iodine-(KI)	10^{-4}	10^{-4}	100
Group IV			
Alloxan	No contr.	10^{-3}	47
N-Ethylmaleimide	10^{-6}	10^{-4}	76
Patulin	10^{-7}	10^{-4}	90
N-Phenylmaleimide	No contr.	10^{-4}	84
Group V			
Cysteine			0
Glyclyl-L-serine			0
Inosine			0
Lecithin	No contraction	Normal ACh contraction	0
Serine			0
Sorbitol hydrate			0
Sucrose			0
Urea			0

* Comparison of columns A and B illustrates those cases of test compounds which at low concentration cause at least 10% of the maximal contraction produced with acetylcholine alone, and at higher concentrations (column B) prevent the acetylcholine contraction (see text).

† Compounds (10^{-3} M) were treated with cysteine (2×10^{-4} M) in acetate buffer, pH 5·8 containing Versene, and the rate of reaction was determined by the indophenol method[10]; 100%–SH represents 2·00 μM cysteine.

studies of the effects of the same chemical agents on the spontaneous contractility and response to acetylcholine of isolated segments of rat intestine in a tissue bath.

EXPERIMENTAL

Chemical reagents were Eastman, Baker, or Fisher reagent grade except as follows: California Corp. for Biochemical Research: acetylcholine chloride, alloxan. K and K Laboratories: N-phenylmaleimide. Nutritional Biochemicals Corp.: dimercaptopropanol (BAL), inosine, iodoacetamide, L-cysteine, L-serine, N-ethylmaleimide, sodium *p*-hydroxymercuribenzoate, sorbitol hydrate. Patulin, kindly supplied by Dr. Emmett W. Bassett of this institution.

Test compounds were treated with standard sulfhydryl-containing reagents including L-cysteine and glutathione. Sulfhydryl content was assayed at various intervals to determine rates of sulfhydryl loss chiefly by means of a modification of the indophenol method.[10] With this technique the reactivity of a given reagent toward sulfhydryl-containing compounds is readily evaluated even though the exact mechanisms or products may be unknown (Table 1). Detailed chemical aspects of the determination of reactivities towards the sulfhydryl group, including data and special problems related to the glutathione assay, will be published elsewhere.

To evaluate the effects of chemical agents on spontaneous contractility of isolated segments of rat intestine the simple apparatus illustrated in Fig. 1 was devised.

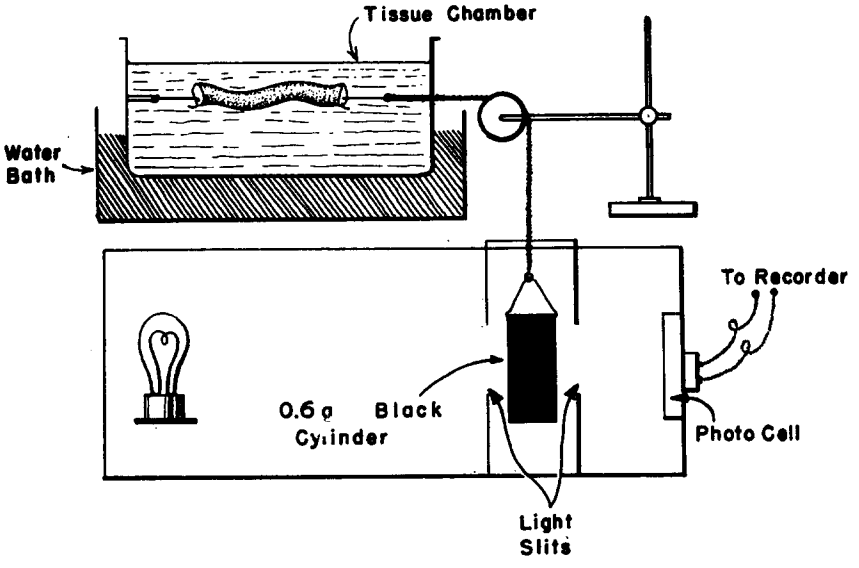

FIG. 1. Apparatus for measuring isotonic contraction of intestinal smooth muscle.

This device, designed to measure isotonic contractions, differs from others previously described;[11] utilizing a small load (0·6 g) and a light pulley (Harvard Apparatus Co., no. 233), it records very feeble contractions with high sensitivity. The segment is suspended in Ringer's solution in the tissue bath by loops of surgical silk tied to each end. One end of silk is anchored to a fixed notch at one end of the chamber,

and the other passes through a hole (1-mm diameter) in the end of the polyacrylic plastic chamber 5 mm below the surface of the liquid and is connected over a pulley to a cylindrical, opaque weight made of exposed photographic film, which rides vertically between parallel rectangular light slits and intercepts a light beam from a 12-W, 110-V frosted lamp falling on a selenium photovoltaic cell (GE type PV 2). Surface tension prevents loss of solution through the hole in the chamber. The current output of the photo cell, which is directly proportional to the area exposed to illumination, was recorded on a GE photoelectric potentiometer recorder, type CE5. Test compounds were introduced in a volume of 1·00 ml at the base of the tissue bath by a tuberculin syringe attached to a polyethylene capillary. Concentrations of reagents upon dilution varied from 10^{-2} M to $5{\cdot}7 \times 10^{-9}$ M. Mixing was by syringe action as well as by action of the rising stream of gas bubbles. The pH of the complete system (Ringer's solution with test compound) remained between 5 and 6 without added buffers. This mildly acidic pH was found desirable not only to enhance the spontaneous motility characteristics of the gut, but also to focus on its sulfhydryl as opposed to its amino or hydroxyl group reactivity. For in proteins above pH 7 the nucleophilic groups –SH, $-NH_2$, and –OH may all react with strongly electrophilic reagents such as the alkylating agents,[12] the halodinitrobenzenes, and others, whereas below pH 7 (in the protonated form) only the –SH group has appreciable nucleophilic potential.

Experimental animals were young male and female rats of the Sprague–Dawley strain with weights ranging from 120–220 g; the entire small intestine was excised from the freshly killed animal and was stored in Tyrode–Ringer's solution at 25° until used. Motility measurements were made in Ringer's solution to keep the number of extraneous reagents to a minimum; 30-mm segments were cut from the terminal ileum and from other regions of the small gut when required. A plastic millimeter scale cemented to the bottom of the polystryene tissue chamber was used to measure segments at intervals during and at the end of each experiment. On the basis of chart calibration, the length at any time during the entire experiment as well as frequency and amplitude of contraction was readily determined. A 5-min equilibration period in Ringer's solution at 37° was generally adequate to achieve a steady state of spontaneous rhythmic contraction. Wet weights of segments were between 0·15 and 0·30 g. The contents of the segments were gently expressed while immersed in Tyrode–Ringer's solution, and the segment was transferred to the tissue chamber. The tissue bath, maintained at 37°, contained 175 ml of Ringer's solution with a very fine stream of O_2–CO_2 (95:5) or air continuously bubbling through, although this was not essential for activity. Segments were generally suspended with the lumen open; closed ends were also used, but results were not significantly changed.

Experiments on a given compound were repeated at least three times for each concentration or until reproducible results were obtained; a fresh segment was used for each experiment. Mean quantitative values for contractile response were calculated in terms of work performance (ergs/sec per cm of gut segment); replicate results were in reasonable agreement with variations within $\pm 10\%$.

To minimize the influence of extraneous electrolytic effects, the segment was suspended by silk loops instead of metallic hooks. Excessive stretching or pressure during initial removal or during the experiment may partially or totally inactivate the gut segment. Agitation of the suspending medium may also affect the contractile pattern.

The motile characteristics of the small intestine of the rat are accentuated in this metastable functional state, making this a versatile system for the study of the mechanisms of smooth muscle contractility. The presence of buffers or glucose tends to mask the contractile characteristics here noted.

The freshly removed intestine may be kept viable in Tyrode–Ringer's solution at 25° for about 4 hr, but it gradually loses tonus. Segments were discarded if initial gross amplitude was less than 1 mm.

RESULTS AND DISCUSSION

With this preparation the frequency and amplitude of spontaneous contraction of 'normal' and treated segments of rat intestine were recorded. Two distinct types of normal periodic contraction were noted, one having a mean frequency of 18 cycles/min with a mean amplitude of 1·2 mm (4% of initial length); 14 ergs/sec per cm) and another superimposed on the first, with a higher mean amplitude of 5 mm (17%) and mean frequency of 1·3 c/min (4·3 ergs/sec per cm) in Ringer's solution at 37°.*

The effects of various groups of chemical reagents highly reactive as well as these that do not react with the organic sulfhydryl group have been determined and are illustrated by specific recordings from each class in Figs. 2–7 and Table 1.

Representative members of the following groups of compounds were used: metal mercaptide-forming reagents, organic electrophilic reagents, oxidizing agents, and others reacting with the sulfhydryl group by a variety of mechanisms. These were compared with compounds that do not react with the sulfhydryl group (Fig. 2).

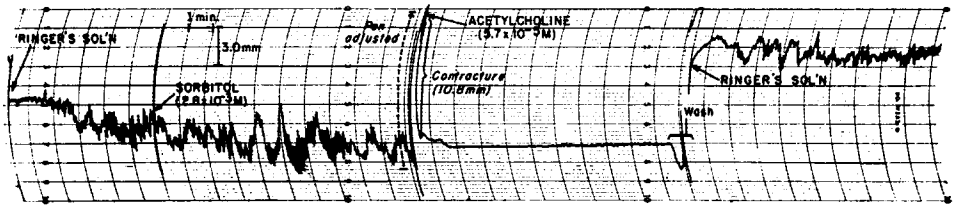

FIG. 2. Sorbitol ($2{\cdot}8 \times 10^{-3}$ M) does not alter the normal pattern of spontaneous motility and has no effect on ACh contraction. Normal motility is restored when ACh is washed out.

Group I. Metal mercaptide reagents: lead acetate, zinc chloride, cadmium chloride, and *p*-hydroxymercuribenzoic acid. All these react very rapidly *in vitro* with the cysteine sulfhydryl group (Table 1). They also react rapidly with the isolated gut segment, causing inactivation at concentrations of 10^{-3} to 10^{-4} M (Fig. 3, 7) with inhibition of the normal acetylcholine-induced contraction. At lower concentrations (10^{-5} to 10^{-6} M) they have no effect or even enhance spontaneous intestinal motility, causing increased amplitude of contraction without inhibition of the acetylcholine (ACh) contraction.

Group II. Organic electrophilic reagents: iodoacetamide, 2,4-dinitrofluorobenzene. These reagents when used in concentrations of 10^{-3} to 10^{-4} M cause initial contraction though at a lower rate than acetylcholine, and inhibit the acetylcholine contraction.

* Energy values are calculated only for the contractile phase of the cycle.

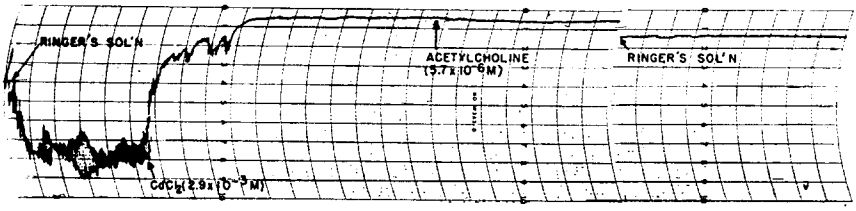

FIG. 3. Cadmium chloride, ($2{\cdot}9 \times 10^{-3}$ M) causes rapid loss of spontaneous motility, total relaxation of segment, and prevents the acetylcholine contraction. Motility is not restored upon washing.

Group II reagents, however, do not enhance spontaneous motility at any concentration. Iodoacetamide prevents the ACh contraction when used at a concentration of $5{\cdot}7 \times 10^{-3}$ M but allows a normal contraction at $5{\cdot}7 \times 10^{-4}$ M. (The more dilute solution still causes reduced amplitude of spontaneous contractions, and the ACh contraction is of shorter than normal duration; Fig. 4.)

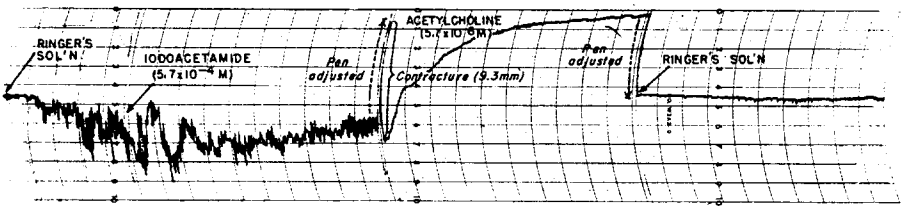

FIG. 4. At a concentration of one-tenth that required for complete loss of intestinal motility, iodoacetamide ($5{\cdot}7 \times 10^{-4}$ M) causes reduced amplitude of contraction and permits a normal ACh contraction followed by relaxation and loss of activity,

Group III. Oxidizing agents: hydrogen peroxide, iodine in KI. The effects of these compounds on spontaneous gut motility are in general quite similar to those of Group II. They produce a slow initial contraction and they prevent the ACh contraction. KI in the absence of I_2 does not distort the normal contractility pattern (Fig. 5).

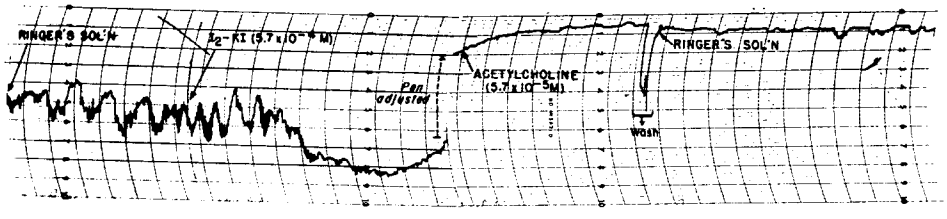

FIG. 5. Iodine in KI ($5{\cdot}7 \times 10^{-4}$ M) produces a slow contraction with loss of spontaneous motility and prevents the acetylcholine contraction. The segment remains virtually inactive upon washing.

Group IV. Miscellaneous sulfhydryl reagents: alloxan, N-ethylmaleimide, patulin, N-phenylmaleimide. Effects of these compounds on gut motility are quite similar to those of Group II. Alloxan and N-ethylmaleimide are well known sulfhydryl reagents[13] and react rapidly in the present assay (Table 1). The antibiotic, patulin,

has been previously reported to have 'spasmolytic action'[14] which was interpreted in terms of a structural antimetabolite mechanism. Present results suggest that its high sulfhydryl reactivity (Table 1) might be the basis not only for its effects on intestinal contraction (Fig. 6) but also for its toxicity. Preincubation of gut segments with cysteine or glutathione was effective in preventing or reducing the inhibitory effects of various of the compounds reactive towards the SH group.

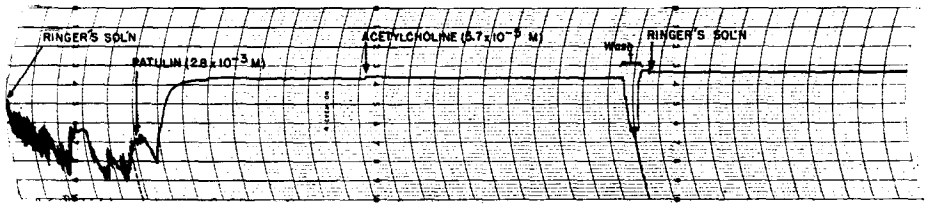

FIG. 6. Patulin ($2{\cdot}8 \times 10^{-3}$ M) results in loss of spontaneous motility and abolishes the contractile response to ACh.

Group V. Compounds unreactive towards the sulfhydryl group: urea, sorbitol hydrate, inosine, serine, sucrose, lecithin, glycyl-L-serine. These compounds are examples of a large number found essentially inert even at high concentrations (10^{-2} M) with respect to spontaneous rhythmic contractions and the acetylcholine-induced contraction, as illustrated by sorbitol hydrate (Fig. 2).

The contractile effects of compounds here noted occurred generally within a few seconds after introduction of the reagent, even at high dilution (10^{-4} to 10^{-8} M). These results are paralleled by the rapid rates of sulfhydryl reactions *in vitro* at comparable concentrations of reagents (Table 1).

Various compounds other than those listed in Table 1, having high reactivity toward the sulfhydryl group, have been found to alter the intestinal contractile patterns. Further studies on these substances will be the subject of a future communication.

Present results demonstrate that reagents of widely varied structures but with high reactivity toward the sulfhydryl group profoundly affect the spontaneous motility of the small intestine (Table 1 and Fig. 7) for all sulfhydryl reagents here studied, and at appropriate concentrations (from 10^{-2} to 10^{-5} M), can completely inhibit spontaneous contractility of the gut. All can block the acetylcholine-induced contraction; many can induce contraction.

Each reagent has a characteristic pattern of activity. Some, such as lead acetate or cadmium chloride at low concentrations, tend to enhance the amplitude of contraction, whereas others such as N-ethylmaleimide at similarly low concentrations merely allow a nearly normal contractile pattern (Fig. 7). Since the contractile effects of compounds here included occur whether or not the gut segments are closed at both ends, it seems likely that the chief site of action involves the serosa rather than the mucosa of the small intestine.

To attribute a major role in the process of smooth muscle contraction to one type of chemical functional group, must obviously involve an oversimplification. It is well known that many reagents can affect intestinal motility and yet appear to be unrelated to sulfhydryl reactivity. Nevertheless, the search for sites of reaction

of pharmacological reagents with protein molecules has often implicated the protein sulfhydryl group.[3, 15]

The present study supports the view of Bacq[3] that those compounds which deprive the normal cell of vital sulfhydryl groups (the 'thioloprives') interfere with normal functional activity of the cell. In this case the 'thioloprives' cause a profound interference with the contractile properties of intestinal smooth muscle.

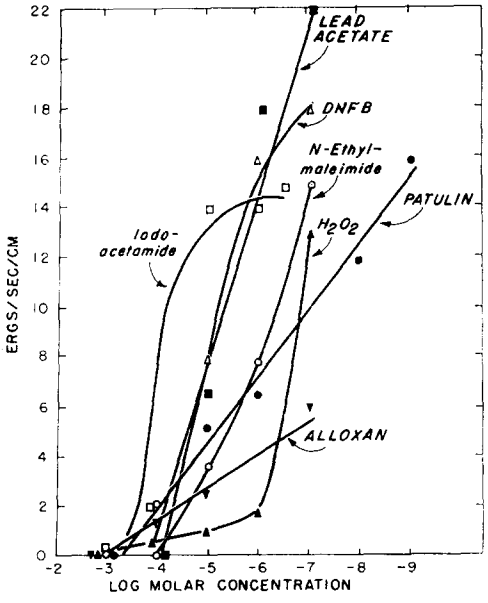

FIG. 7. Mean values for energy expended in acetylcholine ($5{\cdot}7 \times 10^{-6}$ M)-induced contraction. Sulfhydryl reagents were added as illustrated in Fig. 4, at various concentrations. Energy is calculated in ergs per second per centimeter of intestinal segment. Normal ACh contraction in the absence of SH reagents is from 14 to 18 ergs/sec/per/cm.

Present results strongly suggest that the sulfhydryl group is involved in the process of intestinal contraction. It remains to be ascertained whether the sulfhydryl groups involved in contractility are part of the smooth muscle protein or the intrinsic nerve plexi, or are components of vital enzyme systems involved in muscle metabolism, or are part of all of these.

Recent reports linking cell permeability with cell membrane sulfhydryl reactivity[13, 16, 17] lead us to suggest that such permeability changes particularly involving intracellular cation concentrations might form a basis for the interpretation of results reported in this paper.

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