INFLUENCE OF SULFHYDRYL REAGENTS AND COMPOUNDS ON THE CONTRACTILE RESPONSE TO ACETYLCHOLINE IN ISOLATED RAT SMALL INTESTINE

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A slightly significant augmentation of Ach (10^{-6}M)-induced contraction in the rat small intestine was observed by pretreatment with NEM (10^{-7}M), MC (10^{-6}M) or PA (10^{-5}M). Furthermore, pretreatment in high concentration of NEM or PCMB (2 \times 10^{-4} - 10^{-3}M), PA (10^{-3}M) or MC (10^{-5} - 10^{-3}M) caused a marked inhibition of contractile response to Ach.

There were a certain degree of suppression of the Ach uptake by the tissue and a marked increase of Ach level in the organ bath after incubation with NEM (10^{-7}M) or MC (10^{-6}M). These results seem to indicate that these SH-R have an affinity for the terminal cell of cholinergic nerve and the effector cell simultaneously, and they block the uptake of Ach to the former and inhibit the action of Ach to the latter.

On the other hand, contractile response to Ach was slightly increased by addition of ME, CY or PE (10^{-5}M), while the response was depressed after treatment in high doses of them (10^{-4} - 10^{-3}M).

Increase of Ach content in the tissue was observed after incubation of CY (10^{-5}M) and decrement of Ach level in the organ bath was noted after incubation of CY or PE (10^{-5}M).

From these results, it is likely that these SH-C affect on the terminal cell of cholinergic nerve and the effector cell, and they augment the uptake of Ach to the terminal cell and also accelerate the action of Ach to the effector cell.

It has been described in several reports that the cardioinhibitory effect of Ach on the isolated frog heart and rabbit atrium after administration of sulfhydryl reagents (SH-R; NEM and PCMB). As for response of the smooth muscle, on the other hand, the only finding reported to date is by Stubbins et al. that high concentration of SH-R suppressed the Ach-induced concentration of isolated rat ileum. In their experiment, however, only two SH-R (NEM and PCMB) were tested and no attempts were made of assessing the dose-related change of response on the underlying mechanism of action.

The purpose of this investigation was to make observation of the effect of various SH-R and sulfhydryl compounds (SH-C) at increasing concentration on the Ach-induced contraction of isolated rat small intestine and also to clarify the mechanism of actions of these drugs upon the periphery of cholinergic nerves.
MATERIALS AND METHODS

The observation of intestinal motility:

A length, approx. 1-2 cm, of isolated small intestine was dissected and cut off from rat of Wistar strain weighing 150-300g in both sexes. The intestine was suspended with a strain gauge (Nikon Kohden, SB-1T), straining by about 500 mg in an organ bath containing 30 ml Tyrode solution of Magnus apparatus.

The movement and tension of intestine were traced with a constant amplification (Nikon Kohden, RUP-10 or RUP-2-5 and AD-2-22), using an ink-writing recorder (Nikon Kohden, WI-130).

All drugs were diluted in Tyrode solution, and their molar doses determined as final concentration in the organ bath. In the first test, Ach was administered with 10 min-interval and the organ bath was washed out 2 or 3 times with warm Tyrode solution after each observation of the effect of Ach.

As described under experimental results, SH-R or SH-C was treated after the response of intestine to the second dose of Ach had been examined, and, 10 min later, another dose of Ach was administered.

Measurements of the acetylcholine content and activity of cholinesterase:

The whole length of an isolated rat small intestine was divided into three equal segments and each segment was further divided into three equal portions. These segments of intestine were then rearranged into three groups of three each so that each group uniformly contained one of an upper, middle and lower portions of the intestine. The experiments were carried out with the intestinal segments suspended in the bath of Tyrode solution in the same manner as tracing of movements of isolated intestine. These three groups were assigned to none (untreated control), Ach $10^{-4}$M alone and Ach $10^{-4}$M plus SH-R or SH-C, respectively. The intestinal segment in the control group was placed in the bath for 25 min. The segment in the Ach treatment group were placed in the bath for 15 min and then incubated with Ach for 10 min. On the other hand, the tissue in the Ach plus SH-R or SH-C group was exposed to SH-R or SH-C for 10 min after a 5 min-preincubation, followed by addition of Ach to the bath and continuation of incubation for 10 min more. Thus the segments in the three groups were removed from the bath after the same to total length of incubation for 25 min, and subjected to tests.

Activity of cholinesterase (ChE) and Ach assays were performed in accordance with the procedure described by Miyazaki et al. for the former and by Miyazaki et al. modifying Hestrin's method modifying Hestrin's method for the latter.

The following drugs and their concentrations were used: acetylcholine (Ach) $10^{-6}$M; N-ethylmaleimide (NEM), parachloromercuribenzoic acid (PCMB), monoiodoacetic acid (MA), iodoacetamide (IA), mercuric chloride (MC), phenylmercuric acetic acid (PA), mercaptoethanol (ME), penicillamine (PE), cysteine (CY), reduced type glutathion (GL) $10^{-8}$ to $10^{-2}$M.

RESULTS

1. Changes of contractile response to repeated doses of acetylcholine

The most adequate contraction of the intestine was noted when Ach was applied in a dose of $10^{-6}$M. From this observation, Ach in this dose level was used throughout the following experiments.

As shown in Table 1 the contraction of isolated rat small intestine was found to be approx. 20 % increase on the
average at the second dose of Ach in comparison with that noted after the first dose of this drug, when applied with 10 min-interval. Contractile responses to the third dose of Ach, however, were not significantly different from that observed after the second dose of the drug.

From these findings, the degree of contractile response produced by the second dose of Ach was used as the control level in the following experiments carried out to see the effect of pretreatment with SH-R or SH-C.

**TABLE 1**

<table>
<thead>
<tr>
<th>Number of administrations</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contractions</td>
<td></td>
<td>+20.3*</td>
<td>+19.9</td>
<td>+24.4</td>
</tr>
<tr>
<td>% changes from control</td>
<td></td>
<td>±</td>
<td>±</td>
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<tr>
<td></td>
<td>10.7</td>
<td>12.1</td>
<td>14.5</td>
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</table>

Changes of contractile response to repeated doses of acetylcholine.

Acetylcholine 10^{-6}M

*Mean per cent change from control (mean ± s.e.), 12 experiments.

2. Influence of sulfhydryl reagents on the acetylcholine-induced contraction

Most of SH-R failed to exert any significant effect on the movement of small intestine when applied singly at concentration not higher than 10^{-4} M, but only PA caused a slight contraction in some cases. At higher concentrations 2×10^{-4}-10^{-3}M of SH-R, virtually no change occurred after application of MA, whereas IA proved to produce a slight relaxation of some intestines. MC and PA at these concentrations were found to give rise to a slight contraction in several cases, which was followed by a little relaxation in a few cases. With NEM and PCMB a sustained contraction developed in a number of cases, and some of the intestine treated with PCMB subsequently exhibited a slight relaxation.

Pretreatments with 10^{-8}M of NEM, PCMB or IA and less than 10^{-7}M of MC or PA did not significantly affect the Ach-induced intestinal contraction, however, showed about 5-25% increase in the contraction on the average after they had been exposed to 10^{-7}M of NEM, PCMB, MA or IA, 10^{-6}M of MC and 10^{-5}M of PA. The changes in contractile response to Ach after treatment with PCMB, MA or IA, however, were not significant as compared with the untreated control.

The slightly significant enhancements of Ach effect were evident in the case of NEM, PA or MC. In a few cases those effects of various SH-R were reversed to some extent by washing the intestines to restore consequently the contractile effect of Ach to the control level, whereas in many cases, the enhanced Ach effect after treatment with SH-R persisted without significant diminution even in spite of washing. The contraction by Ach was generally not influenced by 10^{-6} - 10^{-4}M of NEM or IA, 10^{-5}-10^{-4}M of PCMB or MA and 10^{-4}M of PA, although a slight inhibition of the contraction was noted in some cases. With further elevation of the SH-R concentration, progressively increasing inhibition of the Ach effect became obvious, and at 2×10^{-4}-10^{-3}M of NEM or PCMB caused significant 70-100% or 60-100% inhibition of the contraction by Ach respectively. Furthermore, 60% or 20-100% inhibition of the contractile response to Ach was noted after 10^{-3}M of PA or 10^{-5}-10^{-3}M of MC. No such significant suppression of the contraction by Ach did occur even after treatment with high doses of MA or IA.

The inhibition of the Ach action by these SH-R could scarcely be reversed washing with Tyrode solution.
Fig. 1 shows the influence of NEM on the contractile response to Ach and Fig. 2 illustrates the mean dose-effect curves of SH-R on the contractile response to Ach.

Practically comparable patterns of dose-effect relationship were observed with NEM, PCMB, MC and PA, viz. a slightly enhanced Ach effect after low concentration of these SH-R and a marked inhibition of the Ach action after high concentration. In contrast, MA and IA caused only slight augmentation of the Ach effect after low concentration, but they failed to produce any significant inhibition of the Ach effect at high concentration.

Meanwhile, the above-described enhancement or suppression by SH-R of the Ach-induced contraction of the intestine was noted to diminish or disappear after treatment of SH-C, $10^{-7}$M or higher concentration of ME, CY or PE in particular, as well as treatment of such SH-C and SH-R concomitantly at comparable concentrations. Even addition of such SH-C at the above-mentioned concentration to the bath after washing, following observation of the response to SH-R, similarly resulted in diminution or disappearance of the SH-R effect.

3. Influence of sulfhydryl compounds on the acetylcholine-induced contraction

Single administration of SH-C at concentration in $10^{-8} - 10^{-5}$M failed to exert any significant changes on the intestinal movement. The contractile response to $10^{-6}$M of Ach was not significantly influenced by pretreatment of ME, CY or PE at concentration not higher than $10^{-6}$M. About 20% increase of the contraction produced by Ach was observed after treatment of ME, CY or PE at concentration in $10^{-6}$M. These enhancing effects of SH-C was diminished or disappeared after washing with Tyrode solution in a few cases while the effects tended to be sustained even after washing in many cases. Moreover, pretreatments with $10^{-4}-10^{-3}$M of CY or PE and $10^{-8}-10^{-5}$M of GL did not cause significant change of Ach-induced contraction, while the contractile response to Ach reduced about 10-30% from control after treatment of ME in concentrations of $10^{-4} - 10^{-3}$M.

The above-mentioned effects of SH-C on the contractile response to Ach were noted to diminish or disappear after treatment of SH-R concomitantly. The same diminution and disappearance of the effects of SH-C were found even...
Fig. 2 Mean dose-effect curves of sulfhydryl reagents on the contractile response to acetylcholine. Each value was obtained from 3-22 experiments. Bars show a standard error of main value.
Fig. 4 Mean dose-effect curves of sulfhydryl compounds on the contractile response to acetylcholine. 
Each value was obtained from 3-14 experiments.
Bars show a standard error of main value.
when SH-R was added to the bath after washing, following observation of the effect to SH-C.

Fig. 3 shows the influence of CY or PE on the contractile response to Ach and Fig. 4 illustrates the mean dose-effect curves of SH-C on the Ach-induced contraction.

4. Influence of sulfhydryl reagents and compounds on the changes of acetylcholine content in intestinal tissue and the organ bath after acetylcholine incubation

There were 14.5 - 25.0 % increase in intestinal tissue Ach content and 14.0 - 65.5 % elevation of Ach level in the bath-medium, after incubation of 10^{-4} M of Ach. When the tissue had been pretreated with SH-R, addition of the same dose of Ach, however, caused practically no increase in tissue Ach level but brought about an approx. three-fold increase of Ach in the medium. In the case of tissues pretreated with MC, nevertheless, nearly the same degree of increase in tissue Ach content as that observed with untreated tissue occurred while there was about a four-fold increase of Ach in the medium. No significant change of Ach levels could be observed by using other kinds of SH-R.

Ach content in the tissue after treatment with 10^{-5} M of CY increased about two-fold, but that in the medium decreased slightly in comparison with the level in control which had been treated Ach only. After treatment with 10^{-5} M of PE Ach content in the tissue was not changed, although Ach content in the medium increased slightly, following incubation of Ach 10^{-4} M after PE in comparison with control.

In other experiments Ach content in the bath medium was only found to be three-fold increase after incubation of MA 10^{-5} M or 10^{-4} M.

Table 2 represents the mean per cent changes of Ach content in the intestinal tissue and organ bath after treatment of Ach, and after incubations of NEM (10^{-7} M) and MC (10^{-6} M) or CY and PE (both 10^{-5} M) followed by Ach.

5. Influence of sulfhydryl reagents and compounds on the activity of cholinesterase

There was no significant alteration on the activity of cholinesterase following the incubation of NEM (10^{-7} and 2\times10^{-4} M), CY (10^{-6} M) or MA (10^{-6} M).

### Table 2

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<thead>
<tr>
<th>Drugs and dosages</th>
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<tr>
<td>Acetylcholine 10^{-4} M</td>
<td>+25.1 ± 8.6</td>
<td>+60.8 ± 11.0</td>
<td>+22.1 ± 6.2</td>
<td>+16.5 ± 5.4</td>
<td>+14.5 ± 5.3</td>
<td>+13.9 ± 4.0</td>
<td>+22.0 ± 5.3</td>
<td>+65.5 ± 16.4</td>
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<tr>
<td>SH reagents or SH compounds</td>
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<tr>
<td>NEM 10^{-7} M</td>
<td>+1.0 ± 2.6</td>
<td>+162.5 ± 37.9</td>
<td>+29.9 ± 8.4</td>
<td>+62.5 ± 7.0</td>
<td>+27.4 ± 7.4</td>
<td>-13.0 ± 3.9</td>
<td>-3.7 ± 2.2</td>
<td>+49.3 ± 16.6</td>
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<td>MC 10^{-6} M</td>
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<td>CY 10^{-5} M</td>
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<td>PE 10^{-5} M</td>
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Mean per cent changes of acetylcholine content in the intestinal tissue and organ bath after treatment of acetylcholine alone, and after incubations of N-ethylmaleimide and mercuric chloride or cysteine and penicillamine followed by acetylcholine.

* Mean per cent change from control (mean ± s.e.), 3-5 experiments.

I: intestinal tissue, O: Organ bath.
DISCUSSION

It is generally recognized that ChE is an SH-related enzyme after the report by Nachmansohn et al. Supposing that ChE activity is inhibited by pretreatment with SH-R, it would be eventually the case that the effect of Ach is enhanced after treatment with SH-R. The enhanced contraction of intestine by Ach after treatment with SH-R is likely to have no bearing upon inhibition of ChE activity, nevertheless, in that no appreciable suppression of ChE activity was demonstrable even in the presence of high concentration of NEM which seem to combine most actively with the sulphydryl group among the various SH-R tested in these experiments.

It was reported by Keswani et al., on the other hand, that SH-R exerted effects on release from and uptake by the nerve terminal cell of noradrenaline granules.

It seems probable that, at the ending of a cholinergic nerve as well, Ach incorporation into the terminal cell is inhibited by SH-R. In the present study, indeed, increased uptake by the tissue of exogenous Ach was demonstrated to be inhibited by pretreatment with SH-R added at a concentration as it proved elsewhere to enhance the effect of Ach. The concentration of Ach in the bath medium increased concomitantly. It follows that the enhanced Ach effect after treatment with low concentration of SH-R may perhaps be related to inhibition by SH-R of Ach uptake at the nerve ending. However, when a high dose of SH-R alone was given, a sustained contraction of the small intestine (probably a direct action on the effector cell) was observed in some instance when the effect of Ach significantly diminished. This suggests that SH-R acts upon the terminal cell and at the same time affects the effector cell as well in inhibiting the action of Ach. This, moreover, may probably account for the facts that a large amount of Ach in the medium, resulting from the inhibition by SH-R of Ach incorporation into the terminal cell, fails to produce marked excitation of the effector cell and that inhibition of the Ach effect alone follows the pretreatment with high concentration of SH-R. Furthermore, this can also be inferred from the finding that addition of Ach after treatment with SH-R resulted in a marked elevation of Ach concentration in the bath. Namely, it is likely that, after treatment with SH-R, the exogenous Ach uptake by the nerve ending is suppressed, the response of effector cell to Ach is simultaneously inhibited by SH-R with reduced breakdown and dissipation of Ach, and consequently a large quantity of Ach accumulates in the medium. Energy changes taking place at the cell membrane are considered to be associated essentially with such events as incorporation of Ach into the terminal cell and excitation of the effector cell.

It is generally believed that changes in permeability of ions through the cell membrane are basically energy changes derived from ATP breakdown by the action of ATPase. Accordingly, the action of ATPase as well as changes of other substances occurring in the vicinity of the cell membrane probably are involved in the Ach uptake and its action on the effector cell. ATPase has been shown to be an SH-related enzyme since the reports by Polis et al. and Singer et al. According to a recent study by Jean and his coworkers, activity of ATPase extracted from the kidney was enhanced by low concentration of SH-R but inhibited by the same compound at high concentration in vitro. The inhibition by SH-R
of Ach incorporation into the terminal cell, as observed in the present investigation, probably may be a consequence of diminished Ach incorporation into the terminal cell caused by suppressed energy changes at the cell membrane which is brought about by inhibition of ATPase activity nearby the cell membrane as well as other inhibitory factors. Provided that the observed slight enhancement of Ach-induced contraction of the small intestine after treatment with low concentration of SH-R is related to increased depolarization of the cell membrane of the effector cell caused, as Jean et al. described, by augmented ATPase activity, considerably marked enhancement of the shown Ach effect have occurred as a result of it as well as the inhibition by SH-R of Ach uptake by the nerve ending. No such conspicuous enhancement occurred, however. Therefore, it is yet to be determined whether the ATPase activity is significantly increased by low concentration of SH-R in intact tissue as in these experiments. Instead, most profoundly related may be the inhibition of the Ach effect by high concentration of SH-R and suppression of Ach-induced energy changes at cell membrane resulting diminished ATPase activity.

Meanwhile, Holton et al. described that "Ach activity diminishes after replacement of the ethyl group by N-methyl radical". It is therefore conceivable that NEM which has an ethyl group is capable of inhibiting the action of Ach, whereas such cannot be applied to the other SH-R. This mechanism with NEM, moreover, is considered to be unlikely when viewed with respect to the fact that the effect of Ach is enhanced after treatment with low concentration of NEM.

In addition, after treatment with certain concentration of SH-C the contraction of isolated small intestine by Ach was observed to be augmented and at the same time increased Ach uptake by the terminal cell and diminished Ach content in the medium occurred. The most probable events that are taking place seem to be facilitation by SH-C of Ach uptake by the terminal cell, increase by SH-C of activity of substances involved in membrane permeability to ions of the effector cell, especially ATPase by addition of SH group, with consequent increased depolarization of the effector cell, thereby enhancing the effect of Ach. This chain of events as well as the reverse actions of SH-R, again seem to indicate the close relationship of cell membrane permeability and sulfhydryl group to Ach uptake by the nerve ending. Concomitant application of SH-R and SH-C to isolated small intestine results in aborption of the respective enhancing or inhibitory effect of these compounds on Ach-induced contraction because they offset each other.

The mechanism of actions of SH-R and SH-C has thus been discussed in particular reference to their actions on cell membrane permeability, especially the relevant activity of ATPase. However, the drugs used in these experiments have nonspecific actions and not merely act on ATPase alone but are considered to react also with membrane and intracellular SH-related substances including proteins. The effects observed on the Ach-induced contraction of isolated small intestine, therefore, would have to be interpreted as manifestations of collective inhibition or facilitation of a number of SH-related substances in the terminal cell of cholinergic nerve and effector cell.
REFERENCES


