Effects of Various Enzymes and Chemical Modification Reagents on the Na⁺- and Cl⁻-dependent Responses of the Ganglion Cells to Acetylcholine

Makoto SATO, Juro MARUHASHI,* Hideko YAI,**
and Masanori SHOZUSHIMA

*Department of Physiology, School of Medicine,
Iwate Medical University, Morioka, Iwate, 020 Japan*

Abstract Using the abdominal ganglion cells of *Aplysia*, we analyzed the effects of various enzymes and chemical modification reagents on the acetylcholine (ACh)-induced responses of the excitatory (Na⁺-dependent) and inhibitory (Cl⁻-dependent) types. (1) Phospholipase A (2 mg/ml) caused no appreciable effects on either type of response. (2) Phospholipase C (2 mg/ml) markedly depressed both types of response. These suggested that the phosphoryl group of the phospholipid is an important site related to the binding of ACh, common to both types of ACh-receptors. (3) Carboxypeptidase A (10 mg/ml) caused no observable effects on either type of response. (4) Carboxypeptidase B (10 mg/ml) depressed the inhibitory type of response without affecting the excitatory one. (5) Pyridoxal-5'-phosphate (1 mM) also depressed the inhibitory response without affecting the excitatory one. These findings (4, 5) suggested that the Cl⁻-channel in the inhibitory ACh-receptor complex includes a C-terminal lysine which may play an active role in the movement of Cl⁻ across the receptor membrane. (6) L-Leucine aminopeptidase (1 mg/ml) depressed the excitatory response without altering the inhibitory one. (7) *p*-Nitrothiophenol (1 mM) also depressed the excitatory response without affecting the inhibitory one. These findings (6, 7) suggested the presence of a certain N-terminal amino acid near a glutamate or aspartate residue within a molecular moiety of Na⁺-channel included in the excitatory ACh-receptor complex.

Key Words: ACh-receptor, ionic channels, nicotinic receptors.

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* Present address: Department of Biology, Daiichi Pharmaceutical College, Fukuoka, 815 Japan

** Present address: Department of Physiology, Saitama Medical College, Moroyama, Saitama, 350-04 Japan

佐藤 誠, 丸橋寿郎, 屋井ヒデ子, 小豆島正典

Numerous studies have been made on the structural analysis of acetylcholine (ACh) receptors using purified receptor membranes or their fragments *in vitro* (O'BRIEN *et al.*, 1971; BOURGEOIS *et al.*, 1972; OLSEN *et al.*, 1972; NICKEL and POTTER, 1973; KARLIN and COWBURN, 1974; DE ROBERTIS, 1974; MEUNIER *et al.*, 1974; COHEN and CHANGEUX, 1975; ELDEFRAWI *et al.*, 1975). However, only a few studies have been made with intact receptor membranes *in vitro*. CHANGEUX *et al.* (1967) examined the effects of *p*-(trimethylammonium)-benzenediazonium fluoroborate (TDF) on the voltage response of electroplax to cholinergic ligands and observed that this compound irreversibly depressed receptor sensitivities to agonists. The diazonium group of TDF is known to form a single covalent bond mainly with tyrosine, histidine, or lysine residues of proteins. This effect was prevented by *d*-tubocurarine and flaxedil, which are typical receptor inhibitors, but no interaction was observed with diisopropyl fluorophosphate (DFP) and phospholine.

The presence of sulfhydryl groups and disulfide bonds in the cholinergic receptor moiety was first suggested by Karlin and his co-workers (KARLIN and BARTELS, 1966; KARLIN, 1969) by their striking experiments with an eel electroplax *in vivo*. They discovered that dithiothreitol, an effective S-S reducing agent, depressed the depolarizing responses (ΔV) of the electroplax to ACh. Characteristically, however, this depression is completely reversed by dithio-bis-nitrobenzoate (DTNB), an SH-reoxidizing agent. Reoxidization by DTNB was prevented by pretreatment with *N*-ethylmaleimide (NEM), an SH-alkylating agent, thus DTNB produced no recovery in ΔV after DTT and NEM.

The presence of a disulfide bond has been confirmed with other ACh-receptor preparations: rat denervated muscle (ALBUQUERQUE *et al.*, 1968), frog rectus (MITTAG and TORMAY, 1970), chick biventer cervix (RANG and RITTER, 1971), frog sartorius (DEL CASTILLO *et al.*, 1971; BEN-HAIM *et al.*, 1973), and leech muscle (ROSS and TRIGGLE, 1972). However, it should be noted that the ACh-receptors studied so far were all excitatory, and curare-sensitive type. Little work has been done on the ACh-receptors of the inhibitory or atropine-sensitive type. SATO *et al.* (1976) confirmed the presence of a disulfide bond in the curare-sensitive, inhibitory receptors of the *Aplysia* ganglion cells. They concluded that the disulfide bond is a crucial element in all curare-sensitive ACh-receptors regardless of whether they are of the excitatory or inhibitory type (SATO and SATO, 1976). On the other hand, the muscarinic receptors do not seem to include such a crucial disulfide bond; KUHNNEN-CLAUSEN (1975) observed rather negative results with the muscarinic excitatory receptors of the intestinal muscles, and SATO *et al.* (1976) described no depressing effect of dithiothreitol on the tetraethylammonium (TEA)-sensitive, slow inhibitory receptors of the *Aplysia* ganglion cells.

It is evident that there are at least four types of ACh-receptors, each one having characteristic binding sites and ion selective channels. Certain chemicals may affect only the excitatory ACh-receptor activities regardless of whether they are

nicotinic or muscarinic, whereas other chemicals may alter only the nicotinic responses irrespective of their excitatory or inhibitory natures. For instance, hexamethonium (Hex) has long been known as a competitive inhibitor of the nicotinic receptors. However, TAUC and GERSCHENFELD (1962) described that Hex does not block the inhibitory response of the nicotinic or Cl^- -dependent type. Later, we confirmed this and found that Hex blocks the arecoline-sensitive, K^+ -dependent inhibitory response instead of the nicotinic or Cl^- -dependent one (SATO *et al.*, 1973). Previously, we found that a dilute concentration of various mercurials can selectively block the inhibitory ACh-responses regardless of whether they are Cl^- - or K^+ -dependent (SATO *et al.*, 1977). The acting site of a particular reagent within a receptor complex including the binding site and ionic channel may be analyzed more conveniently when the effects are compared among different types of ACh-receptors.

In the present study, our interest was focused on the molecular structures which characterize the ion selective behavior of different ACh-receptors. The ionic mechanisms underlying each receptor activity have been well established in the *Aplysia* ganglion cells by SATO *et al.* (1968) and KEHOE (1972). Previously, we demonstrated that the nicotinic excitatory responses are produced by an increase in the permeability of the receptor membrane mainly toward Na^+ , whereas the nicotinic inhibitory responses are produced by an increase in membrane permeability toward Cl^- exclusively (SATO *et al.*, 1968). The relative importance of phospholipid and amino acid components within the molecular moiety of the nicotinic receptors was examined by analyzing the effects of various enzymes and chemical modification reagents on the ACh-induced responses. The effects were continuously compared between the excitatory and inhibitory types in order to determine whether or not they were specific to the particular ion channels. A part of this paper has been published previously (SATO *et al.*, 1979).

METHODS

Materials and preparation. The abdominal ganglia of the *Aplysia californica* and *Aplysia kurodai* were dissected and mounted in a perfusing chamber. The temperature of the perfusing solution was maintained at 10°C throughout the entire experiments. The connective tissues covering the dorsal surface were carefully removed to expose the underlying cells directly to the perfusing media. The effective perfusing volume of the chamber was 0.2 ml and the rate of perfusion was 5 ml per min. This enabled us to change the entire volume of the perfusing media within 2.4 sec. Cells were normally perfused with artificial *Aplysia* Ringer (SATO *et al.*, 1968); Na^+ 587, K^+ 12, Cl^- 671, Ca^{2+} 14, and Mg^{2+} 52 mM. The pH was adjusted between 7.3 to 7.5 with tris(hydroxymethyl)aminomethane (Tris) and HCl unless otherwise specified.

Application of ACh. ACh and other chemicals were dissolved to various

concentrations in normal Ringer's solution before application, and applied through the perfusing media to which the cell surface was directly exposed. The duration of ACh application was adjusted to give only the onset of the maximal plateau response for a given concentration of ACh. This was normally less than 20 sec for ACh higher than 0.1 mM and 60 sec for ACh of 1 μ M. The iontophoretic application of ACh was not adopted in this experiment because of the following reasons; difficulty in estimating the actual concentration of ACh at the cell surface when the perfusing media is continuously flowing, and the instability of the response due to a slight movement of the cell with respect to the tip of the iontophoretic electrode when a perfusing medium is switched from one to another. In fact, we previously confirmed that the results obtained from these cells with the perfusion method are qualitatively the same as those obtained with the iontophoretic method unless the cells are perfused with an extraordinarily high concentration of ACh (SATO *et al.*, 1968). All results were obtained from 84 D-type cells and 63 H-type cells (see Table 1). A statistical treatment of the data was not attempted in the presentation of the dose-response curves because each neuron had a different threshold and the maximum response to ACh. Tables including our entire data were omitted because all data were qualitatively the same as those illustrated in the figures.

Types of ACh-receptors. There are two major types of cholinceptive neurons in the abdominal ganglion of the *Aplysia*; one is depolarized, the other is hyperpolarized by ACh. The former is called the "excitatory type" or simply "D-type," and the latter is called the "inhibitory type" or "H-type" (TAUC and GERSCHENFELD, 1962). GERSCHENFELD and TAUC (1964) have further subdivided each type by their pharmacological analysis and classified D, DILDA, DINHI for D-type and H, HILDA for H-type. The cells described as D-type and H-type in this paper may correspond to their DILDA and HILDA types because most of these cells had both spontaneous EPSP's and IPSP's. They are all localized in the right dorsal surface of the ganglion within the clusters R_B and R_C after FRAZIER *et al.* (1967). A classic pharmacology describes two types of ACh-receptors, namely nicotinic and muscarinic. However, the use of these names has been controversial since later studies on the ACh-receptors of various cells and species including *Aplysia*, revealed that these receptors rarely satisfied all the pharmacological requirements to be classified as either type. Many investigators have used different names to avoid this classic terminology. However, this seems to be creating an additional confusion, again. In this paper, we decided to use classic names just for descriptive convenience. The ACh-receptors which were activated by a dilute nicotine, and responses of which to ACh were readily blocked by *d*-tubocurarine, were simply called the "nicotinic" type. Similarly, the receptors which were activated by a dilute arecoline and whose ACh-responses were readily blocked by tetraethylammonium (TEA) or phenyltrimethylammonium (PTMA), were called the "muscarinic" type. A more exact nature of each type

was not our present concern. Usually the nicotinic D-type cells are identified in the R_B group, whereas the nicotinic H-type cells are in the R_C group as described previously (SATO *et al.*, 1976). Our interest in this study was to compare the structural significance of ACh-receptors between the excitatory and inhibitory types and not between the nicotinic and muscarinic. Accordingly, ACh-receptor membranes used in this experiment were all of the nicotinic type regardless of whether they are excitatory or inhibitory. The nicotinic nature of each receptor membrane could be identified from the fast time course of the response. However, more direct identification was done by comparing the receptor sensitivity to nicotine and arecoline or to *d*-tubocurarine and TEA. The cells which showed both nicotinic and muscarinic responses were discarded.

Measurement of conductance change. Two microelectrodes filled with 1.8 M potassium citrate were inserted into a single cell under binocular microscopic control. A constant inward current pulse (I) of 500 msec was applied through one of the electrode at a frequency of 0.2 Hz, and the membrane resistance (R) was continuously recorded from another electrode as the potential drop across the membrane (IR). This pulse duration was usually long enough to attain the plateau voltage since the time constant of the cell membrane ranged from 100 to 200 msec as reported by FESSARD and TAUC (1956). The conductance change (ΔG) produced by ACh was determined by

$$\Delta G = G_A - G_0 = (1/R_A) - (1/R_0),$$

where G_A and G_0 are the overall conductances of the membrane during the ACh-response and the resting state, respectively. R_A and R_0 are resistances corresponding to G_A and G_0 .

Chemicals and enzymes. The following chemicals and enzymes were used in this experiment. Acetylcholine chloride (Sigma Chemical Company), pyridoxal 5'-phosphate and pyridoxine-HCl (Nutritional Biochemicals Corporation), *p*-nitrothiophenol (K & K Laboratories, Incorporated), phospholipase A (Sigma Chemical Company; approximately 10 units per mg protein), phospholipase C (Sigma Chemical Company; approximately 5 units per mg protein), carboxypeptidase A (Sigma Chemical Company; approximately 50 units per mg protein), carboxypeptidase B (Sigma Chemical Company; approximately 175 units per mg protein), L-leucine aminopeptidase (Sigma Chemical Company; approximately 40–50 units per mg protein). All these chemicals and enzymes were freshly dissolved in the *Aplysia* Ringer, and pH was adjusted between 7.3–7.5 by Tris and HCl unless otherwise specified. ACh-induced responses were tested before, during, and after the application of these chemicals and enzymes. Each testing of ACh-induced responses was done after a complete washing of the preparation with the *Aplysia* Ringer at least for 5 min. This period of washing was previously determined by a careful observation of the recovery time course (see RESULTS).

RESULTS

1) *Phospholipase A* (2 mg/ml)

Both the excitatory and inhibitory types of receptor membranes were markedly depolarized as much as 10–20 mV after a 10 min exposure to this enzyme solution (see Table 1). The resting conductance increased 3–5 times the control value during this depolarization. Despite these changes in the resting membrane, the ACh-induced responses, measured as the relative increase in membrane conductance (ΔG), were not greatly altered by this enzyme solution. Practically no effect on either the excitatory (D) or inhibitory (H) response was confirmed when the responses were examined after cancelling the enzyme-induced depolarization by passing an inward current across the receptor membrane (see Fig. 1).

2) *Phospholipase C* (2 mg/ml)

Neither the excitatory nor the inhibitory receptor membrane was depolarized by exposure to this enzyme solution. Instead, a 2–4 mV hyperpolarization associated with a slight increase (5–10%) in membrane conductance was often observed after a 10–20 min exposure (see Table 1). Despite these mild effects on the resting membrane, both types of ACh-induced responses were markedly depressed to 25–30% of the control values after a 20 min exposure to this enzyme solution. When the reciprocal of the control ΔG was taken as unity, and its change

Table 1.

Chemicals	Resting membrane		ACh-response (ΔG)	
	Cell type:	D_{Na}^* and H_{Cl}^{**}	D_{Na}	H_{Cl}
Phospholipase A (2 mg/ml)		Depolarized (15 ± 5 mV)	No effect	No effect
Phospholipase C (2 mg/ml)		Hyperpolarized (3 ± 1 mV)	Depressed	Depressed
Carboxypeptidase A (10 mg/ml)		No effect	No effect	No effect
Carboxypeptidase B (10 mg/ml)		No effect	No effect	Depressed
Pyridoxal 5'-phosphate (1 mM)		No effect	No effect	Depressed
Leucine aminopeptidase (1 mg/ml)		No effect	Depressed	No effect
<i>p</i> -Nitrothiophenol (1 mM)		Hyperpolarized (3 ± 1 mV)	Depressed	No effect

* D_{Na} , D-type neurons which are depolarized by ACh. Depolarization is produced by a permeability increase mainly toward Na^+ . Twelve neurons of this type were examined for each chemical or enzyme. ** H_{Cl} , H-type neurons which are hyperpolarized by ACh. Hyperpolarization is produced by a permeability increase exclusively toward Cl^- . Nine neurons of this type were examined for each chemical or enzyme.

Fig. 1. Effects of phospholipase A (2 mg/ml) and C (2 mg/ml) on ACh-induced responses. Each point represents the average of five receptor membranes of H- or D-type. ACh-induced responses were evaluated by the increase in membrane conductance (ΔG). The reciprocal of the control ΔG was taken as unity and its relative change was plotted on the ordinate. The abscissa is the cumulative time of exposure to the enzyme in min. Each point was determined after 2 min washing with Ringer. The resting membrane potentials (RMP's) were artificially adjusted to be the same as those recorded before exposure to the enzyme (see the text). RMP's, 42–48 mV; ACh, 1 mM.

was expressed in a relative unit, the data showed a straight line relationship with an increase in cumulative time to exposure to this enzyme, as shown in Fig. 1. It should be noted that the data obtained from both D- and H-type membranes fell on the same straight line when plotted in this manner. The depressing effect was irreversible for several hours after the application of this enzyme.

3) Carboxypeptidase A (10 mg/ml)

Neither the resting membrane nor the ACh-induced responses of either type was significantly altered by a 3 hr application of this enzyme solution (see Table 1 and Fig. 2).

Fig. 2. Effects of carboxypeptidase A (10 mg/ml) on ACh-induced responses of the D- and H-types. Results were represented by one D-type and one H-type membrane. ACh-induced responses (ΔG) of either type remained unaffected. RMP's, 45–49 mV; ACh, 0.1 mM for the D-type, and 1.0 mM for the H-type membrane.

4) *Carboxypeptidase B* (10 mg/ml)

The ACh-induced responses of the excitatory type (D) were not altered by this enzyme solution even after 1 hr application. On the other hand, the ACh-induced responses of the inhibitory type (H) were significantly depressed by a 10–30 min exposure to this enzyme solution (see Fig. 3-left). The time course of this depression was linear when the ACh-induced conductance increase (ΔG) was plotted in a logarithmic scale. A given time of exposure produced a certain extent of depression which remained constant over a period of 1–2 hr after a continuous washing with normal Ringer's solution. Therefore, it was possible to study the effect of a single exposure on the dose-response curve with varying ACh-concentrations. The dose-response curves, thus obtained, showed a systematic shift as the cumulative time of exposure increased. The slope and the maximal plateau gradually declined, but the ACh concentration that produced a half maximal response (D_{50}) remained unchanged (see Fig. 3-right). All the depressing effects

Fig. 3. Left: effects of carboxypeptidase B (10 mg/ml) on ACh-induced responses of the D- and H-types. Relative change in ΔG was expressed by the percent of the control. ACh, 1 mM for both types of membranes; RMP's, 45–48 mV. Note the ordinate expressed in logarithmic scale. Right: dose-response curves ($\log [ACh]$ - ΔG) obtained from an H-type membrane with increasing time of exposure to carboxypeptidase B (10 mg/ml). Each curve was obtained after a complete washing with Ringer following each exposure to the enzyme (see the text). The cumulative time of exposure is shown on each curve in min. RMP, 46 mV.

were irreversible. It should be noted that neither the resting potential nor the resting conductance was appreciably altered by the exposure to this enzyme, and that the cells were fully capable of normal spike firings, while the ACh-induced responses were blocked almost completely (see Table 1).

5) Pyridoxal 5'-phosphate (1 mM)

Neither the resting potential nor the resting conductance of either type of receptor membrane was significantly altered by a 40 min exposure to this solution. The ACh-induced responses of the excitatory type (D) were not appreciably changed during and after a 40 min exposure to this solution (see Fig. 4-left). On the other hand, the ACh-induced responses of the inhibitory type (H) were gradually depressed as the exposure time increased. At pH 7.5, ΔG decreased to 50–60% of the control within 20–25 min of the exposure. At pH 8.0, ΔG decreased to 20–30% of the control within the same exposure period. Further

Fig. 4. Left: effects of 1 mM pyridoxal 5'-phosphate (PLP) on ACh-induced responses of the D- and H-types. Results were represented by a D-type and an H-type membranes. Other nomenclatures are the same as in Fig. 3. RMP's, 44 mV for the D-type, 40 mV for the H-type membrane; ACh, 0.1 mM for both types of the membranes; pH, 7.5. Right: dose-response curves (log [ACh]- ΔG) obtained from an H-type membrane with increasing time of exposure to 1 mM PLP. The cumulative time of exposure to the enzyme is shown on each curve in min. Each curve was obtained after a 5-20 min washing following each exposure to PLP. RMP, 40 mV; pH, 7.5.

exposure, however, did not increase the depression. The initial depression was linear when log ΔG was plotted against the cumulative time of exposure. During the initial depression, the log [ACh]- ΔG curves showed a systematic shift, *i.e.*, a decrease in slope with an increase in exposure time to 1 mM pyridoxal-5'-phosphate (see Fig. 4-right). After being depressed, the inhibitory ACh-receptors gradually regained their responsiveness to ACh after 2-3 hr of continuous washing in normal Ringer's solution. A similar, but milder effect was observed with pyridoxine-HCl, though the results are not shown.

6) *L-Leucine aminopeptidase* (1 mg/ml)

The application of this enzyme solution caused no appreciable effect on either the resting potential or the resting conductance in both types of receptor membranes (see Table 1). The ACh-induced inhibitory responses (H) were not significantly altered during a 3 hr exposure to this solution (see Fig. 5-left).

Fig. 5. Left: effects of L-leucine aminopeptidase (1 mg/ml) on ACh-induced H-type responses. The relative changes in ΔG were expressed in % of the control in logarithmic scale. The data were obtained from three different H-type membranes, each shown by different symbols. Each point was determined after a 2 min washing following each exposure to the enzyme. RMP's, 40–48 mV; ACh, 1 mM. Right: same as the above but on ACh-induced D-type responses. The data were obtained from three different D-type membranes, each shown by different symbols. RMP's, 43–47 mV; ACh, 1 mM.

In contrast, the ACh-induced excitatory responses (D) were significantly depressed by this enzyme even after a 30 min exposure. A 3 hr exposure depressed ΔG to 40–50% of the control (see Fig. 5-right). The depression was linear when the relative change in ΔG was plotted in a logarithmic scale against the cumulative time of exposure. It should be noted that the data obtained from three different cells with the excitatory receptors fell on the same straight line when plotted in this manner. The extent of depression produced by a single exposure to this enzyme remained constant over a period of 1–2 hr after a continuous washing with normal Ringer's solution. This enabled us to study the effect of each exposure on the dose-response curve with varying ACh concentrations. The dose-response curves obtained from an excitatory receptor membrane showed a gradual decline in their slopes as the exposure time increased (see Fig. 6). The depressing effect of this enzyme was irreversible but the cells were still capable of firing normal spikes even after a 3 hr exposure.

7) *p*-Nitrothiophenol (1 mM)

The resting membranes of both D- and H-type cells were slightly hyperpolariz-

Fig. 6. Dose-response curves ($\log [\text{ACh}]-\Delta G$) obtained from a D-type membrane with increasing time of exposure to L-leucine aminopeptidase (1 mg/ml). Other nomenclatures are the same as in Fig. 3. RMP, 45 mV. Each curve was obtained after a complete washing with Ringer's solution following each exposure to the enzyme (see the text).

ed by as much as 2–4 mV after a 40 min exposure to this solution (see Table 1). The resting conductance was not greatly altered, but tended to decrease to only 5–10%. The ACh-induced responses of the inhibitory type (H) were not appreciably altered even after a 60 min exposure. In contrast, the ACh-induced responses of the excitatory type (D) were irreversibly depressed after a brief exposure to this solution. A 5 min exposure decreased ΔG to 60–70% of the control and an additional 10 min exposure further decreased ΔG to 30–40% of the control (see Fig. 7-left). However, a further increase in exposure time did not increase the extent of depression, reaching a final plateau around 30% of the control. The depression was linear when $\log \Delta G$ was plotted against the cumulative time of exposure. During the initial depression, the dose-response curves showed a systematic shift, *i.e.*, decrease in slope with increase in exposure time (see Fig. 7-right).

Fig. 7. Left: effects of 1 mM *p*-nitrothiophenol (NTP) on ACh-induced responses of each type. Other nomenclatures are the same as in Fig. 4. RMP, 43 mV for the D-type, 38 mV for the H-type membrane; ACh, 1.0 mM for the D-type, 0.1 mM for the H-type membrane. Right: dose-response curves ($\log [\text{ACh}]$ - ΔG) obtained from a D-type membrane with increasing time of exposure to 1 mM NTP. Other nomenclatures are the same as in Fig. 4. RMP, 46 mV.

DISCUSSION

Previously, we investigated the ionic mechanism underlying various types of ACh-induced responses of the *Aplysia* ganglion cells (SATO *et al.*, 1968). We concluded that the excitatory (D) responses of the nicotinic type were produced by a selective permeability increase of the receptor membrane mainly toward Na⁺ ions, and that the inhibitory (H) responses of the nicotinic type were produced by a permeability increase toward Cl⁻ ions, exclusively (SATO *et al.*, 1968). All the ACh-responses examined in the present experiment were identified to be of the nicotinic type. Accordingly, the excitatory (D) and inhibitory (H) responses may be called in this discussion as "Na⁺-dependent" and "Cl⁻-dependent" responses, respectively.

A large structural complex including the ACh-binding sites and neighboring ion-channels was considered as a whole receptor complex in this paper. Our kinetic study of the receptor-inhibitor interaction revealed that some chemicals used in this experiment depressed the binding between ACh and its receptor, whereas other chemicals impaired the opening mechanism of either Na⁺- or Cl⁻-

channel included in each receptor complex, as described below.

1) *The relative role of phospholipids in ACh-receptor activities.* Certain phospholipids such as lecithin and phosphatidyl serine are known to be important elements of the electrically excitable membrane. The high electrical resistance of the resting membrane has been ascribed to the hydrophobic chain of fatty acids which are attached to the glycerol component of lipids. Phospholipase A is an enzyme which hydrolyzes lecithin to release fatty acids from the glycerol component. As expected, this enzyme increased the resting conductance 3–5 times the control, and depolarized the resting membranes of both D- and H-types by as much as 10–20 mV. These changes are probably due to a non-specific increase in the membrane permeability to all ion species. Despite these marked changes in the resting membrane, the ACh-induced response (ΔG) of either Na^+ - or Cl^- -dependent type was not depressed appreciably by this enzyme. This suggested that the aliphatic chain of the phospholipid is not directly involved in the function of either receptor.

Phospholipase C is an enzyme which hydrolyzes lecithin to release phosphoryl choline from the glycerol component leaving the fatty acids intact. Prolonged exposures to this enzyme caused a minimal effect on the resting membrane of either type. Despite this minimal effect on the resting membrane, the same enzyme caused a marked depression on both types of ACh-responses. The relative amount of depression and its time course found in the Na^+ -dependent responses were exactly the same as those found in the Cl^- -dependent responses, both showing a second order reaction. It is unlikely that the enzyme-induced depressions of the ACh-responses were due to the changes in the resting potential or ionic equilibrium across the resting membrane. The above findings indicated that the phosphoryl group of the phospholipid is an important site common for the binding of ACh to both the excitatory and inhibitory receptors. However, the relative importance of this phosphoryl group may not be specific to ACh-receptors since the binding of GABA with the GABA-receptor in *Onchidium* neurones was suggested to also be depressed after exposures to phospholipase C (OOMURA *et al.*, 1982b).

It is worth comparing the above results with those obtained from the ACh-receptors of fractionated or solubilized membranes. The fractionated membranes of *Electrophorus* are known to reseal themselves into closed vesicles of 0.1–1.0 μm in diameter (KASAI and CHANGEUX, 1971a, b). MCNAMEE and MCCONNELL (1973) successfully incorporated spin-labelled lipids into these vesicles and observed no change in the spectral properties during the response to cholinergic ligands. They questioned the relative role of lipids concerning the permeability change at the vesicular membrane. The affinities of isolated receptor proteins to cholinergic ligands are known to be different when bound with lipid components within the membrane. The receptor proteins of *Electrophorus* solubilized by detergents showed rather higher affinities than those bound to the membrane (MEUNIER *et al.*, 1974). In contrast, the receptor proteins of *Torpedo* isolated by

detergents showed much lower affinities than those of non-solubilized preparations (COHEN and CHANGEUX, 1975). It is most likely that certain receptor structures are distinctly altered during fractionation and solubilization procedures.

2) *C-terminal lysine or arginine at the Cl^- -channel in the ACh-receptor complex.* In order to assess the relative role of the C-terminal amino acid residues in the ACh-receptor activities, we examined the effects of carboxypeptidase A and B on both the Na^+ - and Cl^- -dependent responses. There was no observable effect with enzyme A on either type of receptor membrane, but a distinct effect was seen with enzyme B when applied to the inhibitory receptor membrane. Carboxypeptidase B is known to hydrolyze peptides with C-terminal lysine or arginine. Exposures to this enzyme did not appreciably alter the resting membrane of either type. In contrast, ACh-induced responses of the Cl^- -dependent type were irreversibly depressed by exposures to this enzyme while those of the Na^+ -dependent type were not affected significantly. The time course of this depression suggested a first order reaction. A systematic shift of the dose-response curves after exposures to this enzyme suggested that the depression of the response is not due to the lowered affinity of ACh-receptors, but to their lowered "intrinsic activity." This pharmacological term "intrinsic activity" may include the conformational change of the receptor molecule which is related to the opening of the Cl^- -channel. Our results suggested the presence of a C-terminal lysine or arginine in the vicinity of the Cl^- -channel of this receptor complex, and that these amino acid residues play an important role for the opening mechanism of this channel.

Pyridoxal 5'-phosphate is a well known coenzyme which is included in various vitamin B₆ holoenzymes. In these enzymes, pyridoxal phosphate is bound to the ϵ -amino group of the lysyl residues in an apoprotein forming "Schiff's base" (DUNATHAN, 1966; METZLER *et al.*, 1954). Pyridoxal phosphate can also react with ϵ -amino groups of the lysyl residues in several enzymes that do not require this compound as a coenzyme for catalytic activity (KALDOR and WEINBACH, 1966; ANDERSON *et al.*, 1966). For example, phosphogluconic dehydrogenase and aldorase of rabbit muscle (RIPPA *et al.*, 1967; SHAPIRO *et al.*, 1968) and D-amino acid oxidase of hog kidney (MIYAKE and YAMANO, 1970), are partially inactivated by the treatment with pyridoxal phosphate. Rabbit kidney fructose 1, 6-diphosphatase, is desensitized to AMP inhibition and also partially inactivated when treated with pyridoxal phosphate (KRULWICH *et al.*, 1969). All these reactions have been shown to involve the Schiff's base formation with lysyl residues of the enzymes. Our present study demonstrated that the Cl^- -dependent responses of the ACh-receptor were selectively depressed by a brief exposure to 1 mM pyridoxal 5'-phosphate. The depressing effect was pH dependent, being greater at higher pH's. The Schiff's base formation is also known to be stabler at higher pH's (MORINO and SNELL, 1967; JENKINS and SIZER, 1957). These findings again suggested that the lysine residues are important in controlling the opening of Cl^- -

channels at this receptor complex.

The depressing effects of both carboxypeptidase B and pyridoxal 5'-phosphate, observed in this experiment, may not be specific to the ACh-receptors of this type. Instead, the results could be due to the impairment on the movement of Cl^- -ions through the channels at the receptor membrane. It is conceivable that the Cl^- -channels of any receptor complexes generally include C-terminal lysine or arginine residues in the vicinity of their openings, and the ϵ -amino groups are somehow controlling the Cl^- -movement through the channels. There is a supporting evidence that the Cl^- -dependent responses of both GABA and glycine receptors in *Onchidium* are also depressed by carboxypeptidase B and pyridoxal 5'-phosphate, according to OOMURA *et al.* (1982b). It should be noted, however, that pyridoxal 5'-phosphate or pyridoxine-HCl did not block the inhibitory responses completely. The depressing effect maintained a maximum level after 15 min of exposure, and the maximum depression was only 20–30% of the control at pH 8.0. This may indicate that some other amino acid residues besides lysine, are partially involved in the permeability increase toward Cl^- -ions.

3) *N-terminal amino acid and glutamate or aspartate residues at the Na^+ -channel in the ACh-receptor complex.* L-Leucine aminopeptidase is an N-terminal exopeptidase. It hydrolyzes a variety of peptides with N-terminal amino acid residues related to L-leucine or L-alanine, but it does not hydrolyze substrates with an acylated amino group, NH_2 -terminal alanyl, and aminoisobutyl residues. The ACh-induced Cl^- -dependent responses were not appreciably altered during a 3 hr exposure to this enzyme. In contrast, the ACh-induced Na^+ -dependent responses were significantly depressed by this enzyme even after a 30 min exposure. The time course of this depression suggested that the enzymatic effect was produced by a first order reaction. It is significant that the relative rate of depression was exactly the same for three different D-type neurons examined. When the depressing effect was demonstrated by the dose-response curve ($\log [\text{ACh}]$ - ΔG), the slope of the curve decreased as the exposure time increased, a fact suggesting that this enzyme decreased the "intrinsic activity" of the excitatory receptor. It is postulated from these findings that the excitatory receptor moiety may include certain N-terminal amino acids related to L-leucine or alanine, and that these residues are somehow involved in the permeability increase toward Na^+ -ions at the excitatory receptor membrane.

p-Nitrothiophenol is known to form stable bonds with myosin A at a neutral pH in the presence of Mg^{2+} and ATP (KITAGAWA *et al.*, 1964). The analysis of amino acid composition suggested that *p*-nitrothiophenol makes an acylthiol bond with a carboxyl side chain of glutamate or aspartate residue in myosin A. It is also known that the ATP-induced superprecipitation of actomyosin in the presence of Mg^{2+} is completely abolished after the treatment of myosin with *p*-nitrothiophenol (TONOMURA and KANAZAWA, 1965; TONOMURA *et al.*, 1969). The structure of the actomyosin may be entirely different from that of the post-

synaptic receptors. However, a comparative study of the reactive sites in many enzymatic structures may lead to the elucidation of basic mechanisms such as ionic requirement, conformational change, and the triggering of the permeability increase, *etc.* The present experiment demonstrated that exposure of the receptor membranes to 1 mM *p*-nitrothiophenol markedly depresses the Na⁺-dependent responses without affecting the Cl⁻-dependent responses. The time course of the depression suggested that the binding of this reagent with the receptor was of the first order reaction. A systematic shift of the log [ACh]-*ΔG* curves, with an increase in time of exposure to *p*-nitrothiophenol, indicates that the mode of receptor depression was noncompetitive. These findings suggested the presence of glutamate or aspartate residues in the excitatory receptor moiety. These residues are probably important for the activation of permeability increase toward Na⁺-ions. It may be postulated that the Na⁺-channels of any receptor complexes generally include glutamyl or aspartyl residues in the vicinity of their openings, and the carboxyl groups are somehow controlling the movement of Na⁺-ions through the channels. According to OOMURA *et al.* (1982a), Na⁺-dependent responses of glycine receptors in *Onchidium* neurons are also depressed after exposures to *p*-nitrothiophenol. However, it should be noted that the *p*-nitrothiophenol did not completely block the excitatory responses in either preparation. Probably, more amino acid residues other than glutamate and aspartate are involved in the activation process of Na⁺-channels. Further study is needed with a single channel analysis in order to examine the validity of our model.

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