Effects of Onium Derivatives on Na-, Cl-, and K-dependent Acetylcholine Receptors

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Abstract Various types of acetylcholine (ACh) receptor in the pleural or abdominal ganglion cells of *Aplysia* were classified into three types depending on the ion species involved in the electrogenesis of their responses. The Na-dependent receptor was designated the D_{Na}-type since the response was a depolarization (D) produced by an increase in permeability, mainly toward Na⁺, although other ions were partially involved. Similarly, the Cl- or K-dependent receptor was designated the H_{Cl}- or H_K-type since the response was a hyperpolarization (H) produced by an exclusive increase in permeability toward Cl⁻ or K⁺. ACh-induced responses were evaluated by an increase in membrane conductance.

The D_{Na}-type receptor could be readily activated by tetramethylammonium (TMA) and choline, but not by decamethonium (Deca). Instead, it was blocked competitively either by hexamethonium (Hexa) or by Deca. The H_{Cl}-type receptor could be readily activated by TMA, choline, and Deca. Hexa did not block this type of receptor. The H_K-type receptor was neither activated nor blocked by TMA, choline, Hexa, and Deca.

The above findings suggested that there is a certain structural similarity in the ACh-binding site between D_{Na}-type and H_{Cl}-type receptors, although the ion species involved in the electrogenesis of each response were totally different. The structure of the ACh-binding site at the H_K-type receptor seemed considerably different from that of either D_{Na}-type or H_{Cl}-type.

The pharmacological properties of the acetylcholine (ACh)-binding sites in the nicotinic receptors of the endplate or electroplax have been examined by many investigators using various ACh-analogues, such as mono- or bis-quaternary ammonium ions. Alkyltrimethylammonium $[(CH_3)_3N(CH_2)_nCH_3]$ is known to be an activator, though the activating potency suddenly decreases when n increases

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beyond $n=8$ (ARIËNS *et al.*, 1955). Polymethylene-bis-trimethylammonium $[(CH_3)_3N(CH_2)_nN(CH_3)_3]$ is generally known as an inhibitor except the case with $n=10-16$ (WIEN *et al.*, 1952; BARLOW and ZOLLER, 1964). Decamethonium ($n=10$) acted as an activator on nicotinic receptors in the endplate or electroplax (DEL CASTILLO and KATZ, 1957b; CHANGEUX and PODLESKI, 1968), whereas it showed the blocking action at the superior cervical ganglion of the cat (BARLOW and ZOLLER, 1964).

The discrepancy among these results may indicate the structural difference in preparation, however, it may also reflect the difference in methods of evaluating the effects. For example, some investigators used muscular contraction whereas others used membrane depolarization as the criteria for ACh-induced responses. These criteria are rather indirect because usually they are produced secondarily as the results of the primary change in membrane conductance. More direct measurement of the change in membrane conductance produced by ACh is desirable for the analysis of the mechanisms in the receptor activity and molecular structure of the receptor.

In fact, the previous work on ACh-receptors was all performed on the excitatory or Na-dependent type, but little work was done on the inhibitory or K-, Cl-dependent type. The aim of this paper is to study the primary actions of oniums on ACh-receptor membranes of both excitatory and inhibitory types by evaluating the change in membrane conductance, and to compare the properties of ACh-binding site characteristics to each type of receptor. Preliminary report of this work has been presented by MATSUMOTO *et al.* (1979).

MATERIALS AND METHODS

Preparation and perfusing solution. The abdominal or pleural ganglion of *Aplysia kurodai* was dissected out and placed in a perfusing chamber. The cells were exposed to the perfusing solution after removal of the connective tissue by means of binocular microscopic surgery. The cells were normally perfused with artificial *Aplysia* blood (SATO *et al.*, 1968); Na^+ 587, K^+ 12, Cl^- 671, Ca^{2+} 14, and Mg^{2+} 52 mM. The effective perfusing volume of the chamber was 0.2 ml, and the rate of perfusion was 5 ml per min. The temperature of the perfusing solution was maintained at 15°C. The pH of the perfusing solution was adjusted to 7.4 with tris(hydroxymethyl)aminomethane and HCl. The ACh-esterase activity was continuously blocked by 0.05 mM neostigmine throughout the entire experiment.

Drugs. The following drugs were used for the experiment: acetylcholine chloride (ACh); choline chloride; tetramethylammonium chloride (TMA); hexamethonium dichloride (Hexa); decamethonium dibromide (Deca); nicotine hydrochloride; arecoline hydrochloride; neostigmine. These drugs were dissolved in the artificial *Aplysia* blood and directly applied to the cell membrane by means

of perfusion.

Measurement of the membrane conductance. Two glass microelectrodes filled with 2.0 M potassium chloride or 1.8 M potassium citrate (5–15 M Ω) were inserted into a single cell, the receptor characteristics of which had usually been identified. One of the electrodes was connected to a high input impedance pre-amplifier (gain 1) for the purpose of recording the membrane potential. The other was connected to a current source in order to control the level of the membrane potential and to estimate the membrane conductance (G). The activation or inhibition of the ACh-receptors was evaluated by the change in conductance (ΔG) of the receptor membrane. The conductance change was calculated in two ways. One was to measure the potential drop across the receptor membrane by supplying a constant inward current pulse of 500 msec every 5 sec. This method was used for the identification of receptor type, and for the qualitative measurement. The other was to measure the drug-induced current or the artificial current flow across the receptor membrane under the voltage clamp. During the application of agonist, the membrane potential was clamped at the resting level or at an any set level which was modulated by a small periodical hyperpolarizing pulse of 2–3 mV with 500 msec every 5 sec. This method was used for all quantitative measurements.

Acetylcholine (ACh)-induced responses in the presence of inhibitor were often expressed in percent of the control response obtained in the absence of it. The relative change in ACh-induced response was plotted against the concentration of inhibitor in logarithmic scale. This may be called "dose-inhibition curve."

Determination of receptor type. Classically, ACh-receptors have been classified into two types "nicotinic and muscarinic." This classification was, however, controversial because very few ACh-receptors were found later to satisfy all the strict pharmacological requirements for the exact nicotinic or muscarinic type. This fact has been confirmed with ACh-receptors in the ganglion cells of *Aplysia* (KEHOE, 1972a, b). Accordingly, we divided the ACh-receptors into three types simply by the ion species involved in the activation of each receptor. The identification of ion species involved was done by controlling the ionic composition of the perfusing solution and by observing the resultant change in reversal potentials of the ACh-induced responses.

RESULTS

I. Effects on the D_{Na} -type of receptors

Many cells in R_B group of the abdominal ganglion are depolarized by an application of acetylcholine (ACh). The depolarization is associated with rapid increase in membrane conductance. The ACh-receptors which produce this type of response may be called " D_{Na} -type" since the characteristic depolarization (D) has been known to be Na-dependent (SATO *et al.*, 1968). The receptors of this

type were readily activated by nicotine but not by arecoline.

Receptor activation by TMA and choline. Both tetramethylammonium (TMA) and choline showed potency in activating the D_{Na} -type receptor as shown in Fig. 1. Relative potencies of the receptor activation were found to be greatest in ACh,

Fig. 1. The activating effects of tetramethylammonium (TMA), and choline on the acetylcholine (ACh) receptor of the D_{Na} -type. All records were obtained from a single cell with the D_{Na} -type receptors. The downward lines appearing periodically from the base line are the index of the membrane resistance (See MATERIALS AND METHODS). The horizontal bar on the left shoulder of each trace indicates extracellular potential level which is virtually grounded. The thick portion of the bottom line indicates the time of drug application. The upper part of the spike discharge was clipped. This nomenclature applies to all other recordings in this paper.

Fig. 2. The dose-response curves which were obtained from a single receptor membrane of the D_{Na} -type, with acetylcholine (ACh), tetramethylammonium (TMA), and choline. All responses were evaluated by the increase (ΔG) in membrane conductance under the voltage clamp at the resting membrane potential, -45 mV.

Fig. 3. Inhibitory effect of hexamethonium (Hexa) on the acetylcholine (ACh) induced response of the D_{Na} -type. All records were obtained from a single cell with the D_{Na} -type receptor. The response to 1 mM ACh is shown in the left as a control. The middle trace shows a little effect of 50 mM Hexa by itself. The response to 1 mM ACh mixed with 50 mM Hexa is shown in the right.

but smallest in choline. A more quantitative comparison was made in the dose-response curves as shown in Fig. 2. Their relative potencies of the receptor activation were found to be ACh:TMA:choline $\approx 1:0.033:0.001$.

Receptor inhibition by Hexa. Figure 3 shows the effect of hexamethonium (Hexa) on the D_{Na} -type response. Neither the resting membrane potential nor the resting membrane conductance was changed appreciably by application of 50 mM Hexa. The effect of Hexa on ACh-induced response of this type was examined and is shown on the right in Fig. 3. The ACh-induced response was markedly depressed by the presence of Hexa. However, the depression was reversible showing complete recovery after 20 min washing with normal artificial *Aplysia* blood. The mode of inhibition of Hexa on D_{Na} -type receptor is shown in Fig. 4. The ACh-induced responses (ΔG) were evaluated in the presence of Hexa of different concentrations and expressed in the relative size (%) of the control (Fig. 4a). ACh-induced responses were depressed by the presence of Hexa, the greater depression with the higher concentration of Hexa. All data were taken from 5 different cells and results were normalized for the statistical presentation. It should be noted that the curve shifted to the right as the concentration of ACh increased. Another way of evaluating the mode of inhibition is Lineweaver-Burk type of plotting. A typical D_{Na} -type of receptor membrane was chosen for this experiment. As shown in Fig. 4b, an increase in [Hexa] augmented the slope of the straight line but did not change the point of intercept on the ordinate. The systematic change shown in this figure was confirmed to be the same qualitatively for all other D_{Na} -type of the receptor membranes.

Receptor inhibition by Deca. Figure 5 shows the effect of decamethonium (Deca) on the D_{Na} -type response. Neither the resting membrane potential nor the resting membrane conductance was changed significantly by application of 50 mM Deca. The effect of Deca on ACh-induced response of this type was ex-

Fig. 4. a: Dose-inhibition curves with hexamethonium (Hexa), obtained from the D_{Na^+} -type receptor membranes. Acetylcholine (ACh)-induced responses were measured in the presence of Hexa and expressed in percent of the control measured in the absence of Hexa. Each point indicates the average response of 5 cells to a given concentration of ACh and vertical bars denote S.D. b: Lineweaver-Burk type of plotting obtained from a single receptor membrane of the D_{Na^+} -type. The acetylcholine (ACh)-induced responses (ΔG) were measured in the presence of Hexa, the concentrations of which were shown on each line in mM. All measurements were performed under the voltage clamp at -45 mV.

Fig. 5. Inhibitory effect of decamethonium (Deca) on the acetylcholine-induced response of the D_{Na^+} -type. Other nomenclature is the same as in Fig. 3.

amined and shown on the right in Fig. 5. The ACh-induced response was considerably depressed by the presence of Deca. However, the depression was reversible showing complete recovery after 15 min washing with normal artificial *Aplysia* blood. The mode of inhibition of Deca on D_{Na^+} -type receptor was shown in Fig. 6. The ACh-induced responses (ΔG) were evaluated in the presence of

Fig. 6. a: Dose-inhibition curves with decamethonium (Deca), obtained from the D_{Na} -type receptor membranes. b: Lineweaver-Burk type of plotting obtained from a single receptor membrane of the D_{Na} -type. Other nomenclature is the same as in Fig. 4a, b.

Deca of different concentrations and expressed in the relative size (%) of the control (Fig. 6a). ACh-induced responses were depressed by the presence of Deca, the greater depression with the higher concentration of Deca. All data were taken from 5 cells and results were normalized for the statistical presentation. It should be noted that the curve shifted to the right as the concentration of ACh increased. Another way of evaluating the mode of inhibition is Lineweaver-Burk type of plotting. A typical D_{Na} -type of receptor membrane was chosen for this experiment. As shown in Fig. 6b, an increase in [Deca] augmented the slope of the straight line but did not change the point of intercept on the ordinate. The systematic change shown in this figure was confirmed to be the same qualitatively for all other D_{Na} -type of the receptor membranes.

II. Effects on the H_{Cl} -type of receptors

Many cells in R_c group of the abdominal ganglion are hyperpolarized by application of acetylcholine (ACh). The hyperpolarization is associated with a characteristic rapid increase in membrane conductance. The reversal potential of this response is often found to be approximately -60 mV, which is very close to the equilibrium potential of Cl^- (TAUC and GERSCHENFELD, 1962). The ACh-receptors which produce this type of response may be called " H_{Cl} -type" since the characteristic hyperpolarization (H) has been known to be produced by the selective increase in permeability of the receptor membrane toward Cl^- exclusively (SATO *et al.*, 1968). The receptors of this type were readily activated by nicotine but not by arecoline. This pharmacological feature was the same as that observed

Fig. 7. The activating effects of tetramethylammonium (TMA), choline, and decamethonium (Deca) on the acetylcholine (ACh) receptor of the H_{Cl} -type. All records were obtained from a single cell with the H_{Cl} -type receptors.

Fig. 8. The dose-response curves which were obtained from a single receptor membrane of the H_{Cl} -type, with acetylcholine (ACh), tetramethylammonium (TMA), choline, and decamethonium (Deca). All responses were evaluated by the increase (ΔG) in membrane conductance under the voltage clamp at the resting membrane potential, -55 mV.

Fig. 9. Non-effectiveness of hexamethonium (Hexa) on the H_{Cl} -type receptor. Other nomenclature is the same as in Fig. 3.

in the D_{Na} -type receptors.

Receptor activation by TMA, choline, and Deca. Certain oniums, such as tetramethylammonium (TMA), choline, and decamethonium (Deca), showed potency in activating the H_{Cl} -type receptor as shown in Fig. 7. The relative potencies of receptor activation seemed to be greatest in ACh but smallest in choline or Deca. More quantitative comparison was made in the dose-response curves as shown in Fig. 8. Their relative potencies of the receptor activation were found to be ACh: TMA: choline or Deca $\approx 1: 0.01: 0.002$.

Non-effectiveness of Hexa. Neither the resting membrane potential nor the resting membrane conductance was changed appreciably by application of 50 mM hexamethonium (Hexa), as shown in Fig. 9. The effect of Hexa on ACh-induced response of this type was examined and shown on the right in Fig. 9. The ACh-induced response was not affected at all by the presence of Hexa.

III. Effects on the H_K -type of receptors

A few cells in the left pleural and abdominal ganglions are hyperpolarized by the application of acetylcholine (ACh). The hyperpolarization is always associated with a slow increase in membrane conductance. The reversal potential of this response is often found to be approximately -80 mV which is very close to the equilibrium potential of K^+ (KEHOE, 1972a). The ACh-receptors which produce this type of response may be called " H_K -type" since the characteristic hyperpolarization (H) recorded in these cells has been known to be produced by a selective increase in permeability of the receptor membrane toward K^+ , exclusively (KEHOE, 1972a).

Non-effectiveness of choline, TMA, Hexa, and Deca. None of the following drugs, 100 mM choline, 10 mM TMA, 50 mM Hexa, and 50 mM Deca, produced a significant response when applied to the resting membrane. Further, the ACh-induced response of this type was not appreciably altered by the presence of these drugs, as shown in Fig. 10.

Fig. 10. Non-effectiveness of choline, tetramethylammonium (TMA), hexamethonium (Hexa), and decamethonium (Deca) on the H_K-type receptor. The records shown in the upper two rows were obtained from the same cell whereas those in the bottom two rows were from another cell. Other nomenclature is the same as in Fig. 3.

DISCUSSION

D_{Na}-type receptors. Hexamethonium (Hexa) is generally known as a reversible, competitive inhibitor of the ACh-receptor in the electroplax (KARLIN and WINNIK, 1968), in the superior cervical ganglion of the cat and in the neuromuscular junction of the rabbit (WIEN *et al.*, 1952). In the ganglion cells of *Aplysia kurodai*, Hexa selectively inhibited the ACh-induced response of D_{Na}-type

but not that of H_{Cl} - or H_K -type. Similarly, the selective inhibition of Hexa on the Na-dependent response in *Aplysia californica* has been reported by KEHOE (1972b).

Since both decamethonium (Deca) and Hexa belong to the bis-trimethylammonium group, Deca may be expected to have an inhibitory effect on the activity of Na-dependent receptor. Deca, however, is known to act as an activator on this type of receptors in the frog endplate (DEL CASTILLO and KATZ, 1957a), and in the electroplax (CHANGEUX and PODLESKI, 1968). In spite of this activating potency of Deca by itself, DEL CASTILLO and KATZ (1957b) also observed that the ACh-induced response is markedly depressed by the presence of lower concentration of Deca. The inhibitory effect of weaker or slowly acting agonists may be satisfactorily explained by the hypothesis of competitive antagonism by partial agonists (ARIËNS, 1954; ARIËNS and SIMONIS, 1954; ARIËNS and DE GROOT, 1954; ARIËNS *et al.*, 1955).

However, it should be noted that Deca (50 mM) did not activate the D_{Na} -type receptor in our preparation at all. In Fig. 6a, the dose-inhibition curve shifted to the right with an increase in Deca concentration. This type of shift indicated that the mode of inhibition by Deca on the D_{Na} -type receptor was competitive. The apparent dissociation constant of Deca was about 35 times greater than that of ACh, since 1 mM Deca depressed the response to 0.03 mM ACh into a half of the control. If Deca should have acted as an activator on this type of receptor, dose-response curve of Deca would have fallen in between two curves by ACh and TMA shown in Fig. 2. Nevertheless, we failed to detect any activating potency of Deca even in a concentration of 100 mM. It was suggested that the intrinsic activity of Deca for this type of receptor was much smaller than that of ACh or TMA. In our electrophysiological experiment, Deca may be considered to act as a simple, competitive inhibitor on the D_{Na} -type receptor in ganglion cells of *Aplysia*. The inhibitory effect of Deca on ACh-induced response was also observed in *Helix* by WALKER and HEDGES (1967).

Despite the Na-dependent nature as well as the nicotinic nature of the D_{Na} -type receptor in our preparation are the same as those of the ACh-receptors in endplate and electroplax, the effects of Deca on the former were found to be different from the latter two. This difference suggested that there is a slight structural variation even in the same Na-dependent, nicotinic type of receptors. Additional difference among these receptors may be demonstrated in the comparison of activating potencies of tetramethylammonium (TMA); 1/400 of ACh potency in endplate (ARIËNS *et al.*, 1955), 1/1,000 in electroplax (KARLIN, 1969), and 1/60 in *Aplysia* ganglion cell. Further structural difference among them may be seen in the Hill coefficient; 3 for the receptor in the frog endplate (DREYER and PEPER, 1975), 2 in the electroplax (KARLIN, 1967, 1969), and 1 in the *Aplysia* ganglion cell (SATO *et al.*, 1976).

H_{Cl}-type receptor. KARLIN and his co-worker (1968, 1969) proved that the

disulfide bond was localized around the anionic site of the ACh-receptor in the electroplax by employing site-directed thiol. SATO *et al.* (1976) analyzed the effect of S-S bond reduction at the receptors of three types in ganglion cells of *Aplysia*. They reported that the disulfide bond was localized around the anionic site in both receptors of H_{Cl} - and D_{Na} -type. The activating action of nicotine and blocking action of *d*-tubocurarine have been confirmed in both types of receptors, despite the ionic mechanism underlying the activation of H_{Cl} -type receptor is totally different from that of D_{Na} -type receptor (KEHOE, 1972b). These findings suggested that the structure of ACh-binding site of H_{Cl} -type receptor is similar to that of D_{Na} -type.

The structural similarity of ACh-binding site between D_{Na} - and H_{Cl} -type receptors was also confirmed in our experiment since the activating potency of TMA and choline on both type receptors were found to be approximately the same. Deca acted as an activator on the H_{Cl} -type receptor though it acted as an inhibitor on the D_{Na} -type one. This activating effect of Deca suggested that the ACh-binding site of H_{Cl} -type receptor in the ganglion cells of *Aplysia* is similar in structure to that of Na-dependent receptors in the frog endplate and electroplax. It should be noted that Deca makes binding with the common anionic sites in the ACh-receptors, of both D_{Na} - and H_{Cl} -types, though it inhibits one but activates another.

Hexamethonium did not possess the affinity to the ACh-binding site of H_{Cl} -type receptor, since it did neither activate nor inhibit the receptor of this type. The lack of blocking action with Hexa on the H_{Cl} -type responses has been observed in other preparations; Navanax (LEVITAN and TAUC, 1972), and *Aplysia californica* (KEHOE, 1972b). These findings suggested that there is a certain structural difference in the vicinity of the ACh-binding site between the receptors of H_{Cl} - and D_{Na} -type.

H_K-type receptors. The ACh-binding site of the K-dependent receptor is known to be different from that of either Na- or Cl-dependent receptor in ganglion cells of *Aplysia*, since this type of receptor is not activated by nicotine and not inhibited by *d*-tubocurarine (KEHOE, 1972b). SATO *et al.* (1976) also reported that K-dependent receptor in the ganglion cells of *Aplysia* did not possess the disulfide bond at the vicinity of the ACh-binding site. In our experiment, the oniums, including TMA, choline, Hexa, and Deca, seemed to possess no affinity to ACh-binding site of H_K -type receptor, since they showed neither activation nor inhibition on this type of receptor. ACh was found, so far, to be the only activator of this receptor. This suggested that the H_K -type receptor had a higher selectivity for the binding of trimethylated ammonium group than did other two.

Recently, it has been proposed that the functional receptor unit is composed of a moiety containing the transmitter binding site and a moiety containing the conductance mediated entities (ionic channel). According to this hypothesis, we divided the ACh-receptors into three types by the ion species involved in the

activation of each receptor. This classification depended on the difference in the property of the ionic channel of each receptor. Our attempt was to find out some characteristic properties of ACh-binding site specific to each receptor classified under the ionic specificities. Our results indicated that the ACh-binding sites at various types of ACh-receptors in the ganglion cells of *Aplysia kurodai* may be classified pharmacologically into two types, though each type under this classification did not exactly correspond to the classical "nicotinic or muscarinic" type.

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