Microelectrophoretic Application of Cholinergic Compounds, Protein Oxidizing Agents, and Mercurials to the Chemically Excitable Membrane of the Electrophox

(acetylcholine/*o*-iodosobenzoate/*p*-chloromercuribenzoate/Mg⁺⁺/electro-osmotic technique)

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ABSTRACT Cholinergic compounds, protein oxidizing agents, heavy metal ions, and organic mercurials were microelectrophoretically (or electro-osmotically) applied to the innervated surface of the isolated electrophox of *Electrophorus electricus*. Phasic potential changes, resembling those observed under similar conditions in frog muscle, followed not only pulses of the common cholinergic drugs, but were also elicited by *o*-iodosobenzoate, *p*-chloromercuribenzoate, and Hg⁺⁺ ions. These results suggest that cholinergic receptors of both the electrophox and amphibian endplates behave in essentially the same manner. The discrepancies previously observed can be attributed mainly to the different techniques of drug application.

The electric organ of *Electrophorus electricus* and, in particular, isolated electrophox preparations (1, 2) has been used to study the effects of chemical modification of cholinergic receptors by externally applied sulfhydryl reagents (3-5). Evidence has been accumulated indicating the presence of disulfide bonds in the vicinity of the anionic site of the cholinergic receptor. The chemical sensitivity of the receptor protein to activating compounds is, to a large extent, dependent on whether these S-S groups are in an oxidized or reduced state.

Similar experiments were performed on the cholinergic receptors of motor endplates of frog muscle (6); reducing and oxidizing reagents, as well as alkylating agents, were applied with the aid of the microelectrophoretic (or electro-osmotic) technique (7, 8). Although the results obtained in both membranes are in general agreement, differences were observed concerning the optimum state of oxidation of the receptor protein necessary for activation. The results in the electrophox experiments suggested that the SH groups have to be in the disulfide form before the protein can undergo activation. However, the observations made in frog muscle indicated that receptor activation by acetylcholine (AcCh) is probably accompanied by a partial oxidation of the protein; in other words, reduced SH groups should be present for activation to occur. In fact, receptor activation can be achieved by oxidizing free SH groups by pulses of *o*-iodosobenzoate and other oxidants.

Since it is widely assumed that electrophox and endplate membranes have fundamentally similar properties in their

response to chemical excitation, it appeared pertinent to find out whether the discrepancies observed are due to differences in either the nature of the receptor protein or the activation mechanism, or whether they can be attributed to the techniques of drug application. For this purpose we have studied the effects of electrophoretic application of several compounds to the innervated surface of isolated electrophox preparations. Although our results, obtained in a relatively small number of preparations taken from four different eels, should be regarded as preliminary, they indicate that the postsynaptic membranes of electrophox and frog muscle have similar chemical properties and react to electrophoretic application of cholinergic compounds and protein reagents in essentially the same manner.

METHODS

Preparation. The dissection of the electrophox was as described (1), except that three rows of cells instead of one were used and the connective tissue membrane was removed on all three cells. The resulting three electrophox (innervated side up) were attached with thin entomological pins to the bottom of a chamber that contained a layer of translucent silicone rubber.

The eel Ringer's solution used had the following composition in mM: NaCl, 160; KCl, 5; CaCl₂, 2; MgCl₂, 2; NaH₂PO₄, 0.3; Na₂HPO₄, 1.2. The pH of the bathing solution was 7.0 unless otherwise specified.

Electrical Recording. The transmembrane potential was recorded with conventional KCl-filled glass micropipettes connected, by a high input impedance preamplifier, to the upper beam of a cathode ray oscilloscope. The lower beam was used to monitor the electric current flowing through the drug micropipettes, as the voltage drop caused across a 10 kΩ resistor interposed between the bath and ground.

Microelectrophoretic Drug Application. Drugs and reagents were delivered to the cell membrane with treble-barrelled glass micropipettes that were drawn by hand. One of the barrels was filled routinely with AcCh or carbamylcholine and was used to locate the best position of the microelectrode tip with reference to the receptor surface before the other drugs were applied (see ref. 6).

RESULTS

Spontaneous Miniature Endplate Potentials. The innervated surface of the isolated electrophox is coated by a thick layer of

Abbreviations: AcCh, acetylcholine; Bis-Q, 3,3'-bis[α-(trimethylammonium)methyl]-azobenzene bromide; QBr, 3-(α-bromo-methyl)-3'-[α-(trimethylammonium)methyl]-azobenzene bromide.

FIG. 1. Spontaneous discharges, equivalent to the miniature endplate potentials of frog muscle, externally recorded from the innervated surface of the electroplax (see *text* for details). Calibrations: vertical, 1 mV for A–D; horizontal, 50 msec for A and B, 100 msec for C, and 10 msec for D.

connective tissue, nerve fibers, and interweaving terminal arborizations. This layer offers considerable resistance to the movements of a recording microelectrode. In fact, in most insertions, the tip of the recording microelectrode produces a deep “dimple” or indentation on the cell surface.

Just before the microelectrode tip penetrates into the cytoplasm, fast negative-going spontaneous discharges are frequently observed. By carefully adjusting the position of the microelectrode tip it was possible to record such discharges for up to 1 min or more.

These discharges are characterized by a fast initial phase followed by a slower decay (Fig. 1A). They closely resemble the externally recorded miniature endplate potentials from the frog neuromuscular junction. A further similarity between these discharges and the miniature endplate potentials of skeletal muscle is the fact that their frequency greatly increases under the influence of hypertonic solutions. This is shown by the record of Fig. 1B (obtained at the same site as Fig. 1A) after the addition of a drop of eel Ringer's solution made hypertonic with sucrose in the vicinity of the site of impalement. This effect was a transitory one; the frequency of the discharges returned to about the initial value after the sucrose diffused away into the bulk of the solution.

Although no statistical tests were performed, it seems likely that these discharges were generated by the random release of multimolecular packets of AcCh. As in frog muscle, the amplitude of these miniature endplate potentials range from a fraction of a mV to almost 10 mV. Fig. 1C shows some of the largest external miniature endplate potentials actually recorded, the largest one about 4 mV, and Fig. 1D shows the time course of one of the miniature endplate potentials recorded at the same site as in Fig. 1C, on a faster time base (10 msec/cm).

Although external miniature endplate potentials were frequently recorded, internal miniature endplate potentials were observed only on two or three occasions, but were not photographed. They were very small, less than 0.5 mV, and their frequency was low. The fact that internal miniature endplate potentials are difficult to observe can be attributed to the low effective resistance of the cell and the correspondingly short space constant. Our observations suggest that only those large

miniature endplate potentials occurring in the immediate vicinity of the microelectrode tip can actually be recorded as a potential variation across the membrane.

Depolarizing Action of AcCh and Carbamylcholine. The electrophoretic application of compounds to the receptors of the isolated electroplax proved to be technically more difficult than in motor endplates, in spite of the fact that cholinergic receptors can be found virtually at any point of the innervated surface of this cell.

Due to the low space constant of the electroplax, the potential changes elicited by electrophoretic drug application can only be recorded if the tip of the recording microelectrode is only a few micrometers away from the tip of the drug micropipette. The amplitude of the recording potentials decays very rapidly with the distance between the two tips. If they are separated by a distance of more than about 50 μ m, only small and slow chemical potentials can be observed after the application of very long current pulses (see Fig. 4).

An additional difficulty is introduced by the layer of connective tissue and nerve fibers, which prevents the tip of the drug micropipette from reaching the receptor surface. The best way of obtaining fast electrophoretic potentials was to first insert the recording microelectrode into the cell and then direct the tip of the drug micropipette towards the tip of the recording microelectrode in the bottom of the dimple created by the impalement. Careful manipulation of the multibarrelled electrode is then necessary to obtain fast drug potentials, which often leads to dislodgement of the recording microelectrode.

It could be shown in this manner that the electrophoretic application of AcCh and carbamylcholine gave rise to phasic potential changes similar to those observed in frog muscle. Fig. 2 illustrates two AcCh and carbamylcholine potentials. AcCh potentials with a rising phase of less than 10 msec have been occasionally recorded.

In Fig. 3A, the first potential was produced by a pulse of tetramethyl ammonium, whereas the second potential was

FIG. 2. Phasic depolarizations elicited by the electrophoretic applications of AcCh (A) and carbamylcholine (B). In this and the following figures, the *upper traces* represent the potential difference across the cell surface membrane. The *lower traces* monitor the currents flowing through the drug microelectrode. Calibrations: vertical, 1 mV for A and 2 mV for B, and 1 μ A for A and B; horizontal, 100 msec for A and B.

produced by carbamylcholine. Fig. 3B illustrates the blocking action of *d*-tubocurarine on the AcCh potentials. In this particular record the AcCh potentials were very slow due to the long distance between the tip of the drug microelectrode and the receptors. Positive pulses of almost 0.2 sec in duration were necessary to build up a concentration of AcCh sufficient to elicit a depolarization of about 4 mV. After the first pulse, giving rise to the largest AcCh potential photographed, the dc braking current used to prevent spontaneous diffusion of *d*-tubocurarine from the pipette was switched off. This caused first a reduction and then complete block of the AcCh-induced depolarization. In addition, a lowering to the baseline can be observed, indicating that, before application of *d*-tubocurarine, the cell did not completely repolarize between the AcCh pulses.

Two other compounds that elicited phase depolarizations of the electoplax membrane were cholinethiol (9) and 3,3'-bis[α -(trimethylammonium)methyl]-azobenzene bromide (Bis-Q) (10). Both compounds depolarize the electoplax membrane when added to the bath before reduction of the disulfide bonds of the receptor. Of the two compounds, Bis-Q is particularly active, its potency exceeding that of carbamylcholine by two orders of magnitude.

Two potentials elicited by cholinethiol and Bis-Q are illustrated in Fig. 4A and B, respectively. Compared with Fig. 2, these potentials are slow. Their long time course is a consequence of the long distance between the tips of the two microelectrodes.

Effects of the Electro-Osmotic Application of Oxidizing Reagents. SH oxidants electro-osmotically applied to the endplate membrane give rise to potential changes similar to those produced by AcCh and carbamylcholine. Since oxidants are inactive when applied even at much higher concentrations to the extrajunctional areas of the membrane, it was concluded that their depolarizing action was due to the activation of the cholinergic receptors (6).

The oxidant agent that exerts the most powerful depolarizing effect on the endplate is *o*-iodosobenzoate, which seems to be highly specific for the sulfhydryl groups of proteins. Often the depolarizing potency of *o*-iodosobenzoate pulses expressed

FIG. 3. (A) a tetramethylammonium and carbamylcholine potential are seen. (B) the depolarization produced by long AcCh pulses is blocked by *d*-tubocurarine diffusing from the other barrel of the drug micropipette. Calibrations for A and B: vertical, 1 mV and 1 μ A; horizontal, 100 msec.

FIG. 4. Slow potential changes elicited by pulses of cholinethiol (A) and Bis-Q (B). Calibrations for A and B: vertical, 2 mV and μ A; horizontal, 2 sec.

in terms of mV/nC has exceeded that of carbamylcholine. In general, the time course of the *o*-iodosobenzoate potentials is faster than those elicited by AcCh and carbamylcholine pulses ejected from the same multiple micropipette.

The electro-osmotic application of *o*-iodosobenzoate to the electoplax membrane also gave rise to phasic potentials as shown in Fig. 5A, which shows two potentials elicited, respectively, *o*-iodosobenzoate and AcCh. Even if the rising phases are about equal in duration, the decay of the *o*-iodosobenzoate potential is faster than that of AcCh.

Fig. 5B illustrates the effect of the negative dc braking current on the release of *o*-iodosobenzoate from the pipette and the time course of the subsequent potential changes. In these records, long pulses of positive current were applied to the *o*-iodosobenzoate barrel. The negative braking current was initially adjusted so that no *o*-iodosobenzoate was ejected by the positive outward current pulse. The first of the traces

FIG. 5. (A) two potentials are shown, elicited by *o*-iodosobenzoate (left) and AcCh (right). (B) long positive pulses were applied to the *o*-iodosobenzoate barrel while the intensity of the negative braking current was gradually reduced (see text). The second pulse on each sweep was AcCh. Calibrations for both A and B: vertical, 1 mV and 1 μ A; horizontal, 100 msec.

FIG. 6. Two potentials elicited by *p*-chloromercuribenzoate (left) and by AcCh (right). Calibrations: vertical, 1 mV and 1 μ A; horizontal, 100 msec.

superimposed in Fig. 5B was recorded under those conditions. The lack of any concomitant changes in membrane potential across the electroplax membrane shows that the depolarizations caused by the following pulses were not due to current injection into the cell. After this first trace was photographed, the intensity of the negative braking current was gradually reduced with each successive pulse. As a result, it can be seen that *o*-iodosobenzoate is first ejected at the end of the pulse. As the braking current is further decreased, the delay between the onset of the pulse and the beginning of the depolarization is seen to decrease.

Effects of Organic Mercurials and Hg^{++} Ions. Endplate receptors can also be activated by the electrophoretic application of organic mercurials and divalent metal ions, particularly Hg^{++} ions, all of which bind to SH groups of proteins. Similar results have been obtained in the electroplax. Fig. 6 illustrates the depolarizing action of a pulse of *p*-chloromercuribenzoate. The depolarization caused by Hg^{++} on the electroplax seemed to be less powerful than on muscle endplates, and also less reversible. Whereas in frog endplates Hg -potentials can be elicited many times, in the

FIG. 7. (A) In the first sweep, a pulse of AcCh alone was applied resulting in a potential of over 4 mV. In the second sweep a pulse of choline disulfide was applied simultaneously, resulting in an almost complete block of the AcCh potential. (B) shows the blocking effect of a similar pulse of choline disulfide interposed on a series of AcCh potentials. Calibrations: vertical for A and B, 1 mV and 1 μ A; horizontal, 100 msec for A, 2 sec for B.

FIG. 8. Depolarizing and AcCh-blocking action of pulses of QBr (see text for details). Calibrations for A and B: vertical, 1 mV and 1 μ A; horizontal, 100 msec.

electroplax they could be observed only after the first few pulses of each series before the receptors in that area became desensitized.

Effects of Choline Disulfide and 3-(α -Bromomethyl)-3'-[α -(trimethylammonium)methyl]-azobenzene Bromide (QBr). Several compounds so far not tested on frog muscle but extensively used on electroplax preparations were applied electrophoretically to electroplax preparations to see if the effects obtained in this manner were consistent with those obtained by adding them to the bath.

One of these compounds is choline-disulfide, which exerts a curare-like action on the electroplax before the reduction of the S-S bonds of the receptor (11). The curarizing action of this compound was confirmed with the present technique (Fig. 7A and B). In Fig. 7A the effect of superimposing a long pulse of choline-disulfide on an AcCh pulse is illustrated. As can be seen, the choline-disulfide pulse leads to an almost total block of the AcCh potential. In Fig. 7B a pulse of choline-disulfide was interposed in a series of AcCh pulses so that the time course of the recovery could be seen.

The other compound we are referring to is QBr (10), which behaves both as an activating and as a blocking agent. Both effects have also been observed with the electrophoretic technique. In Fig. 8A the pulse of QBr elicits a small, but prolonged, depolarization followed by a reduction in the amplitude of the AcCh potential. The first trace of Fig. 8B shows an AcCh potential alone. Between this trace and the following one a pulse of QBr was applied, which caused a depolarization. The following QBr pulse is shown in the next trace in which the effects of AcCh are almost completely blocked.

DISCUSSION

This study was aimed at testing the effects of electrophoretic application on the electroplax of compounds studied with the same technique on muscle endplates and with standard procedures on the electroplax. One pertinent result is the finding that the postsynaptic membranes of both electroplax and frog neuromuscular junctions respond to electrophoretic application of cholinergic compounds and some protein reagents in essentially the same manner. The differences observed between the two preparations must be attributed mainly to the different techniques. Electrophoretic

application revealed effects that are different from those observed with standard procedures.

Many compounds are active at micromolar concentrations. Such a high potency is due both to a high chemical affinity for the receptor, which results in a high number of molecular interactions per unit time, as well as to a high efficacy or intrinsic activity, i.e., the ability of the compound to induce activation of the receptor.

With the microelectrophoretic technique, high concentrations of the compounds tested can rapidly be built up in the environment of a relatively small number of receptor sites. When used with potent agents, the electrophoretic technique has the advantage of allowing a great spatial and temporal resolution. This technique is also helpful to demonstrate interactions between different compounds, although it does not lend itself well to quantitative studies.

Another useful application of the electrophoretic technique is the study of the reaction of the membrane to compounds that have either a very low affinity for the receptor or a very high affinity for chemical groups and functions found not only in the receptor, but also in many other proteins and enzymes. Neither of these two types of compounds can be studied by adding them to the bath. The first group will act only at high concentrations, which exert a variety of unspecific disruptive effects on the preparation acting on areas of the cell not related to the cholinergic receptor. The second group produces widespread effects difficult to interpret in terms of specific receptor interaction. The electrophoretic method can be used to bring these compounds directly to the receptor environment while keeping the concentration in the bulk of the solution at virtually zero level. When, for instance, *p*-chloromercuribenzoate is added to the bath surrounding an isolated electrophax preparation in concentrations as low as 0.5 mM, its most prominent effect is an inhibition of the chemical sensitivity of the cell. This inhibition is not affected by repeated washing of the preparation but can be reversed by 5 mM of 2-mercaptoethanol or 5 mM of cysteine (3). However, electrophoretically applied *p*-chloromercuribenzoate causes a rapid and completely reversible depolarization of the postsynaptic membrane.

Receptor activation seems to be the result of a large concentration pulse, which results in the sudden binding of a large number of free SH groups at a rate much higher than that which prevails when the compound is added to the bath. The rapid reversibility of this action is more difficult to explain and requires further investigations.

The differences between the effects of *o*-iodosobenzoate when either added to the bath or electrophoretically applied can also be explained in a similar way. There is evidence showing that bath concentrations as low as 50 μ M of *o*-iodosobenzoate restore the sensitivity of the electrophax receptors reduced by dithiothreitol. *o*-Iodosobenzoate at much higher concentrations has no effect on the resting potential (Bartels, E., unpublished observations). However, *o*-iodosobenzoate pulses applied electrophoretically to the membrane exert, both in the electrophax and the endplate, an intense depolarizing action.

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