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# THE ACETYLCHOLINE RECEPTOR

# ISOLATION OF A BRAIN NICOTINIC RECEPTOR AND ITS PRELIMINARY CHARACTERIZATION IN LIPID BILAYER MEMBRANES

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## SUMMARY

The technique of affinity chromatography with the curarizing neurotoxins of *Naja naja* venom has been employed to extract nicotinic acetylcholine receptors from the brain tissues of mouse and hog. Both carbachol and hexamethonium were used as linear or step gradients to elute the receptor and its properties were investigated in lipid bilayer membranes. Of particular interest is the observation that discrete quanta of conductance could be observed across an NaCl gradient of 1.0:0.1 M. By switching the voltage-clamp across the bilayer between a positive and negative 80 mV, the separate $Na^+$ and $Cl^-$ conductances of these quanta could be estimated and the following conductances of the smallest discrete quanta were observed: $3.7 \cdot 10^{-11}\ \Omega^{-1}$ ($Na^+$) and $5.9 \cdot 10^{-11}\ \Omega^{-1}$ ($Cl^-$) for mouse brain receptors; $3.8 \cdot 10^{-11}\ \Omega^{-1}$ ($Na^+$) and $4.7 \cdot 10^{-11}\ \Omega^{-1}$ ($Cl^-$) for hog brain receptors. Large aggregates of receptors appeared to activate and deactivate as multiples of a basic conductance size, although there is evidence that they may not represent the actual gating of ion channels. A "background noise" that is not within the temporal capability of the recording system is also present at an intensity that seems to parallel the number of activated receptors, and in view of recent electrophysiological evidence that the relaxation lifetime of the open channel state is of a millisecond duration, it may be that this "noise" actually represents the channel gating.

## INTRODUCTION

In a recent report [1] we noted the presence of quantal changes of conductance in lipid bilayer membranes following the addition of isolated acetylcholine receptors to the surrounding bath. Although the biochemistry of the nicotinic acetylcholine receptor has been studied in some detail [2–8], the properties of the receptor in a reconstituted membrane system are poorly known. Such a system is necessary so that the receptor may be in an environment closer to its native state in the muscle or nerve

membrane and the artificial lipid bilayer would appear to offer the best approach towards achieving this goal that is currently available.

There is a report of an estimated conductance of the single receptor protein in liposomes of $10^{-15}\ \Omega^{-1}$ based on attempted measurement of ion flow and radiochemical quantitation of toxin-binding sites [9]. De Robertis and coworkers [10–12] report that the incorporation of a "proteolipid" receptor into lipid bilayers causes conductance changes following acetylcholine application but the resolutions are not sufficient to clearly distinguish the molecular events associated with the conductance changes. Their work is also complicated by various criticisms and difficulties in reproducing the results [13–15]. There is also a report that acetylcholinesterase preparations from electroplax will induce conductance increases in lipid bilayers [16].

It is probable that the report indicating a single receptor conductance of $10^{-15}\ \Omega^{-1}$ represents an erroneous figure based on recent estimates of the unit channel conductance of the receptor from electrophysiological analysis. Katz and Miledi [17–19] have studied the random noise fluctuations in muscle endplates during iontophoretic application of acetylcholine and have found from statistical studies that they may represent discrete conductance changes of about $10^{-10}\ \Omega^{-1}$ with average lifetimes of approx. 1 ms. Anderson and Stevens [20] studied the Fourier transform spectra of endplate current "noise" and estimate that the unit channel conductance is $3.2 \cdot 10^{-11}\ \Omega^{-1}$ with an average relaxation lifetime for the open-channel state at between 6 and 11 ms, depending on temperature and voltage. No clear values are reported for isolated receptors in this range other than in our earlier report [1] and it thus appears that little is known concerning the nature of these microscopic events.

Nicotinic receptors have been shown to exist in brain tissue [21–23], although there is not agreement over the actual numbers of receptors present. There are estimates of $0.6 \cdot 10^{-12}$ moles/g, $3.4 \cdot 10^{-12}$ moles/g and $17.5 \cdot 10^{-9}$ moles/g, depending on study and brain source. However, as has been shown for gramicidin A and other antibiotics [24–27], one can detect permeability changes in a bilayer membrane at concentrations in the surrounding bathing medium down to $10^{-12}$ M, so that the quantities of receptors found above should be sufficient for bilayer studies. Earlier we noted conductance fluctuations of $3.7 \cdot 10^{-11}\ \Omega^{-1}$ (1.0 M $Na^+$) and $5.9 \cdot 10^{-11}\ \Omega^{-1}$ (1.0 M $Cl^-$) when acetylcholine receptors from mouse brain were incorporated into lipid bilayers. It is of interest that these fluctuations are of the same order of magnitude as those calculated on different grounds by Katz and Miledi [17–19] and Anderson and Stevens [20] and that they are also near the unit channel conductance value for gramicidin A as observed in lipid bilayers [28]. We have now extended these studies using nicotinic receptors isolated from both hog and mouse brain and report some unusual properties of possible biological and biophysical significance. First, there is an apparent tendency for groups of receptors to turn on and off simultaneously possibly indicating the presence of cooperative effects during receptor function. Second, the presence of a "background noise" is noted that appears to increase in intensity as more receptors turn on or activate. Since the recording system had an insufficient bandwidth ($\Delta f < 10\ s^{-1}$), this noise could not be clearly resolved and quantified.

## METHODS

*Extraction of receptors*

The extraction of acetylcholine receptors from brain tissues was similar to that used in other laboratories for isolating electroplax receptor. Affinity chromatography using the curaric neurotoxins of *Elapid* snakes appears to be the most promising method for rapidly obtaining nicotinic receptors that retain a binding capability for cholinergic ligands [4–8] and their immunological identity [29]. Furthermore binding studies to particulate matter of brain using $^{131}$I-labeled α-bungarotoxin show that there is only one toxin-binding site and that this binding is antagonized by tubocurarine chloride [22]. Thus the extraction procedures used in these studies should be specific for the (pharmacologically defined) nicotinic acetylcholine receptor.

The d-toxin of *Naja melanoleuca* was used as the affinity chromatography ligand in early experiments [1]. In later studies, the venom of *Naja naja* was fractionated into its protein components on CM-cellulose and Sephadex G-50. The fractions that did not elute in the G-50 void volume were tested for their potency at inducing flaccid paralysis in mice followed by respiratory failure (e.g. a curare-like effect) and those fractions with such effects were combined and saved. It should be noted that the d-toxin of *N. melanoleuca* and several of the *N. naja* toxins have had their amino acid sequences determined [30–32] and it is clear that they are homologous to α-bungarotoxin [33]. The combined *N. naja* toxins (100 mg) were attached to an *N*-hydroxysuccinimide derivative of agarose [34] (Affi-Gel; Bio-Rad Laboratories) using the manufacturer's recommended procedures. 100 mg of toxin were dissolved in 25 ml of 0.2 M sodium phosphate buffer, pH 7.4, and then a placed on shaker and allowed to react for 4 h after which the swollen gel was poured into a 2.0 cm × 5.0 cm column. The gel was washed with 500 ml of 6.0 M guanidinium chloride to remove non-covalently bound protein. (These snake toxins, due to their possession of four or five disulfide bridges and their small size, are difficult to denature without first reducing the disulfides, even after boiling in water [35].) After a further wash with 500 ml of 1.0 M NaCl, the column was allowed to equilibrate in a 0.05 M phosphate buffer, pH 7.4, containing 0.1% Triton X-100. (As this is the standard buffer used throughout the extraction of receptors, we subsequently refer to it as the phosphate–detergent buffer).

To extract receptors from mouse brain, the following technique was used: 25 whole brains were obtained from freshly decapitated 90-day-old mice and immediately placed in phosphate–detergent buffer in an ice bath. Fresh buffer was then added to obtain a ratio of brain tissue to buffer of 10 (w/v) and the tissue was thoroughly homogenized. The suspension was centrifuged at 10 000 × *g* for 1 h to remove particulate matter and the supernatant was poured into a 200-ml syringe barrel connected at the bottom to the affinity chromatography column containing the toxin–agarose gel. Hog brain was homogenized and centrifuged by essentially the same methods except that a Waring blender was first used to chop up the brain tissue. Hog brain was obtained from a local slaughter house. The supernatant from the centrifugation was allowed to flow through the gel at a rate of about 10 ml/min. Following this, the column was washed with 100 ml of 1.0 M NaCl to remove non-specifically bound material. Elution was then performed with either carbachol or hexamethonium, both of which give identical and reproducible results in terms of bilayer activity. Both a gradient or a 0.2 M step elution proved successful.

*Manufacture of bilayers*

To study the acetylcholine receptor in lipid bilayers with resolution sufficient to identify single molecular events, modifications of previously published methods were used [25–27]. The principle concern is to obtain background conductances across the bilayer membrane sufficiently low to allow accurate resolution of conductance changes below $10^{-10}\ \Omega^{-1}$ and to remove contaminating impurities that might result in artefactual conductance changes. Previous experience has shown that soybean lecithin (Sigma) is an excellent lipid for achieving these standards provided the following procedures for bilayer preparation are used. All glassware is baked at 400 °C for sterilization. A Kel-F fluorochlorocarbon chamber holding 4 ml of solution is used to make the bilayer and is boiled in glass distilled water prior to use. The lipid is dissolved in decane at 30 mg/ml and prior to each experiment this solution is passed, under pressure, through a 0.5 cm × 10 cm column containing neutral alumina (Bio-Rad AG. 7). After setting up the bathing solution the lipid is spread across a 1.4-mm diameter hole (1.5 $mm^2$ area) in the Kel-F chamber using a flattened polyethylene tube connected to a 100-$\mu$l syringe mounted on a microminipulator (the spreader). Current measurements were performed using calomel electrodes (Radiometer) that were in electrical contact with the bilayer baths through agar-filled Teflon tubes. The thinning of the membrane was monitored electrically on an X–Y plotter by measuring the current developed by a triangle wave voltage-clamp from a low impedence source by observing the steadily increasing current due to the rising capacitance as the lipid thins into a bilayer. The final capacitance after thinning was approximately $0.42 \pm 0.02\ \mu f \cdot cm^{-2}$ and the average background conductance was around $2 \cdot 10^{-9}$–$3 \cdot 10^{-9}\ \Omega^{-1} \cdot cm^{-2}$. The current amplification was performed by a Keithley 427 amplifier using current feedback amplification techniques to ensure electronic shot-noise levels well below $10^{-11}\ \Omega^{-1}$. After thinning, the steady-state conductance was recorded on a fast-response strip chart recorder with a clamp maintained at + or

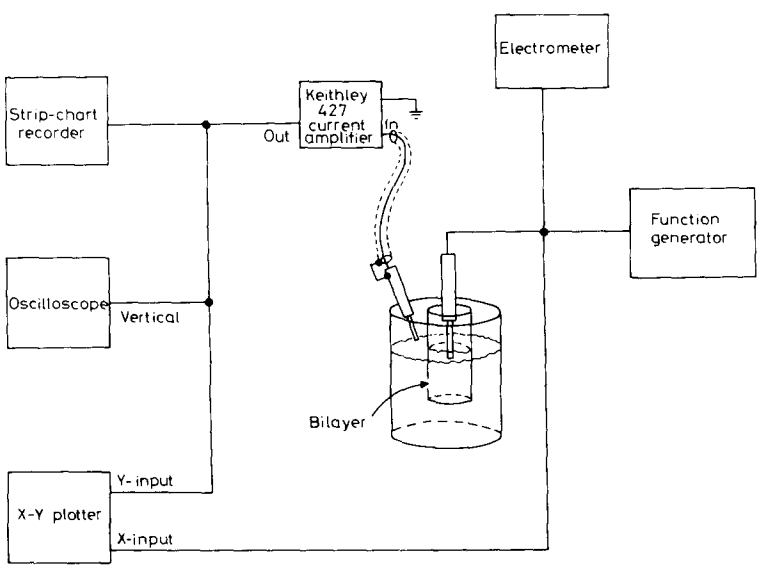


Fig. 1. Schematic of the arrangement for monitoring the electrical properties of lipid bilayers.

−80 mV. Fig. 1 shows a rough schematic outline of the experimental arrangement.

To allow separate estimates of cationic and anionic conductances, a salt gradient was used across the bilayer. The inside of the chamber contained 4 ml of 0.1 M NaCl (the *cis* side) and the outside (*trans*) contained 1.0 M NaCl. The elutant from the affinity column (e.g. presumed samples of acetylcholine receptors) were injected 10 $\mu$l at a time on the *cis* side, thus diluting the sample 400:1. Depending on the polarity of the clamp and given the above solution compositions, the separate $Na^+$ or $Cl^-$ conductances could be estimated by reversing the transmembrane potential. All experiments were performed at 25±1 °C.

## RESULTS AND DISCUSSION

As mentioned earlier, both hexamethonium and carbachol were found to be effective in desorbing the receptor from the column, as judged from bilayer activity. Absorbance was detected by relative ultraviolet monitoring at 280 nm followed by protein determination but the amount of protein it could represent was clearly under 25 $\mu$g/ml and may be due to changing concentration of eluting ligand. Considering that hog brains weight from 100 to 150 g, it appears that these extraction techniques do not yield the quantities of protein expected from Bosmann's data [23]. In view of the specificity of the extraction procedure, it would seem that most receptor protein should be obtained, since the batch of Affi-gel that we used as reported by the manufacturer can be substituted to the extent of approx. 6 $\mu$M/ml of swollen gel. It thus seems likely that the estimates of about $10^{-12}$ moles/gm of tissue may represent a more consistent estimate [21, 22]. The eluant was stored at 4 °C in phosphate-detergent buffer for further use.

When the receptor extracts from mouse brain were placed in the bilayer bath, conductance fluctuations were usually observed within 30 s after 10 $\mu$l of the eluant was added to the *cis* side. These conductance changes were of the basic size described earlier [1], although multiples of this size were often observed. Fig. 2 shows a record in which mouse brain receptor was added to the bathing medium of the bilayer bath. When 10 $\mu$l of the hog brain receptor was added to the *cis* side, conductance fluctuations were observed with values of $3.8 \cdot 10^{-11}\ \Omega^{-1}$ (1.0 M $Na^+$) and $4.7 \cdot 10^{-11}\ \Omega^{-1}$ (1.0 M $Cl^-$).

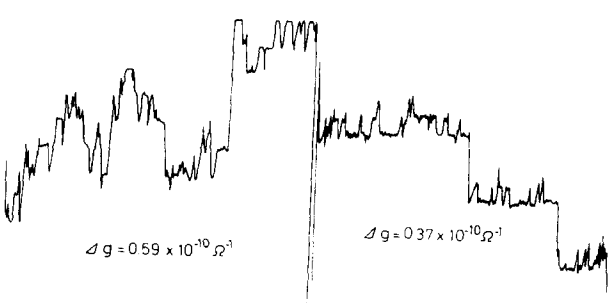


Fig. 2. Response of lipid bilayer electrical properties to the addition of mouse brain acetylcholine receptor extract to the *cis* side of the bilayer. The smallest conductance size observed appeared to be $3.7 \cdot 10^{-11}\Omega^{-1}$ to $Na^+$ and $5.9 \cdot 10^{-11}\ \Omega^{-1}$ to $Cl^-$ ions, both at 1 M concentrations.

In most studies there was approximately $5 \cdot 10^{-4}$ M carbachol or hexamethonium present in the bilayer bath that came from the desorbing gradient. Under these circumstances, quantal changes of conductance tended to occur as large multiples of the basic size. This was also seen with receptor from either hog or mouse brain and preliminary studies also indicate that similar effects may be present with electroplax receptors (Romine, Goodall, Bradley and Reich, unpublished data). These aggregates of a basic conductance change appear to be suppressed by tubocurarine chloride or atropine at competitive concentrations as was the general membrane conductance [1]. Effects became prominent when $2.5 \cdot 10^{-3}$ M of agent was used. It was noted that these conductances could occur in either the direction of the receptor turning on or in the direction of the receptor turning off (e.g. increasing or decreasing conductance). This is illustrated in Figs 4B and 5 and it would seem that there are definitely cooperative interactions involved.

It is also of interest that in addition to the large resolved conductance changes, there appeared an increase in the "noise" level due apparently to fluctuations of fast events beyond the temporal resolved conductance changes. This is especially apparent in records shown in Figs 3 and 5 where high frequency response is maximized. Although it is not clear what the nature of this "noise" is, electrophysiological experiments [17–20] with muscle endplates show that the relaxation time of the channel gate following application of acetylcholine is in the order of milliseconds. From the

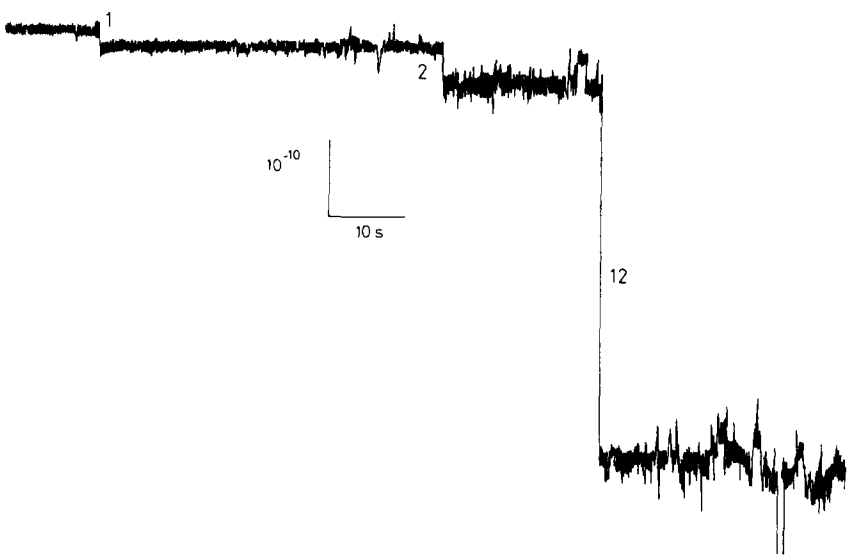


Fig. 3. Bilayer response after addition of receptor extract from hog brain tissue following elution with hexamethonium. In this particular record, note how there is an increase in "background noise" as more quantal jumps of conductance occur. The record shows a smallest size conductance increase to $Na^+$ of $3.8 \cdot 10^{-11}\ \Omega^{-1}$ (1 M) and the larger jumps are integral multiples of this smallest size. In view of the evidence of Anderson and Stevens [20] that the acetylcholine receptor open-channel lifetime is on the order of milliseconds, it would seem that these basic quanta reflect an average of the open- and closed-channel conductance times rather than the true single-channel conductance, whereas the "background noise" represents the statistical fluctuations described by the above investigators and Katz and Miledi [19].

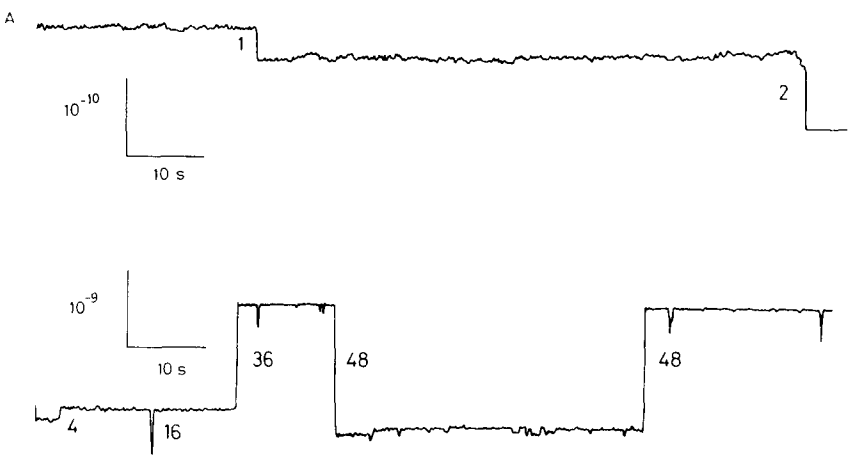


Fig. 4. This record is similar to that of Fig. 3 except that the receptor extract has been first dialyzed against buffer–detergent for 72 h in order to remove most of the hexamethonium. Note that the activity appears to be much less. Huge quanta which appear to be multiples of the basic quanta described earlier appear spontaneously. As the electrical damping is much greater in this record than in the previous record, the "background noise" is not seen.

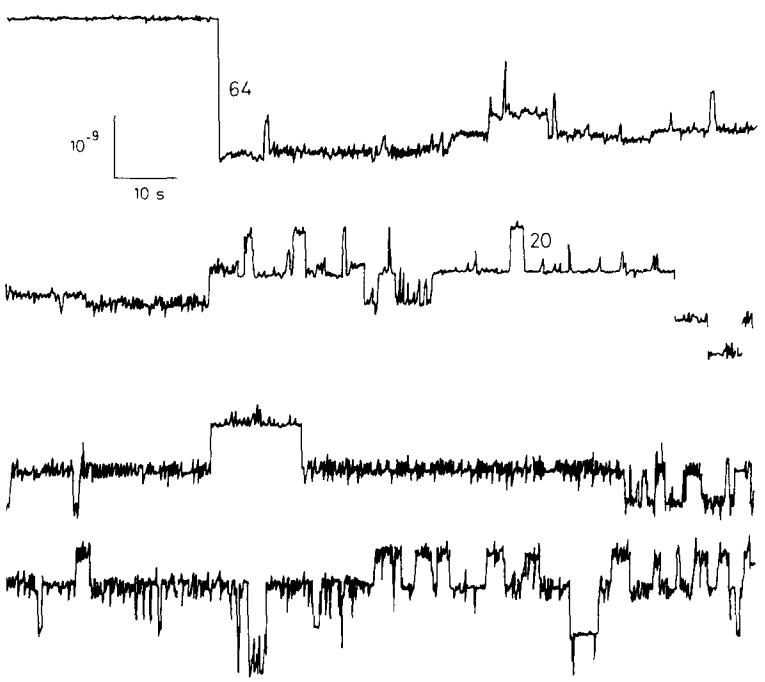


Fig. 5. This record is similar to Fig. 3 but shows a contiguous 8-min uninterrupted recording of the effect at a reduced scale. Of particular interest is that in this record and in the record shown in Fig. 4, the aggregates mostly occur as multiples of 4 times the basic quanta. This might be related to the various speculations that the receptor exists as a tetramer.

physical model suggested by Katz and Miledi [19] and Magleby and Stevens [36, 37] in which the rate-limiting step is the channel gating process and the binding of acetylcholine to the receptor is a very rapid process, one might expect our recorded "noise" to represent rapid gating between the open and closed channel states.

Shortly after the addition of the receptor to the bilayer bath, an equilibrium conductance was reached (Fig. 5). Note that after this equilibrium was obtained, large multiple conductance changes still occurred. It is probable that this is not due to receptors leaving the membrane once entering for several reasons. First, we have found that detergent removal rapidly denatures the receptor as judged by bilayer activity. Since the 400:1 dilution of receptor eluant would reduce the detergent concentration to 0.0003 %, it is likely that the receptor that does not enter the bilayer within the first few seconds is denatured, explaining why equilibrium is rapidly reached. Second, if one considers that the ionophore should be highly lipophilic then it would seem thermodynamically unfavorable for the receptor to leave the bilayer once inside. Third, there is no evidence that receptors spontaneously leave the membrane in nerve and muscle and, although the lipid composition that we use is not identical with the above tissue membranes, it is unlikely that what we observe is the disassociation of the receptor from the bilayer.

It is possible that the slow conductance-change quanta might represent a process parallel to the desensitization process at the synapse. It may be noteworthy that in Fig. 4, where the ligand used to elute the receptor was first removed by dialysis for 72 h, the incidence of these quanta is greatly reduced.

Other possible reasons for these aggregates must also be considered. Although the aggregation of the basic quanta would indicate that there are receptors in close proximity to each other, they may be due to other factors. First, it may be that there is a constant exchange of lipid between the bilayer itself and the lipid on the edges of the hole over which the bilayer is spread. If the receptor were carried with it, then this might explain the sudden quantum jumps in conductance. The main argument against this conclusion is that there is a constancy of membrane conductance once equilibrium is reached, despite the expectation that the receptor rapidly denatures once the detergent is removed. Another possibility is that the extraction procedure or the artificial nature of the system (e.g. the lipid and solution composition) may cause the receptor to have different properties than those found in the nerve membrane. Clearly, more studies of this aggregation phenomenon are needed and it might be of particular interest to see if the phenomenon exists with other receptors.

The use of a 1.0 M NaCl solution instead of Ringer to determine the ionic conductance changes is based on the problems due to non-sterility of the bathing medium. Experience has shown that minute amounts of bacteria, organic impurities and dust particles will cause major electrical artifacts in the membrane. Furthermore, the use of a 10:1 gradient across the membrane allows a straight-forward estimate of separate ionic conductances. It is important to note that Triton X-100 alone, from previous experience in this lab, does not cause electrical artifacts at the concentrations used.

There are a number of experiments that need to be done to clarify and verify the data presented in this paper. First, are the aggregations that we observe representative of events that occur at the synapse, that is, do receptors activate and deactivate cooperatively, possibly as part of a phenomenon such as desensitization? This question

might be investigated by examining "shot-noise" recordings at muscle endplates for conductance changes well above the basic quanta. In fact, considering the size and lifetime of some of our observed quanta, under equilibrium conditions of acetylcholine application at concentrations high enough to induce significant desensitization, such conductance changes might be observable without the necessity of resorting to statistical means.

Another question arises as to whether we are in fact observing the pharmacologically defined nicotinic receptor. Our assumption is based on the known specificity of the extraction procedure for nicotinic receptors coupled to the use of a competitive cholinergic ligand as the desorbing agent. It is noteworthy that we have yet to fail to find bilayer activity with the extraction procedures used in this paper, provided that the receptor was used within 2 weeks following isolation. Furthermore, the quanta that we observe with electroplax receptors ($2.7 \cdot 10^{-11}\ \Omega^{-1}$, Romine, Goodall, Bradley and Reich, unpublished data) are similar in size to that observed for the brain receptors. Further data supporting its identity as a nicotinic receptor might come from pharmacological studies in the bilayer.

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