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Phospholipase A_2 hydrolysis of membrane phospholipids causes structural alteration of the nicotinic acetylcholine receptor

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Thermal perturbation techniques have been used to probe structural alteration of the nicotinic acetylcholine receptor as a function of perturbations of its native membrane environment. Differential scanning calorimetry and a technique involving heat inactivation of the α-bungarotoxin-binding sites on the receptor protein reveal that there is a profound destabilization of the acetylcholine receptor structure when receptor-containing membranes are exposed to phospholipase A_2. The characteristic calorimetric transition assigned to irreversible denaturation of the receptor protein and the heat inactivation profile of α-bungarotoxin-binding sites are shifted to lower temperatures by approx. 7 and 5 C°, respectively, upon exposure to phospholipase A_2 at a phospholipase/neurotoxin binding site molar ratio of about 1:100. The effects of phospholipase A_2 on receptor structure can be (i) reversed by using bovine serum albumin as a scavenger of phospholipase hydrolysis products of membrane phospholipids, and (ii) stimulated by incorporation into the membranes of free, polyunsaturated fatty acids. In particular, linolenic acid (18:3($n-3$)) causes detectable destabilization of the α-bungarotoxin binding sites on the receptor at free fatty acid/receptor molar ratios as low as 10:1. Furthermore, alteration of receptor structure by added phospholipase occurs very rapidly, which is consistent with the observation of rapid in situ phospholipase A_2 hydrolysis of membrane phospholipids, particularly highly unsaturated phosphatidylethanolamine and phosphatidylserine. Based on previously published data on the inhibition of acetylcholine receptor cation-gating activity caused by the presence of either phospholipase A_2 or free fatty acids (Andreasen T.J. and McNamee M.G. (1980) Biochemistry 19, 4719), we interpret our data as indicative of a correlation between structural and functional alterations of the membrane-bound acetylcholine receptor induced by phospholipase A_2 hydrolysis products.

Introduction

The abundance of the nicotinic acetylcholine receptor in the electroplax of several species of electric fish and the availability of specific neurotoxins and other molecular probes have allowed significant progress in the understanding of this cation-channel-linked membrane receptor (for reviews, see Refs. 1–4). The acetylcholine receptor from *Torpedo* is a pentameric glycoprotein ($M_r \approx 270\,000$) [5], composed of four different types of polypeptide chain (α, β, γ and δ) in a 2:1:1:1

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stoichiometry [6,7]. Such a complex spans the postsynaptic membrane and therefore contains extracellular (synaptic), transmembrane and cytoplasmic domains (for review, see Ref. 8). Binding of cholinergic agonists to synaptic domains on the α-subunits of the receptor protein elicits the formation of a transient cation channel by which ion translocation through the membrane occurs, thus initiating postsynaptic membrane depolarization.

Using detergent solubilization followed by reconstitution of the acetylcholine receptor into well-defined liposomes [9,10] we can characterize the dependence of various functional aspects of the receptor upon the chemical composition and physical properties of the lipid matrix. Such a dependence is believed to affect (i) receptor ion-gating activity upon agonist binding, (ii) receptor 'desensitization', by which ion permeability becomes blocked by prolonged exposure to the activing agonist and, (iii) the binding parameters corresponding to the interaction with cholinergic agonists. Some of the above functional manifestations of the acetylcholine receptor presumably involve conformational changes within the receptor protein. Therefore, the acetylcholine receptor appears to be a suitable model system not only for the study of lipid–protein interactions, but also to explore possible structure/function relationships in an integral membrane protein of physiological significance.

In this communication, we use attack by phospholipase A_2, a specific membrane perturbation known to produce inhibition of acetylcholine receptor-mediated cation flux [11–13], to monitor the occurrence of structural changes on the receptor protein. For this, we have applied the techniques of differential scanning calorimetry and heat inactivation of α-bungarotoxin-binding sites, both of which provide specific monitoring of structural alterations of the membrane-bound acetylcholine receptor [14,15].

Materials and Methods

Free arachidonic and linolenic fatty acids were purchased from Supelco. Fatty-acid-free bovine serum albumin was from Sigma. Phospholipase-free α-bungarotoxin was purified from *Bungarus multicinctus* venom (Miami Serpentarium) as previously described [16]. ^{125}I-labeled α-bungarotoxin was either purchased from New England Nuclear or prepared by radioiodination (Enzymobead Reagent, Bio-Rad) of our purified toxin.

Phospholipase A_2 purified from *Naja nigricollis* venom [17], was a generous gift from Dr. Herbert J. Evans at the Medical College of Virginia. This purified material was homogeneous by polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate, had an apparent molecular weight of about 15 000 and a neutral isoelectric point, and was devoid of any α-neurotoxin activity. Moreover, long-term exposure of receptor-containing membranes to the purified phospholipase did not result in degradation of the receptor protein, as indicated by full preservation of the characteristic polypeptide pattern of the acetylcholine receptor upon polyacrylamide gel electrophoresis in presence of SDS. The specific activity of the purified phospholipase was approx. 14 I.U. (μmol phospholipid cleaved/min) per mg protein, as determined by an assay using autoclaved radiolabeled *Escherichia coli* as a substrate [18]. All the experiments described in this paper regarding treatment of receptor membranes with the purified phospholipase were carried out at a constant phospholipase/α-bungarotoxin-binding sites molar ratio of about 1 : 100. According to previously published data on the lipid/protein and phospholipid/cholesterol ratios in our acetylcholine receptor-containing membranes [19], the above molar ratio is equivalent to a phospholipase/phospholipid molar ratio of about 1 : 30 000.

Live *Torpedo marmorata* were obtained through local fishermen during the early spring months. The electric tissue was rapidly excised and maintained frozen in liquid nitrogen until prior to use. Electroplax membranes highly enriched in acetylcholine receptor were prepared by a procedure which involved alkaline extraction of peripheral membrane proteins [20] as previously described [19]. The resulting membranes exhibited typical specific activity values of 30–35 μg of α-bungarotoxin bound/mg membrane protein, and were finally suspended in a 10 mM Hepes buffer (pH 7.4)/100 mM $NaNO_3$. Also, since phospholipase A_2 requires Ca^{2+} for optimal activity, all experiments described in this paper were

carried out in the presence of 5 mM $CaCl_2$. The previously reported inhibition of the characteristic agonist-induced, cation-gating activity of receptor membranes, caused by either treatment with phospholipase or incorporation of free fatty acids [11–13], was confirmed by using a 'fluorescence quenching/stopped flow' technique of thallium influx [16,19,24].

'Heat inactivation' refers to monitoring the characteristic loss of α-bungarotoxin binding by acetylcholine receptor-containing membranes as heat is supplied to the samples. Series of 50 μl aliquots of membranes (about 1–2 mg of protein/ml) were heated in an immersion water bath at a linear rate of about 1 C°/min. Vigorous stirring ensured consistency of the temperature within the water bath. Temperature was measured with a partially immersed thermometer (0.1 C° accuracy), positioned with a geometry similar to that of the samples. Aliquots were removed at selected temperatures, diluted with an equal volume of ice-cold buffer containing 2% Triton X-100, placed on ice for a minimum of 1 h, then submitted to ^{125}I-labeled α-bungarotoxin-binding determination [21]. Alternatively, membrane samples were heated at a constant temperature for selected periods of time (usually 1–40 min), then submitted to α-bungarotoxin-binding analysis.

Differential scanning calorimetry of acetylcholine receptor membranes was performed on a Microcal MC1 or MC2 microcalorimeter. Differences in the heat capacities between 1-ml aliquots of membranes at 6–10 mg of protein/ml (contained in the 'sample' cell of the calorimeter) and buffer alone ('reference' cell) were obtained by raising the temperature at a linear rate of about 1 C°/min [14]. Reported transition temperatures (T_D) correspond with those at which there is a maximum differential heat capacity as observed in the thermograms.

The procedures used for the extraction, fractionation and characterization of membrane lipids were as previously described [22,23], except that a Hewlett-Packard 5880 A gas chromatograph was used for quantitative analysis of methyl ester derivatives of fatty acids.

Results

Differential scanning calorimetry studies of acetylcholine receptor-containing membranes, conducted in the presence of phospholipase A_2, reveal that exposure to the phospholipase causes a profound alteration in the calorimetric profile characteristic of control, untreated membranes (Fig. 1). The main 59–60 °C thermal transition observed in the thermograms, which has been assigned to irreversible denaturation of the receptor protein [14], is shifted to lower temperatures by about 7 C°. Also, the observed endotherm is wider and lower than that corresponding to untreated receptor membranes at identical protein concentration. A second thermal transition ($T_D \approx$ 78 °C), characteristic of calorimetric scans of untreated receptor membranes, is also shifted to lower temperatures as a consequence of phospholipase treatment. Simultaneous destabilization of both major endotherms is also observed when the membranes are exposed to nonspecific membrane perturbants such as detergents at low concentrations [15]. In contrast, the presence of cholinergic agonists affects only the 59–60 °C thermal transition [14].

Studies of heat inactivation of α-bungarotoxin binding sites conducted in the presence of phospholipase are illustrated in Fig. 2. Heat inactivation of α-bungarotoxin-binding sites is believed to be one of the thermal events accounting for the main 59–60 °C thermal transition observed in differential scanning calorimetry of receptor membranes [15,24]. Exposure of the membranes to phospholipase at a phospholipase/receptor ratio identical to that used for the calorimetric studies causes thermal destabilization of the α-bungarotoxin-binding sites. This is indicated by a T_{50} value (temperature at which there remains a 50% of the initial capacity to bind α-bungarotoxin) of about 55 °C, i.e., approx. 5 C° lower than the characteristic T_{50} value of 60 °C observed for control membranes, in the absence of added phospholipase. It is also observed that the temperature range during which heat inactivation of toxin sites occurs is wider than that exhibited by control, untreated receptor membranes. Results similar to those described above are obtained when the membranes are preincubated with phospholipase

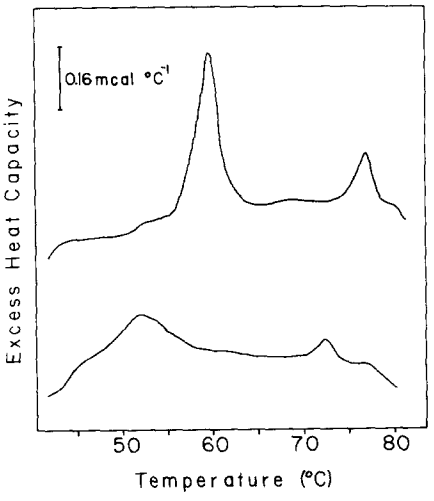

Fig. 1. Representative differential scanning calorimetry thermograms of acetylcholine receptor membranes (about 8 mg of protein/ml) in 10 mM Hepes buffer (pH 7.4)/100 mM $NaNO_3$/5 mM $CaCl_2$. Prior to the calorimetric measurement, the membranes were incubated overnight at 4° C, with (lower trace) or without (upper trace, control) phospholipase A_2.

at either 4° C or room temperature for periods of time ranging from zero to 15 h prior to the heat-inactivation experiment, suggesting therefore, that the effects of phospholipase on the membranes are exerted rapidly compared to the time-scale of the assay (since the starting temperature for these assays is usually 25° C, there is an approx. 30 min period during which membrane samples are exposed to the presence of the added phospholipase, prior to reaching temperatures at which heat inactivation can be observed). In an attempt to estimate the rate at which heat inactivation of α-bungarotoxin-binding sites occurs, experiments were conducted in which receptor membranes were preincubated with a fixed amount of phospholipase for defined periods of time and then kept at a constant temperature at which heat inactivation readily occurs (Fig. 3). Under these conditions, an increase in thermal lability of the α-bungarotoxin binding sites as a consequence of preincubation

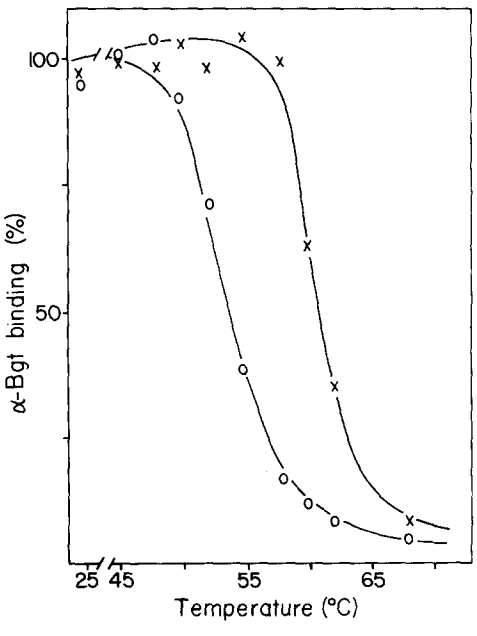

Fig. 2. Heat inactivation of α-bungarotoxin-binding sites in acetylcholine receptor membranes (about 1 mg protein/ml) preincubated overnight, at 4° C, in the presence (○) or in the absence (×) of phospholipase A_2. Data is given as the percentage of α-bungarotoxin binding with respect to unheated acetylcholine receptor membranes, in the absence of added phospholipase A_2.

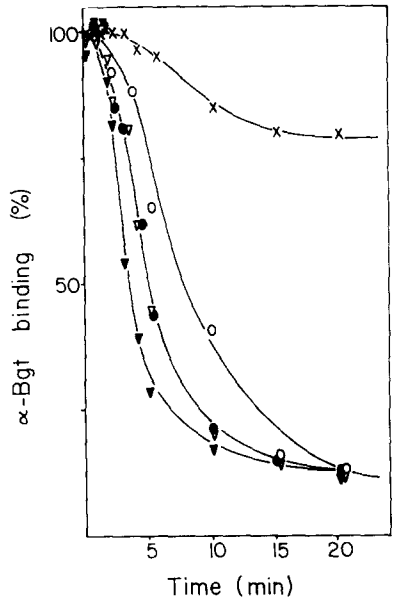

Fig. 3. Effects of heating at a constant temperature on the rate of heat inactivation of α-bungarotoxin-binding sites. Acetylcholine receptor membranes (about 1 mg protein/ml) were preincubated at room temperature with or without (×, control) phospholipase A_2, for 2 (○), 5 (▽), 10 (●) and 20 (▼) min, then submitted to heat inactivation at 57° C. Membrane aliquots were withdrawn from the 57° C water-bath at the indicated times and their capacity to bind α-bungarotoxin was determined. Data are presented as in Fig. 2.

with the phospholipase is translated into a faster, time-dependent, decay of the α-bungarotoxin binding capacity. Choosing a constant temperature of 57 °C for these experiments is convenient, since it is between the observed T_{50} values for untreated ($T_{50} \approx 60$ °C) and phospholipase-treated ($T_{50} \approx 55$ °C) membranes (Fig. 2). At 57 °C, loss of α-bungarotoxin-binding sites by untreated receptor membranes is a fairly slow process ($t_{1/2} \approx$ 40 min, assuming a single exponential decay) and therefore any increase in the rate of heat inactivation induced by phospholipase can easily be monitored. As shown in Fig. 3, preincubation of receptor membranes with the phospholipase for as little as 2 min already produces a large increase in the rate of heat inactivation of toxin-binding sites.

Thermal destabilization of α-bungarotoxin-binding sites induced by treatment with phospholipase can be reversed by repeated washings of the phospholipase-treated receptor membranes with solutions containing fatty-acid-depleted bovine serum albumin (Fig. 4). Albumin binds fatty acids and can be used to shuttle these compounds in and out of membranes. The presence of albumin does not affect the hydrolytic activity of the phospholipase. Likewise, repeated washes of phospholipase-treated membranes using aqueous buffers without albumin are not able to produce any reversal in the observed phospholipase effects. Treatment of control receptor membranes with albumin under conditions identical to those used for phospholipase-treated samples does not alter the characteristic T_{50} value for heat inactivation of α-bungarotoxn-binding sites. Nevertheless, an apparent decrease in the initial toxin-binding capacity of albumin-treated membranes to about 80–90% of that exhibited by untreated membranes is observed (Fig. 4B). This is probably due to an overestimation of the membrane protein concentration caused by presence of albumin remaining from the treatment (see legend to Fig. 4). Albumin scavenging of free fatty acids from phospholipase-treated receptor membranes was reported to be equally effective in reversing the inhibition of acetylcholine receptor cation-gating activity induced by phospholipase treatment [13].

Incorporation of unsaturated free fatty acids into acetylcholine receptor membranes produces effects on the thermal stability of α-bungarotoxin-binding sites which resemble those observed

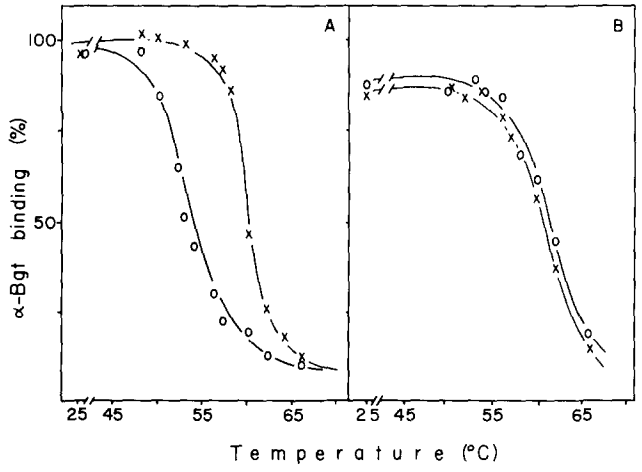

Fig. 4. Effects of bovine serum albumin on reversing the alterations induced by phospholipase A_2 on the profile of heat inactivation of α-bungarotoxin-binding sites. Acetylcholine receptor membranes were first preincubated for about 2 h, at room temperature, in the presence (O) or in the absence (×) of added phospholipase A_2, and then incubated overnight, at 4 °C, with buffer containing (B) or lacking (A) 10 mg/ml of added albumin as scavenger. Membrane samples were then pelleted by centrifugation at 43000 × *g* for 30 min and resuspended in the same buffers. The previous step was repeated once again, except that incubation was at room temperature for about 2 h. Finally, all membrane samples were washed three times in buffer without albumin, resuspended at a membrane protein concentration of about 1 mg/ml and submitted to the heat inactivation procedure as in Fig. 2. Data are presented as the percentage of α-bungarotoxin binding with respect to control membranes which were not submitted to the washing procedure described above.

upon phospholipase treatment (Fig. 5). Furthermore, incorporation of unsaturated free fatty acids into the membranes results in differential scanning calorimetry thermograms indistinguishable from those obtained in the presence of phospholipase.

Thermal destabilization induced by incorporation of free fatty acids into the membranes is a concentration-dependent phenomenon, which can be detected at concentrations of added fatty acid as low as 50–100 μM. We have used two different fatty acids as representatives of the $n-3$ and the $n-6$ polyunsaturated series to which most of the polyunsaturated fatty acids present in *Torpedo* membranes belong [22]. Our observation is that either fatty acid perturbs the thermal stability of the acetylcholine receptor protein. However, the $n-3$ linolenic acid perturbs the heat inactivation profile of α-bungarotoxin-binding sites more efficiently than the $n-6$ arachidonic acid. Minimal free fatty acid/receptor molar ratios at which there is a detectable perturbation of the α-bungarotoxin binding sites can be as low as about 10 : 1 for linolenic and about 40–50 : 1 for arachidonic acid. It seems unlikely that these fatty acid/receptor ratios would be high enough to account for the formation of a complete lipid annulus around the receptor protein, and therefore there is a suggestion that the transmembrane portion of the acetylcholine receptor could have specific sites which are preferentially perturbed by the presence of free fatty acids. A similar suggestion was reported based on electron spin resonance studies [28].

The effects of phospholipase A_2 in in situ hydrolysis of the phospholipids present in the membranes were also monitored (Fig. 6). Acetylcholine receptor membranes from *Torpedo* contain phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine as the major phospholipid components [22]. Treatment of receptor membranes with phospholipase under conditions identical to those used in the heat inactivation studies results in hydrolysis of all three major phospholipids. Nonetheless, the extent and the rate of hydrolysis, both of which should be limited by the accessibility of the different membrane-bound phospholipids to the added phospholipase, are different for each phospholipid type. In agreement with previous reports [13], we found that phosphatidylethanolamine is more extensively and more rapidly hydrolyzed than either phosphatidylcholine or phosphatidylserine. Half-times estimated for the 'in situ' hydrolysis of these phospholipids are approx. 7, 19 and 14 min, respectively, for the ethanolamine, serine and choline

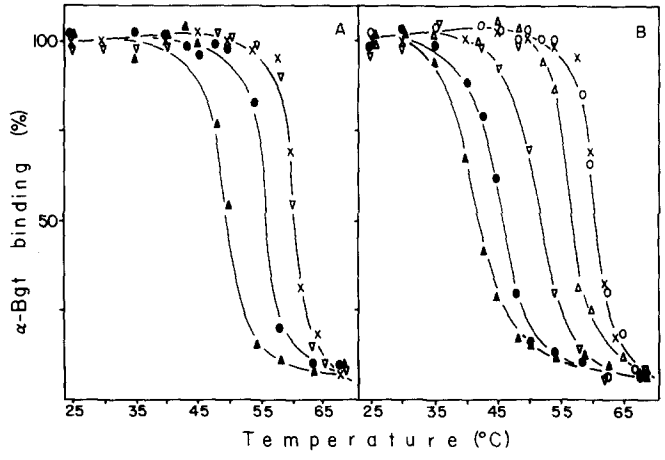

Fig. 5. Effect of added arachidonic (A) and linolenic (B) fatty acids on the profile of heat inactivation of α-bungarotoxin-binding sites. Prior to the heat inactivation assay, acetylcholine receptor membranes at about 2 mg protein/ml were preincubated for about 30 min, at room temperature, in the presence of added aliquots from a sonicated suspension of the free fatty acids in aqueous buffer. Final concentrations of free fatty acids in the incubation mixture were zero (Control, ×), 0.05 (○), 0.1 (△), 0.2 (▽), 0.5 (●) and 1 (▲) mM.

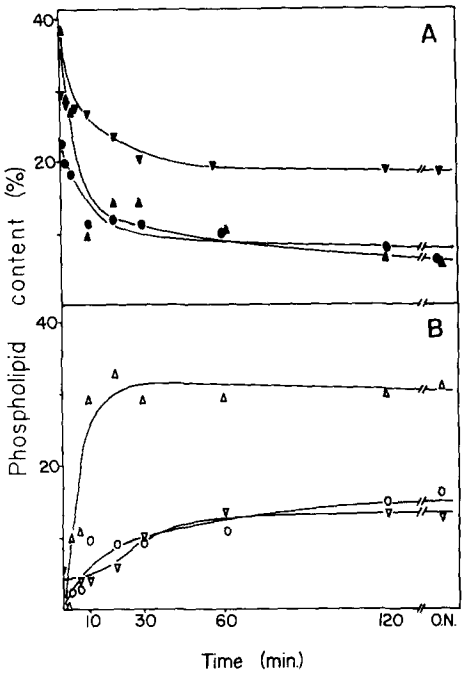

Fig. 6. Time-course for 'in situ' hydrolysis of membrane phospholipids by phospholipase A_2. Acetylcholine receptor membranes were incubated at room temperature with phospholipase A_2. At the indicated times, the reaction was stopped by addition of 2 vol. of chloroform/methanol (2:1, v/v), thus initiating the extraction of membrane lipids (see Ref. 22). The lipid extracts from each time point were fractionated by two-dimensional high performance thin layer chromatography and the different lipid classes were quantitated by gas-liquid chromatography of the methyl ester derivatives of their fatty acids, by using an internal standard. Data are presented as the percentage of phosphatidylcholine (▼), phosphatidylethanolamine (▲), phosphatidylserine (●), lyso-phosphatidylcholine (▽), lyso-phosphatidylethanolamine (△) and lyso-phosphatidylserine (○), with respect to total phospholipid contents.

phospholipids. It should be noted that hydrolysis of the *sn*-2 glycerol position of the highly unsaturated phosphatidylethanolamine and/or phosphatidylserine from *Torpedo* results in the release of a free fatty acid population largely composed of polyunsaturated arachidonic (20:4($n-6$)) and docosahexaenoic (22:6($n-3$)) fatty acids [22]. Based on the observed half-times for the hydrolysis and on the concentration of the different phospholipids within the membrane [19,22], it can be estimated that periods of exposure to the phospholipase as short as 1–2 min are sufficient to produce free fatty acids at concentrations comparable to those used for the experiments described in Fig. 5.

Discussion

Exposure of acetylcholine receptor membranes to phospholipase A_2 from different sources results in the loss of the ability of the membrane-bound receptor to promote rapid cation fluxes upon binding of cholinergic agonists [11,25–27]. Moreover, a series of studies by McNamee's group [11–13] demonstrated that such an effect on acetylcholine receptor function is due to the presence of 'fluid' free fatty acids produced as a consequence of phospholipase hydrolysis of membrane phospholipids. Also, using a variety of spin-labeled fatty acids, it was proposed that the effects of such free fatty acids, at temperatures above their melting points, are related to disruption of specific lipid–protein interactions which directly or indirectly affect the cation channel of the acetylcholine receptor [12,28].

Our approach in this paper has been to use phospholipase treatment of native acetylcholine receptor membranes to determine whether the previously reported loss of receptor function can be correlated with the occurrence of receptor structural alterations. Monitoring of structural parameters of membrane proteins, however, is frequently prevented by the inherent qualities of biological membranes. For instance, the application of spectroscopic techniques, such as circular dichroism or Raman [29–31] is restricted by either excessive membrane scattering or lipid contributions, and is mostly limited to the study of membrane proteins in a detergent-solubilized form or in reconstituted vesicles containing single lipid components. Recently, a differential scanning calorimetry study of acetylcholine receptor membranes was reported [14] in which the native, membrane-bound receptor exhibited a characteristic thermal transition at 59–60 °C which was assigned to irreversible denaturation of the receptor protein and to a complete loss of receptor function, including binding of specific α-neurotoxins. The observed thermal transition was sensitive to presence of cholinergic ligands, thus supporting its assignment to specific structural alteration of the acetylcholine receptor.

Using differential scanning calorimetry as well as other techniques of thermal perturbation to probe receptor structure [15], we have shown that the patterns of heat inactivation of α-neurotoxin-binding sites on the receptor protein provide information similar to that gathered by calorimetry. The heat-inactivation procedure provides also specific monitoring of structural features of the acetylcholine receptor, since it is based on the preservation of the binding sites for highly specific neurotoxins, and is sensitive to the presence of cholinergic ligands and membrane perturbants.

Monitoring of phospholipase effects on receptor membranes by either procedure (Figs. 1 and 2) indicates a strong structural destabilization of the receptor protein as a consequence of phospholipase attack. The observed effects of phospholipase treatment resemble those caused by incorporation into the membrane of unsaturated free fatty acids (Fig. 5). It should be noted that the observed effects of either the phospholipase or the free fatty acids implies that perturbations at the transmembrane portion of the acetylcholine receptor protein induced by those agents can alter the neurotoxin-binding sites, which are located 40–50 Å above the membrane surface [32]. Similar long-range perturbations transmitted through the receptor protein were previously proposed from studies of the effects of cholinergic agonists on a labeled membrane preparation in which a spectroscopic probe was covalently attached to the receptor protein at the lipid/protein interface [33]. Additional evidence to support the role of free fatty acids as responsible for the observed receptor structural alteration induced by phospholipase is the reversal of such effects when albumin is used as a fatty acid scavenger (Fig. 4). Our conclusion is that the parallels observed between the above evidence and that reported by others on the functional perturbation of the acetylcholine receptor induced by phospholipase treatment under similar conditions [11–13] strongly supports the notion of a structure/function relationship for the phospholipase-treated receptor in its native membrane environment.

The effects of phospholipase treatment on the receptor structure must be exerted very rapidly, since short exposures of the membranes to the purified phospholipase are translated into a large decrease in the thermal stability of the α-bungarotoxin binding sites (Fig. 3). This is consistent with the observed rapid hydrolysis of membrane phospholipids (Fig. 6). Our interpretation is, therefore, that structural perturbation of the acetylcholine receptor induced by the phospholipase must be mediated by the release of free polyunsaturated fatty acids from rapidly hydrolizing membrane phospholipids.

Acknowledgements

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