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Effects of Heating on the Ion-Gating Function and Structural Domains of the Acetylcholine Receptor†

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ABSTRACT: The ion-gating ability and the protein electrophoretic band patterns of the acetylcholine receptor from *Torpedo californica* electroplax were examined after receptor-enriched membrane vesicles were progressively heated. The ion translocation function was lost over a temperature range of 40–55 °C. Previous results have shown that the stoichiometry of α-bungarotoxin binding is not affected by these temperatures, although bound toxin reversibly dissociates within this temperature range, and that toxin binding is irreversibly lost at somewhat higher temperatures [Soler, G., Farach, M. C., Farach, H. A., Jr., Mattingly, J. R., Jr., & Martinez-Carrion, M. (1983) *Arch. Biochem. Biophys. 225*, 872]. Thermal gel analysis [Lysko, K. A., Carlson, R., Taverna, R., Snow, J., & Brandts, J. F. (1981) *Biochemistry 20*, 5570], a sodium dodecyl sulfate–polyacrylamide gel electrophoretic procedure which detects thermally induced aggregation of the components of multimeric systems, was applied to heated acetylcholine receptor enriched membranes. This technique suggests two structural domains susceptible to thermal perturbation within the receptor molecule, one consisting of the M_r 50 000 and the two M_r 40 000 subunits and the other consisting of the M_r 60 000 and 65 000 subunits. Heat disrupts molecular events linking agonist binding with ion-channel opening in the acetylcholine receptor molecule.

The abundance of acetylcholine receptor (AcChR)[1] in the electric tissue of *Torpedo californica* has allowed significant progress in the understanding of the structure of this ion-gating, transmembrane protein. The elementary functional unit of the AcChR is a pentameric protein with an $\alpha_2\beta\gamma\delta$ stoichiometry (Reynolds & Karlin, 1978) with apparent subunit molecular weights of 40 000, 50 000, 60 000, and 65 000 and with an overall molecular weight of 270 000 (Martinez-Carrion et al., 1975). In vivo, these elementary units appear to exist as dimers linked by a disulfide bond between δ subunits

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[1] Abbreviations: AcChR, acetylcholine receptor; DSC, differential scanning calorimetry; α-Bgt, α-bungarotoxin; CARB, carbamylcholine; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DEAE, diethylaminoethyl; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; NEM, *N*-ethylmaleimide.

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(Chang & Bock, 1977; Hamilton et al., 1979). A variety of electron microscopic, chemical cross-linking, and X-ray scattering experiments (Kistler et al., 1982; Hucho, 1981; Karlin et al., 1983) indicate a cylindrical arrangement of five subunits about a central pit; however, there is continuing controversy over the relative disposition of the subunits. The bulk of the receptor protein extends from the extracellular side of the membrane while a much smaller portion extends from the intracellular face of the membrane (Devine-Strader et al., 1979; Wennogle & Changeux, 1980; Kistler et al., 1982; Froehner, 1981; Anderson & Blodel, 1981). These observations of gross structural features have been recently complemented by the elucidation of the entire primary sequence of all five subunits (Noda et al., 1983). Amino acid sequences of the individual subunits have a high degree of homology which possibly extends to higher orders of structure. Extensive predictions of secondary structure have ensued (Kosower, 1983; Guy, 1983; Noda et al., 1983), including identification of peptide segments spanning the membrane, lining the putative ion channel, and composing the extra- and intracellular domains of the receptor. Predictions have extended to speculative mechanisms for opening and closing the ion channel in response to ligand binding (Kosower, 1983; Guy, 1983).

The scope of recent predictions belies the poor understanding of how structural features correlate with functional events mediated by the AcChR. One approach to these questions is the application of thermal perturbations to probe the receptor structure in relation to its function. In previous differential scanning calorimetric (DSC) studies of AcChR-enriched membranes (Farach & Martinez-Carrion, 1983), two resolved endotherms were observed. The process described with a transition temperature of about 59 °C was ascribed to irreversible denaturation of the receptor protein. Further examination of the effects of heating on the functional and structural characteristics of the membrane-bound AcChR has shown this denaturation to be more complicated than originally thought and has thereby provided information on the interaction of AcChR subunits in the function of ion gating. In this study, we describe the effect of heating on the ion translocation function of the AcChR which we measure on a rapid time scale. We also report the results of thermal gel analysis, a procedure which examines the thermally induced aggregation of the AcChR subunits.

Materials and Methods

Excised electroplax tissue from *Torpedo californica* was purchased from Pacific Biomarine Supplies Co. (Venice, CA) and was stored at approximately −70 °C until use. ^{125}I-α-Bungarotoxin (25.8 mCi/mg) (^{125}I-α-Bgt) was from New England Nuclear. Peroxidase-conjugated anti-goat IgG was from Cappel Laboratories. Thallium nitrate (ultrapure) was from Alfa Products; sodium 1,3,6,8-pyrenetetrasulfonate was from Eastman Kodak. Reagents for SDS–polyacrylamide gel electrophoresis and immunoblotting were products of Bio-Rad. Other reagents were of the highest purity available from either Sigma, Aldrich, or Fisher.

AcChR-Enriched Membranes. AcChR-enriched membrane fragments were prepared from frozen tissue by modification of published procedures (Lindstrom et al., 1980; Elliot et al., 1980; Neubig et al., 1979). Frozen electroplax tissue (200 g) was sliced into 1-cm cubes, placed in homogenization buffer (300 mL of 10 mM sodium phosphate, 10 mM NaN_3, 15 mM EDTA, 5 mM EDTA, 5 mM iodoacetamide, and 5 mM PMSF, pH 7.6), and then homogenized with a Brinkmann Polytron for 2 × 90 s at power level 7. The homogenate was then centrifuged for 10 min at 3500 rpm in a Sorval GS3 rotor, and the resultant pellet was homogenized again with 100 mL of buffer, and this second extract was centrifuged as before. The pooled supernatants were centrifuged for 30 min at 30000 rpm in a Beckman type 35 rotor. This pellet was resuspended in water, adjusted to 0.6–1 mg of protein/mL, rapidly brought to pH 11 by addition of 1 N NaOH while gently stirring, and incubated for 1 h at 4 °C with continued stirring. These alkaline-extracted membrane fragments were pelleted by centrifugation for 30 min at 30000 rpm in the type 35 rotor. The pellet was resuspended in 10 mM sodium phosphate, 100 mM NaCl, and 0.02% NaN_3, pH 7.4, or 10 mM HEPES and 100 mM $NaNO_3$, pH 7.4, for stopped-flow experiments. Specific α-Bgt binding activities were determined by using the DEAE filter disk assay previously described (Schmidt & Raftery, 1973) and typically ranged from 3 to 4 nmol of α-Bgt bound per mg of protein. Protein concentrations were determined by the procedure of Lowry et al. (1951) with bovine serum albumin as a gravimetrically prepared standard.

More recent shipments of electroplax did not yield sufficiently enriched membrane fragments when subjected to this first procedure. Further modifications in our purification procedure yielded similar AcChR enrichments with the inferior tissue. The ionic strength of the homogenization buffer was increased by the inclusion of 0.4 M NaCl, and the crude tissue extract was centrifuged for 10 min at 5000 rpm in the Dupont-Sorval GS-3 rotor. The crude membrane pellet was suspended in 200 mL of 34% sucrose, 10 mM sodium phosphate, 0.2% NaN_3, 400 mM NaCl, and 1 mM EDTA, pH 7.6. Of this suspension, 40 mL was layered onto 20 mL of 50% sucrose in the same buffer and centrifuged for 2 h at 32000 rpm in a Beckman type 35 rotor. The membrane fragments collecting at the gradient interface were pooled, diluted 5-fold with 10 mM phosphate, 100 mM NaCl, and 0.02% NaN_3, pH 7.6, and centrifuged for 30 min at 30000 rpm in a type 35 rotor. The resulting pellet was extracted at pH 11 as before. The specific activities of these preparations were similar to those obtained by the simpler procedure.

Thermal Gel Analysis. Thermal gel analysis refers to the specific procedure of SDS–PAGE applied to the analysis of thermally perturbed proteins by Lysko et al. (1981). Aliquots of 200–400 μL of AcChR-enriched membranes were heated in a GCA precision water bath at a rate of 1.0–1.2 °C/min, a rate similar to that used in previous DSC studies (Farach & Martinez-Carrion, 1983). Temperature was measured with a total immersion, general use, grade of laboratory thermometer partially immersed in the bath with approximately the same geometry as the membrane samples; no attempt was made to calibrate the accuracy of this temperature measurement system. Previous results have shown that the effect of heating under these conditions is comparable to that of heating in the microcalorimeter (Farach & Martinez-Carrion, 1983). Aliquots were removed at selected temperatures and either kept on ice for 3 h or cooled to room temperature, maintained at that temperature for 30 min, and then kept on ice for 2.5 h.

After incubation, samples were solubilized in SDS by addition of an equal volume of sample buffer without 2-mercaptoethanol and incubation at room temperature for 30 min. Samples were then electrophoresed on SDS–polyacrylamide gels with a 5–15% acrylamide gradient. The solubilization buffer, the remaining reagents, and the electrophoresis and staining procedures are those of Laemmli (1970). Gels were destained in 7% acetic acid in the presence of Dowex MR-12.

The intensities of the stained protein bands were measured by using an EC910 scanning densitometer and a strip chart

recorder. The area corresponding to each AcChR subunit was approximated by triangulation.

Alkylation of AcChR. *N*-Ethylmaleimide was added to 3 mM, and membranes were subjected to thermal perturbations.

Functional Assay of AcChR Membrane. The AcChR-mediated ion flux was measured on a millisecond time scale by using a modified version of the stopped-flow fluorometric assay (Moore & Raftery, 1980). This assay is based upon (1) trapping a hydrophilic fluorophore, 1,3,6,8-pyrenetetrasulfonate, within the AcChR-containing, sealed membrane vesicles (instead of the 8-amino1,3,6-naphthalenetrisulfonate previously used), (2) rapidly mixing these vesicles with Tl^+, an analogue of alkaline metal ions such as Na^+ or K^+, which is also a collisional quencher of the fluorophore, and (3) recording the time-dependent decrease in fluorescence which is a direct measure of the passage of Tl^+ through the membrane into the intravesicular space. The rate of Tl^+ influx in the absence of AcChR agonists is presumed to reflect a nonspecific, non-receptor-mediated ion translocation through the membrane by an as yet ill-defined process. Simultaneous mixing of membranes with agonist and Tl^+ results in a rate of ion translocation enhanced by several orders of magnitude over the nonspecific rate.

Vesicles were loaded with fluorophore by rapidly freezing a 2–3-mL sample containing 3–4 mg/mL protein, 4.3 mM 1,3,6,8-pyrenetetrasulfonate, 10 mM HEPES, and 100 mM $NaNO_3$, pH 7.4, in liquid N_2 and slowly thawing the sample overnight on ice. After vigorous homogenization (Polytron, setting 5, 1 min), extravesicular fluorophore was removed by gel filtration. These membranes were mixed with 30 mM $TlNO_3$, 70 mM $NaNO_3$, and 10 mM HEPES, pH 7.4, with or without carbamylcholine, and the fluorescence decay was recorded by a modified Durrum-Gibson stopped-flow spectrometer. Data reduction employed an iterative least-squares algorithm implemented for an Apple II microcomputer (courtesy of Dr. R. Benyon, University of Liverpool) by using the relation between fluorescence decay and rate of Tl^+ influx described (Dunn et al., 1983).

Immunoblotting. The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was carried out by the method of Towbin et al. (1979). Coat Fab against purified receptor (Farach & Martinez-Carrion, 1983) was used as the first antibody with horseradish peroxidase conjugated, rabbit anti-goat IgG as the second antibody. The electrophoretic transfer of the proteins was performed in a modified gel-destaining apparatus for 3 h at 200 mA with cooling. To develop the color reaction, the nitrocellulose-bound antibody–antigen complexes were soaked in 0.3 mg/mL diaminobenzidine, 0.005% H_2O_2, and 50 mM Tris-HCl, pH 7.5, and 2–5 min. The reaction was stopped by washing with distilled water containing 0.02% sodium azide.

Results

Effect of Heating on Carbamylcholine-Induced AcChR-Mediated Ion Flux. In the previous DSC study of AcChR-enriched membranes (Farach & Martinez-Carrion, 1983), attempts to correlate functional disruptions with thermal transitions were limited by the slow ^{22}Na efflux assay available at the time. We have since developed the rapid ion influx assay which allows us to measure the ion translocation properties of the AcChR on a millisecond time scale (Moore & Raftery, 1980). In the absence of AcChR-activating ligands (passive flux), the rate of Tl^+ influx is rather slow, $t_{1/2} \approx 6$ s, and does not vary by more than about ±25% from preparation to preparation. Simultaneous addition of Tl^+ and an agonist, carbamylcholine (CARB), results in a marked increase in the rate of influx which is dependent (Moore & Raftery, 1980) on the concentration of agonist. For these experiments, we chose a single agonist concentration for which we could reliably measure the rate of influx, 87.5×10^{-6} M CARB, $t_{1/2} \approx 33$ ms. On the basis of this type of experiment, CARB has been shown to bind to the AcChR in a cooperative fashion with $K_d = (2\text{–}4) \times 10^{-4}$ M and a Hill coefficient of 1.8 (J. M. Gonzalez-Ros, J. Ferragut, and M. Martinez-Carrion, unpublished results).

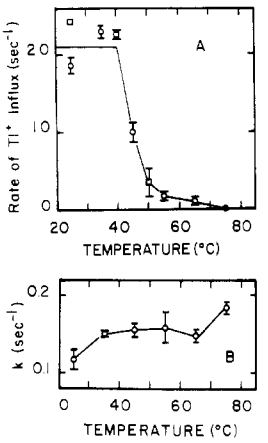

FIGURE 1: Effect of thermal exposure on ion permeability of AcChR-enriched membrane vesicles. Membranes were loaded with fluorophore as described under Materials and Methods and then heated to the indicated temperature. This buffer differs from that used in the remainder of these studies in containing 100 mM $NaNO_3$ and 4.3 mM pyrenetetrasulfonate. However, this buffer does not affect the thermal susceptibility of the AcChR as demonstrated in experiments such as shown in Figure 4 (data not shown). After the membranes were heated and cooled, they were subjected to gel filtration, and the rate of Tl^+ influx was determined as described under Materials and Methods. The rate of influx is an average over the membrane vesicle population. The protein concentration was the same for all samples. Error bars indicate the average deviation for three replicate determinations of the rate constant for a single sample. The data were obtained from two separate membrane preparations (□, ○). Panel A shows the rate of Tl^+ influx in the presence of 87 μM CARB; panel B shows that rate in the absence of CARB.

As shown in Figure 1A, heating AcChR membranes to 40 °C has no effect on the ability of CARB to increase the rate of ion influx. However, after the membranes are heated above 40 °C, CARB stimulation of the receptor is rapidly diminished. Essentially all of this activity is lost between 40 and 55 °C, most of the loss occurring between 40 and 50 °C.

It is interesting to note in Figure 1B that the increased rate of ion influx in the absence of CARB (passive influx) is only slightly greater than experimental uncertainty over the entire temperature range studied. The passive rate is essentially unchanged over the range of 40–60 °C that encompasses the loss of the two attributes of AcChR functionality we have measured. Up to almost 40 °C, there is no significant change in the agonist-induced rate of flux, while the passive influx does change somewhat. We suggest that changes on this magnitude are probably not significant in light of the experimental uncertainty of these data.

Thermally Induced Aggregation of AcChR Subunits. It has been shown that SDS–PAGE under nonreducing conditions can be utilized to correlate DSC thermal transitions with denaturation of given components of complex systems (Lysko et al., 1981; Brovillette et al., 1982). This is based on the observation that thermal denaturation can be accompanied by aggregation, presumably mediated by disulfide bond formation or re-formation. As a control experiment, AcChR-enriched membranes were heated for increasing lengths of time at 59 °C, the calorimetric transition temperature of these

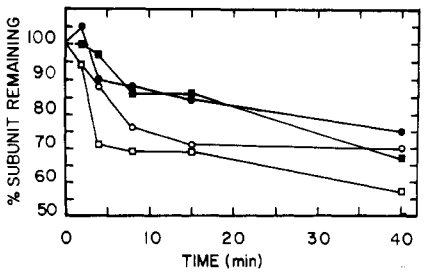

FIGURE 2: Time-dependent effect of heating on the SDS–PAGE (+mercaptoethanol) patterns of AcChR-enriched membranes under reducing conditions. Membranes were heated at 60 °C for varying times, cooled, solubilized with SDS in the presence of mercaptoethanol, and subjected to SDS–PAGE. Stained gels were scanned to give the integrated intensities shown. The values are expressed as a percentage of the control at time 0. (○) 40K, (●) 50K, (□) 60K, (■) 65K.

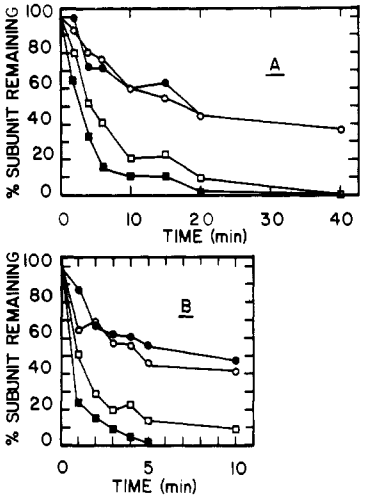

FIGURE 3: Time-dependent effect of heating on the SDS–PAGE patterns of AcChR-enriched membranes under nonreducing conditions. Membranes were heated at 58 (A) or 68 °C (B), cooled, solubilized with SDS in the absence of mercaptoethanol, and subjected to SDS–PAGE analysis as described for Figure 2. (○) 40K, (●) 50K, (□) 60K, (■) 65K. Band intensities are presented as a percentage of the intensity of the sample of AcChR which was not heated above room temperature. This does not correct for the intensity loss seen for the control (gels run under reducing conditions).

membrane vesicles. After being heated, the entire sample was solubilized by addition of SDS at room temperature, reduced with mercaptoethanol, and subjected to SDS–PAGE analysis. Figure 2 shows an initial rapid decrease in the amount of all subunits, the 40K and 60K subunits disappearing more rapidly than the 50K and 65K subunits. Even after 40 min of heating, however, there is only a 20–40% loss in AcChR subunits. Others have shown that heating AcChR in SDS similarly alters the apparent subunit ratios obtained from SDS–PAGE (Sobel et al., 1980; Criado & Barrantes, 1982; Barrantes et al., 1980). The loss in subunits seen here is not accompanied by the appearance of high molecular weight species and is consistent with the previously proposed decreased capacity to bind stain after heating.

By contrast, the SDS electrophoretic behavior markedly differs if the same heating experiment is carried out but the electrophoretic analysis is performed in the *absence* of mercaptoethanol (Figure 3). The only intersubunit disulfide cross-link observed is that between the 65K subunits. These nonreducing SDS–PAGE experiments show a more extensive loss in all subunits on a percentage basis of the sample not heated. Intensity is lost more rapidly in the 60K and 130K (2 × 65K) bands, with complete disappearance after 15 min. The data obtained in Figure 3A, resulting from heating at 59

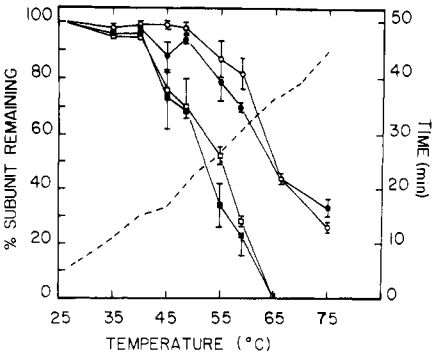

FIGURE 4: Effect of progressively increasing temperature on the SDS–PAGE pattern of AcChR. Samples were heated and analyzed as described for Figure 3. Error bars represent the average deviation of data from experiments with three separate membrane preparations. (■) 130K, (□) 60K, (●) 50K, (○) 40K.

°C, were treated as if the time-dependent loss of each subunit was a first-order process. This describes the disappearance of the 60K and 130K bands reasonably well, with half-lives of about 2.2 min for the 130K subunit and about 4.4 min for the 60K subunit. The semilog plot for loss of the 40K and 50K bands is linear to only about 50% of the intensity remaining; this initial loss has a $t_{1/2}$ = 18 min. A similar experiment, performed at a higher temperature, 68 °C, gave the results of Figure 3B. All bands lose intensity at a greater rate; the 60K and 130K bands lose intensity faster than the 40K and 50K bands, which still retain about 50% of their intensity at this higher temperature.

The data in Figure 3, as well as those in Figure 4, do not consider the underlying loss of intensity seen in the control experiment (Figure 2, gel run in the absence of mercaptoethanol). We do not believe that his omission seriously alters our interpretation. Figure 2 shows that the loss of intensity of all receptor bands occurs at about the same rate, though the magnitude of the loss is distinct for the 60K and 65K bands in comparison to the loss in the 40K and 50K bands. If corrections for the control were included in Figure 3, only the amplitude of the loss in subunits would be diminished. The difference in susceptibility to thermally induced loss would still be observed, which is the important observation we wish to convey.

The experiments of Figures 2 and 3, which were performed at temperatures at or above the apparent transition temperature of the AcChR, indicate that the 60K and 130K subunits are more susceptible to thermally induced aggregation mediated through a disulfide mechanism. If instead of heating at fixed temperatures for prolonged periods, we monitor the process as a consequence of progressive temperature increase, electrophoretic protein patterns can be more closely correlated with DSC thermograms, Tl^+ flux, and α-Bgt release experiments all carried out under identical conditions. Figure 4 illustrates the dependence of apparent subunit aggregation on increased temperature. The onset of aggregation of the 60K and 130K subunits appears to occur simultaneously with the loss in the ion-gating function of the AcChR, starting approximately at 40 °C. The disappearance of these subunits is not complete until the membrane vesicles have been heated to 65 °C. The wide temperature range of this effect is uncharacteristic of the sharp, cooperative transition seen in the loss of toxin binding ability and to some extent in the loss of ion-flux activity. Much higher temperatures are required to detect loss of intensity of the 40K and 50K subunit bands, and the temperature range over which this occurs is similarly wide. The loss of intensity of the 40K and 50K subunits begins at 45–50 °C and is only 70% complete at 75 °C. At the midpoint

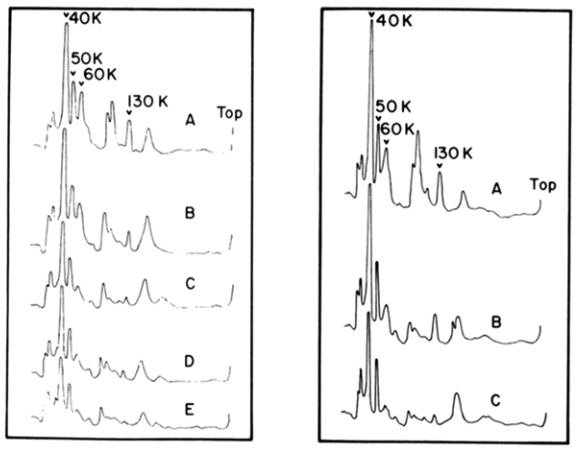

FIGURE 5: Densitometer scans of SDS–polyacrylamide gels of heated AcChR membranes. Membranes were heated at 59 °C for (A) 0, (B) 4, and (C) 10 min, at 68 °C for (D) 2 and (E) 4 min (left panel), and at different temperatures: (A) 25, (B) 59, and (C) 65 °C (right panel). The area of each band was approximated by triangulation. The limited resolution of our densitometer does not accurately reflect the resolution of the AcChR bands we achieve. Howeve, the approximations of band areas we make give a reproducibility of about ±15% from gel to gel. The accuracy of the method is indicated by the correct receptor band stoichiometry which we obtain.

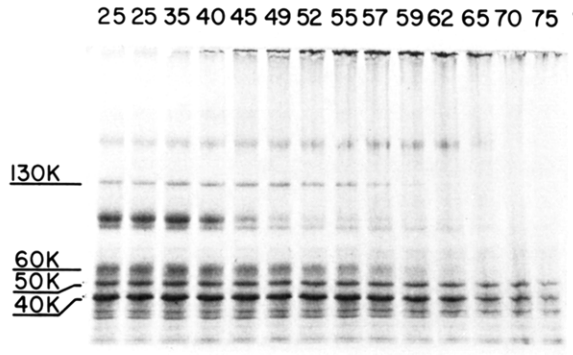

FIGURE 6: Representative SDS–polyacrylamide gel of heated AcChR membranes. Membranes were heated to the indicated temperature, cooled, and subjected to thermal gel analysis as described under Materials and Methods (SDS–PAGE in the absence of mercaptoethanol). This is a representative gel from which densitometer scans such as shown in Figure 5 were made to give the intensity data such as presented in Figures 3 and 4.

temperature at which toxin binding is irreversibly lost, 60 °C, 70–80% of the 40K and 50K subunit intensities remain. At the midpoint temperature at which α-Bgt is released from its complex with AcChR (Farach & Martinez-Carrion, 1983), 49 °C, there are no apparent changes in the electrophoretic bands for the 40K and 50K subunits, while there is already disappearance of the 60K and 65K (as 130K dimer) bands (Figure 5).

The gradual and selective disappearance of the AcChR subunits with heating is the result of aggregation to very high molecular weight species seen in Figure 6 as material remaining at the origin of the gel slab. When similar gels were subjected to immunoblotting, these new, heavy species as well as the remaining 40K, 50K, 60K, and 130K species were stained, indicating the presence of material originating from the AcChR in these aggregates. The mechanism for such aggregation seems to require the presence of free sulfhydryl residues at some point in the process, since inclusion of the alkylating reagent, *N*-ethylmaleimide, during the heating process totally protects all subunits against the aggregation observed in these gels. Heating the membranes under an argon atmosphere did not give any significantly different results in the thermal gels.

Discussion

These studies indicate that the ability of the AcChR to facilitate transmembrane ion flux in response to agonist is almost completely destroyed by heating to 54 °C. The correlation of these results with our previous findings suggests that certain ligand binding properties are left intact after heating to 54 °C (Farach & Martinez-Carrion, 1983; Soler et al., 1983). The equilibrium binding of α-Bgt is essentially unperturbed after the membrane-bound receptor to is heated 54 °C. In addition, it was shown (Farach & Martinez-Carrion, 1983) that AcChR-enriched membranes heated to 54 °C still exhibit the ability to undergo desensitization as expressed in a decreased rate of toxin binding after exposure of AcChR to CARB. These observations require careful consideration on several points. Equilibrium binding of α-Bgt may not be a true index of the integrity of the ligand binding function of the AcChR. The affinity of α-Bgt for the AcChR is very high, apparently because of multiple points of contact (Kosower, 1983; Low, 1979). Subtle perturbations at perhaps only a few of these binding subsites may not significantly affect the extent of toxin binding at equilibrium. On the other hand, the rate of toxin binding may be more informative. Not only is CARB a cholinergic ligand competitive with bungarotoxin but also its presence leads to pharmacological-like desensitization, which is manifested, in part, by a decreased rate of α-Bgt binding as well as by a conformational change which increases the affinity of AcChR for ligands. The effect of CARB on the rate of α-Bgt binding is an indirect measure of agonist affinity for AcChR. On the basis of this information, it appears that the functionally significant ligand binding properties of the receptor, at least for α-Bgt and CARB, are not *drastically* perturbed by temperatures that serverely perturb the ion-flux activity of the receptor. This suggests that heating can be utilized to disrupt the mechanism linking agonist binding with ion-channel opening.

It is interesting to note that irreversible loss of toxin binding occurs with a midpoint temperature of about 60 °C but dissociation of previously bound toxin occurs at a lower temperature, the midpoint temperature, 49 °C. Therefore, it is possible to heat the toxin–AcChR complex as high as 54 °C, release the bound toxin, cool the sample, and observe its ability to rebind the toxin (i.e., a reversible process). It is as if the thermal event(s) which lead(s) to irreversible loss of ion-flux activity also reversibly perturb(s) the α-Bgt binding domains such that toxin dissociates. That is, the perturbation in the binding of toxin and perhaps agonist is reversed upon cooling while the ion-gating function is irrecoverable.

The differences in susceptibility to thermally induced aggregation suggest two domains of AcChR subunits. In the absence of mercaptoethanol, the more sensitive domain seems to be composed of the 60K and 65K subunits and should possibly be considered more properly as an $\gamma_2\delta_2$ domain with likely contributions from both units of the dimeric AcChR. However, the temperature-dependent loss in the 60K and 65K subunits of the monomeric AcChR is indistinguishable from that of the unmodified dimeric AcChR (data not shown). Under appropriate conditions, all of the components of this more labile domain undergo the aggregation process. The domain that seems to form less irreversible aggregates under the experimental conditions consists of the 40K and 50K subunits. In addition to differing in the greater temperature required to produce the observed aggregation of these subunits, complete aggregation or loss of the 40K and 50K subunits was

never observed under any of our conditions. It is unclear whether aggregation occurs in some reversible process and is not detected by our SDS–PAGE treatment or whether it indeed is a slower process. On the other hand, it does seem clear that the occurrence of the detectable aggregation process is secondary to two distinct primary protein conformational events—loss of ion-flux activity and loss of ligand binding. Whatever the mechanism, it appears that only minor changes in structure may be responsible for loss of the latter two properties. Indeed, most ion-flux activity is lost between 40 and 50 °C, while only about 30% of the $\gamma_2\delta_2$ domain is lost over that temperature range. Similarly, α-bungarotoxin binding is irreversibly lost between 58 and 62 °C while only 20–30% of the $\alpha_2\beta$ domain is lost.

The suggestion that the aggregation process follows some primary conformational changes poses the question of whether the differences in the rates of aggregation actually reflect the structural organization of the receptor. We favor a model in which a primary thermal event abolishes a functional property of the AcChR and simultaneously activates a distinct region in the receptor structure. Maintenance of this activated state by continued application of thermal energy allows a time-dependent aggregation of similarly activated regions. Existing data impose certain constraints upon this model. No transitions are detected by DSC for the alkaline-extracted AcChR-enriched membranes in the temperature range over which loss of ion-flux activity is observed. This could imply that the change in heat capacity associated with this process is small and undetectable by our instrumentation or that it is obscured by a compensating lipid or protein effect. For water-soluble proteins, the magnitude of the change in heat capacity upon denaturation may be related, in part, to the epoxure of hydrophobic sites to aqueous solvent (Kauzmann, 1959). If the perturbation that disrupts ion-flux activity occurs in a region of the receptor that is hydrophilic, changes in H bonding and internal vibrational modes (Sturtevant, 1977) may compensate those due to hydrophobic to hydrophilic solvent exposure and could account for the lack of an endothermic signal in DSC scans. On the other hand, the thermal event responsible for loss of toxin binding is apparently detected by DSC and presumably results in a more extensive perturbation in receptor structure.

The efficacy of NEM in preventing thermally induced aggregation suggests that the process involves intermediate sulfhydryl groups. But this experiment does not distinguish between an activation of preexisting thiol residues which allows the formation of new disulfide bridges or the disruption of existing disulfide bonds which then form new, altered disulfide bridges. If the aggregation does procede through preexisting thiols, these sulfhydryl residues must not be reactive toward the iodoacetamide present at the initial stages of membrane purification. We do not know the composition of the aggregates which appear as the receptor subunits disappear. We do know, however, that the high molecular weight species do contain AcChR subunits. These membrane vesicles, though enriched in AcChR, contain only 40–50% of their total protein as AcChR. The predominant nonreceptor proteins appear with polypeptides of M_r 90K and 95K. The heavier of these bands disappears more rapidly than any of the receptor bands while the 90K band is as persistent as the 40K and 50K receptor bands. Because of the differing time scales of apparent loss in various subunits in SDS–PAGE gels, it appears that the $\gamma\delta$ subunits aggregate with $\gamma\delta$ subunits. However, the $\alpha\beta$ subunits may aggregate with like subunits or, less likely, with non-AcChR membrane components.

The size or total composition of the aggregates remaining at the SDS–PAGE gel origin has not been accurately determined. Our previous study showed that after being heated to 54 °C, all the AcChR protein was solubilized by Triton X-100, and the subunit stoichiometry was unchanged when examined by SDS–PAGE under disulfide reducing conditions. At this temperature, about 50% of the γ and δ subunits are lost. The sum of these results suggests that the receptor with a $\gamma_2\delta_2$ aggregate is detergent soluble. After being heated to 61 °C, none of the AcChR protein is soluble in Triton X-100 solutions while 80% of the γ and δ subunits and 30–40% of the $\alpha\beta$ subunits are lost. These results could suggest that aggregation initially occurs within the AcChR dimers but increasing temperature leads to disulfide cross-linking between dimeric AcChR molecules. The differences in aggregation of the two receptor domains could be only a result of the relative abundance of reactive sulfhydryl residues or their relative proximities. Inspection of the sequence of the AcChR shows that the α subunit is richest in cysteinyl residues, the β subunit is the poorest, and the γ and δ subunits are intermediate (Noda et al., 1983). However, if the predicted distribution of sulfhydryl residues is correct (Guy, 1983), the γ subunit has almost twice as many cysteinyl residues in its transmembrane segments as does the α subunit. It is possible that sulfhydryl groups in certain regions of the receptor structure are responsible for the differential aggregation of subunits.

These results point out the pitfall of reliance on α-Bgt binding, either its stoichiometry at equilibrium or its time dependence, as a criterion for the native state or the functionality of receptor preparations. Indeed, it has been shown that the isolated 40K subunit can still specifically bind α-Bgt, albeit with much lower affinity than in its native state (Haggerty & Froehner, 1981; Oblas et al., 1983). Similarly, the ability of cholinergic agents to retain their functional role to induce a desensitized-like state of the receptor is also an incomplete criterion for the integrity of the native structure of the receptor. The decoupling of the receptor's α-Bgt binding ability from ion-flux capability is now achievable through applicaton of thermal energy. The structural elements responsible for ion translocation are more susceptible to irreversible, thermally induced conformational changes at temperatures that cause limited, reversible conformational changes in protein domains which directly or indirectly affect cholinergic binding sites.

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Effects of Anesthetic and Nonanesthetic Steroids on Dipalmitoylphosphatidylcholine Liposomes: A Calorimetric and Raman Spectroscopic Investigation†

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ABSTRACT: The effects of anesthetic and nonanesthetic steroids on dipalmitoylphosphatidylcholine liposomes were studied by use of high sensitivity scanning calorimetry and Raman spectroscopy. Calorimetric measurements indicated that both anesthetic and nonanesthetic steroids depressed and broadened the gel to liquid-crystalline phase transition. There was no correlation between the perturbations by the steroids on the primary gel to liquid-crystalline phase transition temperature and anesthetic potency. The magnitudes of the steroid-induced transition broadening and lowering of the pretransition temperature, however, correlated well with anesthetic potency. This effect appeared to arise from the projection from the plane of the D ring of substituents at the C(17) position of the steroid nucleus. Raman spectroscopic measurements demonstrated that the steroid molecule is localized within the acyl region of the bilayer and that effects of the steroid do not extend to either the head-group or interface regions of the lamellae. The data are consistent with unitary hypotheses relating general anesthesia to lipid perturbations. For model systems, perturbations to the subtle structural and dynamical properties of the bilayer pretransition may provide a more sensitive marker than the main phase transition in assessing the significance of lipid mediation in inducing anesthetic action.

General anesthesia is widely believed (Roth, 1979, 1980; Janoff & Miller, 1982; Mountcastle et al., 1978; Trudell, 1977; Seeman, 1972) to result from a lipid-mediated perturbation of protein function in excitable membranes. Evidence supporting this hypothesis consists primarily of numerous correlations between anesthetic potency, lipid solubility, and lipid effects (Janoff & Miller, 1982; Meyer, 1899; Overton, 1901; Janoff et al., 1981; Seeman, 1966, 1972). Various investigators (Janoff & Miller, 1982; Trudell et al., 1973a,b; Mountcastle et al., 1978; Hill, 1978) have shown that general anesthetics lower and broaden the main bilayer phase transition of phosphatidylcholine liposomes and that these effects, like general anesthesia, may be reversed by pressure. General anesthesia both in mice and in tadpoles results from inhalation anesthetics at concentrations that induce approximately 1 °C decrease in the phase transition temperatures of dipalmitoylphosphatidylcholine (DPPC) liposomes (Janoff & Miller, 1982); this corresponds to anesthetic concentrations in the DPPC bilayers of about 4 mol % (Hill, 1978). Despite

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