123.
Segregation of phosphatidic acid-rich domains in reconstituted acetylcholine receptor membranes

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Reconstitution of the purified nicotinic acetylcholine receptor (AChR) from *Torpedo* into artificial membranes has shown that the presence of lipids such as cholesterol and phosphatidic acid (PA), are important in preserving an optimal cation channel activity of the protein.[1,2] However, no satisfactory explanation has been given for the molecular events by which specific lipids exert such effects on AChR activity. Several hypotheses have been entertained, including (i) indirect effects of lipids through alteration of bilayer properties, such as the fluidity or the membrane curvature and lateral pressure or (ii) direct effects through binding of lipids to transmembrane sites on the protein. The latter led to postulation of a role for lipids as peculiar 'allosteric' ligands of the AChR.

Previous Fourier-transform infrared spectroscopy studies using AChR reconstituted into lipid mixtures containing dimyristoyl phospholipids as probes to explore the effects of the protein on phospholipid organization[3] suggested that the AChR specifically directs lateral phase separation of the monoanionic phosphoryl form of the dimyristoyl phosphatidic acid (DMPA) probe, causing the formation of phosphatidic acid-rich domains that become segregated from the bulk lipids. We are now reporting on further details of the protein dependence of such phenomena.

Differential scanning calorimetry (DSC) studies of AChR reconstituted at ~1:3500 protein to phospholipid molar ratio in egg phosphatidylcholine, cholesterol, and different dimyristoyl phosopholipids (2:1:1, by mole) show that in the absence of AChR protein, none of the vesicles exhibited thermal transitions within the temperature range studied, as expected from ideal (random) mixing of the lipid components. In the presence of AChR, only vesicles containing DMPA out of all the different dimyristoyl phospholipids attempted (DMPC, DMPS, DMPG, and DMPA) show a small and narrow endotherm, at ~41–43°C. A wider endotherm at lower temperatures (below 35–40°C), is also seen in these samples preceding the cooperative transition at 41–43°C (Fig. 123.1). At higher temperatures a very small and wide endotherm appears centered at 55–57°C resulting from thermal denaturation of the AChR protein which, because of its irreversible nature, disappears from the thermogram in a second thermal scan.

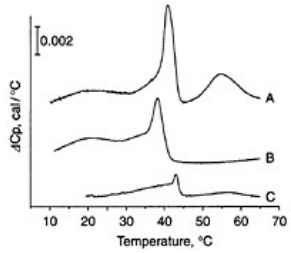

Figure 123.1 DSC studies on the effect of the AChR protein on lipid organization in reconstituted vesicles.

The thermograms shown correspond to reconstituted AChR vesicles prepared from lipid mixtures containing 50 mole percent of egg PC, 25 mole percent of cholesterol and 25 mole percent of DMPA at a protein to phospholipid molar ratio of either 1:560 (A and B) or 1:2500 (C). Thermogram B corresponds to the second thermal scan of sample A.

Specific segregation (lateral phase separation) of DMPA induced by the protein appears as the obvious candidate to account for the observed calorimetric phenomena.

To further study the dependence of the observed DMPA segregation with the presence of AChR protein, reconstituted vesicles were prepared at higher protein to phospholipid ratios. Fig. 123.1 compares the DSC thermograms from samples reconstituted at 1:2500 and 1:560 protein to phospholipid ratios. Despite the fact that the DMPA concentrations in these samples are essentially identical (i.e. 10 mM), the DMPA-related endotherms in the sample at higher protein concentration become much more noticeable. This suggests that at the lower ratio, only a fraction of the available DMPA becomes segregated into a membrane domain. This also means that the extent of the DMPA-related domain is limited by the amount of protein present. A gross estimation of the size of such domains from the heat involved in the thermal events indicates that ~220 and 120 moles of DMPA were segregated per mole of AChR protein, respectively, in the samples prepared at low and high protein to phospholipid molar ratios. These estimates exceed the capacity of the transmembrane region of the AChR to bind phospholipids (~45 phospholipid-binding sites per AChR monomer[4]) and, therefore, our observations can not be understood solely in terms of hydrophobic lipid-protein interactions.

We also use fluorescence resonance energy transfer between the protein tryptophan residues as energy donors and different fluorescence probes (diphenylhexatriene, DPH and *t*-parinaric acid, *t*-PnA) as acceptors, to determine whether the protein is within Förster distance from the membrane domains into which these probes partition. AChR reconstituted at a protein to phospholipid ratio of 1:560, in egg PC: cholesterol: DMPA (2:1:1) and egg PC: cholesterol: DMPC (2:1:1) was used in these experiments, at temperatures below the DMPA-rich domain transition temperature. To analyse the energy transfer data, we used the Gutierrez-Merino model for two-dimensional systems,[5] in which changes in transfer efficiency with acceptor surface density depend on three variables: H, the distance between the plane containing the donor and that of the acceptor, normal to the membrane; r, the distance of closest approximation between donor and acceptor; and K_r the apparent dissociation constant for a putative lipid domain surrounding the protein.[5] Based on information on the location of tryptophan residues in the transmembrane portion of the AChR, we arbitrarily fixed H=10 Å. Also, since DPH does not show any preference for partitioning into gel or fluid phases, we assumed K_r=1, which according to the model corresponds to random distribution of the fluorophore within the membrane.[5] Under these assumptions, the best fit of the experimental data from the tryptophan-DPH transfer to the Gutierrez-Merino model, is that corresponding to r=30 Å. This value of r=30 Å was then used, along with the same value of H=10 Å, to calculate K_r for the *t*-PnA probe. Energy transfer data for the egg PC: cholesterol: DMPA reconstituted membranes show that the best fit corresponds to K_r=0.6 ±0.1. On the other hand, in the egg PC: cholesterol: DMPC, in which there is no domain segregation, the best value corresponds to K_r=1±0.1. Values of K_r lower than 1, indicate a preferential location of the *t*-PnA probe in membrane regions immediately surrounding the protein, within Förster distance of the tryptophan donors; while a K_r value of 1 indicates random distribution.[5] As *t*-PnA partitions preferentially in gel-like lipid phases, these results further support the existence of a gel-like, DMPA-rich membrane domain induced by the AChR, but more importantly, they strongly suggest that the protein itself is included in such domain.

In summary, the segregated domain should be formed by units of AChR molecules, each surrounded by several PA-rich ordered lipid shells. Such units are expected to coalesce into much larger domains to provide the reported macroscopically observable behavior.

ACKNOWLEDGMENT

Supported by a grant from the DGESIC of Spain (PM1998–0098).

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