Extraction of peripheral proteins is accompanied by selective depletion of certain glycerophospholipid classes and changes in the phosphorylation pattern of acetylcholine-receptor-rich-membrane proteins

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The widely used alkaline treatment of acetylcholine-receptor (AChR)-rich membranes from *Torpedo marmorata* (electric fish) and *Discopyge tschudii* (a marine ray) results not only in the extraction of non-receptor peripheral proteins but also in that of glycerophospholipids (~ 13%). Minor acidic phospholipids, notably phosphatidic acid and polyphosphoinositides, are particularly enriched in the NaOH extracts. When electrocytes or receptor-rich membranes are incubated with [³²P]P_i or [γ -³²P]ATP, polyphosphoinositides accumulate most of the label (~ 45% in *D. tschudii*; 96% in *T. marmorata*) and exhibit the highest specific radioactivity. Furthermore, more than 50% of these phosphorylated lipids are extracted by NaOH together with the peripheral membrane proteins. NaOH treatment also results in modification of the phosphorylation pattern of AChR membrane proteins. Phosphorylation decreases in the M_r-43 000 group of peripheral proteins and in the γ -subunit of the receptor. The results indicate that polyphosphoinositides constitute a metabolically very active lipid pool in the postsynaptic membrane, and that a substantial proportion of these phospholipids are preferentially released from the membrane together with other acidic phospholipids upon peripheral-protein extraction. The conclusion is drawn that membranes submitted to the above treatments can no longer be considered equivalent to native ones in terms of their phospholipid composition and phosphorylation characteristics.

INTRODUCTION

The nicotinic cholinergic receptor (acetylcholine receptor, AChR) is an intrinsic membrane glycoprotein. It is by now the most exhaustively characterized endogenous receptor protein (Karlin *et al.*, 1986; Barrantes, 1986). In addition to the AChR protein, purified receptor-rich postsynaptic membranes from electric-organ tissue contain substantial amounts of other non-receptor proteins whose function is largely unknown, most conspicuously various different proteins of M_r about 43 000 (Sobel *et al.*, 1978; Hamilton *et al.*, 1979; Barrantes *et al.*, 1980; Gysin *et al.*, 1981). Their only common feature appears to be their extraction by procedures known to release loosely bound peripheral proteins from membranes (Neubig *et al.*, 1979; Elliot *et al.*, 1980). Although several effects have been reported consequent on the depletion of these proteins from the membrane, such as alterations in the thermal stability (Saitoh *et al.*, 1979) and freedom of motion of the AChR (Lo *et al.*, 1980; Bartholdi *et al.*, 1981), only one specific function has been unambiguously ascribed to a particular species of this group of proteins, when it was demonstrated that the pI 6.5–6.8 M_r-43 000 polypeptides are isoenzymes of creatine kinase (Barrantes *et al.*, 1983a,b, 1985; Gysin *et al.*, 1983).

In the present work we have applied the NaOH

extraction procedure to AChR membranes and found that not only are peripheral proteins released from such membranes, but polyphosphoinositides, minor lipids not previously quantified in these membranes, are preferentially depleted from the bulk lipid. Depletion of these lipids and peripheral protein is also accompanied by changes in the pattern of phosphorylation of the AChR and non-receptor proteins. The importance of these findings is to be assessed in the light of the widespread use of the above-mentioned procedures in current research on the AChR. The possible association of specific phospholipids with peripheral proteins and the implications of such association on postsynaptic membrane structure are discussed.

EXPERIMENTAL

Animals and materials

Electric fish (*Torpedo marmorata*) were obtained from the Biological Marine Station at Arcachon, France, and *Discopyge tschudii* (a marine ray) were provided by the Institute of Marine Biology at Mar del Plata, Argentina. Membrane fragments rich in AChR were prepared as previously described (Barrantes, 1982a), frozen in liquid N₂, and kept at –80 °C until use. NaOH was obtained from Merck (Darmstadt, Germany). Lithium 3,5-diiodosalicylate, cyclic AMP, ouabain and β -mercapto-

Abbreviations used: AChR, acetylcholine receptor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PPI, polyphosphoinositides; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; CL, cardiolipin (diphosphatidylglycerol); SPH, sphingomyelin; PA, phosphatidic acid.

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ethanol were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents used were analytical grade and purchased from Merck. $\text{Na}_2\text{H}^{32}\text{PO}_4$ (sp. radioactivity 387 mCi/mmol) was obtained from the Argentine Atomic Energy Commission, Buenos Aires, Argentina; $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 8.66 Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A.

Peripheral protein extraction from AChR membranes

T. marmorata AChR membranes frozen at -196°C immediately after they were obtained and subsequently stored at -80°C were washed by centrifugation to eliminate sucrose before use. After resuspension in distilled water at a final concentration of 2 mg of protein/ml, 0.1 M-NaOH was added until the pH was adjusted to 11.0. The samples were maintained at this pH for 1 h at 4°C , i.e. as described in Barrantes' (1982b) modification to the procedure of Neubig *et al.* (1979). The membranes were then centrifuged at 120000 *g* for 30 min in a Ti-80 rotor using a Beckman LS-85 ultracentrifuge. The supernatant was precipitated overnight with trichloroacetic acid (final concn. 10% w/v) at 4°C and then centrifuged as described above. Analogous steps were followed for the treatment with 10 mM-lithium 3,5-di-iodosalicylate, which was performed in 10 mM-Tris/HCl buffer, pH 8.0, for 30 min at 4°C as described by Criado & Barrantes (1984). After centrifugation as described above, the supernatant was precipitated with trichloroacetic acid, and both precipitated protein and residual membrane remnants were washed by two additional centrifugation steps and adjusted to pH 7.4 before lipid extraction. Control samples were submitted to similar incubation and centrifugation steps, but only 10 mM-Tris/HCl buffer, pH 7.4, was used.

Labelling of electrocyte lipids *in vitro*

Stacks of *D. tschudii* electrocytes not more than a few cells wide were dissected immediately after killing the fish. They were incubated with $\text{Na}_2\text{H}^{32}\text{PO}_4$ in microwell plates in a physiological saline solution with the following composition: 275 mM-NaCl/7.6 mM-KCl/4.3 mM- CaCl_2 /1.4 mM- MgCl_2 /glucose (2.5 mg/ml)/50 mM-Hepes buffer, pH 7.4. The medium was supplemented with 415 mM-sucrose in order to preserve osmotic balance. Electrocyte stacks were incubated with 125 μCi of precursor in a total volume of 120 μl . The temperature was kept at about 15°C . The pH of the samples was checked and found to be maintained even after 28 h of incubation. Membrane fractions were prepared from the electrocyte stacks as described by Elliot *et al.* (1980).

Phosphorylation of purified AChR-rich membranes

The standard medium adopted for the direct phosphorylation of native and NaOH-extracted AChR-rich membranes from *T. marmorata* was 20 mM-Tris/HCl buffer (pH 6.8)/0.1 mM-EGTA/20 mM- MgCl_2 /0.5 mM-ouabain/10 mM- β -mercaptoethanol. The membrane samples were preincubated in this medium for 2 min at 30°C and the phosphorylation assay was started by the addition of 5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, plus various concentrations of unlabelled ATP and other cofactors as required, to give a final volume of 250 μl . After the

desired incubation time, 2 ml of 5 mM-ATP and 2.5 ml of 10% (w/v) trichloroacetic acid were added to stop the reaction. The precipitated material was centrifuged in a benchtop centrifuge, and lipids were extracted as described below. In the case of calmodulin-dependent phosphorylation conditions, the incubation medium described by Hayashi & Amakawa (1985) was used.

Duplicate samples were submitted to polyacrylamide-gel electrophoresis as described by Laemmli (1970), with the modifications introduced in previous work (Barrantes *et al.*, 1980; Barrantes, 1982b) in order to avoid the deleterious effect of heating on AChR chains. This was of particular importance in the present work, given the observed thermal lability of the γ -subunit (Barrantes *et al.*, 1980; Criado & Barrantes, 1982). Samples were incubated for 30 min at room temperature in 5% (w/v) SDS/375 mM-Tris/HCl (pH 6.8)/30% (v/v) glycerol/15% (v/v) 2-mercaptoethanol/0.05% (v/v) Bromophenol Blue. After electrophoresis the gels were stained with 0.25% Coomassie Blue in 45% methanol/9% acetic acid/water (45:9:46, by vol.), destained in 50% methanol/75% acetic acid/water (2:3:35, by vol.) and dried. Autoradiography was carried out using 3M Medical X-ray film type R and a Gevaert intensifying screen at -70°C (Laskey & Mills, 1977). The ^{32}P incorporated by individual polypeptide chains was determined by cutting out the bands in wet stained gels, eluting the acrylamide slices in H_2O_2 at 70°C overnight, and counting their radioactivity by liquid scintillation in a Beckman 8000 instrument.

Analytical procedures

Lipids were extracted by the methods of Folch *et al.* (1957) and Bligh & Dyer (1959). In the case of polyphosphoinositides the method of Dawson & Eichberg (1965) was employed, but only one extraction was carried out at 37°C .

Phospholipid quantification was done as described by Rouser *et al.* (1970) after isolation and identification by two-dimensional t.l.c. using 500 μm -thick silica-gel H plates. Polyphosphoinositides present in neutral and acid extracts were separated by one-dimensional t.l.c. on 1% potassium oxalate-impregnated silica-gel H plates, which were developed as described by Shaik & Palmer (1977); they were identified by comparing their R_F values with those of authentic standards.

Non-esterified fatty acids were separated by one-dimensional t.l.c. on silica-gel G, with hexane/ethyl ether/acetic acid (400:600:23, by vol.) as solvent. The region corresponding to non-esterified fatty acids was located with the use of appropriate standards, scraped off, and submitted to methanolysis with 2 ml of 14% BF_3 in methanol. Methyl esters were separated and identified by temperature-programmed g.l.c. in a Varian 3700 chromatograph using two 2 m $\times$ 0.2 cm (internal diameter) glass columns packed with 15% OV-275 on Chromosorb WAW, 80-120 (Varian, Sunnyvale, CA, U.S.A.). The columns were connected to a flame-ionization detector operated in the dual-differential mode. The initial and final oven temperatures were 160° and 220°C respectively, an increase rate of $5^\circ\text{C}/\text{min}$ being used. Injector and detector temperatures were 220° and 230°C respectively, and N_2 (30 ml/min) was the carrier gas.

Protein concentration was determined as described by Lowry *et al.* (1951), with bovine serum albumin as

Table 1. Peripheral protein and phospholipid extraction on NaOH treatment of AChR-rich membranes from *T. marmorata*Results are means $\pm$ S.D. for three separate experiments, except for the supernatant of hypo-osmotic buffer ($n = 2$).

	Protein		Lipid phosphorus		Lipid/protein (w/w)
	(mg)	(%, w/w)	(nmol)	(%, w/w)	
Native membranes	2.55 $\pm$ 0.2	100	1404.1 $\pm$ 179.4	100	0.50 $\pm$ 0.07
Hypo-osmotic buffer (20 mM-Tris/HCl, pH 7.4)					
Supernatant	0.015	0.6 $\pm$ 0.1	24.8 $\pm$ 0.3	1.8 $\pm$ 0.2	1.10 $\pm$ 0.02
Pellet	2.50 $\pm$ 0.2	99.4 $\pm$ 0.1	1355.7 $\pm$ 179.2	98.1 $\pm$ 0.2	0.48 $\pm$ 0.06
NaOH					
Supernatant	0.42 $\pm$ 0.1	18.1 $\pm$ 6.0	158.0 $\pm$ 12.7	13.0 $\pm$ 0.4	0.31 $\pm$ 0.01
Pellet	1.80 $\pm$ 0.3	82.2 $\pm$ 6.2	1280.5 $\pm$ 176.7	87.0 $\pm$ 0.4	0.60 $\pm$ 0.02

Table 2. Effect of alkali treatment on the distribution of phospholipids in AChR-enriched membranes from *T. marmorata*

Data are expressed as means $\pm$ S.D. for three separate experiments. The statistical significances of differences between values for lipids distributed in supernatant and pellet after NaOH extraction, estimated by Student's *t* test, were: * $P < 0.005$; ** $P < 0.001$.

Phospholipid	Control AChR-rich membranes		NaOH-treated			
			Pellet		Supernatant	
	(nmol/mg of protein)	(mol %)	(nmol/mg of protein)	(mol %)	(nmol/mg of protein)	(mol %)
PC	205.3	38.1 $\pm$ 2.8	215.2	35.4 $\pm$ 2.0	131.9	34.9 $\pm$ 1.6
PE	173.1	32.1 $\pm$ 0.7	188.8	31.1 $\pm$ 0.6	66.2	17.5 $\pm$ 1.2
PS	67.5	12.5 $\pm$ 2.7	86.6	14.3 $\pm$ 0.6	43.6	11.5 $\pm$ 0.9
SPH	25.7	4.8 $\pm$ 1.1	42.0	6.9 $\pm$ 0.5	10.5	2.8 $\pm$ 0.8
PI	26.0	4.8 $\pm$ 0.1	28.6	4.6 $\pm$ 0.6	8.8	2.3 $\pm$ 1.3
CL	9.4	1.7 $\pm$ 0.2	10.6	1.7 $\pm$ 0.4	5.2	1.4 $\pm$ 0.6
PA	5.6	1.0 $\pm$ 0.4	4.3	0.7 $\pm$ 0.1*	5.5	1.5 $\pm$ 0.2*
Lyso-PC	4.8	0.9 $\pm$ 0.1	7.7	1.3 $\pm$ 0.4**	21.9	5.8 $\pm$ 0.4**
Lyso-PE	12.1	2.2 $\pm$ 0.2	17.6	2.9 $\pm$ 0.8**	51.4	13.7 $\pm$ 1.0**
PIP	6.9	1.3 $\pm$ 0.4	4.2	0.7 $\pm$ 0.05**	20.0	5.3 $\pm$ 0.5**
PIP ₂	2.4	0.4 $\pm$ 0.1	2.1	0.3 $\pm$ 0.05**	13.3	3.5 $\pm$ 1.0**

standard. Radioactivity was measured by liquid-scintillation counting.

RESULTS

Selective depletion of certain phospholipids by NaOH treatment of AChR-rich membranes

Exposure of AChR-rich membranes to NaOH results in the extraction of peripheral membrane proteins (Neubig *et al.*, 1979; Elliot *et al.*, 1980). When the membrane-released proteins in the alkaline extracts were precipitated with trichloroacetic acid, and subsequently submitted to organic-solvent extraction, it was found that the extracts of these protein precipitates contained more than 10% of the membrane phospholipids (Table 1), whereas no lipids were found in the aqueous phase remaining after protein precipitation. Although this cannot be taken as evidence for any particular type of attachment between peripheral proteins and lipids depleted from the AChR-rich membrane, the recovery of the lipid moiety together with the extracted protein on precipitation of the latter suggests some degree of

association between the two. The phospholipid/protein ratio in native membranes and in those depleted of peripheral proteins fell within the range reported by other authors for receptor-rich membranes of *T. californica* (Gonzalez-Ros *et al.*, 1982) and *T. marmorata* (Popot *et al.*, 1978). Total lipid extracts of native and NaOH-treated membranes were also analysed for non-esterified fatty acids (see the Experimental section). No saponification of membrane lipids could be found, as evidenced by the lack of fatty acid accumulation during the 1 h NaOH treatment of the membranes.

Table 2 shows an analysis of the content of individual phospholipids in AChR-rich control membranes and in those extracted with NaOH. PC and PE, amounting together to 70%, were the major phospholipids in native membranes, followed by PS (12%), and PI and SPH (5% each). This profile is similar to that of extracted membranes, and differs from the phospholipid composition reported for *T. californica* (Gonzalez-Ros *et al.*, 1982) in two respects: (1) the relative contribution of PC and PE, which is exactly the opposite in the two *Torpedo* species, and (2) the presence of minor phospho-

Table 3. Incorporation of [32 P]P_i and [γ - 32 P]ATP into phospholipids of *D. tschudii* and *T. marmorata* AChR-rich membranes *in vitro*

Phospholipid compositions are means $\pm$ S.D. for three separate samples used in the present series of experiments. The percentage composition of *T. marmorata* lipids differs slightly from that of Table 2; the two sets of data fall within statistical limits of each other. [32 P]P_i labelling of *D. tschudii* electrocyte stacks was carried out for 28 h. AChR-rich membranes were prepared from this source, and lipid extracts were obtained from such membranes. [γ - 32 P]ATP labelling of AChR-rich membranes from *T. marmorata* was carried out for 5 min. Data are means $\pm$ S.D. for three separate experiments. Conditions are given in the Experimental section.

Phospho- lipid	Phospholipid composition (mol %) (A)		Incorporation of [³² P]P _i				Relative specific radioactivities [(B)/(A)]	
			(pmol/mg of protein)		(%) (B)			
	<i>D. tschudii</i>	<i>T. marmorata</i>	<i>D. tschudii</i>	<i>T. marmorata</i>	<i>D. tschudii</i>	<i>T. marmorata</i>	<i>D. tschudii</i>	<i>T. marmorata</i>
PC	36.3±2.4	40.7±0.9	0.8±0.2	—	1.3	—	0.03	—
PE	32.4±2.1	33.9±0.9	0.1±0.05	—	0.3	—	0.01	—
PS	15.2±3.8	12.6±1.6	5.6±2.4	—	9.4	—	0.62	—
SPH	11.2±1.7	4.8±1.1	—	—	—	—	—	—
CL	0.7±0.3	1.2±0.3	—	—	—	—	—	—
PA	2.0±0.4	1.0±0.4	17.7 (2)	0.07±0.01	29.8	4.4	14.9	4.4
PI	1.8±0.4	4.4±0.5	8.6±3.6	—	14.5	—	8.1	—
PIP	0.3±0.1	0.8±0.4	10.1±5.3	1.08±0.11	16.9	67.5	56.3	84.4
PIP ₂	0.1±0.01	0.5±0.1	16.6±3.3	0.45±0.03	27.8	28.1	278.0	56.2

acylglycerols (PI, lyso-PE and PPI), which we could identify and quantify (Table 2). Differences are also apparent within the same species. Other authors have not reported the presence of SPH, CL or PPI in *T. marmorata* membrane (e.g. Popot *et al.*, 1978), as shown here. Comparison of the phospholipid composition of treated membranes and NaOH extracts further revealed that the protein loss from the membrane was accompanied by the preferential release of certain phospholipids (Table 2). Thus the proportion of PA, PPI and lysophospholipids was significantly higher in the NaOH supernatants than in the corresponding residual and non-extracted membranes.

The enrichments in PA, PIP and PIP₂ in the supernatant are about 2-, 8- and 12-fold respectively (Table 2).

In order to test whether the pH and not the peripheral-protein extraction was associated with the liberation of lipids from AChR-rich membranes, control experiments were undertaken with lithium 3,5-di-iodosalicylate, a chaotropic agent known to deplete peripheral proteins from AChR-rich membranes (Elliot *et al.*, 1980) at pH 8.0. The major peripheral protein released from AChR-rich membranes, namely the 43000-*M_r* protein, is nearly isoelectric at this pH. The treatment was carried out at 4 °C in order to restrict the differences with the NaOH procedure to a minimum of variables (pH 8.0 as against pH 11.0). This resulted in a diminished protein extraction ($\sim$ 5.5%) as compared with the usual 15% protein released at room temperature (Steck & Yu, 1973; Criado & Barrantes, 1984), but the important fact is that lipids were also extracted at pH 8.0. The lipid phosphorus/protein ratio was about 0.2 for lithium 3,5-di-iodosalicylate extracts, indicating that the net amount of lipid phosphorus extracted was less than for NaOH extraction. The observed ratio of PPI (6 nmol) mass to all other phospholipids (23 nmol) in lithium di-iodosalicylate extracts was 0.26 in comparison with 0.1 in NaOH extracts. This indicates that the latter reagent extracted proportionately more PPI than did

NaOH, and that factors other than charge alone must be responsible for the enrichment of these two acidic phospholipids in the aqueous phase, since a higher ionization of the PPIs would be achieved at pH 11.0 than at pH 8.0.

The polyphosphoinositide pool in electrocytes and AChR-rich membranes

Given the known lability of the PPIs and their extremely high turnover (Hawthorne & Pickard, 1979; Van Rooijen *et al.*, 1985), their finding in the AChR-rich membranes, which undergo a relatively long purification procedure, suggests that at least part of these phospholipids withstood such manipulations and constitute a rather stable pool. In order to test this hypothesis, experiments *in vivo* were undertaken using 32 P labelling of freshly dissected electrocyte stacks from the electric organ of the marine ray *Discopyge tschudii* and [γ - 32 P]ATP labelling of AChR-rich membranes of *T. marmorata* (both members of the family Torpedinidae). Preliminary experiments indicated a steady incorporation of 32 P into the electrocyte lipids up to 28 h incubation with the sole addition of glucose as energy supply. AChR-rich-membrane fractions were subsequently purified from the electrocyte stacks.

Table 3 compares the labelling profile of *Discopyge tschudii* and *Torpedo marmorata* AChR-rich membranes. The overall compositions of the two preparations are rather similar, except for the slightly higher inositol phosphoacylglycerol content in *T. marmorata* AChR-rich membranes. Inositol phosphoacylglycerols displayed the highest incorporation of 32 P in comparison with all other lipids in AChR-rich membranes under the two experimental conditions used. In both species the major phospholipid constituents displayed little if any 32 P incorporation; acidic phosphoacylglycerols, such as PPI and PA, accumulated most of the label. The phosphorylation of the latter had not been reported in this experimental system. Under basal incubation conditions the 32 P labelling in *D. tschudii* was highest in PA,

Table 4. Extraction of [^{32}P]-labelled glycerophospholipids from AChR-rich membranes of *D. tschudii* by alkaline treatment

^{32}P labelling of living electrocyte stacks was carried out for 28 h *in vitro*. AChR-rich membranes were obtained from pools of the electrocyte stacks. Data are the means for two separate samples.

Phospholipid	Radioactivity					
	Control pellet		NaOH			
			Supernatant		Pellet	
	(%)	(d.p.m.)	(%)	(d.p.m.)	(%)	(d.p.m.)
PC	1.1	554	0.5	25	0.9	449
PE	0.3	143	—	—	0.2	102
PS	22.7	10788	17.0	859	18.9	8933
PI	0.4	173	4.0	205	0.1	45
PA	26.6	12657	32.8	1658	24.0	11445
Lyso-PC	0.3	139	—	—	0.2	109
PI 4-phosphate	12.4	5914	13.7	690	14.5	6920
PI 4,5-bisphosphate	34.0	16234	31.8	1606	38.2	18179
Amount (mg) of protein (%) . . .	1.833 (99.1)		0.333 (17.4)		1.583 (82.6)	

followed by PIP_2 and PIP ; in the case of *T. marmorata* membranes, 95% of the labelling was restricted to the PPI pool, PIP attaining higher levels of phosphorylation (Table 3). When the mass of the individual phospholipids was taken into account, relative specific activities could be calculated (Table 3). PPI stands out among all other phosphorylated phospholipids in AChR-rich membranes of the two species.

Co-extraction of radiolabelled phospholipids and peripheral proteins

Given the relatively high specific radioactivity achieved by labelling of *D. tschudii* electrocytes *in vitro*, stacks of these cells were incubated for 28 h with the precursor ($\text{Na}_2\text{H}^{32}\text{PO}_4$), AChR-enriched membranes were purified therefrom and submitted to NaOH extraction. No label was detected in total lipid extracts of control supernatants. Lipid radioactivity, accumulated predominantly in acidic phospholipids, was recovered in the alkali extract of NaOH-treated membranes (Table 4). PPI accounted for more than 50% of the extracted radioactivity.

Alkaline extraction modifies the phosphorylation pattern of AChR-rich-membrane proteins

The conditions used in the present study to phosphorylate AChR membrane lipids resulted in the labelling of three AChR subunits (β , γ , δ) and non-receptor membrane proteins (Figs. 1a and 1a'). Alkali stripping of AChR-rich membranes extracts principally two groups of proteins, in the M_r -100000 and -43000 regions respectively (Neubig *et al.*, 1979; Elliot *et al.*, 1980; Fig. 1b). Under such conditions a net decrease was observed in the level of phosphorylation of these two groups of proteins in autoradiograms and in the total ^{32}P incorporation into AChR-rich-membrane proteins (Fig. 1b'). Concomitantly, the AChR γ -subunit displayed decreased levels of phosphorylation (Fig. 1b'). When the amount of ^{32}P incorporated into each chain was quantified by scintillation counting of the individual bands excised from polyacrylamide gels, it was found that the radioactivity associated with the γ -chain

decreased by about 40% on NaOH treatment, whereas that of the two other labelled chains was not significantly affected. Two cycles of NaOH extraction resulted in an even more pronounced decrease of the phosphorylation levels (Figs. 1d and 1d'). Additions of $1\text{ }\mu\text{M}$ -cyclic AMP, up to $200\text{ }\mu\text{M}$ - Ca^{2+} , with or without $100\text{ }\mu\text{M}$ -calmodulin, under the conditions reported to stimulate cyclic AMP- (Huganir & Greengard, 1983; Smilowitz *et al.*, 1981; Hayashi & Amakawa, 1985) and Ca^{2+} /calmodulin-stimulated protein kinases were without effect on the phosphorylation pattern. On the other hand, Mn^{2+} over the concentration range 5–10 mM was found to increase up to 4-fold the phosphorylation levels of alkali-stripped AChR-rich membranes. Attempts to restore the level of ^{32}P incorporation by co-incubation of alkali-stripped membranes with the neutralized NaOH supernatant (Figs. 1c and 1c') were unsuccessful.

DISCUSSION

Treatment of membranes with the NaOH and lithium 3,5-di-iodosalicylate extraction procedures for AChR-rich membranes has been shown to result in the depletion of non-receptor peripheral membrane proteins (Neubig *et al.*, 1979; Elliot *et al.*, 1980). Similar results have been obtained on extraction with 2,3-dimethylmaleic anhydride and 3,4,5,6-tetrahydrophthalic anhydride (Eriksson *et al.*, 1983). Here we have shown that not only proteins, but also glycerophospholipids and predominantly certain classes thereof, are depleted from AChR-rich membranes of *T. marmorata* and *D. tschudii* when these procedures are applied. Reference has previously been made to the possibility that alkaline treatment affected membrane lipids (Johansson *et al.*, 1981), but experimental evidence produced subsequently dispelled this indirect proposal (Neugebauer & Zingsheim, 1982).

PPI, which had not been either found or reported as not being present in this type of membrane (e.g. Gonzalez-Ros *et al.* 1982), have been identified in AChR-rich membranes and extracted together with peripheral proteins. Though the mass of the PPI pool

Fig. 1. Coomassie Blue (a–f) and autoradiographic (a'–d') patterns of [32 P]ATP-labelled AChR-rich membranes from *T. marmorata*

(a, a') Native membrane; (b, b') NaOH-extracted membrane; (c, c') NaOH-extracted membrane phosphorylated in the presence of neutralized alkali extract (i.e. supernatant, track e); (d, d') membrane submitted to two successive NaOH extractions (see Barrantes, 1982b); (f) M_r standards (myosin, β -galactosidase, phosphorylase *b*, bovine serum albumin and carbonic anhydrase). The right panel (a'–d') is a 48 h autoradiograph overexposed, in order to show changes in minor phosphorylated bands. The arrow points to the M_r -43000 proteins. The inset shows the decreased phosphorylation of the AChR γ -subunit after alkali stripping. The four AChR chains (α , β , γ , δ) and the non-receptor ν and ζ proteins and actin (A) are indicated.

with respect to the total phospholipids was small, its relative proportion in the extracts was significantly high (Table 2). Furthermore, the high activity of this minor pool became apparent in two types of experiments.

(1) The 32 P incorporation in *D. tschudii* living electrocyte stacks incubated *in vitro* with $\text{Na}_2\text{H}^{32}\text{PO}_4$ for 28 h and subsequently yielding AChR-rich membranes by subcellular fractionation revealed that about 45% of the total label was accounted for by the polyphosphoinositide pool (Table 3). In the case of *T. marmorata*, purified AChR-rich membranes were incubated directly with [γ - 32 P]ATP for 5 min; the labelling pattern was essentially the same (Table 3), the consequences of wider implication. Thus the results of the first series of 'in vitro' labelling experiments make clear that PI kinase and PIP kinase must be very active in the electrocyte, and that the EDTA present in the homogenization medium suffices to inhibit phosphodiesterase activity during subsequent purification of the membranes. The increased synthesis of PA, probably via diacylglycerol kinase, does not appear to involve a concomitant production of PI and/or zwitterionic phospholipids like PC or PE.

(2) The second series of experiments with purified *T. marmorata* AChR membranes indicate that the specific

kinases involved in PPI phosphorylation display different activities in the intact electrocyte and in the AChR-rich membrane, PIP and PIP₂ accumulating most of the label in each case (Table 3). The PPI pool as a whole remains the most active one in any case.

Changes were observed in the pattern of protein phosphorylation of the γ AChR chain on alkali stripping (Fig. 1). This AChR subunit is a substrate for two protein kinases in AChR membranes: a cyclic AMP-dependent kinase and a tyrosine-specific kinase (Huganir & Greengard, 1983; Huganir *et al.*, 1984). Alkaline stripping of the membranes diminished phosphorylation of this AChR chain without affecting the Mn^{2+} -stimulated reaction; the bivalent cation still increased the labelling of the chain 4-fold in alkaline-extracted membranes. Mn^{2+} stimulation of phosphorylation has been interpreted to imply the involvement of a tyrosine-dependent kinase (Huganir *et al.*, 1984, and references therein). If this interpretation is correct, the endogenous tyrosine kinase activity exhibited by these membranes should be associated with a tightly-membrane-bound protein. In the case of some hormone receptors, e.g. the insulin receptor, tyrosine kinase activity is displayed by the receptor protein itself.

Attempts to restore other phosphorylation reactions by addition of alkali extracts to the AChR membranes were unsuccessful (Fig. 1).

Determination of the location of AChR structural domains by means of immunochemical techniques requires permeabilization of the otherwise-sealed membrane vesicles after treatment of the membranes with lithium 3,5-di-iodosalicylate (Froehner *et al.* 1983; Lindstrom *et al.*, 1985) or NaOH (Criado *et al.*, 1985b). The effectiveness of the latter procedure was attributed to the formation of 'holey' membranes and/or extraction of peripheral proteins by alkaline treatment (Criado *et al.*, 1985a). Two comments are pertinent in this context. Firstly, although, as found here, depletion of phospholipids by these treatments is not massive, and nor is the lipid/protein ratio significantly different on alkaline treatment, receptor-receptor interactions are altered in these membranes (Barrantes *et al.*, 1980; Criado & Barrantes, 1984), and regions of the AChR otherwise hidden in the bilayer may become accessible. Secondly, although alkaline and lithium di-iodosalicylate extractions may be without apparent effects on ligand-binding (Neubig *et al.*, 1979) and ion-translocation (Elliot *et al.*, 1980) properties of the AChR, the increased rotational (Lo *et al.*, 1980; Bartholdi *et al.*, 1981) and translational (Criado *et al.*, 1982) mobility of the AChR on NaOH treatment suggests changes in the local lipid environment of the receptor protein. Indeed, recent Raman-spectroscopy studies indicate the acquisition of a temperature-dependent phase transition in the AChR-rich membranes after NaOH treatment (Aslanian & Negrier, 1985). Caution should be exercised in the interpretation of experimental work concerned with the availability of antibodies or ligands on the AChR protein and with the influence of the lipid environment on receptor function. As shown here, it is no longer possible to consider AChR-rich membranes submitted to peripheral-protein extraction as equivalent to native ones in terms of lipid composition, some dynamic properties of the AChR protein itself, and the pattern of phosphorylation of AChR chains.

It is known that some peripheral membrane proteins interact with specific types of lipid. The specificity can be rather wide, as is the case with myelin basic protein, or absolute, as in the case of the B-subunit of cholera toxin and ganglioside G_{M1} (Fishman, 1982). The fact that particular types of lipid are involved has an important consequence for membrane structure: the protein-lipid interaction affects not only the specific protein and lipid class involved, but the membrane in general. Lateral phase separation of lipid may occur, as documented in the case of vesicular-stomatitis-virus matrix protein (Wiener *et al.*, 1983): membrane regions in contact with peripheral proteins are enriched in a particular lipid, with a concomitant relative depletion of the latter in the rest of the membrane bilayer. Since the function of most peripheral proteins extracted from AChR-rich membranes is still unknown, it is too early to speculate on the specificity of the associations reported here and on the particular proteins involved. We can only surmise that such associations already exist in the native membrane between non-receptor proteins and lipid classes, and that they do not necessarily appear after peripheral-protein depletion. The structural implications may be analogous to those discussed above for other membrane systems; their functional consequences remain to be explored.

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