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RECONSTITUTION OF ACETYLCHOLINE RECEPTOR FROM *TORPEDO CALIFORNICA* WITH HIGHLY PURIFIED PHOSPHOLIPIDS: EFFECT OF α-TOCOPHEROL, PHYLLOQUINONE, AND OTHER TERPENOID QUINONES

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SUMMARY: Acetylcholine receptor from *Torpedo californica* can be incorporated by the cholate dialysis procedure into liposomes prepared with crude soybean phospholipids (asolectin). Vesicles reconstituted with asolectin depleted of neutral lipids or with a mixture of pure phospholipids, are less active in catalyzing carbamylcholine-sensitive Na^+ flux. Inclusion of α-tocopherol or certain quinones such as coenzyme Q_{10} or vitamin K_1 during reconstitution yields vesicles with carbamylcholine-sensitive Na^+ flux which, under optimal conditions, was considerably higher than that observed with vesicles reconstituted with crude phospholipid mixtures.

The lipid environment of the acetylcholine receptor in the electric organ may be an important determinant of the receptor's properties. Studies have shown that delipidation of the receptor leads to changes in the affinity of the receptor for agonists (1,2) and that treatment with phospholipase A_2 inhibits ion fluxes in membrane vesicles (3). Experiments with phospholipases are often difficult to evaluate, however, because of side effects by hydrolysis products. For example, Na^+ efflux from vesicles prepared from *Torpedo californica* electroplax was inhibited by unsaturated fatty acids (3). An alternative approach to the role of phospholipids is reconstitution experiments (4). Among the limitations of such experiments is the difficulty in differentiating whether a lipid is required for biological activity or for reconstitution.

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In the present paper we show that the reconstitution of the acetylcholine receptor by the procedure of Epstein and Racker (5), originally performed with a crude mixture of soybean phospholipids, can be carried out with purified phospholipid. However, to obtain active vesicles either α-tocopherol, coenzyme Q_{10} or certain other quinones have to be present during reconstitution.

MATERIALS AND METHODS

Crude soybean lipids (asolectin) and coenzyme Q_{10} were purchased from Sigma Chemical Co., St. Louis, MO. Chromatographically pure plant phosphatidylethanolamine and phosphatidylcholine and bovine phosphatidylserine were obtained from Avanti Biochemical, Birmingham, AL. Synthetic α-dl- tocopherol, phylloquinone (vitamin K_1), menadione, phytol, and isophytol were gifts from Hoffmann-La Roche, Nutley, NJ. Tocopheryl quinone was prepared by oxidation of α-tocopherol in gold chloride solution (6). ^{22}Na-sodium chloride (carrier-free) was purchased from New England Nuclear, Boston MA. Partially purified soybean phospholipids were prepared by washing asolectin with dry acetone and by extracting with ether (7). Preparation of liposomes, reconstitution of acetylcholine receptor and uptake of ^{22}Na measurements were performed as described previously (5). All assays were performed at the "10 sec" point with carbamylcholine concentration of 2×10^{-4}M. Lipid concentrations were 25 mg/ml and the ratio of lipid to protein was 16 (w/w).

RESULTS AND DISCUSSION

In the original experiments (5) crude asolectin was used for the reconstitution of the acetylcholine receptor. We have now observed that after extraction of asolectin with acetone to remove neutral lipids (7), the Na^+ flux activity of reconstituted vesicles was greatly reduced. The neutral lipid extract, when added during the reconstitution procedure, restored activity. Purification of the active ingredient proved difficult, however, because of great losses in activity during handling and inhibition of Na^+ flux when excess neutral lipid was used.

As can be seen from Fig. 1, α-tocopherol at high concentrations (20 to 40%) effectively replaced the neutral lipid fraction during the reconstitution of vesicles. Several other quinones such as coenzyme Q, phylloquinone (vitamin K_1), and tocopherylquinone, were also active, but menadione, phytol, isophytol, and butylated hydroxytoluene or hydroxyaniline had little or no activity.

Appreciable carbamylcholine-stimulated Na^+ fluxes were observed with vesicles reconstituted with phosphatidylethanolamine and phosphatidylserine

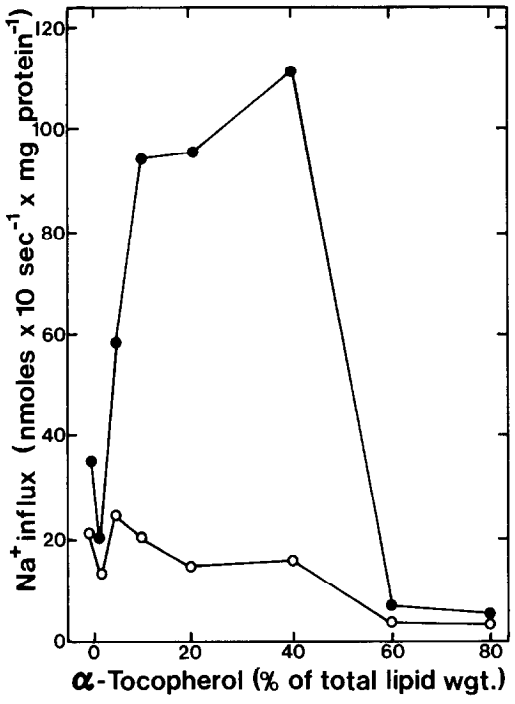

Fig. 1. Effect of α-tocopherol on reconstitution of acetylcholine receptor with partially-purified soybean phospholipids. Reconstitution and assay were performed with partially purified phospholipids as described under Materials and Methods. Values for crude asolectin were 80 and 25 nmoles Na^+/mg protein with and without carbamylcholine, respectively.
● Na^+ influx in presence of carbamylcholine
0 Na^+ influx without carbamylcholine

in a ratio of 3:1 (Table I). The activity varied with different batches of lipids and membrane preparations ranging from 30 to 70% of the activity with asolectin. The activity of these vesicles was, however, markedly increased when α-tocopherol, phylloquinone or coenzyme Q_{10} were incorporated during reconstitution (Table II). This stimulation by α-tocopherol or quinones was always observed at appropriate concentrations though some variability was noted with respect to optimal concentrations. For example, in the experiments shown in Fig. 1, 60% α-tocopherol was highly toxic, while 40% was tolerated; in the experiments shown in Table I, 40% α-tocopherol or 10% phylloquinone was toxic. In the case of crude neutral lipid fractions from asolectin, careful titrations were necessary because excesses markedly impaired the activity of the reconstituted vesicles.

TABLE I

Effect of phylloquinone and tocopherol on reconstitution of acetylcholine receptor with highly purified phospholipids

	Neutral lipid, % of total lipid weight														
	None			Phylloquinone (5%)			Phylloquinone (10%)			α-Tocopherol (20%)			α-Tocopherol (40%)		
	-carb	+carb*	Δ	-carb	+carb	Δ	-carb	+carb	Δ	-carb	+carb	Δ	-carb	+carb	Δ
	Na^+ influx, nmoles/mg protein														
PE	19	14	-	10	10	-	10	10	-	10	10	-	10	10	-
PE+PS(3:1)	20	39	19	21	195	174	10	35	25	20	147	127	24	40	16
PC+PE(1:1)	16	17	-	10	10	-	25	25	-	18	43	25	10	10	-
PC+PS(3:1)	96	87	-	34	36	-	16	25	-	10	18	-	28	114	86
PC	138	149	-	104	115	-	27	29	-	15	16	-	10	18	-
PC+PE+PS (1.5:1.5:1)	20	15	-	10	30	20	10	31	21	12	47	35	24	89	65

* 2×10^{-4} M carbamylcholine

Sum of weight of phospholipid and neutral lipid was constant and reconstitution and assay was performed as described under Materials and Methods. Na^+ influx for asolectin vesicles were 74 and 23 nmoles Na^+/mg protein with and without carbamylcholine, respectively.

TABLE II

Effect of α-tocopherol, phylloquinone and coenzyme Q_{10} on reconstitution of the acetylcholine receptor with phosphatidylethanolamine (PE) and phosphatidylserine (PS) at a ratio of 3:1.

	% of total lipid weight	Na^+ influx (nmoles x 10 sec^{-1} x mg $protein^{-1}$)		
		- carb	+ carb*	Δ
Asolectin		21	111	89
		24	113	
PE + PS (3:1)		12	48	33
		19	50	
+ α-Tocopherol	10	17	123	106
	20	25	198	173
	30	24	196	172
	40	21	204	183
+ Phylloquinone	2	24	92	68
	4	18	157	139
	6	30	198	168
	8	29	138	109
	10	26	128	102
+ Coenzyme Q_{10}	4	20	144	124
	6	31	167	136
	8	32	207	175

*2×10^{-4} M carbamylcholine

Desensitization (5) of carbamyl-sensitive Na^+ flux and sensitivity to curare was observed with proteoliposomes reconstituted with purified lipids and quinones.

Since butylated hydroxytoluene or hydroxyaniline were ineffective, it seems unlikely that α-tocopherol acts as an anti-oxidant (8). The high concentration of α-tocopherol or the quinones required for reconstitution suggest that these neutral lipids affect the packing of the phospholipid bilayer. This interpretation is supported by the observation that α-tocopherol is also required for the incorporation of the Excitability-Inducing Material into black lipid membranes (9). Attempts to incorporate the quinone after reconstitution of the vesicles has been completed have not yielded reproducible results. This approach needs to be explored further in order to differentiate between an effect of the quinones on the reconstitution process or on the activity of the reconstituted receptor protein (4).

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