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Conductance Changes Produced by *l*-Norepinephrine on Lipid Membranes Containing a Proteolipid from the Bovine Spleen Capsule

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SUMMARY

A proteolipid isolated from bovine spleen capsule, with a high affinity for binding [7-^{3}H]*dl*-norepinephrine, has been incorporated into artificial lipid bilayer membranes that separate two aqueous phases containing ions. To one of these phases several drugs were added, and the electrical conductance changes were recorded. The addition of *l*-norepinephrine produced a transient conductance change whose amplitude was related to the dose. *l*-Isoproterenol gave a smaller response, and *d*-norepinephrine had no effect. Membranes made with large amounts of phosphatidylcholine also reacted to *l*-norepinephrine. Since this phospholipid is absent from the proteolipid which binds norepinephrine, it is concluded that phosphatidylcholine gives a nonspecific reaction. Several control experiments and the observation that the conductance change toward *l*-norepinephrine is blocked with phentolamine suggest that this proteolipid may play a receptor role in the spleen capsule.

INTRODUCTION

Studies from this laboratory have demonstrated that certain proteolipids (1) (that is, hydrophobic lipoproteins) extracted from the cerebral cortex or from nerve-ending membranes of central synapses show high affinity for binding *d*-tubocurarine (2, 3), serotonin (4), atropine (5), and adrenergic blocking agents (6, 7). Furthermore, a special proteolipid with cholinergic ligand-binding properties has been isolated from the electric organs of *Torpedo* and *Electrophorus* (8). In these studies only the first step of the drug-receptor interaction, a high-affinity binding, was demonstrated. The second step, eliciting a response, has been explored recently by Parisi *et al.* (9), with the incorporation of the "cholinergic proteolipid" from *Electrophorus* into artificial lipid membrane bilayers (i.e., black) separating two water compartments containing ions. After the local application of acetylcholine there was a sudden increase in conductance across the membranes, which was transient and reversible.

Fiszer de Plazas and De Robertis have isolated from bovine spleen capsule a proteolipid peak with a high affinity for binding [7-^{3}H]*dl*-norepinephrine; in contrast, other proteolipid peaks, isolated from the same tissue, showed no binding.[1] It thus seemed

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[1] S. Fiszer de Plazas and E. De Robertis, unpublished observations.

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pertinent to carry out a similar study on artificial lipid membranes into which these proteolipids could be incorporated. These membranes responded with a transient conductance change to *l*-norepinephrine but not to *d*-norepinephrine, while membranes containing the other proteolipids gave no reaction. Membranes containing large concentrations of phosphatidylcholine were also sensitive to *l*-norepinephrine; however, this phospholipid was absent from the peak binding [7-^{3}H]*dl*-norepinephrine.

METHODS

Isolation of proteolipids. Bovine spleens were obtained from the slaughterhouse, packed on ice, and processed in the laboratory. After sectioning into small slabs, the spleen capsule was separated from the parenchyma by mechanical teasing. This procedure gave reddish fragments of capsule, which were subjected to three consecutive washings in distilled water and homogenized in a Waring Blendor for 1 min at high speed. In each case the tissue fragments were filtered through a Büchner filter. The final suspension contained small, yellowish fragments of capsule that were practically free of parenchymal cells as checked with histological sections. Such fragments were frozen, lyophilized, and stored under vacuum over a desiccant. Two grams of this material were homogenized in 50 ml of chloroform–methanol (2:1, v/v) in an Ultra-Turrax (Karl Kolb, Frankfurt). After standing at room temperature for 5 min, the extract was filtered through Whatman No. 2 filter paper and the residue was washed with 10 ml of chloroform–methanol (2:1, v/v). The final volume of the extract was noted, and half the volume of chloroform was added. The extract was then evaporated under vacuum at room temperature to a final volume of 3 ml.

For studying the binding of [7-^{3}H]*dl*-norepinephrine (6.6 Ci/mmole, New England Nuclear Corporation), the chloroform–methanol extract was incubated (final concentration of ligand, 10 nM) for 15 min at room temperature and then subjected to chromatography on a column of Sephadex LH-20 (2.1 × 18 cm) that had been equilibrated overnight in chloroform.

Elution was performed with 80 ml of chloroform followed by chloroform–methanol (15:1, 10:1, 6:1, and 4:1, v/v) (20 ml of each). The eluate was monitored at 278 nm with an LKB Uvicord ultraviolet absorption meter. The flow rate was controlled at 0.5 ml/min, and samples were collected as 2-ml fractions. Chemical determinations and radioactivity measurements were performed as described by La Torre *et al.* (8).

The elution pattern showed four proteolipid peaks eluted with chloroform (subsequently designated peaks 1–4) and one with chloroform–methanol, 4:1 (peak 5). Only the first peak showed a high affinity for binding the catecholamine and radioactivity eluted coincident with this peak. The chromatographic pattern was also reproducible with higher concentrations of ligand and in the absence of the drug. At each concentration of norepinephrine, control experiments were performed by adding the radioactive drug dissolved in chloroform–methanol, 2:1. No radioactivity was detected in the eluate unless an additional 120 ml of chloroform-methanol 2:1 were added to the column. Details of the binding of [7-^{3}H]*dl*-norepinephrine to the various fractions, as well as data on protein recovery, will be presented in a forthcoming paper.

Membranes were made by incorporating one of the five proteolipid peaks into the membrane-forming solution to give a final concentration of 3.5 μg/ml. In peaks 1 and 5 the phospholipids were separated and determined by thin-layer chromatography on silica gel (10) (see Table 1).

TABLE 1

Proteolipid protein and phospholipid content of two peaks used to make artificial membranes

The peaks were obtained by Sephadex LH-20 column chromatography of bovine spleen capsules. Results are the means of two experiments.

	Peak 1 proteolipid	Peak 5 proteolipid
	μg/ml	*μg/ml*
Protein	8.0	1.6
Phosphatidylcholine		4.5
Phosphatidylinositol	10.0	3.7
Phosphatidylethanolamine	50.0	3.0

TABLE 2

Composition of artificial membranes, their resistance at 75 mV, and mean conductance changes produced by injection of various concentrations of l norepinephrine

Results are means ± standard errors. 0 0 0

Membrane composition	n^a	Resistance	Conductance change		
			0.5 mM	5 mM	50 mM
		ohms/cm²		*nanoampere*	
Cholesterol, 10 mg/ml	15	9.4×10^6			
Cholesterol, 10 mg/ml; peak 1 proteolipid, 3.5 μg/ml	7	7.6×10^6	0.04 ± 0.002	0.08 ± 0.001	0.12 ± 0.03
Cholesterol, 10 mg/ml; peak 2, 3, 4, or 5 proteolipid, 3.5 μg/ml	6	3.9×10^6	0	0	0
Cholesterol, 10 mg/ml; phosphatidylinositol, 100 μg/ml	6	3.8×10^5	0	0	0
Cholesterol, 10 mg/ml; phosphatidyl ethanolamine, 100 μg/ml	5	7.6×10^5	0	0	0
Cholesterol, 10 mg/ml; phosphatidyl choline:					
10 μg/ml	4	8.0×10^6	0	0	0
100 μg/ml	5	7.3×10^5	0.07 ± 0.003	0.09 ± 0.004	0.11 ± 0.02
5000 μg/ml	5	8.3×10^5	0.10 ± 0.003	0.17 ± 0.003	0.19 ± 0.02

[a] Number of experiments.

Composition of membranes. Chloroform and methanol were obtained from Merck, A.G., and were redistilled before use; tetradecane, from British Drug Houses; cholesterol (99 standard for chromatography) and α-DL-phosphatidylcholine, from Sigma Chemical Company; and phosphatidylinositol and phosphatidylethanolamine, from Mann Research Laboratories. The compositions of the artificial membranes are summarized in Table 2. The standard solution, containing chloroform–methanol–tetradecane (1.0:0.8:0.4 by volume), was employed as the solvent for cholesterol and phospholipids. The proteolipids were added in the corresponding solvent in which they were eluted from the column. The solutions were freshly prepared, gassed with N_2, preserved from humidity, and discarded after 2 days of use.

Membrane formation and apparatus. The artificial membranes were made by brushing the membrane solution across a 1-mm hole in a Teflon septum separating two chambers containing 100 mM NaCl and 50 mM Tris buffer (pH 7.2) (Fig. 1). As described by

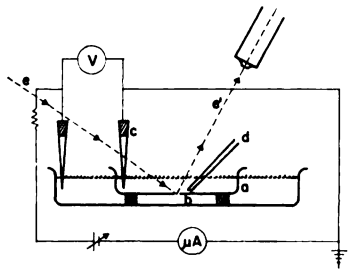

FIG. 1. *Apparatus used to study action of drugs on artificial membranes*

A Teflon cup (*a*) is immersed in a Petri dish. The membranes are made in a 1-mm hole (*b*); *c*, calomel electrodes; *d*, micropipette for injection of 50 μl of drug solution. A light beam (*e*, *e'*) reflects on the membrane and is observed with the stereomicroscope.

Vásquez *et al.* (11), lipid film made on a horizontal septum behaved in the same way as that brushed on a vertical partition. The circuit was similar to that of Ehrenstein *et al.* (12); a voltage difference across the membrane was maintained constant by a direct-current source and was measured via calomel electrodes, with agar bridges, using

a Keithley d.c. voltmeter, Model 200 B. The current was measured with a Keithley 150 A microammeter and recorded with a Heath EUW servorecorder. Since the applied voltage remained constant, the recorded d.c. potential change reflected variations in membrane conductance. The resistance of the various membranes at 75 mV is indicated in Table 2. Membrane formation was followed with a stereomicroscope; the membranes became black within 5–10 min. The drugs, dissolved in water, were applied by means of a fine capillary tube ending about 2 mm from the positively charged side of the membrane (Fig. 1).

The following drugs were tested: *l*- or *d*-norepinephrine bitartrate monohydrate, *l*-isoproterenol bitratrate dihydrate (Sterling-Winthrop), acetylcholine chloride, histamine dihydrochloride, serotonin creatine sulfate, and pilocarpine hydrochloride (Sigma); hexamethonium bromide (Squibb); *d*-tubocurarine chloride and theophylline (Calbiochem); Dibenamine hydrochloride (Szabó); propranolol hydrochloride (Ramón) and phentolamine mesylate (Ciba). For all drugs the concentration is expressed below in moles per liter. In each case a 50-μl aliquot from these solutions was injected. The adrenergic blocking agents phentolamine and propranolol were diluted in the bathing solution prior to membrane formation, after which the agonists were applied to the membrane.

RESULTS

In an artificial membrane containing only cholesterol, the current-voltage (I/V) curve showed an ohmic relationship between 0 and 175 mV. Above this voltage the relationship was not linear, and above 200 mV breakage of the membrane invariably occurred. Similar I/V curves were with all the membranes employed throughout the study. For the study of conductance changes by drugs, the membranes were maintained at 75 mV.

Figure 2A shows the effect of *l*-norepinephrine (50 μl of 0.5 mM solution) applied to cholesterol membranes containing 3.5 μg/ml of the norepinephrine-binding peak from spleen capsule. Injection of the drug resulted in a sudden increase in conductance, which returned to the original level in 20–40 sec.

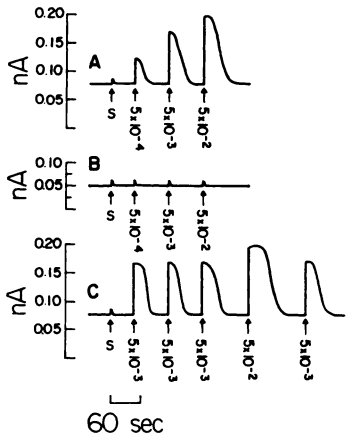

FIG. 2. *Conductance changes produced in artificial membranes by injection of 50 μl of l-norepinephrine at different concentrations*

S, injection of bathing solution. The arrows indicate the time of injection. *A*. Membrane containing the proteolipid of peak 1 (see the text). *B*. Membrane containing proteolipid of peak 5. *C*. Membrane containing the proteolipid of peak 1 treated with five consecutive doses of *l*-norepinephrine (see the text).

Both the amplitude and length of the response increased with the dose, reaching a maximum with the injection of 50 μl of 50 mM *l*-norepinephrine. This response was reproducible in films obtained from freshly prepared membrane-forming solutions properly gassed with nitrogen. Injection of the optical isomer *d*-norepinephrine gave completely negative results. Experiments with peaks 2, 3, 4, and 5, which do not bind [7-^{3}H]*dl*-norepinephrine, gave negative results with *l*-norepinephrine (Fig. 2B).

Since the proteolipid of peak 1 contains relatively large amounts of phospholipids, it was of interest to determine whether the conductance change was due to the protein or to the lipid moiety. This problem became even more important when it was observed that membranes containing phosphatidylcholine (100 or 5000 μg/ml in the membrane-forming solution) gave a conductance change with *l*-norepinephrine somewhat similar to that given by the proteolipid (Table 2). A study of the phospholipids present in the proteolipid of peak 1 revealed that phosphatidylcholine was absent. Furthermore, the small amount of phosphatidylcholine present

in peak 5 (see Table 1) was not enough to give a reaction with *l*-norepinephrine. In fact, incorporating as much as 10 μg/ml of phosphatidylcholine into the membrane-forming solution produced no reaction. The same result occurred in films made from solutions containing only cholesterol or in those in which 100 μg/ml of phosphatidylinositol or phosphatidylethanolamine had been incorporated (Table 2). Other control experiments, which gave negative results, included the application of saline solutions, distilled water, or 10 mM sodium bitratrate.

Figure 2C shows the effect of succesive applications of *l*-norepinephrine on the membrane made from cholesterol and containing the norepinephrine-binding proteolipid (peak 1) of the spleen capsule. Five consecutive doses, one of them 10 times greater than the others, did not change the pattern of response. Figure 3 shows a dose-response curve in which the amplitude of the conductance change is plotted against log *l*-norepinephrine concentration (concentration refers to the original solution from which a 50-μl aliquot was applied). The response tended to become saturated with the injection of 50 μl of 50 mM *l*-norepinephrine.

Table 3 shows the response of membranes containing the proteolipid of peak 1 to the injection of various drugs. The cholinergic drugs acetylcholine, hexamethonium, and *d*-tubocurarine gave negative results. *l*-Isoproterenol, a *beta*-adrenergic agonist, gave responses significantly lower than those of *l*-norepinephrine ($p < 0.01$). The adrenergic antagonists also produced a response, but that of the *beta*-adrenergic blocking agent propranolol was smaller than that given by phentolamine ($p < 0.01$). Other drugs giving a response after injection were pilocarpine, histamine, and serotonin (Table 3).

When the adrenergic antagonist phentolamine was incorporated into the bathing

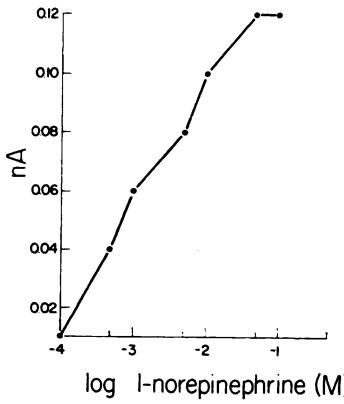

FIG. 3. *Amplitude of conductance change as a function of log l-norepinephrine concentration*

Concentration refers to the original solution from which a 50-μl aliquot was taken. In each case the drug was applied to the membrane containing the proteolipid of peak 1. Each point is the mean of five experiments.

TABLE 3

Conductance changes in artificial membranes produced by injection of l-norepinephrine and other drugs

In each case the mean response (± standard error) of seven experiments is shown. All membranes contained 10 mg/ml of cholesterol and 3.5 μg/ml of the proteolipid of peak 1.

Drug	Concentration	Conductance change
	mM	*nanoampere*
Adrenergic agonists		
l-Norepinephrine	0.5	0.04 ± 0.002
	5	0.08 ± 0.001
	50	0.12 ± 0.03
d-Norepinephrine	0.5	0
	5	0
	50	0
l-Isoproterenol	0.5	0.01 ± 0.01
	5	0.02 ± 0.01
	50	0.05 ± 0.03
Adrenergic antagonists		
Phentolamine	0.8	0.10 ± 0.03
Propranolol	0.8	0.04 ± 0.01
Cholinergic agonists		
Acetylcholine	1	0
	10	0
Pilocarpine	10	0.12 ± 0.01
Cholinergic antagonists		
Hexamethonium	1	0
	10	0
d-Tubocurarine	1	0
	10	0
Other drugs		
Histamine	1	0.05 ± 0.02
	10	0.12 ± 0.02
Serotonin	0.1	0.07 ± 0.01
	1	0.10 ± 0.07
Theophylline	10	0

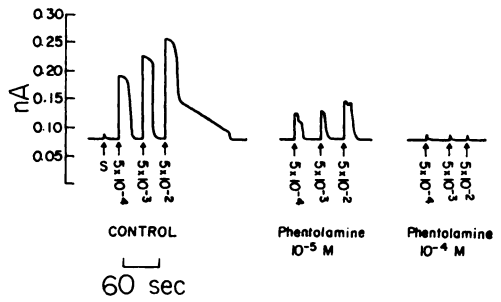

FIG. 4. *Effect of phentolamine on response of artificial membranes containing peak 1 proteolipid to l-norepinephrine*

The antagonist was incorporated into the bathing solution, at the concentrations indicated, prior to membrane formation. The agonist was injected in 50-μl aliquots at the concentrations indicated. The arrows indicate the time of injection.

solution prior to the formation of the artificial membrane containing the proteolipid of peak 1, there was an evident blockade of the effect of *l*-norepinephrine (Fig. 4). Phentolamine at a concentration of 10 μM greatly reduced the response, and at 0.1 mM completely abolished it. Similar results were obtained with propranolol for the response given by *l*-isoproterenol. In membranes containing 100 μg/ml of phosphatidylcholine in the original solution, there was no blockade of the response to *l*-norepinephrine with phentolamine at 0.1 mM.

DISCUSSION

The use of artificial lipid membranes for the study of the "cholinergic proteolipid" from *Electrophorus* opened the possibility of studying conductance changes resulting from drug-receptor interactions (9). In the present study also, the possibility of contamination with material of bacterial origin similar to the excitability-inducing material of Mueller and Rudin (13) could be ruled out. The proteolipid was made from freshly dissected spleen capsules, lyophilized, and extracted with organic solvents. Furthermore, the proteolipid exhibited conductance changes only when excited by specific chemical agents. We also ruled out changes in conductance that might have resulted from sudden variations in hydrostatic pressure between the two sides of the membrane, as observed by Parisi and Rivas (14) when more than 50 μl of solution were applied.

The membranes made with the "cholinergic proteolipid" contained a larger proportion of protein (10 μg/ml), as well as cholesterol and total phospholipids from the cerebral cortex, and their resistance was about 5×10^4 ohms/cm^2 (9). In the present study membranes containing total phospholipids from the cerebral cortex could not be used, because the large phosphatidylcholine content "masked" the response produced by the proteolipid of peak 1. Therefore we used artificial membranes made from cholesterol or from cholesterol and pure phospholipids, which had 10 times the resistance of those containing total phospholipids from cerebral cortex (Table 2). However, our experimental conditions have not permitted us to establish exactly the concentration of the drug reaching the membrane surface, which may be influenced by factors such as convection currents or uneven distribution of the drug.

The results described here reveal that artificial membranes made from cholesterol and containing small amounts of the norepinephrine-proteolipid of the spleen capsule undergo transient conductance changes with the application of *l*-norepinephrine. Evidence is also presented that the conductance change may be a consequence of the protein moiety and does not depend on the phospholipids present in the peak (see Tables 1 and 2). Furthermore, membranes made with proteolipids which do not bind norepinephrine did not react to the injection of *l*-norepinephrine.

The fact that phosphatidylcholine, at much greater concentrations than the proteolipid, induces a reaction toward *l*-norepinephrine is of interest because of a possible role of phospholipids in adrenergic mechanisms. Belleau (15) has suggested that phosphatidylcholine could provide a hydrophobic cavity for the adrenergic binding site. Our results indicate that this phospholipid probably gives a rather unspecific response with *l*-noreinephrine. This interpretation is also supported by the lack of blockade of this response with phentolamine.

The fact that histamine, serotonin, and pilocarpine gave a response with the proteolipid of peak 1 poses some interesting problems, since these drugs are able to induce contraction or relaxation of the smooth muscle cells of the spleen capsule (16). It may be that the various receptors present in this tissue are different molecular entities that are separated in the same region of the chromatogram, but this supposition needs further work to be proven. Another interesting possibility is that, while in solution, the adrenergic drugs, as well as histamine, serotonin, and pilocarpine, may have a common β-ethylamine group which may be important in the interaction with the macromolecular receptor (17).

Strongly in favor of a specific conductance response toward *l*-norepinephrine is the fact that the optical *d*-isomer gave negative results. The blockade of the reaction to *l*-norepinephrine obtained with phentolamine is also in line with this interpretation.

The results reported here do not allow us to draw conclusions about the *alpha* or *beta* nature of the response obtained with the proteolipid of peak 1 of the spleen capsule. The fact that a purely *beta*-agonist, *l*-isoproterenol, produced a conductance change much smaller than that given by *l*-norepinephrine would be more in accord with the well-known pharmacological evidence that *alpha*-adrenergic receptors predominate in the spleen capsule.

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REFERENCES

1. J. Folch and M. Lees, *J. Biol. Chem.* **191**, 807 (1951).
2. E. De Robertis, S. Fiszer and E. F. Soto, *Science* **158**, 928 (1967).
3. E. De Robertis, S. Fiszer, J. M. Pasquini and E. F. Soto, *J. Neurobiol.* **1**, 41 (1969).
4. S. Fiszer and E. De Robertis, *J. Neurochem.* **16**, 1201 (1969).
5. E. De Robertis, J. González-Rodríguez and D. N. Teller, *FEBS Lett.* **4**, 4 (1969).
6. S. Fiszer and E. De Robertis, *Life Sci.* **7**, 1093 (1968).
7. E. De Robertis and S. Fiszer de Plazas, *Life Sci.* **8**, 1247 (1969).
8. J. L. La Torre, G. S. Lunt and E. De Robertis, *Proc. Nat. Acad. Sci. U. S. A.* **65**, 716 (1970).
9. M. Parisi, E. Rivas and E. De Robertis, *Science* **172**, 56 (1971).
10. V. P. Skipski, R. F. Peterson and M. Barclay, *Biochem. J.* **90**, 374 (1964).
11. C. Vásquez, M. Parisi and E. De Robertis, *J. Membrane Biol.* In press.
12. G. Ehrenstein, H. Leccar and R. Nossal, *J. Gen. Physiol.* **55**, 119 (1970).
13. P. Mueller and D. O. Rudin, *Nature* **213**, 603 (1967).
14. M. Parisi and E. Rivas, *Biochim. Biophys. Acta* **233**, 469 (1971).
15. B. Belleau, *Ann. N. Y. Acad. Sci.* **139**, 580 (1967).
16. P. de Franciscis, *Agg. Fisiol.* **6**, 79 (1962).
17. W. T. Salter, "A Textbook of Pharmacology." Saunders, Philadelphia, 1952.