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In Vitro Excitation of Purified Membrane Fragments by Cholinergic Agonists

I. Pharmalogical Properties of the Excitable Membrane Fragments

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Summary. Excitation of membrane fragments by cholinergic agonists is measured *in vitro* by a filtration technique. Membrane fragments which contain high levels of the enzyme acetylcholinesterase and presumably originate from the innervated excitable faces of electroplax are first purified from homogenates of electric organ of *Electrophorus electricus* by centrifugation in a sucrose gradient. Then the fragments, which make closed vesicles or microsacs, are equilibrated overnight with a medium containing $^{22}Na^+$. After equilibration of the inside of the microsacs with the outside medium, the suspension is diluted into a nonradioactive medium. The $^{22}Na^+$ content of the microsacs as a function of time is then followed by rapid filtration on Millipore filters. In the presence of cholinergic agonists, the time course of $^{22}Na^+$ release changes: the rate of $^{22}Na^+$ release increases. This increase is blocked by *d*-tubocurarine and is absent with microsacs derived from the non-innervated inexcitable membrane of the electroplax. The response to cholinergic agonists is thus followed on a completely cell-free system, in a well-defined environment. The dose-response curves to cholinergic agents obtained *in vitro* agree, quantitatively, with the dose-response curves recorded *in vivo* by electrophysiological methods. In particular, the dose-response curve to agonists is sigmoid, the antagonism between *d*-tubocurarine and carbamylcholine competitive, and the antagonism between tetracaine and carbamylcholine noncompetitive. The effects of two different affinity labeling reagents on the response to agonists and on the catalytic activity of acetylcholinesterase are followed in parallel on the same microsac preparation. The effects of dithiothreitol and of gramicidin A on the microsacs are studied and are found to be similar to those observed *in vivo* with the isolated electroplax.

Up to now the excitability of biological membranes has been almost exclusively studied with whole cells using electrophysiological techniques. Although this approach has yielded considerable information and will certainly continue to do so, the molecular basis of this important regulatory mechanism remains largely unknown. New methods and simpler preparations are required.

First attempts to obtain a subcellular excitable preparation included those of Oikawa, Spyropoulos, Tasaki and Teorell (1961) and of Baker, Hodgkin and Shaw (1961) who showed that squid giant axons are still excitable after removal of the cytoplasm when perfused internally with a physiological solution. Experimentation with this preparation presents difficulties however; the external surface of the excitable membrane is covered by Schwann cells, an important fraction of neural cytoplasm embeds the internal surface (Hoskin, 1966), and electrical measurements are still essential.

In this series of papers, we shall describe and analyze the main properties of an entirely *acellular* system which appears particularly convenient for the study of chemical excitation. It consists of membrane fragments which are purified from crude homogenates of electric organ of *Electrophorus electricus*. In our preparation, these membrane fragments form closed vesicles, or "microsacs", of very small size (average diameter, 0.1 μm and the permeability of these microsacs to radioactive ions can easily be followed by rapid filtration on Millipore filters in a well-defined ionic environment. The principle advantage of this preparation resides in the fact that the microsacs which derive from the excitable membranes of electric tissue retain their excitability *in vitro*: they respond to cholinergic agonists by an increase of permeability to cations. We are thus able to follow chemical excitation (references in Nachmansohn, 1959, 1971) on a completely acellular system in a well-defined environment by a simple, direct and quantitative measure of ion flux.

In the first paper, we shall be concerned with the general description of the system and its pharmacological properties. We shall compare extensively the sensitivity of the excitable microsacs to a variety of effectors – including cholinergic agents – with that of the excitable membrane of the isolated electroplax. In general, the *in vitro* and the *in vivo* data agree, and, moreover, the *in vitro* measurements are more reliable and quantitative than the electrical potential measurements on the whole cell.

In paper II of this series, the ionic permeability of the excitable microsacs and the effects of cholinergic agonists and ionic environment on this permeability are analyzed in some detail. In paper III, we compare quantitatively the selective increase of permeability and the amount of cholinergic agonist specifically bound to the cholinergic receptor integrated in the microsacs Finally, paper IV describes the ultrastructure, at high resolution, of two classes of microsacs.

A preliminary presentation of this work has already been published (Kasai & Changeux, 1970).

Materials and Methods

The electric eels, *Electrophorus electricus*, were bought alive from Paramount Aquarium (Ardsley, New York), and were stored in Paris in the Tropical Aquarium of the Musée des Arts Africains et Océaniens (thanks to the generous help of Mr. Goussef and Mr. Denise).

Membrane fragments which originate from the *innervated faces* of the electroplax were purified, as a concentrated suspension, on the basis of their high acetylcholinesterase (AcChE) content, following a method described by Changeux, Gautron, Israel and Podleski (1969). A 10 g portion of fresh electric organ was cut with scissors in fragments of approximately 1 cm^3 and suspended in 50 ml of 0.2 M sucrose in distilled water. The suspension was homogenized with a Virtis apparatus in a 250 ml glass vessel, carefully maintained at 0 °C with crushed ice, for 1 min and 30 sec at 75% of maximal speed. The homogenate was then centrifuged at $5.000 \times g$ (6,500 rpm) for 20 min in a rotor L of a Servall centrifuge. The supernatant fluid was collected and centrifuged at high speed in a SW 25 rotor of a Beckman LH 20 ultracentrifuge. In general, the 30 ml centrifuge tube contained, from the bottom to the top, 5 ml of 1.0 M sucrose, 5 ml of 0.4 M sucrose, and 20 ml of low-speed supernatant, carefully layered on top of each other. Sometimes 25 ml of low-speed supernatant was disposed on 5 ml of 1.4 M sucrose. In this latter case, the suspension of membrane was more concentrated but was contaminated by soluble cytoplasmic proteins. The gradients were centrifuged for 5 to 7 hr at $64,000 \times g$ (25,000 rpm) immediately after preparation. Fractions of 1 ml were collected after perforation of the bottom of the tubes with a needle. The specific activity of AcChE and the response to carbamylcholine was measured in each fraction. The fourth and (or) fifth tubes collected usually had the highest activity in AcChE and contained the most excitable fragments. A detailed profile of AcChE, protein, flux and excitability in a typical sucrose gradient after centrifugation is given in Fig. 7 of paper II of this series. The recovery of AcChE in the membrane fragments was generally 80 to 100% of the total amount of AcChE added on top of the gradient.

The specific activity of AcChE in the fraction used in the flux experiments usually ranged between 0.5 and 3 moles acetylthiocholine (AcTCh) hydrolyzed per hr per g protein. The concentration of AcChE was between 3 and 5 moles of AcTCh hydrolyzed per hr per liter of microsacs suspension, and the concentration of membrane proteins in the suspension was between 2 and 5 mg per ml. In the method formerly used by Changeux *et al.* (1969), 5 instead of 20 ml of low-speed supernatant was added on top of the sucrose gradient. As a consequence, the total quantity and the concentration of membrane fragments in the recovered fractions were much lower, although the specific activity of AcChE in the purified fractions sometimes reached 7 moles of AcTCh hydrolyzed per hr per g protein.

Success in the flux measurements was found to depend on the concentration of AcChE-rich microsacs in the membrane fractions. This concentration is directly related to the concentration of AcChE-rich microsacs in the low-speed supernatant, which is itself determined by the homogenization procedure and the quality of the organ. Increasing the speed of the blade of our old Virtis apparatus from 75% up to 90% gave marked improvement in the recent purifications of excitable membrane fragments.

As mentioned later, flux experiments appeared to be highly reproducible within a preparation. However, the absolute values of the rates measured vary significantly from one preparation to the other. As a consequence, we shall identify a given group of experiments carried out with a particular preparation of microsacs by the number of the preparation (from 1 to 24).

Microsacs which originate from the *non-innervated membrane* were isolated following the procedure originally described by Bauman, Changeux and Benda (1970) on the

basis of their high content in the Na^+, K^+-activated, ouabain-sensitive ATPase. Homogenization and low-speed centrifugation were carried out in the same manner as for the excitable microsacs. The low-speed supernatant was then centrifuged at high speed in the following manner: 20 ml of low-speed supernatant was layered on a discontinuous gradient of 5 ml of 1.4 M sucrose and 5.0 ml of 1.0 M sucrose in a 30 ml lusteroid tube. The gradients were then centrifuged in a SW 25 rotor for 3 hr at 25,000 rpm in a Beckman preparative ultracentrifuge. The ATPase-rich fragments accumulated at the interface between 1.4 and 1.0 M sucrose. They were collected after perforation of the bottom of the tube, pooled, and then diluted twofold with distilled water and recentrifuged, for the same length of time and at the same speed, above a bottom layer of 5 ml of 1.4 M sucrose. The purified microsacs formed a thin layer at the interface between the supernatant and the 1.4 M sucrose solution; they were again collected after perforation of the bottom of the tube. Recovery of ATPase in this fraction was approximately 50% of the total ATPase added on top of the first sucrose gradient. The specific activity of ATPase in these fragments was 300 to 500 μmoles of ATP hydrolyzed per hr per mg protein at 37 °C, and that of AcChE was 0.080 moles of AcTCh hydrolyzed per hr per g protein at 27 °C.

AcChE was assayed with AcTCh as the substrate by the method of Ellmann, Courtney, Andress and Featherstone (1961). The assay mixture contained 5×10^{-4} M AcTCh, 5×10^{-4} M dithiobis-dinitrobenzoic acid in 5×10^{-2} M sodium phosphate buffered at pH 7.0. The total volume was 1.0 ml. The assay was carried out at 27 °C in a Zeiss PMQ spectrophotometer. The reaction was started by the addition to the assay mixture of, for example, 10 μliters of membrane suspension diluted 100-fold in 5×10^{-2} M sodium phosphate buffer, pH 7.0. The increase of optical density was recorded at 412 nm.

Protein concentration was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951), using bovine serum albumin as the standard.

Sucrose concentrations were measured by refractometry with a Zeiss Abbe's refractometer.

Measurement of the permeability of the microsacs to $^{22}Na^+$ was routinely performed in the following manner: 1 ml of membrane suspension in 0.7 to 0.8 M sucrose containing 2 to 5 mg of membrane protein per ml was mixed with 0.3 ml of an aqueous solution of $^{22}NaCl$ (prepared by the Radiochemical Centre, Amersham, Great Britain) containing 0.1 mC/ml of $^{22}Na^+$ with a specific activity close to 75 mC/mg. To the radioactive solution was then added 3 M NaCl (approximately 20 μliters) and enough distilled water (approximately 0.2 ml) to make the solution 8×10^{-6} M $^{22}NaCl$, 10^{-2} M nonradioactive NaCl, and 0.5 M sucrose. The pH of the suspension was, in the absence of added buffer, close to 7.0. The suspension was then stored at 4 °C, in the refrigerator, for 10 to 20 hr (overnight). The flux measurement was started by the 50-fold dilution of the radioactive microsacs suspension (0.1 in 5.0 ml) into a nonradioactive "dilution buffer" at room temperature (22 °C) containing 1.7×10^{-1} M KCl, 2×10^{-3} M $CaCl_2$, 1×10^{-3} M sodium phosphate, pH 7.0, with (or without) the cholinergic effector. In these conditions the osmotic pressure of the dilution medium was slightly lower than that of the equilibrium medium. At given times 1.0 ml samples were rapidly filtered on Millipore filters (HAW P 02500, 25ea, HA 0.45 μ, white plain, 25 mm) and washed three times with 3 ml of cold (0 °C) dilution buffer. Under these conditions 95% of the microsacs remain on the Millipore filter although their size is much smaller than that of the pores of the filter. The Millipore filters were then dried and soaked in 10 ml of scintillation counting medium containing 3 g of PPO and 0.3 g of POPOP in 1 liter of toluene. The flasks were counted for 10 min in either a Packard scintillation counter model 3003 on channel 3 with a gain of 25% and a window width of 0.050 to infinity or in an Intertechnique "spectromètre à scintillation liquide" ABAC SL 40 on channel C with a window width of 0 to 10. The

counting efficiency was 60 to 70%. The radioactivity remaining on the Millipore filter in the absence of microsacs (from 50 to 200 cpm depending on the batch of Millipore) was always subtracted from the total number of counts. Since the dilution of the concentrated microsacs suspension was always finite (approximately 50-fold), the equilibrium concentration of $^{22}Na^+$ in the microsacs after dilution was expected to be that of the diluted suspension. Therefore we routinely subtracted as well the number of counts corresponding to the total radioactivity remaining on the Millipore divided by the dilution factor. The radioactivity remaining on the filters which was associated with the presence of microsacs varied from one experiment to another from 200 to 2,000 cpm. This amount was always a linear function of the concentration of microsacs present in the dilution medium.

In the great majority of experiments, the permeability measurements were carried out on microsacs prepared from fresh electric tissue. Interestingly, microsacs which still present permeability properties and excitability can be prepared from frozen tissue following the standard procedure.

Results

The in Vitro Response of Purified Membrane Fragments to Cholinergic Agonists

In the eel electroplax, the surfaces of the cell which are excitable are distinct from those which carry the active transport of Na^+ and K^+ (reference in Nachmansohn, 1959, and in Changeux, Podleski, Kasai & Blumenthal, 1970). The active transport takes place at the level of the rostral part of the cell surface: the non-innervated membrane. The enhanced flux of ions caused by excitation is exclusively restricted to the caudal surface of the electroplax which receives the nerve terminals and is referred to as the innervated membrane. As shown by Bauman *et al.* (1970), fragments which belong to each of these two classes of cytoplasmic membranes are easy to separate, *in vitro*, by ultracentrifugation of crude homogenates of electric tissue. They are purified on the basis of their different enzymatic compositions. Fragments of the non-innervated membrane are expected to be rich in the active transport enzyme – the ouabain-sensitive, Na^+, K^+-activated ATPase. On the other hand, fragments of the innervated membrane should have a high content in AcChE, which is commonly associated with excitability and which, in fact, is present almost exclusively on the innervated surface of the cell (*see* Benda, Tsuji, Daussent & Changeux, 1970). Indeed, fragments with high levels of ouabain-sensitive ATPase but low levels of AcChE are found to migrate to a higher density of sucrose ($d=1.14$; 1.15 M) than those with low levels of ATPase but high levels of AcChE ($d=1.09$; 0.65 M).

Both classes of membrane fragments make closed vesicles, or microsacs, but their permeability behavior and their sensitivity to cholinergic agents are different.

Our first step in the study of the sensitivity of these fragments to cholinergic agonists was the measure of their permeability to cations: we selected $^{22}Na^+$ as a particularly convenient radioisotope. The experimental procedure that we routinely used (*see* Methods) had two steps: (1) an overnight incubation in the presence of $^{22}Na^+$ to equilibrate the microsacs with the radioactive permeant, and (2) a dilution of the equilibrated suspension into a nonradioactive "dilution medium".

The $^{22}Na^+$ content of the microsacs as a function of time was then measured by rapid filtration of the diluted suspension on Millipore filters (Fig. 1). The curves of equilibration obtained are characterized by the following two parameters:

(1) *The time for half equilibration* (τ_0). This time was, in general, close to 20 min for $^{22}Na^+$, but depended on the nature of the permeant ion. A significant variation of τ_0 from one membrane preparation to another was encountered (from 15 to 24 min), but with a given preparation of microsacs the error of τ_0 was always smaller than 5%.

(2) *The apparent volume* (V_{app}). The extrapolation of the curve of equilibration at zero time gives an amount of radioactivity (R_0) which corresponds to $^{22}Na^+$ either trapped within the microsacs or irreversibly bound to their membrane. Extrapolation back to zero time was, in a few cases, done by fitting the experimental points by the equation $N(t)=N_0\left(\frac{t_0}{t+t_0}\right)^v$, where t is the time, and v a constant (0.45 ± 0.05). In general, extrapolation was done by eye and gave almost the same results. We define the apparent volume as the quantity:

$$V_{app}=\frac{R_0}{R\times p}$$

where p is the mass of membrane proteins filtered on the Millipore, and R is the radioactivity of the solution of incubation per unit volume. This quantity represents a volume per unit mass and more rigorously might be called a specific volume. The apparent volume usually ranged between 0.5 and 2 µliters/mg protein. In the experiment reported in Fig. 1, the apparent volume was 1.1 µliters/mg protein.

In paper II of this series, we shall extensively study and discuss the transport properties of the microsacs. Briefly, we show that the amount of radioactivity which remains on the Millipore filter after overnight equilibration with $^{22}Na^+$ corresponds to $^{22}Na^+$ trapped within the microsacs. The time course of $^{22}Na^+$ release from the microsacs is shown to be insensitive to 10^{-3} M ouabain and thus corresponds to a passive efflux of $^{22}Na^+$. Using a very simple filtration technique, we are able to measure the permeability of membrane fragments to Na^+ in a well-defined environment.

Fig. 1a shows that the AcChE-rich microsacs are excitable *in vitro*. After overnight equilibration with $^{22}Na^+$, the microsacs were diluted in the presence of a cholinergic agonist, 10^{-4} M carbamylcholine (Carb). Under these conditions, the apparent volume did not change but the rate of $^{22}Na^+$

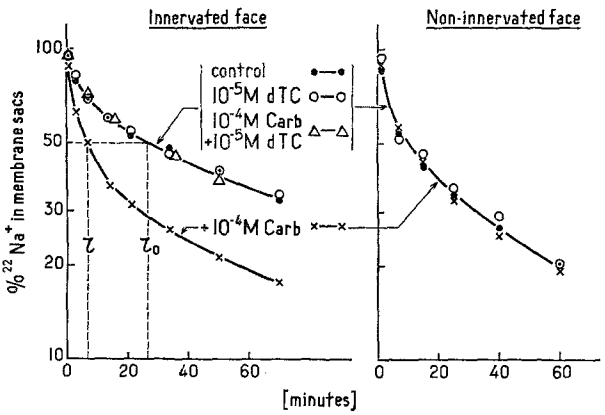

Fig. 1. Specific effect of a cholinergic agonist, carbamylcholine (Carb), on the efflux of $^{22}Na^+$ from excitable microsacs. *Left:* Excitable AcChE-rich microsacs derived from innervated membranes. dTC is the abbreviation for *d*-tubocurarine. *Right:* Non-excitable ATPase-rich microsacs derived from non-innervated membranes.—The method of preparation of the membrane fragments and the measurement of $^{22}Na^+$ efflux are described in the text. The concentration of proteins in the suspension filtered on Millipore filters was 89 µg/ml (innervated face) and 46 µg/ml (non-innervated face). The specific activity of AcChE in the preparation of excitable microsacs (no. 1) was only 0.8 mmoles of AcTCh/hr/mg protein at 27 °C. τ and τ_0 are the times for half equilibration in the presence and in the absence of cholinergic agonist respectively

exit increased three- to fourfold. This increase was blocked by 10^{-5} M *d*-tubocurarine (*d*-tubo) which, in the absence of Carb, had no significant effect on $^{22}Na^+$ efflux. The observed effect thus corresponds to a permeability response of the microsacs to Carb. In order to evaluate the specificity of the response to Carb, we then studied the behavior of the ATPase-rich microsacs. Fig. 1 illustrates that, after overnight equilibration, the ATPase-rich microsacs, like the AcChE-rich ones, retained $^{22}Na^+$ and released it upon dilution. The rate of equilibration was somewhat faster than that of the AcChE-rich microsacs but did not change in the presence of cholinergic agonists or antagonists. As expected from their origin, the ATPase-rich microsacs are *not* excitable *in vitro*. Only the microsacs which are derived from the innervated face of the electroplax are excitable.

Quantitative Comparison of the Dose-Response Curves to Cholinergic Agonists Obtained in Vitro and in Vivo

In the preceding paragraph, we have seen that, in a *qualitative* manner, the physiological response to cholinergic agonists still operates, *in vitro*, on isolated membrane fragments. An important question is then to what

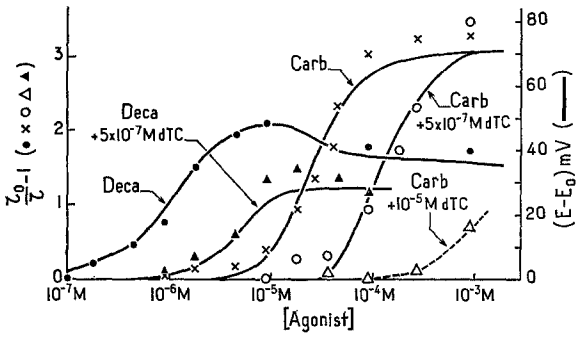

Fig. 2. Comparison of the dose-response curves obtained *in vivo* by measuring steady-state membrane potentials with a single isolated electroplax and *in vitro* by following $^{22}Na^+$ efflux. Carb and Deca represent carbamylcholine and decamethonium, two specific cholinergic agonists of the electroplax *in vivo*. dTC is the abbreviation for *d*-tubocurarine, a typical cholinergic antagonist. The electrophysiological data are from Changeux and Podleski (1968). Preparation no. 1

extent is there *quantitative* agreement between the data obtained *in vitro* and those obtained *in vivo* by electrophysiological techniques?

We used as a measure of the *in vivo* response the measurements of the electric potential of the isolated electroplax of Changeux and Podleski (1968) recorded intracellularly, by the technique of Higman, Podleski and Bartels (1964). We have taken as the measure of the response to a given dose of cholinergic agonist the difference $(E-E_0)$ between the resting potential (E_0) and the *steady-state* potential (E) observed after a 1 to 5 min bath application of cholinergic agonist. In Fig. 2 we have reported these data as a continuous line and superimposed on them (dots) the *in vitro* results.

In vivo, as *in vitro*, the experiments were performed at room temperature and in a dilution medium with ionic strength ($\Gamma/2=0.177$) very close to that of the physiological Ringer's solution ($\Gamma/2=0.180$). We have taken as the measure of the *in vitro* response the relative increase of the rate of $^{22}Na^+$ exit: $\frac{\tau_0}{\tau}-1$, τ_0 and τ being the times for half equilibration of ^{22}Na between the inside and the outside of the microsacs in the absence and in the presence of cholinergic agonists, respectively. In order to allow quantitative comparison between both sets of results, we have normalized all the data to the same maximal response to decamethonium (Deca).

The general shapes and positions of the dose-response curves are very similar. Almost the same midpoints and thus the same "apparent affinities" are obtained with the two agonists tested, Carb and Deca. In addition, both *in vitro* and *in vivo*, the maximal response (or "intrinsic activity") to Carb is larger than that to Deca. Moreover, *d*-tubo, a typical cholinergic

antagonist, acts quantitatively in exactly the same manner *in vitro* and *in vivo*. In the presence of *d*-tubo, the dose-response curves to Carb and Deca are shifted to the right and almost the same apparent inhibition constant for *d*-tubo is measured. Podleski and Changeux (*unpublished results*) have noticed that, with the isolated electroplax, the antagonism between *d*-tubo and Carb is simply competitive, whereas that between *d*-tubo and Deca is accompanied by a decrease of the maximal response to Deca, i.e. is not strictly competitive. Interestingly, *d*-tubo presents, both qualitatively and quantitatively, the same behavior *in vitro*.

The agreement is good enough to allow a comparison of some characteristic details of the shape of the dose-response curves. For instance, as first found by Higman *et al.* (1964), and further confirmed and extended by Changeux and Podleski (1968), the dose-response curves to various agonists recorded *in vivo* with the isolated electroplax systematically deviate from simple Langmuir isotherms. Their early parts show a slight but significant convexity to the abscissa (or sigmoid shape); they are converted into straight lines in the double logarithmic plot of Hill (Brown & Hill, 1922), and in this plot their slopes (or Hill coefficient) are systematically different from one (Fig. 3a). Depending on the agonist considered, the Hill coefficients range from 1.6 to 2.0 (Changeux & Podleski, 1968). Fig. 3 and the Table show that the same effects are present *in vitro*. The Hill coefficients measured in these conditions are very close, if not identical, to those reported *in vivo*. Further confirmation of the presence of cooperative effects in the response to cholinergic ligands was offered by the analysis of the shape of the dose-response curve to an antagonist, *d*-tubo, measured in the presence of a fixed concentration of Carb (10^{-4} M) as the agonist. Fig. 3b shows that, here again, the shape of the response curve to increasing concentration of *d*-tubo is sigmoid and has a Hill coefficient of 1.5. Apparent cooperative effects are thus present with both cholinergic agonists and cholinergic antagonists. Additional evidence in favor of this conclusion is offered by the occurence *in vitro* of the same conversion of shape of the dose-response curve to a given agonist in the presence of another agonist as observed by Changeux and Podleski (1968) on the isolated electroplax. Indeed, Fig. 4 shows that the early part of the dose-response curve to Carb is concave to the abscissa in the absence of Deca, but convex in its presence. The two curves cannot be converted by simple translation in a semilogarithmic plot. The effects of Carb and Deca are not additive but cooperative.

Finally, in the Table we have compared the results obtained with a variety of cholinergic agonists and antagonists on the microsacs with those available in the literature for the same compounds on the isolated

Table. *Parameters characteristic of the dose-response curves to various cholinergic agonists and antagonists obtained in vivo and in vitro*

Agonists	*In vivo*					*In vitro*			
	Temp. (°C)	K_{app} (M)	R_{max} (mV)	$\frac{R_{max}}{R_{max\ Deca}}$	n_H	Temp. (°C)	K_{app} (M)	$\frac{R_{max}}{R_{max\ Deca}}$	n_H
Decamethonium (C. & P.)	22	1.2×10^{-6}	50	1	1.63 ± 0.02	22	1.2×10^{-6}	1	1.7
						4	1.2×10^{-6}	1	1.7
Carbamylcholine (C & P.)	22	2.6×10^{-5}	69.5	1.39	2.0 ± 0.1	22	3.3×10^{-5}	1.56	1.7
(H.P & B.)		3.0×10^{-5}	60.0	1.20	1.8 ± 0.1		4×10^{-5}	1.87	1.5
							5×10^{-5}	1.68	1.7
						4	1.2×10^{-4}	1.6	1.5
							1.0×10^{-4}	1.65	1.7
Phenyltrimethyl ammonium (C. & P.)	22	1.2×10^{-5}	50	1	1.77 ± 0.05	22	2×10^{-5}	1.57	1.7
		1.3×10^{-5}	50	1	1.66 ± 0.05				
						4	4×10^{-5}	1.57	2.0
Acetylcholine $+5\times10^{-5}$ M eserine (M.B. & W.)	22	3×10^{-6}				22	4×10^{-6}	1.70	1.6
Acetylthiocholine $+5\times10^{-5}$ M eserine (M.B & W.)		5×10^{-5}				22	1.3×10^{-4}	1.55	1.0

Antagonists	*In vivo*		*In vitro*	
	Temp. (°C)	Ki_{app}	Temp. (°C)	Ki_{app}
d-Tubocurarine (agonist carbamylcholine) (C. & P.) (H.P. & B.)	22	1.6×10^{-7}	22	1.5×10^{-7}
			4	1.6×10^{-7}
d-Tubocurarine (agonist decamethonium) (C. & P.)	22	1.6×10^{-7}	22	1.5×10^{-7}
			4	1.5×10^{-7}
Flaxedil (agonist carbamylcholine) (C. & P.)	22	3.0×10^{-7}	22	3.3×10^{-7}
			4	3.5×10^{-7}
Hexamethonium (K. & W.)	22	3.0×10^{-5}	22	6.2×10^{-5}

K_{app} is the apparent dissociation constant of the considered agonist: it is the concentration of agonist which gives a response which is half that of the maximal response R_{max}. The presence of cooperative effects is neglected in this estimate. Ki_{app} is determined from the curve of antagonism of the response to a fixed concentration of agonist at increasing concentration of antagonist. In the *in vitro* measurements, the concentrations of agonist were $a=10^{-4}$ M Carb, and $A=3\times10^{-6}$ M Deca. The concentration of antagonist giving 50% inhibition of the response (I_{50}) was measured, and Ki_{app} was estimated from these measurements by the relation: $Ki_{app}=\dfrac{I_{50}}{1+\dfrac{A}{K_{app}}}$. We took for Carb: $K_{app}=4\times10^{-5}$ M, and for Deca: $K_{app}=1.20\times10^{-6}$ M. The term n_H is the slope of the straight line obtained by plotting the data in the system of coordinates of Hill (Brown & Hill, 1923). The *in vivo* data are from Changeux and Podleski (1968) (C. & P.), Higman, Podleski and Bartels (1964) (H.P. & B.), Mautner, Bartels & Webb (1966) (M.B. & W.), and Karlin and Winnik (1968) (K. & W.).

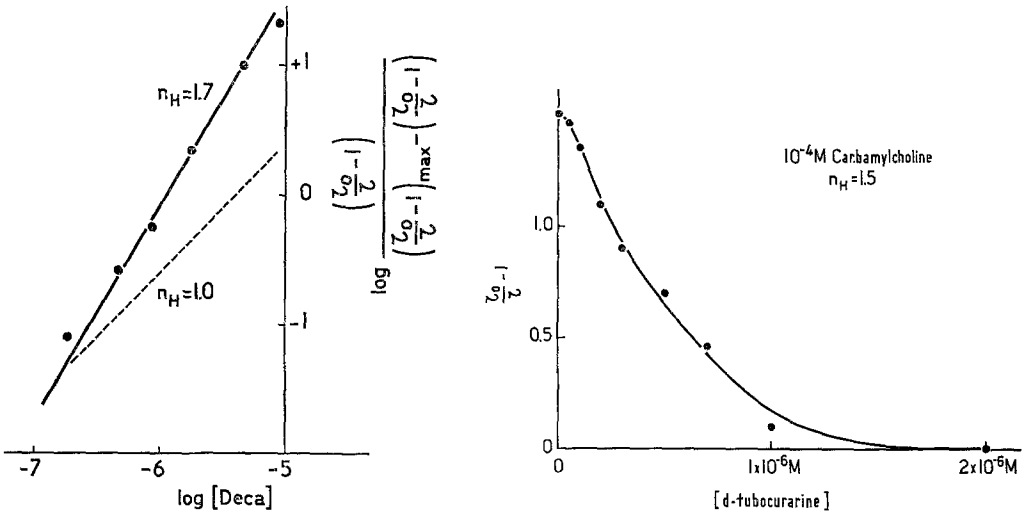

Fig. 3a and b. Cooperative effects associated with the *in vitro* response to decamethonium (Deca) (a) and the antagonism by *d*-tubocurarine of the response to Carb (b). (a) Hill plot of the same data as for Fig. 1. (b) Overnight incubation was carried out in the presence of 10^{-2} M NaCl, 5×10^{-1} M sucrose, and $^{22}Na^+$. The concentration of proteins was 5.1 mg/ml. The suspension was supplemented 30 min before dilution with 10^{-4} M Carb. The suspension was then diluted 80-fold in 1.7×10^{-1} M KCl, 2×10^{-3} M $CaCl_2$, 10^{-3} M Na-phosphate buffer, pH 7.0, supplemented with 10^{-4} M Carb, and the indicated concentration of *d*-tubocurarine. The apparent K_i for *d*-tubocurarine was found to be 1.3×10^{-7} M with $K_D=4\times10^{-5}$ M for Carb. Preparation no. 13

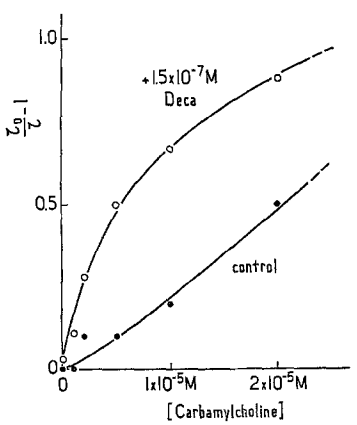

Fig. 4. Conversion of shape of the dose-response curve to Carb by Deca. The concentrated suspension of microsacs (no. 3) containing 4.4 mg of protein per ml, was incubated overnight with $^{22}Na^+$ in the standard conditions. Before dilution the suspension was preincubated with 1.5×10^{-7} M Deca for 50 min. Dilution was carried out in the standard dilution medium in the presence of 1.5×10^{-7} M Deca and the indicated concentration of Carb. In the presence of 10^{-4} M Carb, the maximal responses recorded were 1.8 and 2.0 in the presence and in the absence, respectively, of 1.5×10^{-7} M Deca

electroplax. There is quantitative agreement between the two series of results.

A slight but significant discrepancy should, however, be mentioned. As shown in Fig. 2 and in the Table, there is a difference between the *absolute values* of the maximal responses to Carb recorded *in vitro* and *in vivo*. When the curves are normalized to the same maximal response to Deca, the maximal response to Carb measured *in vitro* is 20 to 40% larger than that measured *in vivo*.

In Vitro Study of Some Characteristic Properties of the Excitable Membrane of the Electroplax

In this section we shall be concerned with some characteristic properties of the innervated membrane of the electroplax which are related only *indirectly* to the typical response to cholinergic agonists. Here again, we shall see that the *in vitro* behavior of the excitable microsacs is almost exactly the same as that of the excitable membrane *in situ*.

Noncompetitive Antagonism Between Carb and Tetracaine. Podleski and Bartels (1963) have reported that tetracaine blocks the response to Carb in a manner different from that of *d*-tubo. In the presence of tetracaine, the maximal response to Carb is lower; this antagonism between Carb and tetracaine is not competitive. Fig. 5 shows that this was found to be true also with the isolated membrane fragments, and that the concentration of local anesthetic which blocked the response *in vitro* was very similar

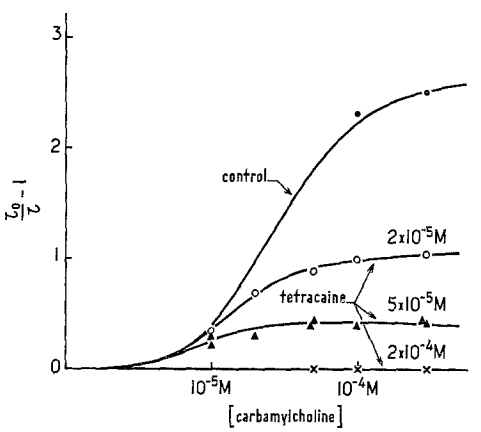

Fig. 5. Noncompetitive antagonism between a cholinergic agonist (Carb) and a local anesthetic (tetracaine). Overnight incubation was carried out in the standard conditions: the concentration of proteins was 6.6 mg/ml. The suspension was supplemented 10 min before dilution with tetracaine at the indicated concentration. The concentrated suspension was diluted 94-fold in the standard dilution medium supplemented with the indicated concentration of Carb and tetracaine. Preparation no. 6

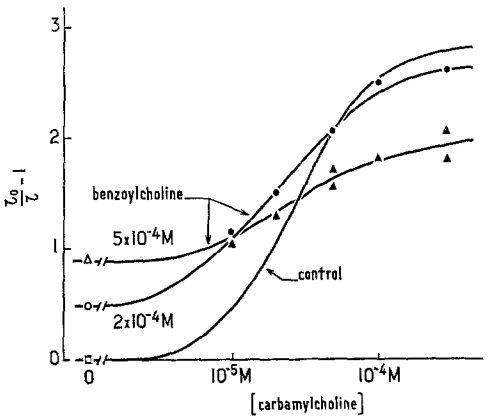

Fig. 6. Mixed agonistic and antagonistic effects of benzoylcholine. Exactly the same conditions as for Fig. 4 except that benzoycholine was used instead of tetracaine. Preparation no. 6

to that which blocks the response *in vivo*. About 4×10^{-5} M tetracaine was needed to reduce the response to 10^{-4} M Carb by 50% *in vivo*; the same result was obtained *in vitro* with 2×10^{-5} M tetracaine. *In vivo*, as *in vitro*, tetracaine behaves like a noncompetitive antagonist.

Mixed Agonistic and Antagonistic Effects of Benzoylcholine. Bartels (1965) has shown that, *in vivo*, benzoylcholine might act like either an agonist or an antagonist. It is an agonist since it causes the depolarization of the excitable membrane of the isolated electroplax. However, the maximal response, or intrinsic activity, of benzoylcholine is considerably lower than that of Carb or even Deca ($E_{max} - E_0 = 25$ mV, about 40% of the maximal response to Carb). It is an antagonist as well since, at saturating levels, it antagonizes the response to Carb as long as the Carb response is larger than its own maximal response. Fig. 6 shows that this particular behavior could be repeated *in vitro* and thus that benzoylcholine has mixed agonistic and antagonistic effects on the excitable membrane.

Covalent Binding of Affinity Labeling Reagents. A variety of affinity labeling reagents have been used by several groups of workers to identify the cholinergic receptor macromolecule. The first to be tested on the isolated electroplax was *p*-(trimethylammonium) benzene diazonium fluoroborate (TDF), a compound initially designed by Fenton and Singer (1965) to label the active sites of antibodies directed against the phenyl trimethylammonium hapten. As shown by Changeux, Podleski and Wofsy (1967), TDF acts on the isolated electroplax as an irreversible antagonist. Fig. 7 shows that it

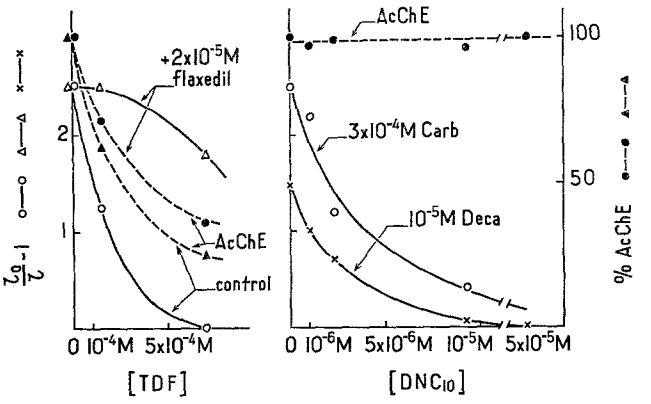

Fig. 7. Compared effects of two affinity labeling reagents on the *in vitro* response of excitable microsacs to Carb and Deca and on the activity of AcChE present in the same microsacs. TDF is for trimethyl ammonium benzene diazonium difluoroborate, DNC 10 M for dinaphthyl decamethonium mustard (*see* Rang & Ritter, 1969). Overnight incubation was carried out in the presence of 10^{-2} M NaCl, 5×10^{-1} M sucrose and $^{22}Na^{+}$. The concentration of protein was 4.4 mg/ml. The experiment was started by the addition to the microsac suspension, equilibrated at 22°, of TDF or DNC 10 M at the indicated concentration. The length of exposure was 10 min for DNC 10 M and 20 min for TDF. When TDF was used, the microsac suspension was adjusted to pH 8.0 by addition of Tris-HCl buffered at pH 8.0 up to a final concentration of 10^{-2} M Tris. The suspension was then diluted 70-fold in the usual dilution medium supplemented with either 3×10^{-4} M Carb (TDF and DNC 10 M) or 10^{-5} M Deca (DNC 10 M only). AcChE was assayed in the diluted suspension. In the case of the TDF experiment, exposure to TDF of the concentrated suspension was performed either in the presence of 2×10^{-5} M flaxedil or in its absence (control). Preparation no. 11

has the same action *in vitro*. Furthermore, in agreement with the observation of Changeux *et al.* (1967), flaxedil, a reversible antagonist, both *in vivo* and *in vitro* protects against the irreversible blockade by TDF of the response to Carb. The use of TDF to isolate the receptor protein meets, however, with a serious problem of specificity. As shown by Wofsy and Michaeli (1967) and by Meunier and Changeux (1969), TDF reacts as well with the catalytic site of AcChE and even with some "allosteric" sites on the same enzyme (Changeux, 1966). It would be possible to distinguish without ambiguity between these various classes of sites if the kinetics of reaction of TDF with each class of site was measurable. A considerable advantage of the microsac assay is that such a measurement is feasible. We have followed in parallel the effect of TDF on the activity of AcChE present in the fragments and the abolition of the permeability response to Carb. Fig. 7 shows that, in the presence of TDF, both the activity of AcChE and the response to Carb were strongly inhibited. As expected, TDF reacted with both the

cholinergic receptor site and the catalytic site of AcChE. However, the TDF concentration dependence of the rates of the two reactions differed strikingly. This difference was amplified in the presence of an antagonist such as flaxedil, which presents a high affinity for the cholinergic receptor site and a low affinity for the catalytic center of AcChE. Indeed, 2×10^{-6} M flaxedil strongly protected against the inactivation of the response to Carb, but had only little effect, at the concentration used, against the inactivation of AcChE. The catalytic center of AcChE and the active site of the cholinergic receptor are, unambiguously, distinct structures (*see* Podleski, 1967, and Changeux *et al.*, 1969).

This last conclusion is supported by studies carried out with another affinity labeling reagent discovered by Rang and Ritter (1969), the dinaphthyl decamethonium mustard (DNC 10 M). This compound is again, like TDF, an irreversible curare *in vivo*. Fig. 7 shows that it plays the same role *in vitro*. Interestingly, as shown in the same figure, the mustard had strictly no effect on the catalytic activity of AcChE. DNC 10 M is thus a much more specific labeling reagent of the cholinergic receptor site than TDF.

In Vitro Alteration by Dithiothreitol of the Response of the Microsacs to Cholinergic Agents. Karlin and associates (Karlin & Bartels, 1966; Karlin & Winnik, 1968) have shown that *in vivo* exposure of the innervated membrane of the electroplax to dithiothreitol (DTT), a reducing agent, is accompanied by characteristic perturbations of the cell response to cholinergic agents. In particular, the amplitude of the response to Carb decreases, and hexamethonium, a typical antagonist, becomes an agonist after DTT treatment. The same observation could be repeated *in vitro* (Fig. 8). After *in vitro* exposure of the microsacs to 10^{-3} M DTT, the response to Carb decreased and hexamethonium then behaved like Carb: it increased the efflux of Na^+ ions.

Again, the microsacs react in exactly the same manner as the excitable membrane of the cell. The molecular mechanisms which account for the process of excitation are thus integrally preserved after isolation and purification of the microsacs.

Effect of Gramicidin A on the Permeability of the Microsacs. Podleski and Changeux (1969) have shown with the isolated electroplax that the polypeptide antibiotic, gramicidin A (Gra), causes an irreversible increase in the permeability of the innervated membrane to Na^+ ions.

Fig. 9 illustrates that *in vitro* exposure of microsacs to Gra was accompanied, as well, by an increase of $^{22}Na^+$ release. In addition, Gra was active

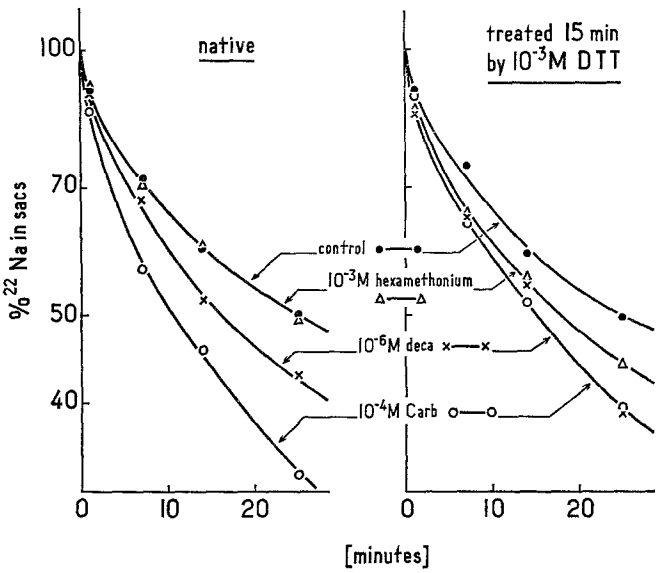

Fig. 8. Effect of dithiothreitol (DTT) on the response of excitable microsacs to hexamethonium, Carb and Deca. The concentrated suspension of microsacs containing 4.9 mg of protein per ml was exposed overnight to $^{22}Na^+$ in the presence of 10^{-2} M NaCl and 5×10^{-1} M sucrose. The experiment was started the next morning by adding DTT to the concentrated microsac suspension, equilibrated at 15 °C at a final concentration of 10^{-3} M DTT in the presence of 10^{-2} M Tris buffer, pH 8.0. The mixture was then diluted 60-fold in the standard dilution medium supplemented with the indicated concentration of Carb, Deca or hexamethonium. Preparation no. 2

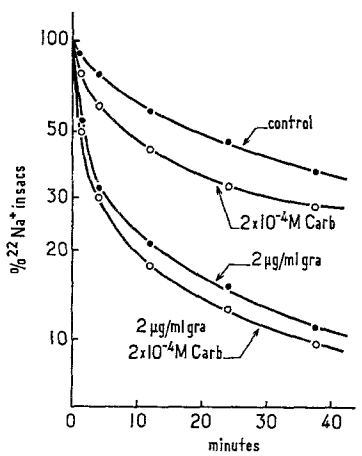

Fig. 9. Sensitivity of the excitable microsacs to 2×10^{-4} M Carb after exposure to gramicidin A (Gra). The concentrated suspension of microsacs (preparation no. 6) containing 6.6 mg/ml of membrane protein was incubated overnight with $^{22}Na^+$ in the standard conditions. It was preincubated with 16.7 µg/ml Gra for 10 min and then diluted 84-fold in the standard dilution medium supplemented or not with Carb; the final concentrations of Carb or Gra are indicated on the figure

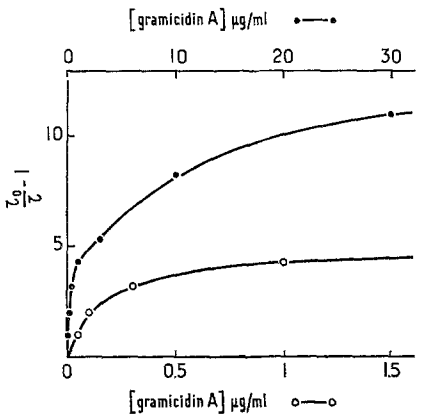

Fig. 10. Dose-response curve of excitable microsacs to Gra. The two curves correspond to two different scales for the Gra concentration. The concentrated suspension containing 6.1 mg of membrane protein per ml was first equilibrated with $^{22}Na^+$ in the standard conditions and subsequently diluted 60-fold in the standard dilution medium with or without Gra at the indicated concentration. Preparation no. 4

in the same concentration range both *in vitro* and *in vivo* (Fig. 10). Interestingly, at high levels of Gra, the rates of $^{22}Na^+$ release became much larger than those measured at saturating concentrations of Carb. This suggests that, in agreement with the interpretation of Podleski and Changeux (1969), new channels or pores for Na^+ ions, which are distinct from those associated with the cholinergic receptor appear after exposure of the microsacs to Gra.

In the presence of Gra, the microsacs still responded to Carb but, again in agreement with the Podleski and Changeux results, the amplitude of the response to Carb was markedly reduced (Fig. 9). However, in contrast with these authors' findings, the *apparent* cooperative effects observed *in vivo* have not yet been detected *in vitro* (Fig. 10).

Finally, in order to test the reversibility of the effect of Gra, we carried out the dilution experiment represented in Fig. 11. The microsacs were first exposed to 0.3 μg/ml Gra and then diluted 20-fold. The rates of $^{22}Na^+$ release of a suspension of microsacs treated by Gra were exactly the *same before* dilution and *after* dilution; dilution did not reverse the effect of Gra. We thus confirm the result of Podleski and Changeux (1969) that Gra acts as an *irreversible* membrane effector. This effect is strikingly different from the action of Carb, which, as extensively discussed in paper II, is always reversible.

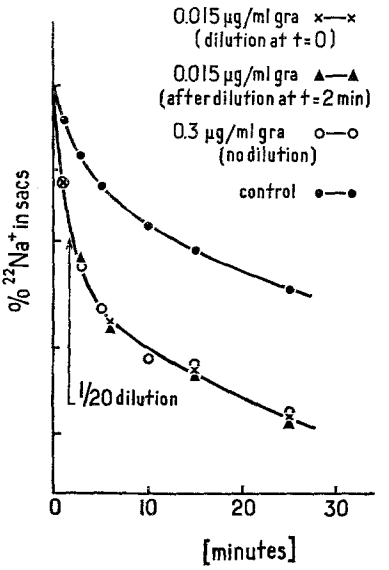

Fig. 11. Irreversible effect of Gra on the permeability of the microsacs to $^{22}Na^+$. A concentrated suspension of microsacs containing 4.8 mg of protein per ml was exposed overnight to $^{22}Na^+$ in the standard incubation medium, then diluted 20-fold in the standard dilution medium supplemented or not with Gra at the indicated concentration. The diluted suspension was then diluted a second time, 20-fold, at $t=0$ and $t=2$ min (indicated by the vertical arrow). Preparation no. 7

Discussion

Membrane fragments purified from the electric organ of *E. electricus* respond *in vitro* to cholinergic agonists by an increase of permeability to $^{22}Na^+$. This effect is blocked specifically by cholinergic antagonists. The dose-response curves measuring $^{22}Na^+$ efflux *in vitro* superimpose almost exactly with those recorded *in vivo* by measuring steady-state electrical potentials. The same apparent affinities for the cholinergic effectors, as well as the same shapes for the response curves, are found with the two methods. The competitive and noncompetitive effects observed *in vivo* between cholinergic agonists and antagonists are the same with the isolated microsacs.

A slight discrepancy was noticed, however, when the maximal responses to various agonists *in vitro* were compared with those recorded *in vivo*. For instance, when the dose-response curves to Carb and Deca are normalized to the maximal response to Deca, the maximal response to Carb is significantly larger *in vitro* than *in vivo*. The question is then, which of these two measurements is the more reliable? The electrical potential measurements could plausibly give an underestimation of the maximal response to Carb. Indeed, at high concentration of Carb, the membrane potential

decreases down to −15 mV, a domain of potential where the membrane permeability might change without being accompanied by parallel changes of electrical potential. It is very likely that, under these conditions, the flux measurements become more reliable than the measurements of electrical potential.

We have never been able to demonstrate without ambiguity the phenomenon of receptor desensitization with the excitable microsacs. As clearly shown in paper II of this series, the effect of cholinergic agonists (at the concentrations tested) is entirely reversible. The peak in sensitivity, which seems to occur in the dose-response curve to Deca around 10^{-5} M (10 times its apparent dissociation constant) (Fig. 2), might be considered as an indication of receptor desensitization. However, alternative interpretations, e.g., a weak antagonistic effect of Deca, cannot be ruled out.

The sigmoid shape of the dose-response curve to various agonists, which was extensively studied by Changeux and Podleski (1968) on the isolated electroplax, is preserved *in vitro* with the microsacs. The apparent cooperativity observed *in vitro* rules out electrical artifacts as a cause of this observation and makes more plausible a theoretical structural cooperativity in the assembly of the cholinergic receptors. These might possibly be grouped in oligomeric clusters within the excitable membrane. On the other hand, we have not been able to show *in vitro* that cooperative effects accompany the irreversible action of Gra.

A local anesthetic, tetracaine, blocks the response of the microsacs to Carb in a noncompetitive manner. This result, which confirms the observation of Podleski and Bartels (1963) on the isolated electroplax, might be accounted for by several different interpretations. Podleski and Bartels, for instance, following Keynes and Martins-Ferreira (1953), have distinguished two classes of excitable membranes in the innervated face of the electroplax: a subsynaptic membrane and a conductive membrane. The noncompetitive effect of tetracaine would be due to its preferential action on the conducting membrane. An alternative interpretation is that the local anesthetic binds to a site *on* the cholinergic receptor protein, or in its immediate vicinity, which is, at least partially, *distinct* from the active site of the receptor macromolecule.

A similar interpretation might be valid for benzoylcholine, which behaves, *in vitro*, as *in vivo*, both as an agonist and as a noncompetitive blocking agent. Benzoylcholine would bind to both the active site of the cholinergic receptor where it acts as an agonist and to the "local anesthetic receptor site" where it acts, like tetracaine, as a noncompetitive antagonist.

The simple fact that the preparation consists of a suspension of small membrane fragments has proved to be extremely convenient for quantitative

biochemical and physicochemical studies. For example, the catalytic activity of AcChE and the permeability response to cholinergic agonists can be measured in parallel on the same membrane fragments. Experiments in which the effect of affinity labeling reagents was followed on both the activity of AcChE and the response to cholinergic agonists strongly support the conclusion that the catalytic site of AcChE and the receptor site of the receptor protein are entirely distinct entities.

In relation to the effect of affinity labeling reagents, we would like to mention some recent results of Changeux, Kasai and Lee (1970). These authors have demonstrated that a snake venom toxin, α-bungarotoxin, is *in vivo* as well as *in vitro* a highly specific and irreversible reagent of the cholinergic receptor site. By using the excitable microsacs, they have even been able to offer a quantitative estimate of the number of α-bungarotoxin sites, and thus of cholinergic receptor sites, per mass of membrane proteins. The microsac preparation is thus particularly convenient for a quantitative binding study (*see* paper III).

In addition, the microsac suspension presents several important advantages over all the physiological preparations used up to now: (1) the membrane fragments constitute a well-defined subcellular preparation; (2) the environment of the excitable membrane fragments can be controlled *ad libitum* on both faces; and (3) the permeability to a single ionic species and the permeability changes caused by the cholinergic agonists can be measured quantitatively.

It is expected that this technique shall be extended to the study of the excitatory process in tissues other than the electric tissue of the eel. Neuroblasts or myoblasts in culture, or suspensions of neurons isolated from brain, might be convenient biological materials for this purpose. The only technical prerequisite is a sufficiently concentrated suspension of membrane fragments consisting of closed vesicles.

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