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Incorporation of chromaffin granule membranes into large-size vesicles suitable for patch-clamp recording

Serge Picaud, Alain Marty[+], Alain Trautmann[+], Odile Grynszpan-Winograd[°] and Jean-Pierre Henry*

Institut de Biologie Physico-Chimique, 13, rue P. et M. Curie, 75005 Paris, [+]*Laboratoire de Neurobiologie, Ecole Normale Supérieure, 46, rue d'Ulm, 75005 Paris,* and [°]*Laboratoire de Cytologie, Université P. et M. Curie, 7, Quai St Bernard, 75005 Paris, France*

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Incubation of chromaffin granules with excess liposomes at pH 6.0 resulted in the formation of cell-size structures, which were purified by centrifugation on sucrose gradients. Experiments with fluorescein-labeled granules indicated incorporation of granule membrane to these structures. The preparation contained various vesicular structures with a diameter up to 15 μm. The largest elements were studied by the 'patch-clamp' technique. 'Cell-attached' and 'whole-cell' recordings indicated the presence of currents corresponding to unitary conductances ranging from 100 to 500 pS.

Chromaffin granule *Membrane fusion* *Liposome* *Patch-clamp* *Ion channel* *Electron microscopy*

1. INTRODUCTION

Several hypotheses [1,2] recently proposed to explain the mechanism of exocytosis in adrenal medulla involve ion motions through the chromaffin granule membrane. The ionic permeability of this membrane has been investigated by rather indirect biochemical approaches [3,4]. It was concluded that the membrane is impermeable to cations and has some permeability to anions, possibly through specific sites [5]. An electrophysiological approach should give more direct information on the characteristics of this membrane. The technique of patch-clamp recording is well suited to the study of ion channels [6]. Recently, the properties of the acetylcholine receptor purified from *Torpedo* electric organ has been analyzed by this technique, after its incorporation into large-size liposomes [7]. Here, we describe the incorporation of bovine chromaffin granule membranes to large-size lipidic structures suitable for patch-clamp analysis.

* To whom correspondence should be addressed

Abbreviations: FITC, fluorescein isothiocyanate; DBH, dopamine β-hydroxylase

2. EXPERIMENTAL PROCEDURES

Bovine chromaffin granules were prepared either by differential centrifugation (crude granules) or by centrifugation on Percoll gradients (purified granules), as in [8,9], respectively. Granule membranes were prepared as in [10] and stored at $-80°C$.

Liposomes (small unilamellar vesicles) were prepared by sonicating a suspension (35 mg/ml) of purified asolectin (Associated Concentrates, Woodside, NY) in 0.15 M KCl/10 mM Hepes buffer (pH 7.4) as described [11]. The milky solution was kept in ice under argon and centrifuged at $40000 \times g$ for 20 min before use. Liposome-granule fusion was performed as in [11]. Chromaffin granules or granule membranes (about 0.12 mg protein) were suspended in 1 ml of KCl/Hepes buffer maintained at 30°C under constant stirring.

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Liposomes (0.33 ml) were added and the pH lowered to 6.0 with dilute HCl solutions. The mixture was incubated for 15 min. Two additional aliquots of liposomes were added at 15-min intervals and the pH was adjusted at 6.0. The pH of the mixture was then raised to 7.4 and the sample centrifuged at 27000 × *g* for 15 min.

The presence of large-size structures in the incubation mixture was controlled by phase contrast microscopy and by counting dilutions of the medium in a Coulter Counter (Coultronics, Margency, France), operating generally with 3–4 and 6–13 μm windows.

The pellet of crude large-size structures was resuspended in 1.0 ml of KCl/Hepes buffer and layered onto a discontinuous sucrose gradient in 10 mM Hepes (pH 7.4):0.2 M/1.2 ml:0.4 M/1.4 ml:0.6 M/1.4 ml. The tubes were centrifuged at 50000 rpm in a SW 65 rotor for 3 h and fractions collected from the bottom of the tubes. The distribution of large-size structures was followed with the Coulter Counter.

For patch-clamp recording an aliquot (2–5 μl) of the preparation collected at the 0.4–0.6 M interface of the sucrose gradient was added to a 1-ml chamber containing the recording solution. In some cases, further elimination of small liposomes was obtained by slowly flushing the chamber with saline, leaving the larger structures on the bottom of the dish. The pipette and bath solutions contained a 250 mM NaCl/5 mM Hepes buffer (pH 7.2). $CaCl_2$ (1 mM) was usually added to both solutions, but in some cases Ca was buffered to about 10 nM with a 5.5 mM EGTA/0.5 mM Ca buffer. Electrical recordings were performed at room temperature using standard patch-clamp techniques [6]. Either the currents were recorded directly on intact structures ('cell attached' configuration), or they were collected from whole structures after breaking through the initial patch ('whole cell' configuration).

Electron microscopy was performed after fixation by 2.5% buffered glutaraldehyde. After washing in the buffer, pellets were postfixed for 1 h in 2% osmium tetroxide, dehydrated and embedded in Araldite. Sections were stained with uranyl acetate and lead citrate.

FITC-labeled granules were prepared by resuspending crude granules (6.3 mg protein/ml) in 0.3 M sucrose/20 mM bicarbonate buffer (pH 9.0) and adding 4 μl of a 0.5% solution of FITC in ethanol. After 1 h incubation at 0°C, the mixture was centrifuged at 27000 × *g* for 15 min, resuspended in 0.3 M sucrose/10 mM Hepes (pH 7.0) and purified on a Percoll gradient.

Protein was determined according to [12] and DBH activity as in [13].

3. RESULTS

The addition of liposomes to a suspension of chromaffin granules, followed by the incubation of the mixture at pH 6.0, resulted in the formation of some rounded large-size structures. The presence of these structures was followed by phase contrast microscopy and by measurements with a cell counter (table 1). The number of structures

Table 1

Fusion experiment

Experimental conditions	Number of 6–13 μm particles × 10^{-3}/ml	
	Buffer	Buffer + chromaffin granules
Before liposome addition (pH 7.4)	6	12
First liposome addition	24	71
Second liposome addition	38	70
Third liposome addition	37	118
Adjustment to pH 7.4	41	143

The experiment was performed with either crude chromaffin granules (right) or buffer alone (left). Fusion was followed by counting dilutions of the incubation mixture in the cell counter with a 6–13 μm window. Figures are means of 3 measurements

counted in the 6–13 μm window increased when the additions were repeated. In the absence of chromaffin granules, the number of these structures was largely decreased (table 1). Crude granules (purified by differential centrifugation) and purified granules (purified by centrifugation on Percoll gradients) gave similar results. Chromaffin granules could also be substituted by membranes derived from the granules by osmotic lysis.

The large-size structures (macrogranules) were separated from the bulk of liposomes by centrifugation at 27000 × *g* for 15 min and purified by centrifugation on a discontinuous sucrose gradient (fig.1). They banded at the 0.4–0.6 M sucrose interface, as indicated by the finding of a protein peak at the 0.4–0.6 M interface (fig.1). This peak was not observed when large-size structures were prepared in the absence of chromaffin granules. Some DBH activity was detected at the 0.4–0.6 M interface (not shown). Remaining liposomes were found at the 0.2–0.4 M interface and chromaffin granules were found in the pellet.

To gain information on the presence of chromaffin granule membranes in macrogranules,

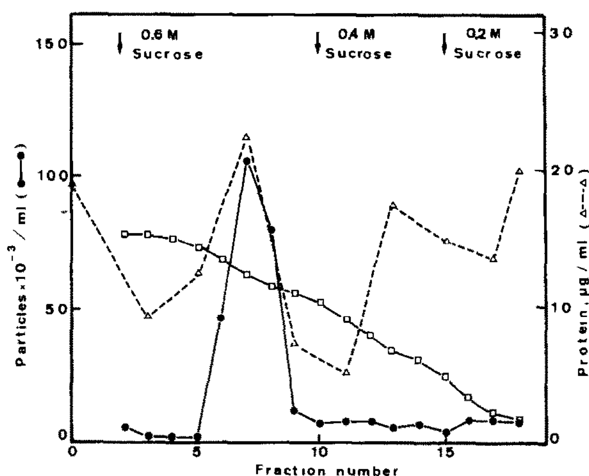

Fig.1. Centrifugation of a crude macrogranule preparation on discontinuous sucrose gradient. The distribution of macrogranules was followed by counting aliquots through the 6–7 μm window of the Coulter counter (○—○). Protein (△—△) and sucrose (□—□) concentrations are indicated. The phospholipid concentration of tube 7 is 0.76 mM. The 0.4–0.6 M sucrose interface is distributed between tubes 6–8.

granules were labeled with FITC and the fusion experiment was followed with a fluorescence microscope. Granules were present as small bright dots. As the fusion reaction proceeded, larger spots with various levels of fluorescence were observed which were associated with macrogranules (fig.2). It should be noted that the fluorescence of the macrogranules was uniformly distributed.

Pellets of the material found at the 0.4–0.6 M sucrose interface were examined by electron microscopy (fig.3). The material was heterogeneous with many empty vesicles ranging from 0.2 to 2 μm. Larger structures (2–5 μm diameter) which were filled with small (about 50 nm) vesicles were also seen. The largest structures, presumably the so-called macrogranules, were vesicles reaching a diameter of 15 μm and limited by a membrane composed of one or several lamellae. These structures were either empty or contained myelin-like figures, often unrelated to the external membrane.

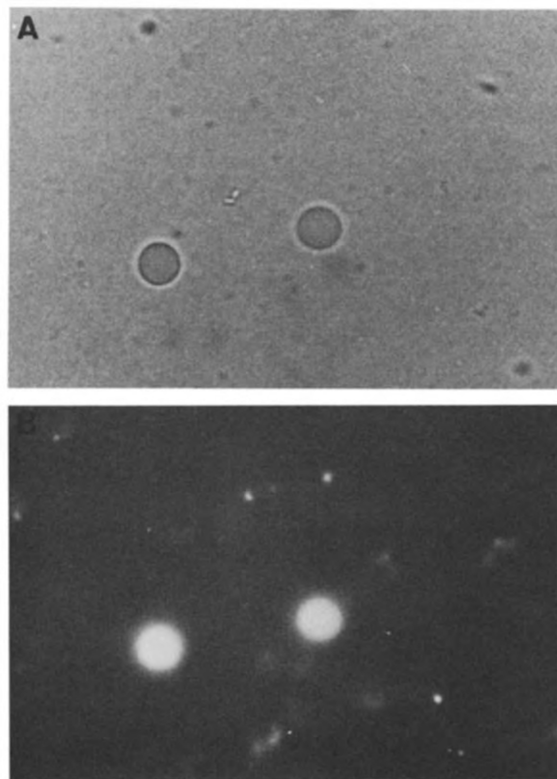

Fig.2. Large-size structures prepared with FITC-labeled chromaffin granules and observed with (A) Nomarski and (B) fluorescein equipment (× 800).

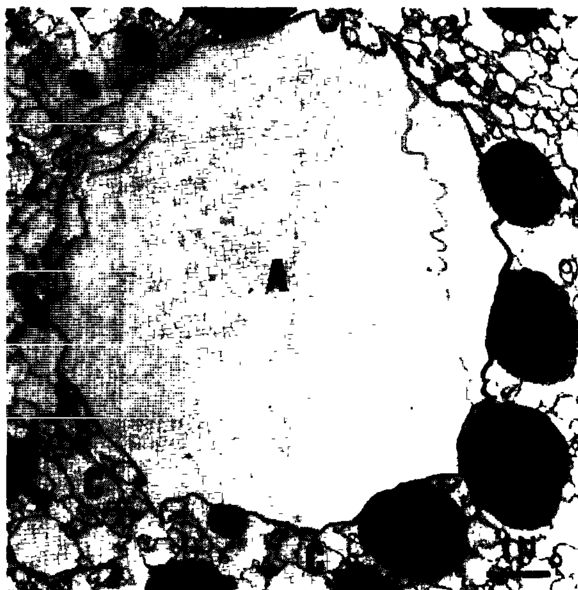

Fig.3. Electron micrograph showing the 3 main types of vesicles contained in the pellet. (A) Large vesicles limited by a membrane presumably composed of several sheets. The few internal membranes appear to be detached from the limiting membrane. (B) Smaller spherical structures crowded with minute vesicles or perforated membranous structures. (C) Empty vesicles ranging from 0.2 to 2 μm. Their membrane is likely to comprise only one sheet (× 7600).

Patch-clamp experiments conducted on intact macrogranules revealed large current steps of various amplitudes, corresponding to unitary conductances ranging from 100 to 500 pS. In one patch, where a single 500 pS channel was present, several conductance substates were apparent. This suggested that, in spite of the heterogeneous current step sizes, most transitions observed in 'cell attached' recordings may have originated from a single class of channels with large unitary conductance and numerous conductance substates.

'Whole-cell' type recordings were also obtained on the macrogranules. In the experiment shown in fig.4, repetitive 100 mV voltage jumps were given from a holding potential of 0 mV. Responses to 4 such jumps, where the inside of the macrogranule was made positive with respect to the bath potential, are displayed in fig.4. Current steps of 30–50 pA are present in each record, corresponding to unitary conductances of 300–500 pS.

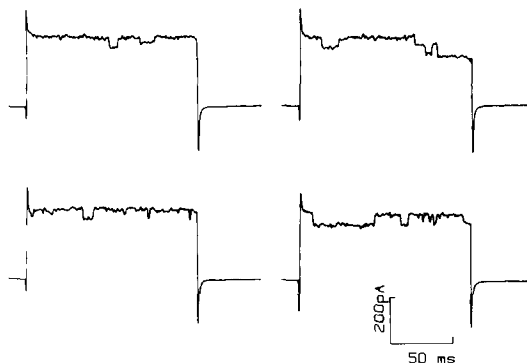

Fig.4. Patch-clamp recording from a macrogranule. A macrogranule was studied with the 'whole-cell' recording technique which allows measurement of current flowing through the entire structure. The holding potential was 0 mV. The interior of the macrogranule was dialysed with a pipette solution containing 250 mM NaCl/5 mM Hepes–NaOH (pH 7.2)/5.5 mM EGTA–0.5 mM Ca (free Ca around 10 nM). The bath solution contained 250 mM NaCl/5 mM Hepes–NaOH (pH 7.2)/1 mM $CaCl_2$. Current responses to 4 voltage jumps to + 100 mV (inside positive) are shown. The current steps seen correspond to conductance drops lasting from a few ms to 50 ms, presumably because of transient closures of channels with unit conductances of 300–500 pS.

Recordings obtained on one liposome preparation where chromaffin granules were omitted failed to give any current step.

4. DISCUSSION

The observed macrostructures did not derive from large multilamellar vesicles preexisting in the sonicated asolectin suspension since this asolectin suspension was centrifuged just before use. The large-size structures thus result from the incubation at acidic pH of liposomes with chromaffin granule membranes. These structures are certainly rare events since their frequency, as determined in the 6–13 μm window of the counter is in the range 8–24 × 10^4 vesicles/ml, whereas the estimated initial number of chromaffin granules is about 10^{10}/ml.

The mechanism of formation of such large structures is unknown. Other instances of fusion occurring at acidic pH have been described. Liposomes fuse with mitochondrial inner membranes at pH 6.5 [11]. Several proteins mediate an

acid-induced liposome fusion [12–14], and recently it has been shown that palmitoylhomocysteine mediates such a fusion in the absence of protein [14].

The main evidence for the presence of chromaffin granule membrane elements in the macrogranules are the experiments with FITC-labeled granules. These granules are likely to be labeled on membrane components, protein and phospholipid. The homogeneous fluorescence of the macrogranules suggests some fusion of chromaffin granule membrane components with the membrane of these structures. In accord with this hypothesis, protein and DBH activity have been found at the 0.4–0.6 M sucrose interface.

The current steps illustrated in fig.4 are most probably due to membrane proteins of the initial chromaffin granule preparation, since liposomes prepared without adding chromaffin granules did not give comparable signals. At present, we cannot exclude that the current steps may have derived from contaminating membranes (for instance, of mitochondrial origin) in the chromaffin granules preparation. Nevertheless, the results raise the interesting possibility that chromaffin granules contain a specific class of ion channels with large unitary conductance. Further characterization of these channels (notably concerning their ion selectivity) and elucidation of their possible functional role must await future investigation.

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