ACETYLCHOLINE-INDUCED IN VITRO FUSION BETWEEN CELL MEMBRANE VESICLES AND CHROMAFFIN GRANULES FROM THE BOVINE ADRENAL MEDULLA

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1. Introduction

Catecholamine (CA) secretion from the adrenal medullary chromaffin cell (CC) occurs by means of exocytotic membrane fusion [1]. In studies at the plasma membrane fusion with the chromaffin granule (CG) and subsequent efflux of CA and associated granule components, it is useful to have an in vitro system in which such a process can be demonstrated. The 'leaky' CC has been proposed for this purpose [2].

Here we report data indicating that fusion between isolated CC plasma membranes and intact CG in suspension can be induced by acetylcholine (ACh), the natural cholinergic agonist of the adrenal medulla. The method is based on our observation that the fluorescence of 6-carboxy-fluoresceine (CF) is quenched by relatively high CA concentrations. Emission quenching of the parent compound, fluoresceine, by a variety of phenolic compounds, was reported in [3]. Our approach is different to previous applications of CF where increases in fluorescence occurring when liposome-contained CF was transferred into intact lymphocytes and other cells were recorded, with a consequent 'relief of self-quenching' [4–6].

Changes in CF emission yield, in a suspension containing intact CG and CC plasma membrane vesicles loaded with CF, can occur for different reasons. In the absence of fusion between the two different types of vesicles, the fluorescence intensity of the suspension will remain constant if the CF-loaded plasma membrane vesicles are tight. If they are leaky, a progressive increase in fluorescence will occur, owing to CF release into the medium. In the event that fusion does occur, two things may happen:

(1) If fusion is 'leaky' and both CF and CA are released into the medium, there will be a fluorescence increase, since the CA concentrations in the medium are below the level required for observable CF quenching (see below).
(2) In the case of 'tight' fusion, CF is released into the intragranular space, where it is quenched by the high concentration of CA, so that the fluorescence will decrease.

If several of these processes occur simultaneously, they may partially cancel. 'Tight' fusion is thus indicated only if there is a net fluorescence decrease. This is shown to occur in the experiments below.

2. Materials and methods

Bovine adrenal glands were used to prepare the chromaffin cell plasma membranes, according to [7], with the modifications applied in [8]. The material collected from the interface, after centrifugation on gradient II [7], was subjected to a final centrifugation at 100 000 × *g* for 1 h in 0.3 M sucrose/10 mM Tris–HCl, to yield the plasma membranes. Chromaffin granules were obtained from a separate batch of adrenal glands, as in [9]. CG membranes were prepared by lysis in 10 mM Tris, (pH 7.4). Acetylcholinesterase (EC 3.1.1.7), catecholamines and proteins were determined by the methods in [10], [11] and [12], respectively. The mean value (±SD) of the acetylcholinesterase activity, from 7 different plasma membrane preparations, was 242.5 ± 46.4 nmol thiocholine formed . min^{-1} . mg $protein^{-1}$. Catecholamine content of the granules varied from 0.5–2.0 μmol/mg granule protein in 6 different preparations. The CC plasma membranes were loaded

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with CF, by passing the membranes through the French Press (American Instrument Corp.) for 4 times, at 20 000 lb.in^{-2}, in the presence of a self-quenched concentration (150 mM) of the dye, according to [13]. Subsequently, the suspension was passed over a Sephadex G-25 column (3 X 40 cm) to separate vesicles from non-entrapped dye. Electron micrographs of negatively-stained preparations showed a rather uniform population of vesicles with mean diam. ~80 nm.

The fluorescence measurements were made in a Perkin-Elmer MPF-44A spectrofluorimeter with thermostated cell holder. The emission at 520 nm ($\Delta\lambda$ = 5 nm), for excitation at 470 nm ($\Delta\lambda$ = 5 nm) of 2 ml suspensions of plasma membranes and chromaffin granules in either high potassium or high sodium buffer containing the appropriate additions, was monitored as a function of time.

Purified CF [4,6] was a generous gift of Professor M. Yatvin, University of Madison, WI. ACh-chloride was from Merck, hexamethonium bromide from Sigma, decamethonium bromide from ICN Pharm. and Prostigmin from Hoffmann La-Roche. Buffers, made of analytical grade salts, contained either 150 mM KCl–10 mM Tris–HCl (pH 7.4) (high K^+ buffer) or 135 mM NaCl, 10 mM KCl, 10 mM Glc and 5.7 mM Hepes (pH 7.4) (high Na^+ buffer).

The experiments were mostly run at 120 μg CC plasma membrane protein/ml and 240 μg CG protein/ml. For mean particle diameters of 80 nm (this work) and 200 nm [14] and protein weight fractions of 0.62 [7] and 0.42 [15] of the dry weight, respectively, and taking the specific gravity of both to be 1.2, one calculates particle densities of 1.0×10^{12} CC membrane vesicles/cm^3 and 3.5×10^{11} CG/cm^3 and volume fractions of 2.8×10^{-4} and 1.45×10^{-3}, respectively. The total CF concentration in the suspensions was determined from the fluorescence intensity of a sample to which 0.1% (v/v) Triton X-100 had been added, which resulted in lysis of the vesicles and complete release of entrapped dye. The maximal dye retention, calculated from this concentration and the value of the vesicle volume fraction given above, was ~8%.

3. Results and discussion

The fluorescence of free CF, at concentrations similar to those used subsequently, is substantially quenched by CA. Fig.1 shows the dependence of the uncorrected fluorescence intensity of CF on CA concentration. The CA concentration (~5 mM), which gives almost total quenching of the CF fluorescence, is still by a factor of 50–100 lower than that present in the intact CG. Experiments with higher CA concentrations could not be run on account of precipitate formation in the suspensions. Fig.2 depicts the time course of CF fluorescence changes in a typical experiment. Mixtures of CF-loaded vesicles

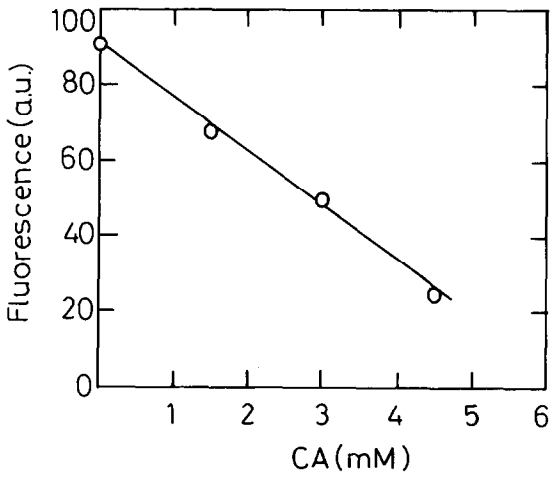

Fig.1. Quenching of the fluorescence emission (arbitrary units) of 6-carboxy-fluorescein by catecholamine mixtures (75% epinephrine, 25% norepinephrine). CF 4 mM; λ_{exc} 470 nm; λ_{em} 520 nm; T 25°C.

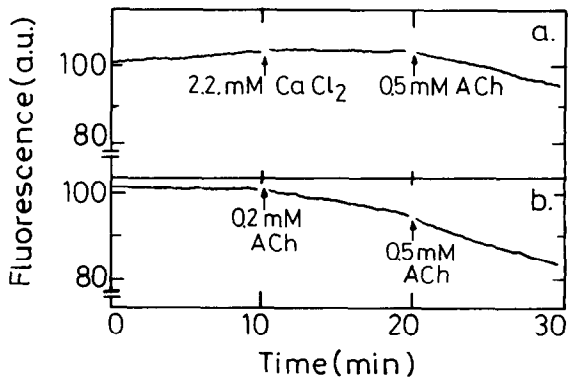

Fig.2. Experimental traces of ACh-induced fluorescence changes in suspensions of CF-loaded plasma membrane vesicle (120 μg/ml) and intact chromaffin granule (240 μg/ml) in high K^+-buffer (see text): (a) in presence of 2.2 mM Ca^{2+}; (b) in absence of Ca^{2+} at two different ACh concentrations.

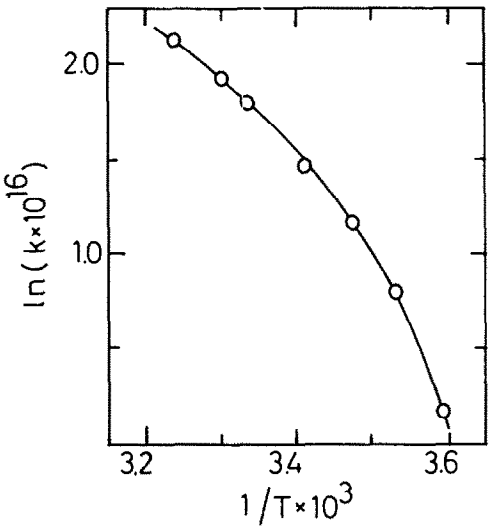

Fig.3. Arrhenius plot of the temperature dependence of ACh-induced in vitro fusion between CC plasma membrane vesicles and chromaffin granules. The points are derived from linear regression analysis (correlation coefficient, $r = 0.984$) of the cumulative data ($n = 16$) obtained from 3 independent experiments. ACh 6×10^{-4} M. Further details, see text.

and CG in 150 mM KCl–10 mM Tris buffer usually show a small fluorescence increase with time, which we believe to be due to some leakage of CF. After addition of CA^{2+} (2.2 mM final conc.) the slightly ascending slope does not change appreciably or, in some cases, decreases. Upon addition of ACh, the natural secretagogue of the adrenal medulla, we observe a linear decrease in the fluorescence. This fluorescence change is not significantly dependent on the presence of added Ca^{2+}, and on the time and order of Ca^{2+} and ACh addition. The rather high activity of the membrane bound acetylcholinesterase notwithstanding, only ~0.2% of the total ACh are hydrolysed during an experiment, and this does not influence the result, as could be tested by control runs in the presence of the acetylcholinesterase inhibitor Prostigmin.

The following control experiments were performed to test for the specificity of the response:

(i) CF-loaded plasma membrane vesicles plus ACh in either the presence or absence of calcium;
(ii) Free CF plus intact CG in the presence, as well as absence, of ACh and/or calcium;
(iii) CF-loaded plasma membrane vesicles plus empty CG-membrane ghosts, with or without ACh and/or calcium.

None of these experiments resulted in fluorescence responses similar to that shown in fig.2. The intragranular CA present in the suspension, if totally released into the medium, would not exceed 125 μM in most experiments. Fig.1 shows that this concentration would give negligible quenching of the CF fluorescence. Therefore, the observed effect cannot be due to extragranular CA. Intragranular CA concentration, on the other hand, varies from 85–330 mM (see section 2), which is ample for complete CF quenching. Coalescence of the intragranular volume of CG and one or more plasma membrane vesicles, which are ~1/10 of its size, would not change the CA concentration to any significant extent. Thus, the most likely interpretation of the fluorescence decrease observed is that it stems from non-leaky fusion events between the CC membrane vesicles and the CGs. While added calcium seems to influence the extent of fusion only marginally, ACh enhances it significantly. Fig.2 also indicates that the change in fluorescence signal increases with increasing ACh concentration. The quantitative features of the dose-dependency are at present being further investigated. The data of fig.2 were recorded in a high K^+ medium, intended to roughly simulate the intracellular cytoplasmic K^+ concentration. However, very similar results were obtained in high Na^+ buffer. In the latter, the plasma membrane vesicles seemed to be more leaky, as judged by more steeply ascending slopes of the fluorescence increase with time in the control runs. The effect of ACh appeared in that case as a decrease in the slope, i.e., a smaller rate of increase in fluorescence intensity. The net decrease was calculated as the difference between the slopes of test vs control runs. The cholinergic nature of the effect was further tested in experimental runs, with 0.5 mM ACh by addition of the nicotinic antagonists hexamethonium and decamethonium. While the former, at 0.5–4 mM gave a variable and limited ($\lesssim$30%) reduction of the fluorescence change, the latter at 2.5 mM, completely abolished the ACh-induced fusion. This order in effectiveness corresponds to that found with respect to the inhibition of α-bungarotoxin binding to isolated CC plasma membranes by the two antagonists [16].

Fig.3 shows an Arrhenius plot of the ACh-induced rate of fusion. The apparent energy of activation varies with temperature, from ~10 kcal/mol over 10–20°C to ~6 kcal/mol at >30°C. This decrease may be linked to a temperature-induced fluidity increase in the interacting membranes [8], which

enhances their ability to fuse with each other. The rate constant, k, calculated for a biparticle reaction, is 4.38×10^{-16} cm^3/particle . s at 20°C, under the specified experimental conditions. This value may be taken as a lower limit estimate, since the initial fluorescence intensity was not corrected for:

(i) The contribution of free CF, present in solution at time zero; and

(ii) Another, quite small, contribution due to vesicle and granule scattering.

An apparent peculiarity of the reconstituted system is the absence of a significant sensitivity to Ca^{2+}. While no data are available on CC plasma membrane-bound Ca^{2+}, the CG intragranular calcium concentration is quite high, i.e., 76 nmol/mg protein [15]. Random Ca^{2+} leaks from the CG, and perhaps also Ca^{2+} set free from CC membranes by ACh stimulation [17], may elevate local concentrations of Ca^{2+} in the environment of the colliding particles to the μM level that is apparently necessary for exocytosis [2]. On the other hand, although Ca^{2+} is required for CA secretion [18], there is so far no definite evidence that it is indeed involved in the fusion process proper.

We also note that, in order to permit exocytotic fusion, the sidedness of the CC membrane vesicles has to be the reverse of that of the intact cell. It has, therefore, to be assumed that a sizeable fraction of the vesicle preparation is oriented inside-out. This leads to an apparent paradox, since current views place the ACh-binding site of the cholinergic receptor on the outer surface of the plasma membrane [18] and, therefore, the vesicle with inside-out orientation could not be responsive to ACh stimulation. However, it is not improbable that the agonist may permeate through the vesicle membrane, particularly in view of our observation that the vesicles are somewhat leaky also to the highly water-soluble CF.

Several other laboratories have tried to isolate specific membrane and/or cytoplasmic proteins, believed to be involved in exocytotic CA release from the chromaffin cell [19–21]. In contrast, we have deliberately worked with whole plasma membrane vesicles of intermediate purity (see section 2). This preparation may still contain membrane-associated cytoskeletal, and possibly other components, that could be essential for exocytotic CA release. Having found it to be active, we are currently attempting to define the specific requirements for fusion in greater detail. The experimental system described seems to provide us with an excellent opportunity for elucidating the existence and role of the, so far, hypothetical site [22] for activation of the exocytotic event.

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