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Synexin-mediated fusion of bovine chromaffin granule ghosts. Effect of pH

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Synexin induces chromaffin granule ghosts to fuse one to another, a process which is followed continuously and quantitatively by monitoring the mixing of the intragranular aqueous compartments. A freeze-thaw technique was used for preparing chromaffin granule ghosts loaded with a self-quenching concentration of the fluorescent, high molecular weight probe FITC-Dextran. When the loaded ghosts were mixed with empty ghosts in the presence of synexin, the two compartments fused, resulting in the dilution of the probe with the concomitant increase in fluorescence. So as to suppress possible leakage signals, anti-fluorescein antibodies which quench probe fluorescence were present in the reaction media. Synexin-mediated fusion of freeze-thaw (F/Th) ghosts and binding of ^{125}I-synexin to these membranes were found to be dependent on Ca^{2+} concentration, but only in a partial manner. However, these two synexin-mediated properties were demonstrably sensitive to $[H^+]$ in the medium. A detailed pH profile of fusion revealed an apparent midpoint of activation at approx. pH 5.2, with asymptotic values at pH 4 (maximum) and pH 7.2 (minimum). In our attempt to determine whether the pH effect was on the synexin or on the membranes, we found that fusion was blocked only by treatment of the membranes with the membrane-impermeant carboxyl group modifier 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. These data suggest that membrane fusion evoked by synexin seems to be promoted by rendering the F/Th membranes relatively less negatively charged while the synexin becomes more positively charged. The fusion process was entirely dependent upon synexin concentration; the $k_{1/2}$ under optimal conditions of p*Ca* and pH was 85 nM. Similar to what has been previously found with intact granules, an anti-synexin polyclonal antibody partially (48%) blocked fusion, as did pretreatment of the chromaffin granules ghosts with trypsin (30%). We conclude that the coincident p*Ca* and pH sensitivity of synexin-mediated binding to chromaffin granule membranes and their subsequent fusion might be associated with physiological changes in the concentration of both cations in the cytoplasm of secreting chromaffin cells.

Introduction

Synexin is a cytosolic protein of 47 kDa, which causes native isolated chromaffin granules to aggregate by a process which is entirely dependent on the free calcium ion concentration and is promoted by lowering the pH to approx. 6.0 [1]. Fusion of these chromaffin granule aggregates can be induced by further addition of low concentra-

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid); FITC, fluorescein-isothiocyanate; EAC, 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)-carbodiimide.

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tions of arachidonic acid [2]. The details of the mechanism of synexin-dependent fusion and the relative importance of H^+ and Ca^{2+} in the process are not known. However, the physiologic role of synexin may be to promote membrane fusion during exocytosis in chromaffin cells and perhaps in the wide variety of other cell types where synexin is also found [3].

In pursuit of the above information, we recently devised a fusion assay for chromaffin granule ghosts in which FITC-Dextran was loaded into ghosts at self-quenching concentrations by freeze-thawing in liquid nitrogen [4]. Upon exposure to synexin these chromaffin granule membrane ghosts were found to fuse with each other rather rapidly (aggregation constant of $5 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ and fusion rate constant of 2 s^{-1}). In the present study we found that all fusogenic properties of F/Th chromaffin granule ghosts were completely dependent upon the addition of synexin. We also found that synexin itself could bind to the F/Th ghost membranes, thus explaining the modest calcium requirements we later found for membrane fusion. Finally, we observed that synexin-driven fusion was absolutely dependent upon an acidic pH, the pH effect residing on membrane localized carboxyl groups. We concluded that by comparing fusion properties of F/Th ghosts with intact granules we had perhaps unmasked previously hidden properties of granule membranes relevant to fusion processes in vivo.

Materials and Methods

Preparation of chromaffin granule ghosts. Chromaffin granules were prepared as described elsewhere [5]. Briefly, the granules were prepared from bovine adrenal medullary tissue, purified by centrifugation over a 1.6 M sucrose step gradient and then lysed in either distilled water or 5 mM Tris-maleate (pH 7.2). The resulting chromaffin granule ghosts were then washed and resuspended in a medium of the following composition (mM): 140 KCl, 20 Hepes and 0.1 EGTA (pH 7.2).

FITC-Dextran was incorporated into the chromaffin granule ghosts by repeated rapid freezing and slow thawing at a concentration which gives fluorescence self-quenching (0.1 mM [6]). The loaded chromaffin granule ghosts were subsequently filtered through a Sephacryl S-300 column, in order to remove excluded FITC-Dextran [4].

Measurement of membrane fusion. The membrane fusion assay monitored the dilution of the probe (FITC-Dextran) from loaded into blank (empty) chromaffin granule ghosts, which occurs following the membrane fusion event. The dilution results in an increase in fluorescence intensity due to relief of the concentration-dependent self-quenching [4]. Fluorescence of FITC-Dextran leaking into the extravesicular space was quenched by the addition of an anti-fluorescein antibody which ensures that only non-leaky fusion events are registered. The amount of anti-fluorescein antibody required for each experiment was obtained from the calibration experiments. The antibody was added to the medium before the addition of the chromaffin granule ghosts [4].

All the experiments were performed in a standard medium containing 140 mM KCl, 20 mM Hepes. The pH of the solution was adjusted with 10 M HCl or 2 M KOH to the desired values. For experiments performed at a pH below 6.0, Hepes was replaced by equimolar amounts of Pipes. The free Ca^{2+} concentration (pCa) and pH were determined as reported [7]. In order to account for the pH dependency of the fluorescence emission of fluorescein, the fluorescence values were calibrated for each individual pH value measured. The experiments were run at 37 °C. The ghosts were mixed at a 1 : 10 ratio (loaded/empty, respectively) and the total amount of chromaffin granule ghost protein per experiment in this case was 10 μg in 2 ml. Synexin was added 30 s after mixing the vesicles, in the presence of absence of the anti-fluorescein antibody. The reaction mixture was stirred continuously and the fluorescence was monitored in a Spex Fluorolog II spectrophotofluorimeter equipped with a digital plotter and a microprocessor (DM 1B) (Spex Industries, Metuchen, NJ). The excitation and emission wavelengths were 465 and 520 nm, respectively. A cutoff filter (498 nm) was regularly used. After 5 min, detergent (Nonidet P-40, British Drug House, 0.1% final concentration) was added so as to obtain the infinite dilution condition and to confirm that the amount of anti-fluorescein antibody present was adequate to correct for any leakage.

Synexin preparation and anti-synexin antibodies. Bovine liver synexin was purified as described [9] and the anti-synexin antibodies were raised in goat as reported previously [10].

Modification of the chromaffin granule ghosts and of synexin. Chromaffin granule ghosts were incubated with trypsin (Sigma, type I) at a concentration of 1 mg/ml for 30 min at 37 °C (pH 7.2). The reaction was stopped by adding an equal amount of soybean trypsin inhibitor (1 mg/ml, Sigma). The chromaffin granule ghosts were then washed three times and resuspended in a medium, containing 140 mM KCl, 20 mM Hepes and 0.1 mM EGTA (pH 7.2), prior to the fusion experiments initiated shortly after the trypsinization.

Chemical modification: EAC was synthesized by methylation of 1-ethyl-3-(dimethylaminopropyl)carbodiimide, according to Ref. 11.

Synexin was iodinated following the procedure described in Ref. 12.

Data analysis. The calibration experiments (n = 2, for each experimental condition) as well as the fusion experiments ($n = 3$, for each experimental condition) were averaged point-by-point. The fluorescence signals were corrected for leakage by subtracting both averaged signals. The final extent of fluorescence, F_u, is given by

$$F_u = [(F_i - F_o)/(F_t - F_o)]$$

in which F_i = fluorescence intensity after i seconds upon adding synexin; F_o = basal fluorescence intensity; and F_t = fluorescence intensity after the addition of the detergent for obtaining the infinite dilution condition in the calibration experiments.

Results

Time course for fusion induced by synexin

Chromaffin granule ghosts were loaded with a self-quenching concentration of FITC-Dextran by the freeze-thaw (F/Th) technique. Synexin, at different concentrations, was then added to this reaction mixture which contained also anti-fluorescein antibodies at amounts far in excess to the amount required for fully quenching the probe present in the medium. In these conditions only minor increases (< 8%) in the fluorescence signal were observed (not shown) and they are likely to represent volume changes due to fusion between probe-laden ghosts [13] and/or dilution of ghost-associated probe. In contrast, when a 10-fold excess of probe-free ghosts was also included, the fluorescence increased up to 70% (Fig. 1). The fusion with empty ghosts was readily detected over a 2 min time period (Fig. 1B), with rates and extents increasing proportionally from 50 to 360

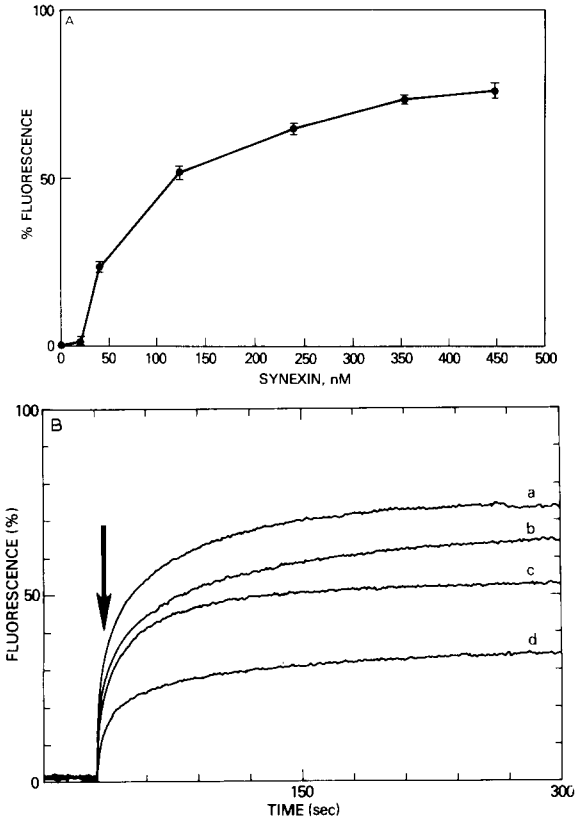

Fig. 1. (A) Fluorescence increase of FITC-Dextran as a function of synexin concentration. The final extent of the fluorescence increase was obtained from the traces depicted in (B). The synexin concentration giving 50% of fluorescence dequenching is approx. 85 nM. (B) Time course and synexin dependence of the fusion reaction as detected by the volume mixing assay. The arrow indicates the time at which synexin was added. Trace (a) corresponds to a synexin concentration of 355 nM, (b) 240 nM, (c) 124 nM and (d) 40 nM. The total ghost concentration was 5 μg protein/ml and the total reaction volume was 2 ml, pH 6, pCa 7 and 37 °C. Each trace corresponds to an average of two experiments and leakage has been subtracted. The ghosts (loaded/blank) were mixed at a 1 : 10 ratio and synexin was added (t = 30 s). This ratio of 1 : 10 was the same for all the experiments. Dequenching of the FITC fluorescence follows upon addition of synexin.

nM synexin. Using constant ghost concentration, the minimal concentration of synexin required to trigger fusion was 40 nM, while an increase in the concentration above 450 nM, without further addition of ghosts resulted in no further increase of the fluorescence signal.

The synexin-induced granule membrane fusion proceeds without latency and can be divided into an initial phase of rapid fusion, followed by a phase of relatively slower rate. This reflects a complex behavior which has not been demonstrated before for the synexin-induced chromaffin granule aggregation. Preliminary kinetic analysis of the overall reaction indicates that the rate constant of fusion, k_f, is relatively fast ($k_f = 2\ s^{-1}$), and that the aggregation rate constant, k_c, had a value close to that of a diffusion controlled process ($k_c = 5 \cdot 10^9\ M^{-1} \cdot s^{-1}$). These observations indicate that the potential barrier for close approach between vesicles can be practically ignored [13].

Synexin-concentration dependence of membrane fusion

Granule ghost membrane fusion was examined at different synexin concentrations, as shown in Fig. 1. From this plot we calculated the half-maximal concentration of synexin to be approx. 85 nM. This number is similar to the concentration of synexin which gives half-maximal aggregation of intact granules, approx. 77 nM [14], and also to the average estimated concentration of synexin in cells (approx. 150 nM).

Influence of Ca^{2+} on synexin-induced fusion

Earlier experiments had shown synexin to fuse chromaffin granule ghosts at relatively low concentrations of free calcium [4]. This is shown in Fig. 2, where a significant level of fluorescence (approx. 38%) was obtained at p*Ca* 9. However, elevation of free calcium ion concentration to p*Ca* 8 induced a further increase in the extent of fluorescence. This effect saturated at about p*Ca* 7 and dropped down drastically at p*Ca* > 6. The modulatory effect of Ca^{2+} on the fusion process was observed at all the synexin concentrations studied (40–500 nM). On the other hand, Ca^{2+} alone (1 μM to 10 mM), in the absence of synexin failed to induce fusion. In addition, Mg^{2+} alone (1 μM to 10 mM) in the presence of synexin had no demonstrable effect on the fusion of chromaffin granule ghosts.

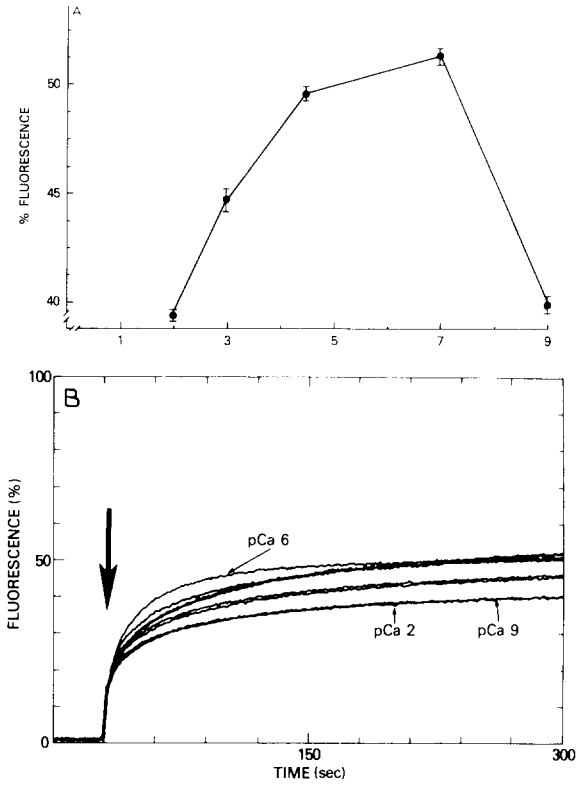

Fig. 2. Effect of Ca^{2+} on the fusion reaction. (A) The final extent of the fluorescence increase is plotted against the free calcium concentration (p*Ca*). In these experiments the pH was kept at 6. The maximal increase in the final extent of fluorescence (approx. 12%) was achieved at p*Ca* 7. (B) The arrow indicates the time at which synexin was added. Actual traces of the experiments. Both lowermost traces correspond to a p*Ca* of 2 and 9.

Binding of ^{125}I-synexin to chromaffin granules and granule ghosts

Binding of synexin to chromaffin granule ghosts is the initial step in the process of fusion induction. So as to examine the dependence of synexin binding to chromaffin granule ghosts as a function of modulatory parameters, synexin was labeled with ^{125}I by the Bolton-Hunter method [12]. As shown in Table I, although synexin bound in a Ca^{2+}-dependent manner to intact chromaffin granules it also showed substantial Ca^{2+}-independent binding to the F/Th chromaffin granule

ghosts. Thus, the freeze/thawed chromaffin granule ghosts seem to have an intrinsic affinity for synexin distinct from that of the native granules. This finding is in line with the fusion experiments presented above. We also noted (see Table I) that synexin binding to the F/Th chromaffin granule ghosts was enhanced by lowering the pH of the medium, a finding not observed with intact chromaffin granules.

Influence of pH on the synexin-induced fusion

The pH effect observed in the binding experiments led us to predict that if the binding of ^{125}I-synexin to F/Th chromaffin granule ghosts were relevant to the fusion process, then fusion itself might be enhanced at lower pH values. A similar pH response has been reported for some viral-mediated fusion of membranes [15–17]. To test this hypothesis we examined the effect of pH on the fusion reaction elicited by a single pulse of synexin (90 nM, 37 °C, p*Ca* 7). It should be noted that fluorescein fluorescence decreases rather than increases at low pH, a fact which may yield underestimated values in these experiments. As shown in Fig. 3B, almost no fusion occurs at pH 7.2 and by lowering the pH the fluorescence signal increases markedly and in a non-linear fashion, saturating at about pH 4.0. The pH profile of the fusion process as depicted in Fig. 3A was of a sigmoidal shape, resembling a pH titration curve. The inflection point, which represents the pH at half-maximal fusion, was approx. 5.2.

This pH value is in the range of p*K* values for carboxylic groups, suggesting to us the possible involvement of these very groups in the observed process. To test this assumption we subjected the granule ghosts to chemical modification by the membrane impermeant $COOH^-$ group modifier, EAC (0.1 mM for 30 min at 37 °C). After extensive washing, the membranes showed a > 80% reduction in their ability to undergo fusion (Table II). By contrast, treatment of synexin with the same agent was without effect. We thus conclude that the pH sensitivity of the synexin-induced fusion reaction and binding of ^{125}I-synexin reflect a property of the vesicles, possibly acquired by the freeze/thaw procedure shown above.

Specificity of the synexin-induced fusion

In order to investigate the question of specificity of synexin-induced fusion, we also tested the effect of non-related proteins. As seen in Fig. 4, first arrow, bovine serum albumin (Sigma, fraction V) at a concentration of 250 nM did not induce fusion of the vesicles. Moreover, bovine serum albumin did not promote aggregation of chromaffin granule ghosts, as followed by absorbance at 540 nm (not shown). In addition, non-specific goat IgG supported neither aggregation nor fusion (data not shown). On the other hand, synexin, heat denatured at 70 °C for 10 min, neither elicited fusion of the ghosts (Fig. 4, second arrow), nor

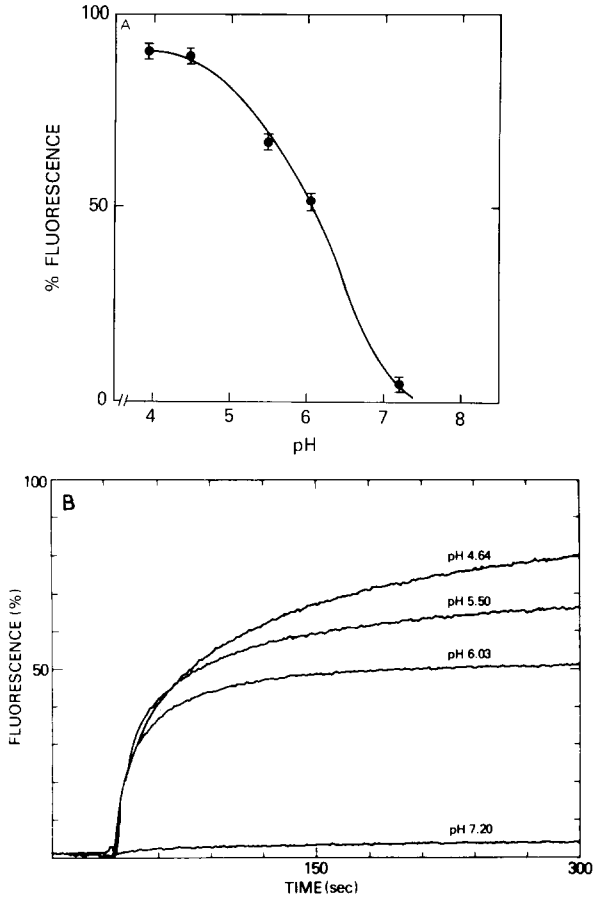

Fig. 3. The pH dependence of the fusion reaction. (A) Fluorescence increase as a function of extravesicular pH. Each point is the average of three experiments. The fluorescence values were obtained from traces, such as shown in (B). The inflection point of the curve is approx. pH 5.2. (B) The experiments were run at a synexin concentration of 124 nM, at 37 °C, p*Ca* 7. The granule ghosts were allowed first to equilibrate with the different media prior to the addition of synexin.

TABLE I

^{125}I-SYNEXIN BINDING TO INTACT CHROMAFFIN GRANULES AND TO FREEZE/THAWED (F/Th) CHROMAFFIN GRANULES GHOSTS AT DIFFERENT pH AND p*Ca* COMBINATIONS

Synexin was iodinated (specific activity of 526 cpm/pmol) using the Bolton-Hunter reagent. An aliquot (0.5 mg protein/ml) of either intact granules or ghosts was washed and incubated for 30 min with radioactive synexin (approx. 70 pmol) and 5% bovine serum albumin at room temperature, followed by 20 min centrifugation at 20000 rpm over a 15% sucrose cushion. After the centrifugation the radioactivity was measured in the supernatant and in the pellet. Control (no membranes) and background were subtracted. The radioactivity was normalized for protein content found in the pellet.

Conditions		10^3 cpm/mg protein (mean ± S.E.)			
p*Ca*	pH	Chromaffin granules		F/Th chromaffin granules ghosts	
		Pellet	Supernatant	Pellet	Supernatant
2	6	25.75 ± 0.52	4 ± 0.25	19 ± 0.73	7 ± 0.48
7	6	2.25 ± 0.61	27 ± 0.83	19.2 ± 0.41	6.5 ± 0.39
7	4.6	2.75 ± 0.40	26.5 ± 0.71	25.6 ± 0.85	1 ± 0.69

hindered the activity of native synexin (Fig. 4, third arrow).

The question of a putative protein moiety on the surface of chromaffin granule ghosts which may act as a specific receptor for synexin was also approached [18]. We treated the F/Th chromaffin granule ghosts with trypsin and then tested their ability to undergo synexin-induced fusion. Both populations of F/Th chromaffin granule ghosts (loaded and blank) were subjected to proteolysis (see Materials and Methods). As shown in Table II, the fusion capacity of proteolyzed F/Th ghosts was significantly reduced (approx. 30%). When

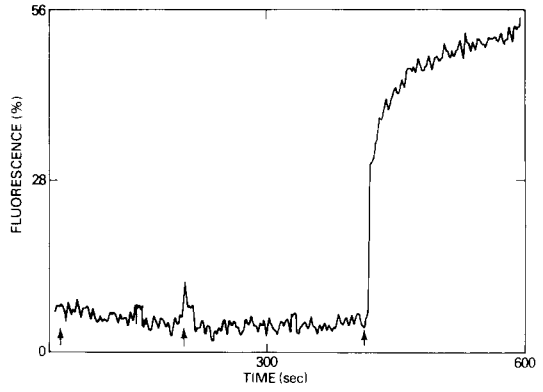

Fig. 4. Effect of proteins and heat-denatured synexin on the fusion reaction. The addition of bovine serum albumin (250 nM) is shown by the first arrow. The addition of a heat-denatured sample of synexin (124 nM, heated at 70 °C for 10 min) is depicted by the second arrow. Addition of an active sample of synexin (124 nM) is indicated by the third arrow.

TABLE II

EFFECT OF CHEMICAL AND BIOCHEMICAL MODIFICATION ON SYNEXIN-INDUCED MEMBRANE FUSION

Treatment	Inhibition of fluorescence (% of control) [d]
EAC [a]	82.1 ± 6.3
Trypsin [b]	31.6 ± 2.4
Anti-synexin antibodies [c]	40.3 ± 5.8

[a] The membranes were pre-treated with 0.1 mM EAC for 30 min at 37 °C, prior to initiation of fusion with synexin.

[b] The membranes were pre-treated with trypsin (1 mg/ml) for 30 min at 37 °C, washed and then mixed with soybean trypsin inhibitor (1 mg/ml), prior initiation of fusion with synexin.

[c] The anti-synexin antibodies were added at $t = 0$ to the fusion mixture.

[d] $n = 2$ for each condition, ± standard error.

polyclonal antibodies against synexin were included in the fusion assay system, the final extent of fluorescence increase could be specifically reduced, although to a maximum of approx. 40%, irrespective whether or not an excess concentration of antibody was used in this assay (Table II).

Discussion

Fusion of chromaffin granule ghosts to one another by synexin is clearly shown in this paper

to be dependent not only on calcium but also on the pH of the medium. Synexin-driven aggregation and fusion of native chromaffin granules, is likewise dependent on these two ions, but with quantitative differences. However, these differences seem to be very instructive in terms of the mechanisms by which synexin interacts with and fuses native membranes, especially since the details of pH dependence appear to be experimentally accessible in the ghost fusion system. In particular, the pH dependence of fusion seems to be interpretable in terms of titration of carboxyl groups on the ghost membranes. The evidence for this is that fusion depends on pH with an apparent pK of 5.2, and that the membrane-impermeant modifier of COOH groups, EAC, blocks the ability of the ghost membranes to fuse. Since pretreatment of synexin itself with EAC has no effect on the fusion activity of synexin, it is apparent that the site of action of both protons and EAC is likely to be on membrane-specific COOH groups. The molecular identity of the membrane molecules bearing these COOH groups remain to be determined. Since stimulated cells undergo changes in both intracellular calcium concentration and pH [19], it is likely that these sites may eventually prove not to be only of biochemical interest but also of some physiological importance as well.

The pH dependence of synexin-driven chromaffin granule aggregation is somewhat different from that described here for ghost fusion, where the optimal pH is 6.0 and no further increments are observed at lower pH values (data not shown). It is apparent, as we will expand on below, that ghost membranes are in fact different in many ways from membranes of native chromaffin granules. For example, the calcium dependence of synexin-driven ghost fusion differs from the calcium dependence of synexin-driven aggregation of native chromaffin granules. While calcium does promote synexin-induced fusion of the F/Th ghosts, the contribution to the overall level of fusion is modest. In addition, maximum fusion is observed at 0.1 μM calcium, and higher values actually inhibit the fusion event. Also, the calcium effect is not on the kinetics, but on the final extent of fusion. This might mean that a fraction of the F/Th chromaffin granule ghosts may retain their capacity to be fused by calcium-activated synexin. However, it is also clear, that the larger fraction of F/Th chromaffin granule ghosts do not require the activation of synexin by calcium per se in order to fuse. Our results are therefore consistent with a modulatory effect of Ca^{2+} on the system, and could also perhaps explain the well known basal secretion of catecholamines from chromaffin cells occurring at relatively low intracellular Ca^{2+} concentrations.

Yet another difference between F/Th chromaffin granule ghosts and native granules is that the F/Th chromaffin granule ghosts do not require arachidonic acid to fuse [2]. This observation might clearly reflect the different fusion capabilities of both membranes. Thus, the freeze-thaw treatment seems to take the majority of the granule membranes to a fusion-prone state, perhaps similar to a condition found in native granules after binding of calcium-activated synexin, or similar to synoxin-induced fusion of some liposomes [20,21].

There are, however, some profound similarities between the synexin-driven fusion in both systems. The dose-response curve showed a half-maximal fusion concentration for synexin of 85 nM and a saturating concentration of 450 nM. These values are similar to the values for synexin-induced aggregation of native chromaffin granules (77 nM, Ref. 14) indicating that the action of synexin on intact granules or ghosts is exerted primarily but not necessarily exclusively at the aggregation step. The main difference lies, therefore, in the fusion step.

A possible similar situation is seen in the case of red blood cell ghosts. In order for red blood cell ghosts to undergo viral-mediated fusion, pretreatment with SH-reagents is necessary. However, treated and non-treated ghosts are capable of undergoing viral-mediated aggregation [22]. This differential behavior might be explained by the fact that the membrane matrix has to relax in order to be able to undergo fusion. Hence, an analogous explanation could also be possible here when comparing fusion capabilities of native granules and granule ghosts.

The likely importance of this fusion process also rests on the specificity of the reaction for synexin. We found that the anti-synexin goat antibody blocked the synexin-induced fusion by ap-

prox. 40%. This blocking effect has been also found in native granules [10]. The fact that total inhibition could not be achieved with the anti-synexin antibodies might be due to either the heterogeneity of the polyclonal anti-synexin antibodies and/or to partial masking of synexin domains associated with fusion, so that only a fraction of them inhibit the fusion reaction.

This synexin-driven fusion process also requires a native protein, since heat-denatured synexin was unable to support fusion. In addition, bovine serum albumin was not able to elicit any fusion event, a fact previously shown with intact granules. Therefore, the bovine serum albumin experiments, the heat-denatured synexin experiments and the anti-synexin experiments suggest that the synexin-driven fusion event is specific.

Synexin-induced fusion of intact granules is, however, not trypsin sensitive, while fusion of F/Th chromaffin granule ghosts is. Previous controversy arose due to residual trypsin effect on synexin [18,20]. This is not the case in our experiment, and the difference might actually reside on the properties of the membranes. For example, the freeze-thaw technique might expose protein residues involved in fusion, which are normally buried in the membrane, thus making the fusion event sensitive to trypsin [23]. The involvement of protein(s) is also suggested by experiments using anti-idiotype antibodies to synexin monoclonal antibodies. Preliminary data show a 180000 mol. wt. protein which is specifically isolated from chromaffin graule membranes by immunoprecipitation (unpublished results).

We therefore anticipate that further studies on native granules and F/Th chromaffin granule ghosts will continue to yield insight into the mechanisms of synexin-mediated fusion. For example, the present studies have served to emphasize the importance of the state of membrane in synexin fraction, a perception missing in previous studies of synexin action.

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