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Effect of synexin on aggregation and fusion of chromaffin granule ghosts at pH 6

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Fusion of chromaffin granule ghosts was induced by synexin at pH 6, 37°C, in the presence of 10^{-7} M Ca^{2+}. To study the kinetics and extent of this fusion process we employed two assays that monitored continuously mixing of aqueous contents or membrane mixing by fluorescence intensity increases. In both assays chromaffin granule ghosts were either labeled on the membrane or in the included aqueous phase. The ratios of blank to labeled chromaffin granule ghosts were varied from 1 to 10. The results were analyzed in terms of a mass action kinetic model, which views the overall fusion reaction as a sequence of a second-order process of aggregation followed by a first-order fusion reaction. The model calculations gave fare simulations and predictions of the experimental results. The rate constants describing membrane mixing are more than 2-fold larger than those for volume mixing. The analysis also indicated that the initial aggregation and fusion processes, up to dimer formation, were extremely fast. The rate constant of aggregation was close to the limit in diffusion-controlled processes, whereas the fusion rate constant was about the same as found in fastest virus-liposome fusion events at pH 5. A small increase in volume was found to accompany the fusion between chromaffin granule ghosts. Using ratios of synexin to chromaffin granule ghost protein of 0.13, 0.41 and 1.15 indicated that the overall fusion rate was larger for the intermediate (0.41) case. The analysis showed that the main activity of synexin was an enhancement of the rate of aggregation. At intermediate or excessive synexin concentrations it, respectively, promoted moderately, or inhibited the actual fusion step.

Introduction

The most rigorous criterion for membrane fusion involves measurement of the mixing of the contents of one vesicle with the contents of a second vesicle. Wilschut et al. [1] developed an assay based on volume mixing that has been applied to numerous studies on vesicle fusion and is amenable to analysis [2]. At low pH another assay [3] has been developed. However, the problems of inefficient encapsulation and excessive leakage through biological membranes have restricted the application of these assays to a few biological systems. A less rigorous solution has been to measure the mixing of membrane components of fusing vesicles [4].

Hoekstra et al. [5] labeled biological membranes with the fluorescent probe octadecyl rhodamine (R_{18}) and monitored fusion by relief of self-quenching of the fluorescent probe in the

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Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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presence of unlabeled membranes. The R_{18} method is most suitable because of the ease of incorporation of the probe into biological membranes and its minimal exchange through diffusion.

However, Stutzin [6] has recently developed a fusion assay suitable for biological membranes such as chromaffin granules that monitors volume mixing and avoids the problem of leakage. The procedure involves loading of a population of vesicles with self-quenching concentrations of fluorescein, chemically linked to high molecular weight dextran. Upon fusion of the dye-loaded vesicle with a blank vesicle dilution and dequenching occur. A specific anti-fluorescein antibody [7] present in the medium suppresses the signals due to leakage.

In the present work we have therefore attempted to use both the new volume mixing method and the R_{18} method for membrane mixing to analyze the mechanism of synexin-induced fusion of chromaffin granule membranes. Synexin is a widely distributed 47 kDa calcium binding protein, which causes isolated chromaffin granules to aggregate [8] or to fuse, if certain fatty acids are added [9,10] to the compound exocytotic structures observed by electron microscopy in secreting chromaffin cells [11]. Synexin has also been shown to promote the overall rate of Ca^{2+}-induced fusion of acidic vesicles [12–14] and to reduce significantly the required concentration of Ca^{2+}.

We have also attempted to supplement the experimental technique developed by Stutzin [6] with a theoretical analysis. This analysis enables us to translate the increase in fluorescence into percent of fusion and to provide an estimate of the concentration distribution of aggregation-fusion products.

Materials and Methods

Preparation of chromaffin granules. Bovine chromaffin granules were prepared by differential centrifugation and purified over a 1.6 M sucrose density gradient, by a modification of a method previously reported [15].

The isolation medium was sucrose 300 mM, Hepes-K 40 mM, EDTA 1 mM, dithiothreitol 1 mM and phenylmethylsulfonyl fluoride 1 mM (pH 7.2) [16]. The final granule suspension was stored at −80 °C or used immediately for preparing chromaffin granule ghosts. Protein content was measured as in Ref. 17 with bovine serum albumin as a standard [18].

Chromaffin granule ghost preparation. The chromaffin granules were lysed in a 25-fold volume of either distilled water or 5 mM Tris-maleate (pH 7.2) for 10 min at 4 °C, followed by a 30-min incubation at 37 °C. The resulting chromaffin granule ghosts were then washed twice and resuspended in the experimental medium containing KCl 140 mM, Hepes-K 20 mM and EGTA 0.1 mM (pH 7.2).

Incorporation of FITC-Dextran into chromaffin granule ghosts. An aliquot of the chromaffin granule ghosts was resuspended in 0.5 ml of the experimental buffer containing 0.1 mM FITC-Dextran, mol. wt. 20000 (Sigma). Another chromaffin granule ghost aliquot was resuspended in the same buffer but without FITC-Dextran. Both samples were submerged in liquid nitrogen for 1 min and subsequently allowed to thaw at room temperature. This cycle was repeated three times to increase entrapping of the probe by the vesicles [19]. The FITC-Dextran-loaded chromaffin granule ghosts were then washed twice by centrifugation in buffer and subsequently passed through a Sephacryl S-300 Super-fine column (Pharmacia), to remove the excess dye. The non-loaded vesicles were treated similarly. The protein contents of both samples were adjusted to 0.3 mg/ml.

Incorporation of octadecyl rhodamine B (R_{18}) into chromaffin granule ghosts. Octadecyl rhodamine (R_{18}) (Molecular Probes, Junction City, OR) was dissolved at 10 mg/ml in ethanol, and the probe was incorporated into the membranes at a 5% molar lipid ratio [5]. The membranes were then incubated for 30 min at 37 °C and subsequently passed through a Sephadex G-25 M column (20 × 1 cm, Pharmacia) to remove the excess R_{18}.

Anti-fluorescein antibodies. Anti-fluorescein antibodies were prepared as described in Ref. 7. The conjugation protein was Keyhole limpet hemocyanin, KLH (Calbiochem, CA), and the molar ratio FITC/KLH was 40 : 1.

Fluorescent measurements. Continuous monitoring of the fluorescence was carried out in a Spex Fluorolog 2 spectrophotofluorimeter (Spex Industries, Metuchen, NJ), equipped with a digital

plotter and a microprocessor (DMIB). For experiments with FITC-Dextran the excitation and emission wavelengths were 465 and 520 nm, respectively. In the R_{18} experiments the respective wavelengths were 540 nm and 580 nm. A cutoff filter (498 nm for FITC-Dextran and 565 nm for R_{18}) was regularly used. The sample chamber was equipped with a magnetic stirrer and the temperature was controlled with a thermostated circulating water bath.

Conditions for fusion experiments. The medium used was 140 mM KCl/20 mM Hepes-K (pH 6), p*Ca* 7 and the experiments were run at 37°C. Synexin was purified as described in Ref. [20]. The experiments to calibrate the extent of fusion and the necessary amounts of anti-fluorescein antibody to quench the leakage signal were run as follows: the vesicles were mixed in the given ratio (1 : 1 or 1 : 10; loaded/blank, respectively), in the absence of anti-fluorescein antibodies. The total amount of chromaffin granule ghost protein per experiment was 2.2 μg, 16 μg or 100 μg per 2 ml. When the signal was stabilized, synexin was added. Nonidet P-40 (British Drug House) (0.1% final concentration) was then added and the fluorescence at infinite dilution was obtained. The disruption of the vesicles was followed by the addition of small aliquots of the anti-fluorescein antibodies, to quench the fluorescence to background levels. In each fusion experiment, the vesicles were mixed at the above mentioned ratio simultaneously with an excess amount of anti-fluorescein antibodies, as calculated from the calibration experiments. Once the system was stabilized, synexin was added. Nonidet P-40 was added at the end of the experiment to confirm that the concentration of anti-fluorescein antibodies was adequate to correct for any leakage.

Calibration of intensity increase with changes in surface or volume probe concentration. The percent increase in fluorescence intensity, I, per molecule of probe, due to relief of self-quenching of R_{18} molecules upon probe dilution, is given by a linear relationship [5]

$$I = (1 - X)100 \tag{1}$$

in which X is the relative surface concentration of the probe.

Dilution of FITC-dextran results in a relief of self-quenching of the probe. The increase in intensity per molecule was linear over the range of concentrations from 10^{-4} M to $2.5 \cdot 10^{-5}$ M. We found the most convenient representation of the data in this range to be

$$I_R = 2.56 - 1.56y \tag{2}$$

in which I_R is the fluorescence intensity per molecule relative to the initial value of fluorescence at a probe concentration of 10^{-4} M, and y is probe concentration relative to the initial value of 10^{-4} M.

Final levels of R_{18} fluorescence intensity. In a population of labeled and blank particles whose number ratio is L/k complete fusion to products consisting of n particles yields a fluorescence intensity, I, of

$$I = 100 \cdot [k/(k + L)] \cdot (n - 1)/n \tag{3}$$

As n becomes large Eqn. 3 coincides with the expression for a homogeneous mixture [21,22]. For fusion up to doublets ($n = 2$) the cases $L/k = 1$ and $L/k = 1/10$ (1/10 population) yield $I = 25$ and 45.5, respectively.

Analysis of fusion kinetics. The analysis followed the mass-action kinetic model [2,23–27], which views the overall fusion reaction as a sequence of two gross steps: (1) aggregation; (2) fusion, i.e., membrane destabilization and merging. Dissociation of aggregates was explicitly considered. The calculations were performed by introducing certain modifications into the program described in Ref. 22 which was used here for aggregation-fusion products consisting of up to eight particles. The program calculates the distribution of aggregation-fusion products and the corresponding values of increase in fluorescence intensity, I or I_R for both membrane mixing and volume mixing processes. The rate constants of aggregation, fusion and dissociation are C_{ij} ($M^{-1} \cdot s^{-1}$), f_{ij} (s^{-1}) and D_{ij} (s^{-1}), respectively, where $i = j = 1$ refers to aggregated dimers or fused doublets. The procedure usually involves first the determination of C_{11} by simulating the results for dilute vesicle suspensions where aggregation, which is of second order in vesicle concentrations is the

rate limiting step. A criterion for this case is [23–25]

$$K = f_{11}/(C_{11}V_o) \gg 1 \quad (4)$$

in which V_o is the initial molar concentration of particles. Usually this limit corresponds to $K \geq 100$. Next, the rate constant of fusion is determined by simulating I values obtained with a concentrated suspension. The rate constant $D = D_{11}$ is determined by focussing on later stages of the reaction.

The calculations allowed for a certain factor of volume increase associated with fusion according to Eqn. 5 below. The relative concentration of FITC-dextran, y, in a fusion product consisting of I labeled and J blank particles is

$$y = I/[(I + J) \cdot B] \quad (5)$$

where B is a parameter giving the factor of increase in volume. If $B = 1$ then $y = 1$ in a fusion product consisting entirely of labeled particles, where $J = 0$, and consequently fusion between labeled particles would not result in fluorescence increase. The parameter B was determined from one set of fusion results in a population consisting entirely of labeled particles.

Results

Chromaffin granule ghosts at pH 6 and 37 °C were mixed with synexin and the resulting fusion was monitored continuously either by means of mixing of encapsulated contents using the FITC dextran assay (Fig. 1) or by mixing of membranes using the R_{18} assay (Fig. 2). From the extent of fluorescence increase after 5 minutes it can be deduced that practically all the particles underwent at least one round of fusion and that almost all of them are active, i.e., capable of fusing. For example, in the case of the R_{18} membrane mixing experiment (Fig. 2) we computed that a homogeneous mixture formed from a 1/1 population of R_{18}-surface labeled and blank chromaffin granule ghosts would yield a percent of increase in fluorescence intensity $I = 50$, and a complete fusion to doublets would yield $I = 25$ (see Eqn. 3), whereas curve b of Fig. 2 gives $I = 47$. Similarly, the relative increase in fluorescence intensity due to

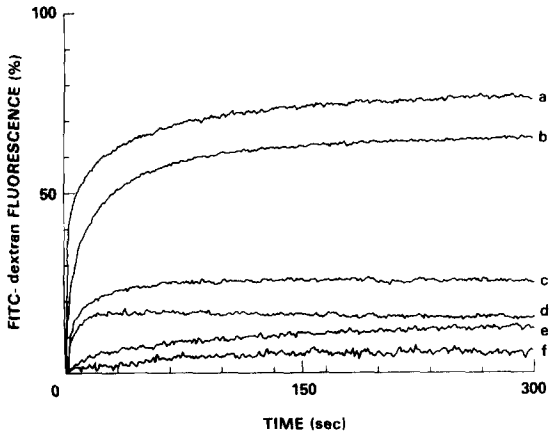

Fig. 1. Synexin-induced fusion of chromaffin granule ghosts detected by the volume mixing assay. The experiment was performed at 37 °C, pH 6.0 and p*Ca* 7 in a total volume of 2 ml. In all these experiments the ratio between synexin and ghosts was 0.41 (expressed in terms of amount of protein). Each curve represents the average of two experiments, and leakage has been subtracted. Curves a and b show the effect of synexin on the fluorescence signal when the total ghost protein was 16 μg. Curve a corresponds to a 1/10 labeled/blank ratio and curve b to a 1/1 ratio. In curves c and d the total amount of ghost protein is 2.2 μg; in curve c the ratio labeled/blank is 1/10 and in curve d the ratio is 1/1. Traces e and f show the effect of synexin on the fluorescence signal when no blank ghosts were present. The cuvettes in curve e contained a total ghost protein of 16 μg, while those in curve f contained 2.2 μg.

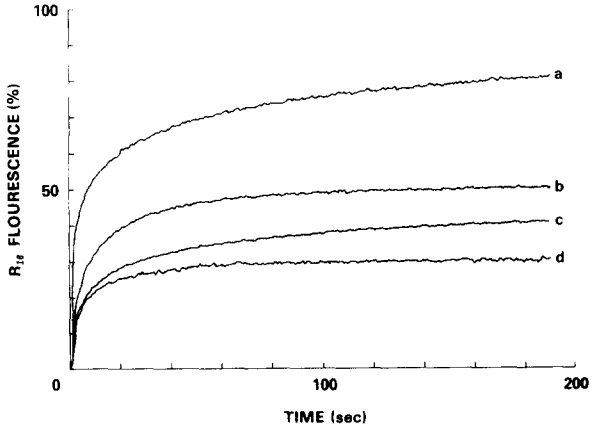

Fig. 2. Synexin-induced fusion of chromaffin granule ghosts detected by the membrane mixing assay (Hoekstra et al., 1984). The experimental conditions were the same as for the volume mixing assay (Fig. 1). The R_{18} molar ratio was 5% and the ratio between synexin and ghosts was 0.41. Curves a and b correspond to a total ghost protein of 16 μg; in curve a the ratio labeled/blank was 1/10 and in curve b the ratio was 1/1. Traces c and d show the effect of decreasing the total ghost protein to 2.2 μg. The labeled/blank ratio is 1/10 in curve c and 1/1 in curve d.

volume mixing (Fig. 1) in a 1/1 population fusion up to doublets would be 1.39 (see Eqn. 2), whereas Fig. 1b gives a value of 1.49.

According to the equations of mass action an increase in particle concentration should result in a faster fusion process as monitored by volume or membrane mixing assays, either in a 1/1 or 1/10 population. We would also expect that in dilute suspensions the overall fusion process would be rate limited by the aggregation step. Indeed in a 1/1 population whose protein content is 2.2 μg/2 ml (Fig. 1d) the concentration of chromaffin granule ghosts is $5.16 \cdot 10^{-12}$ M making the product $V_o C_{11} = 0.01$, a value 100-fold smaller than f (see Eqn. 4 and Table I). Under such conditions the increase in fluorescence intensity in the initial stages should obey a simple rule that the value in a 1/10 population is (10/11)/(1/2) times the value in a 1/1 population [22]. This relation is indeed satisfied by the experimental results obtained with both assays. Table II demonstrates in this case that the concentration of free particles decreases linearly over time.

As previously noted [6] fusion between R_{18} surface labeled chromaffin granule ghosts in the absence of unlabeled ghosts, did not result in an increase in fluorescence intensity. In contrast, curves e and f in Fig. 1 demonstrate a slow increase in fluorescence intensity, up to $I_R \leq 1.1$, in a population consisting exclusively of volume labeled chromaffin granule ghosts. These experiments thus provide information on the increase in volume associated with the fusion process. For example, if two spherical vesicles fuse into a spherical vesicle, then their total interior volume must increase by a factor $B = 1.4$; for spherical fusion products consisting of n spherical primary vesicles B equals approximately $\sqrt{n}$ [2]. A simulation of curve e of Fig. 1 was achieved by using $B = 1.1$, and the prediction for curve f was good. A comparison of curves e and f in Fig. 1 lends further support to the fact that the observed in-

TABLE I

RATE CONSTANTS DESCRIBING AGGREGATION AND FUSION OF CHROMAFFIN GRANULE GHOSTS AT pH 6 AND 37 °C

The estimated uncertainties in D_{11}, f_{11} and C_{11} are 100%, 30% and 20%, respectively, except where explicitly indicated. The calculations employed $\gamma = 0.1$ s^{-1} (see Eqn. 6).

Fusion assay	Ratio synexin CGG protein (w/w)	C_{11} ($M^{-1} \cdot s^{-1}$)	f_{11} (s^{-1})	D_{11} (s^{-1})
Membrane mixing	0.41	$5 \cdot 10^9$	≥ 10	0.5
Volume mixing	0.41	$3.2 \cdot 10^9$	1.3(1.6 [a])	0.5
Volume mixing	1.15	$(2\text{–}4) \cdot 10^9$	0.1–0.3	0.5
Volume mixing	0.13	$4 \cdot 10^8$	1	0.5

[a] The value in parenthesis was used when Eqn. 7 was employed with $\gamma = 0.11$ s^{-1}.

TABLE II

A SAMPLE FROM A DISTRIBUTION OF AGGREGATION-FUSION PRODUCTS OF CHROMAFFIN GRANULE GHOSTS

	A concentrated [a] suspension 1 : 10 $T = 30$ s	A dilute [b] suspension 1 : 1 $T = 30$ s
%A(1,0) [c]	35.6 [c]	82.3 [c]
%A(1,1)	2.4	1
%A(1,2)	0.1	0.009
%A(1,3)	0.007	$8 \cdot 10^{-5}$
%A(3,4)	10^{-8}	$2 \cdot 10^{-6}$
%F(1,1)	25	6.6
%F(1,2)	13.2	0.4
%F(1,3)	7.1	0.02
%F(2,0)	1.3	3.3
%F(2,3)	0.5	0.002
I_R	1.55	1.08

[a] The protein content of chromaffin granule ghosts is 16 μg/2 ml, which corresponds to a lipid concentration of 4.55 μM, or molar concentration of chromaffin granule ghosts of $3.76 \cdot 10^{-11}$ M, assuming a radius of 60 nm. In all cases in this table the calculations pertain to a synexin/chromaffin granule ghosts protein ratio of 0.41. See curve a of Fig. 4.

[b] In this case the lipid concentration is 0.625 μM, which corresponds to $5.16 \cdot 10^{-12}$ M of chromaffin granule ghosts. See curve c of Fig. 4.

[c] The table gives percents of the given aggregation-fusion products relative to initial concentration of labeled chromaffin granule ghosts or A(1,0) at $T = 0$. The quantities A(I, J) and F(I, J) denote aggregation and fusion products consisting of I labeled and J blank chromaffin granule ghosts. The program calculates additional structures as described in Ref. 22. The rate constants used are $C_{11} = 3.2 \cdot 10^9$ $M^{-1} \cdot s^{-1}$, $f_{11} = 1.3$ s^{-1}, $D = 0.5$ s^{-1}.

[d] The calculations employed $\gamma = 0.1$ s^{-1} (see Eqn 6).

crease in volume is indeed that associated with the fusion process, since it proceeds faster in more concentrated suspensions.

We have also compared the rates of volume mixing and membrane mixing in fusing chromaffin granule membranes. A comparison of curves a to d in Fig. 1 with the corresponding ones in Fig. 2 indicates an apparent faster kinetics of membrane mixing than volume mixing. Our analysis treated each set of data independently and provided the rate constants of aggregation, fusion and dissociation which gave the best simulations of experimental results. Table I shows that C-values describing membrane mixing are almost 2-fold larger than those used for volume mixing, and the difference in f values is even larger.

In order to elucidate more details of the effect of synexin on the aggregation and fusion of chromaffin granule ghosts, similar experiments to those presented in Fig. 1 were carried out for additional synexin concentrations. In all these experiments we maintained the ratio of synexin to chromaffin granule ghost protein constant for all chromaffin granule ghost concentrations. The rationale was that at a given ratio of synexin to chromaffin granule ghost protein, below saturation, most of the synexin was bound as determined by employing ^{125}I-synexin (Stutzin, Lelkes, Cabantchik, Rojas and Pollard, unpublished data). The ratio between synexin and chromaffin granule ghost protein in the experiments described in Figs. 1 and 2 was 0.41 (6.5 : 16, w/w). Fig. 3 demonstrates the effect of synexin contents on the overall fusion kinetics for a given concentration of chromaffin granule ghosts.

In planning the experiments with a low synexin content it was anticipated that the rate of aggregation would be reduced. In order to include cases with a larger product of $C_{11}V_o$, the concentrated suspensions used in this case were 100 μg chromaffin granule ghost protein/2 ml. For brevity only the summary of these results in terms of the rate constants is given in Table I. The reduction in the amount of synexin from a ratio of 0.41 to 0.13 per chromaffin granule ghost protein resulted in a significant reduction in the rate of overall fusion reaction. However, a further increase in synexin ratio above 0.41 resulted in inhibition in the overall fusion reactions. Thus the overall fusion rate

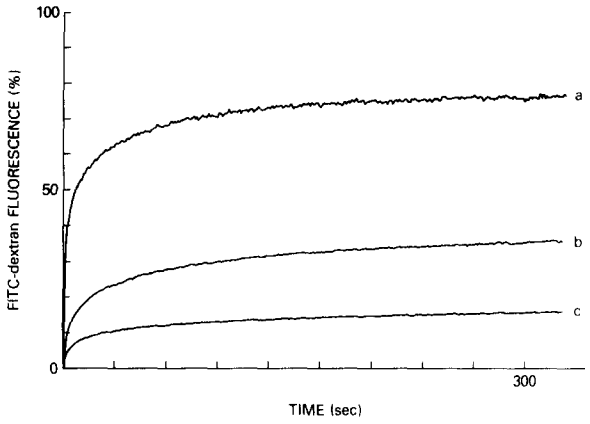

Fig. 3. Effect of synexin content on the overall kinetics. The experimental conditions are the same as for Fig. 1. In these experiments the ratio labeled/blank vesicles was kept constant at 1/10 and the synexin/ghost protein ratio was modified. The total ghost protein is 16 μg. In trace a the ratio synexin/ghost protein is 0.41, in trace b 1.15 and in trace c 0.13.

exhibits a maximum as a function of synexin concentration. We have not attempted to locate this maximum.

The calculations based on a mass action model which views the overall fusion step to consist of a second order process of aggregation followed by a first order process of membrane destabilization and merging, enable us to deduce more details of the effect of synexin on the fusion of chromaffin granule ghosts. Figs. 4 and 5 illustrate the fit of calculated (continuous lines) to experimental (symbols) I values for two chromaffin granule ghosts concentrations and ratios between labeled and blank vesicles for synexin/chromaffin granule ghost protein ratio of 0.41. Curve a of Fig. 4 gives the fusion kinetics for a 1/10 mass ratio of labeled to unlabeled vesicles, while curve b is for a 1/1 mass ratio. Curve c is similar to curve b except for a lower concentration of chromaffin granule ghosts. Curve d of Fig. 4 illustrates the predictions for a system consisting only of labeled particles.

The calculations (see Ref. 22 and Materials and Methods) gave good fit to the I_R values of volume mixing up to $t = 15$ s. At later times the calculations give overestimates which increased with time. Fig. 1 demonstrates that the overall rate of fluorescence increase decays with time and an apparent plateau occurs at about 3 min. In fact,

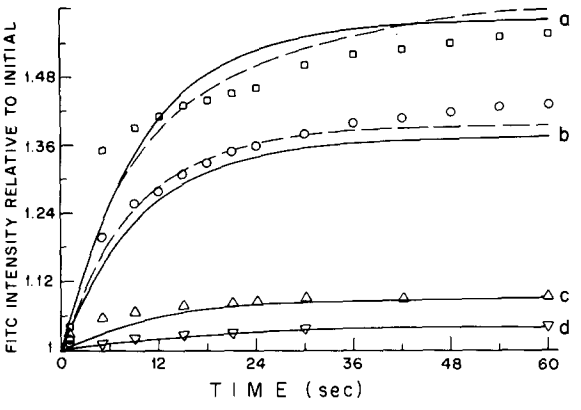

Fig. 4. Simulation and prediction of kinetics of volume mixing of chromaffin granule ghosts induced to fuse by synexin at pH 6. The lines denote calculated values employing rate constants given in Table I for a synexin/chromaffin granule ghost protein ratio of 0.41. Curves a, b, c and d are under the same conditions as curves a, b, d and f of Fig. 1, respectively. Chromaffin granule ghost molar concentrations are $3.76 \cdot 10^{-11}$ (a,b) and $5.16 \cdot 10^{-12}$ M (c). Curve a corresponds to a 1/10 labeled/blank ratio and curves b and c to a 1/1 labeled/blank ratio. In curve d no blank chromaffin granule ghosts were present. The solid and broken lines were calculated with Eqn. 6 or 7, respectively.

there is some moderate increase up to about 3 h. Another feature of the curves in Fig. 1 is that the extent of fluorescence increase at $t = 300$ s in

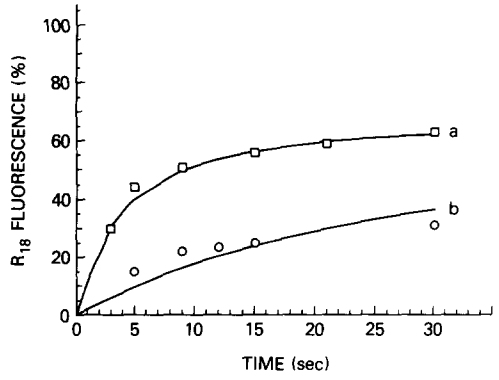

Fig. 5. Simulation of kinetics of R_{18} fluorescence increase during fusion of chromaffin granule ghosts. The lines denote calculated values employing rate constants given in Table I for membrane mixing. The molar concentrations of chromaffin granule ghosts are $3.76 \cdot 10^{-11}$ and $5.26 \cdot 10^{-12}$ M in curves a and b, respectively, which correspond to curves a and c in Fig. 2. The ratio between labeled and blank particles is 1/10. The synexin/chromaffin granule ghosts protein ratio (w/w) is 0.41.

curves c and d is lower than at the corresponding curves a and b at 15 s despite the fact that chromaffin granule ghost concentrations in curves c and d are smaller than those of curves a and b by only a factor of 8. Furthermore, the final extents at later times, e.g., 1 h and 3 h are significantly lower in more dilute suspensions. In order to account for this decay of the rates of fluorescence increase with time we introduced a decay of the fusion rate constants according to either

$$f(t) = f(0) \exp - \gamma t \tag{6}$$

or

$$f(t) = f(0)/(1+\gamma t)^2 \tag{7}$$

The values used for γ were 0.1 and 0.11, respectively.

The implication of Eqns. 6 or 7 is that there is time-dependent inactivation of synexin-induced fusion capacity of chromaffin granule ghosts, similarly to an inactivation recently found for influenza firus [28,29] which will be elaborated on in the Discussion. An attempt by us to account for the overestimates at later times by reducing the higher order rate constants, i.e., those employed for fused particles, was not successful. With the use of Eqn. 6 or 7 the calculations (see Fig. 4) provided good simulations and predictions for the kinetics of the fluorescence increase.

Table II illustrates distributions of aggregation-fusion products for two concentrations of chromaffin granule ghosts. In the more dilute suspension most of the aggregation-fusion products at times up to 30 s are aggregated dimers and fused doublets. In the case of the more concentrated suspension there is a significant fraction of higher order fusion products.

Discussion

The results in Figs. 1 and 2 and Table I show that the rate constants describing membrane mixing of chromaffin granule ghosts are several times larger than those for volume mixing. The interpretation is that in a certain fraction of events that lead to a close apposition of chromaffin granule ghosts, and possibly to membrane destabilization, mixing of external monolayers can occur without

complete fusion. Eventually complete vesicle fusion as monitored by volume mixing is accomplished, but it proceeds at a slower rate than membrane mixing. This interpretation has been previously considered [30] to explain a similar phenomenon in Ca^{2+}-induced fusion of PS vesicles, where changes in the interior volumes have a minor effect on the outcome of the analysis. Results of Ca^{2+}-induced fusion of acidic vesicles [30,31] have shown that in certain cases the overall fusion rates and the rate constants were practically similar, whereas in other cases membrane mixing was a faster process. Clearly, volume mixing is a more strict criterion for fusion, but the availability of results with the application of the two assays adds details of the aggregation-fusion process.

In the presence of optimal synexin concentration the rate constants found (Table I) indicate that both aggregation and fusion of chromaffin granule ghosts are extremely fast processes, and this is probably due to the freeze and thaw procedure employed. Van Dijck et al. [32] demonstrated that repeated cycling through the phase transition temperature induced fusion of DMPC vesicles. A similar phenomenon was reported in Refs. 33 and 34. However, it should be noted that in our case the freeze and the thaw procedure was applied during the labeling of chromaffin granule ghosts, and that no fusion occurred prior to addition of synexin. Furthermore, we noted [6] that fusion of chromaffin granule ghosts is a rather non-leaky process (10% leakage) at least for large molecules such as dextran, M_r 20000. It has been reported [35] that application of electric pulses to erythrocyte ghosts in suspension resulted in enhanced fusion activity at the moment of their aggregation, even up to 5 min after termination of these pulses. Thus, in that case as well as for freeze and thawed chromaffin granule ghosts we encounter a phenomenon of long-lived fusion-susceptible states of membranes.

The rate constant of aggregation is close to the ultimate value in diffusion controlled processes (approx. $5 \cdot 10^9$ $M^{-1} \cdot s^{-1}$, (Smoluchowski [36]) indicating that there is no potential barrier for the close approach of chromaffin granule ghosts in the presence of synexin at pH 6. The value of the fusion rate constant, $f = 1$ s^{-1}, is also among the largest reported for vesicle fusion [14,23–27,30, 31,37,38] or for virus-liposome fusion [22].

Fig. 3 demonstrates that the overall fusion rate of chromaffin granule ghosts at pH 6 is optimal for a certain synexin concentration, and that an excess of synexin leads to inhibition. Such a phenomenon was recently recorded by Gad et al. [37,38] who studied the effect of polylysine on the Ca^{2+}-induced fusion of cardiolipin/PC large vesicles. The conclusion in the latter case [37] was that excess polylysine caused a reduction in the fusion rate constant, whereas it did not result in a reduction in the rate or extent of vesicle aggregation. At lower polylysine ratios the value of f_{11} was unaffected. Previous studies [14] on the effect of synexin on Ca^{2+}-induced aggregation and fusion of vesicles concluded that synexin had no significant effect on f_{11}, and its effect on enhancing the overall rate of fusion was by increasing the rate of aggregation, i.e., C_{11} values. Table I demonstrates that a 3-fold increase in synexin contents (from a ratio of 0.13 to 0.41 synexin/chromaffin granule ghost protein) resulted in 8-fold increase in C_{11} and in 30% increase in f_{11}. A further three-fold increase in synexin resulted in 5–10-fold decrease in f_{11}, and no reduction in C_{11}. The conclusion that emerges is that the main action of synexin is in promotion of the aggregation step. The inhibition of fusion activity by excess protein is still not well understood. We have found such an inhibition when the amount of synexin in the system exceeds that of chromaffin granule ghost protein. Hence, it is possible that the excess of protein simply restricts the rearrangements to unstable intermediate states of the lipids. In support of this point of view we note that the inhibition of fusion activity by excess of synexin is only partial. In contrast, it has been found that fusion products between virus particles and liposomes do not fuse with additional virions or among themselves unless the ratio (w/w) between virus and liposomes becomes small [21,22,39–41].

However, at optimal and suboptimal synexin concentrations, synexin also moderately promotes the actual fusion step of chromaffin granule ghosts. This action of synexin may be explained by recent findings on the sequencing of synexin, which elucidated the existence of lengthy hydrophobic regions (Pollard et al. [42]). This finding may be

analogous to similar sequences found in intrinsic membrane proteins and viral glycoproteins, which may promote penetration into the target membranes and thus be responsible for their fusion activity [43–51].

Synexin-induced fusion of chromaffin granule ghosts is an extremely fast process, but the fusion capacity is rapidly decaying with time. A similar phenomenon of inactivation of hemolytic [29] and fusogenic [28,52] activity of influenza virus has been reported. Preincubation of the virus alone at optimal conditions for fusion, i.e., at pH 5 and 37 °C, resulted in immobilization and loss of activity of haemagglutinin [29]. Junankar and Cherry [29] proposed that acidic pH induces a conformational change in haemagglutinin which exposes hydrophobic segments suitable for interactions with target membranes. In the absence of apposed target membranes these exposed hydrophobic regions cause aggregation of haemagglutinin with loss of mobility and activity. While molecular details for the inactivation with time of chromaffin granule ghost fusion capacity are not available, we have evidence for its occurrence. Thus, when the chromaffin granule ghost concentration increases, more extensive fusion can proceed before inactivation has occurred. The fact is that in all cases, there is fast reduction in the overall rate of fusion and the final extents after very long times of incubation increase with chromaffin granule ghost concentration.

The exponential decay with time of the fusion rate constant (Eqn. 6) can correspond to a first-order transformation of certain molecules essential for the fusion process. The decay according to Eqn. 7 is similar to the reduction in time of monomers upon initiation of aggregation [36]. The decay (Eqn. 6 or 7) does not have to terminate in vanishing values of f. For instance, if the process reaches an equilibrium, then a residual fusion activity will be retained. Although it appears that a slightly better fit is obtained by the use of Eqn. 7, implying clustering as a possible mode of inactivation [29], our current results cannot adequately discriminate between the two modes of inactivation.

The study of fusion of chromaffin granule ghosts might mimick in certain respects the situation of compound exocytosis. The combination of low pH and synexin in the presence of a very low Ca^{2+} concentration may have biological relevance. There are examples indicating that a decrease in extracellular (and intracellular) pH results in enhancement of secretion [53]. The fusion of several viruses with the endosomes also accurs at low pH and does not require Ca^{2+} [50].

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