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KINETICS OF CATION-INDUCED AGGREGATION OF *TORPEDO* ELECTRIC ORGAN SYNAPTIC VESICLES

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Summary

Synaptic vesicles from the *Torpedo* ray can be induced to aggregate in the presence of Ca^{2+} and K^+ in the 4 mM and 50 mM range, respectively. The reactions are strikingly similar to those of chromaffin granule membranes reported previously (Morris, S.J., Chiu, V.C.K. and Haynes, D.H. (1979) Membrane Biochem. 2, 163—202). The Ca^{2+}-induced reaction includes dimerization and higher order aggregation, and is shown to be due to electrostatic screening interactions and binding to negatively-charged groups on the membrane surface. The K^+-induced reaction includes only dimerization and is shown to be due to screening interactions alone.

The kinetics of the dimerization reactions were studied using the stopped-flow rapid mixing technique. The Ca^{2+}-induced reaction has a 'bimolecular' rate constant of $4.77 \cdot 10^8\ M^{-1} \cdot s^{-1}$ while the value for the K^+-induced reaction is $7.05 \cdot 10^9\ M^{-1} \cdot s^{-1}$. These values are close to the limit of diffusion control ($8.03 \cdot 10^9\ M^{-1} \cdot s^{-1}$), indicating that no large energy barriers or structural barriers to aggregation exist. Arrhenius plots for the Ca^{2+}-induced aggregation showed a break at 5°C. Above this temperature, the activation energy is low (+0.65 kcal/mol), consistent with the above. Below this temperature, the activation energy is high, consistent with a membrane structure change increasing the energetic and structural barriers. This information, and the observation of a high stability constant of the complex, were taken as evidence for the involvement of 'recognition sites' on the membrane surface.

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The results were analyzed in terms of an encounter complex model in which vesicles with separations of 26—126 Å are considered capable of transformation into a stable complex. The rate constant of the transformation step is $1.4 \cdot 10^3\ s^{-1}$ for Ca^{2+} and approx. $1.6 \cdot 10^5\ s^{-1}$ for K^+. The values are compared with previous results for chromaffin granule membranes and for phospholipid vesicles derived from chromaffin granule lipids and from acidic phospholipids. The half-time for Ca^{2+}-induced transformation of the encounter complex into the stable complex is 435 μs. It is concluded that the recognition sites are almost as optimally deployed as the vesicle plasma membrane recognition sites involved in exocytotic release.

Introduction

Calcium-triggered exocytosis in acetylcholine release at the neuromuscular junction [1] is a good example of a rapid kinetic process of high specificity. The process includes the diffusion of the vesicle several tens of ångströms to the plasma membrane, establishment of membrane-membrane contact and fusion of the membranes. Recent experiments of Llinas and Steinberg [2] have established that the half-time for this sequence of processes is less than 200 μs. It has not yet been possible to demonstrate these processes in aqueous suspensions although the correlation of electrophysiological and electron micrographic studies [3], theoretical considerations [4,5] and a good deal of speculation have given us a fairly detailed picture of these events [6,7].

Previously we have sought to model exocytotic events and to gain an understanding of the problems involved in establishing membrane-membrane contact by studying the cation-induced aggregation of chromaffin secretory granule membranes (ghosts) derived from the adrenal medulla [8—10]. We have shown that the cation-induced reactions result from charge screening (monovalent and divalent cations) and charge neutralization (divalent cations) interactions. Our equilibrium and kinetic studies showed evidence for a high degree of structural specificity of the reaction, leading us to the conclusion that recognition sites exist on the membrane surface. The cation-induced aggregation is extremely rapid, approaching the limit of diffusion control [8,9]. The K_m for the reaction with respect to Ca^{2+} was 4.5 mM. Much higher concentrations of KCl were necessary to induce the reaction. The results were analyzed in terms of a three step model involving: (a) rapid membrane-cation interaction, (b) encounter-complex formation and (c) transformation to give a stable complex. The encounter-complex formation

$$\text{vesicle}^* + \text{vesicle}^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{encounter complex} \qquad (1)$$

(where vesicle * represents the vesicles perturbed by the added ions) was considered to occur with a diffusion-controlled rate constant (k_1). The encounter complex was considered to exist when the vesicle bilayer surfaces are between two arbitrary distances of separation (26 and 126 Å), the lower limit being the distance at which repulsive interactions of phosphatidylcholine bilayers become appreciable [11]. The upper limit was chosen for the purposes of modelling

the Ca^{2+}-triggered exocytotic process (cf. Ref. 7). The transformation step

$$\text{encounter complex} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{stable complex} \qquad (2)$$

requires the membranes to diffuse the remaining distance, overcome any energetic or structural barriers, and react. The analysis showed that the energy barriers were low and that k_2 was of the order of $3 \cdot 10^2\ s^{-1}$. This value is close to the reciprocal time for diffusion over the remaining distance. If the process of exocytotic fusion of chromaffin granules with the plasma membrane were equally facile and if similar distances were involved, a delay time of approx. 2 ms would be expected between Ca^{2+} entry and release of the stored contents.

In the present communication we report divalent cation-induced aggregation of acetylcholine storage vesicles from the electric organ of the ray *Torpedo marmorata*, and subject the phenomenon to a similar analysis. The results indicate that synaptic vesicle aggregation is more rapid than chromaffin granule aggregation. Striking similarities were observed in the behavior of the two types of membrane.

Materials and Methods

Synaptic vesicles from the electric organ of *Torpedo* were prepared either by the method of Tashiro and Stadler [12] using zonal centrifugation or by the method of Breer et al. [13] using density gradient centrifugation followed by chromatography on CPG-10-3000 porous glass beads. Vesicles prepared by both of these methods have been well characterized in terms of their protein, lipid, acetylcholine and ATP composition [12—15]. They appear by electron-microscopic examination of thin sections as membrane bound spheres of 80—100 nm diameter with electronlucent centers. Contamination by larger membrane bound vesicles is very low (cf. Fig. 1a of Ref. 15). Preliminary studies established that vesicles prepared by either procedure gave essentially identical results in either the rapid mixing or Tb^{3+}-binding experiments. Since the vesicles prepared by zonal centrifugation [12] have been better characterized in terms of lipid and protein composition [12,14,15], they were used for all the stopped-flow mixing experiments reported here. In other preliminary experiments it was shown that the Tb^{3+}-binding properties reported here could be detected in the vesicle peak from the glass bead column elutions but not in the void volume peak. Ca^{2+}-induced aggregation was not observed in the crude mitochondrial fraction.

The progress of the aggregation reaction was followed by recording the optical transmittance at 320 nm after rapid mixing. These experiments were carried out on a custom designed stopped-flow apparatus at the Max-Planck-Institut and on an Aminco-Morrow apparatus in Dr. Haynes' laboratory. The methods of analysis of the data are the same as described previously [8,9], except that the complete progress curve was subjected to computer analysis as the sum of second-order processes. Calculations were done on the Prophet Computer System using the FITFUN program.

Tb^{3+} titration studies were performed in 2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) pH 7.0, 20°C as described by Morris and Schober [16] using glass bead column-purified vesicles which had been lysed in

10 mM HEPES to remove acetylcholine and ATP [14]. Protein determinations were performed by the Coomassie Blue binding method of Bradford [17].

Results and Discussion

Cation-induced vesicle aggregation

Torpedo synaptic vesicles show cation-induced aggregation behavior which is very similar to that of chromaffin granule membranes. In this communication we will compare the two systems. The previous communications [8—10] provide a fuller description of the mechanisms for the chromaffin granule. All data reported below are similar to the behavior of chromaffin granule membranes unless a statement is made to the contrary. (Table I compares the results for the two types of neurotransmitter storage particles and phospholipid vesicles.)

Torpedo synaptic vesicles show increases in turbidity when mixed with 10 mM Ca^{2+}. The progress curve for the reaction on the time scale of seconds is given in Fig. 1. The progress curve could be fitted as two processes with second-order kinetics, with half-times of 4.8 s and 600 s. Mg^{2+}-induced aggregation was also observed with similar results. The rates of these processes increase with

TABLE I

COMPARISON OF CATION-PROMOTED AGGREGATION OF *TORPEDO* SYNAPTIC VESICLE MEMBRANES, CHROMAFFIN GRANULE MEMBRANES AND LIPID VESICLE MEMBRANES

k_{app}, measured bimolecular rate constant; PA, phosphatidic acid; PS, phosphatidylserine; CG, chromaffin granule.

	Torpedo [a]	CG membrane [b]	CG lipid vesicles [b]	PA and PS vesicles [c]
1. Aggregation by Ca^{2+}	k_{app} approaches diffusion control K_m = 4.0 mM	k_{app} approaches diffusion control K_m = 4.5 mM	k_{app} approx. 10^2 lower than diffusion control (k_1) K_m not measured	k_{app} approx. 10^2 lower than diffusion control (k_1) K_m = 4.0 mM
2. Aggregation by K^+	dimers only $k_{app} \approx k_1$ faster processes seen	dimers only $k_{app} \approx k_1$	dimers only $k_{app} < 10^{-2}\ k_1$	none
3. Ca^{2+}/Mg^{2+} selectivity	none	none	Ca^{2+} 3-times more effective	PA none PS some [d]
4. Structural transition at 5—10°C	yes	yes	no	no
5. Ca^{2+}-binding-sites	yes [e] (low affinity)	yes [e] (low affinity/ high affinity <7°C)	yes	yes
Tb^{3+}-binding-sites	yes	yes	not studied	not studied

[a] This study.
[b] From Ref. 9.
[c] From Ref. 22.
[d] L. Westine and D.H. Haynes (in preparation).
[e] Inferred from multimer formation.

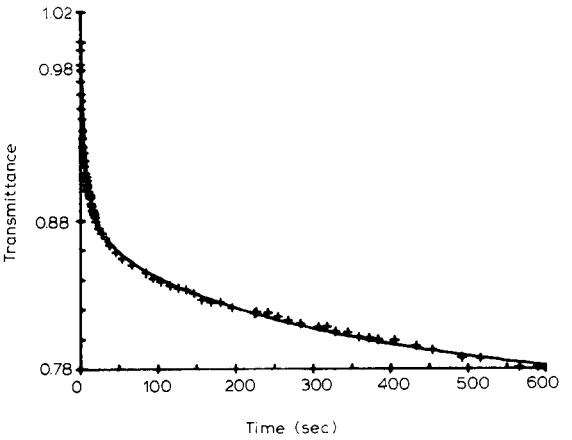

Fig. 1. Progress curve for dimerization and higher-order aggregation processes. The figure shows the relative transmittance decreases observed at 320 nm after rapid mixing of Ca^{2+} and synaptic vesicles to final concentrations of 10 mM and $4.7 \cdot 10^{-3}$ mg/ml protein, respectively. The reaction conditions were pH 7.2 (10 mM Hepes buffer) and 22°C. The experimental points were taken off of the strip chart record using the digitization program. The continuous line is the result of least-squares minimization as described in Table I.

increasing vesicle concentration (not shown) indicating that they represent aggregation processes. This is in agreement with the mechanism implied by a second-order (vs. exponential) fit as was the case with the chromaffin granule aggregation [8—10]. The first phase corresponds to vesicle dimerization and the second process represents the sum of all of the higher order aggregation processes. An additional fast process shown in Fig. 2 with a first-order

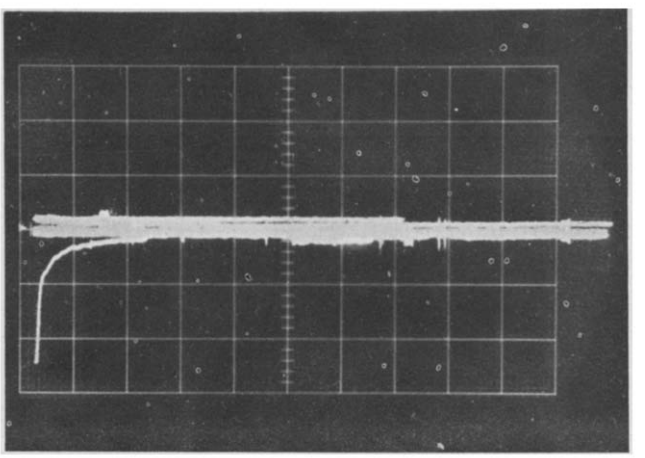

Fig. 2. Oscillograph trace showing a fast decrease in transmittance at 320 nm. The conditions were identical to those of Fig. 1. The vertical sensitivity was 0.1 unit per scale division, transmittance decreasing in the upwards direction. The horizontal sensitivity was 10 ms per scale division (first sweep) and 5 s per scale division (three subsequent sweeps). The first sweep shows a fast decrease in transmittance. The three subsequent (horizontal) sweeps are partially overlapping. Information was taken on the slow time scale using a strip chart recorder at higher sensitivity.

TABLE II

KINETIC LIGHT SCATTERING CHANGES

The results are the maximal changes in transmittance at 320 nm (normalized to 1.0) associated with the reaction induced by mixing with the perturbants to the final concentration given. The reaction conditions were pH 7.2 (10 mM Hepes buffer) and 22°C. The protein concentration after mixing was $4.7 \cdot 10^{-3}$ mg/ml. The choice of 'exponential' or 'second order' was based on the best fit of the data. The oscillograph traces and strip chart recordings were digitized by tracing with the light-pen of the Prophet Computer System. Least squares regression analysis were run on the system using the EXPFIT (exponential) or FITFUN programs. Second order processes were analyzed according to the equation $\Delta T = \Delta T_{max(1)}(k_{r(1)}t/(1.0 + k_{r,1}t)) + \Delta T_{max(2)}(k_{r(2)}t/(1.0 + k_{r(2)}t))$ where ΔT is the time-dependent transmittance decrease, $\Delta T_{max(1)}$ and $\Delta T_{max(2)}$ are the maximal values of the contributions of the first and second reactions, where $k_{r(1)}$ and $k_{r(2)}$ are constants related to the bimolecular rate constants k_{app} for dimerization and higher order aggregation, respectively, and where t is the time elapsed after mixing. The equation is equivalent to Eqn. 7 of Ref. 22, rearranged to predict the dependence of the experimentally measured ΔT value on the time of reaction, taking two consecutive reactions into account. Our experience has been that the progress curves for aggregation reactions can be approximated well as the sum of two second-order processes, the first representing dimerization and the second being the net result of all of the higher order processes. The first-order processes were analyzed by $\Sigma\Delta T_{max} \exp(-k_r T)$. The value of k_r is related to k_{app} (second-order) by $k_r = 2 \cdot k_{app}[P]/N_p$ where k_{app} is the apparent bimolecular rate constant for vesicle dimerization, where [P] is the vesicle concentration (g/l) and where N_p is the number of grams of protein per mol vesicles. The standard deviations of the fitted parameters were less than 5% of the given values.

Perturbant	Reaction; Amplitude; $k_r(ms^{-1})$	Reaction; Amplitude; $k_r(ms^{-1})$	Reaction; Amplitude; $k_r(s^{-1})$	Reaction; Amplitude; $k_r(s^{-1})$	Reaction; Amplitude; $k_r(s^{-1})$	Net amplitude
10 mM $CaCl_2$	Exponential; −0.114; −0.199	—	Second-order; −0.117; 0.299	Second-order; −0.154; −0.0024	—	−0.385
50 mM KCl	Exponential; −0.126; ~0.334	Exponential; −0.081; 0.046	Second-order; −0.0321; 4.25	Second-order; +0.0068; 0.0178	—	−0.292
100 mM sucrose	—	Exponential; −0.109; 0.0597	Exponential; −0.0385; 1.41	Sigmoidal; +0.11; (10—90 s) [a]	Exponential; −0.083; 0.0071	−0.121

[a] Time region encompassing 5—95% extent of reaction.

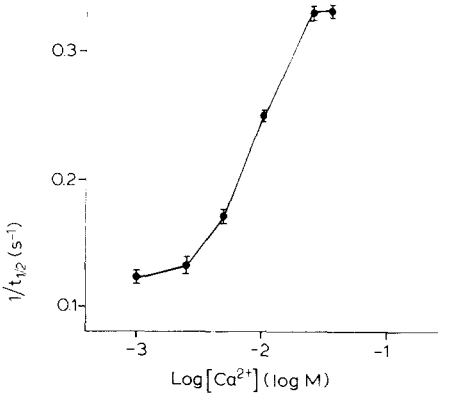

Fig. 3. The Ca^{2+}-concentration dependence of the dimerization rate. The conditions and medium were as in Fig. 1.

(exponential) time constant of approx. 5 ms was observed. Due to its extremely rapid rate and lack of dependence on vesicle concentration we conclude that it must represent an effect of Ca^{2+} on the structure of the vesicle. This process was not observed with chromaffin granule membranes.

The synaptic vesicles could also be aggregated by 50 mM KCl. This shows that the aggregation is made possible by the effect of cations to diminish the membrane surface potential and that full surface charge neutralization via direct cation binding is not necessary for aggregation [8—10]. The kinetic behavior of KCl-induced aggregation is compared with that of Ca^{2+}-induced aggregation in Table II. As with the chromaffin granule membranes, KCl-induced dimerization is faster than Ca^{2+}-induced dimerization and KCl is ineffective in inducing higher order aggregation. In contrast to the behavior of chromaffin granule membranes, two fast phases of transmittance decrease were observed, with exponential time constants of approx. 3 ms and 22 ms. The first process may be related to the 5 ms process observed with Ca^{2+}. Comparison with the results for the osmotic control (100 mM sucrose, Table II) suggests that an osmotic shape-change may be involved in the 22 ms process. *Torpedo* synaptic vesicles have been shown to be good osmometers [14] and fixation of vesicles in high osmotic strength buffers results in a collapse of many vesicle profiles into flattened discs. Such shape changes will increase the turbidity of isolated vesicles [8,9]. However, the small amplitude of the reversal phase (the slowest process) indicates that the dimerization process is not affected by changes in osmotic pressure across the membrane (cf. Ref. 9).

Study of the Ca^{2+} concentration dependence of the rate of the dimerization reaction showed results similar to the chromaffin granule membranes. Fig. 3 shows that increasing the Ca^{2+} concentration increases the rate of the reaction and that a constant rate is obtained at high concentrations. This observation shows that the charge neutralization and screening exert their maximal effects at these concentrations and that the rates of the charge screening and Ca^{2+}-binding reactions are fast compared to the rate of the dimerization reaction. The Ca^{2+} concentration for half-maximal effect is 4 mM, a value close to that

observed for chromaffin granule membranes (4.5 mM). Experiments were carried out to determine whether direct Ca^{2+}-binding to the membrane was involved.

Ca^{2+}-binding experiments

Ca^{2+}-binding to the membrane proteins was measured using Tb^{3+} as a fluorescent probe of Ca^{2+}-binding-sites (cf. Ref. 18 for review). Morris and Schober [16] demonstrated that chromaffin granule membranes contain a single Tb^{3+}-binding-site which was competitively inhibited by Ca^{2+} and Mg^{2+}. Since the mechanism for Tb^{3+}-fluorescent enhancement runs via excited triplet-triplet transfer of energy from tryptophan, tyrosine and phenylalanine moieties, the binding-site demonstrated by this method is almost certainly a protein. Electron micrographic observations placed the binding-site on the external surface of the isolated granules.

Fig. 4 shows that membranes of purified *Torpedo* synaptic vesicles also have a single binding-site for Tb^{3+} with $K_d \approx 2.56$ μM at 20°C. Ca^{2+} and Mg^{2+} are competitive inhibitors of Tb^{3+}-binding with K_I values of approx. 1.00 and 1.56 mM respectively [19]. Since these results are taken from studies of CPG-10-3000 glass bead purified vesicles it is highly unlikely that the binding-sites are on contaminating membranes.

Direct studies of Ca^{2+}-binding to chromaffin granule membranes demonstrated a lack of high affinity binding-sites at 20°C although a single high affinity site was seen at temperatures below 10°C. The present study only confirms the existence of protein binding-sites on the synaptic vesicle membrane for Tb^{3+} at 20°C which have low affinity for Ca^{2+} or Mg^{2+}. It leaves unanswered the questions as to whether low affinity non-protein sites or high affinity sites at low temperatures exist in the vesicle membrane. Such studies

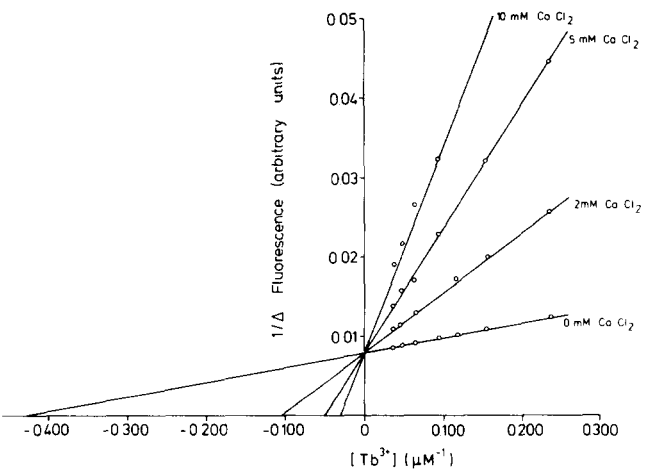

Fig. 4. Titration of *Torpedo* synaptic vesicle membranes with Tb^{3+}. Equal quantitites of CPG-10-3000 column purified vesicle membranes were resuspended in 1.50 ml of 2 mM Hepes pH 7.0 and titrated by adding μl vols. of 9.75 mM $TbCl_3$ to the cuvette. When present, Ca^{2+} was added to the samples before Tb^{3+}.

utilizing spectroscopic indicators such as calcein [9] or arsenazo III [20] require pure vesicles in quantities in excess of those available by present preparative methods.

Although divalent cation binding is not necessary for the dimerization reaction, the presence of divalent cations is necessary for higher order aggregation to occur. Mechanisms to explain this effect were discussed in detail in our previous communications [8—10]. Briefly it is thought that K^+-induced aggregation requires lateral movement of negatively charged lipids out of the contact region, thus raising the net negative potential of the remaining membrane surface and making the approach of a third granule more difficult. Ca^{2+} can bridge between negatively charged lipids, reducing the necessity of the lateral phase separation.

Temperature dependence of dimerization rate

Fig. 5 shows an Arrhenius plot of the dimerization rate. The data are very similar to those of the chromaffin granule membrane. A constant temperature dependence is observed between 5 and 35°C. At 5°C the plot shows a break indicating a structural transition in the membrane at this temperature. With the chromaffin granule membranes, the break was found at 7°C. The activation energy obtained in the high temperature range is +0.65 kcal/mol, which is smaller than the values obtained for the chromaffin granule membrane and for aqueous diffusion (+4.4 and +4.6 kcal, respectively). The activation energy below 5°C is large (72 ± 25 kcal/mol), indicating that gross rearrangements of membrane structure are necessary for aggregation to occur in this temperature range.

Calculation of k_{app}

The overall rate constant k_{app} for the dimerization reaction (processes of Eqns. 1 and 2) can be determined from the rate data of Table II. The value of the tabulated rate constant k_r is equal to $2 \cdot k_{app}\,[P]/N_p$, where [P] is the protein concentration in g/l and where N_p is the number of grams of protein per mol synaptic vesicle.

The value of N_p can be estimated from the known vesicle diameter (900 ±

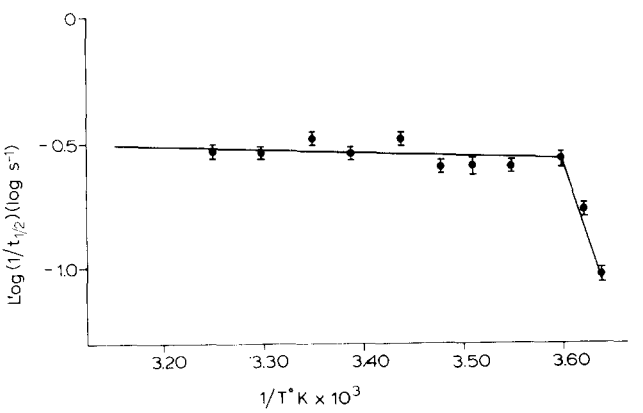

Fig. 5. Arrhenius plot of Ca^{2+}-induced dimerization rate data. The conditions and media are as in Fig. 1.

100 Å; Ref. 18) and from the known lipid composition of the vesicles (1.6 μmol cholesterol/mg protein and 3.82 μmol phospholipid/mg protein; Ref. 12) using the peripheral protein model of vesicle structure (see Ref. 9, Appendix) which assumes all protein to be adherent to the membrane surface. Each 60 $Å^2$ is considered to be occupied by two lipid molecules (one outside and one inside). The number of lipids per vesicle is $8.48 \cdot 10^4$ and N_p becomes $1.56 \cdot 10^7$ g protein/mol vesicles. The value of k_{app} is calculated from the N_p value, the measured protein concentration and from the k_r values of Table I. The value for Ca^{2+}-induced aggregation is $4.77 \cdot 10^8\ M^{-1} \cdot s^{-1}$, one order of magnitude lower than the value predicted for a diffusion-controlled reaction (approx. $8.03 \cdot 10^9\ M^{-1} \cdot s^{-1}$; Refs. 9 and 22). The value for K^+-induced aggregation is $7.05 \cdot 10^9\ M^{-1} \cdot s^{-1}$, which is close to the theoretical limit. If we considered all membrane protein to be integral the estimates would be approx. 25% smaller. The vesicles for these studies were prepared by zonal centrifugation after extraction of the tissue with 0.4 M NaCl containing 3.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) [12]. Wagner et al. [21] have isolated *Torpedo* vesicles from 10 mM EGTA containing solutions with phospholipid/protein ratios of 6.2 μmol/mg. Since it is presumed that surface (non-integral) proteins have been stripped by the higher EGTA concentration, we can assume that at least 50% of the protein of our preparations is surface protein and the value for N_p is accurate to ±10%.

Experiments with chromaffin granule membranes showed no change in aggregation kinetics with membranes prepared from granules isolated in 1 mM EDTA containing solutions or membranes washed with or without EDTA or EGTA-containing solutions. Therefore the proteins responsible for granule-granule contact are not easily removed from the membrane. In these experiments, the very high EGTA concentrations [21] were not used since in our experience they lead to loss of vesicle integrity, leakage of stored acetylcholine and ATP [12] and, upon long exposure, dissociation of the membrane (Giompres, P. and Morris, S.J., unpublished).

Table III shows that the results for *Torpedo* vesicles are very similar to the results for the chromaffin granule membrane, while the rates for phospholipid vesicle aggregation are much lower. The high values of k_{app} suggest a pre-existing complementarity between the surface elements of the two membranes. We have presented detailed arguments [8—10] leading to the intuitively-expected conclusion that such high rates cannot be accounted for by aggregation of lipid bilayer portions of the membranes. The Ca^{2+}-induced aggregation of lipid vesicles requires the rearrangement of the various phosphatidylcholine and acidic phospholipid head groups on the membrane surface [9,22] and we have recently shown that short-range dipolar repulsion interactions between zwitterionic head groups also must be overcome [23]. We have suggested that these difficulties are circumvented in secretion granules by protein recognition sites which are regularly arranged and which protrude 10—20 ångströms from the bilayer surface [9,10,16]. This model allows for aggregation of membranes bearing net charge and moderate surface potential. The break in the temperature dependence can be explained by redistribution of the recognition sites. A further proof of the existence of these sites lies in the high value of the stability constant of the dimer. The present data allow us to calculate a value of

TABLE III

COMPARISON OF KINETIC CONSTANTS FOR AGGREGATION OF SEVERAL TYPES OF VESICLE

The k_1/k_{-1} and k_1 values were calculated as described previously [10] using R_o = 450 Å (synaptic vesicles), R_o = 1500 Å (chromaffin granules) and R_o = 125 Å and 250 Å (phospholipid vesicles). The encounter complex was considered to be formed for separations of 26—126 Å. The k_1/k_{-1} values were $3.60 \cdot 10^5$ M^{-1}, $3.58 \cdot 10^6$ M^{-1} and $(4.05—12.58) \cdot 10^4$ M^{-1}, respectively. The k_1 values were $8.03 \cdot 10^9$ $M^{-1} \cdot s^{-1}$, $7.02 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ and $(8.09—9.26) \cdot 10^9$ $M^{-1} \cdot s^{-1}$. The k_2 values were calculated from Eqn. 3 of the text.

Vesicle	Perturbant	k_{app} ($M^{-1}s^{-1}$)	k_{app}/k_1	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{app}/k—app (M^{-1})
Synaptic vesicle	10 mM Ca^{2+}	$4.77 \cdot 10^8$	0.059	$2.23 \cdot 10^4$	$1.40 \cdot 10^3$	$\geqslant 6.67 \cdot 10^9$
Synaptic vesicle	50 mM KCl	$7.05 \cdot 10^9$	0.877	$2.23 \cdot 10^4$	$\sim 1.60 \cdot 10^5$	$\geqslant 6.67 \cdot 10^9$
Chromaffin granule	20 mM Ca^{2+}	$(0.865—1.04) \cdot 10^9$	0.123—0.148	$1.96 \cdot 10^3$	$(2.75—3.41) \cdot 10^2$	$\geqslant 4 \cdot 10^{10}$
Chromaffin granule	100 mM KCl	$(3.31—4.01) \cdot 10^9$	$>(0.471—0.571)$	$1.96 \cdot 10^3$	$\geqslant (1.74—2.61) \cdot 10^3$	$\geqslant 4 \cdot 10^{10}$
Chromaffin granule lipid vesicle *	H^+ + 20 mM Ca^{2+}	$(0.58—2.32) \cdot 10^7$	$(0.72—2.50) \cdot 10^{-3}$	$(0.74—2.00) \cdot 10^5$	$(1.43—1.84) \cdot 10^2$	$\geqslant 2 \cdot 10^8$
Chromaffin granule lipid vesicle *	H^+ + 500 mM KCl	$(0.21—0.84) \cdot 10^7$	$(0.26—0.91) \cdot 10^{-3}$	$(0.74—2.00) \cdot 10^5$	$(0.52—0.66) \cdot 10^2$	$\geqslant 2 \cdot 10^8$
Phosphatidic acid vesicle **	100 mM Ca^{2+}	$(0.99—3.9) \cdot 10^7$	$(1.22—4.21) \cdot 10^{-3}$	$(0.72—2.00) \cdot 10^5$	$(2.44—3.10) \cdot 10^2$	$\geqslant 2 \cdot 10^8$
	500 mM KCl	no aggregation	no aggregation	no aggregation	no aggregation	no aggregation
Phosphatidylserine vesicle **	100 mM Ca^{2+}	$(1.7—6.9) \cdot 10^7$	$(2.10—7.49) \cdot 10^{-3}$	$(0.74—2.00) \cdot 10^5$	$(4.19—5.48) \cdot 10^2$	$\geqslant 2 \cdot 10^8$
	500 mM KCl	no aggregation	no aggregation	no aggregation	no aggregation	no aggregation

* Data from Refs. 9 and 10.

** Data from Ref. 22.

$6.7 \cdot 10^9\ M^{-1}$ for the lower limit of the synaptic vesicle dimerization constant. This is over four orders of magnitude larger than the stability constant of the encounter complex which will be calculated in the next section.

Rate of aggregation of closely apposed membranes

Our results can be applied to the problem of synaptic delay-time by use of the encounter complex model described in a previous communication [10]. A number of theoretical calculations have been made for this problem [24]. Vesicles with distances of separation of less than arbitrary distance $2\Delta R$ are assumed to exist in an encounter complex. The rate and equilibrium constants for the encounter complex are as defined in Eqn. 1. The encounter complex can transform into a stable complex according to the process described in Eqn. 2. The rate constant k_2 is the average value for the reciprocal of the time taken for the membranes to come into effective contact as the result of diffusion across the encounter complex region. If $k_{app} << k_1$, then k_2 will be determined primarily by an energy barrier. The width of the encounter complex region, ΔR, can be chosen to approximate the synaptic vesicle-plasma membrane case, but should not be so large that $k_2 \approx k_{-1}$ for the $k_{app} << k_1$ case.

The value of k_2 can be calculated from k_{app} using

$$k_2 = k_{-1}(k_{app}/k_1)/(1 - (k_{app}/k_1)) \quad (3)$$

The values of k_1 are calculated for the case of the diffusion-controlled rate constant (cf. Refs. 9, 10, 22). They are relatively insensitive to the choice of ΔR. The values of k_1/k_{-1}, calculated from our encounter complex model [10], are quite sensitive to the choice of ΔR.

The values of k_2 for the 26—126 Å separation case are given in Table III for synaptic vesicles, chromaffin granules and phospholipid vesicles. The values for the Ca^{2+}-induced dimerization of synaptic vesicles are over four times as large as the values for the chromaffin granule. The latter are somewhat greater than the values for vesicles derived from chromaffin granule lipids but are about equal to the values for vesicles made from acidic lipids (phosphatidic acid and phosphatidylserine) in the pure form. For the case of KCl-induced aggregation the differences are more pronounced. The four types of membranes can also be compared on the basis of another parameter, k_{app}/k_1, the ratio of successful collisions to total collisions. For Ca^{2+}-induced aggregation, the chromaffin granules are about twice as effective as the synaptic vesicles. The 'success' rates for these vesicles are about two orders of magnitude greater than for the phospholipid vesicles.

The considerations above point to two important factors which determine the rates of aggregation of closely apposed membranes which are quantitated by k_2: (a) vesicle diffusion rates, and (b) the degree of match of the two surfaces. The chromaffin granule membrane has a higher value of k_{app}/k_1 than the synaptic vesicle but diffuses more slowly owing to its larger size. The net result is a somewhat slower rate. This may have significance for the biology of exocytosis. Larger vesicles with larger volume to surface-area ratios would be most efficient means of transmitter storage, but the requirement of rapid fusion of the vesicles, which is necessary for transmission at neuromuscular junctions, will dictate an upper limit for the vesicle size. The phospholipid

vesicles have low values of k_{app}/k_1, but can compensate for this by higher diffusion rates, with the result that their k_2 values can approach those of the chromaffin granule membrane.

Relevance to exocytosis

Recently, Parsegian [24] put forward three critical tests for mechanisms coupling Ca^{2+} entry into the cell with exocytotic release of vesicle contents: (a) the mechanism must occur within 200 μs, (b) it must show high specificity for Ca^{2+} over Mg^{2+}, and (c) it must eventually cause release of vesicle contents into the synaptic cleft rather than simply into the cell cytosol by non-specific leakage or lysis. In this study we show that the half-time for establishment of contact between closely apposed (<126 Å) synaptic vesicles is approx. 435 μs. We take this to mean that the vesicle-vesicle surface recognition sites are almost optimally deployed as the vesicle-plasma membrane sites involved in exocytosis. Thus our results suggest that approx. 100 μs are needed to fuse the vesicle and plasma membrane after contact. Cells normally contain high (>100 mM) concentrations of K^+. This would greatly reduce the vesicle-surface potential, dimerize a fraction of the vesicles or allow some vesicles to approach closely or even bind to their release-sites. However the contact would not be expected to result in release of vesicle contents, since hyperpolarization of synapses or depolarization in the absence of Ca^{2+} which would raise or lower internal K^+ does not change the rate of miniature end plate potentials. In any case high K^+ would lower the apparent K_m for Ca^{2+} promoted aggregation.

Although not discussed in detail in this communication, Mg^{2+} also aggregates vesicles (Table I). Heuser (Ref. 6, Fig. 37 and Ref. 7) has shown by electron-microscopy that elevated Mg^{2+} seems to 'freeze' the exocytosis process at the point where the bilayers of the vesicle and the cell membrane are in close contact. Thus as discussed previously [6] the yet to be elucidated Ca^{2+}-specificity would seem to lie in a process of fusion which takes place subsequent to the establishment of close contact, mediated by a Ca^{2+}-specific enzyme [24] or a highly Ca^{2+}-specific protein which regulates the binding of the storage vesicle to the membrane [25]. This point and point (c) above require real time studies on the interaction of the vesicles with their plasma membrane release sites.

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