Biochimica et Biophysica Acta, 693 (1982) 425–436
Elsevier Biomedical Press

BBA 71455

CALCIUM-PROMOTED RESONANCE ENERGY TRANSFER BETWEEN FLUORESCENTLY LABELED PROTEINS DURING AGGREGATION OF CHROMAFFIN GRANULE MEMBRANES

STEPHEN J. MORRIS [a,b]*, THOMAS C.SÜDHOF [b] and DUNCAN H. HAYNES [c]

[a] *Neurotoxicology Section, NINCDS, National Institutes of Health, Bethesda, MD 20205 (U.S.A.),* [b] *Department of Neurochemistry, Max Planck Institute for Biophysical Chemistry, D-3400 Goettingen (F.R.G.) and* [c] *Department of Pharmacology, University of Miami School of Medicine, Miami, FL 33101 (U.S.A.)*

(Received July 26th, 1982)

Key words: Membrane protein; Membrane aggregation; Fluorescence resonance energy transfer; Fluorescent label; Ca^{2+}; (Chromaffin granule)

Proteins of the chromaffin granule membrane were covalently labeled in situ with sulfhydryl-specific fluorophores. Using MIANS (maleimide iodoaminonaphthyl sulfonate) as the donor and fluorescein mercury acetate or fluorescein-5-maleimide as the acceptor, Förster fluorescence resonance energy transfer (FRET) could be employed to measure the degree of inter-membrane and intra-membrane protein-protein contact upon Ca^{2+}-induced aggregation of the membranes. The four major findings were: (1) Raising the Ca^{2+} concentration to approx. 500 μM causes the proteins to aggregate in the plane of the membrane. This is demonstrated by Ca^{2+}-induced increases in the fluorescence resonance energy transfer in double labeled membranes. This effect is not protein-concentration dependent and occurs at calcium concentrations too low for granule aggregation, implying intra-membrane protein clustering or patching. To our knowledge this is the first direct demonstration of the fluid mosaic nature of subcellular organelles. (2) If two sets of granules are labeled separately, Ca^{2+}-induced aggregation brings at least 74% of the labeled proteins into close transmembrane proximity. This effect is also observed at 10–100-fold slower rates in the absence of calcium and can be greatly reduced by depleting the granule membrane of labeled peripheral proteins. It is enhanced if the granules are aggregated by Ca^{2+} or K^+. We conclude that (some) peripheral proteins can transfer from one membrane surface to another. (3) Aggregation of separately labeled sets of membranes by Ca^{2+} also produces transmembrane energy transfer since: (a) the K_m for Ca^{2+}-induced quantum transfer is in the same range as the K_m for aggregation; (b) the reaction is protein-concentration dependent; (c) reversal of aggregation also (partially) reverses donor quenching. (4) A kinetic analysis of the transmembrane effect shows it to be 5–10-fold slower than aggregation itself, supporting earlier suggestions (Haynes, D.H., Kolber, M. and Morris, S.J., (1979) J. Theor. Biol. 81, 713–743) that lipid and protein rearrangements are secondary to granule membrane aggregation.

Introduction

Ca^{2+}-triggered exocytosis of hormones and transmitters involves the association of storage vesicles with the plasma membrane and the fusion of the two membranes to release the vesicle contents. In our studies of the properties of the chromaffin granule and synaptic vesicle membranes,

* Correspondence to S.J. Morris: NIH, Building 9, Room 1E127, Bethesda, MD 20205, U.S.A.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MIANS, maleimide iodoaminonaphthyl sulfonate.

0005-2736/82/0000–0000/$02.75

we have observed that the vesicles can be aggregated by the addition of cations [1,2] and that granule membranes will fuse in the presence of Ca^{2+} [3,4]. This evidence suggests that granule-granule interactions may be used as a model for membrane fusion events [5,6]. Limited association (dimerization) of chromaffin granule and synaptic vesicle ghosts also can be brought about by the addition of K^+ to final concentrations in the 100 mM range while dimerization and higher order aggregation can be brought about by divalent cations in the 5 mM range [1,2]. We have studied these reactions as a means of elucidating the properties of the membranes and as a model for the vesicle-plasma membrane association.

Aggregation of isolated granule membranes is an extremely rapid process occurring at nearly diffusion-controlled rates [1,2], while aggregation of phospholipid vesicles prepared from their extracted lipids is about 500-fold slower than predicted by diffusion control. This can be explained on the basis of protein-protein interaction. Direct contact of proteins between membranes is considered to be the first event in the aggregation reaction, with intermembrane lipid-lipid interaction playing a secondary role in stabilizing the aggregate. This is supported by electron microscopic studies of the distribution of the integral proteins as represented by membrane-associated particles (MAPs). The membrane-associated particles are normally distributed in a random fashion but are excluded from the contact region of granules aggregated either by Ca^{2+} [3,7] or K^+ [8] and come to lie in patches in the region just adjacent to the contact area.

Our previous aggregation studies were carried out by monitoring increases in light scattering [9]. Although this method is convenient for the study of isolated vesicles, it is impractical for the study of the association of vesicles with plasma membranes. The method records increases in aggregate 'molecular' weight, but cannot distinguish between self-aggregation and vesicle-plasma membrane aggregation. Furthermore, light scattering changes which involve only a small fraction of the total membranous material are not detectable.

In attempting to increase the specificity of readout of membrane association reactions we have investigated the use of fluorescence resonance energy transfer (FRET) between covalently-attached fluorescent probes [10,11]. The Förster theory [10,12] predicts that if an excited donor is in close proximity to an acceptor then it will transfer energy in a radiationless process and the acceptor will fluoresce. For an isolated pair, the rate constant for the transfer has an inverse sixth power dependence on the distance of separation. Quantum transfer can be measured either by the loss of fluorescence at the donor emission wavelength or by the gain of fluorescence at the acceptor emission wavelength. The present study shows that these techniques have the ability to report vesicle-vesicle aggregation and other processes which bring membrane proteins into close proximity. The method consists of choosing a donor-acceptor pair with sufficient overlap between the fluorescent emission spectrum of the donor and the excitation spectrum of the acceptor.

Materials and Methods

Preparation of labeled chromaffin granule ghosts. All steps were carried out at 0–4°C. Chromaffin granules (more than 95% pure) were prepared as described previously [13]. The thrice washed pellets were resuspended in 0.26 M sucrose, 10 mM Na-Hepes, pH 7.4 (buffered sucrose), at 2.5–3.5 mg protein/ml. 4 ml of suspension was treated with 100 μl of 0.5 mM dye dissolved in acetone and incubated for 2 h at 0°C. (Preliminary experiments had established that coupling of the dyes to the granules saturated at less than 10 μM). The suspensions were treated with 50 ml of 10 mM Hepes (pH 7.4) which lysed more than 99% of the particles, then centrifuged 30 min at 23000 rpm ($63000 \times g_{max}$) in a Beckman AH35 rotor. The supernatants were discarded and the pellets resuspended in 4.0 ml of buffered sucrose, and layered on top of a discontinuous gradient consisting of 2.5 ml 0.4 M sucrose in ^2H_2O and 4.0 ml 0.4 M sucrose in tubes for the Beckman SW41Ti swing out rotor. The gradients were centrifuged 60 min at 32500 rpm [14]. The lysed, resealed chromaffin granule ghosts were recovered at the interface between the two 0.4 M sucrose solutions, while the unsealed ghosts, unlysed granules, mitochondria and lysosomes pelleted to the bottom of the tube [14]. The ghosts were diluted with 5–10 volumes of

0.26 M sucrose, 10 mM Hepes (pH 7.4) and pelleted at 16000 rpm ($30000 \times g_{max}$) in a Sorvall SS-1 rotor. The samples were resuspended in 5 ml of buffered sucrose, the protein concentrations were again determined and each sample was diluted with buffered sucrose to a protein concentration of 0.100 mg/ml.

Labeling of membrane protein. Fluorescent maleimides were obtained from Molecular Probes, Plano, TX. To ensure that only the protein components of the granule membranes were being analyzed, fluorescent derivatives specific for free sulfhydryl groups were employed [15–18]. The following fluorescent maleimides did not label the chromaffin granule membranes: 4- and 5-maleimidylsalicylic acid and *N*-(1-naphthyl)maleimide. Other maleimide derivatives tested were eosin-5-maleimide, chrysene-6-maleimide, *N*-(1-pyrenyl) maleimide and 2,5-dimethoxystilbene-4′-maleimide. These labeled the membranes but were not employed due to low quantum yields when coupled to the membranes, excessive photobleaching or poor overlaps with a second fluorescent compound to serve as a donor or acceptor. MIANS (maleimide iodoaminonaphthyl sulfonate) proved to be an acceptable donor for fluorescein-5-maleimide, fluorescein mercury acetate (Eastman, Rochester, NY), or eosin-5-maleimide as acceptors. The last was not employed due to its relatively lower quantum yield. Fluorescein mercury acetate and fluorescein-5-maleimide have approximately the same quantum yield. Therefore it was easily established by the ratio of fluorescence to protein concentration that fluorescein mercury acetate labeled more than twice the number of sites labeled by fluorescein-5-maleimide. MIANS was sensitive to photobleaching, which was seen as a slow decrease in fluorescence with a rate which was proportional to the excitation slit width. For this reason the excitation slits were set as small as feasible (1.0–1.5 nm) and, if bleaching exceeded 3% in 30 min, the amplitude in donor fluorescence was corrected for this effect.

Peripheral proteins were extracted by repeated pelleting of the ghosts followed by resuspension and incubation in buffered sucrose. Chromatography of the supernatant extracts on Sephadex G-25 showed that the fluorescence was excluded from the gel, indicating that the dyes were attached to macromolecular structures of molecular weight greater than 2500. Since proteins were being removed by this process, the final pellets were resuspended to their original volumes to keep the particle concentration constant. Most experimentation was done on peripheral-protein deficient membranes prepared in this manner. In some cases this procedure was omitted and experiments were carried out on peripheral protein-rich vesicles.

Labeling of granule lipids. To test for the specificity of the fluorescent sulfhydryl reagents, the lipids were extracted from fluorescein mercury acetate and MIANS-labeled granule ghosts [19]. The chloroform-methanol extracts contained some fluorescent material; however, this was almost entirely removed by partitioning three times against an aqueous phase containing 0.1 M NaCl. We calculate that less than 0.02% of the original fluorescent label remained in the washed chloroform-methanol extracts.

Other analytical procedures. Protein concentration was determined by the Coomassie blue binding assay of Bradford [20], using bovine serum albumin as a standard.

Fluorescence spectra and changes in fluorescence were measured in a Perkin-Elmer MPF4 fluorimeter in a thermostatically controlled cell holder. Changes in turbidity at 320 nm were measured in a Zeiss PMQIII spectrophotometer also fitted with a thermostatically controlled cell holder. Measurements were initiated by adding small volumes of labeled membranes to the cuvettes containing sucrose pre-equilibrated to 30°C or 4°C. Other perturbants were added as small volumes of concentrated materials, rapidly by hand. Amplitude changes were normalized to values at zero time ($\Delta F/F_0$ or $\Delta A/A_0$). With practice, mixing times of 2–3 seconds were achieved. These were fast enough to resolve the half-times of the fluorescence changes. Aggregation as measured by changes in turbidity was too rapid to be resolved into its two phases (dimerization followed by multimerization) [1,9] by this method. We previously demonstrated that particles the size of granule ghosts should show an increase in turbidity of approx. 28% when going from monomers to dimers [2,9]. Therefore, a first approximation of the half-time of the fast phase (dimerization) was taken as the time for the particles to increase their

apparent absorbance by 14% ($t_{1.14}$). Membrane concentrations were chosen which yielded values of $t_{1.14} > 3$ s and several repetitions generally gave half-times with deviations of less than 10–15%. Although this approach was not exact enough for detailed analysis, the difference in initial velocities of the dimerization reaction and the fluorescence changes were large enough to easily establish that the latter was almost an order of magnitude slower than the former (see results below).

The 'bimolecular' rate constant for Ca^{2+}-promoted dimerization, V_{max}, and the apparent rate constant for the development of quantum transfer, $k_{Qt,max}$ at saturating [Ca^{2+}] were calculated from the y-intercept of double reciprocal plots ($t_{1/2}$ vs. $1/Ca^{2+}$) and the relationship $k_{Qt,max}$ (or V_{max}) = 0.69 $(1/t_{1/2})_{max}$. The K_m values for the calcium dependence of the reactions were determined as the negative reciprocal of the x-intercept of these same plots.

Results

Characteristics of fluorescence changes

Fig. 1 shows the fluorescence excitation and emission spectra of chromaffin granule ghosts labeled with MIANS and fluorescein mercury acetate. The spectrum of fluorescein-5-maleimide was qualitatively identical to fluorescein mercury acetate. The figure demonstrates that the MIANS emission centered at 420 nm has good overlap with the fluorescein excitation, although the contribution of MIANS emission at peak fluorescein mercury acetate or fluorescein-5-maleimide emission is appreciable.

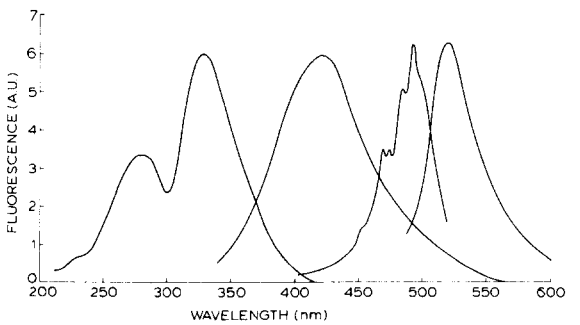

Fig. 1. Excitation and emision of MIANS- and fluorescein mercury acetate-labeled chromaffin granule membranes. Spectra from left to right are uncorrected excitation and emission of MIANS-labeled membranes and excitation and emission spectra of fluorescein mercury acetate-labeled membranes. Ordinate is in arbitrary units which differ for the two fluorophores. Protein concentration was 0.100 mg/ml.

Fig. 2 shows that the quantum transfer is induced when separately labeled MIANS- and fluorescein mercury acetate-labeled membranes are added together and aggregation promoted by the addition of 7.1 mM Ca^{2+}. The reduction in MIANS fluorescence (donor quenching) is measured (excitation at 328 nm; emission at 420 nm). The Ca^{2+} addition brought about a slow decrease in the donor fluorescence which eventually reaches 36% of its initial value with a $t_{1/2}$ of approx. 70 s for the change. The same process could be measured by the increase in acceptor (fluorescein mercury acetate) fluorescence, but with less sensitivity. It should be noted that, in the absence of acceptor-labeled membranes, Ca^{2+} produced no change in donor fluorescence.

The increase in energy transfer shows that the proteins of the chromaffin granule vesicles are brought together to distances of less than 50 Å (see discussion of R_0 below). This could be accomplished either by vesicle aggregation or fusion. In previous studies [1,9] we have shown that dimerization results in a 28% increase in sample turbidity. To determine if aggregation processes were responsible for the observed effect, the experiment of Fig. 2 was repeated monitoring turbidity at 320 nm. The Ca^{2+} addition brought about a 47% increase in turbidity, indicating that the vesicles

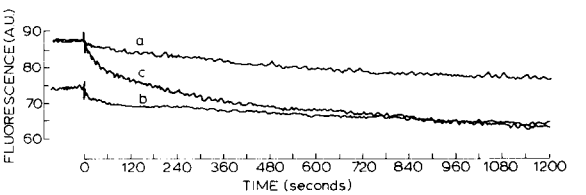

Fig. 2. Progress of donor quenching of MIANS-labeled granule membranes by fluorescein mercury acetate-labeled granule membranes. Shortly before the start of each experiment, the two labeled samples were mixed together and allowed to equilibrate to 30°C for approx. 2 min. At time zero, the perturbing cation was added rapidly by hand. (a) Addition of 10 μl of buffer. (b) After approx. 2500 s, Ca^{2+}, to a final concentration of 7.1 mM, was added to (a). (c) Addition of Ca^{2+} to a final concentration of 7.1 mM.

had been transformed into aggregates of higher order than dimers. A half-time for the dimerization, $t_{1.14}$, of approx. 14 s was measured. Thus, the half-time of the quantum transfer process was 5-times greater than the half-time of the aggregation and the quantum transfer reads out processes slower than aggregation.

We also observed that quantum transfer could occur by coincubation of MIANS- and fluorescein-mercury acetate-labeled granules in the absence of Ca^{2+}. However this occurred more slowly and with smaller amplitudes. The slow, Ca^{2+}-insensitive quantum transfer process was reduced if peripheral proteins were first removed (Table I). Table II and Fig. 2 demonstrate an extremely slow decrease in donor fluorescence ($t_{1/2} = 1120$ s) occurs with coincubation of the two types of labeled membrane (Expt. 2a). That this was not a photobleaching effect was established on parallel samples incubated in the dark. The amplitude of the decrease was about 70 percent of that observed with Ca^{2+}-induced aggregation (Table II, Expt. 1). Expt. 2b shows that subsequent addition of Ca^{2+} brings about a decrease in the fluorescence to levels observed at the end of Expt. 1. The turbidity data show large and equal Ca^{2+}-induced aggregation in both experiments.

Model for quantum transfer

These experiments suggested to us that the proximity of the proteins reported by quantum transfer can occur by a number of mechanisms. These include the exchange of peripheral proteins between membrane and the inter-membrane proximity of both peripheral and integral proteins. A model, presented in Fig. 3 was used as a basis for the interpretation of the above experiments and as a guide in planning further experiments.

The membrane is considered to have two types of protein: (a) loosely-bound peripheral protein which can be transferred from one vesicle to the other and (b) integral protein which can be transferred only by membrane fusion. This model predicts that coincubation of separately labeled vesicles would result in slow randomization of peripheral proteins between vesicles (Fig. 3b). This would give rise to intra-membrane quantum transfer between peripheral and integral proteins. Dimerization by KCl addition would be expected to increase the rate of peripheral protein randomization (Fig. 3c). It would also be expected to increase the extent of quantum transfer by providing integral protein-integral protein and integral protein-peripheral protein quantum transfer between membranes. As noted above, Ca^{2+} addition has been shown to produce high degrees of membrane aggregation and to induce aggregation of membrane associated particles. Thus the model predicts that Ca^{2+}-induced aggregation would give the highest degrees of quantum transfer, as observed (Fig. 3d). The model was tested by further comparing the quantum transfer behavior of vesicles rich and difficient in peripheral protein.

TABLE I

EFFECT OF REMOVAL OF PERIPHERAL PROTEINS ON THE DONOR QUENCHING OF MIANS- AND FLUORESCEIN MERCURY ACETATE-LABELED GRANULES

Experiments were performed using the cation concentrations and temperatures listed. $\Delta F/F_0 = (F - F_0)/F_0$.

Expt. No.	Cation	Temp. (°C)	Peripheral protein-rich membranes (0.100 mg/ml)		Peripheral protein-depleted membranes (0.090 mg/ml)	
			$t_{1/2}$ (s)	$\Delta F/F_0$	$t_{1/2}$ (s)	$\Delta F/F_0$
1	7.1 mM Ca^{2+}	30	50	−0.70	48	−0.28
2	140 mM K^+	30	10	−0.50	49	−0.18
3	7.1 mM Ca^{2+}	4	112	−0.32	1320	−0.22
4	140 mM K^+	4	12	−0.36	60	−0.11

TABLE II

COMPARISON OF CA^{2+} AND K^+ -INDUCED AGGREGATION AND DONOR QUENCHING

Peripheral protein depleted MIANS- and fluorescein mercury acetate-labeled granule membranes (protein concentrations 0.090 mg/ml) were mixed, then concentrated cation chloride solutions as indicated were rapidly added. Temperature = 30°C.

Expt. No.	Cation	$t_{1/2}$ (s)	$\Delta F/F_0$	$t_{1.14}$ (s)	$\Delta A/A_0$
1	7.1 mM Ca^{2+}	48	−0.30	3.4	0.62
2a	none	1120	−0.23	–	0
2b	7.1 mM Ca^{2+}	48	−0.10	3.4	0.62
3	140 mM K^+	49	−0.14	< 2	0.25

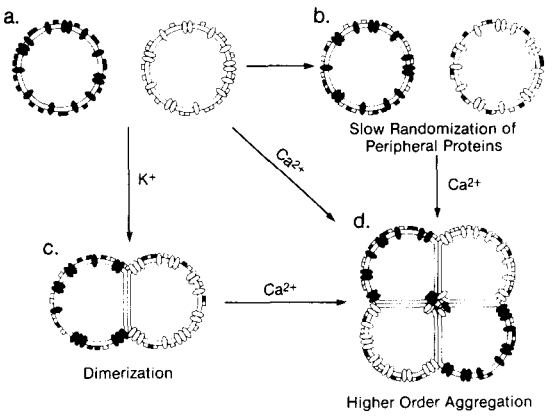

Fig. 3. Model for chromaffin granule protein-protein interactions. The model is based on the following assumptions. (1) The granule membrane is a fluid mosaic consisting of a lipid bilayer containing both donor- and acceptor-labeled integral proteins (= 0=0) and adhering peripheral proteins (□). (2) Some integral proteins are completely free to diffuse in the plane of the bilayer and all integral and peripheral proteins are cleared from regions of contact promoted by K^+ or Ca^{2+}. (3) Ca^{2+} and K^+ also tend to cluster the membrane proteins. Arguments for these assumptions are detailed in the text. (a) Chromaffin granules previously labeled either with MIANS (dark proteins) or fluorescein compounds (no shading) are mixed. (b) If left to stand in the absence of perturbing cations, some peripheral proteins will slowly exchange and randomize, leading to increase in energy transfer. (c) If K^+ is added shortly after mixing, the granules will form dimers. Clearing of the contact region will decrease the average distance between all protein species and some proteins will cluster. Randomization of perpheral proteins will occur more rapidly. Transmembrane interactions will occur between those proteins near the contact region. All of these events will increase energy transfer. (d) If Ca^{2+} is added, higher order aggregates (trimers, tetramers, multimers) will form. Increased numbers of contacts will further decrease the space between proteins and increase the number of proteins involved in transmembrane interactions.

Quantum transfer with peripheral protein-rich ghosts

Table I compares the amplitudes and half-times of chromaffin granules rich and deficient in peripheral protein. The degree of transfer is higher in the peripheral protein-rich granules. The rates for Ca^{2+}-induced quantum transfer were the same for both types of vesicles. However, the rate of K^+-induced transfer was faster for the peripheral protein containing vesicles than for the deficient vesicles, suggesting that the peripheral proteins come into proximity more rapidly than the integral proteins.

The previous experiment suggests that peripheral proteins can communicate between vesicles. An additional experiment establishes this conclusion more firmly. Peripheral proteins were extracted from MIANS- and fluorescein mercury acetate-labeled chromaffin granules and the extracts were incubated with unlabeled granule ghosts. The experimentation of Table I was repeated, and qualitatively similar results were obtained, although the levels of fluorescence and the amplitudes of quantum transfer were greatly reduced in keeping with lower levels of labeled protein per vesicle (data not shown). This experiment demonstrated the ability of peripheral proteins both to bind to and dissociate from chromaffin granules and thus transfer from one granule to another. All changes in fluorescence of the peripheral proteins required the chromaffin granule membrane as a substrate; there was no energy transfer between the extracted peripheral proteins when treated with Ca^{2+} or K^+ in the absence of membranes.

Occlusion of labeled protein in aggregation

Additional experiments were performed to characterize the conditions under which protein transfer and proximity can occur. Table III shows an experiment designed to probe the accessibility of vesicle monomers to Ca^{2+}-aggregated vesicles. Preaggregation of one type of labeled vesicles reduces the quantum transfer observed upon addition of the second type of labeled vesicles under aggregating conditions. The reduction is substantial (50%). Since previous experiments show that peripheral protein is the major contributor to quantum transfer in the peripheral protein-rich vesicles, we conclude that a large portion of the peripheral protein is trapped in self-aggregates on the preaggregated vesicles and is not available for exchange or aggregation with the second type of vesicle. Similar conclusions were reached for experiments in which MIANS-labeled vesicles and fluorescein mercury acetate-labeled vesicles were preaggregated and then exposed to an excess of unlabeled monomer vesicles under aggregating conditions. No diminution of quantum transfer was observed after the addition of unlabeled vesicles (data not shown). These experiments demonstrate that labeled proteins will not transfer to another membrane once the vesicles have been aggregated by Ca^{2+} at mM concentrations.

Reversibility of quantum transfer

Further experiments were performed to determine to what extent the quantum transfer could be reversed by removal of aggregating cations. Quantum transfer resulting from aggregation per se should be reversed by disaggregation of the vesicles, whereas peripheral protein randomization and quantum transfer arising from membrane fusion should not. The addition of EGTA approx. 5 min after Ca^{2+} addition and after the full extent of quantum transfer and aggregation was observed, resulted in an approx. 50% reversal of the turbidity increase but only a 30% reversal of donor quenching (data not shown). The experiments of Table II show that peripheral protein randomization accounts for approximately two-thirds of the quantum transfer. This process is not expected to be reversed by Ca^{2+} removal and vesicle disaggregation. Thus the remaining one third of the quantum transfer amplitude which is reversible is most likely associated with vesicle aggregation (increased intra- and inter-membrane quantum transfer). Further experiments demonstrated that the maximal effect on fluorescein mercury acetate-labeled and MIANS-labeled vesicles observed by simultaneous addition of Ca^{2+} and K^+ was identical to that observed by Ca^{2+} addition followed by K^+ addition or K^+ addition followed by Ca^{2+} addition (Table III). This indicates that the degree of energy transfer obtained is independent of the route by which the aggregates are formed (continuous aggregation vs. dimerization followed by higher order aggregation).

Ca^{2+} concentration dependence of the phenomenon

Experiments described above show that quantum transfer involving the peripheral proteins can occur slowly under non-aggregating conditions. In this section we will describe experiments which show how the effects of inter-membrane quantum transfer and intra-membrane quantum transfer can be differentiated by a comparison of quantum transfer and turbidity data.

The degree of aggregation can be adjusted by varying either the Ca^{2+} concentration or the vesicle concentration. Fig. 4a compares the extents of quantum transfer (fluorescence) and vesicle aggre-

TABLE III

EFFECT OF PREAGGREGATION ON QUANTUM TRANSFER OF PERIPHERAL PROTEIN RICH VESICLES AT 30°C

FMA, fluorescein mercury acetate.

Initial condition	Perturbing addition	$\Delta F/F_0$	$t_{1/2}$ (s)
MIANS vesicles FMA vesicles	7.1 mM Ca^{2+} added immediately	−0.74	− 80
MIANS vesicles + 7.1 mM Ca^{2+}	FMA vesicles added after 15 min	−0.37	− 60
FMA vesicles + 7.1 mM Ca^{2+}	MIANS vesicles added after 15 min	−0.44	− 30

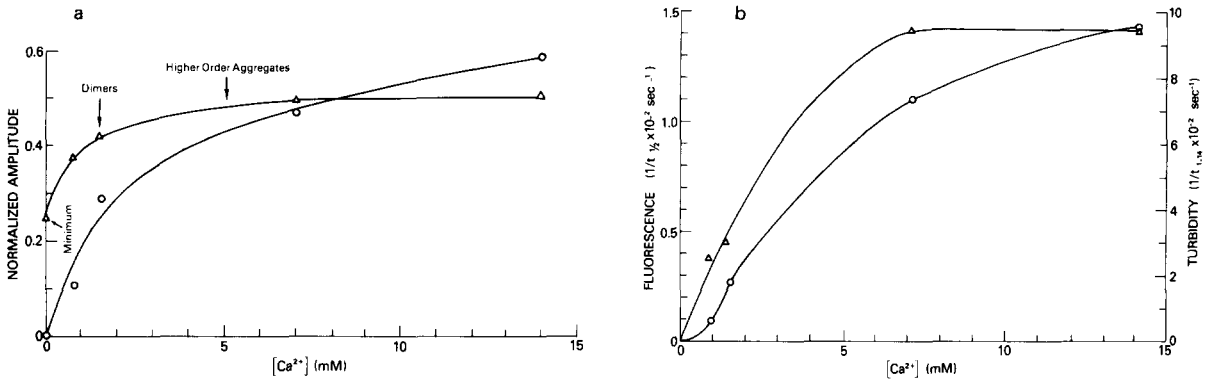

Fig. 4. Dependence of the steady state and rates of aggregation and quantum transfer processes on Ca^{2+} concentrations. (a) Dependence of the maximal quantum transfer amplitude, $-\Delta F/F_0$ (△) and the turbidity amplitude, $\Delta A/A_0$ (○) on the calcium concentration. (b) Dependence of the rates of the quantum transfer process ($1/t_{1/2}$) (○) and the dimerization process ($1/t_{1.14}$) (△) on Ca^{2+} concentration. Experiments were carried out at 30°C with aggregation of MIANS- and fluorescein mercury acetate-labeled 'peripheral protein-rich' vesicles at 30°C, protein concentration was 0.100 mg/ml. The reaction was initiated by the addition of Ca^{2+}.

gation as a function of the Ca^{2+} concentration. As shown above, substantial but not complete quantum transfer occurs at zero Ca^{2+} concentration. The reaction is extremely slow ($t_{1/2} > 1000$ s). Fig. 4a also shows that the extent of quantum transfer increases with increasing Ca^{2+} – concentration in a manner qualitatively similar to aggregation. The Ca^{2+}-dependent portion of the fluorescence change is about two-thirds maximal at the Ca^{2+} concentration which gives about 100% dimerization. The remaining portion of the aggregation dependent quantum transfer occurs in the transition from dimer to higher aggregates. Fig. 4b compared the Ca^{2+}-dependent rates of the quantum transfer and aggregation changes. The rates of the two processes show similar Ca^{2+} dependencies. However, the rate of the quantum transfer is 10-times slower than that of the dimerization process. Comparison of Figs. 4a and 4b at the Ca^{2+}-concentration range which gives full dimerization suggests that a Ca^{2+}-dependent structural change in the aggregated vesicles makes the greatest contribution to the Ca^{2+}-dependent portion of the quantum transfer increase.

Fig. 5a shows that the vesicle concentration dependencies of the aggregation and quantum transfer reactions are qualitatively similar at saturating levels of Ca^{2+}. Both Figs. 4a and 5a show that aggregation above dimers ($\Delta A/A_0 > 0.28$) gives rise to additional increases in $\Delta F/F_0$, suggesting that higher order aggregation is necessary for maximal quantum transfer. Fig. 5b shows that the rate of the quantum transfer enhancement increases with increasing vesicle concentration, but saturates at high vesicle concentrations. This is in line with our conclusion that slow rearrangements and structural changes in the highly aggregated vesicles are necessary for maximal quantum transfer changes.

The data for the effect of Ca^{2+} concentration on the rate of both dimerization and quantum transfer were subjected to analysis using double reciprocal plots. Determinations were made for the K_m, V_{max} and the rate constant for the maximal rate of the quantum transfer at saturating Ca^{2+} concentrations ($k_{Qt,max}$). Table IV compares the average V_{max} and K_m values of peripheral protein-rich membrane with peripheral protein-depleted membranes. Peripheral protein depletion reduces the value of $\Delta F/F_0$, as shown above. The K_m values for the depleted membranes are lower than those of the peripheral protein rich membrane. The value of $k_{Qt,max}$ is lower for deficient granules than for peripheral protein rich granules. Premixing of separately labeled granules reduces the $\Delta F/F_0$ value observed upon Ca^{2+} addition. The finding of Ca^{2+}-aggregation dependence in the peripheral protein-depleted vesicles suggests contact and mixing of integral proteins. The vesicle concentration dependence of the phenomenon

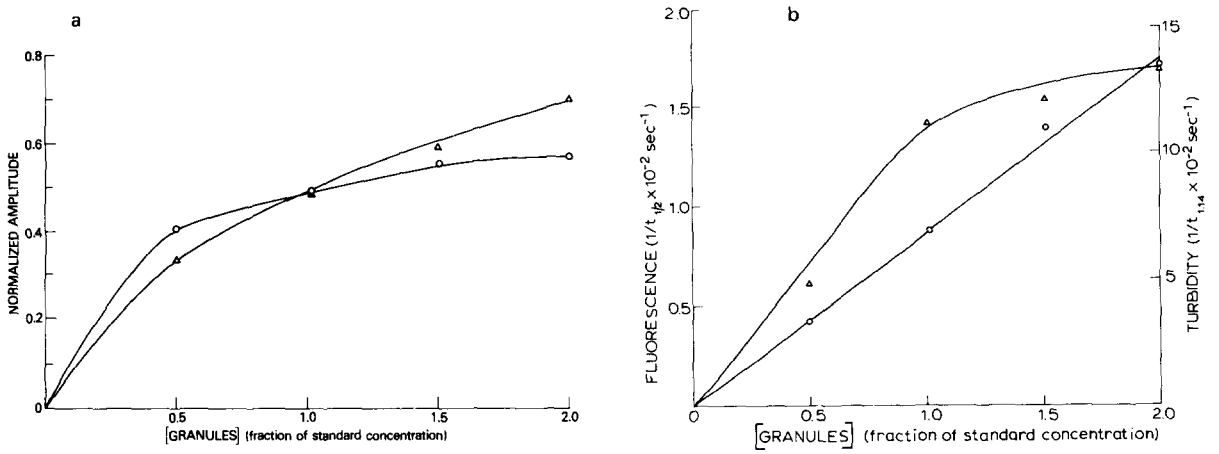

Fig. 5. Dependence of steady state and rates of quantum transfer and aggregation processes on the membrane concentration. Amplitudes, ratios and symbols are as defined for Fig. 4. Experiments were carried out at 30°C by adding Ca^{2+} to 'peripheral protein-rich' chromaffin granule membranes. The membrane concentration is expressed as a fraction of the standard concentration which was 0.100 mg/ml.

suggests that both inter-membrane and intra-membrane quantum transfer occurs between labeled integral proteins. Table IV shows that premixing does not have an appreciable effect on the energy transfer K_m or $k_{Qt,max}$ values (Expts. 2 and 3) suggesting that the rearrangements of peripheral protein and integral protein following vesicle aggregation must be of a similar nature.

Vesicles which are treated with both labels also show Ca^{2+}-induced quantum transfer increases, with $\Delta F/F_0$ values which are comparable to those of separately labeled and premixed vesicles (Table IV, Expt. 4). Since the double labeled vesicles have both fluorophores on the same membranes, their

TABLE IV

KINETICS OF Ca^{2+}-PROMOTED AGGREGATION AND DONOR QUENCHING OF GRANULE MEMBRANES LABELED WITH FLUORESCENT DERIVATIVES

Experimental error is approx. 15% of the presented values. The rate constants and Ca^{2+} K_m values for dimerization and development of quantum transfer as defined in Methods were calculated from double reciprocal plots.

Expt. No.	Donor Acceptor	Peripheral protein (rich/ depleted)	Labeling configuration	Fluorescence			Turbidity (dimerization)	
				K_m (mM)	$k_{Qt,max}$ (s^{-1})	$(\Delta F/F_0)_{max}$	K_m (mM)	V_{max} (s^{-1})
1	MIANS/FMA	rich	labeled separately; not premixed	4.0	0.0138	−0.74	10	0.115
2	MIANS/F5M	depleted	labeled separately; not premixed	0.82	0.0055	−0.38	12	0.383
3	MIANS/F5M	depleted	labeled separately; premixed	0.66	0.086	−0.22	10	0.552
4	MIANS/F5M	depleted	double labeled	0.55	0.0143	−0.20	10	0.345

large Ca^{2+} response can only be the result of aggregation of proteins in the plane of the membrane.

The results of Table IV can be summarized as follows. Peripheral proteins seem to be responsible for 70% of the quantum transfer observed. Reactions involving integral proteins probably account for the remaining 30% of the quantum transfer increase. The distribution of both types of protein is changed dramatically in processes which are an order of magnitude slower than vesicle aggregation.

Discussion

The most important finding presented in this study is that aggregation brings a substantial fraction of the membrane-associated proteins into proximity. The maximal fractions of quantum transfer ($\Delta F/F_0$) observed were -0.74 for peripheral protein-rich membranes and -0.38 for peripheral protein-deficient membranes. These numbers reveal extensive co-mingling of proteins, as shown in the following example. The Förster theory [10,12] predicts that if each donor molecule had one acceptor molecule located at a distance R_0, the distance where half-maximal transfer occurs, the value of $\Delta F/F_0$ would be equal to -0.5. Although R_0 was not determined in this study, it can be estimated to be in the neighborhood of 27 Å by analogy with the quantum transfer calculations for the 1-aminonaphthalene/Rhodamine B pair [21,22] which has spectral overlap similar to that presented in Fig. 1. R_0 has a 1/6th power dependence on the spectral overlap, donor lifetime and acceptor extinction coefficient, making our R_0 estimation very insensitive to these parameters. It is most probable that R_0 value of our pairs is no smaller than 16 Å and no larger than 38 Å. The latter number will be used here. The rate constant for transfer in a fixed donor/receptor pair also varies as the inverse 6th power of the distance of separation (R), such that the probability of transfer is 91% for $R/R_0 = 0.71$ and is 10% for $R/R_0 = 1.4$. If we consider distribution of donor and acceptor molecules in terms of these two extremes, a $\Delta F/F_0$ value of -0.74 (peripheral proteins) can either be taken to mean (a) that all of the donors have one acceptor located at a distance 0.91 R_0 (< 35 Å) or (b) that 74% of donors have one or more acceptors located at distances which gave 100% transfer ($R_0 < 27$ Å) with the remaining 26% of two donors having no acceptors within transfer distance ($R_0 > 52$ Å). One can also postulate a number of models between these extremes, and the phenomenon could in fact be modeled as a two-dimensional solution, if detailed information about the surface density of the donors and acceptors were available. Such considerations are outside the scope of the present study, and we will simply characterize the phenomenon as follows: Ca^{2+}-induced aggregation brings about a high degree of co-mingling of the proteins of separately-labeled vesicles, with at least 74% of the donor molecules of peripheral protein-rich membranes having at least one acceptor molecule within 27 Å or less. For peripheral protein-deficient membranes, 38% of the donor molecules are brought to within this distance.

In all cases studied, the rate of the quantum transfer increase was at least an order of magnitude slower than the rate of vesicle dimerization, implying a membrane structural change for reorganization which proceeds slower than the rate of establishment of membrane-membrane contact. Analysis of the Ca^{2+}-dependence of the quantum transfer increase shows that it occurs by both aggregation-dependent and aggregation-independent processes. The rate of the aggregation independent process is very slow. Its amplitude can be decreased by removal of peripheral proteins, and it can be reproduced by adding peripheral protein extracts to unlabeled granules. There is an absolute requirement for the presence of granule membranes; the proteins do not show energy transfer in their absence. Our studies show that peripheral protein can communicate between vesicles on a time scale of approx. 1000 s; that it represents a substantial portion of the labeled protein in the normal chromaffin granule membrane preparation; and that upon vesicle aggregation the peripheral protein comes into close proximity with each other and with integral proteins. Comparison of Expts. 1 and 2 of Table IV suggests the loosely associated peripheral protein accounts for about 70% of the Ca^{2+}-induced quantum transfer observed in the normal (peripheral protein-rich) preparation.

The aggregation-dependent quantum transfer involves the establishment of proximity between both integral and peripheral proteins. It accounts for about 50% of the quantum transfer observed in the peripheral protein-deficient preparation (Fig. 4a). It occurs at rates which are about 7% as great as the process of aggregation (Table II), and it most probably involves substantial reorganizations of the proteins in the plane of the membrane.

Our findings with quantum transfer agree well with freeze-fracture electron microscopy (EM) studies of the effect of ions on the distribution of membrane-associated particles (MAPs). We have previously demonstrated using glycerol-replacement freeze-fracture techniques [7] that when isolated chromaffin granules are aggregated by Ca^{2+}, the membrane-associated particles are excluded from the region of membrane-membrane contact. However, clusters of membrane-associated particles are observed in the region just adjacent to contact points. similar results have been reported for exocytosis from intact chromaffin cells during exocytosis [23], as well as for exocytosis of mast cell storage granules [24] and the granules of other secreting cells [25]. Our observations were recently repeated on unfixed material using rapid freeze-fracture techniques on unfixed tissue [18]. It is probable that the peripheral proteins are also aggregating at the edges of the contact regions. Freeze-fracture electron microscope studies are presently under way to look at Ca^{2+} and K^+ redistribution of membrane-associated particles.

In earlier communications, we have postulated that the initial contact between vesicles is made via protein-protein interaction, and that the region of contact is extended by diffusion of aggregation-promoting phospholipids (e.g. phosphatidylethanolamine and phosphatidylserine) into the region of contact, to establish lipid bilayer-lipid bilayer contact in the central region of contact and exclusion of the membrane-associated particles [2,3]. The role of peripheral proteins in this process has not been elucidated, but is amenable to study using the present techniques. If granule aggregation is mediated through 'recognition' proteins, the failure to find an energy-transfer process with the same rate constant as aggregation could be due either to lack of sulfhydryl groups on the proteins or to there being sufficient distance between the SH-groups after contact that insignificant energy transfer occurs.

We have also demonstrated by kinetic analysis of turbidity changes that K^+ will aggregate chromaffin granules to a limited extent [1]. This result was verified by Schuler et al. [8] using rapid-freezing techniques. A new and important finding of the present study is that peripheral protein transfers slowly between isolated vesicles, and that the rate of transfer is accelerated by elevated KCl concentrations. Transfer of cell membrane proteins to phospholipid vesicles has previously been observed by Cook et al. [26]. The transfer can be explained either by the dissociation and reassociation of peripheral proteins with the vesicles or by protein transfer resulting from transient contact during vesicle collisions. Similarly, the enhancement effect of KCl on the rate of transfer could be due to the decreased energy barries to vesicle collision resulting from K^+-diminished membrane surface potential. This question deserves further study in the context of peripheral protein binding and characterization studies. We have suggested that the K^+-induced dimerization was the result of surface potential reduction by the cation to the extent that two vesicles could approach each other and make contact through putative recognition proteins. Tighter association could be produced by migration of negatively-charged phospholipids from the region of contact and migration of phosphatidylethanolamine into the region of contact [2,3]. Rand [27] has recently presented detailed reasoning for such rearrangement of phosphatidylethanolamines. The degree of K^+-aggregation is limited to dimer formation; the increased negative surface potential of the remainder of the surface discourages further contacts between vesicles. In contrast, Ca^{2+} enhancement of quantum transfer in premixed vesicles is partially irreversible and is associated with high degrees of aggregation. As noted above this can be partially attributed to the co-mixing of a fraction of the integral proteins and may indicate that vesicle fusion has occurred.

We have remarked upon our previous electron microscopic observations of Ca^{2+}-induced granule membrane fusion [3,4,5]. Using the techniques presented here, we are presently studying the time course of quantum transfer between fluorescently labeled membrane lipids as a test for vesicle fu-

sion. Finally, we will note that since donor and acceptor populations can be labeled separately, the methodology presented here constitutes a means for monitoring the aggregation and fusion of chromaffin granules either with each other or with plasma membranes and provides a method to study the highly specific process of Ca^{2+}-triggered exocytosis.

Acknowledgements

We wish to thank Professor V.P. Whittaker and Dr. John H. Phillips for helpful discussion of these studies. This work was supported in part by NIH grant No. GM23990 and a grant from the Florida Heart Association to DHH and a travel grant from the North Atlantic Treaty Organization to SJM during the period 1979–1980.

References

1 Morris, S.J., Chiu, V.K.C. and Haynes, D.H. (1979) Membrane Biochem. 2, 162–202
2 Haynes, D.H., Kolber, M. and Morris, S.J. (1979) J. Theor. Biol. 81, 713–743
3 Morris, S.J., Costello, M.J., Robertson, J.D., Südhof, T.C., Odenwald, W.F. and D.H. Haynes (1983) J. Auton. Nerv. Syst. 7, 19–33
4 Morris, S.J. (1974) in Biochemistry of Sensory Function (Jaenicke, L., ed.), pp. 535–540, Springer Verlag, Berlin
5 Morris, S.J., Südhof, T.C. and Haynes, D.H. (1982) Biophys. J. 37, 117–118
6 Creutz, C.E. (1981) J. Cell Biol. 91, 247–256
7 Schober, R., Nitsch, C., Rinne, U. and Morris, S.J. (1977) Science 195, 495–497
8 Schuler, G., Plattner, H., Aberer, W. and Winkler, H. (1978) Biochim. Biophys. Acta 513, 244–254
9 Morris, S.J., Hellweg, M.A. and Haynes, D.H. (1979) Biochim. Biophys. Acta 553, 342–350
10 Fung, B.K.-K. and Stryer, L. (1978) Biochemistry 17, 5241–5248
11 Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–3099
12 Förster, T. (1951) Fluoreszenz organischer Verbindungen, p. 85, Vanderhoeck and Ruprecht, Goettingen
13 Cahill, A.L. and Morris, S.J. (1979) J. Neurochem. 32, 855–867
14 Apps, D.K., Pryde, J.G., Sutton, R. and Phillips, J.H. (1980) Biochem. J. 190, 273–282
15 Gupte, S.S. and Lane, L.K. (1979) J. Biol. Chem. 254, 10362–10367
16 Burgum, A.A. and Matthews, K.S. (1978) J. Biol. Chem. 253, 4279–4286
17 Heitz, J.R. (1973) J. Biol. Chem. 248, 5790–5793
18 Dissing, S., Jesaitis, A.J. and Fortes, P.A.G. (1979) Biochim. Biophys. Acta 553, 66–83
19 Fretten, P.M., Morris, S.J., Watts, A. and Marsh, D. (1980) Biochim. Biophys. Acta 598, 247–259
20 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
21 Berman, I.B. (1971) Handbook of Fluorescence Spectra of Aromatic Molecules, 2nd Edn., p. 343, Academic Press, New York
22 Berman, I.B. (1973) Energy Transfer Parameters of Aromatic Compounds, p. 323, Academic Press, New York
23 Aunis, D., Hesketh, J.E. and DeVilliers, G. (1979) Cell Tissue Res. 197, 433–441
24 Lawson, D., Raff, M.C., Gomperts, G., Fewtrell, C. and Gilula, N.B. (1977) J. Cell Biol. 72, 242–259
25 Orci, L., Amherdt, M., Montesano, R., Vassalli, P. and Perrelet, A. (1981) Phil. Trans. R. Soc. London B 296, 47–54
26 Cook, S.I., Bouma, S.R. and Huestis, W.H. (1980) Biochemistry 19, 4601–4607
27 Rand, R.P. (1982) Annu. Rev. Biophys. 10, 277–314