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20 May 1980; revised 14 July 1980

# Micromolar $Ca^{2+}$ Stimulates Fusion of Lipid Vesicles with Planar Bilayers Containing a Calcium-Binding Protein

Abstract. *Fusion of phospholipid vesicles with a planar phospholipid bilayer membrane that contains a calcium-binding protein appears to mimic the essential aspects of cytoplasmic-vesicle fusion with plasma membranes (exocytosis) in that (i) there is a low basal rate of fusion in the absence of $Ca^{2+}$, (ii) this basal rate is enormously increased by micromolar ($\sim$ 10 $\mu$M) amounts of $Ca^{2+}$, and (iii) this rate is not increased by millimolar $Mg^{2+}$. Essential to this process is an osmotic gradient across the planar membrane, with the side containing the vesicles hyperosmotic to the opposite side. Similar osmotic gradients or their equivalent may be crucial for biological fusion events.*

Exocytosis, the fusion of intracellular vesicles with plasma membranes and the subsequent extracellular discharge of vesicular contents, is fundamental to such diverse biological phenomena as transmitter release at synapses (*1*), secretion by endocrine and exocrine glands (*2, 3*), and raising of the fertilization membrane after union of sperm and egg (*4*). Recently, we demonstrated that this process is modeled by the interaction of multilamellar phospholipid vesicles with planar phospholipid bilayer membranes (*5, 6*). In this model system, the existence of an osmotic gradient across the planar membrane is a prerequisite for fusion; the *cis* compartment containing the vesicles must be hyperosmotic with respect to the opposite (*trans*) compartment (*6*). We noted (*6*) that this osmotic condition, or its equivalent, may be required for biological exocytosis, and that there are basic similarities between fusion of phospholipid vesicles with planar phospholipid bilayer membranes, on the one hand, and fusion of cytoplasmic vesicles with plasma membranes, on the other. There is a major difference, however, between these two systems in the $Ca^{2+}$ requirement. Whereas millimolar amounts of any divalent cation ($Mg^{2+}$, $Ca^{2+}$, $Ba^{2+}$) stimulate fusion in the model system (*6*), micromolar amounts specifically of $Ca^{2+}$ are necessary to stimulate biological exocytosis (*7, 8*). This difference is not surprising, since phospholipids alone have neither the sensitivity nor the specificity for $Ca^{2+}$ manifested in exocytosis (*9, 10*); $Ca^{2+}$ sensitivity and specificity must arise from other components, presumably a protein or proteins, in the plasma or vesicle membrane. We now report that fusion of multilamellar phospholipid vesicles with planar phospholipid bilayer membranes is catalyzed by 10 $\mu M$ $Ca^{2+}$, but not by 1 m$M$ $Mg^{2+}$, if a calcium-binding protein extracted from synaptic membranes is included in the planar membranes.

Upon addition of vesicles, under appropriate conditions, to one of the two aqueous compartments on either side of a planar membrane, fusion is seen by two independent criteria: (i) the transfer of vesicular contents across the planar membrane into the *trans* aqueous compartment (*5*) and (ii) the incorporation of a vesicular membrane marker into the planar membrane (*6*). The latter criterion is used in the present study. We chose as the membrane marker the intrinsic membrane protein, VDAC (the voltage-dependent anion channel), obtained from outer mitochondrial membranes (*11, 12*). As reported previously (*6*), fusion events are characterized by sudden jumps in conductance of the planar membrane; because vesicles can contain more than one channel in their outermost lamella, some of these jumps result from "simultaneous" (within 200 $\mu$sec) incorporation of several channels into the planar membrane.

Figure 1A demonstrates the effect of $Ca^{2+}$ on the fusion rate of multilamellar vesicles with a planar phospholipid bilayer membrane that contains the calcium-binding protein. This water-insoluble, membrane-associated protein which is partially purified from calf brain, has a molecular weight of $\sim$ 16,000 and a Michaelis constant ($K_m$) for $Ca^{2+}$ of $\sim$ 15 $\mu M$ (*13*). In the complete absence of $Ca^{2+}$, there is a low but finite rate of fusion (not shown in this record) when the *cis* compartment is hyperosmotic with respect to the *trans* side. (The osmotic gradient is here established by 100 m$M$ glucose in the *cis* compartment.) This basal fusion rate is increased by several orders of magnitude on addition to the *cis* compartment of $Ca^{2+}$ to a concentration of 10 $\mu M$.

The requirement for an osmotic gradient is shown in Fig. 1B. In the presence of 10 $\mu M$ $Ca^{2+}$, no fusion occurs until the *cis* compartment is made hyperosmotic to the other side. An effective osmotic gradient can be established either by adding an osmoticant (solute that creates the osmotic gradient) to the *cis* compartment or by removing one from the *trans* compartment.

When calcium-binding protein is present in the planar membrane, the fusion process also shows $Ca^{2+}$ specificity. Under otherwise appropriate conditions 1 m$M$ $Mg^{2+}$ does not stimulate fusion, but subsequent addition of 100 $\mu M$ $Ca^{2+}$ initiates fusion events (Fig. 1C). The presence of calcium-binding protein does not alter the effect of $Mg^{2+}$ on fusion; concentrations > 5 m$M$ are still required for $Mg^{2+}$ alone to promote fusion events. Nor does the calcium-binding protein alter any other conditions for fusion of multilamellar vesicles to planar bilayer membranes; only sensitivity and specificity for $Ca^{2+}$ are enhanced. An osmotic gradient across the planar membrane (*cis* side hyperosmotic) is still required; indeed this alone, in the complete absence of divalent cation, causes fusion, albeit at a low rate.

Figure 2 depicts our proposed mechanism for fusion of a phospholipid vesicle with a planar membrane. The figure shows a single-walled vesicle because this is a simpler physical system to analyze than the multilamellar vesicles used in the present study; we have in fact obtained fusion of single-walled vesicles with planar membranes using conditions very similar to those we describe for multilamellar vesicles.

For the sake of clarity, let us assume that both compartments as well as the vesicles initially contain 100 mos$M$ glucose in addition to 200 mos$M$ salt (*14, 15*). If glucose is now removed from the *trans* compartment, water flows across the planar membrane from the *trans* to


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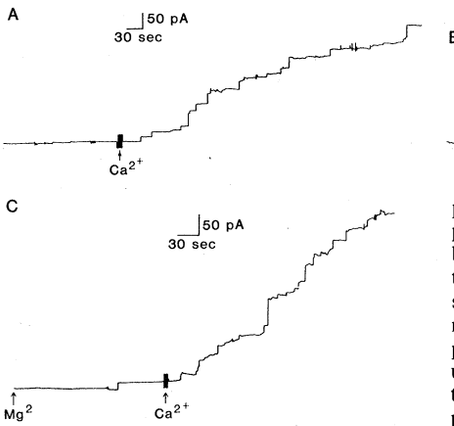


Fig. 1. (A) The effect of $Ca^{2+}$ on the rate of fusion of phospholipid vesicles with a planar bilayer membrane containing calcium-binding protein. A membrane was formed at room temperature by the brush technique (*20*) across a 1-$mm^2$ hole in a Teflon partition; the membrane-forming solution contained 4.5 percent asolectin, 0.5 percent diphytanoyl phosphatidylcholine (DPPC), and 0.5 percent calcium-binding protein. (The calcium-binding protein was dispersed in the membrane-forming solution by brief sonication immediately before we made the membrane. Within minutes the protein settled out of the decane solution and had to be redispersed before we made a new membrane.) The membrane separates solutions containing 100 m*M* NaCl, 10 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.1 m*M* EDTA, *p*H 6.0 in both compartments, and, in addition, 104 m*M* glucose in the *cis* compartment. The membrane potential (*V*) is clamped at 10 mV (*trans*-side positive). Several minutes prior to the beginning of the record, VDAC-containing multilamellar vesicles were added to the *cis* compartment to a concentration of $\sim 3 \times 10^8$ vesicles per milliliter. [The vesicles were prepared as previously described (*6*); their lipid composition was approximately 80 percent egg phosphatidylcholine (PC) and 20 percent bovine phosphatidylserine (PS), and their phospholipid-to-protein ratio was 25:1 (by weight).] Fusion events are characterized by jumps in the current record, which represent the insertion of ion-conducting channels (VDAC) into the planar membrane (*6*). At the arrow, $CaCl_2$ is added to the *cis* compartment to a total concentration of 100 $\mu M$; the concentration of free $Ca^{2+}$, as measured with a calcium-selective electrode, was 10 $\mu M$. Numerous fusion events follow this addition. The different heights of the jumps reflect differences in the number of channels in the outermost lamella of the fusing vesicles. Records such as this can proceed for as long as 20 minutes without a plateau in fusion rate; membranes containing calcium-binding protein are not long-lived and generally break after this time. [Asolectin (lecithin type II-S from Sigma) was washed with acetone and ether to remove neutral lipids (*21*); DPPC, egg PC, and bovine PS were from Avanti Biochemicals.] (B) Demonstration of the requirement of an osmotic gradient for fusion of phospholipid vesicles with a planar bilayer membrane containing calcium-binding protein. The membrane, clamped at $V = 10$ mV, separates symmetrical solutions (100 m*M* NaCl, 10 m*M* MES, 0.1 m*M* EDTA, *p*H 6.0). At the first arrow, vesicles (liposomes) are added to the *cis* compartment to a concentration of $\sim 3 \times 10^8$ vesicles per milliliter, and at the second arrow $Ca^{2+}$ is added to the *cis* compartment to a free ion concentration of 10 $\mu M$. Note that no fusion events occur in either the 3-minute period prior to $Ca^{2+}$ addition or in the 4-minute period following $Ca^{2+}$ addition. Within 30 seconds after addition of glucose (third arrow) to the *cis* compartment to a concentration of 104 m*M*, fusion proceeds at a rapid rate. The compositions of the planar membrane and vesicles are the same as in (A). (C) Demonstration of the specificity of $Ca^{2+}$ in stimulating fusion of phospholipid vesicles with a planar bilayer membrane containing calcium-binding protein. The membrane initially separated symmetrical solutions (100 m*M* NaCl, 10 m*M* MES, 0.1 m*M* EDTA, *p*H 6.0). Vesicles were added to the *cis* compartment to a concentration of $\sim 3 \times 10^8$ vesicles per milliliter; glucose was then added to that compartment to a concentration of 104 m*M*; just prior to the beginning of the record $MgCl_2$ was added to the *cis* compartment to a concentration of 1 m*M*. Note that one fusion event occurs during the 3.5-minute period following $Mg^{2+}$ addition. At the second arrow, $CaCl_2$ is added to the *cis* compartment to a concentration of 200 $\mu M$, which results in a free $Ca^{2+}$ concentration of $\sim 100$ $\mu M$. Within 15 seconds after this addition, fusion proceeds at a rapid rate. Thus, whereas 1 m*M* $Mg^{2+}$ did not stimulate fusion, the subsequent 100 $\mu M$ $Ca^{2+}$ did. The compositions of the planar membrane and vesicles are the same as in (A).

the *cis* side because of the resulting osmotic gradient. When a vesicle comes in close contact with the planar membrane, the combined planar and vesicular membranes are functionally one, and water flows, at the region of contact, from the *trans* compartment into the vesicle, causing it to swell. As its contents are diluted, water flows osmotically out of the vesicle, across those parts of its membrane not in contact with the planar membrane, into the *cis* compartment; in the steady state, the vesicle is swollen, and the osmolarity of its contents are intermediate to those of the *cis* and *trans* compartment. (For simplicity we neglect solute movement into or out of the vesicle either through the bilayer proper or through channels, such as VDAC.) If, however, the vesicles are almost completely swollen before the osmotic gradient is established across the planar membrane, the subsequent swelling bursts the vesicle and planar membrane at the region of contact before a steady state is reached, and the vesicular contents are expelled into the *trans* compartment (*16*).

Establishing the osmotic gradient by adding osmoticant to the *cis* compartment rather than removing it from the *trans* compartment complicates the situation. If the osmoticant is somewhat permeant (for example, glycerol), then the initial shrinkage of the vesicles is followed by rapid swelling (within milliseconds, because of their small size) back to their original size, and fusion proceeds as described above (*17*). If the osmoticant is impermeant, then the vesicles remain shrunken, and subsequent swelling upon contact with the planar membrane will not be sufficient to produce fusion (*18*).

The driving force for the fusion process, as we describe it, is the osmotic

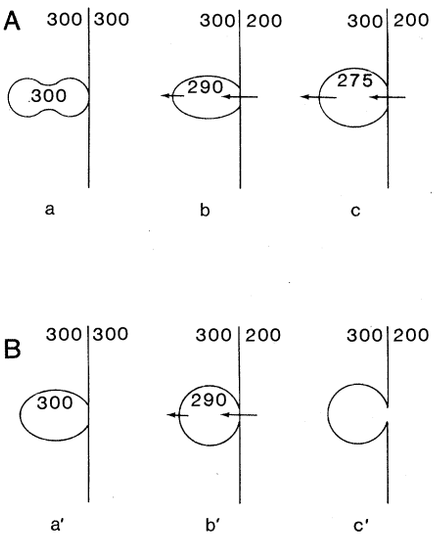


Fig. 2. Schematic diagram of our proposed osmotic mechanism for fusion of a phospholipid vesicle with a planar membrane. (A) (a) A shrunken vesicle is in contact with the planar membrane; the osmolarity on the two sides of the planar membrane and within the vesicle is 300 m*M*. (b) The osmolarity of the *trans* compartment has been diluted to 200 m*M*. Subsequent water entry into the vesicle has caused some swelling of the vesicle and dilution of its osmolarity to 290 m*M*. The rate of osmotic entry of water into the vesicle from the *trans* compartment (long arrow) exceeds the rate of osmotic exit of water from the vesicle into the *cis* compartment (short arrow), and the vesicle will continue to swell. (c) The vesicle has further swollen, and its contents are now diluted to 275 m*M*. A steady state exists with the rate of water entry into the vesicle equal to the rate of exit (arrows of equal length). No fusion occurs. (Note that the value of 275 for the osmolarity of the vesicle contents at steady state is hypothetical. The actual value will be a function of both the ratio of the vesicle area in contact with the planar membrane to the area not in contact, and the ratio of the permeabilities to water of those two areas.) (B) Same as (A) except the vesicle in (a′) is not so shrunken to start with as the vesicle in (a). Thus, in (b′) the vesicle is maximally swollen, but the rate of water entry is still greater than the rate of exit. Consequently, the vesicle bursts and fuses (c′).

gradient. We believe that divalent cations catalyze the process by increasing both the probability of vesicle-membrane contact and the duration of that contact, thus allowing time for the osmotic swelling to occur. For phospholipids, millimolar amounts of any divalent cation are required, whereas the presence of calcium-binding protein in the planar membrane lowers this requirement to the micromolar range of $Ca^{2+}$, specifically. Still lower $Ca^{2+}$ concentrations might produce fusion if calcium-binding protein were present in both planar and vesicular membranes. Limitations in the amount of protein available to us thus far have precluded experiments along this line.

The calcium-binding protein used here was obtained from synaptic membranes, but it is not clear at present whether it either promotes transmitter release at synapses or is particularly unique in its properties. Conceivably, many such binding proteins exist in neuronal or other membranes and function in related or different capacities. Its molecular weight of ~ 16,000, four calcium-binding sites, and $K_m$ for $Ca^{2+}$ of ~ 15 $\mu M$ are strikingly similar to the properties of calmodulin (*19*); further work is required to determine if the two molecules are related.

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14. If small vesicles have a membrane of finite surface energy, the law of Young and Laplace [see (*15*)] requires that a hydrostatic pressure difference exist across the vesicle membrane; in order for the chemical potential of water within the vesicle to equal that of water outside the vesicle, the contents of the vesicle must be hyperosmotic. In this circumstance fusion is possible by the same mechanism described in the text without an osmotic gradient across the planar membrane (*6*).
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16. We do not discuss here our speculations concerning the molecular rearrangements of phospholipid molecules during incorporation of vesicle membrane into planar membrane, nor why vesicle bursting occurs preferentially at the region of contact with the planar membrane.
17. If the planar membrane is also slightly permeable to the osmoticant (as is the case for glycerol), it can be shown both theoretically and experimentally that fusion proceeds less effectively than if it were not, and if the vesicular and planar membranes are too permeable, fusion does not proceed at all.
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8 May 1980; revised 28 July 1980

## Microencapsulated Islets as Bioartificial Endocrine Pancreas

Abstract. *Single implantation of microencapsulated islets into rats with streptozotocin-induced diabetes corrected the diabetic state for 2 to 3 weeks. The microencapsulated islets remained morphologically and functionally intact throughout long-term culture studies lasting over 15 weeks.*

Many techniques have been used to circumvent the problem of immune rejection, a major difficulty in the transplantation of pancreatic islets, or B cell tissue. Lacy *et al.* (*1*) reported that allografts survived 100 days in rats if the islets were cultured for 7 days at 24°C before they were transplanted and the rats received a single injection of antiserum to lymphocytes immediately before the transplantation. Sutherland and coworkers (*2*) achieved varying degrees of success with the use of dispersed neonatal pancreatic tissue which contained few or no exocrine cells as a result of prior treatment of the donor rats with DL-ethionine. Mullen *et al.* (*3*) avoided transplantation rejection by using fetal pancreas. Other investigators (*4-10*) have used diffusion chambers and hollow fiber units to act as mechanical barriers between islets (or B cells) and the host's immune cells and molecules and to achieve normalization of glucose homeostasis in experimental diabetic animals.

We have obtained prolonged survival of islets in vitro and in vivo by using a novel microencapsulation procedure

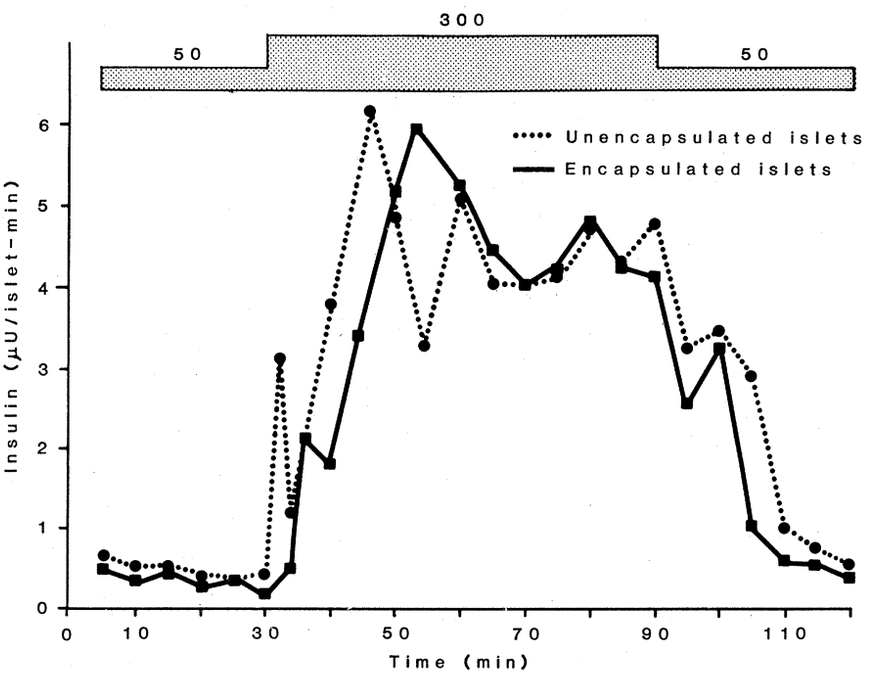


Fig. 1. Insulin release patterns of encapsulated and unencapsulated islets (~ 1 week in culture) in perifusion study. Both groups showed comparable response to high and low glucose concentrations. Microencapsulated islets appeared to show a slight delay in response initially (see text). The shaded area indicates the level and duration of glucose content (numbers are expressed in milligrams per 100 ml) in the perifusing culture medium.


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