Vol. 137, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1986 Pages 101-107

# SYNERGISTIC EFFECTS OF MICROMOLAR CONCENTRATIONS OF $Zn^{2+}$ AND $Ca^{2+}$ ON MEMBRANE FUSION

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Received March 25, 1986

SUMMARY: Resonance Energy Transfer between N-(7-nitro-2,1,3 benzoxadiazol-4 yl) phosphatidyl ethanolamine and N-Lissamine-Rhodamine B sulfonyl) phosphatidyl ethanolamine embedded in two different populations of small unilamellar vesicles made of phosphatidyl serine has been used to study the fusion process induced by $Zn^{2+}$ and $Ca^{2+}$. Lipid intermixing demonstrating fusion of liposome membranes can already be observed at 125 and 250 µmol/l of $Zn^{2+}$. After short time pre-incubations with micromolar concentrations of $Zn^{2+}$ as low as 150 µmol/l, $Ca^{2+}$ induces an instantaneous increase of vesicle fusion. The lipid intermixing induced by micromolar concentrations of $Ca^{2+}$ (250-500 µmol/l) could be increased up to 4 times when pre-incubated with 150 or 200 µmol/l of $Zn^{2+}$. The effect of 1 mM of $Ca^{2+}$ alone on lipid intermixing can be mimicked by 150 µmol/l of $Zn^{2+}$ followed by 500 µmol/l of $Ca^{2+}$. Our data demonstrate that $Zn^{2+}$ and $Ca^{2+}$ act synergistically to affect cation-induced membrane fusion. We suggest that $Zn^{2+}$ specifically alters the physical state of phospholipid membranes making them more prone to calcium-triggered fusion. 

The involvement of Zinc in biochemical and physiological processes (e.g. cofactors of metallo-enzymes) has been known for years (1). More recently the idea that zinc might also play an important role in maintaining membrane structure and function has been put forward (2,3).

High amounts of $Zn^{2+}$ are located in brain tissue and in particular in the terminals of hippocampal neurones (mossy fibers) (4-6). Electrical stimulation of hippocampal slices has been reported to result in concomitant release of both $Zn^{2+}$ and neurotransmitter substance (7). Uptake by the nerve terminals of

Abbreviations used: Ptd Ser: Phosphatidyl serine; Ptd Etn: PE: phosphatidyl ethanolamine; NBD: (7 nitro-2,1,3 benzoxadiazol-4 yl); N-Rh-Ptd Etn: N(Lissamine-Rhodamine B- sulfonayl) Ptd Etn; RET: Resonance Energy Transfer.

0006-291X/86 $1.50

$^{65}Zn^{2+}$ added to the medium (8) suggests that the cation serves a specific functional role in synaptic transmission. This is further supported by findings demonstrating that chronic zinc deficiency alters the neuronal function of hippocampal mossy fibers (9).

When stimulated, nerve terminals release neurotransmitter substance via a process involving the fusion of intracellular vesicles containing the neurotransmitter and the cytoplasmic nerve membrane. This fusion process is $Ca^{2+}$ dependent and probably triggered by inflow of $Ca^{2+}$ subsequent to depolarization of the nerve terminal (10).

Numerous studies have demonstrated that calcium provokes membrane fusion in different biological and model systems (10-13). More recently it has also been shown that zinc, although at concentrations at least twice those encountered in brain tissue, induces fusion of phospholipid membranes (14). To our knowledge, however, the possibility that $Zn^{2+}$ might serve to facililate $Ca^{2+}$ induced fusion in biological systems where both cations are present, has not been investigated.

The aim of the present study was therefore to investigate the possible effect of low concentration of $Zn^{2+}$ on calcium triggered fusion of phospholipid membranes.

## MATERIALS AND METHODS

Bovine brain phosphatidyl serine (Ptd Ser) was purchased from SERVA (Heidelberg, Germany). N-NBD-Ptd Etn and N-Rh-Ptd Etn were purchased from AVANTI POLAR LIPIDS (Birmingham, Al, USA). The metal salts used were all standard chloride of high grade purity from UCB Chemicals (Brussels, Belgium). Small unilamellar vesicles (SUV) made of Ptd Ser were obtained as previously described (15-16) in 120 mM NaCl, 20 mM Tris-HCl buffer at pH 7.4 by successive ultrasonications under nitrogen flux at 4°C from a multilamellar vesicle suspension (5 mg phospholipids in 2 ml) containing 2 mol % of N-NBD Ptd Etn or 2 mol % of N-Rh-Ptd Etn (12,17-18). 20 µmol/l of each type of liposomes were suspended in a cuvette containing 2000 µl of buffer and thermostatted at 24°C. An SLM 4800 Aminco spectrofluorometer (SLM-Aminco-Urbana,Il.) was used.

A fusion index was calculated by measuring the ratio R of N-NBD-Ptd Etn emission at 533 to the N-Rh-Ptd Etn emission at 586nm (12, 18-19) which is a sensitive measure for the efficiency of Resonance Energy Transfer between the probes. Repeated emission spectra were taken from 480 to 630nm for each sample at various time intervals. In the absence of fusion between the two vesicles populations, the maximal value of R is 2.09 while a complete mixing of the probes resulting in vesicles containing 1 mol % of each probe gives a value of 0.21. The percent of liposome fusion index used in this study is defined as % fusion index =

$$100 \frac{R_{max} - R_o}{R_{max} - R_{fused}} = 100 \frac{(2.09 - R_t)}{1.88}$$

where $R_t$ is the ratio of fluorescence at time t (19).

## RESULTS AND DISCUSSION

The effect of increasing concentrations of $Zn^{2+}$ on the resonance energy transfer between N-NBD-PE and N-Rh-PE incorporated in two different vesicles populations, is demonstrated in figure 1. It can be seen that long time periods were required to

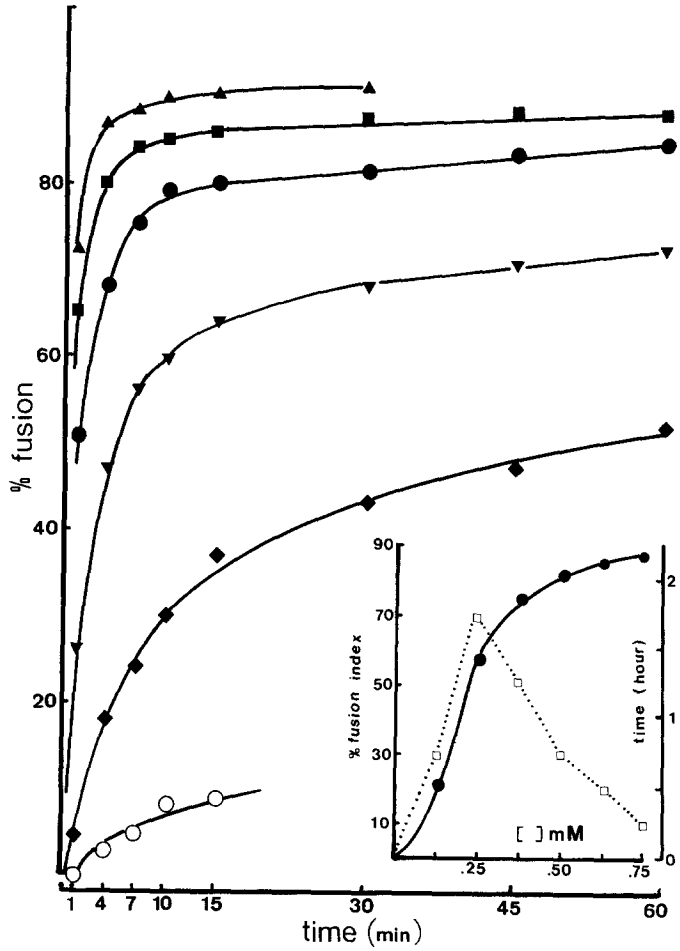


FIGURE 1: Kinetics of $Zn^{2+}$-induced fusion of unilamellar Ptd Ser vesicles incubated in the presence of various concentrations of the cation at 24°C after short incubation times. 40 mmol of N-NBD-Pts Etn: Ptd Ser (2:98) were incubated with 40 nmol of N-Rh-Ptd Etn: Ptd Ser (2:98) in 2 ml of buffer and were allowed to stand for 2 min before addition of the cation. Final concentrations of $Zn^{2+}$ were 125 (○), 250 (♦), 375 (▼), 500 (●), 625 (■) and 750 µmol/l (▲). Each point is the mean of 3 experiments. Insert: plateau values of $Zn^{2+}$-induced RET between the two vesicle populations as a function of the cation concentration the dotted line (□) represents the time to reach plateau values of fusion index for the different $Zn^{2+}$ concentrations.

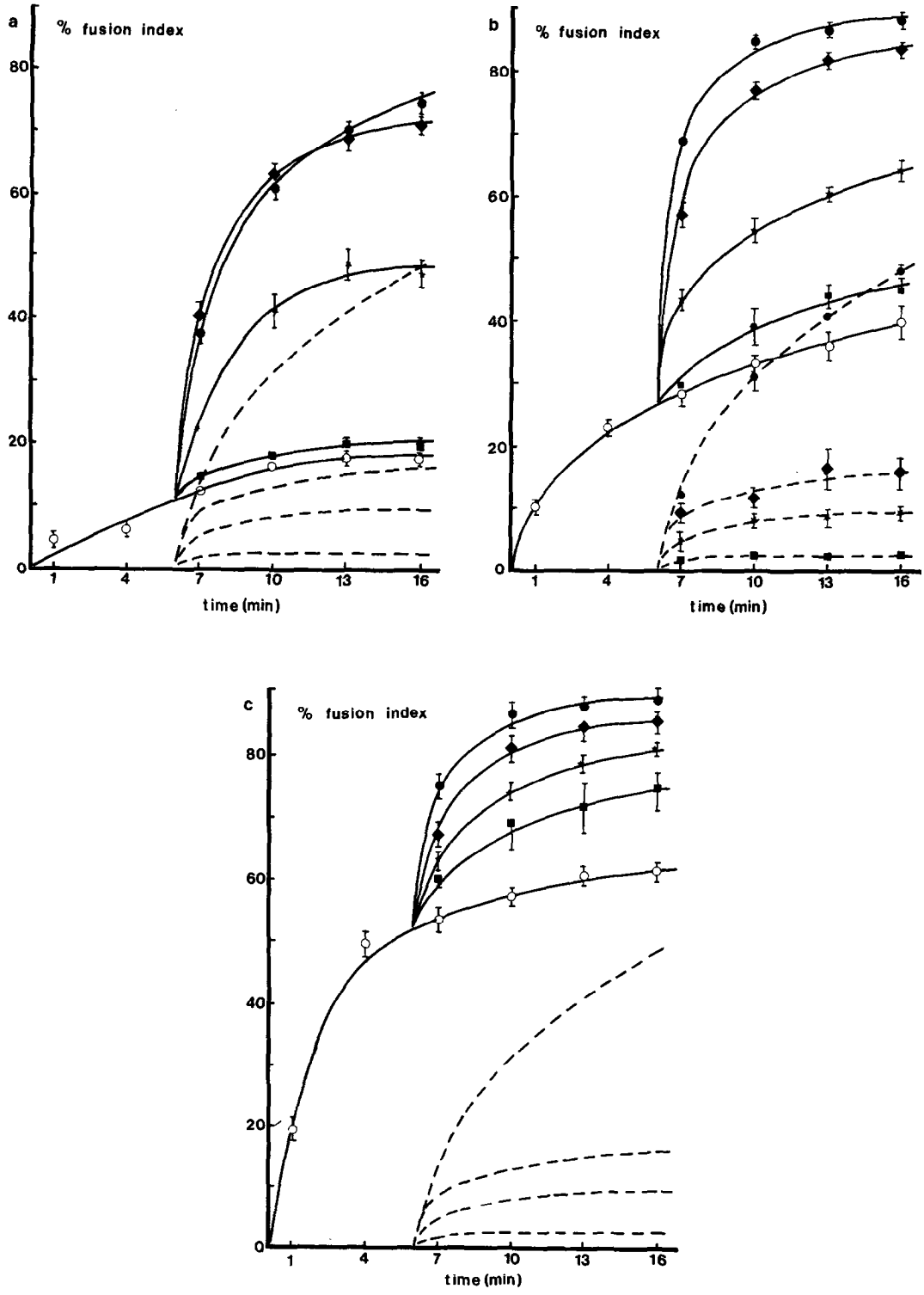


FIGURE 2: Kinetics of $Zn^{2+}$ and $Ca^{2+}$ induced fusion of Ptd Ser vesicles in the presence of various concentrations of $Ca^{2+}$ alone (dashed lines) or of various concentrations of $Ca^{2+}$ after pre-incubation with 150 (fig. 2a), 200 (fig.2b) or 300 (fig.2c)

reach plateau values for the fusion index when using low concentrations (250 μM)of the cation. The insert shows the maximal fusion index at different concentrations of $Zn^{2+}$ and the time required to reach maximal fusion.

When the mixture of the vesicle suspension was incubated with $Zn^{2+}$ (150, 200 or 300 μM) and then afterwards with $Ca^{2+}$,a rapid and highly pronounced increase in lipid intermixing could be seen (figure 2 abc). This synergistic effect is more pronounced at the beginning of the experiment (time = 1 or 4 minutes after addition of $Ca^{2+}$) than at the end (t = 10 min after addition of $Ca^{2+}$). The magnitude of the cooperative effect of the two cations at different time intervals is shown in table 1.

The data demonstrate that zinc provokes almost complete (90 %) fusion of lipid vesicles when monitoring lipid intermixing (12). This effect is by far more extensive than effects reported with other cations.

According to these findings $Zn^{2+}$ also appears to be the most potent divalent cation in inducing membrane fusion with a half maximal effect ($ED_{50}$) at 200 μM (insert fig.1). This may be explained at least in part by its higher capacity to bind to Ptd Ser (20) than $Ca^{2+}$. This high binding capacity does not however explain the time required for developing the fusion phenomenon at low and intermediate $Zn^{2+}$ concentrations. Although not directly comparable, data have been published indicating that $Zn^{2+}$ also affects the packing of phospholipid membranes quite differently from other cations (21).

Our data also show that a short time pre-incubation of Ptd Ser vesicles with $Zn^{2+}$ obviously increases the lipid intermixing by subsequent addition of low concentrations of $Ca^{2+}$. The potentiation of $Ca^{2+}$ triggered vesicles fusion by $Zn^{2+}$ indicates that this cation induces alterations in the membrane thus favouring the effect of $Ca^{2+}$. We have already demonstrated similar synergistic effects on lateral lipid phase segregation in membranes (22).

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micromol/l of $Zn^{2+}$ (○) (full lines). The $Ca^{2+}$ concentrations were respectively from top to bottom 1 (●), 0.75 (♦), 0.50 (*) and 0.25 (■) mmol/l (see figure 2b for Ca alone). Each point is the mean of 4 experiments (± s.e.m.)

TABLE 1

| $Zn^{2+}$ (microM) | $Ca^{2+}$ (milliM) | Time (minutes) 1 | 4 | 7 | 10 |
|---|---|---|---|---|---|
| | .25 | 100 | 100 | 112 | 125 |
| | .50 | 191 | 331 | 342 | 345 |
| 150 | .75 | 318 | 380 | 355 | 341 |
| | 1.00 | 203 | 144 | 128 | 118 |
| | .25 | 166 | 300 | 333 | 300 |
| | .50 | 278 | 291 | 280 | 277 |
| 200 | .75 | 318 | 348 | 311 | 284 |
| | 1.00 | 326 | 162 | 124 | 101 |
| | .25 | 430 | 550 | 566 | 540 |
| | .50 | 200 | 221 | 223 | 220 |
| 300 | .75 | 164 | 187 | 172 | 153 |
| | 1.00 | 171 | 89 | 70 | 58 |

Cooperative effect of $Zn^{2+}$-induced, $Ca^{2+}$- triggered fusion of Pts Ser vesicles at different time intervals after $Ca^{2+}$ addition. The results are expressed in % of the effect of $Ca^{2+}$ alone which is always taken as 100 %. The vesicles were first incubated for 3 minutes with different $Zn^{2+}$ concentrations as indicated.

The possibility that similar phenomena may be involved in $Ca^{2+}$ dependent and triggered relase of neurotransmitters is therefore a tempting one. A biological correlate of our data could be that membrane bound $Zn^{2+}$ plays a role in maintaining specific lipid domains of the nerve terminal membrane in a fusion-prone state. Inflow of $Ca^{2+}$, subsequent to depolarization of the membrane, could be the final trigger event for exocytosis and release of neurotransmitter and zinc.

Indeed, recent studies have demonstrated that both $Zn^{2+}$ and neurotransmitters are released during electrical stimulation of hippocampal slices (7). Specific uptake of exogenous zinc by the nerve terminal (8) also pinpoints the role of membrane zinc in maintaining integrity and function of synaptic transmission.

## REFERENCES

1. Underwood, E.J. (1971) Trace elements in human and animal nutrition. 3rd Ed. Academic Press. New York.
2. Bettger, W.J. and O'Dell, B.L. (1981) Life Sciences 28, 1425-1428

3. Bevan, D.R., Warrell, W.J. and Barfield, K.D. (1983) Colloids and Surface 6, 365-376
4. Maske, H. (1955) Naturwissenschaften 42, 424-430
5. Dauscher, G. and Zimmer, J. (1978) Histochem. 55, 27-40
6. Frederickson, C.J., Kiltenik, M.A., Manton,W.I. and Kirkpatrick, J.B. (1983) Brain Res. 273, 335-339
7. Assaf, S.Y. and Shin-Ho Chung (1984) Nature 308, 734-736
8. Howell, G.A., Welch, M.G. and Frederickson, C.J. (1984) Nature 308, 736-738
9. Hesse, G.W. (1979) Science 205, 1005-1007
10. Rubin, R.P. (1975) Calcium and the secretory process, Plenum Press, N.Y.
11. Papahadjopoulos, D., Portis, A. and Pangborn, W. (1978) Ann. N.Y. Acad.Sci. 308, 50-66
12. Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093-4099
13. Hoekstra, D., Wilschut, J. and Scherphof, G. (1985) Eur. J. Biochem. 146, 131-140
14. Barfield, K.D. and Bevan, D.R. (1985) Biochem. Biophys. Res. Commun. 128, 389-395
15. Deleers, M., Servais, J.P. and Wülfert, E., (1985) Biochim. Biophys. Acta 813, 195-200
16. Deleers, M. (1985) Res. Commun. Chem. Pathol. Pharmacol. 49, 277-294
17. Hoekstra, D. (1982) Biochemistry 21, 2833-2840
18. Deleers, M., Servais, J.P. and Wülfert, E. (1986) Biochim. Biophys. Acta in press
19. Connor, J., Yatvin, M.B. and Huang, L. (1984) Proc. Natl. Acad. Sci. USA 81, 1715-1718
20. Blaustein, M.P. (1967) Biochim. Biophys. Acta 135, 653-668
21. Inouye, H. and Kirschner, D.A. (1984) Biochim. Biophys.Acta 776, 197-208
22. Deleers, M., Servais, J.P. and Wülfert, E. (1986) Biochem. Biophys. Res. Commun. 136, 476-481.