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Neurotoxic cations induce membrane rigidification and membrane fusion at micromolar concentrations

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The effect of the neurotoxic cations aluminum, cadmium and manganese on membranes was examined in sonicated unilamellar vesicles containing phosphatidylserine and compared to the effect of Ca^{2+}. Fusion of membranes was monitored by assessing the resonance energy transfer between *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine and *N*-(lissamine-rhodamine B-sulfonyl)phosphatidylethanolamine. Self-quenching of high concentrations of carboxyfluorescein in liposomes was used to demonstrate the release of molecules entrapped in liposomes to compare the kinetics of leakage and intermixing of lipid. Rigidification of membranes was evaluated by monitoring the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene embedded in membranes containing phosphatidylserine and dipalmitoylphosphatidylcholine. Cation-induced lipid intermixing of vesicles membranes and release of dye from the vesicles occurred in the same concentration range. With aluminum, these effects were observed with concentrations less than 25 μM. Significant rigidification of vesicle membranes was apparent with less than 25 μM of Al^{3+}. Similar effects could only be observed with concentrations of Cd^{2+} and Mn^{2+} at least one order of magnitude higher (200 and 400 μM, respectively).

Introduction

Neurotoxic effects of aluminum, cadmium and manganese have been well documented [1–5]. Moreover, aluminum has been demonstrated to accumulate in the brain of patients with Alzheimer's disease, specifically in areas where neurotic plaques and neurofibrillary tangles are located [2,3,6]. Administration of aluminum to laboratory animals has also been shown to induce profound cognitive impairments associated with the development of plaques and tangles in the brain [3,7,8].

These findings have renewed interest in the biochemical mode of action of Al^{3+}-induced brain damage and raised the possibility that aluminum could be a possible etiological agent or risk factor in the development of certain types of dementia [4,9–11]. It has been known for many years that chronic exposure to manganese increases the incidence of Parkinson type dementia in mine workers. However, it has also been reported recently that manganese concentrations increase in the brain with ageing, but not in patients with Alzheimer's disease [12,13]. Inhibition of choline transport in red blood cells and in synaptosomes [14,15] as well as the inhibition of release of neurotransmitters [16] and glycolysis in nerve cells [17] by aluminum, cadmium and manganese suggest an elective effect of these metals on neuronal structures.

Abbreviations: *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine-rhodamine B-sulfonyl)phosphatidylethanolamine; PS, phosphatidylserine; DPPC, dipalmitoylphosphatidylcholine.

We have recently shown that aluminum, cadmium and manganese induce membrane phase separation and membrane aggregation of phosphatidylserine-containing liposomes at micromolar concentrations [18,19]. The possibility might therefore arise that these cations induce fusion of cell membrane structures in the brain, leading to the rupture of nerve cells and to nerve degeneration.

The aim of this study was therefore to investigate the possibility that aluminum, cadmium and manganese induce membrane fusion at concentrations having been reported in the literature to be neurotoxic.

Materials and Methods

Materials

Bovine brain phosphatidylserine (PS), dipalmitoylphosphatidylcholine (DPPC) and 1,6-diphenyl-1,3,5-hexatriene were purchased from Sigma (Saint Louis, MO). *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine-rhodamine B-sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL) and 6-carboxyfluorescein from Eastman Kodak (Rochester, NY). The metal salts studied were all standard chloride of high grade purity.

Methods

Small unilamellar vesicles (SUV), made of PS or PS/DPPC, were obtained in 120 mM NaCl/20 mM Tris-HCl buffer at pH 7.4 by three successive ultrasonications of 60 s at 75 W with a Branson Sonifier B_{12} (Branson Co., Soest, The Netherlands) under nitrogen flux at 4°C (or 44°C) from a multilamellar vesicle suspension (5 mg phospholipids in 2 ml) containing either 2 mol% of *N*-NBD-PE or *N*-Rh-PE [20–24]. The concentration of liposomes was 20 mol/l of each type of liposomes in all lipid-intermixing experiments. Vesicles are mixed in a cuvette and fusion was initiated by addition of Al^{3+}, Cd^{2+}, Mn^{2+} or Ca^{2+}.

An Aminco spectrofluorometer SLM-4800 was used. Repeated emission spectra were taken from 480 to 630 nm for each sample at 1, 4, 7 and 10 min. The samples were excited at 468 nm and slits were set at 2 nm. The ratio R of *N*-NBD-PE emission at 533 nm to the *N*-Rh-PE emission at 586 nm is a sensitive measure of the efficiency of resonance energy transfer between both probes [20–23].

In the absence of lipid intermixing between the two vesicle populations, the maximum value of R is 2.09, while complete mixing of the probes resulting in vesicles containing 1 mol% of each probe gives a value of 0.21. A qualitative estimation of liposome fusion may be defined as:

$$\text{fusion index} = 100\frac{R_{max} - R_t}{R_{max} - R_{fused}} = 100\left(\frac{2.09 - R_t}{1.88}\right)$$

where R_t is the ratio of fluorescence at time t [23].

We used liposomes containing self-quenched 6-carboxyfluorescein to determine leakage during fusion by monitoring fluorescence at 550 nm when exciting at 490 nm. Liposomes were obtained by ultrasonication (300 s at 75 W) of multilamellar vesicles (10 mg/ml) in a buffer consisting of 100 mM carboxyfluorescein adjusted to pH 7.4 with NaOH. Liposomes were separated by passage through an equilibrated Sephadex G-75 column as previously described [24]. Liposomes are diluted to a final concentration of 30 μM of phospholipids.

The fluorescence polarization of liposomes containing DPPC and PS (8:2, mol/mol) labelled with 1,6-diphenyl-1,3,5-hexatriene (1 mol% lipids) was measured between 25 and 45°C with the SLM-4800 equipped with a Glan-Thompson polarizer in the T-format mode.

Rigidification was assessed as the increase in temperature (ΔT) needed to obtain the polarization value of half of the height of the polarization curve ($P = 0.250$). Fluorescence lifetimes were obtained by the phase and modulation measurements [25] to assess that the polarization variations were not due to diphenylhexatriene lifetime modifications.

Results

The effect of increasing concentrations of aluminum, cadmium and of three different concentrations of calcium on lipid intermixing of two vesicle populations, monitored by resonance energy transfer of the probes used, is shown in Figs. 1 and 2. High values of fusion index were obtained

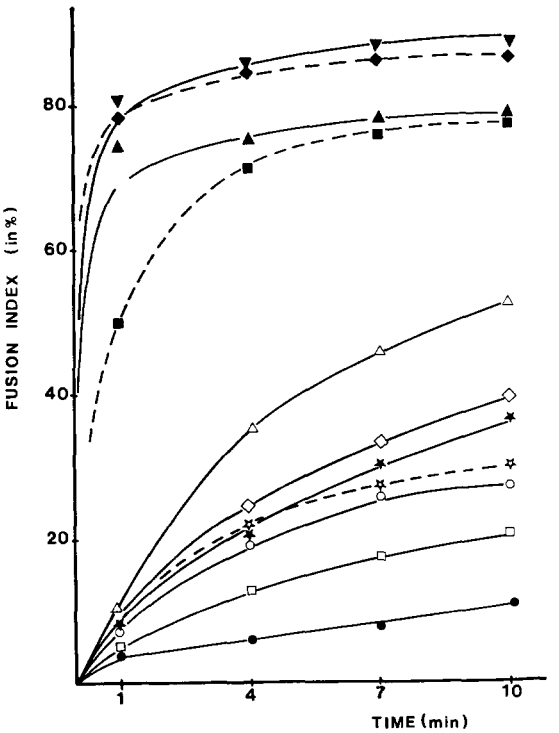

Fig. 1. Kinetics of Al^{3+}- and Ca^{2+}-induced fusion of unilamellar PS vesicles incubated in the presence of various concentrations of the cations at 24°C. 40 nmol of *N*-NBD-PE/PS (2:98) were suspended with 40 nmol of *N*-Rh-PE/PS (2:98) in 2 ml of buffer and were allowed to stand for 2 min before addition of the cation. Fusion index is calculated as described in Materials and Methods. Final concentrations of Al^{3+} (full line) were 12.5 (●), 25 (□), 37.5 (○), 50 (★), 62.5 (◇), 75 (△), 100 (▼) and 125 mol/l (▲). Final concentrations of Ca^{2+} (dashed line) were 1 (☆), 1.5 (■) and 2 mmol/l (◆). Each point is the mean of at least two experiments.

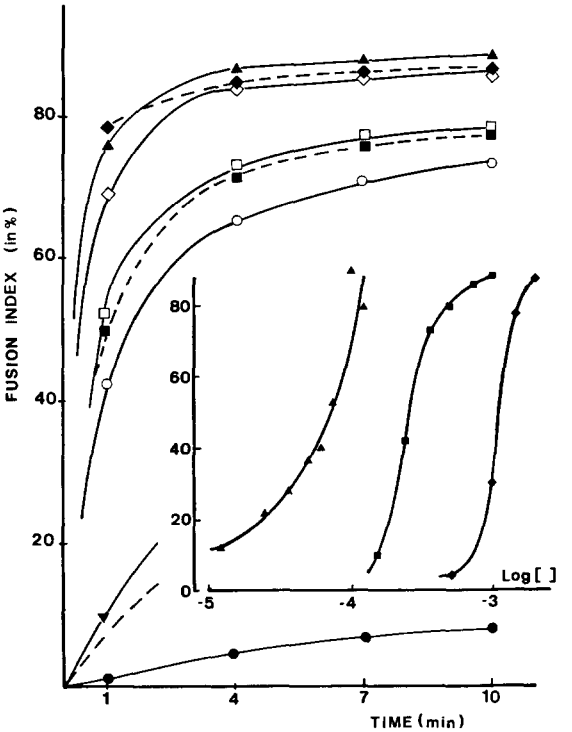

Fig. 2. Kinetics of Cd^{2+}- and Ca^{2+}-induced fusion of unilamellar PS vesicles incubated in the presence of various concentrations of the cations in the same conditions as in Fig. 1. Fusion index is calculated as described in Materials and Methods. Final concentrations of Cd^{2+} (full line) were 125 (●), 250 (▼), 375 (○), 500 (□), 750 (◇) and 1000 mol/l (▲). Final concentrations of Ca^{2+} (dashed line) were the same as in Fig. 1. Each point is the mean of at least two experiments. Inset: Summary of the effects of increasing concentrations of Al^{3+} (▲), Cd^{2+} (■), Ca^{2+} (◆) (expressed in log of concentration) on lipid intermixing index of unilamellar PS vesicles at 24°C.

for high aluminum (100 and 125 μM), cadmium (500–1000 μM) and calcium (1.5 and 2 mM) concentrations after 1 min. Plateau values are stable within 10 min. At lower concentrations of the three cations, plateau values were not achieved within 10 min. The inset in Fig. 2 shows the effect of increasing concentrations of Al^{3+}, Cd^{2+} and Ca^{2+} (expressed in log of concentration) on fusion values obtained after 10 min of incubation.

Fig. 3 shows the release of carboxyfluorescein from the same liposomes within the same time period and demonstrate the dependency of release on the concentration of aluminum. Cadmium and manganese induce a same pattern of carboxyfluorescein release (not shown). Plateau values for the release of carboxyfluorescein are obviously reached within 4 to 5 min at all concentrations studied. Half-maximal effects were obtained at 9 min with 62.5, 400 and 800 μM of Al^{3+}, Cd^{2+} and Mn^{2+}, respectively.

The increment on temperature required to reach a polarization value of 0.250 (half of the height of the polarization curves – see inset in Fig. 4) for unilamellar liposomes of DPPC/PS (8:2) is shown in Fig. 4. Aluminum and cadmium induced comparable maximal increments in temperature (ΔT). However, this effect required a concentration of

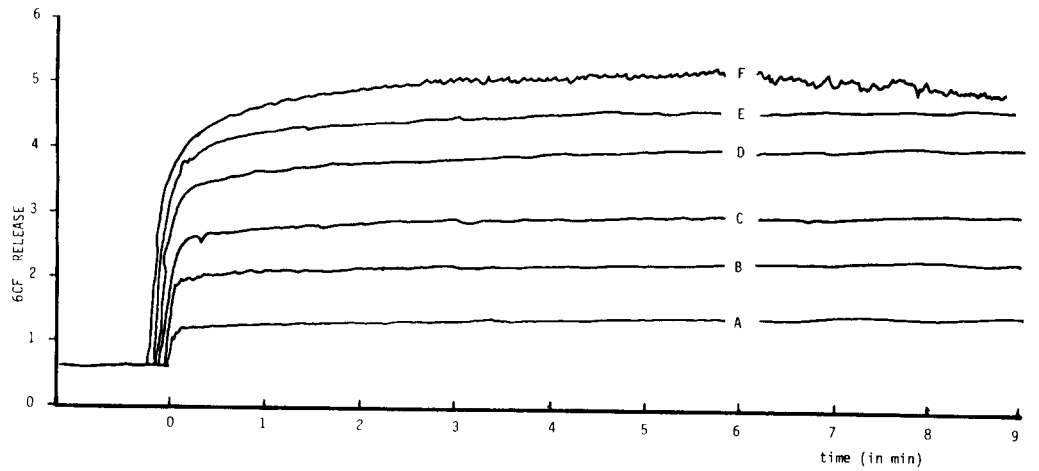

Fig. 3. Kinetics of carboxyfluorescein (6CF) release from sonicated PS vesicles (30 μmol/l) after addition of Al^{3+} at various concentrations: 25 (A), 50 (B), 75 (C), 87.5 (D), 100 (E), 125 μmol/l (F). Maximum fluorescence intensity was obtained following addition of Triton X-100 (final concn. 0.1%) to a vesicle suspension and set at 8 arbitrary units.

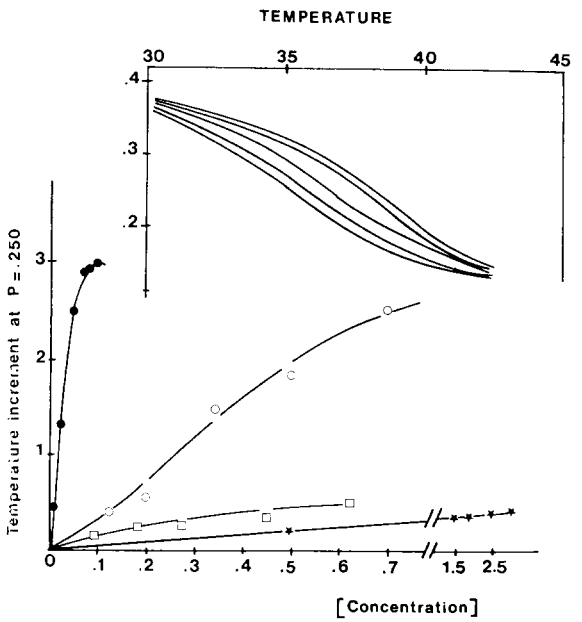

Fig. 4. Increment of temperature required to maintain a polarization value of 0.25 (apparent microviscosity = 2.38 P, Ref. 31) of diphenylhexatriene in DPPC/PS (8:2) with Al^{3+} (●), Cd^{2+} (○), Mn^{2+} (□), Ca^{2+} (★) as a function of their concentrations (expressed in mmol/l). Each point is the mean of 2 or 3 experiments (entire polarization curve). Inset: Polarization curves of diphenylhexatriene embedded in unilamellar vesicles: transition temperatures for control vesicles (left curve) or vesicles incubated in the presence of 12.5, 25, 50 and 75 μmol of Al^{3+} (from left to extreme right). Lipid concentration was 300 μmol/l. Unilamellar and multilamellar vesicles made of DPPC/PS (8:2) gave almost the same polarization curves.

aluminum approx. 7-times lower than with cadmium (100 μM Al^{3+} vs. 700 μM Cd^{2+}). It is interesting to note that neither Mn^{2+} nor Ca^{2+} induced similar changes even at a concentration as high as 2.5 mM. The inset in Fig. 4 shows the dramatic effect of micromolar concentrations of aluminum on diphenylhexatriene polarization. We have confirmed that the fluorescence lifetime of diphenylhexatriene remained unaltered in these membranes at increasing concentrations of aluminum (not shown).

Discussion

The data presented here demonstrate that aluminum and cadmium produce rapid release of dye from PS-containing unilamellar vesicles followed by intermixing of membrane lipids as assessed by resonance energy transfer. Both effects were observed at micromolar concentrations of the cations. The effect of aluminum could be observed at concentrations less than 25 μM. Whereas dye release appeared to be a fast process reaching almost maximum plateau values in less than 3 min after addition of the ion (Al^{3+} or Cd^{2+}), lipid intermixing, although developing rapidly at high concentrations, proceeded more slowly at low concentrations. After 10 min of incubation, plateau values for lipid mixing could only be obtained with concentrations of aluminum exceeding 100

μM and concentrations of cadmium exceeding 500 μM. This is not surprising and has already been reported by others for Ca^{2+} concentrations less than 1 mM [22]. With large unilamellar vesicles, plateau values could not be reached after 2 min [26] even with 5 mM Ca^{2+}.

We have recently demonstrated that Al^{3+} and Cd^{2+} promote rapid lipid phase segregation in PS membranes containing NBD-C_6-PC [18,19]. In these studies, the apparent kinetics of quenching of NBD-C_6-PC, used to assess phase separation, and the formation of aggregates were similar to the kinetics of dye release observed here.

The simultaneous appearance of all these phenomena (phase separation, dye release and aggregation) does not preclude the possibility that dye release is an independent process not preceded by lipid phase separation. Answering this question will however require further studies.

The significance of the increase of diphenylhexatriene polarization seen here with aluminum and cadmium and not with manganese and calcium in the process of membrane fusion is not clear. This apparent rigidification does not appear to be involved in membrane fusion since calcium, which has high fusion activity between 0.5 and 2.5 mM [20–22,27], did not show any rigidification effect in our experiments. This rigidification might well be responsible of the toxic effects of Al^{3+} and Cd^{2+}.

Whatever the processes induced by aluminum and cadmium might be, we have however, clearly demonstrated here that these cations profoundly perturb membrane structure at concentrations reported to inhibit choline transport in red blood cells or release of nerve transmitters from synaptosomes [14–6].

The lowest concentration of aluminum, which induced significant measurable lipid intermixing, aggregation or rigidification in our experiments, was 25 μM. Crapper et al. [2] determined the amount of aluminum to be 15 μg/g dry brain tissue in cats with aluminum-induced neurofibrillary degeneration. This amount is close to 120 μM of the cation.

More recently, Garruto et al. [10] also demonstrated the accumulation of high levels of aluminum in patients with Parkinson Dementia of Guam. The amount of aluminum showed here to induce membrane fusion and aggregation of phospholipid vesicles is therefore compatible with observations from several groups associating brain levels of aluminum and neurofibrillary degeneration.

The data presented here clearly demonstrate that the neurotoxic cations studied profoundly alter the membrane of phospholipid vesicles.

To the extent that such membranes do constitute a relevant model for the study of biological membranes, similar effect could be anticipated in the neuronal circuitry. Although this has to be demonstrated, it is a tempting hypothesis for studying the molecular mechanism of the neurotoxicity of Al^{3+}, Cd^{2+} and Mn^{2+}.

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