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THE ASYMMETRIC EFFECT OF LANTHANIDES ON Na^+-GRADIENT-DEPENDENT Ca^{2+} TRANSPORT IN SYNAPTIC PLASMA MEMBRANE VESICLES

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Lanthanides (La^{3+}, Pr^{3+} and Tb^{3+}) inhibit Na^+-gradient-dependent Ca^{2+} influx into synaptic plasma membrane vesicles. 50% inhibition is obtained by 7 μM lanthanide concentration. The inhibition of the Na^+-gradient-dependent Ca^{2+} uptake exhibits competitive kinetic behaviour. The apparent K_m of the Ca^{2+} influx is increased from 50 μM in the absence of lanthanides to 118 μM in the presence of La^{3+}, 170 μM in the presence of Pr^{3+} and 130 μM in the presence of Tb^{3+}. The maximal reaction velocity is not altered (8.35 nmol Ca^{2+} transported per mg protein per min in the absence of lanthanides and 8.16 nmol/mg per min in the presence of lanthanides). Lanthanides also inhibited Na^+-gradient-dependent Ca^{2+} efflux from synaptic plasma membrane vesicles that were preloaded with Ca^{2+} in a Na^+-gradient-dependent manner. Introduction of La^{3+} into the interior of the synpatic plasma membrane vesicles by rapid freezing of the vesicles in liquid N_2 and slow thawing had no effect on either Na^+-gradient-dependent Ca^{2+} influx or efflux. Synaptic plasma membrane vesicles can be preloaded with Ca^{2+} also in an ATP-dependent manner. This form of Ca^{2+} uptake is also inhibited by La^{3+} though at higher concentrations than the Na^+-gradient-dependent Ca^{2+} uptake. Na^+-gradient-dependent efflux from synaptic plasma membrane vesicles preloaded in an ATP-dependent fashion ('inside-out' vesicles) unlike efflux from synaptic plasma membrane vesicles preloaded in a Na^+-gradient-dependent manner was not inhibited by La^{3+}. These findings suggest that the inhibition by La^{3+} is manifested asymmetrically on both sides of the synaptic plasma membrane. Lanthanides are probably not transported via the Na^+-Ca^{2+} exchanger since Tb^{3+} entry measured by fluorescence of Tb^{3+}-dipicolinic acid complex formation occurred at high Tb^{3+} concentrations only (1.5 mM or above) and was not Na^+-gradient dependent.

Introduction

Lanthanides have been shown to affect in a dual fashion neurotransmitter liberation at the neuromuscular synapse [1]. Their overall effect has been attributed to changes in intracellular calcium ion concentration caused by a combination of the inhibitory effect of the lanthanides on the voltage-dependent Ca^{2+} entry process [2,3] and the inhibition of the respiration-dependent mitochondrial Ca^{2+} uptake in the nerve terminal [4]. At high La^{3+} or Pr^{3+} concentrations (200 μM) there is a rapid abolition of the evoked endplate potential (e.p.p.) and an increase in the spontaneous release of transmitter [1]. However, at lower Pr^{3+} concentrations (below 100 μM), there is an initial decrease in e.p.p. followed by an increase in the e.p.p. Between 10–30 μM Pr^{3+}, only an increase in the amplitude of the e.p.p. is observed [1].

In addition to the voltage dependent Ca^{2+} channel, two other Ca^{2+} transporting proteins present in the presynaptic plasma membrane take part in contributing to cellular Ca^{2+} homeostasis:

the Na^+-Ca^{2+} exchanger [5,6] and an ATP-dependent Ca^{2+} pump [7,8]. Previous studies [9–11] have shown that La^{3+} inhibited both Na^+-Ca^{2+} exchange and ATP-dependent Ca^{2+} pump in sarcolemmal and synaptic plasma membrane vesicles. Therefore, we thought that in order to have a better understanding of the effects of lanthanides on synaptic activity, a more detailed study regarding the mechanism of action of lanthanides on the Na^+-Ca^{2+} exchanger and the synaptic plasma membrane Ca^{2+} pump should be performed. Since the neuromuscular synapse cannot be used for biochemical studies due to small size of the nerve relative to the muscle, we chose for this purpose synaptic plasma membrane vesicles prepared by differential centrifugation from lysed rat brain synaptosomes. Synaptic plasma membrane vesicles, like other membrane vesicle preparations [12], permit the formation of well-defined intravesicular and extravesicular ionic environments by choice and the transport across the vesicles' membrane can be measured free of cytosolic or metabolic perturbations.

Besides these general advantages, the preparation of synaptic plasma membrane vesicles has also the specific advantage that both transport systems the Na^+-Ca^{2+} exchanger and Ca^{2+} pump, are present in it and each of them can be demonstrated separately. The Na^+-Ca^{2+} antiport system is investigated by measuring Ca^{2+} influx under conditions of preformed outward oriented Na^+ gradient (see Methods) in the absence of ATP. The ATP-dependent Ca^{2+} pump is studied in the absence of a Na^+ gradient and in the presence of added ATP. ATP-dependent Ca^{2+} influx in synaptic plasma membrane vesicles is probably exhibited by a fraction of the vesicles that were resealed after hypoosmotic treatment of the synaptosomes in an inverted fashion, with the internal face of the synaptic plasma membrane on the outside ('inside-out' vesicles).

The Na^+-gradient-dependent Ca^{2+} transporter can transport Ca^{2+} across the synaptic membrane in both directions depending on the direction of the Na^+ gradient. The direction of the Na^+-gradient-dependent Ca^{2+} flux with respect to the orientation of the membrane can be distinguished by selectively preloading with Ca^{2+} only the 'inside-out' vesicles in the absence of a Na^+ gradient and in the presence of ATP. Ca^{2+} efflux from these vesicles can be initiated via the Na^+-Ca^{2+} exchanger by increasing the Na^+ concentration in their external medium.

In this work, we examine in detail the effects of lanthanides on the Na^+-Ca^{2+} antiport system of rat brain synaptic plasma membrane vesicles. The results obtained suggest that the inhibitory effect of lanthanides on the Na^+-Ca^{2+} antiport process is not symmetrically manifested on both sides of the membrane, the cytosolic side ('inside') and the extracellular side ('outside).

Methods

Preparation of synaptic plasma membrane vesicles. Synaptic plasma membrane vesicles were isolated from 14-day-old rats as described by Rahamimoff and Spanier [13]. Each synaptic plasma membrane preparation consisted of 30 rat brains. The experiments presented here were repeated several times with at least three different synaptic plasma membrane vesicle preparations.

Possible mitochondrial contamination of the synaptic plasma membrane vesicle preparation was determined from the detectable specific activity of glutamate dehydrogenase (EC 1.4.1.3). Glutamate dehydrogenase activity was measured by the α-ketoglutarate coupled oxidation of NADH at 340 nm, in a reaction mixture containing: 0.1 M immidazole buffer (pH 7.9), 0.00025 M ammonium acetate, 0.012 M NADH, 0.0025 M EDTA, 0.002 M ADP, 0.012 M α-ketoglutaric acid and about 50 μg membrane protein in the presence or absence of 0.1% Triton X-100. In none of the synaptic plasma membrane vesicle preparations tested could more than 10% mitochondrial contamination be detected. This estimate is based on comparing the specific activity of glutamate dehydrogenase activity of gradient purified brain mitochondria [14] and the synaptic plasma membrane both measured as such, or after Triton solubilization.

Ca^{2+} transport measurements. Na^+-gradient-dependent Ca^{2+} transport studies were done on synaptic plasma membrane vesicles pre-equilibrated by incubation at 37 °C with a 0.15 M NaCl-10 mM Tris-HCl (pH 7.4) solution. The Na^+ preloaded vesicles were concentrated by centrifugation at $27\,000 \times g$ for 20 min and sus-

pended into a small amount of the same medium as used for preincubation. Na^+-gradient-dependent Ca^{2+} transport was initiated by diluting 3 μl of these vesicles (about 30 μg protein) into 250 μl of a solution without Na^+ (0.15 M KCl, 10 mM Tris-HCl (pH 7.4)) and containing 50 μM $^{45}CaCl_2$ (0.1 μCi). Control experiments were done to determine the amounts of Ca^{2+} associated with the vesicles in the absence of Na^+ gradient. 3 μl of the same NaCl preloaded vesicles were diluted into 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), 50 μM $^{45}CaCl_2$ (0.1 μCi). The values obtained were subtracted from those obtained in the presence of a Na^+ gradient. They did not exceed 15% of the total Ca^{2+} uptake (Fig. 1). The reaction was stopped by rapidly diluting the entire reaction mixture with 2 ml of ice-cold 0.15 M KCl and the vesicles were collected by filtration through 0.85

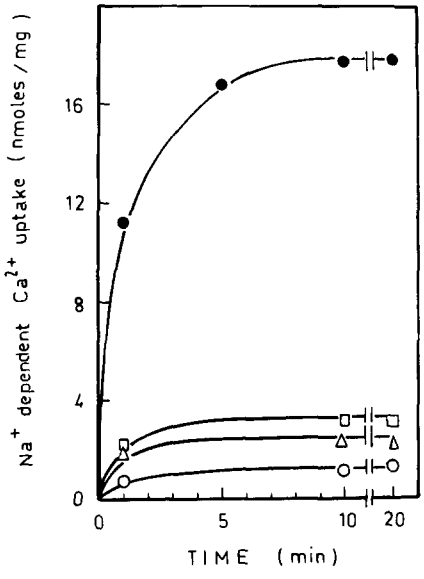

Fig. 1. Na^+-gradient-dependent Ca^{2+} uptake in synaptic plasma membrane vesicles. Ca^{2+} transport was measured by diluting 3 μl (about 30 μg protein) of synaptic plasma membrane vesicles preloaded with 0.15 M NaCl/10 mM Tris-HCl (pH 7.4) into: 0.15 M KCl/10 mM Tris-HCl (pH 7.4)/50 μM $^{45}CaCl_2$ (0.1 μCi) (●———●); or the same external medium except that 10 μM A23187 was also included (△———△); or 0.15 M NaCl/10 mM Tris-HCl (pH 7.4)/50 μM $^{45}CaCl_2$ (0.1 μCi) (○———○); Ca^{2+} transport in 0.15 M KCl/10 mM Tris-HCl (pH 7.4) preloaded vesicles diluted into 0.15 M NaCl/10 mM Tris-HCl (pH 7.4)/50 μM $^{45}CaCl_2$ (0.1 μCi) (□———□) is also shown.

BA 0.45 μM Schleicher and Schull filters. The filters were washed with the same solution two more times, dried and counted in a 25% Lumax-toluene scintillator solution.

ATP-dependent Ca^{2+} transport was determined by diluting 3 μl (about 30 μg protein) of synaptic plasma membrane vesicles into 0.15 M KCl, 10 mM Tris-HCl (pH 7.4), 10 μM $^{45}CaCl_2$ (0.1 μCi), 5 mM $MgCl_2$ and 2 mM ATP. ATP-independent $^{45}Ca^{2+}$ uptake was determined by diluting the vesicles into identical medium except that it did not contain ATP. These values were subtracted from the data obtained in the presence of ATP. They did not exceed 25% of the total Ca^{2+} uptake.

Preloading synaptic plasma membrane vesicles with solutes by 'freeze-thaw' cycle

Solutes of limited permeability were introduced into synaptic plasma membrane vesicles by rapid freezing of the vesicles with the desired medium in liquid N_2 followed by slow thawing of the frozen mixture at 4°C [15]. Presumably, upon freezing the closed membraneous structures open and during slow thawing they reseal, while entrapping within them the desired 'in' medium.

Determination of Tb^{3+} transport

Measurements of Tb^{3+} transport have been performed by preloading synaptic plasma membrane vesicles by 'freeze-thaw' cycle with 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4) and 0.1–10 mM dipicolinic acid. These vesicles were diluted into 0.15 M KCl, 10 mM Tris-HCl (pH 7.4) and 0.03–1.5 mM $TbCl_3$. Tb^{3+}-dipicolinic acid complex formation has been measured spectrofluorometrically as described in detail [16,17]. Calibration of the method was done by preloading synaptic plasma membrane vesicles with 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4) and a combination of 0.1–10 mM dipicolinic acid and 0.03–1.5 mM $TbCl_3$ by 'freeze-thaw' method. The Tb^{3+} (dipicolinic acid)$^{3-}$ containing vesicles were passed through a Sephadex G-50 mini-column to remove externally bound Tb^{3+} or dipicolinic acid. The loaded vesicles obtained by this procedure were stable, and neither internal Tb^{3+} nor dipicolinic acid leaked out of the vesicles for at least 2 h. The Tb^{3+} (dipicolinic acid)$^{3-}$ containing vesicles were diluted into 0.15 M KCl, 10 mM Tris-HCl (pH

7.4) and emitted fluorescence at 491 nm (excitation at 276 nm) was measured.

Determination of the intravesicular space has been done by measuring the ratio of ^3H_2O and [^{14}C]inulin spaces as described by Ref. 18. The average intravesicular space of our preparation of synaptic plasma membrane vesicles is 7 μl/mg protein. Protein was determined by the method of Lowry et al. [19].

Radiochemicals were purchased from the Radiochemical Center, Amersham, U.K., and Biochemicals from Sigma, Israel. Lumax was purchased from Lumac, The Netherlands. All other reagents used were of analytical grade.

Results

1. Inhibition of Na^+-gradient-dependent Ca^{2+} uptake by lanthanides

Synaptic plasma membrane vesicles take up Ca^{2+} in response to an outward-oriented Na^+ gradient. Fig. 1 shows the Ca^{2+} uptake when buffered NaCl preloaded synaptic plasma membrane vesicles (closed circles) are rapidly diluted into an isoosmotic medium without Na^+ (KCl in this case) and containing Ca^{2+}. The Ca^{2+} taken up by the vesicles reaches a steady-state level of 17 nmol Ca^{2+}/mg protein per 5 min. In the absence of an outward-directed Na^+ gradient when $[Na^+]^{in} = [Na^+]^{out}$ (Fig. 1, open circles) or when the Ca^{2+} ionophore A23187 is added to the external KCl medium (Fig. 1, open triangles) or when the direction of the Na^+ gradient is reversed (Fig. 1, open squares), very little Ca^{2+} is taken up by the vesicles. The K^+ gradient (created by the external K^+-containing solution) is not essential for Na^+-gradient-dependent Ca^{2+} uptake since it can be replaced by external choline chloride or sucrose solutions [15]. Lanthanides, La^{3+}, Pr^{3+} and Tb^{3+} are inhibitory to the Na^+-gradient-driven Ca^{2+} transport. Fig. 2 shows the inhibition of Ca^{2+} transport obtained by adding increasing concentrations of either of the lanthanides La^{3+}, Pr^{3+} or Tb^{3+} to the extravesicular reaction medium. Lanthanide concentrations as low as 5 μM are already inhibitory to the Na^+-gradient-dependent Ca^{2+} transport system. The inhibition increases rapidly with increasing lanthanide concentrations and 50% inhibition of the Na^+-gradient-driven Ca^{2+} transport is reached at about 7 μM lanthanide concentration. No significant differences in relative inhibitory potency of the three lanthanides investigated was observed, therefore, an average curve of three determinations of each of the lanthanides is presented. In order to examine the kinetic nature of the inhibition of Ca^{2+} transport by lanthanides, 5 μM of each lanthanide was added to 10–500 μM Ca^{2+} in the external buffered KCl medium. Initial velocities of the Na^+-gradient-dependent Ca^{2+} uptake at $t = 0.5$ min were measured and the reciprocal of the initial velocity was plotted against the reciprocal of the Ca^{2+} concentration. The K_m, K_i and V_{max} of the control and the inhibited reactions were calculated. In Table I, the calculated kinetic data for the Na^+-gradient-dependent Ca^{2+} uptake in the presence of 5 μM $LaCl_3$, $PrCl_3$ and $TbCl_3$ are presented. It can be seen that addition of lanthanides did not significantly alter the maximal reaction velocity, 8.35 ± 0.78 in the absence of lanthanides and 8.16 ± 1.36 (average) in the presence of lanthanides. However, addition of

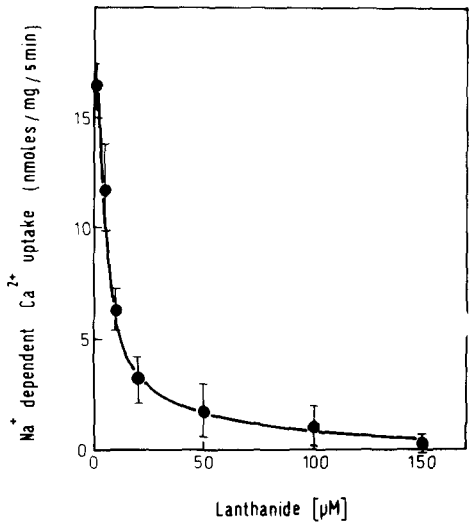

Fig. 2. The inhibition of Na^+-gradient-dependent Ca^{2+} uptake by lanthanides. 3 μl (about 30 μg protein) of 0.15 M NaCl/10 mM Tris-HCl (pH 7.4) preloaded synaptic plasma membrane vesicles were diluted into 250 μl 0.15 M KCl/10 mM Tris-HCl (pH 7.4)/50 μM $^{45}CaCl_2$ (0.1 μCi) and 0–150 μM La^{3+}, Pr^{3+} or Tb^{3+} (●———●). Ca^{2+} transport was measured as described in Methods. Zero-time controls and Ca^{2+} associated with the vesicles in the absence of Na^+ gradient has been subtracted.

TABLE I

THE KINETIC CONSTANTS OF THE INHIBITION OF THE Na^+-GRADIENT-DEPENDENT Ca^{2+} UPTAKE BY LANTHANIDES

3 μl of synaptic plasma membrane vesicles (30 μg protein) preloaded with 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), were diluted into 250 μl of 0.15 M KCl, 10 mM Tris-HCl (pH 7.4), 10–500 μM $^{45}CaCl_2$ (0.1 μCi) in the presence or in the absence of 5 μM La^{3+}, Pr^{3+} or Tb^{3+}. Initial rate of Ca^{2+} transport was measured (at 0.5 min).

Kinetic constants	Additions			
	None	La^{3+}	Pr^{3+}	Tb^{3+}
K_m (μM)	50.1 ±11.9	118	171.8	130.7
V_{max} (nmol/mg/min)	8.35± 0.78	7.09±1.2	9.7±0.8	7.69±0.7
K_i (μM)	–	2.8	2.56	2.72

lanthanides did change the K_m of the reaction from 50.1 ± 11.9 μM to 118 μM (La^{3+}), 171.8 μM (Pr^{3+}) and 130.7 μM (Tb^{3+}). The apparent K_i values of the three lanthanides were 2.8 μM (La^{3+}), 2.56 μM (Pr^{3+}) and 2.72 μM (Tb^{3+}). Thus, the inhibition by lanthanides of the Na^+ gradient dependent Ca^{2+} transport is a competitive inhibition.

2. Do lanthanides also inhibit Na^+-gradient-dependent Ca^{2+} efflux from synaptic plasma membrane vesicles?

In the experiments represented in Fig. 3, we examined the effect of added La^{3+}, Pr^{3+} and Tb^{3+} on Ca^{2+} efflux from synaptic plasma membrane vesicles. In these experiments, the synaptic plasma membrane vesicles were initially preloaded with Ca^{2+} in a Na^+-gradient-dependent manner (not shown). At $t = 5$ min of Ca^{2+} uptake, the direction of the Na^+ gradient was reversed by diluting the entire reaction mixture 5-fold with isoosmotic buffered NaCl in the presence or in the absence of different concentrations of lanthanides. Addition of the lanthanides to the efflux medium led to inhibition of Na^+-gradient-driven Ca^{2+} efflux. The extent of inhibition of Na^+-gradient-driven Ca^{2+} efflux by lanthanides was concentration dependent and exhibited similar values as those obtained for inhibition of Na^+-gradient-driven Ca^{2+} influx. No significant differences were observed between the three lanthanides. Addition of La^{3+} did not damage the synaptic plasma membrane and thus impair the Na^+-gradient-dependent Ca^{2+} efflux since addition of La^{3+} at $t = 3$ min after initiation of the Na^+-gradient-driven Ca^{2+} uptake led to immediate inhibition of the transport process but did not affect the Ca^{2+} already present within the vesicles (Fig. 4).

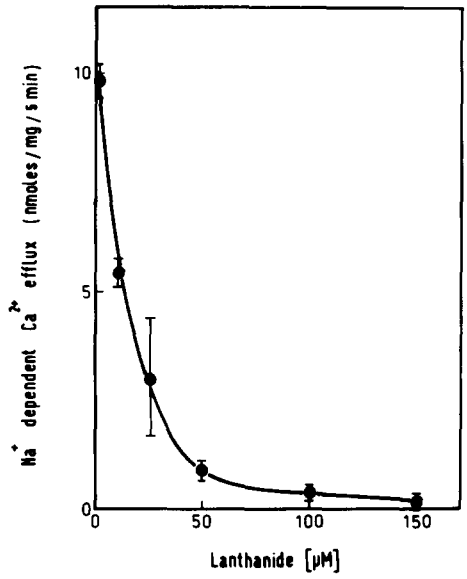

Fig. 3. The inhibition of Na^+-gradient-dependent Ca^{2+} efflux by lanthanides. 3 μl (about 30 μg protein) of 0.15 M NaCl/10 mM Tris-HCl (pH 7.4) preloaded synaptic plasma membrane vesicles were diluted into 0.15 M KCl/10 mM Tris-HCl (pH 7.4)/50 μM $^{45}CaCl_2$ (0.1 μCi). At $t = 5$ min, when the Ca^{2+} uptake reached 11.6 nmol Ca^{2+}/mg protein, the entire reaction mixture was diluted 5-fold with 0.15 M NaCl/10 mM Tris-HCl (pH 7.4) in the absence or in the presence of 10–150 μM La^{3+}, Pr^{3+}, or Tb^{3+} (●———●). Ca^{2+} content of the vesicles was measured after initiation of efflux and subtracted from the Ca^{2+} load before the dilution. Zero-time controls were also subtracted.

TABLE II

THE INHIBITION OF Na^+-GRADIENT-DEPENDENT Ca^{2+} TRANSPORT BY La^{3+} INTRODUCED TO THE INSIDE OR ADDED TO THE OUTSIDE OF SYNAPTIC PLASMA MEMBRANE VESICLES

Synaptic plasma membrane vesicles preloaded with either 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4) or 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4) and 100 μM $LaCl_3$ by 'freeze-thaw' technique (see Methods). 3 μl of these vesicles (30 μg protein) were diluted into 250 μl of 0.15 M KCl, 10 mM Tris-HCl (pH 7.4), 50 μM $^{45}CaCl_2$ (0.1 μCi) with or without 100 μM $LaCl_3$. Ca^{2+} transport was measured as described. Figures are presented as means ± S.D.

$[Medium]^{in}$	Time (min)	Ca^{2+} uptake (nmol/mg protein); $[Medium]^{out}$	
		KCl	KCl + 100 μM $LaCl_3$
NaCl	1	8.3 ± 3.7	1.7 ± 1.00
	5	13.07 ± 3.6	2.48 ± 1.5
NaCl	1	8.19 ± 5	1.74 ± 0.54
100 μM $LaCl_3$	5	11.95 ± 4.5	2.85 ± 0.78

3. Are lanthanides inhibitory to the Na^+-Ca^{2+} exchange process also from 'inside' the vesicles?

Since lanthanides were found inhibitory to both Na^+-gradient-dependent Ca^{2+} influx or Na^+-gradient-dependent Ca^{2+} efflux when added to the external medium of the vesicles, we decided to investigate the question whether the orientation of the membrane had a role in mediating this inhibition. To do so, we introduced La^{3+} into synaptic plasma membrane vesicles by two techniques: One was preincubation of synaptic plasma membrane vesicles with a 50-fold excess by volume of the buffered NaCl solution with different La^{3+} concentrations at 37 °C for 30 min, followed by concentration of the vesicles by centrifugation and resuspension in a minimal volume of the same solution as used for preincubation. The second method involved a 'freeze-thaw' cycle (see Methods) of synaptic plasma membrane vesicles in a solution identical in composition to the desired 'in' medium. Table II summarizes the results of five such experiments, all done by the 'freeze-thaw' procedure. The results obtained by preincubation of the vesicles with NaCl and La^{3+} were identical and therefore, they are not shown. It can be seen that freezing and thawing synaptic plasma membrane vesicles does not impair their capacity to take up Ca^{2+} in response to an outward-directed Na^+ gradient, nor is the inhibitory action of La^{3+} impaired when added to the 'outside' (the Ca^{2+}-facing side in these experiments) of the synaptic plasma membrane vesicles. However, addition of La^{3+} to the inside of the vesicles (the Na^+-facing side in this case) did not lead to inhibition of the Na^+-gradient-driven Ca^{2+} uptake, even when 500 μM $LaCl_3$ (not shown) was introduced into the vesicles.

4. Is the inhibitory effect of the lanthanides related to the orientation of the synaptic plasma membrane?

As shown in Figs. 2 and 3, lanthanides are effective inhibitors of both Na^+-gradient-dependent Ca^{2+} influx and efflux in synaptic plasma membrane vesicles. On the other hand, introducing La^{3+} into synaptic plasma membrane vesicles had no effect on Na^+-gradient-dependent Ca^{2+} influx (Table II). There are several ways to explain this apparent discrepancy. One such explanation would fit well with the hypothesis that the inhibitory effect of La^{3+} on Na^+-Ca^{2+} antiport is manifested asymmetrically across both sides of the synaptic plasma membrane. The population of synaptic plasma membrane vesicles is a heterogeneous mixture of two types of vesicles: those that were resealed following their formation as in the native synaptosome (right side out) and those that were resealed in an inverted fashion (inside-out). If our mixture of vesicles consisted mainly of the right-side-out population and if the Na^+-Ca^{2+} antiport process would be inhibited by La^{3+} added only to the extracellular side of the membrane, we would expect that in the 'inside-out' vesicle population the Na^+-gradient-dependent Ca^{2+} efflux would not be inhibited by La^{3+} added to the outside. This indeed happens and is shown in Fig. 5. Fig. 5 compares the effect of 100 μM La^{3+} on

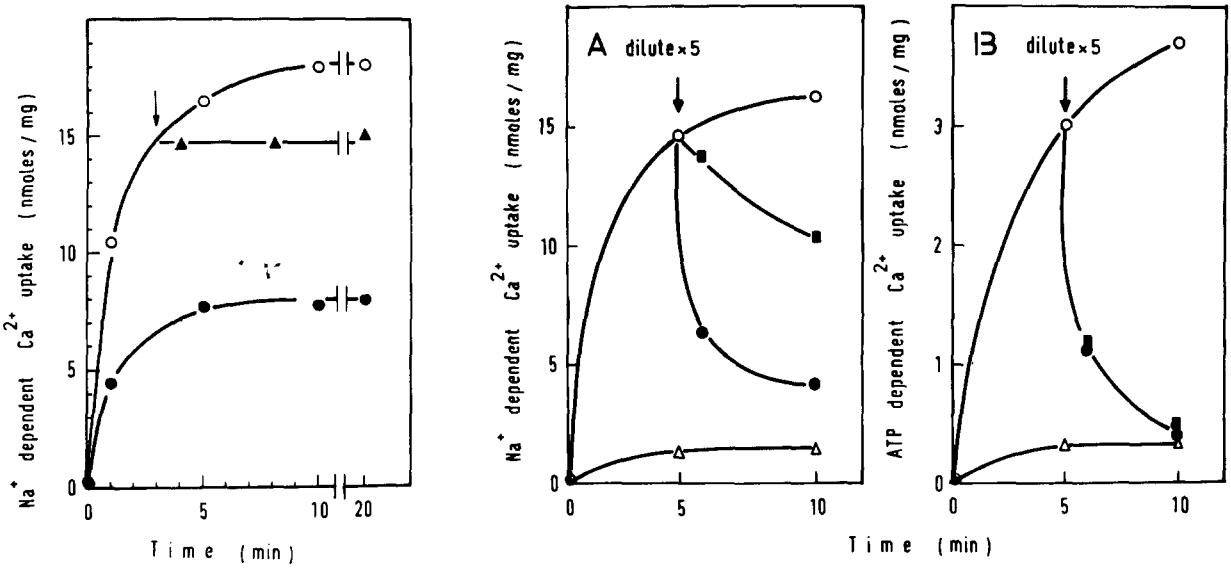

Fig. 4. The effect of La^{3+} on the Ca^{2+} content of synaptic plasma membrane vesicles. 3 μl (30 μg protein) of synaptic plasma membrane vesicles were preloaded with 0.15 M NaCl/10 mM Tris-HCl (pH 7.4) and diluted into 0.15 M KCl/10 mM Tris-HCl (pH 7.4)/50 μM $^{45}CaCl_2$ (0.1 μCi). Ca^{2+} transport has been measured at time points indicated (○——○). At t = 3 min after initiation of the Ca^{2+} transport, 30 μM La^{3+} has been added (marked by arrow) to the reaction mixture (▲——▲). In addition, Na^+-gradient-dependent Ca^{2+} transport in the presence of 30 μM La^{3+} added initially is also shown (●——●).

Fig. 5. Na^+-gradient-dependent Ca^{2+} efflux from Ca^{2+} preloaded synaptic plasma membrane vesicles. (A) 3 μl of synaptic plasma membrane vesicles (about 30 μg protein) were preloaded in 0.15 M NaCl/10 mM Tris-HCl (pH 7.4) and diluted into 250 μl of 0.15 M KCl/10 mM Tris-HCl (pH 7.4)/50 μM $^{45}CaCl_2$ (0.1 μCi) (○——○); at t = 5 min, the entire reaction mixture was diluted 5-fold with 0.15 M NaCl/10 mM Tris-HCl (pH 7.4) (●——●) or the same solution with 100 μM La^{3+} (■——■). The Ca^{2+} associated with the vesicles in the absence of Na^+ gradient is also shown (△——△). (B) 3 μl (about 30 μg protein) synaptic plasma membrane vesicles were preloaded with Ca^{2+} in a solution containing: 0.15 M KCl/10 mM Tris-HCl (pH 7.4)/5 mM $MgCl_2$/2 mM ATP/10 μM $^{45}CaCl_2$ (0.1 μCi) (○——○). At t = 5 min, the vesicles were diluted with 0.15 M NaCl (●——●) in an identical fashion to those in Fig. 5A, or with 0.15 M NaCl and 100 μM La^{3+} (■——■). The Ca^{2+} uptake in the absence of ATP is also shown (△——△).

Na^+-gradient-dependent Ca^{2+} efflux. In Fig. 5A, the vesicles were preloaded with Ca^{2+} in a Na^+-gradient-dependent manner (open circles). At t = 5 min, the entire reaction mixture was diluted 5-fold with either 0.15 M buffered NaCl (closed circles) or with 0.15 M buffered NaCl with 100 μM La^{3+} (closed squares). In Fig. 5B, the same experiment has been performed, except that the synaptic plasma membrane vesicles were preloaded in an ATP-dependent manner. In Fig. 5A, the Na^+-gradient-dependent Ca^{2+} efflux is inhibited by the added La^{3+} while in Fig. 5B, addition of 100 μM La^{3+} had no effect on Na^+-gradient-dependent Ca^{2+} efflux.

It should be noted that while 'freeze-thaw' cycle or preincubation of synaptic plasma membrane vesicles can not distinguish between 'inside-out' and 'right-side-out' vesicles present in the preparation, ATP-dependent-Ca^{2+} loading is exhibited only by 'inside-out' vesicles; those that have their ATP binding site oriented outward.

In order to rule out the possibility that the added La^{3+} was inefective due to the presence of either ATP or its hydrolysis products (ADP or inorganic phosphate) the following control experiments were performed: (1) The inhibition of ATP dependent Ca^{2+} influx by La^{3+} has been determined (Table III). (2) The experiment presented in Fig. 5B in which the effect of La^{3+} on Na^+-gradient-dependent Ca^{2+} efflux from 'inside-out' vesicles has been measured was repeated in two stages (Table IV). In the first stage, the synaptic plasma membrane vesicles were preloaded with Ca^{2+} in an ATP-dependent manner as in the

TABLE III

THE INHIBITION OF ATP DEPENDENT Ca^{2+} UPTAKE BY La^{3+}

The medium used to measure ATP dependent Ca^{2+} uptake contained: 0.15 M KCl, 10 mM Tris-HCl (pH 7.4), 2 mM ATP, 5 mM Mg^{2+}, 10 μM $^{45}CaCl_2$ (0.1 μCi) and La^{3+} as specified. The Ca^{2+} uptake in the absence of ATP was subtracted.

Additions to medium La^{3+}	Ca^{2+} uptake (nmol/mg/5 min)	% inhibition
–	5.8	–
30 μM	4.36	25%
100 μM	3.21	45%

experiment presented in Fig. 5B. At $t = 5$ min the vesicles were separated from the 'uptake' medium by centrifugation. In the second stage, the Ca^{2+}-preloaded synaptic plasma membrane vesicles were resuspended in 0.15 M NaCl for determination of Na^+-gradient-dependent Ca^{2+} efflux in the presence or in the absence of La^{3+}. These experiments show that the presence of ATP or its hydrolysis products in the reaction medium were not the cause for the ineffectiveness of La^{3+} as an inhibitor of Na^+-gradient-dependent Ca^{2+} efflux from 'inside-out' vesicles since: (1) the ATP-dependent Ca^{2+} influx is inhibited under these conditions by La^{3+} (Table III) and; (2) separation of the Ca^{2+}-preloaded 'inside-out' vesicles from the reaction medium containing ATP and its hydrolysis products did not alter the results obtained.

5. Are lanthanides transported by the Na^+-Ca^{2+} exchanger?

In order to examine the question whether lanthanides inhibit Na^+-gradient-driven Ca^{2+} uptake by interacting somehow with the carrier in a competitive fashion with Ca^{2+} or are in fact also transported via this carrier, we have performed the experiments summarized in Table V. Synaptic plasma membrane vesicles were preloaded either by preincubation or by rapidly freezing and slowly thawing (see Methods) with buffered NaCl and different amounts of dipicolinic acid. These vesicles were diluted into a medium containing different amounts of $TbCl_3$. Formation of Tb^{3+}-dipicolinic acid complex leads to a 10^4 increase in fluorescence (see Methods and Refs. 16 and 17). Thus, we

TABLE IV

Na^+-GRADIENT-DEPENDENT Ca^{2+} EFFLUX FROM 'INSIDE-OUT' VESICLES

Synaptic plasma membrane vesicles in 0.15 M KCl, 10 mM Tris-HCl (pH 7.4) were preloaded with $^{45}Ca^{2+}$ in the presence of 5 mM Mg^{2+} and 2 mM ATP (see Methods). Before initiation of Na^+-gradient-dependent Ca^{2+} efflux, the vesicles were separated from the uptake medium by centrifugation at 27000 $\times g$ for 20 min. Efflux was initiated by dilution of the Ca^{2+}-preloaded vesicles into 250 μl of 0.15 M NaCl in the presence or in the absence of La^{3+} as specified.

Time (min)	Ca^{2+} content in vesicles (nmol/mg), additions to NaCl medium		
	None	30 μM La^{3+}	100 μM La^{3+}
0	4.03	4.13	3.97
1	2.40	2.57	2.51
5	1.39	1.45	1.45

expected that entry of Tb^{3+} into the vesicles would lead to considerable increase in fluorescence and we should be able to detect even very small amounts of Tb^{3+}-dipicolinic acid complex formation. The experimental conditions were calibrated by preloading the vesicles in buffered NaCl and varying amounts of $TbCl_3$ and dipicolinic acid (keeping a molar ratio of 1 : 3). Externally bound

TABLE V

$TbCl_3$ ENTRY INTO SYNAPTIC PLASMA MEMBRANE VESICLES

Synaptic plasma membrane vesicles were preloaded by 'freeze-thaw' method (see Methods) with 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4) and varying amounts of dipicolinic acid. 5 μl of these vesicles (50 μg protein) were diluted into either 0.15 M KCl or 0.15 M NaCl and 10 mM Tris-HCl (pH 7.4). Emission of fluorescence at 491 nm was measured, excitation was at 276 nm. Results are presented as % of initial arbitrary values.

$[Medium]^{in}$	$[Medium]^{out}$	Change in fluorescence per 20 min
0.1 mM dipicolinic acid	buffered KCl, 30 μM $TbCl_3$	No change
1 mM dipicolinic acid	buffered KCl, 0.3 mM $TbCl_3$	No change
5 mM dipicolinic acid	buffered KCl, 1.5 mM $TbCl_3$	+20%
5 mM dipicolinic acid	buffered NaCl, 1.5 mM $TbCl_3$	+25%
5 mM dipicolinic acid	buffered KCl, 2 mM EDTA	No change

Tb^{3+} (dipicolinic acid)$^{3-}$ was removed by passing the vesicles, before fluorescence measurements, through a Sephadex G-50 mini-column. The results obtained in these experiments indicate that Tb^{3+} is not transported into the vesicles via the Na^+-gradient-dependent Ca^{2+} carrier, since: (1) No increase in fluorescence could be measured over a period of 20 min with Tb^{3+} concentrations effective in inhibition of Na^+-Ca^{2+} antiport. Only $TbCl_3$ concentrations above 1 mM in the extravesicular medium led to an increase in fluorescence. (2) The same increase in fluorescence has been observed with both KCl or NaCl in the extravesicular medium, indicating that the Na^+ gradient did not play a role in mediating Tb^{3+} entry into the vesicles. Thus, Tb^{3+} entry into the vesicles probably takes place by passive entry without the involvement of the Na^+-gradient Ca^{2+} transporter.

Discussion

In this work, we examined in detail the inhibition of the Na^+-gradient-dependent Ca^{2+} transport by lanthanides. All three lanthanides tested (La^{3+}, Pr^{3+} and Tb^{3+}) inhibited the Na^+-gradient-dependent Ca^{2+} transport considerably. Two basic questions were dealt with in our work: (1) The kinetic nature of the inhibition by lanthanides of the Na^+-gradient-dependent Ca^{2+} transport. (2) The mechanism of inhibition by lanthanides of the Na^+-gradient-dependent Ca^{2+} transport.

Examining the kinetic nature of the inhibition of the Na^+-Ca^{2+} exchange process by lanthanides was done by measuring the initial rate of Na^+-gradient-dependent Ca^{2+} uptake at different Ca^{2+} concentrations in the presence and in the absence of added lanthanide. Lineweaver-Burk plots of these results showed that there is no significant difference in the maximal velocity of reaction obtained in the presence or in the absence of added lanthanide. However, the K_m of the reaction was increased considerably by addition of any of the three lanthanides tested.

Synaptic plasma membrane vesicles are capable of carrying out Na^+-gradient-dependent Ca^{2+} transport in both directions across the synaptic membrane, depending on the direction of the Na^+ gradient.

In order to study the mechanism of the inhibitory action of the lanthanides, their effects on the Na^+-gradient-dependent Ca^{2+} efflux was studied. Addition of lanthanides to synaptic plasma membrane vesicles inhibited not only Na^+-gradient-driven Ca^{2+} influx but also Na^+-gradient-induced Ca^{2+} efflux, when the lanthanides were added to the external medium in which the synaptic plasma membrane vesicles were incubated. Introduction of La^{3+} to the inside of synaptic plasma membrane vesicles together with Na^+ did not inhibit Na^+-gradient-dependent Ca^{2+} influx or efflux. There are several possibilities to explain these results. One explanation would be that the synaptic plasma membrane is freely permeable to La^{3+} and therefore, due to the relatively small internal volume of the synaptic plasma membrane vesicles (7 μl/mg protein; 210 nl/30 μg protein, see Methods) compared to the large external volume of the medium (250 μl), the La^{3+} introduced into the vesicles could leak out rapidly. In the external medium, it would be diluted approx. 1000-fold, beyond its effective inhibitory range. Another explanation for our results could be that the lanthanides bind to some sites on the membrane. On the inside of the vesicles, where the total amount of La^{3+} relative to the area of the membrane is much smaller than on the outside, the remaining free La^{3+} concentration could be beyond the effective inhibitory range.

The experiments presented in Fig. 5, Table II, III and IV strongly favour a third explanation for the ineffectiveness of La^{3+} introduced into the vesicles as inhibitor of the Na^+-Ca^{2+} antiporter. These experiments show that La^{3+} is also ineffective as an inhibitor of Na^+-gradient-dependent Ca^{2+} efflux when added to the outside of the vesicles provided that 'inside-out' vesicles are selectively employed. This can be done by initially preloading the synaptic plasma membrane vesicles with Ca^{2+} in the absence of a Na^+ gradient and in the presence of ATP.

Evidence shows that the majority of the synaptic plasma membrane vesicles in our preparation are 'right-side-out' vesicles. In experiments performed on the relationship between Na^+-coupled γ-aminobutyric acid uptake and Na^+ channels [20] in a similar preparation of synaptic plasma membrane vesicles to that used by us it has been shown that about 80% of the Na^+-coupled γ-

aminobutyric acid uptake is inhibited by veratridine and this inhibition is antagonized by tetrodotoxin. Moreover, the entire Na^+ flux in these vesicles was inhibited by veratridine and the inhibition was prevented by tetrodotoxin and the latter of these toxins is acting on the Na^+ channels from the 'outside' (extracellular side) [21]. Reconstitution of the synaptic plasma membrane Ca^{2+}-transport ATPase by solubilization of the synaptic plasma membrane in cholate in the presence of added phospholipids and consequent dialysis leads to a 5-fold increase in specific activity of the ATP-dependent Ca^{2+} transport [22], suggesting that following reconstitution many more vesicles were reoriented with their cytosolic side to the outside than in the native preparation. Thus, is it not unexpected that internally introduced La^{3+} (from the cytosolic side) in our synaptic plasma membrane vesicle preparation had no effect on the Na^+-Ca^{2+} antiport (Table II) in these predominantly 'right-side-out' vesicles.

The last experiments in this paper dealt with the question of whether lanthanides prevent Ca^{2+} from being transported or are transported instead themselves.

Low concentrations of $TbCl_3$ added to the outside of Na^+ preloaded synaptic plasma membrane vesicles containing dipicolinic acid did not lead to an increase in fluorescence as expected, if the lanthanides were transported. Increasing $TbCl_3$ concentration to 1.5 mM did lead to an increase in fluorescence emitted by the vesicles. This fluorescence was not, however, dependent on the presence of the Na^+ gradient. Thus, it seems that lanthanides inhibit the Na^+-Ca^{2+} exchanger without being transported themselves.

The mechanism of inhibition of Na^+-Ca^{2+} antiport by lanthanides is probably quite complicated. Any model proposed for this inhibition has to accomodate two experimental findings: (1) competitive behaviour between lanthanides and Ca^{2+} on the Na^+-gradient-dependent uptake process and; (2) the inhibition of Ca^{2+} efflux from right-side-out vesicles by lanthanides. A model with two lanthanide-sensitive sites, one of which would bind Ca^{2+} and exhibit competitive inhibition with La^{3+} and the other which would be a regulatory site, could fit well with our results. However, further work has to be done before any model can be proposed to understand the mechanism of Na^+-Ca^{2+} antiport and its inhibition by lanthanides. The affinity of the carrier for the lanthanides is very high, and even very small amounts of lanthanides will lead to immediate inhibition of Ca^{2+} extrusion under physiological conditions where the Na^+ gradient is inward oriented. In addition, entry of lanthanides into the cell (either by diffusion or by endocytosis) will lead to inhibition of synaptic plasma membrane Ca^{2+} pump also. Very low concentrations of lanthanides were shown to inhibit respiration-dependent Ca^{2+} accumulation by mitochondria [4]. Inhibition of mitochondrial Ca^{2+} uptake by lanthanides leads to net Ca^{2+} efflux from these mitochondria which in the presence of lanthanides cannot be efficiently extruded by the Na^+-gradient-dependent mechanism or by ATP-dependent Ca^{2+} pumping via the plasma membrane. The consequence of this would explain the physiological observations of an increase in spontaneous transmitter release following addition of lanthanides to the neuromuscular preparation.

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