SODIUM-DEPENDENT CALCIUM UPTAKE IN MEMBRANE VESICLES DERIVED FROM RAT BRAIN SYNAPTOSOMES

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Received 31 May 1979

1. Introduction

In presynaptic nerve terminals an increase in the free intracellular Ca^{2+} concentration leads to an increase in neurotransmitter liberation. This increase in $[Ca]_{in}$ is either due to calcium entry via voltage sensitive channels in the presynaptic membrane, or can originate by release of calcium from intracellular stores [1]. Following synaptic activation a number of processes can participate in lowering of the elevated $[Ca]_{in}$ and thus bring the nerve terminal to the resting steady state. One of the major processes regulating $[Ca]_{in}$ is the sodium-dependent calcium extrusion process described in squid axons [2], brain slices [3], cardiac muscle [4] and isolated synaptosomes [5]. The synaptosomes are complex structures and therefore the data obtained from them are in some respects ambiguous for interpretation [5]. We report here the isolation of a vesicular membrane system derived from synaptosomes, capable of sodium-dependent calcium transport. In addition, a dependence of $^{22}Na^{+}$ uptake on intravesicular Ca^{2+} is shown. These membrane vesicles offer a simpler system for studying the molecular properties of the calcium–sodium antiport in synaptosome membranes.

2. Materials and methods

2.1. *Preparation of membrane vesicles*

Synaptosomes were isolated from day 14 rat brains on discontinuous Ficoll gradients, essentially as in [6]. Isolated synaptosomes were lysed for 40 min in 0.005 M Tris–HCl buffer (pH 7.4)–0.001 M EDTA. Membrane vesicles were isolated from the lysate by centrifugation at 17 000 × *g* for 15 min, suspended in 0.3 M mannitol and either used immediately, or kept frozen in liquid N_2.

2.2. *Uptake assays*

Fresh or frozen membrane vesicles (usually 1–5 mg/ml) were equilibrated by preincubation for 10 min at 37°C in a 20-fold excess (by vol.) of the desired 'in' medium. At the end of the preincubation the vesicles were cooled on ice and centrifuged at 17 000 × *g* for 15 min. The pellet thus obtained was suspended in a very small volume of the 'in' medium to yield ~1–5 mg vesicular protein/ml. For the assay the preloaded vesicles were diluted into a 10-fold excess (by vol.) of the desired 'out' medium. The assays were at 20°C, terminated by dilution with the appropriate buffer and rapid filtration on HA 0.45 μm millipore filters. The filters were washed 3 more times in buffer, dried and counted in a liquid scintillation counter.

Details of the 'in' and 'out' media used in the various experiments are given in the appropriate legends to the figures and tables in section 3. Protein was determined by a micromodification of Lowry [7]. $^{45}CaCl_2$ and $^{22}NaCl$ were purchased from the Radiochemical Centre, Amersham. The calcium ionophore A23187 and Nigericin were the generous gifts of Dr R. J. Hosley of Ely Lilly, Indianapolis, IN. All other agents used were commercial preparations of analytical grade.

3. Results

Figure 1 shows the calcium uptake into vesicles preloaded with 0.1 M Na-phosphate buffer at pH 7.4 and diluted into K-phosphate buffer at pH 7.4 containing $^{45}CaCl_2$. It can be seen, that the uptake is very rapid during the initial 2 min then gradually reaches a steady state. The calcium taken up by the vesicles is not released spontaneously even after 60 min. The reaction is specific for $[Na]_{in}$, since preloading the vesicle in 0.1 M K-phosphate buffer at pH 7.4 does not lead to calcium accumulation within the vesicles after diluting them into Na-phosphate buffer containing $^{45}CaCl_2$. In the presence of internal sodium, the external 0.1 M K-phosphate buffer can be replaced by iso-osmotic mannitol solution, Tris

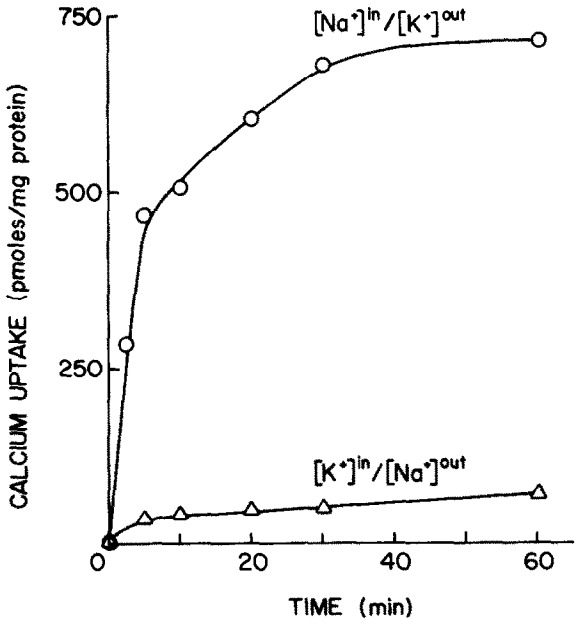

Fig.1. Sodium-dependent calcium uptake by membrane vesicles. Membrane vesicles (~100 μg protein) were preloaded with 0.1 M Na-phosphate buffer (pH 7.4) (as in section 2) and added to a solution containing 0.1 M K-phosphate buffer at pH 7.4 and 2 μM $^{45}CaCl_2$. The rate of uptake was measured as function of time (o–o–o). In another sample the preloading of membrane vesicles was with 0.1 M K-phosphate buffer (pH 7.4) and the uptake medium contained 0.1 M Na-phosphate buffer (pH 7.4) and 2 μM $^{45}CaCl_2$ (Δ–Δ–Δ). All solutions contained 5 mM $MgCl_2$. Note the marked difference in calcium uptake when an outward sodium gradient exists.

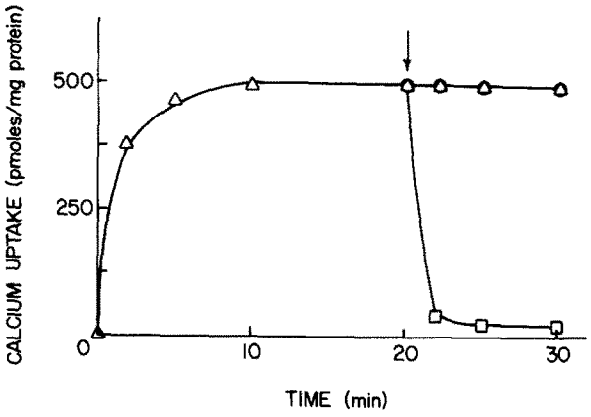

Fig.2. The effect of A23187 and externally added $CaCl_2$ on the sodium-dependent calcium uptake by membrane vesicles. Vesicles were preloaded with 0.1 M Na-phosphate buffer–5 mM $MgCl_2$ as in fig.1. The external medium contained 0.1 M K-phosphate buffer (pH 7.4)–5 mM $MgCl_2$ and 2 μM $^{45}CaCl_2$ (Δ–Δ–Δ). At t=20 min (marked by arrow) one part of the reaction mixture was diluted by 0.1 M K-phosphate buffer (10-fold by vol.) and 2 μM unlabeled $CaCl_2$ (Δ–Δ–Δ). The second part of the reaction mixture was diluted with identical buffer, $CaCl_2$ and 3 μM A23187 (□–□–□). The third part of the reaction mixture was diluted with 0.1 M Na-phosphate buffer (pH 7.4)–5 mM $MgCl_2$ and 2 μM $CaCl_2$ (o–o–o). Only addition of the calcium ionophore A23187 caused a release of calcium from membrane vesicles.

buffer or LiCl without altering significantly the calcium uptake. Figure 2 shows an experiment in which vesicles preloaded in $[Na]_{in}$ are diluted into $^{45}CaCl_2$ containing $[K]_{out}$. At t=20 min one part of the reaction mixture was diluted further with a 10-fold excess of the same $[K]_{out}$ buffer as that used before except that the radioactive $^{45}CaCl_2$ was replaced by equal concentration of unlabeled $CaCl_2$. Another part of the reaction mixture was diluted with the same buffer and identical concentration of unlabeled $CaCl_2$ and with the calcium ionophore A23187. The third part of the reaction mixture was diluted 10-fold into identical concentration of $[Na]_{out}$ as the $[Na]_{in}$ containing the same concentration of unlabeled $CaCl_2$. As shown in fig.2; once calcium is taken up into the vesicles in the sodium-dependent fashion and reaches a steady state it is not released spontaneously, nor does it undergo spontaneous exchange with externally added unlabeled Ca^{2+} at the same concentration. Only the

Table 1
The effect of the sodium gradient on the calcium uptake by membrane vesicles

$[Medium]_{in}$	$[Medium]_{out}$	Calcium uptake (pmol/mg protein/ 5 min)
0.1 M Na^+	0.1 M K^+	469.75
0.05 M[a] Na^+	0.1 M K^+	356.51
0.01 M[a] Na^+	0.1 M K^+	257.81
0.1 M Na^+	0.1 M Na^+	40.2
0.1 M Na^+	0.1 M K^+	178.5
	10 μM nigericin	
0.1 M Na^+	0.1 M K^+	243.5
	0.3 mM gramicidin	

[a] The osmolarity was balanced by mannitol

$[Medium]_{in}$ contained: 0.1 M Na-phosphate buffer (pH 7.4), 0.005 M $MgCl_2$. $[Medium]_{out}$ contained: 0.1 M K-phosphate buffer (pH 7.4), 0.005 M $MgCl_2$. $^{45}CaCl_2$ (2 μM) and additions as specified. Preloaded membrane vesicles (10 μl) containing about 100 μg protein were diluted into 0.1 ml $[medium]_{out}$

calcium ionophore A23187 was capable of releasing the calcium taken up by the vesicles.

The dependency of the calcium uptake on the sodium gradient is shown in table 1. It can be seen that the amount of calcium taken up by the vesicles is related to the preformed sodium gradient. As the gradient decreases either by lowering the concentration of $[Na]_{in}$ or by adding nigericin or gramicidin the amount of calcium taken up by the vesicles decreases as well. In the absence of sodium gradient when the concentration of $[Na]_{in} = [Na]_{out}$ no calcium is taken up into the membrane vesicle.

Calcium-dependent sodium fluxes were shown by trapping 0.1 M $CaCl_2$ inside the membrane vesicles. This was done by adding $CaCl_2$ to the lysing solution of the synaptosomes prior to the sealing of the membrane vesicles. These calcium containing membrane vesicles take up $^{22}NaCl$ from external medium more than control vesicles containing 0.3 M mannitol (fig.3). The initial rate of sodium uptake is much faster in calcium preloaded vesicles than in control vesicles: 90 nmol sodium/mg protein/30 s and 14.6 nmol/mg protein/30 s, respectively. After the initial phase, control vesicles continue taking up calcium at approximately the same rate while calcium preloaded vesicles reach a steady state.

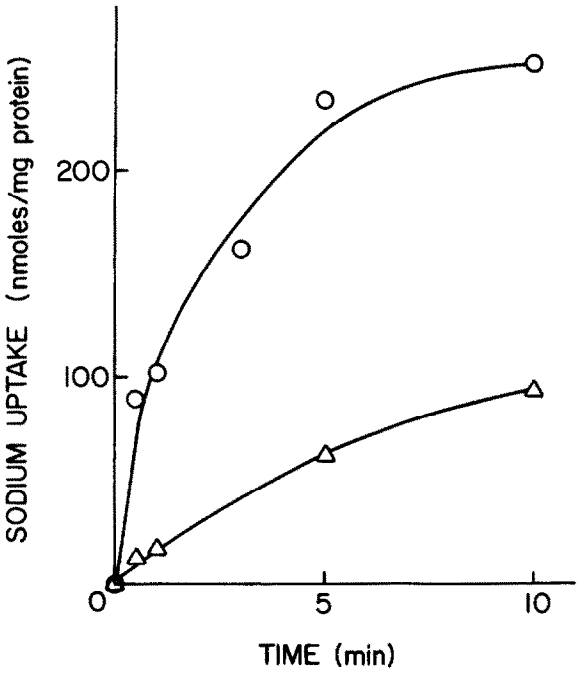

Fig.3. Calcium-dependent sodium uptake by membrane vesicles. Membrane vesicles preloaded with 0.1 M $CaCl_2$ (○–○–○) or 0.3 M mannitol (△–△–△) were suspended in a medium containing 0.1 M $^{22}NaCl$, 5 mM $MgCl_2$ and 10 mM Tris–HCl buffer (pH 7.4). $^{22}NaCl$ uptake was measured as a function of time. For each time point ~100 μg vesicle protein in 10 μl were added to 0.1 ml uptake medium.

4. Discussion

Membrane vesicles have been used successfully in many bacterial and eukaryotic systems to study transport phenomena [8]. They offer closed membraneous structures in which the internal and external composition of the desired media can be manipulated. In this work we used membrane vesicles to study the sodium-dependent calcium transport. This type of transport has been proposed in many excitable membranes to be a major mechanism participating in the regulation of intracellular calcium ion concentration [9]. Calcium efflux from squid axons is largely dependent on the presence of sodium in the extra-axonal medium, both in normal and in cyanide-poisoned preparations [10]. The Ca–Na exchange process is not sensitive to ouabain or tetrodotoxin, thus it does not appear to be mediated by Na-K-ATPase or voltage-sensitive sodium channels [9].

We do not know at present the subcellular localization of the sodium-dependent calcium transporting system. One obvious location is the plasma membrane; another possibility is the mitochondrial membrane, since it has been shown that relatively low extramitochondrial sodium concentrations can induce calcium efflux from mitochondria [11]. An indication that at least part of the sodium-dependent calcium uptake system resides in the plasma membrane comes from experiments that show the parallel location of ouabain inhibited ATP hydrolysis, sodium-dependent GABA (γ-amino butyric acid) uptake and the specific activity of the sodium-dependent calcium uptake (in preparation). Membrane vesicles prepared from rat brain synaptosomes, like intact nerve terminals do not take up significant amounts of calcium. Preloading them with sodium containing buffered media causes a rapid influx of calcium. Calcium uptake into the vesicles is strongly dependent on the presence of sodium in the vesicles, since preloading them with the potassium containing buffers does not lead to calcium influx. The amount of calcium taken up by the sodium containing vesicles is directly proportional to the extent of the sodium gradient across the vesicular membrane. When the sodium gradient across the vesicular membrane is decreased either by lowering $[Na]_{in}$ or by adding sodium ionophores like nigericin or gramicidin, the amount of calcium taken up by the membrane vesicles is smaller.

The calcium taken up by the vesicles in the sodium-dependent fashion is not released spontaneously even after 60 min. It neither undergoes an exchange with externally added calcium at the same concentration. It can be released however from the vesicles by adding the calcium ionophore A23187 to the extravesicular media.

Calcium-dependent sodium uptake can also be demonstrated in the membrane vesicles. Sodium is taken up by the vesicles also in the absence of calcium in the intra vesicular medium. However, trapping calcium within the membrane vesicles during their preparation causes a 6-fold increase in the initial rate of sodium uptake. The present system seems to be very advantageous for studying the molecular properties of sodium-dependent calcium transport and calcium-dependent sodium transport. In order to derive the exact stochiometry of the Na–Ca exchange however, further purification of the system is required.

Acknowledgements

We would like to thank Drs B. Kanner and R. Rahamimoff for many helpful discussions and reading the manuscript. The research was supported in part by a research grant from the Israel Center for Psychobiology.

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