Biochimica et Biophysica Acta, 1028 (1990) 21–24
Elsevier

BBAMEM 74975

Endocytic vesicles contain a calmodulin-activated Ca^{2+} pump that mediates the inhibition of acidification by calcium

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(Received 22 January 1990)
(Revised manuscript received 7 June 1990)

Key words: Calcium ion; Calmodulin; Endocytic vesicle; Acidification; pH; Calcium pump

Endocytic vesicles isolated from rabbit reticulocytes contained an intrinsic Ca^{2+}-pump activity that was dependent on ATP, activated by calmodulin and inhibited by vanadate. $^{45}Ca^{2+}$ uptake and acidification studies indicated that acidification of the endocytic vesicles inversely correlated with the Ca^{2+}-pump activity. Acidification was inhibited by externally added Ca^{2+} and calmodulin and activated by vanadate and EGTA. It is suggested that intravesicular Ca^{2+} can act as a modulator of endocytic vesicle acidification.

Introduction

Acidification of the endosome, brought about by a NEM-sensitive, electrogenic H^+-pump, is necessary for the normal routing of ligands and receptors internalized by endocytosis [1–5]. During our studies on vesicle acidification we noted that acidification was inhibited by extravesicular calcium [6]. Since inhibition of the acidification of endocytic vesicles has a profound effect in the cellular physiology of these vesicles, being the most notorious the stoppage of intracellular vesicle traffic [7–9], we decided to study the possible interactions of Ca^{2+} with the acidification process. We found that the endocytic vesicles have an intrinsic Ca^{2+} pump and that vesicle acidification correlated inversely to Ca^{2+}-pump activity. The above data indicate a possible inhibitory effect of intravesicular Ca^{2+} on the acidification process of endocytic vesicles.

Methods

Vesicle preparation. Endocytic vesicles were prepared from rabbit reticulocytes by a modification of the method of Choe et al. [5] as described in Ref. 10. The composition of the buffer used during the overall preparation was (mM): 50 NaCl, 50 KCl, 1 $MgSO_4$, 10 Hepes-Tris (pH 7.0). This buffer will be referred hereon as preparation buffer. When vesicles were to be used in measurements of intravesicular acidification, the cells were incubated for 5 min at 37 °C with 4 μM FITC-labeled transferrin prior to vesicle preparation (see below).

$^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ uptake was measured at 25 °C. The reaction was started with the joint addition of 100 μM (10 μCi) $^{45}CaCl_2$ and 2 mM ATP-Mg to 50 μg of vesicle suspension in preparation buffer. Reaction was stopped by quick filtration through Millipore GSWP 0.22 μm filters pre-treated with 100 μM $CaCl_2$, 5 mM $MgCl_2$, and 10 mM Hepes-Tris (pH 7.0) [11]. In some experiments the endocytic vesicles were stripped of calmodulin as described [11].

Density shift. For density shift experiments, 50 μg of ^{125}I-transferrin-containing vesicles were pre-equilibrated in 1 ml of preparation buffer containing 5 mM potassium oxalate. Reaction was started with the addition of 100 μM $CaCl_2$, 3.0 μg/ml calmodulin and 2 mM ATP-Mg. In controls calmodulin was replaced by 100 μM vanadate. After incubation for 3 h at 37 °C the mixture was cooled and loaded onto 8 ml of 27% Percoll in 10 mM Hepes-Tris (pH 7.0), 0.25 M sucrose, and was then centrifuged for 30 min at $54000 \times g_{av.}$ in a Beckman type 40 rotor. The gradient was collected from the bottom, and the ^{125}I radioactivity of the fractions was determined in a gamma counter.

Labeling of transferrin. Purified rabbit transferrin [12] was labeled with ^{125}I by the iodine monochloride method

Abbreviations: NEM, *N*-ethylmaleimide; FITC, fluorescein isothiocyanate; FCCP, carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone.

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[13] and then saturated with iron as described [14]. The ^{125}I-labeled ferrotransferrin was reacted with FITC as described by Musgrove et al. [15]. The FITC/transferrin molar ratio was between 4 and 5.

Measurement of vesicle acidification. The acidification of endocytic vesicles was determined at 25°C as the decrease in the fluorescence (excitation 490 and 450 nm; emission 520 nm) of 30–50 μg of FITC-transferrin-containing vesicles [10,16]. The reaction was started by the addition of 1 mM ATP-Mg (pH 7.0) and fluorescence was followed in a Perkin-Elmer MPF-2A instrument with the detection chamber thermostated at 25°C.

Free calcium concentration. The concentration of free Ca^{2+} in the presence of ATP, Mg^{2+}, and EGTA was calculated using a computer program according to Goldstein [17].

Reagents. $^{45}CaCl_2$ (32.33 mCi/mg) was from Dupont (Wilmington, DE). Carrier-free $Na^{125}I$ was from Comision Chilena de Energia Nuclear (Santiago, Chile). FITC, isomer I, and the ionophore A23187 were from Calbiochem (San Diego, CA). Sodium orthovanadate was from Fisher Sci. Co. (Orangeburg, NY). Percoll, calmodulin, ATP, and other biochemical reagents used were from Sigma Chemical Co. (St. Louis, MO).

Results

$^{45}Ca^{2+}$ uptake by endocytic vesicles

$^{45}Ca^{2+}$ uptake experiments demonstrated an ATP-dependent Ca^{2+} pump in reticulocyte vesicles (Fig. 1).

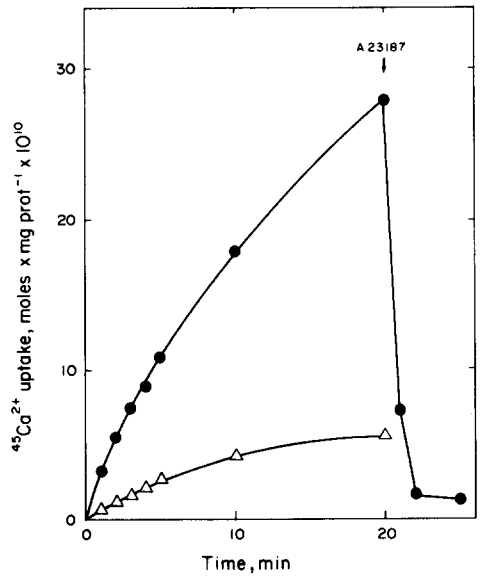

Fig. 1. ^{45}Ca uptake by endocytic vesicles. Vesicles were incubated with $^{14}CaCl_2$ as described in Methods, with (●), or without (△) 2 mM ATP-Mg. At 20 min, as indicated by the arrow, 5 μM A23187 was added to the incubation mixture. Results are expressed as Ca^{2+} incorporated into the vesicles as a function of time. Shown is a representative of four experiments.

TABLE I

Calcium uptake in endocytic vesicles

Vesicles were incubated for 5 min at 25°C under the conditions described in Methods. Calmodulin was added at a concentration of 6 μg/ml and orthovanadate at 100 μM. Values are mean ± S.D. of three experiments.

Condition	$^{45}Ca^{2+}$ uptake (10^{-10} moles/5 min per mg protein)	% of control
Control	12.5 ± 1.6	100
No ATP	1.7 ± 0.7	14
Orthovanadate	3.6 ± 1.0	29
Calmodulin	18.8 ± 1.2	150
Stripped vesicles	12.9 ± 2.4 *	103 *
Stripped vesicles + calmodulin	17.8 ± 1.7 *	142 *

* Activities should be halved if expressed per mg of original vesicle protein. See text for details.

That $^{45}Ca^{2+}$ was being accumulated in the intra-vesicular space and not merely bound to the vesicle membrane was demonstrated by the effect of the ionophore A23187, which quickly depleted the vesicle of the accumulated $^{45}Ca^{2+}$.

The Ca^{2+} pump was inhibited by vanadate and activated by calmodulin (Table I). Stripping of the vesicles reduced the pump activity, that could be partially regained adding calmodulin. Using vesicles that

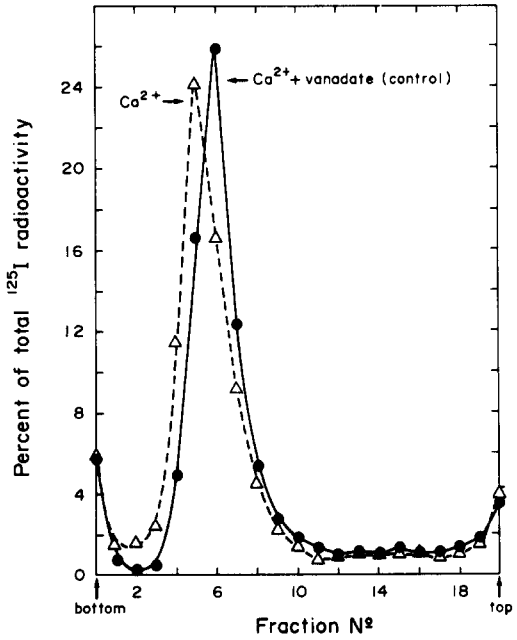

Fig. 2. Density shift after Ca^{2+} loading of endocytic vesicles. Vesicles were loaded with calcium oxalate as described in Methods and the increased density of the loaded vesicles was detected in a 27% Percoll, 0.25 M sucrose density gradient. Gradients were collected from the bottom in fractions of about 0.4 ml. Densities were calculated from density bead standards (Pharmacia) centrifuged in parallel tubes under identical conditions.

contained ^{125}I-labeled transferrin as an internal label, we noticed that the stripping process resulted in the loss of about one half the protein associated with the vesicles: after the stripping process 85% of the transferrin and 41% of the total vesicle associated protein was recovered. Therefore, the specific Ca^{2+}-pumping activity of the stripped vesicles was about $6.4 \cdot 10^{-10}$ and that of stripped plus calmodulin $8.9 \cdot 10^{-10}$ moles/5 min per mg protein when expressed per initial vesicle protein.

To demonstrate that the Ca^{2+} pump was associated with the endocytic vesicles and not with a contaminating structure, density shift experiments were performed by incubation of the vesicles loaded with oxalate in the presence of Ca^{2+} and ATP [19]. The density shift was detected in Percoll gradients using ^{125}I-transferrin as a label for the endocytic vesicles (Fig. 2). After incubation, the transferrin-associated radioactivity shifted to a density of 1.086 ± 0.001 compared with a density of 1.079 ± 0.002 for the control vesicles incubated with 0.1 mM vanadate, and 1.072 ± 0.001 for the non-incubated vesicles. This shift demonstrated that the Ca^{2+} pump was an intrinsic component of the endocytic vesicle. Values are mean $\pm$ S.D. from five experiments with incubated vesicles and three experiments with non-incubated vesicles.

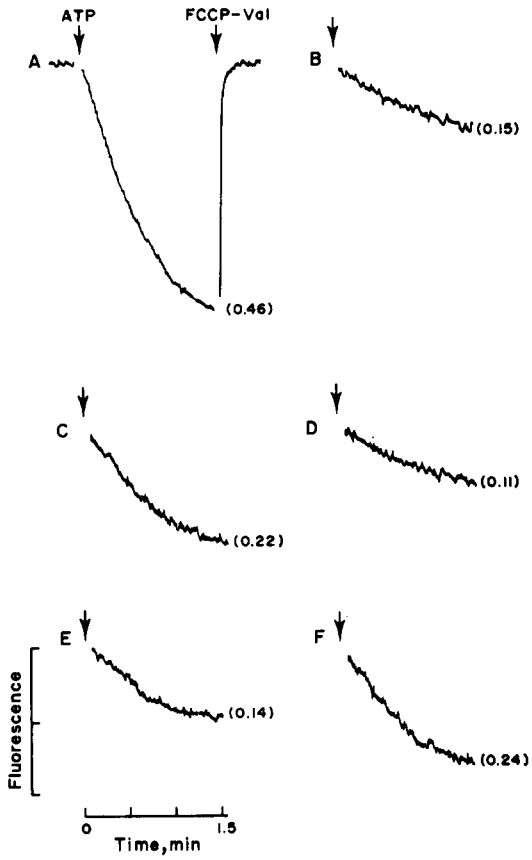

Fig. 3. Acidification by endocytic vesicles. Acidification was determined by the quenching of fluorescence in FITC-transferrin-containing vesicles as detailed in Methods. The intravesicular decrease in pH 1.5 min after the addition of ATP is indicated in parenthesis at the end of each curve. (A) Vesicles prepared with 1 mM EGTA in the preparation buffer; (B) vesicles prepared without EGTA in the preparation buffer; (C) as in (B) but with 1 mM EGTA added to the incubation medium; (D) as in (C) but with 10 μM free Ca^{2+} and 6 μg/ml of calmodulin added; (E) as in (C) with 10 μM free Ca^{2+} added; (F) as in (E) but with 100 μM sodium orthovanadate added.

Vesicle acidification

The effect of Ca^{2+} and some effectors of the Ca^{2+} pump on the kinetics of vesicle acidification is shown in Fig. 3. In the experiment shown, vesicles prepared in a buffer that contained 1 mM EGTA produced, after the addition of ATP, a decrease in fluorescence corresponding to an intravesicular acidification of 0.46 pH units in 1.5 min (Fig. 3A). The addition of 0.5 μM FCCP–0.5 μM valinomycin reversed this acidification. In contrast, vesicles prepared in the buffer without EGTA had a decreased capacity of acidification (Fig. 3B). The acidification capacity of the vesicles prepared without EGTA could be partially restored by adding EGTA to the incubation medium (Fig. 3C), and this reactivation was abolished by adding either Ca^{2+} and calmodulin (Fig. 3D) or Ca^{2+} (Fig. 3E) to the incubation medium with EGTA. In addition, acidification was further activated by vanadate even in the presence of Ca^{2+} (Fig. 3F). Vanadate per se has not effect in the acidification system [2–4]. The inhibitory effect of Ca^{2+} was studied in the concentration range 1–100 μM free Ca^{2+}, and it was found similar to that described in Fig. 3D. Additionally, acidification of vesicles prepared with buffers without EGTA and with 10 μM Ca^{2+} in the external medium, similar to conditions in Fig. 3E, could be enhanced by the addition of 2 μM A23187, suggesting a possible Ca^{2+} (in > out) concentration gradient (results not shown).

Discussion

Blitz et al. [18] reported the presence of a Ca^{2+} pump in endocytic vesicles derived from bovine brain, but a later report by Rubenstein et al. [19] ascribed this, and other ATPase activities, to contaminant smooth vesicles. A high cell Ca^{2+} content has been observed in red blood cells of patients with sickle cell anemia [20], beta-thalassemia [21], and hemoglobin C disease [22]. In these instances the excess cellular Ca^{2+} has been shown to be present in structures similar to endocytic vesicles [21,23].

The present study has shown that most or all the endocytic vesicles derived from rabbit reticulocyte contained a Ca^{2+} pump: in density shift experiments we observed that when incubated with calcium, oxalate, and ATP, all of the ^{125}I-transferrin labeled vesicles migrated to a higher density. These results are in apparent contradiction with those of Rubenstein et al. [19]. The contradiction can be explained if, during the

process of vesicle purification by agarose gel electrophoresis carried out by these authors, the Ca^{2+} pump was inactivated. This inactivation could be due in part to the loss of calmodulin, a factor that we showed could increase the Ca^{2+}-pump activity.

Some preliminary conclusions can be drawn in reference to the mechanisms underlying the observed inhibition by Ca^{2+}. It is unlikely that the decrease in acidification derives from the H^+ dissipation mediated by the Ca^{2+}/H^+ exchange postulated for the Ca^{2+}-ATPase [24], since the Ca^{2+}-pumping activity is a fraction of the H^+-pumping activity, estimated by direct H^+ flux measurement as $(2.09 \pm 0.45) \cdot 10^{-8}$ moles/min per mg protein [25]. Alternatively, the observed decrease in acidification could be mediated by the Ca^{2+}/H^+ antiport. The presence of a Ca^{2+}/H^+ exchanger in renal cortical endosomes has been recently suggested [26], although more experimental evidence is needed for a definitive conclusion.

The fact that acidification can be only partially restored by the external addition of EGTA, and that acidification correlates inversely with the Ca^{2+}-pumping activity, is an indication that the inhibitory entity could be the free intravesicular Ca^{2+} concentration.

The function of the Ca^{2+} pump in endocytic vesicles remains speculative. If the observation that Ca^{2+} inhibits acidification holds for intracellular conditions, then the Ca^{2+} pump-mediated system described here would have a regulatory role in the acidification process, and therefore on the intracellular routing of ligand and receptors. Alternatively, under conditions of high concentrations of intracellular Ca^{2+} the endocytic vesicles could act as Ca^{2+} reservoirs [18], contributing to maintain low intracellular calcium levels.

Acknowledgements

This work was sponsored by Grants from Universidad de Chile (D.T.I. 2200), FONDECYT Chile (1003-88), and National Science Foundation, U.S.A. (8715381). We thanks Drs. Cecilia Hidalgo and Ramón Latorre for helpful comments on the manuscript.

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