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PLASMA MEMBRANE Ca^{2+} TRANSPORT: STIMULATION BY SOLUBLE PROTEINS

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SUMMARY

Inside-out membrane vesicles were prepared from human red blood cells. In the presence of ATP, these vesicles took up $^{45}Ca^{2+}$ against a chemical gradient. The active transport of Ca^{2+} was increased by addition of an activator protein of $(Ca^{2+}+Mg^{2+})$-ATPase isolated from the membrane-free hemolysate of human red blood cells. A closely related protein, the protein modulator of cyclic AMP phosphodiesterase from bovine brain, also increased the rate of active transport of $^{45}Ca^{2+}$. Addition of the calcium ionophore A23187 caused a rapid efflux of $^{45}Ca^{2+}$ from loaded, inside-out vesicles. When La^{3+} was added to the system in the presence of activator protein, the uptake of $^{45}Ca^{2+}$ was inhibited. Results are compatible with the interpretation that activity of the plasma membrane Ca^{2+} pump may be modulated by certain cytoplasmic proteins.

The human red blood cell (RBC) contains an enzyme system that is responsible for the maintenance of low intracellular Ca^{2+} concentrations (1, 2). Membranes made from RBCs contain $(Ca^{2+}+Mg^{2+})$-ATPase activity (3, 4). This activity is thought to be an expression of the Ca^{2+} active transport system in whole cells (1). The $(Ca^{2+}+Mg^{2+})$-ATPase activity of membranes can be enhanced (activated) by the addition of dialyzed membrane-free RBC hemolysate (5). It was shown that this activation is due to a small acidic protein which can be isolated from RBC hemolysates (6). Because of its reversible binding to the membrane and the specificity of its activation of $(Ca^{2+}+Mg^{2+})$-ATPase, it was postulated that the cytoplasmic activator protein functions as a regulator of the plasma membrane Ca^{2+} pump (7). It may be noted that the hemolysate of RBCs enhanced the active transport of Ca^{2+} into inside-out (IO) vesicles of RBC membranes (8); presumably because of the presence of activator protein. The ATPase activation property of the RBC activator protein ("activator") is mimicked by the soluble Ca^{2+} binding protein known as a modulator of cyclic AMP phosphodiesterase (PDE) (9, 10) ("modulator"). In this paper we demonstrate that both the purified $(Ca^{2+}+Mg^{2+})$-ATPase activator from RBC and the PDE protein modulator from bovine brain stimulate active transport of Ca^{2+} into IO vesicles.

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METHODS

IO membrane vesicles from human RBCs were made by the method of Steck (11) as modified by Blostein and Chu (12). The purified IO vesicles were stored in 40 mM Tris-glycylglycine buffer, pH 7.4 containing 0.1 mM $MgCl_2$ at a concentration of approximately 5 mg protein/ml. The vesicles were used within two days. Membrane protein was measured by a modification of the method first described by Lowry et al. (13). The membranes were initially dissolved in sodium dodecyl sulfate (SDS) (0.77% final concentration) before dilution with water to obtain the proper protein range, and bovine serum albumin standards contained 17 mg% SDS.

Ca^{2+} uptake experiments were performed at room temperature in a final volume of 1.675 ml. The standard reaction medium contained 23 mM Tris-glycylglycine (pH 7.4), 0.9 mM $MgCl_2$, 90 µM $CaCl_2$ ($^{45}CaCl_2$ specific activity, 3.691×10^5 cpm/µmole), 0.9 mM ATP, 7.5 mM NaCl, and about 1.2 mg IO vesicles. When ATP was omitted, an equivalent amount of NaCl was substituted. Activator and modulator proteins were made up in NaCl such that when added yielded a NaCl concentration of 7.5 mM. The reaction mixture was constantly stirred in a beaker insulated from the magnetic stirrer with a styrofoam pad. No appreciable temperature drift could be detected. The vesicles were preincubated for 30 min. before the reaction was initiated by the addition of ATP. At specified times, 100 µl samples were removed and quenched in 1.5 ml of ice cold 30 mM Tris-glycylglycine (pH 7.4) with 0.1 mM $MgCl_2$. The IO vesicles were rapidly collected by vacuum filtration on an Amicon microporous filter (25 mm Dia., 0.45 µm) and washed once with 1.5 ml of the same buffer. The filters were placed in 10 ml of Aquasol^R (N.E.F.-934) which completely dissolved the filters within 20 min. at room temperature. Samples were counted in a Beckman LS-9000 with window settings of 100-720. The samples showed good counting efficiency with constant quench of 30 H units. Each vial was counted twice for 10 min. Ca^{2+} standard solutions were counted using the same window settings. The (Ca^{2+}+Mg^{2+})-ATPase protein activator was isolated from RBC hemolysate and purified by the method of Jung et al. (14). Protein modulator was supplied by Dr. T. Vanaman of Duke University.

The calcium ionophore A23187 was 0.1 mM in absolute ethanol. The sodium ionophore gramicidin was 20 µM in absolute ethanol and $LaCl_3$ was 10 mM. $^{45}CaCl_2$ was obtained from ICN Chemical and Radioisotope Division, specific activity of 17.7 Ci/gm. ATP and gramicidin were purchased from Sigma and A23187 was a gift from Eli Lilly and Co.

RESULTS AND DISCUSSION

In confirmation of others (8, 15) we found that IO vesicles are capable of taking up $^{45}Ca^{2+}$ when ATP is present. As shown in Figure 1 (curve C), when ATP was included in the reaction medium, there was a time-dependent increase in $^{45}Ca^{2+}$ associated with the vesicles. When ATP was omitted (curve D) only a small amount of $^{45}Ca^{2+}$ was present in (on) the vesicles. IO vesicles are "tight" to Ca^{2+} over a 90 min. reaction time as demonstrated by the low and constant amount of $^{45}Ca^{2+}$ associated with the vesicles. When ATP was added to these vesicles at 90 min., there was an increase in $^{45}Ca^{2+}$ uptake. Curve C represents uptake of $^{45}Ca^{2+}$ into IO vesicles with ATP but without activator. These vesicles pumped at a rate of 7.36 nmole Ca^{2+}·mg protein^{-1}·hr^{-1}. When purified cytoplasmic activator protein of

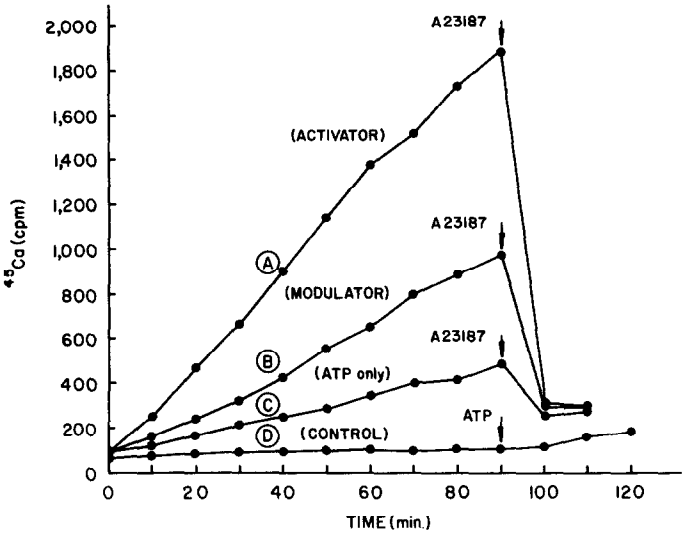

Figure 1. Uptake of $^{45}Ca^{2+}$ into IO vesicles of RBC membrane. All vesicles were preincubated for 30 minutes (see Methods) and uptake was initiated by addition of ATP (except control). Each point represents $^{45}Ca^{2+}$ associated with vesicles in 100 µl of incubation medium removed at the specified time. Curve A: uptake with added activator protein (0.94 µg/ml); Curve B: uptake with added modulator protein (0.60 µg/ml); Curve C: uptake with standard buffer and ATP, no added proteins; Curve D: control uptake without ATP. At 90 minutes, ATP was added to vesicles in Curve D. At 90 minutes, A23187 (3.6×10^{-6} M, final concentration) was added to vesicles in Curves A, B and C.

$(Ca^{2+}+Mg^{2+})$-ATPase was also present (curve A) IO vesicles pumped at a rate of 37.3 nmoles $Ca^{2+}\cdot mg\ protein^{-1}\cdot hr^{-1}$. This represents a 4.1 fold increase in the apparent rate of transport of Ca^{2+}. Activator protein can bind to the RBC membrane and simultaneously increase the activity of $(Ca^{2+}+Mg^{2+})$-ATPase (7). This is presumably the mechanism by which activator increases Ca^{2+} uptake into IO vesicles.

IO vesicles rapidly lost $^{45}Ca^{2+}$ when A23187 (3.6×10^{-6} M, final concentration) was added, as seen in curves A, B and C. When gramicidin was added, there was no effect (data not shown).

An estimate of the vesicular volume was made. The number of counts remaining with the vesicles after treatment with A23187 was assumed to represent the sum of Ca^{2+} inside plus Ca^{2+} bound to the external surface. We assume that, in the presence of ionophore, the free concentration of Ca^{2+} is equal inside and outside. From the difference between ionophore treated vesicles and the background binding of $^{45}Ca^{2+}$ in the absence of

ATP and ionophore, we calculate that the IO vesicles shown in Figure 1 have a "calcium-ionophore accessible volume" of 59 $\mu l \cdot mg\ protein^{-1}$. This is probably an overestimate of vesicular volume since we do not account for any binding inside the vesicles. Assuming an intravesicular volume of 59 $\mu l \cdot mg\ protein^{-1}$, we estimate that in the presence of activator (curve A), these vesicles accumulate Ca^{2+} to a level of at least 0.92 mM at the end of 90 min. This estimate, and the rapid efflux of $^{45}Ca^{2+}$ in the presence of A23187, indicates that Ca^{2+} was pumped against a concentration (and presumably electrochemical) gradient.

When purified protein modulator of cyclic AMP phosphodiesterase was present (0.60 µg/ml) along with ATP (curve B), the rate of $^{45}Ca^{2+}$ uptake was 17.5 nmoles $Ca^{2+} \cdot mg\ protein^{-1} \cdot hr^{-1}$. Under these conditions, modulator thus increased the pump rate by 1.4 fold (compare curves B and C). At the end of 90 min., vesicles accumulated an estimated 0.44 mM Ca^{2+} in the presence of modulator. The unstimulated vesicles accumulated Ca^{2+} to an estimated level of 0.19 mM in the same time period.

Lanthanum is an effective inhibitor of active Ca^{2+} transport (16-18). Therefore, its effect upon activator-stimulated $^{45}Ca^{2+}$ uptake was examined. Figure 2 shows that Ca^{2+} transport into IO vesicles was rapidly inhibited when La^{3+} was added to the reaction medium in a concentration of 0.15 mM (curves E and F). Curve A is the same as in Figure 1 and is used as a point of reference. It is clear from this experiment also that IO vesicles are impermeable to Ca^{2+} in the absence of substrate (curve F); i.e., no diffusion into the vesicles occurred and there was no (or little) outward diffusion from loaded vesicles when transport was arrested (curves E and F).

Data from four different IO vesicle preparations are presented in Table 1. The calcium ionophore accessible volume (see above) ranges from 30.2 to 59.0 $\mu l \cdot mg\ protein^{-1}$. The IO vesicles of Perrone and Blostein (19) had a water permeable space of 35.9 to 36.9 $\mu l \cdot mg\ protein^{-1}$ (whereas their right-side out (RO) vesicles had a water permeable volume in the range of 88.3 to 90.4 $\mu l \cdot mg\ protein^{-1}$). Using the calcium ionophore accessible volume calculated for each preparation, we have estimated the minimum intravesicular Ca^{2+} concentration as above. For example, in experiment 3, at the end of three hours, the intravesicular Ca^{2+} concentration reached 1.1 mM. This corresponds to a concentration gradient of 16.6 (Ca^{2+} inside/Ca^{2+} outside) at the termination of the experiment. From the quantitative variability in the data, it is obvious that our technique for the preparation of IO vesicles needs improvement, and this is underway in our laboratory. Nevertheless, the fundamental qualitative information is unmistakable. Only sealed IO vesicles will take up and concentrate Ca^{2+}, since the Ca^{2+} pump

TABLE 1

Inside-out Vesicle Characteristics and Pump Activities

Exp. #	Calcium-Ionophore Accessible Volume ($\mu l \cdot mg\ protein^{-1}$)	Ca^{2+} Pump Rate (nmoles $Ca^{2+} \cdot mg\ protein^{-1} \cdot hr^{-1}$) Basal Rate	Activated Rate*	Fold Activation	Calculated Intravesicular $[Ca^{2+}]$ at Time Indicated (mM)
1	-	5.48	47.7 (A)	7.7	-
2	59.0	7.36	37.3 (A)	4.1	0.92 (1.5 hr)
			17.5 (M)	1.4	0.44 (1.5 hr)
3	34.7		12.0 (A)		1.10 (3.0 hr)
4	30.2	1.42	6.40 (A)	3.5	0.25 (2.5 hr)
			2.04 (M)	0.44	

*Activator (A) at a final concentration of 0.94 $\mu g \cdot ml^{-1}$. Modulator (M) at a final concentration of 0.60 $\mu g \cdot ml^{-1}$ in experiment 2 and 0.99 $\mu g \cdot ml$ in experiment 4.

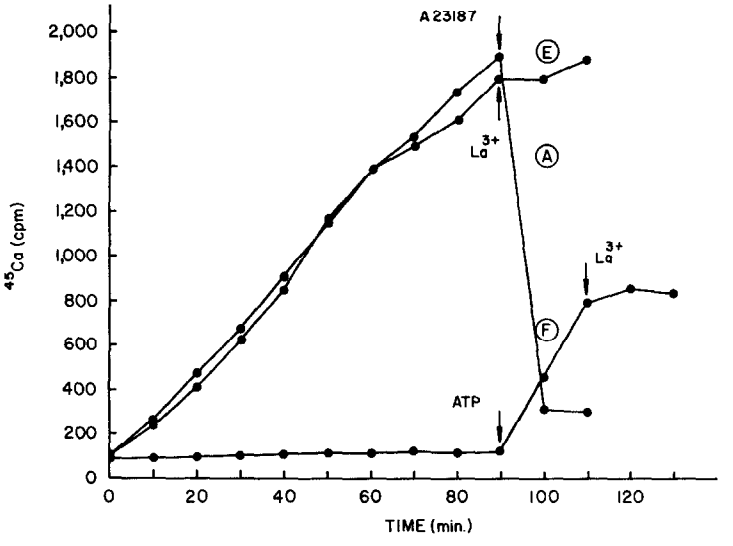

Figure 2. Uptake of $^{45}Ca^{2+}$ into IO vesicles of RBC membrane in the presence of activator protein. Data as in Figure 1. All media contained activator (0.94 $\mu g/ml$). Curve A: same data as in Figure 1; Curve E: addition of $LaCl_3$ (0.15 mM) at 90 min; Curve F: ATP free control, addition of ATP at 90 min and $LaCl_3$ (0.15 mM) at 110 min.

is unidirectional (2). Even if our preparations of IO vesicles are contaminated with broken membrane fragments and RO vesicles, only the IO vesicles should be capable of active Ca^{2+} uptake. Activator can activate the (Ca^{2+}+Mg^{2+})-ATPase (and thus, presumably the Ca^{2+} pump) only when it is present on the cytosolic side of the membrane (20). Therefore, only the IO vesicles would be capable of being activated.

We also found an increase in Ca^{2+} pump activity when PDE modulator was included in the reaction medium. We did not find the same degree of activation with the two proteins. Activator and modulator each maximally activate the (Ca^{2+}+Mg^{2+})-ATPase at similar concentrations (9). In order to clarify this apparent discrepancy, more concentration-effect data will be needed. The specificity of the proteins we tested was not demonstrated, but would be anticipated on the basis of ATPase data (5).

In conclusion, we were capable of measuring the apparently active uptake of Ca^{2+} by IO vesicles. We found that the Ca^{2+} pump would only function when substrate was available. It is not known whether our vesicles contain some bound activator or whether the "basal" uptake of Ca^{2+} represents an "idling" pump. More work on this question is needed. When purified activator or modulator protein was added, the rate of Ca^{2+} accumulation increased above basal. La^{3+} produced rapid inhibition of Ca^{2+} transport in the presence of activator. Active transport is generally assumed to be a function of intrinsic membrane-bound proteins. The present results are compatible with the interpretation that activity of the plasma membrane Ca^{2+} pump may be modulated by soluble cytoplasmic proteins.

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