Biochimica et Biophysica Acta, 643 (1981) 421—434
Elsevier/North-Holland Biomedical Press

BBA 79229

ACTIVE Ca^{2+} TRANSPORT BY MEMBRANE VESICLES FROM PIGEON ERYTHROCYTES

STIMULATION BY AMINO ACIDS, ATP, GTP, P_i AND SOME OTHER CELL CONSTITUENTS

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(Received November 3rd, 1980)

Key words: Ca^{2+} transport; Amino acid stimulation; Nucleotide stimulation; (Pigeon erythrocyte membrane)

Summary

Pretreatment of pigeon erythrocyte membrane vesicles with amino acids, ATP, GTP, P_i and some other simple cell constituents (singly and in combination) causes an increase in ATP-dependent Ca^{2+}-uptake activity of vesicles upon subsequent incubation with $^{45}Ca^{2+}$ after removal of the above agents from the 'i' face. Amino acids augment the stimulation by all stimulatory agents and are required for stimulation by P_i. The effects of amino acids, ATP, GTP and P_i all occur at physiological concentrations. Many if not all of the effects of the mixture of amino acids that occur naturally in the cells can be accounted for by the group transported by the 'ASC' transport system of Christensen (Christensen, H.N. (1975) Biological Transport, 2nd edn., W.A. Benjamin, Inc., Reading, MA), but not by any single amino acid at its physiological concentration. The effects of ATP and GTP are not mimicked by their non-hydrolysable β,γ-imido analogues nor by the corresponding 3′,5′-cyclic monophosphates. None of the effects described appears to involve calmodulin. We suggest that amino acid transport plays a role in metabolic regulation through effects on cell $[Ca^{2+}]$. Analogous effects on cell $[Ca^{2+}]$ may be involved in the action of the many hormones which augment amino acid accumulation by the 'A' amino acid transport system.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; GMPPNP, guanosine 5′-[β,γ-imido]triphosphate tetrasodium salt; AMPPNP, adenosine 5′-[β,γ-imido]triphosphate; NNP, any one of ATP, GTP or phospho*enol*pyruvate.

0005-2736/81/0000—0000/$02.50

Intracellular free Ca^{2+} is presumed to be a 'second messenger' like cyclic AMP. It modulates a variety of biological processes and reactions [1]. The Ca^{2+} concentration is kept in the micromolar range by several Ca^{2+} transport processes, among which is prominent an active expulsion system at the cytoplasmic membrane [2]. This Ca^{2+} transport process is itself a target for regulation [3]. The most extensively studied regulation is that by calmodulin, a protein confering Ca^{2+} responsiveness on this Ca^{2+} transport process [4,5] as well as on a variety of other processes (Refs. 6—8 and references cites therein).

Most of the studies on Ca^{2+} transport by cytoplasmic membrane have used mammalian erythrocyte preparations; whole cells [3,9,10], hemolysed and restored cells [10,11] or membrane vesicles [12] have been used. We described Ca^{2+} transport by membrane vesicles from pigeon erythrocytes [13] with properties similar to those of the mammalian erythrocyte plasma membrane vesicle system. We believe these are fairly pure cytoplasmic membrane vesicles for reasons given earlier [13,14] which include their possession of a number of transport activities of whole cells. These membrane vesicles were prepared by sonication of erythrocytes in a cold isotonic medium. The vesicles were then 'sealed' (made impermeable) by annealing. We unexpectedly observed that a membrane vesicle preparation annealed with 154 mM potassium glutamate showed a subsequent Ca^{2+} transport activity 10-times greater than the usual. Studies were extended to other amino acids at various concentrations, added either singly or in groups corresponding to different amino acid transport systems. A mixture of amino acids at near physiological concentrations for intact pigeon erythrocytes stimulated Ca^{2+} transport 2-fold. Most of the stimulating effect could be accounted for by the amino acids transported by the ASC system. We also found stimulation of Ca^{2+} transport by ATP, GTP and phospho*enol*pyruvate. Stimulation by these agents was increased by amino acids and stimulation by P_i required amino acids. The results suggest that Ca^{2+} transport in vivo may be controlled by amino acids and phosphate compounds.

Materials and Methods

$^{45}CaCl_2$ and $NaB[^3H]H_4$ were obtained from ICN, Irvine, CA. [6,6′-^{3}H]-Sucrose was bought from Amersham/Searle, Arlington Heights, IL. [^{3}H]Maltitol (O-α-D-glucopyranosyl-(1 → 4)-β-D-[1-^{3}H]sorbitol) was prepared by reduction of maltose with $NaB[^3H]H_4$ [15] and purified by passage through a Sephadex G-15 column. GTP was obtained from Calbiochem-Behring, La Jolla, CA. GMPPNP was obtained from ICN. Other nucleotides, phospho*enol*pyruvate and amino acids were purchased from Sigma Chemicals, St. Louis, MO. The scintillation counting cocktail 3a70B was obtained from Research Products International, Elk Grove Village, IL. Inorganic chemicals were analytical reagent grade or better. All water was double deionized and glassware was rinsed with double-deionized water.

Membrane vesicle preparation

Membrane vesicles were prepared from pigeon erythrocytes by a previously described procedure [13,14] with slight modifications: The second sonication was in a solution containing 136 mM KCl, 2 mM EGTA, 1 mM $CaCl_2$, and 10

mM Tes at pH 7.6. After pelleting, the membrane fragments were resuspended in the same solution at either 5 or 15 ml/g wet wt., and 12 ml sonicated at setting 5 for two 45 s intervals. Membrane suspensions were annealed at 25°C for 30 min in the above sonication solution containing 4 mM $MgCl_2$ and adjusted to pH 8. At pH 8, the calculated free [Ca^{2+}] was 10^{-8} M [16]. When amino acids and/or nucleotides were added to the annealing solution, the amount of KCl was decreased to maintain isotonicity. [^{3}H]Sucrose or [^{3}H]maltitol was added to the annealing solution (33 μCi/ml, 0.1 mM) as a trapped-space marker. After annealing, the membrane preparation was stored overnight at 0°C, and reannealed the next day for 10 min at 25°C, chilled, diluted with 10 ml Tes buffer (144 mM KCl and 10 mM Tes, pH 7.6) and centrifuged for 30 min at 30 900 × *g*. The vesicle population contains both inside-out and right-side-out vesicles (see Discussion). Only the former should accumulate Ca^{2+}. Since vesicles are unsealed at the beginning of annealing, both faces are initially exposed to the agents but they are absent from the i face of the active vesicles during Ca^{2+} uptake except where indicated.

Measurement of Ca^{2+} transport

$^{45}Ca^{2+}$ uptake by membrane vesicles was measured by incubating vesicles (about 10 mg wet wt., 0.4 mg protein and 0.2 μl trapped-space per sample) for 5 min at 27°C in 1.0 ml of 145 mM KCl, 10 mM Tes, 0.1 mM $MgCl_2$, 1 mM EGTA, 0.87 mM $^{45}CaCl_2$ (0.526 μCi/ml) with or without 1 mM MgATP. The calculated free [Ca^{2+}] was 1 μM [16] and the pH was 6.9. We presume any pH gradient arising from the difference between annealing and incubation pH was rapidly dissipated by the erythrocyte anion-exchange porter. Where more than 10 mM amino acids were present during annealing, they were present during Ca^{2+} uptake, replacing some KCl. This was done to minimize diffusion potentials that might influence Ca^{2+} transport. For all other experiments, amino acids and other agents were present only during annealing. $^{45}Ca^{2+}$ uptake by vesicles annealed with 26 mM AA_t was similar whether or not 26 mM AA_t was present in the Ca^{2+} uptake media. The membrane suspensions were chilled, diluted with 9 ml Tes buffer and centrifuged for 30 min at 30 900 × *g*. Pellets were washed once with 6 ml Tes buffer, the tubes were drained and the insides wiped dry. Each pellet was transferred to a counting vial containing 7 ml of 3a70B by resuspending and rinsing three times with a total of 1 ml of 0.1% Triton X-100, 1 mM EGTA, 0.5 mM $CaCl_2$ and 1 mM sucrose or maltitol. Samples of annealing and incubation medium were counted. ATP-dependent Ca^{2+} uptake was calculated as nmol Ca^{2+} (with ATP − without ATP)/μl trapped-space of vesicles as before [13]. 'Basal' Ca^{2+} transport activity is the ATP-dependent Ca^{2+} uptake/μl space of membrane vesicles annealed without the addition of any amino acid or phosphate compound. Most of the data are presented as the ratio: (uptake/space)treated ÷ (uptake/space)basal, same experiment, averaged over several experiments. Note, however, that the effects reported are not due to changes in 'space' values.

Amino acid groups and concentrations

The compositions of amino acid mixtures are shown in the following lists. These values were derived from the pigeon erythrocyte amino acid concentra-

tions reported by Eavenson and Christensen [17]. Amino acids were present in the same proportions, but at a slightly higher concentration (26 as against 16 mM total amino acids) than those reported [17]. Classification of amino acids into ASC and L transport groups was according to the method of Christensen [18]. $\overline{ASC}$ represents the total amino acid mixture minus the ASC group. ASC (mM), 2.1 Ala, 1.5 Asn, 0.13 $\frac{1}{2}Cys_2$, 1.5 Gln, 0.25 Pro, 1.3 Ser, 0.7 Thr, 7.5 total ASC; L (mM), 0.15 Ile, 0.3 Leu, 0.10 Met, 0.15 Phe, 0.15 Trp, 0.15 Tyr, 0.25 Val, 1.25 total L; $\overline{ASC}$ (mM), 0.5 Arg, 3 Asp, 4.5 Glu, 8.3 Gly, 0.5 His, 0.15 Lys, +1.25 L, 18.2 total $\overline{ASC}$. The total amino acid concentration of the above lists (ASC + $\overline{ASC}$) is 25.7 mM. AA_t is ASC + $\overline{ASC}$ in the proportions listed above but at some specified concentration.

Results

Stimulation of membrane Ca^{2+} transport by amino acids

Ca^{2+} transport by membrane vesicles annealed in 154 mM potassium glutamate averaged about 5-times that of vesicles annealed in KCl (Table I). 154 mM AA_t or 154 mM AA_t minus glutamate and aspartate acted like 154 mM glutamate. The stimulation of Ca^{2+} transport produced by annealing with various concentrations of AA_t is shown in Fig. 1. Stimulation by 26 mM AA_t was statistically significant (Table I). The basal Ca^{2+} transport activity varied among different membrane preparations as did the stimulation by AA_t (Fig. 2). At the low activity end of the curve (uptake below 2 nmol $Ca^{2+}/\mu l$ per 5 min), stimu-

TABLE I

STIMULATION BY AMINO ACIDS OF Ca^{2+} TRANSPORT BY MEMBRANE VESICLES

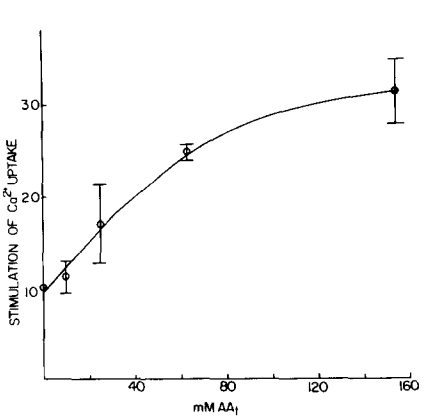
Membrane vesicles were annealed with various concentrations of amino acids. Ca^{2+} uptake was from media (Materials and Methods) with amino acid concentrations equal to those in the annealing media. The values shown are the averages ± S.E. The ratio of ATP-dependent Ca^{2+} uptakes/μl trapped-space by vesicles annealed with and without amino acids (+aa/—aa) was calculated for each experiment, and the probability (*P*aa) that the average ratio from *n* experiments was different from 1 by chance was obtained by Students *t*-test [19]. n.s., not significant.

Amino acid	Concentration (mM)	Ca^{2+} uptake ratio (*n*) (+aa/—aa)	*P*aa
Glu	154	5.01 ± 1.27 (4)	<0.005
AA_t *	154	3.42 ± 0.36 (7)	<0.005
AA_t-Glu-Asp *	109	3.20 ± 0.22 (2)	<0.05
Ala + Ser + Pro	22	2.83 ± 0.28 (2)	<0.025
Pro	2	1.93 ± 0.19 (3)	<0.025
Gly	50	0.97 ± 0.30 (2)	n.s.
AA_t *	25.7	2.37 ± 0.52 (48)	<0.005
Pro	0.25	1.17 ± 0.16 (3)	n.s.
$\frac{1}{2}Cys_2$	0.8	1.06 ± 0.23 (3)	n.s.
CysH **	0.8	0.27 ± 0.11 (4)	<0.005
$\frac{1}{2}Cys_2 + BH_4^-$ ***	0.8	0.78 ± 0.06 (2)	n.s.

* AA_t represents the total amino acid mixture in the proportions listed in Materials and Methods.

** As discussed in Results, this inhibition is probably not due to cysteine itself.

*** 4 mM $\frac{1}{2}Cys_2$ stock solution was freshly reduced with 10 mM $NaBH_4$ for 10 min at room temperature. Residual BH_4^- was quenched with HCl and the solution readjusted to pH 8.0 with KOH. A BH_4^- control blank was prepared; its Ca^{2+} uptake activity was the same as that of the control without BH_4^-.



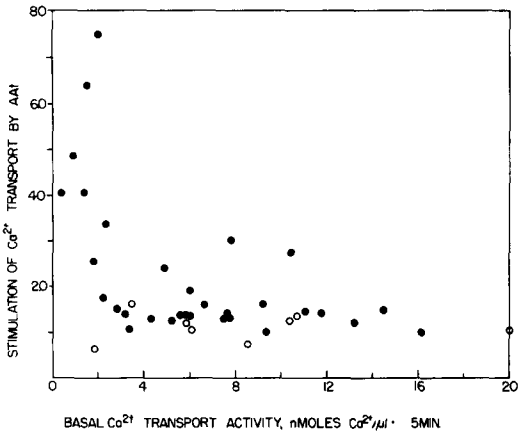


Fig. 1. Stimulation of membrane Ca^{2+} transport by total amino acid mixture. Membrane vesicles were annealed with various concentrations of amino acid mixture (AA_t). The incubation medium for the Ca^{2+} uptake assay contained the same amount of amino acids as those in the annealing medium. Averaged results of three experiments are shown ± S.E. Ordinate, ratio of ATP-dependent Ca^{2+} uptake in the presence of AA_t to the basal uptake in the same experiment; abscissa, concentration (mM) of AA_t. Basal Ca^{2+} transport activity is the ATP-dependent Ca^{2+} uptake of membrane vesicles annealed in the absence of amino acids.

Fig. 2. Correlation between the magnitude of amino acid stimulation and the unstimulated Ca^{2+} transport activity. Membrane was sonicated for the third time at 5 ml/g wet wt. (○) or 15 ml/g wet wt. (●). Vesicles were annealed without or with 26 mM AA_t.

lation by AA_t was high and variable (2.5—7.5-fold). With Ca^{2+} uptake values above 2 nmol $Ca^{2+}/\mu l$ per 5 min, stimulation by 26 mM AA_t was roughly 1.3-fold. Dilution of membrane suspensions during the third sonication tended to reduce the unstimulated Ca^{2+} transport activity but gave vesicles somewhat more responsive to 26 mM AA_t. These data suggest that the variable basal activity may be due to a variable endogenous state of activation. They also show that stimulation can be very large.

Glycine, the most abundant amino acid in pigeon erythrocytes, had no effect on Ca^{2+} transport (Table I). With the exceptions of proline and cysteine (Table I), no single amino acid was active at its concentration in either the 154 or 26 mM AA_t mixtures, nor at 10 mM (data not shown). Stimulation of Ca^{2+} transport by different concentrations of proline is shown in Fig. 3. At 0.25 mM, the concentration of proline in 26 mM AA_t, proline did not stimulate Ca^{2+} transport. Cysteine at 0.8 mM inhibited Ca^{2+} transport by about 70% while cystine did not. Inhibition was produced by two different commercial cysteine hydrochloride preparations and one commercial cysteine preparation. However, cysteine freshly formed from cystine by borohydride or dithiothreitol reduction did not inhibit, nor did cystine plus thioglycolate. Glutathione at 10 mM did not affect Ca^{2+} transport (data not shown). Inhibition by 'cysteine' apparently was not due to the cysteine itself nor to a sulfhydryl group, but to some impurity in the commercial preparations. We also eliminated the possibility that inhibition was caused only by a mixture of cysteine and cystine by adding partially reduced cystine. Because of the inhibition by cysteine, only cystine was included in the amino acid mixtures.

TABLE II

STIMULATION OF Ca^{2+} TRANSPORT BY GTP, PHOSPHO*ENOL*PYRUVATE AND ATP, AND THE EFFECT OF VARIOUS AMINO ACID MIXTURES ON STIMULATION BY THESE AGENTS

Membrane vesicles were annealed with no additions or with 1 mM GTP or 1 mM phospho*enol*pyruvate or 5 mM ATP with or without amino acids. AA_t was 26 mM, amino acid groups (aa) were as listed in Materials and Methods. Conditions of annealing and Ca^{2+} uptake were the same as for Table I. Data are presented as mean ± S.E. (number of experiments). *P* values are probabilities of significance by Student's *t*-test: *P*aa values refer to the comparisons of the effects of amino acid groups to samples with no additions (=basal). *P*NNP values refer to the effects of NNP (GTP, phospho*enol*pyruvate or ATP) in the presence of the amino acids (if any) listed for the row compared with the basal. *P*NNP(aa — 0) values refer to the effects of the NNP (column) in the presence of the amino acid group of that row compared with the effect of the same NNP without amino acid (top row); i.e., the null hypothesis tested by paired difference is whether [(NNP + aa)/aa] — [NNP/basal] = 0. It is rejected when $P < 0.05$. *P*NNP(aa — AA_t) values refer to the effects of the NNP in the presence of the amino acid group in its row compared with the effect of the same NNP in the presence of AA_t (second row). Thus, a significant value for *P*NNP(aa — 0) means the effect of the NNP is augmented by the amino acid mixture indicated while a significant value for *P*NNP(aa — AA_t) means the effect of the amino acid mixture on the NNP stimulation was different from that of AA_t on the NNP effect. In all cases comparisons were made between values obtained within a given experiment and the ratios averaged for *n* experiments. n.s., not significant ($P \geq 0.05$); n.d., not done.

Ca^{2+} uptake ratio		Ca^{2+} uptake ratio, (NNP + aa)/aa		
aa	aa/basal	GTP	Phospho*enol*pyruvate	ATP
None	≡1.000	1.430 ± 0.082 (21) *P*NNP < 0.005	1.081 ± 0.049 (17) *P*NNP n.s.	1.965 ± 0.169 (14) *P*NNP < 0.005
AA_t	2.389 * ± 0.522 (48) *P*aa < 0.005	2.483 ± 0.207 (23) *P*NNP < 0.005 *P*NNP(aa — 0) < 0.005	1.908 ± 0.215 (17) *P*NNP < 0.005 *P*NNP(aa — 0) < 0.005	3.029 ± 0.303 (16) *P*NNP < 0.005 *P*NNP(aa — 0) < 0.025
ASC	1.395 ± 0.159 (8) *P*aa < 0.025 *P*(aa — AA_t) n.s.	2.137 ± 0.162 (8) *P*NNP < 0.005 *P*NNP(aa — 0) < 0.025 *P*NNP(aa — AA_t) n.s.	1.513 ± 0.185 (8) *P*NNP < 0.025 *P*NNP(aa — 0) < 0.05 *P*NNP(aa — AA_t) n.s.	 n.d.
ASC — Pro	1.146 + 0.093 (7) *P*aa n.s. *P*(aa — AA_t) < 0.05	1.711 ± 0.184 (7) *P*NNP < 0.005 *P*NNP(aa — 0) n.s. *P*NNP(aa — AA_t) < 0.05	1.299 ± 0.186 (7) *P*NNP n.s. *P*NNP(aa — 0) n.s. *P*NNP(aa — AA_t) < 0.05	 n.d.
$\overline{\text{ASC}}$	0.709 ± 0.087 (6) *P*aa < 0.025 *P*(aa — AA_t) < 0.05	1.603 ± 0.167 (6) *P*NNP < 0.01 *P*NNP(aa — 0) n.s. *P*NNP(aa — AA_t) < 0.01	1.173 ± 0.189 (6) *P*NNP n.s. *P*NNP(aa — 0) n.s. *P*NNP(aa — AA_t) < 0.005	 n.d.
L	0.968 ± 0.101 (6) *P*aa n.s. *P*(aa — AA_t) < 0.01	1.309 ± 0.172 (6) *P*NNP n.s. *P*NNP(aa — 0) n.s. *P*NNP(aa — AA_t) < 0.005	1.102 ± 0.263 (6) *P*NNP n.s. *P*NNP(aa — 0) n.s. *P*NNP(aa — AA_t) < 0.005	 n.d.

* This value is larger than the ASC value on the line below because it includes more samples from near the ordinate of Fig. 2.

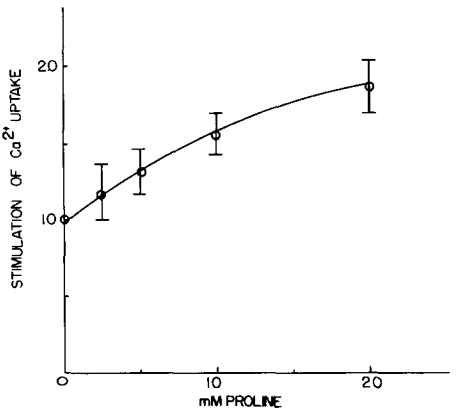


Fig. 3. Effect of proline on membrane Ca^{2+} transport. Averaged results of three experiments are shown ± S.E. Ordinate, ratio of ATP-dependent Ca^{2+} uptake in the presence of proline to the basal uptake; abscissa, proline concentration (mM).

Groups of amino acids, classified by their transport specificities, were tested. The first column of Table II shows the effects of different amino acid groups on Ca^{2+} transport. The amino acid group transported by the ASC system, at 7.5 mM, increased Ca^{2+} transport by about 40%. The difference between the stimulation by the ASC group and AA_t (P(aa − AA_t)) was not statistically significant as tested by paired differences from eight experiments. Deleting proline (0.25 mM) from the ASC group caused loss of stimulating activity. (From Table II, ASC − Pro: Paa n.s., P(aa − AA_t) < 0.05.) However, comparing stimulation of ASC as against ASC − Pro, P(ASC − (ASC − Pro)) was less than 0.10 (n.s.). Note that 0.25 mM proline by itself did not stimulate (Table I). The L group was inert. $\overline{\text{ASC}}$, which is AA_t − ASC, was weakly inhibitory.

Effect of organic phosphate compounds on Ca^{2+} transport by membrane vesicles

The results in the first row of Table II show that annealing membrane vesicles with 1 mM GTP or 5 mM ATP increased their Ca^{2+} transport activities by 1.4—2-fold. The effect of phospho*enol*pyruvate alone was insignificant.

AA_t at 26 mM amplified the stimulatory effect of ATP, GTP and phospho*enol*pyruvate on Ca^{2+} transport. ATP stimulated Ca^{2+} uptake 3-fold in the presence of AA_t compared to 2-fold in the absence of AA_t (Table II). Note that stimulation by ATP plus AA_t relative to the basal averaged 6-fold (from Table II), and was as much as 20-fold in some experiments. The stimulatory effects of GTP and phospho*enol*pyruvate were also increased by 26 mM AA_t; rising from 1.4 to 2.5-fold for GTP and from 1.1 to 1.9-fold for phospho*enol*pyruvate (Table II).

When data from Table II were used to calculate augmentation of AA_t stimulation of Ca^{2+} uptake by 1 mM GTP, phospho*enol*pyruvate or 5 mM ATP, no statistically significant effect was observed. Stimulation by AA_t was 2.389 ± 0.522 (n = 48) in the absence of NNP, and 2.828 ± 0.705 (n = 20), 1.917 ± 0.192 (n = 15) and 3.335 ± 1.053 (n = 14) in the presence of GTP, phospho*enol*pyruvate and ATP, respectively. None of these effects of any other amino

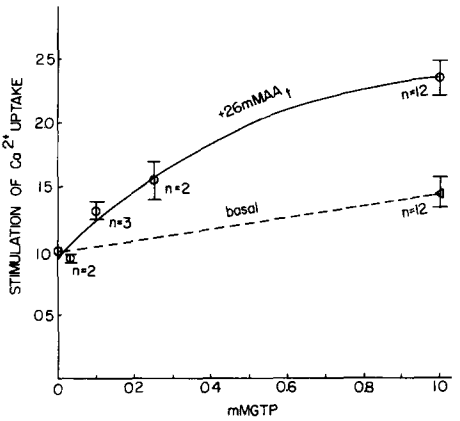


Fig. 4. Stimulation of membrane Ca^{2+} transport by GTP in the presence or absence of 26 mM AA_t. GTP at different concentrations was annealed with membrane vesicles with or without 26 mM AA_t. Stimulation is shown as the ratio of Ca^{2+} transport activity with GTP to activity without GTP. Solid line, AA_t present; dashed line, AA_t absent. Data are the average ± S.E. of *n* experiments.

acid mixture tested was influenced by those agents.

The ASC amino acids alone at 7.5 mM had stimulatory effects on Ca^{2+} transport and also enhanced stimulation by GTP and phospho*enol*pyruvate. This enhancement was similar to the corresponding enhancement by AA_t (Table II, *P*NNP(aa − AA_t) values for aa = ASC were not significant). Omission of proline from the ASC group abolished the amino acid enhancement of GTP and phospho*enol*pyruvate stimulation of Ca^{2+} transport. (Table II, ASC − Pro: *P*NNP(aa − 0) values n.s., *P*NNP(aa − AA_t) values less than 0.05). Comparisons between data for ASC and ASC − Pro gave *P*GTP((ASC − Pro) − ASC) < 0.05 (not listed) but *P*phospho*enol*pyruvate((ASC − Pro) − ASC) was only less than 0.25 (not listed). Proline in the ASC group seems necessary for the full GTP effect, but its requirement for full enhancement of phospho*enol*pyruvate stimulation is not certain. The $\overline{ASC}$ group present alone cause a significant drop in Ca^{2+} transport. In the presence of GTP or phospho*enol*pyruvate, the $\overline{ASC}$ group had no significant effect relative to the basal (*P*NNP(aa − 0) n.s., Table II), suggesting that GTP and phospho*enol*pyruvate might block the weak $\overline{ASC}$ inhibition.

Stimulation of Ca^{2+} transport by various concentrations of GTP is shown in Fig. 4. The augmentation by 26 mM AA_t of the GTP stimulation is evident. In the presence of 26 mM AA_t there is marked stimulation by GTP at 0.33 mM, its physiological [20] concentration.

Nucleotides other than ATP, GTP and phosphoenolpyruvate

The effects of some other nucleotides on Ca^{2+} transport activity are shown in Table III. In the absence of amino acids, AMPPNP inhibited Ca^{2+} transport. In the presence of 26 mM AA_t, stimulation by ADP but not GDP was statistically significant and AMPPNP no longer inhibited significantly. There was no effect of 5′-GMP or the 3′,5′-cyclic monophosphates of guanosine and adenosine or 2′,3′-cyclic GMP either with or without 26 mM AA_t.

TABLE III

EFFECT OF NUCLEOTIDES ON Ca^{2+} UPTAKE ACTIVITY BY MEMBRANE VESICLES

Nucleotides were annealed with membrane vesicles in the presence or absence of 26 mM AA_t. Conditions and format are the same as Table I. n.s., not significant.

Nucleotide	Concentrations (mM)	+ Nucleotide/basal	P	$(AA_t$ + nucleotide)/AA_t	P
ATP	1	0.881 ± 0.019 (3)	<0.025	1.753 ± 0.199 (3)	<0.05
	5	1.965 ± 0.169 (14)	<0.005	3.049 ± 0.303 (16)	<0.005
ADP	5	1.122 ± 0.115 (3)	n.s.	2.656 ± 0.545 (3)	<0.05
AMPPNP	5	0.341 ± 0.031 (4)	<0.005	0.716 ± 0.181 (4)	n.s.
GTP	1	1.430 ± 0.082 (21)	<0.005	2.463 ± 0.207 (23)	<0.005
GDP	1	1.167 ± 0.118 (4)	n.s.	1.358 ± 0.225 (4)	n.s.
GMP	1	1.163 ± 0.082 (4)	n.s.	1.012 ± 0.124 (4)	n.s.
GMPPNP	1	0.877 ± 0.106 (4)	n.s.	0.952 ± 0.103 (4)	n.s.
3′,5′-cyclic AMP	1	0.944 ± 0.226 (4)	n.s.	0.859 ± 0.136 (4)	n.s.
	0.1	1.034 ± 0.062 (2)	n.s.	0.939 ± 0.199 (2)	n.s.
	0.01	0.961 ± 0.045 (2)	n.s.	0.952 ± 0.136 (2)	n.s.
3′,5′-cyclic GMP	1	0.903 ± 0.156 (5)	n.s.	0.829 ± 0.113 (5)	n.s.
	0.1	1.019 ± 0.046 (3)	n.s.	0.975 ± 0.198 (3)	n.s.
	0.01	0.955 ± 0.068 (3)	n.s.	1.024 ± 0.139 (3)	n.s.
2′,3′-cyclic GMP	1	1.149 ± 0.126 (4)	n.s.	1.018 ± 0.050 (4)	n.s.

Effect of orthophosphate on Ca^{2+} transport by membrane vesicles

In the absence of AA_t, P_i up to 13 mM had no significant effect on the Ca^{2+} transport activity of membrane vesicles (Fig. 5). However, in the presence of 26

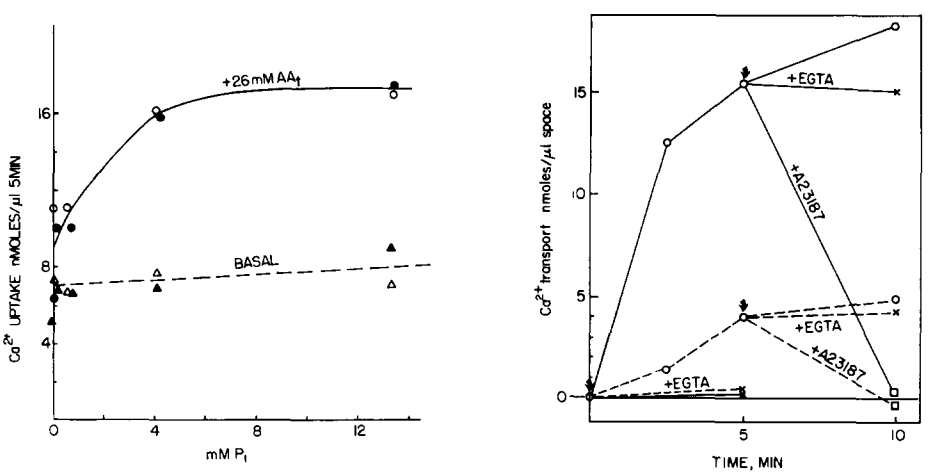


Fig. 5. Stimulation of membrane Ca^{2+} transport activity by orthophosphate in the presence or absence of 26 mM AA_t. Potassium phosphate at various concentrations was annealed with membrane vesicles with (solid line) or without (dashed line) 26 mM AA_t. Other conditions were as described in Materials and Methods. Data from two experiments are shown, distinguished by different symbols.

Fig. 6. Time course of ATP-dependent Ca^{2+} accumulation by membrane vesicles annealed in the presence and absence of 26 mM amino acids and 5 mM ATP. Membrane vesicles were annealed with (solid line) or without (dotted line) 5 mM ATP and 26 mM AA_t as described in Materials and Methods. Membrane vesicles were washed and incubated at 27°C for different time intervals. EGTA, 5 mM (X) or ionophore A23187, 7 μM (□) was added at the times designated by arrows. Data shown are from one representative experiment of three.

mM AA_t, 4 mM P_i stimulated Ca^{2+} transport 2-fold. 13 mM P_i had no more effect than 4 mM P_i. The physiological P_i concentration in avian erythrocytes is about 4 mM [20]. Reciprocal cooperative interaction between P_i and AA_t was observed, 4 mM P_i stimulated Ca^{2+} uptake 1.8-fold in the presence of 26 mM AA_t compared to no stimulation (1.1-fold) in the absence of AA_t, and AA_t stimulated 2.4-fold in the presence of 4 mM P_i compared to 1.3-fold in its absence. Mutual cooperation between P_i and AA_t persisted in the presence of AA_t plus phospho*enol*pyruvate but transport stimulated by GTP or ATP was not further enhanced by P_i either in the presence or absence of 26 mM AA_t, nor did P_i enhance the effect of phospho*enol*pyruvate in the absence of AA_t (data not shown).

Stimulation of Ca^{2+} transport is due to increased transport rate

The Ca^{2+} uptake data have been presented as ratios of ATP-dependent $^{45}Ca^{2+}$ uptake in 5 min per μl trapped-space (treated vesicles) to the corresponding uptake by untreated vesicles in the same experiment. The effects of treatment do not appear to be due to alterations in the percentage of the vesicles sealed nor to differential leakiness to accumulated Ca^{2+}.

Sealing of membrane vesicles as determined by [^{3}H]maltitol trapping was not altered by annealing with amino acids and nucleotide triphosphates. The average space values in μl/pellet (0.4 mg protein/pellet) ± S.E. (number of experiments in parentheses) of vesicle samples are: 26 mM AA_t, 0.182 ± 0.013 (48), corresponding controls 0.185 ± 0.014 (48); 1 mM GTP + 26 mM AA_t, 0.167 ± 0.010 (23), controls 0.167 ± 0.009 (23); 5 mM ATP + 26 mM AA_t, 0.171 ± 0.017 (16), controls, 0.170 ± 0.016 (16).

The time course of ATP-dependent Ca^{2+} uptake is illustrated in Fig. 6. Addition of 5 mM EGTA at 5 min failed to release accumulated $^{45}Ca^{2+}$ although 5 mM EGTA blocked uptake when added at zero time. Therefore, uptake is not limited by efflux in either control or treated vesicles. The $^{45}Ca^{2+}$ was accumulated in both cases, rather than bound as shown by its release by 7 μM ionophore A23187.

'Ca^{2+} transport' in Fig. 6 is the difference between uptake in the presence and absence of ATP. After treatment with A23187 at 5 min, samples pretreated with ATP + AA_t and control samples, whether incubated with or without ATP, had the same $^{45}Ca^{2+}$ associated with them 0.37 ± 0.015 nmol/μl (n = 12). This is taken to represent the maximum passive uptake. The average passive $^{45}Ca^{2+}$ uptake in 10 min for all vesicles in the population (not just those capable of active Ca^{2+} uptake) was estimated from the $^{45}Ca^{2+}$ uptake in the absence of ATP. This passive uptake was the same in the control and ATP + AA_t-treated vesicles. The average value was 0.049 ± 0.003 nmol/μl per 10 min (n = 6) (compared with the maximum passive uptake of 0.37). Thus, passive Ca^{2+} movements in the whole vesicle population, or in the fraction capable of active Ca^{2+} uptake, had no significant effect on our measurements of ATP-dependent Ca^{2+} uptake.

Although the total trapped-space was not altered by treatment with amino acids and/or nucleotides, it was possible that accumulated $^{45}Ca^{2+}$ was released during the washing steps to different degrees by treated and control vesicles. Such an effect does not appear to account for our results. When vesicles are

TABLE IV

PROTECTION OF Ca^{2+} TRANSPORT ACTIVITY BY AMINO ACIDS AND GTP

Membrane vesicles (approx. 20 mg (wet wt.)/ml) were annealed in basal medium as described in Materials and Methods. After washing, they were incubated 60 min at 25°C in unsupplemented medium (136 mM KCl, 2 mM EGTA, 1.628 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM Tes at pH 7.0, calculated free $[Ca^{2+}]$ = 1 μM) or in AA_t medium (75 mM AA_t, 90 mM KCl, 2 mM EGTA, 1.628 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM Tes at pH 7.0) with or without the addition of ATP or GTP. After incubation the membrane suspension was diluted 10-times with Tes buffer and centrifuged. Each sample was divided in two for ATP-dependent Ca^{2+} uptake measurements (Materials and Methods). Results are mean ± S.E. (number of experiments). n.s., not significant.

Addition	Concentration (mM)	Addition/basal	AA_t + addition/AA_t
AA_t	75	1.722 ± 0.325 (6) $P < 0.05$	≡1.000
ATP	5	0.448 ± 0.056 (2) $P < 0.05$	0.348 ± 0.085 (2) $P < 0.05$
GTP	1	0.884 ± 0.513 (2) P n.s.	1.671 ± 0.195 (3) $P < 0.05$

sedimented in a bovine serum albumin gradient, most of the ATP-dependent Ca^{2+} uptake activity is found in the region between 15 and 17% bovine serum albumin [13]. Aliquots of membrane vesicles after Ca^{2+} uptake, were layered on a bovine serum albumin density gradient with steps of 5, 9, 13 and 18%. After centrifugation for 40 min at 275 000 × g in an SW-41 Ti rotor at 5°C in a Beckman L5-65 ultracentrifuge, the vesicle fractions from the bovine serum albumin interfaces were collected and counted. Equal aliquots of vesicles were also harvested by the usual pelleting method. $^{45}Ca^{2+}$ uptake in 5 min (three experiments) in the main active region (banding at the 13—18% interface) was 2.699 ± 0.242 nmol for vesicles annealed with 26 mM amino acids plus 5 mM ATP and 0.745 ± 0.034 nmol for control vesicles, whereas Ca^{2+} uptake measured by pelleting was 2.818 ± 0.234 nmol for vesicles annealed with amino acids and ATP and 0.692 ± 0.063 nmol for controls. Therefore, differential Ca^{2+} loss due to the washing was not significant.

Protection of Ca^{2+} transport activity

Ca^{2+} transport activity deteriorated when previously annealed membrane vesicles were incubated at 25°C for 60 min. Activity was 0.243 ± 0.060 (n = 7) times that of the unincubated vesicles. When 75 mM AA_t was present in the incubation medium, about half of the Ca^{2+} transport activity was retained (1.7 × 0.24) (Table IV). In the presence of both 1 mM GTP and 75 mM AA_t most of the Ca^{2+} transport activity was retained (1.7 × 1.7 × 0.24 = 0.7), but GTP alone did not protect Ca^{2+} transport activity. On the other hand, in the presence of 5 mM ATP, with or without 75 mM AA_t, Ca^{2+} transport activity deteriorated more rapidly than in the absence of ATP. Thus, the effects of GTP and ATP when present only after annealing were markedly different from the effects of these nucleotides present during annealing.

Discussion

Our membrane vesicle population had both inside-out and right-side-out vesicles [13]. Since only inside-out vesicles were capable of active Ca^{2+} accumulation, we were observing the effects of the agents on the inside-out vesicles. In most experiments the agents were removed after the annealing step and thus were absent from the i face of the vesicles during Ca^{2+} uptake. That is, a membrane alteration(s) which affected subsequent Ca^{2+} transport was produced and persisted in the absence of the agents from the i face.

The stimulatory effects were cooperative. The enhancing effect of amino acids and phosphate was mutual. Also, AA_t either enhanced or was required for stimulation by all the other agents. The effect of P_i and phospho*enol*pyruvate absolutely required AA_t. The AA_t requirements for ATP and GTP effects were not absolute, but the percentage stimulation by those agents was at least doubled by 26 mM AA_t. The effects of the amino mixtures themselves showed signs of cooperative interaction between amino acids. Removal of 0.25 mM proline from the ASC group decreased its activity, particularly on the effect of GTP, while 0.25 mM proline alone was not active. The other amino acids of the group used singly could not substitute for the group even at a concentration (10 mM) greater than that of ASC group together (7.5 mM).

It appears (cf. Tables III and IV) that some of these agents, such as GTP and ATP ± AA_t, affect processes governing subsequent activity which are completed during annealing, while some other processes (affected by AA_t) may remain subject to modulation after annealing. This might reflect a difference between modulation of membrane assembly steps and modulation of membrane activity.

Most of the effects were significant at physiological concentrations of the agents (AA_t, ASC, ATP, GTP and P_i). For AA_t, stimulation was a function of concentration across the physiological range, so variations in the AA_t in vivo may have significant effects on $[Ca^{2+}]_i$ in vivo. Some effects were observed only at supraphysiological concentrations of affectors (phospho*enol*pyruvate, glutamate, proline). The co-affector(s) for proline may be some or all of the other ASC amino acids.

Finally, since $[Ca^{2+}]$ was kept below the $[Ca^{2+}]$ required for calmodulin to act, the effects described are unlikely to involve calmodulin. A Sephadex fraction of an extract of our membrane, corresponding to the M_r of calmodulin, stimulated our membrane in the presence of added Ca^{2+}, but not at the $[Ca^{2+}]$ we used for annealing.

Cells of higher animals accumulate amino acids, but the reason is not clear. The obvious explanation, to facilitate protein synthesis, is not satisfying because mammalian amino acid activating enzymes appear to be half-saturated by amino acid concentrations in the 10 μM range [21—24], considerably below the concentrations of the cognate amino acids in plasma [25]. Our observations suggest a different role, that of altering the cell $[Ca^{2+}]$ by modulating the activity of the Ca^{2+} pump of the plasma membrane. The observation that most of the effects are exerted by amino acids transported by the ASC transport system is intriguing because a number of hormones stimulate amino acid accumulation in a number of cell types, and it is usually only the A system which is stimu-

lated [26—28]. Many other alterations in the cell environment trigger the adaptive regulation of amino acid transport by the A system [28,30]. (Pigeon red cells and mammalian reticulocytes have an ASC system which is similar although distinguishable from the A system of most other cell types [18].)

There are examples of particular amino acids serving as signals: Christensen et al. [31] described the stimulation of insulin release by b(—)2-aminobicyclo(2,2,1)heptane-2-carboxylate [31] and stimulation of insulin and glucagon release by 4-amino-1-guanylpiperidine-4-carboxylate [31,32]. These are non-metabolizable analogues of leucine and arginine, respectively (which are also active), and hence show that amino acids can act directly. In muscle, the rate of protein catabolism is inversely related to the cell levels of the branched chain amino acids, particularly leucine [33].

Since the effect of amino acids on Ca^{2+} pump activity is concentration dependent across the physiological concentration range, there is a plausible mechanism for hormones to control cellular activities through stimulation of amino acid accumulation.

In marked contrast to several previously described membrane systems (Refs. 34—36 and references cited therein) [37] GMPPNP is inert; i.e., GTP itself is probably not an affector in our system. 3′,5′-Cyclic AMP and cyclic GMP were inert and AMPPNP was inhibitory. The inactivity of cyclic AMP and cyclic GMP makes it unlikely that the ATP and GTP effects were due to the action of endogenous nucleotidyl cyclases. The lower activity or inactivity of the nucleoside di- and monophosphates makes it unlikely that ATP and GTP are active due to their conversion to the mono- or diphospho compound. This leaves the hypothesis that ATP and GTP phosphorylate (or adenylylate, etc.) some component(s) of the membrane. The stimulation of Ca^{2+} transport activity by prior exposure to ATP and GTP suggests the possibility of an energy requirement in the maintainance of membrane functions and/or structure.

There is a formal similarity between the effects of low molecular weight compounds reported and the effects of low molecular weight affectors at some other central control points, namely the glycogen degradation and synthesis systems [38], pyruvate dehydrogenase [39], and bacterial glutamine synthetase [40,41]. In all these cases control is exerted by the impingement of a large number of low molecular weight affectors on a complex 'enzyme' in a cumulative or cooperative fashion, the response to these affectors is modulated in turn by the state of the central 'enzyme' with respect to some covalently bound moiety (i.e., the phosphorylation or nucleotidylylation state) and the covalent modification process is in turn the target of control signals from outside the immediate system (i.e., outside the cell or (pyruvate dehydrogenase) the organelle).

We suggest this is a general pattern of control for biological systems and that the Ca^{2+} transport system is an example in the cytoplasmic membrane.

Acknowledgements

We thank Cynthia Stryker for excellent technical assistance. This work was supported by Grant HL-13256 from the U.S. Public Health Service and by the

University of Nebraska-Lincoln Research Council and NIH Biomedical Research Support Grant RR-07055.

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