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Modulation of ATP-dependent Ca^{2+} transport in rat parotid basolateral membrane vesicles by $K^+ + Cl^-$ flux

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In basolateral membrane vesicles (BLMV) isolated from rat parotid glands, the initial rate of ATP-dependent Ca^{2+} transport, in the presence of KCl, was approx. 2-fold higher than that obtained with mannitol, sucrose or *N*-methyl-D-glucamine (NMDG)-gluconate. Only NH_4^+, Rb^+, or Br^- could effectively substitute for K^+ or Cl^-, respectively. This KCl activation was concentration dependent, with maximal response by 50 mM KCl. An inwardly directed KCl gradient up to 50 mM KCl had no effect on Ca^{2+} transport, while equilibration of the vesicles with KCl (> 100 mM) increased transport 15–20%. In presence of Cl^-, $^{86}Rb^+$ uptake was 2.5-fold greater than in the presence of gluconate. 0.5 mM furosemide inhibited $^{86}Rb^+$ flux by approx. 60% in a Cl^- medium and by approx. 20% in a gluconate medium. Furosemide also inhibited KCl activation of Ca^{2+} transport with half maximal inhibition either at 0.4 mM or 0.05 mM, depending on whether $^{45}Ca^{2+}$ transport was measured with KCl (150 mM) equilibrium or KCl (150 mM) gradient. In a mannitol containing assay medium, potassium gluconate loaded vesicles had a higher (approx. 25%) rate of Ca^{2+} transport than mannitol loaded vesicles. Addition of valinomycin (5 μM) to potassium gluconate loaded vesicles further stimulated (approx. 30%) the Ca^{2+} transport rate. These results suggest that during ATP dependent Ca^{2+} transport in parotid BLMV, K^+ can be recycled by the concerted activities of a K^+ and Cl^- coupled flux and a K^+ conductance.

Introduction

Neurotransmitter-induced fluctuations in cytosolic Ca^{2+} mediate fluid and electrolyte secretion in rat parotid gland acinar cells [1]. Basolateral membranes possess an ATP-dependent Ca^{2+} transporter [2], which appears to be a primary system for regulating cytosolic Ca^{2+} levels in this secretory cell. This system is distinct from the ATP-dependent calcium transporter of the endoplasmic reticulum [3–5], and exhibits properties similar to calcium transporters described in plasma membranes from other tissues [6].

Recent studies [7–9] have shown that the ATP-dependent Ca^{2+} transporter, associated with membrane vesicle preparations from a variety of sources, mediates an electrogenic flux of Ca^{2+}. Accordingly, the positive charge accumulated in vesicles during Ca^{2+} flux would lead to the inhibition of further transport activity. ATP-dependent transport systems, in both endoplasmic reticulum and plasma membrane vesicles, have been re-

Abbreviations: NMDG, *N*-methyl-D-glucamine; TMA^+, tetramethylammonium.

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ported to be activated by monovalent cations [6,9–12]. In most cases, optimal activity of Ca^{2+} transport is obtained in the presence of KCl and a KCl-concentration dependence of Ca^{2+} transport activity has been described [6]. In some instances it has been reported that the K^+-induced activation is catalytic, due to direct effects on the kinetic properties of the enzyme [13]. The stimulation of Ca^{2+} transport by Cl^- is not yet well understood. The accumulated charge, however, could be compensated by the movement of other ionic species (namely, K^+, Cl^-, Na^+ or H^+) across the vesicular membranes.

We have reported that during ATP-dependent Ca^{2+} transport in rat parotid basolateral membrane vesicles there is a transfer of charge and that the initial rates of Ca^{2+} transport can be modulated by altering the membrane potential, thus indicating an electrogenic Ca^{2+} flux [14,15]. The present study was directed towards assessing monovalent ion movements related to the ATP-dependent Ca^{2+} flux. The data suggest the involvement of a furosemide-sensitive, Cl^--dependent, K^+ flux and a K^+ conductance in ATP-dependent Ca^{2+} transport in parotid BLMV.

Experimental procedures

Materials. Adenosine-triphosphate Tris salt (Tris-ATP), phenylmethanesulfonyl fluoride (PMSF), furosemide, and dithiothreitol (DTT) were purchased from Sigma Chemical Company. $^{45}Ca^{2+}$ (2 mCi/ml) was routinely obtained from Amersham International. All other reagents used were of the highest grade commercially available.

Animals. Animals used in these studies were male Wistar strain rats obtained from Harlan-Sprague Dawley. Rats were given ad libitum water and Purina Chow. The animals were anesthesized with ether and killed by cardiac puncture before the parotid tissue was excised.

Preparation of basolateral membrane vesicles. Basolateral plasma membrane vesicles were prepared as described previously [2], with some minor modifications. The procedure, in brief, was as follows. Glands from at least 15–20 rats were pooled for each membrane preparation. The excised glands were immersed in ice-cold buffer containing 0.25 M sucrose, 0.1 mM PMSF, 1 mM DTT, 10 mM Tris-Hepes (pH 7.5) and homogenized (twice, 10 s each) using a Polytron (Brinkman Instruments) at setting 5 to give a 10% (w/v) homogenate. The homogenate was further diluted to 7.5% (w/v with the same buffer) and then centrifuged at $2600 \times g$ for 15 min. The supernatant was filtered through four layers of cheesecloth and recentrifuged at $23\,500 \times g$ for 20 min. The pellet obtained was re-homogenized (by hand) in the sucrose medium using a teflon-glass homogenizer, mixed with Percoll (12% v/v) and centrifuged at $41\,700 \times g$ for 15 min. The basolateral membrane fraction was collected (Takuma et al., 1985) and washed three times with 100 mM mannitol, 0.1 mM pMSF, 1 mM DTT, 10 mM Tris-Hepes (pH 7.5) by centrifuging at $49\,000 \times g$ for 15 min. Membranes were finally washed in a medium containing 300 mM mannitol, 1 mM DTT, 10 mM Tris-Hepes (pH 7.5) and resuspended in a minimum volume of the same medium. All the procedures described above were carried out at 4 °C. Aliquots of membrane vesicles were quick frozen in liquid nitrogen and stored at −70 °C. Membranes were thawed in an ice-bath before each experiment and kept on ice until used. All membrane preparations were subjected to only one freeze-thaw. This freeze-thaw procedure did not alter calcium transport activity when compared to that of freshly prepared vesicles (data not shown).

Ca^{2+} transport in basolateral membrane vesicles. Calcium transport was measured by rapid filtration through Millipore filters (pore size 0.45 μm, HA). The assay medium typically contained 150 mM KCl, 10 mM Tris-Hepes (pH 7.5), 1 mM magnesium gluconate, and 1 mM Tris-ATP, unless otherwise indicated, in a volume of 200 μl. Ca^{2+} was added at the indicated concentrations and activity was maintained around 0.5 μCi $^{45}Ca^{2+}$/ml assay medium. The protein concentration was approx. 50 μg/ml in all the experiments. Transport was initiated by the addition of membrane vesicles to the assay medium at 37 °C.

The uptake reaction was terminated by a 'quench-filtration' method. To stop the reaction, 1.0 ml of cold reaction medium, without ATP and Ca^{2+}, was added to 200 μl reaction medium at the required time. The sample was then vortexed and filtered. The filter was washed with 3 ml (three

times) of the same medium, air-dried, dissolved in acidified ACS (Amersham International) and the radioactivity determined. Inclusion of 0.1 mM $LaCl_3$ in the wash medium did not alter the values obtained and so $LaCl_3$ was not routinely included in wash procedures. To obtain a 'zero' time point, cold wash medium was added to the reaction mixture before the addition of the membrane vesicles. Initial rates were calculated from linear regression of the values obtained for 0, 10, and 20 s of assay. Each time point was assayed in duplicate or triplicate. Where indicated, S.E. show the variations obtained between experiments with different membrane preparations. Only averages (from triplicate samples) are shown for representative (of 3–6 individual) experiments. Correlation coefficients between 0.98–0.99 were routinely obtained for the transport data. Specific conditions for each experiment have been indicated in the figure legends.

^{86}Rb flux. ^{86}Rb flux in BLMV was determined by a Millipore filtration method. Vesicles (approx. 25–40 μg protein) were incubated at 37°C in a medium (200 μl) containing 10 mM KCl or potassium gluconate, 1 mM magnesium gluconate, 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5); and tracer amounts of ^{86}Rb (approx. 0.5–1.0 μCi) for the required time intervals. The reaction was quenched by the addition of 1.0 ml of cold medium containing 150 mM *N*-methyl-D-glucamine (NMDG) 10 mM Tris-Hepes (pH 7.5). The radioactivity trapped intravesicularly was determined as described above for $^{45}Ca^{2+}$.

Other methods. Protein was measured using the Bio-Rad protein assay kit with bovine serum albumin as the standard.

Results

KCl requirement of ATP-dependent Ca^{2+} transport in BLMV

Basolateral membrane vesicles isolated from rat parotid glands exhibit an ATP-dependent Ca^{2+} transport activity (Fig. 1). In a medium containing 150 mM KCl, 10 mM Tris-Hepes, 1 mM magnesium gluconate, and 1 mM Tris-ATP, there is a rapid uptake of $^{45}Ca^{2+}$ into the vesicles reaching a level close to steady state by two minutes. The initial rate of Ca^{2+} transport calculated from the

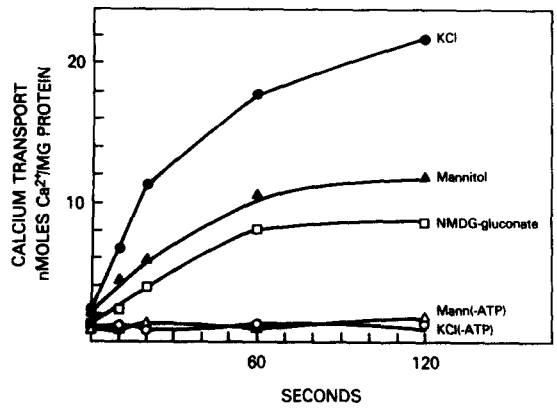

Fig. 1. KCl effects on ATP-dependent Ca^{2+} transport in rat parotid basolateral membrane vesicles. Ca^{2+} transport was assayed in the presence of either 150 mM KCl, (●, ○) 150 mM NMDG-gluconate (□), or 300 mM mannitol (▲, △). The assay media contained 10 mM Tris-Hepes (pH 7.5), 1 mM magnesium gluconate with (●, ▲) or without (○, △) 1 mM Tris-ATP. Ca^{2+} concentration (0.5 μCi $^{45}Ca^{2+}$) in the assay was 12.3 μM (total). The intravesicular medium contained 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5). Protein (50 μg/ml) was added to initiate the reaction, performed at 37°C. Other details of the assay are given in Methods. The data represent the mean results from two experiments performed with different membrane preparations, each point being assayed in triplicate.

first 20 seconds (at 12.5 μM Ca^{2+} total) under these conditions is about 25 ± 8 nmol/mg protein per min, while the vesicles typically accumulate between 20 and 30 nmol Ca^{2+} by 2 min. In the absence of ATP there is a low level of Ca^{2+} uptake (1–2 nmol per mg protein, 10–12-fold less than in the presence of ATP). We have reported earlier that when the calcium ionophore, A23187, is added to vesicles loaded with Ca^{2+} (at steady state), the intravesicular Ca^{2+} is decreased to the level seen in the absence of ATP [14].

When calcium transport is assayed in a non-ionic medium with 300 mM mannitol substituted for 150 mM KCl, both the initial rate of Ca^{2+} uptake and steady-state amount of Ca^{2+} loading are decreased by about 50–60% (Fig. 1). Ca^{2+} uptake in the absence of ATP is not altered. The ATP-dependent Ca^{2+} transport in a medium containing 300 mM sucrose is similar to that in the mannitol medium (data not shown). This decrease in Ca^{2+} transport rate could be due to the absence of an ionic medium. In order to differentiate between non-ionic versus ionic effects, KCl was

replaced with the impermeant ions *N*-methyl-D-glucamine (to replace K^+) and gluconate (to replace Cl^-). As shown in Fig. 1, in a KCl-free, impermeant ionic medium (NMDG-gluconate), the level of ATP-dependent Ca^{2+} transport is similar to that in a KCl-free, non-ionic (mannitol) medium. These data show that the lower level of Ca^{2+} transport seen in the mannitol medium cannot be attributed to the reduced ionic strength of the assay medium, but rather possibly due to the absence of KCl.

Effect of K^+ and Cl^- substitution on Ca^{2+} transport in BLMV

In order to assess the relative roles of K^+ and Cl^- in stimulating the ATP-dependent Ca^{2+} transport activity, a series of experiments were performed, where either K^+ or Cl^- was replaced with an equimolar concentration of various cations or anions. The data are shown in Table I. Both permeant and impermeant ions were tested under two experimental conditions. First, with an inwardly directed ion gradient when the intravesicular medium contained 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5) and the extravesicular medium contained 150 mM of the specified ions, 12.5 μM $^{45}Ca^{2+}$ (total), 1 mM Tris-ATP magnesium gluconate. Second, with an equilibrium condition, when membrane vesicles were allowed to incubate in the various ionic media for 60–80 min at 25–28 °C. In all experiments, the initial rate of ATP-dependent Ca^{2+} uptake was measured as described in Methods, using data obtained during the initial 20 s of transport. The maximum calcium uptake rate was obtained in KCl-equilibrated vesicles, while initial rate with KCl (150 mM) gradient was approx. 20% lower. Anion substitution of Cl^- (in the presence of K^+) was effective in the order $Br^- >$ gluconate, $NO_3^- > SO_4^{2-}$. Maximal level of Ca^{2+} transport (equal to the level in KCl) was seen only in a Br^- medium and this was obtained only after equilibration of the vesicles in a medium containing 150 mM KBr. In the presence of a 150 mM KBr gradient, the initial rate of Ca^{2+} transport was equal to that observed with a medium containing an impermeant anion (potassium gluconate). With potassium gluconate or KNO_3, maximum levels of transport could not be achieved even after equilibration (about 30% and 45% lower activity than in KCl).

Effective substitution of K^+ (in the presence of Cl^-), was achieved in the order $Rb^+ > NH_4^+ >$ TMA^+, $NMDG^+$. Permeant cations, Rb^+ and NH_4^+ elicited Ca^{2+} transport rates equivalent to that mediated by K^+. NH_4^+, however, had to be pre-equilibrated in vesicles in order to obtain optimal Ca^{2+} transport activity. Rb^+, on the other hand, induced maximal Ca^{2+} transport rates even under gradient condition. Impermeant cations like TMA^+ and $NMDG^+$, could not substitute K^+ effectively (approx. 30% lower in either gradient or equilibrium condition).

The data in Table I suggest that no other cation, except Rb^+ can optimally substitute for K^+. No other anion can substitute for Cl^-, except Br^- after prolonged periods of incubation with

TABLE I

THE ABILITY OF VARIOUS CATIONS AND ANIONS TO SUPPORT ATP-DEPENDENT CALCIUM TRANSPORT IN RAT PAROTID BASOLATERAL MEMBRANE VESICLES

Assays were performed at 37 °C, in the presence of various ions (150 mM) in media containing 12.5 μM total Ca^{2+}, 1 mM magnesium gluconate, 10 mM Tris-Hepes (pH 7.5). In the gradient condition, the intravesicular medium contained 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5). In the equilibrium condition, the vesicles were incubated with the various assay media for 60–80 min at 25–28 °C. Initial rates of $^{45}Ca^{2+}$ uptake were determined as described in Methods. Data show averages from two individual experiments or mean ± S.E. from 3–5 experiments performed with different membrane preparations. All assays were performed in triplicate. The data are expressed relative to the initial rate of Ca^{2+} transport in a KCl containing medium; 24 ± 4.0 nmol/mg protein per min (KCl gradient), 30 ± 3.0 nmol/mg protein per min (KCl equilibrium).

Assay medium	Ion substituted	% Calcium transport; ionic condition during assay	
		gradient	equilibrium
KCl	none	100	100
TMA-Cl [a]	K^+	67.3 ± 2.8	45 ± 2.0
NMDG-Cl [a]	K^+	not done	47 ± 2.0
NH_4Cl	K^+	65	106 ± 12.4
RbCl	K^+	108 ± 3.0	not done
K-gluconate [a]	Cl^-	67.3 ± 6.0	72.4 ± 3.0
K_2SO_4 [a]	Cl^-	25	not done
KNO_3	Cl^-	72.5 ± 8.1	55 ± 8.5
KBr	Cl^-	70	110
Mannitol	$K^+ + Cl^-$	40 ± 4.0	36.2

[a] Impermeant substituted ion.

BLMV. Moreover, under gradient conditions, separate substitution of K^+ by TMA and Cl^- by gluconate results approximately in the same decrease in calcium transport rate (approx. 30%), while when both ions (K^+ and Cl^-) are substituted simultaneously (i.e. in a medium with mannitol) the rate of Ca^{2+} transport is reduced by about 60% as compared to that obtained with an inwardly directed KCl gradient. We cannot exclude the possibility that the higher Ca^{2+} transport activity in the potassium gluconate medium, as compared to that in mannitol, maybe due to direct effects of K^+ on the Ca^{2+} pump. However, this still represents only about approx. 50% of the total KCl-induced stimulation of the Ca^{2+} transport in these membrane vesicles.

Effect of KCl concentration on initial rates of Ca^{2+} transport

Since the ion substitution experiments suggested a specific requirement for K^+ and Cl^-, we examined the effect of KCl on the Ca^{2+} transport activity. In these experiments the KCl concentration in the assay medium was varied between 0 and 130 mM. Mannitol was added as required to maintain the osmolarity of the medium at 300 mM. In these experiments the effects of both an inwardly directed KCl gradient and KCl equilibrium conditions on the ATP-dependent Ca^{2+} transport rate were examined. The data are shown in Fig. 2. In case of KCl equilibrium, the Ca^{2+} transport activity increased with increasing [KCl] in the assay medium reaching a maximum at about 50 mM KCl. There was no further change in Ca^{2+} transport activity up to 130 mM KCl. When the effect of KCl was examined under gradient conditions, the Ca^{2+} transport rate obtained up to 50 mM KCl was similar to that seen with KCl at equilibrium. At concentrations higher than 50 mM KCl (100 mM and 130 mM), the rate of Ca^{2+} transport in the presence of an inwardly directed KCl gradient is about 10–20% lower than

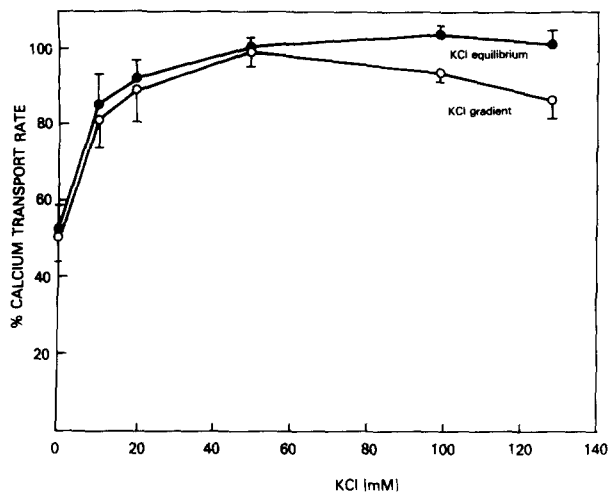

Fig. 2. KCl dependence of ATP-dependent Ca^{2+} transport in BLMV. Parotid BLMV, containing 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5) were incubated for 1 h at 25 °C in media containing various [KCl] (between 5–130 mM), 10 mM Tris-Hepes (pH 7.5) (KCl-equilibrium, ●) or 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5) (KCl-gradient, ○) and then assayed in the presence of 300 mM mannitol (no KCl) or similar varying [KCl] as indicated in the figure. Other assay conditions were similar to that described for Fig. 1. Data are the mean ± S.E. values of three experiments performed (each point assayed in duplicate) with different membrane preparations and have been normalized to the maximal transport rate obtained in each case. For the KCl equilibrium condition, the [KCl] during incubation and assay was similar.

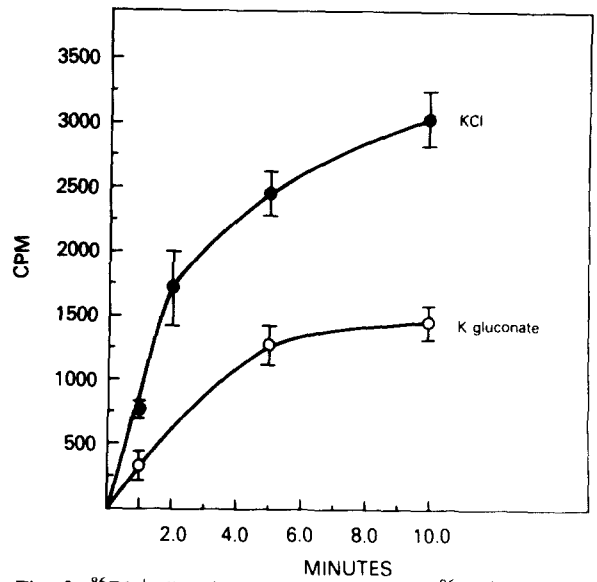

Fig. 3. $^{86}Rb^+$ flux in rat parotid BLMV. $^{86}Rb^+$ flux was assayed by Millipore filtration in media containing 10 mM Tris-Hepes (pH 7.5), 300 mM mannitol and either 10 mM KCl (●) or 10 mM potassium gluconate (○) and 1 mM magnesium gluconate. Vesicles (25–40 μg/ml) were added to 200 μl of the assay mixture at 37 °C to initiate the transport. Cold NMDG-sulfate (2 ml) was added to quench the reaction. Other details of the assay are described in the methods. The figure represents data (mean ± S.E.) from three similar experiments with different membrane preparations, each assay in triplicate.

TABLE II

EFFECT OF FUROSEMIDE ON $^{86}Rb^+$ FLUX

Incubation media contained 10 mM of either KCl or potassium gluconate, 300 mM mannitol, 1 mM magnesium gluconate, 10 mM Tris-Hepes (pH 7.5). Furosemide 0.5 mM was included where indicated. Other details of the assay were as described for Fig. 3. The values have been expressed relative to the uptake observed in the first 10 seconds in the presence of KCl (−furosemide). The number of experiments performed (n), each assayed in triplicate, is indicated.

Incubation medium	Relative uptake of $^{86}Rb^+$	
	−furosemide ($n = 7$)	+furosemide ($n = 2$)
KCl	100 ± 8.0	38 ± 1.0
K-gluconate	40 ± 2.0	33 ± 1.0

that with KCl at equilibrium. We have earlier reported that compared to the Ca^{2+} transport rate in the presence of an inwardly a directed KCl (150 m M) gradient, equilibration of BLMV with 150 mM KCl, induces approx. 20% increase in the initial rate of calcium transport, without any significant change in the steady-state accumulation [14]. We have also shown that the addition of valinomycin under these conditions (inwardly directed 150 mM KCl gradient) further inhibits the initial rate of Ca^{2+} transport by about 25%. These data suggest the possible occurrence of a K^+ conductive pathway in these membranes which is detectable when large K^+ gradients are imposed.

Cl^- dependent $^{86}Rb^+$ flux in BLMV

$^{86}Rb^+$ was used as a tracer to study K^+ flux in BLMV, and to specifically assess its anion requirement. In these experiments, 10 mM K^+ was used in the presence of tracer amounts of $^{86}Rb^+$ and an equimolar concentration (10 mM) of the anion species, either Cl^- (permeant) or gluconate (impermeant). In the presence of Cl^-, there was a time-dependent (Fig. 3) and [Rb]-dependent (not shown) accumulation of $^{86}Rb^+$ in these membranes. When gluconate was substituted for Cl^-, $^{86}Rb^+$ incorporation was approx. 2.5-fold lower (Table II). The flux of $^{86}Rb^+$, in the presence of Cl^-, approached saturation by 5–10 min. In the absence of Cl^-, (i.e. in the gluconate medium), $^{86}Rb^+$ flux was fully saturated by 5 min, and thereafter showed no further increase. These data suggest that a Cl^--dependent K^+ flux occurs in BLMV under conditions similar to those used to follow ATP-dependent Ca^{2+} transport.

To further characterize the Cl^--dependent K^+ flux we assessed the effect of the loop diuretic, furosemide, on the $^{86}Rb^+$ flux in BLMV. Furosemide has been reported to inhibit systems which mediate $Na^+/K^+/Cl^-$ cotransport, KCl symport, and Cl^- flux [16–18]. Since all the experiments described here were performed in the absence of Na^+, the former mode of KCl transport is unlikely to be expressed our experimental system. Table II shows the effect of 0.5 mM furosemide on $^{86}Rb^+$ flux in the presence of 10 mM K^+ and either 10 mM Cl^- or 10 mM gluconate. In a Cl^- containing medium, furosemide inhibited 60% of the $^{86}Rb^+$ flux while in a gluconate containing medium, furosemide was much less effective and only approx. 20% of the $^{86}Rb^+$ flux was inhibited.

Effect of furosemide on ATP-dependent Ca^{2+} uptake in BLMV

In order to examine the association between ATP-dependent Ca^{2+} transport and Cl^--depen-

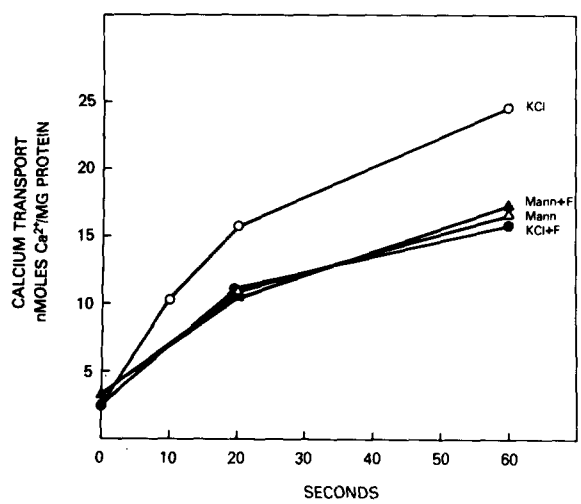

Fig. 4. Effect of furosemide on ATP-dependent Ca^{2+} transport in BLMV. Membrane vesicles were incubated in a medium containing 300 mM mannitol, 10 mM Tris-Hepes (pH 7.5) at 30 °C for 1 h with (●, ▲) or without (○, △) 0.5 mM furosemide. Ca^{2+} transport was assayed in the presence of 150 mM KCl (○, ●) or 300 mM mannitol (△, ▲) 1 mM Tris-Hepes (pH 7.5) and 12.5 μM Ca^{2+} (total). The data are representative of the results from three experiments with different membrane preparations. Other details of assay are as described for Fig. 1.

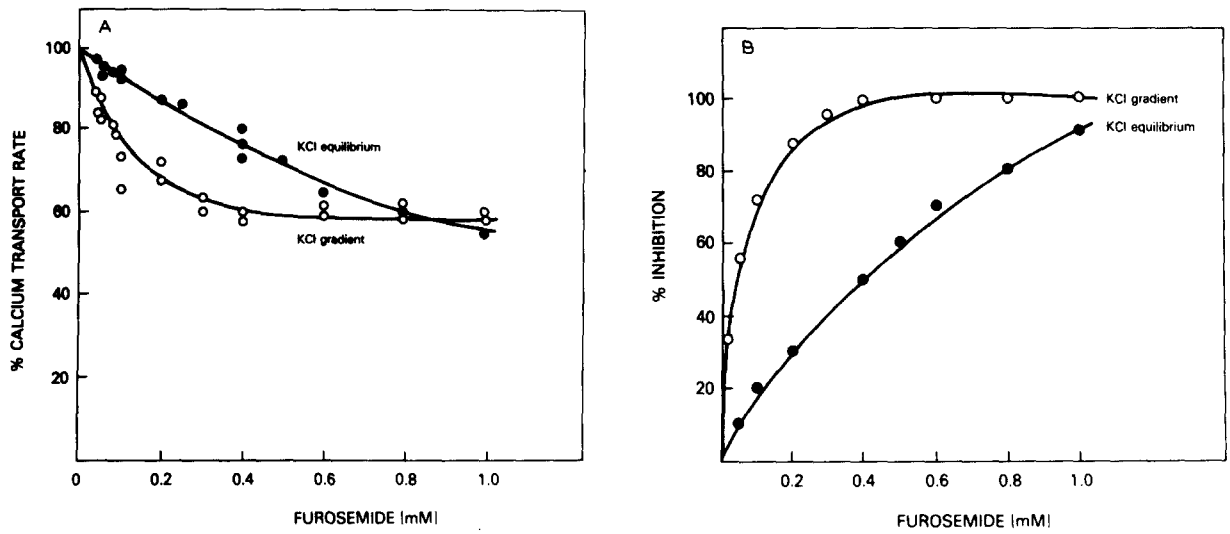

Fig. 5. Effect of various concentrations of furosemide on ATP-dependent calcium transport in the presence of KCl equilibrium and KCl gradient conditions. Membranes were preincubated (1 h at 25 °C) either in a medium containing 300 mM mannitol (○) or 150 mM KCl (●) with various concentrations of furosemide. Thereafter Ca^{2+} transport was assayed in a medium containing 150 mM KCl, 1 mM magnesium gluconate, 1 mM Tris-ATP, 10 mM Tris-Hepes (pH 7.5), 12.5 μM Ca^{2+} (total), and furosemide at a concentration equal to that used during preincubation. (A) Shown are results from experiments which have been normalized to the transport rate obtained in the absence of furosemide in each assay condition, i.e. KCl equilibrium (●) and KCl gradient (○). (B) The data from (A) have been corrected for the Ca^{2+} transport obtained in the absence of KCl and expressed relative to the rate of Ca^{2+} transport at KCl equilibrium (maximal Ca^{2+} transport rate in the absence of furosemide).

dent K^+ flux, we tested the effect of furosemide on the ATP-dependent Ca^{2+} transport activity in BLMV. As shown Fig. 4, furosemide (0.5 mM) induces approx. 40% inhibition of this Ca^{2+} transport activity. The inhibition is apparent both in initial rate as well as steady state measurements. The level of activity retained in the presence of 0.5 mM furosemide is thus equal to the level seen in a mannitol medium. This suggests that furosemide completely inhibits the KCl-stimulated component ATP-dependent calcium transport. Moreover, there is no inhibition of calcium transport by furosemide (up to 1 mM) when the activity is assayed in a KCl-free medium (Fig. 4). We also examined the effect of furosemide on ATP-dependent Ca^{2+} transport assayed in a medium containing potassium gluconate. In this medium, as in the mannitol medium, there was no effect of furosemide (data not shown). Therefore, furosemide-induced inhibition of Ca^{2+} transport appears to be expressed only in the presence of both K^+ and Cl^-. Bumetanide, a specific inhibitor of the $Na^+/K^+/2Cl^-$ cotransport activity [16] was equally effective (at 0.5 mM) in inhibiting both initial and steady-state Ca^{2+} transport activity as 0.5 mM furosemide (data not shown). However, it is not possible, at the present time, to specify the KCl flux system which accounts for the bumetanide and furosemide sensitivities of the ATP-dependent Ca^{2+} transport activity.

The inhibitory effect of furosemide on the initial rates of Ca^{2+} transport in a KCl-containing medium was measured next as a function of furosemide concentration (Fig. 5A). In initial experiments, membrane vesicles were preincubated in a mannitol medium with various concentrations of furosemide for an hour at 25–28 °C, and Ca^{2+} transport rates were then determined (see Methods) in assay medium (see Methods) containing 150 mM KCl and furosemide at concentrations equal to that used during the preincubation. In this KCl-gradient condition half-maximal inhibition was seen approx. 0.05 mM furosemide and maximum inhibition of transport (40%) was achieved by about 0.4 mM furosemide. Increasing the concentration of furosemide up to 1.0 mM did not further inhibit transport. These data suggest that in the presence of a KCl gradient, there is a

flux of KCl into the vesicles which is blocked by furosemide. This observation agrees well with the ability of furosemide to inhibit ^{86}Rb flux in the presence of KCl (above).

To determine if Ca^{2+} transport was associated with $K^+ + Cl^-$ flux under steady-state (KCl equilibrium) conditions, membrane vesicles were preincubated in the presence of 150 mM KCl and furosemide for an hour. Thereafter, calcium transport was measured in 150 mM KCl assay medium, along with the various concentrations of furosemide. Under these KCl-equilibrium conditions, maximum inhibition of Ca^{2+} transport was achieved between 0.8 and 1.0 mM furosemide, while half-maximal inhibition occurred at approx. 0.3 mM furosemide. Thus furosemide is 3–6-times less effective in blocking Ca^{2+} transport measured in a KCl-equilibrium condition than in a KCl-gradient condition. In Fig. 5A, the transport rate obtained in the absence of furosemide has been taken as control (100%), and the activities obtained in KCl-equilibrium and KCl-gradient conditions have been normalized to their respective controls. Fig. 5B depicts the same data, relative to the maximum rate of KCl-dependent Ca^{2+} transport, i.e. in KCl (150 mM) equilibrium after correcting for the KCl-independent (i.e. in mannitol) Ca^{2+} transport activity. When the furosemide inhibition of only the KCl-supported Ca^{2+} transport activity was examined, the ability of furosemide to inhibit calcium transport at KCl equilibrium was 10-fold lower as compared to that with an inwardly directed KCl gradient. Half-maximal inhibition shifted from approx. 0.04 mM with KCl gradient to 0.4 mM in the case of KCl equilibrium. These data suggest that intravesicular KCl, can support a considerable level of calcium transport, even when KCl influx is reduced. However, in the absence of intravesicular KCl, a reduction in KCl influx induces a rapid inhibition of calcium transport.

Evidence for K^+ conductance in BLMV

Our earlier observations [14] are indicative of a transfer of charge during ATP-dependent flux of Ca^{2+} in BLMV. In view of the data presented above, we hypothesized the existence of a K^+ conductive pathway, which could mediate K^+ efflux from vesicles during Ca^{2+} transport, and thus

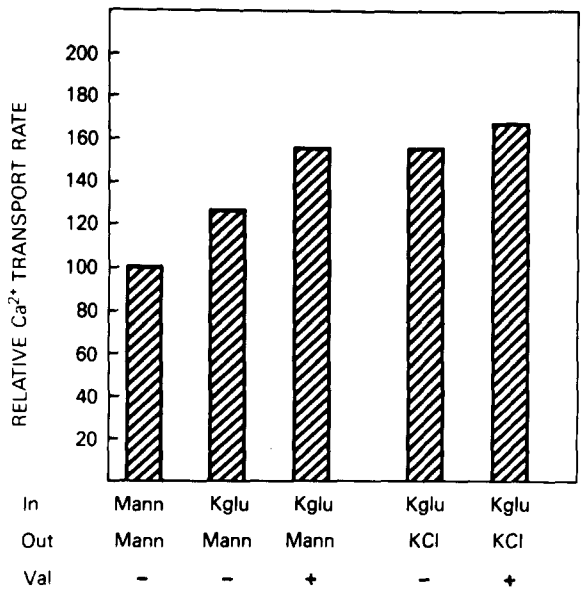

Fig. 6. Effect of outwardly directed K^+ gradients on Ca^{2+} transport. Rat parotid gland BLMV were incubated at 30 °C for 1 h in the presence of either 300 mM mannitol or 150 mM potassium gluconate, and 10 mM Tris-Hepes (pH 7.5). Ca^{2+} transport rates were measured in the specified assay medium (out) containing either 300 mM mannitol or 150 mM KCl and 10 mM Tris-Hepes (pH 7.5), 1 mM magnesium gluconate, 12.5 μM Ca^{2+} (total). Valinomycin, 5 μM, (+) or equivalent (5 μl) ethanol (−) were added to the assays indicated in the figure. Other details of assay were as described in Methods.

compensate, fully or partially, for the charge generated during the ATP-dependent influx of Ca^{2+}.

We assessed the presence of a K^+ conductance, associated with ATP-dependent Ca^{2+} transport, by creating outwardly directed K^+ gradients in the absence of Cl^-. The data are shown in Fig. 6. In these experiments, vesicles were loaded with potassium gluconate or mannitol and then diluted into a medium containing mannitol or KCl. In the mannitol medium, potassium gluconate-loaded vesicles had approx. 25% higher initial rate of Ca^{2+} transport than mannitol-loaded vesicles. This stimulation of Ca^{2+} transport by an outwardly directed K^+ gradient is comparable to the inhibition produced by an inwardly directed K^+ gradient (see Fig. 2). Addition of valinomycin to potassium gluconate vesicles further stimulated the Ca^{2+} transport rate by approx. 30%, i.e. total stimulation of approx. 60%. The initial rate of transport achieved under these conditions (outward K^+

gradient + valinomycin) was similar to that of potassium gluconate loaded vesicles in a KCl medium. In these experiments, KCl-equilibrated vesicles when diluted into a KCl medium showed approx. 70% stimulation over Ca^{2+} transport in a mannitol medium (data not shown). In the absence of valinomycin, the highest Ca^{2+} transport activity was obtained when potassium gluconate loaded vesicles were diluted into KCl containing assay medium. These data show that an optimal initial rate of Ca^{2+} transport can be induced even when the extravesicular $[K^+]$ is < 5 mM (i.e. with mannitol), provided sufficient K^+ is present inside the vesicles.

Discussion

The results presented in this report demonstrate that ATP-dependent Ca^{2+} transport in inverted basolateral membrane vesicles isolated from rat parotid glands is maximally stimulated in the presence of K^+ and Cl^-. More than 95% of the KCl-dependent activation can be inhibited by furosemide, by a mechanism which is independent of an effect on the calcium transport per se. Two observations support this latter conclusion; (i) in the presence of KCl and furosemide, at maximal inhibitory concentrations, the level of calcium transport is equal to that obtained in the absence of KCl (i.e. in mannitol or NMDG-gluconate medium), and (ii) in the absence of KCl, furosemide, even at maximal concentrations, exerts no inhibitory effects on Ca^{2+} transport. Thus, KCl-dependent activation of the ATP-dependent Ca^{2+} transport in parotid BLMV appears to involve a furosemide-sensitive K^+ and Cl^- transport process.

Our data also clearly demonstrate that both the K^+ flux in BLMV and its activation of Ca^{2+} transport, are Cl^- dependent. We are presently unable to distinguish whether this flux represents a coupled K^+/Cl^- cotransport system or coupled K^+ and Cl^- conductance pathways. We have further shown the involvement of a K^+ conductance during ATP-dependent Ca^{2+} transport. However, it appears that this K^+ conductance is distinct from the furosemide-sensitive, Cl^--dependent K^+ pathway, since as shown the data in Fig. 6, it does not require Cl^-.

It has been suggested in other membrane vesicle systems e.g. plasma membrane [6,7,10], sarcoplasmic reticulum [8,11,19] and endoplasmic reticulum [9], that during ATP dependent calcium transport there is an electrogenic flux of Ca^{2+}. We have reported [14] that the ATP-dependent Ca^{2+} transport mechanism in rat parotid BLMV, can be modulated by preimposed membrane potentials and thus involves a transfer of electric charge. These data are suggestive of an electrogenic Ca^{2+} transport mechanism. Therefore, depending on the inherent ion permeability of the membrane electrically coupled ion fluxes could provide charge compensation, and allow the expression of maximal Ca^{2+} transporting activity. Indeed, in sarcoplasmic reticulum vesicles, it has been suggested by Meissner [11] that the flux of secondary ions like Na^+, K^+, Cl^- and H^+ can modulate calcium transport by their charge compensatory effects and channels for Na^+, K^+, and anion and a pathway for $H^+/(OH^-)$ have been identified. Muallem et al. [9] have suggested the presence of such a pathway (i.e. K^+, Cl^-) in rat liver microsomes, which is inhibited selectively by furosemide. They have also reported the presence of a discrete K^+-conductive pathway, sensitive to triethylammonium (an inhibitor of Ca^{2+}-activated K^+ channels), in the same membrane preparations. We have not been able to observe any effect of the K^+ channel blockers, triethylammonium or tetrapropylammonium (data not shown) on Ca^{2+} transport activity. However, this may be due to an effect of the inverted vesicle preparation which limits access of the drugs to the sensitive site. Additionally, furosemide and bumetanide-sensitive KCl cotransport systems, or K^+ and Cl^- coupled conductance pathways, have been identified in plasma membranes from a variety of cell types [17,20]. These Cl^--dependent K^+ fluxes are reportedly quiescent in the resting cells and are specifically activated during a regulatory volume decrease or following modification of membrane sulfhydryl groups [21]. The regulatory volume decrease has been described to involve the loss of K^+ and Cl^- from the cell, with a 1 : 1 stoichiometry, driven by the K^+ concentration gradient. KCl fluxes during regulatory volume decrease in rat parotid acinar cells have not yet been characterized. Thus, the full physiological significance

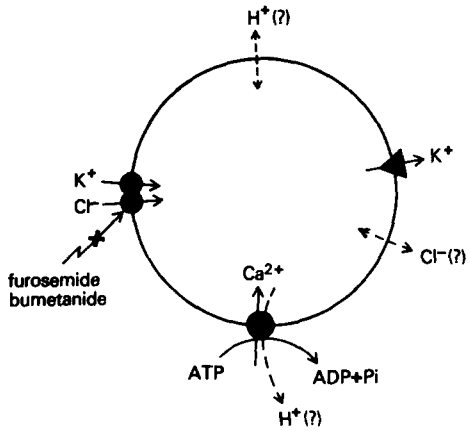

Fig. 7. Proposed ion flux pathways related to ATP-dependent Ca^{2+} transport in BLMV. See text for details.

of the occurrence of such a transporter in our membrane vesicle preparation is as yet unestablished.

Based on the data presented we propose (Fig. 7) that the ATP-dependent influx of Ca^{2+} into BLMV, which is likely mediated by the Ca^{2+}/Mg^{2+}-ATPase, is modulated by K^+ and Cl^- fluxes. In the presence of intravesicular K^+, accumulation of an intravesicular positive charge due to Ca^{2+} influx, would stimulate an efflux of K^+ through a K^+-conductive pathway, possibly accompanied by Cl^- movement. Intravesicular K^+ could be replenished by a Cl^--dependent, furosemide-sensitive, K^+ influx into BLMV. In the absence of intravesicular K^+, H^+ movement could compensate for Ca^{2+} influx although not as effectively as intravesicular K^+ [14], resulting in a lower Ca^{2+} transport rate in the absence of K^+. The possibility exists of a partially electrogenic Ca^{2+} influx, due to H^+: Ca^{2+} exchange catalysed by the Ca^{2+}/Mg^{2+}-ATPase, as suggested for the erythrocyte plasma membrane calcium transporter [10]. However, we have not observed any change in Ca^{2+} transport, either in a KCl or mannitol medium, after the addition of FCCP [14]. Obviously, further studies are needed, e.g. using artificial lipid vesicles with the reconstituted transporter, to provide more direct information regarding the molecular mechanisms of the Ca^{2+} flux and to differentiate between direct effects of ions on the transporter and effects of secondary ion fluxes during ATP-dependent Ca^{2+} transport in situ.

It is nonetheless important to emphasize the relationship between the KCl activation of ATP-dependent Ca^{2+} transport and the furosemide-induced inhibition of ATP-dependent Ca^{2+} transport, in the studies reported here. In view of the widely reported stimulatory effect of KCl on ATP-dependent calcium transport in plasma membranes [6], it is particularly significant that more than 95% of this stimulation appears to be associated with a possible coupled flux of K^+ and Cl^-. This KCl flux, together with a K^+ conductive pathway, would allow the recycling of K^+ across the vesicular membrane (Fig. 7), which could, by electrical coupling provide the necessary compensation for the charge generated during ATP-dependent Ca^{2+} flux.

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