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B L Blitzer, … , B R Hostetler, K A Scott

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Research Article

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Hepatic Adenosine Triphosphate-dependent Ca^{2+} Transport Is Mediated by Distinct Carriers on Rat Basolateral and Canalicular Membranes

Bennett L. Blitzer, Barbara R. Hostetler, and Kimberly A. Scott
Liver Study Unit, Division of Digestive Diseases, Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Abstract

To characterize and localize hepatic plasma membrane ATP-dependent Ca^{2+} transport and Na^+/Ca^{2+} exchange, studies were performed using highly purified rat basolateral and canalicular membrane vesicles. ATP-dependent Ca^{2+} transport activity was present in vesicles from both domains, insensitive to azide, oligomycin, oxalate, calmodulin, and calmidazolium, and virtually abolished at pH 6.8. However, basolateral and canalicular transport differed significantly. While basolateral transport was markedly stimulated by 1 mM Mg^{2+}, canalicular transport was Mg^{2+} independent. Basolateral transport was similar at pH 7.4 and 8.0 but canalicular activity was stimulated fourfold at pH 8.0. Both Ca^{2+} K_m [1.4 ± 0.1 (SE) $\cdot 10^{-8}$ vs. $4.8\pm0.7 \cdot 10^{-8}$ M] and V_{max} (3.6 ± 0.1 vs. 9.0 ± 0.6 nmol mg^{-1} protein min^{-1}) were lower in basolateral than in canalicular vesicles. Basolateral transport was somewhat more nucleotide specific (for ATP) and sensitive to vanadate (IC_{50} 130 vs. 500 μM, respectively) than was canalicular transport. Na^+/Ca^{2+} exchange activity was not detected in membranes from either domain. These studies suggest that hepatic ATP-dependent Ca^{2+} transport is mediated by domain-specific carriers on the basolateral and canalicular membranes.

Introduction

In eukaryotic cells, the concentration of intracellular Ca^{2+} (≈ 0.1 μM) is $\sim$ 10,000-fold lower than that of extracellular Ca^{2+} (≈ 1 mM). Two separate mechanisms have been identified which singly or in combination maintain this steep cation gradient in a wide variety of cell types (1). One mediates Na^+/Ca^{2+} exchange and the other is an ATP-dependent Ca^{2+} transporter.

Although several studies have established the presence of ATP-dependent Ca^{2+} transport in plasma membrane vesicles isolated from mammalian liver (2–5), it is not known whether this process is localized to the basolateral (sinusoidal and lateral) or canalicular (apical) domain of the hepatocyte. It is conceivable that a single transporter could be exclusively localized to either domain or be present on both surfaces. Alternatively, ATP-dependent Ca^{2+} transport could be present at both surfaces but be mediated by functionally distinct carriers.

A recent report of ATP-dependent Ca^{2+} transport by rat basolateral and canalicular liver plasma membrane vesicles concluded that this activity is exclusively localized to the canalicular domain (6). However, due to the relative impurity of the membrane preparations used, the validity of this conclusion is uncertain. In addition, it is not known whether a Na^+/Ca^{2+} exchanger is present on either domain of the liver plasma membrane.

Therefore, in the present study, we utilized recently developed techniques for the preparation of highly purified rat liver plasma membrane vesicles in order to both characterize and localize hepatic ATP-dependent Ca^{2+} transport and Na^+/Ca^{2+} exchange. These studies provide evidence for distinct ATP-dependent Ca^{2+} transporters on the basolateral and canalicular surface of the hepatocyte which differ significantly with respect to several important characteristics. These mechanisms are apparently the sole mode of Ca^{2+} efflux by the liver cell since no evidence for Na^+/Ca^{2+} exchange was found.

Methods

Preparation of basolateral and canalicular plasma membrane vesicles from rat liver. Basolateral and canalicular plasma membrane vesicles were prepared from rat liver by previously described, well-validated techniques. Basolateral membrane vesicles were isolated using a self-generating Percoll gradient method as previously described in this laboratory (7). Based on marker enzyme analysis, 97–98% of the surface membranes present in the preparation are estimated to have arisen from the basolateral membrane domain and only 2–3% from the canalicular surface (7). Canalicular liver plasma membrane vesicles were prepared according to the method of Inoue et al. (8) except that the final ultracentrifuge spin was performed with the suspension buffer described below. Measurement of marker enzyme activities in our laboratory demonstrated substantial enrichment of this preparation with canalicular membranes and a lack of significant contamination by subcellular organelles. The canalicular markers, alkaline phosphatase (n = 30) and Mg^{2+}-ATPase (n = 8), were enriched 99 ± 3 (SE) and 81 ± 6-fold, respectively, compared to homogenate. In contrast, the basolateral marker, Na^+,K^+-ATPase (n = 8), was enriched only 3.4 ± 0.8-fold. The endoplasmic reticulum marker, NADPH-cytochrome c reductase (n = 3), and the mitochondrial marker, succinate-cytochrome c reductase (n = 3), were not enriched (0.46 ± 0.07-fold and 0.47 ± 0.08-fold, respectively). Calculations based upon the enrichments reported here for canalicular and basolateral markers suggest that while 80% of the surface membranes in this preparation are of canalicular origin, 20% are from the basolateral domain.

The final membrane pellets from both the basolateral and canalicular preparations were suspended in 1–4 ml of suspension buffer (10 mM Hepes/KOH, pH 7.5, 300 mM sucrose) by passage through a 23-gauge hypodermic needle three times ($\sim$ 4–8 mg protein/ml). The

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Address reprint requests to Dr. Blitzer, Liver Study Unit, University of Cincinnati College of Medicine, 231 Bethesda Avenue (Room 6560), Cincinnati, OH 45267.

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suspended membranes were stored at −70°C and frozen aliquots were thawed and vigorously resuspended with a Vortex mixer immediately before use. Preliminary studies demonstrated that Ca^{2+} transport was not affected even after storage for several weeks under these conditions.

Determination of vesicle sidedness. The sidedness of canalicular vesicles was determined by quantitating the relative particle density of convex and concave vesicles seen in freeze-fracture electron micrographs as previously described for basolateral vesicles (7). Convex vesicles with relatively high particle densities and concave vesicles with relatively low particle densities were considered to be "right side-out" while vesicles with the opposite particle densities were considered "inside-out." From this analysis, it was determined that ~ 75% of the canalicular vesicles were oriented "right side-out," a value similar to that of 72% for basolateral vesicles previously reported by us (7).

Uptake of ^{45}Ca by basolateral and canalicular vesicles. For studies of nucleotide-dependent Ca^{2+} uptake, membrane vesicles were preloaded with a solution containing 50 mM KCl, ±1 mM $MgCl_2$, 1 mM ouabain, 200 mM sucrose, and 10 mM Hepes/KOH, pH 7.4 by incubating the suspended vesicles with a buffer of appropriate ion and substrate composition for 2 h at room temperature followed by storage on ice until uptake measurements were begun. 5 μl of membrane suspension containing 10–20 μg of protein were then preincubated in a test tube in a 37°C water bath. Uptake of ^{45}Ca at 37°C was initiated by adding 95 μl of incubation buffer (final concentrations: 50 mM KCl, ±1 mM $MgCl_2$, 1 mM ouabain, 0.5 mM EGTA, 200 mM sucrose, 10 mM Hepes/KOH, pH 7.4) containing varying amounts of $^{45}CaCl_2$, unlabeled $CaCl_2$, and nucleotides. A computer program was used to determine the total calcium concentration required to obtain a given free calcium concentration in the presence of varying ATP and cation concentrations and pH (9). The tube was mixed vigorously and returned to the water bath for varying times. Timed uptakes were terminated by the rapid addition of 3.5 ml of ice-cold stop solution (50 mM KCl, 0.5 mM EGTA, 200 mM sucrose, 10 mM Hepes/KOH, pH 7.4) and then immediate vacuum filtration through 0.45 μm Millipore filters (HAWP). The test tube was rinsed with an additional 3.5 ml of stop solution and the contents filtered. Finally, the filter was washed twice with 3.5 ml of stop solution. Filters were rendered transparent with 6 ml of Ready-Solv HP/b (Beckman Instruments, Inc., Fullerton, CA) and counted on a Beckman LS 6800 liquid scintillation counter. All uptakes were corrected for a blank in which ice-cold stop solution was added to the vesicles before the addition of ^{45}Ca. Inhibitors, ionophores and other agents were added to the preincubation and/or incubation media under conditions as described in the figure legends or text.

The effects of Na^+ gradients on calcium transport by basolateral or canalicular vesicles were assessed in three ways. First, vesicles were preloaded with Na^+ or K^+ and the effects of outwardly directed cation gradients on Ca^{2+} uptake were compared. Second, vesicles were preloaded with ^{45}Ca and the effects of inwardly directed Na^+ or K^+ gradients on Ca^{2+} efflux were studied. Finally, after the influx of ^{45}Ca in the presence of ATP and Mg^{2+}, the effects of added external Na^+ or K^+ on Ca^{2+} efflux were assessed.

Statistical methods. Unless otherwise indicated, the figures depict representative experiments in which uptakes were performed in quadruplicate. Experiments were generally repeated at least two to three times with different membrane preparations. Student's t test was used to test the significance of differences among means. For kinetic studies, uptake data were obtained for multiple membrane vesicle preparations and pooled. A weighted least squares fit of the individual data points was performed on a computer as previously described (7, 10). Derived kinetic parameters (K_m and V_{max}) for basolateral and canalicular vesicles were tested for significant differences with a Z test (11).

Chemicals. Phenylmethylsulfonyl fluoride, Percoll, Hepes, ouabain, EGTA, oligomycin, bovine brain calmodulin, nucleotides and sodium orthovanadate were obtained from Sigma Chemical Co. (St. Louis, MO). A23187 was from Calbiochem-Behring Corp. (La Jolla, CA) and calmidazolium from Boehringer-Mannheim Biochemicals (Indianapolis, IN). $^{45}CaCl_2$ was from New England Nuclear (Boston, MA). All other chemicals were of reagent grade or of the highest purity available.

Results

Effects of ATP and Mg^{2+} on Ca^{2+} uptake by basolateral and canalicular vesicles. In basolateral vesicles, ATP (1 mM) in the presence of 1 mM Mg^{2+} markedly stimulated Ca^{2+} (10^{-8} M) uptake (Fig. 1, *left*). Peak uptake (at 5 min) under these conditions was sevenfold higher than that observed in the presence of Mg^{2+} alone. In the absence of Mg^{2+}, ATP stimulated peak uptake only twofold.

To assess whether the stimulation of basolateral Ca^{2+} uptake by ATP in the presence of Mg^{2+} was due to increased transport or binding, the effects of the Ca^{2+} ionophore A23187 (2 μM) were studied (Fig. 2). When this agent was added 3 min after uptake was initiated, vesicle-associated Ca^{2+} fell promptly to levels comparable to those seen when uptake was performed in the absence of ATP. In contrast, dimethyl sulfoxide (final concentration = 0.5%), in which the ionophore was dissolved, did not significantly affect vesicle-associated Ca^{2+} when added alone [1.56±0.11 (SD) vs. 1.64±0.08 nmol mg^{-1} protein at 5 min]. Release of accumulated Ca^{2+} by A23187 implies that ATP and Mg^{2+} stimulated Ca^{2+} transport and not merely binding.

The effects of ATP and Mg^{2+} on Ca^{2+} uptake by canalicular vesicles (Fig. 1, *right*) differed from those observed in basolateral vesicles. While ATP stimulated peak uptake fourfold, Ca^{2+} transport in the presence of both ATP and Mg^{2+} (1 mM) was not greater than in the presence of ATP alone. Further, neither higher (5 mM) nor lower (0.1 mM) Mg^{2+} concentrations stimulated canalicular ATP-dependent Ca^{2+} transport (data not shown). Release of accumulated Ca^{2+} by A23187 similar to that seen in basolateral vesicles was observed in canalicular vesicles (data not shown).

Nucleotide specificity. Basolateral Ca^{2+} uptake was highly specific for ATP (Fig. 3, *left*). Nucleotide-dependent transport

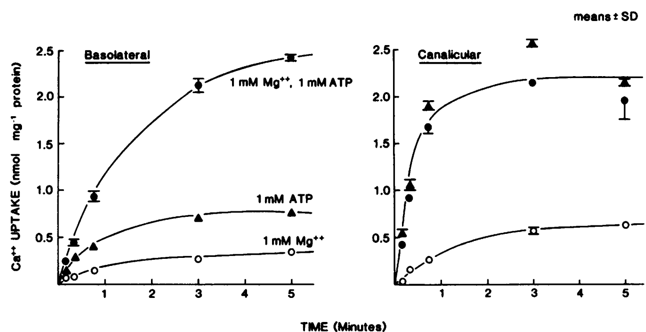

Figure 1. Effects of ATP and Mg^{2+} on Ca^{2+} uptake by basolateral and canalicular vesicles. Vesicles were preloaded with a solution containing 50 mM KCl, 1 mM ouabain, 200 mM sucrose, 10 mM Hepes/KOH, pH 7.4, with (○, ●) or without (▲) 1 mM $MgCl_2$. Uptake of ^{45}Ca at 37°C was then assayed in a medium containing (final concentrations) 50 mM KCl, 1 mM ouabain, 0.5 mM EGTA, 200 mM sucrose, 10 mM Hepes/KOH, pH 7.4, ^{45}Ca and unlabeled Ca^{2+} to yield a free Ca^{2+} concentration of 10^{-8} M, with (○, ●) or without (▲) 1 mM Mg^{2+} and with (▲, ●) or without (○) Na_2ATP. Each point represents the mean±SD of four determinations for a representative membrane preparation. Where error bars are not shown, the SD is contained within the limits of the symbol.

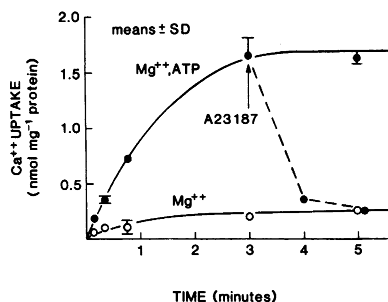

Figure 2. Effects of A23187 (2 μM) on Ca^{2+} (10^{-8} M) taken up by basolateral vesicles in the presence of ATP and Mg^{2+}. Conditions for Ca^{2+} uptake in the presence of Mg^{2+} (1 mM) with (●) or without (○) ATP (1 mM) were as described in the legend to Fig. 1. A23187 was added 3 min after uptake in the presence of ATP was initiated and vesicle-associated Ca^{2+} was measured at 4 and 5 min (- - ● - -).

(calculated as the difference between uptake in the presence of the nucleotide and uptake in the presence of buffer alone) was similar for the disodium and Tris salts of ATP. In contrast, with UTP, GTP, AMP-PNP[1] (a nonhydrolyzable analogue of ATP) and NPP, Ca^{2+} transport rates were only 15, 18, 13, and 2% of ATP-dependent rates, respectively.

Canalicular nucleotide-dependent transport (Fig. 3, *right*) was also similar with the disodium and Tris salts of ATP. However, UTP, GTP, and AMP-PNP stimulated Ca^{2+} uptake to a relatively greater degree than in basolateral vesicles (42, 37, and 28% of ATP-dependent rates, respectively).

Effects of azide, oligomycin, and oxalate (Fig. 4). Neither basolateral nor canalicular ATP-dependent Ca^{2+} transport was significantly inhibited by oligomycin (5 μg/ml) or azide (20 mM), agents known to block mitochondrial Ca^{2+} pumps (12). Further, distinct from microsomal pumps, uptake was not stimulated by 5 mM oxalate (12).

pH studies (Fig. 5). At pH 6.8, at which microsomal Ca^{2+} pump activity is maximal (12, 13), both basolateral and canalicular ATP-dependent Ca^{2+} transport were largely inactivated, falling to only 5 and 10% of that observed at pH 7.4, respectively. However, raising the medium pH to 8.0 affected transport activity quite differently in vesicles from the two membrane domains. While basolateral transport at pH 8.0 was similar to that at 7.4, canalicular transport was fourfold higher at the more alkaline pH.

Kinetic studies. The kinetics of ATP-dependent Ca^{2+} transport were determined by incubating vesicles with varying free Ca^{2+} concentrations ($5 \cdot 10^{-9}$ to $5 \cdot 10^{-7}$ M) in the presence or absence of ATP. At the highest Ca^{2+} concentrations studied ($5 \cdot 10^{-7}$ and $1 \cdot 10^{-7}$ M for basolateral and canalicular vesicles, respectively), uptake was linear through 20 s. In addition, extrapolation of the line relating uptake to time to the y axis yielded an intercept which was only 6% of total uptake at 20 s (data not shown), indicating a relatively small contribution of binding to initial rate measurements determined at 20 s. Accordingly, uptakes at 20 s were used to estimate initial rates of ATP-dependent Ca^{2+} uptake.

In basolateral vesicles, ATP-dependent Ca^{2+} uptake was a saturable function of Ca^{2+} concentration and well described by a rectangular hyperbola (Fig. 6, *left*). The derived kinetic parameters included a Ca^{2+} K_m of 1.4±0.1 (SE) $\cdot 10^{-8}$ M and a V_{max} of 3.6±0.1 nmol mg^{-1} protein min^{-1}. The relationship of canalicular uptake to Ca^{2+} concentration (Fig. 6, *right*) was hyperbolic only over concentrations up to 10^{-7} M. In this range, the Ca^{2+} K_m and V_{max} ($4.8 \pm 0.7 \cdot 10^{-8}$ M and 9.0±0.6 nmol mg^{-1} protein min^{-1}, respectively) were significantly higher than in basolateral vesicles ($P < 0.001$). At Ca^{2+} concentrations above 10^{-7} M (e.g., $3 \cdot 10^{-7}$ M, and $5 \cdot 10^{-7}$ M), canalicular uptake in the presence of ATP and Mg^{2+} was not greater than in the presence of Mg^{2+} alone (Fig. 7). Therefore, canalicular ATP-dependent transport was not detectable in this Ca^{2+} concentration range.

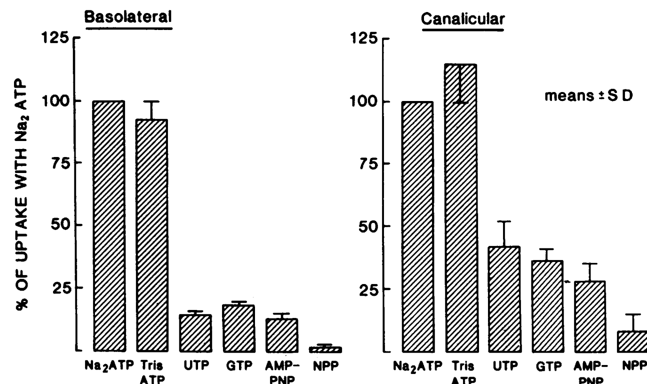

Figure 3. Nucleotide specificity of basolateral and canalicular Ca^{2+} uptake. Ca^{2+} (10^{-8} M) uptake at 20 s was measured in the presence of 1 mM Mg^{2+} under the same conditions outlined in the legend to Fig. 1 except that various nucleotides or nucleotide analogues (1 mM) were substituted for ATP. Nucleotide-dependent uptakes were calculated as the difference between uptake in the presence and absence of the nucleotide and expressed as a percentage of that observed with Na_2ATP. Depicted are the means±SD for four separate basolateral and canalicular vesicle preparations.

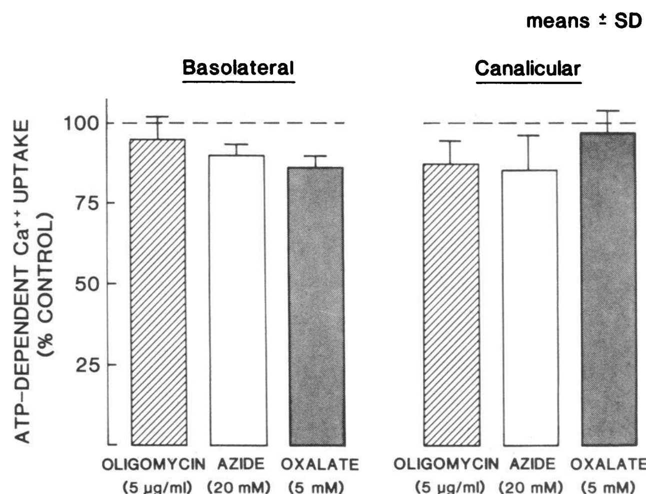

Figure 4. Effects of oligomycin (5 μg/ml), azide (20 mM), and oxalate (5 mM). Calcium (10^{-8} M) uptake at 20 s was measured under the conditions outlined in the legend to Fig. 1 and ATP-dependent uptake calculated from the difference between uptakes measured in the presence and absence of the nucleotide. ATP-dependent Ca^{2+} uptake in the presence of oligomycin, azide, or oxalate is expressed as a percentage of that observed in the absence of these agents. Data are the means±SD for three basolateral and four canalicular vesicle preparations.

1. *Abbreviations used in this paper:* AMP-PNP, 5'-adenylimidodiphosphate; NPP, *p*-nitrophenyl phosphate.

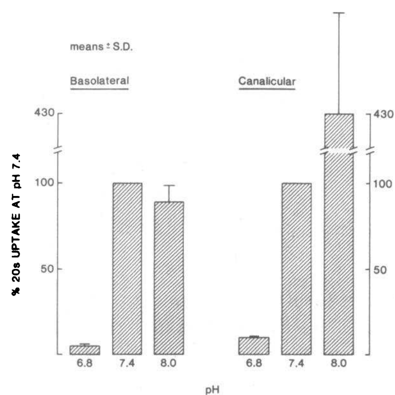

Figure 5. pH studies. ATP-dependent Ca^{2+} ($2 \cdot 10^{-8}$ M) uptake at 20 s was measured as described in the legends to Figs. 1 and 2 except that the pH of the Hepes preincubation and incubation buffers was adjusted to 6.8, 7.4, or 8.0 with varying amounts of KOH. Uptakes are expressed as a percentage of the value at pH 7.4. Data are the means±SD for four separate basolateral and canalicular vesicle preparations.

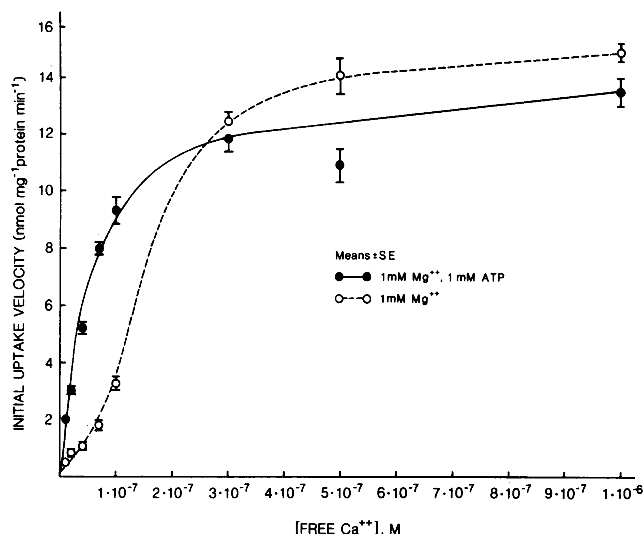

Figure 7. Effects of Ca^{2+} concentration on canalicular uptake (±ATP). Canalicular vesicles were incubated in the presence or absence of ATP and the initial velocity of Ca^{2+} uptake was determined at 20 s over a range of free Ca^{2+} concentrations.

Effects of vanadate, calmodulin, and calmidazolium. Vanadate, a known inhibitor of various ATPases (1, 2), inhibited basolateral ATP-dependent Ca^{2+} transport in a dose-dependent manner (Fig. 8). The vanadate concentration producing half-maximal inhibition (IC_{50}) was ~ 130 μM. Canalicular transport was relatively less sensitive to vanadate (IC_{50} ≈ 500 μM).

Incubation with calmodulin (5 μg/ml) did not affect ATP-dependent Ca^{2+} transport by either basolateral (92±2 and 100±6% control uptake at 20 s or 3 min, respectively) or canalicular vesicles (105±2 and 98±1% control uptake at 20 s or 3 min, respectively). In addition, 2 μM calmidazolium (R24571), a calmodulin antagonist, had no effect on basolateral or canalicular transport (99±10 and 96±4% control uptake at 20 s for basolateral and canalicular vesicles, respectively).

Na^+ gradient studies. Possible effects of Na^+ gradients on Ca^{2+} transport were assessed in several ways. First, an outwardly directed Na^+ gradient (63 mM inside and 3.1 mM outside the vesicle) did not stimulate Ca^{2+} (10^{-8} M) uptake compared to K^+ preloading in either basolateral or canalicular vesicles (Fig. 9). Studies using higher Ca^{2+} concentrations (1 and 20 μM) also failed to demonstrate stimulation of Ca^{2+} uptake by an outwardly directed Na^+ gradient. Second, efflux experiments were performed in basolateral vesicles in which the vesicles were preequilibrated with 25 μM ^{45}Ca and then exposed to inward Na^+ or K^+ gradients (100 mM Na^+ or K^+ outside and 75 mM K^+ inside). Release of ^{45}Ca into the medium which contained 1 mM EGTA was similar under the two conditions. Finally, basolateral or canalicular vesicles were loaded with ^{45}Ca in the presence of ATP and Mg^{2+} for 3 min and then exposed to 50 mM Na^+ or K^+. Again, no difference in the release of ^{45}Ca was observed.

Discussion

The present study demonstrates that ATP-dependent Ca^{2+} transport activity is found on both the basolateral and canalicular domains of the hepatocyte. However, these transport processes appear to be distinct since they differ with respect to

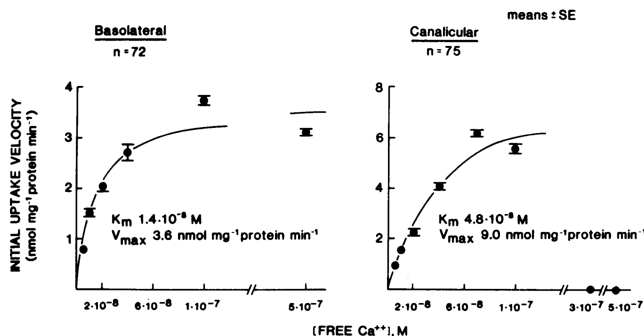

Figure 6. Kinetics of basolateral and canalicular ATP-dependent Ca^{2+} transport. Vesicles were incubated with varying free Ca^{2+} concentrations and the initial velocity of ATP-dependent uptake was determined at 20 s as outlined in earlier figure legends. Weighted least squares fits of the individual data points were performed on a computer yielding the depicted hyperbolas and derived kinetic parameters.

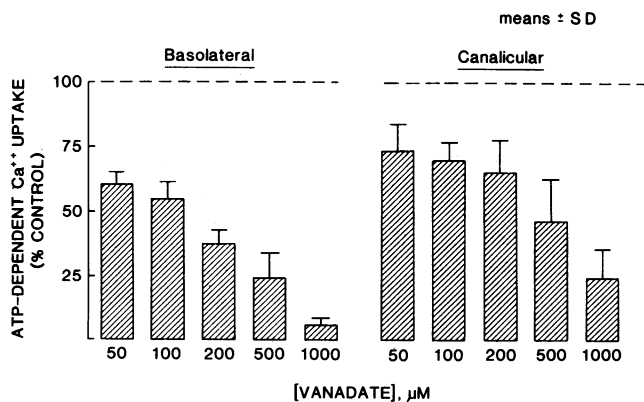

Figure 8. Vanadate inhibition of basolateral and canalicular Ca^{2+} (10^{-8} M) uptake. Vesicles were preincubated with varying vanadate concentrations for 2 h and ATP-dependent uptake at 20 s was determined in the presence or absence of the inhibitor. Uptake data were obtained in three separate basolateral and canalicular vesicle preparations and are expressed as a percentage of the value observed in the absence of vanadate.

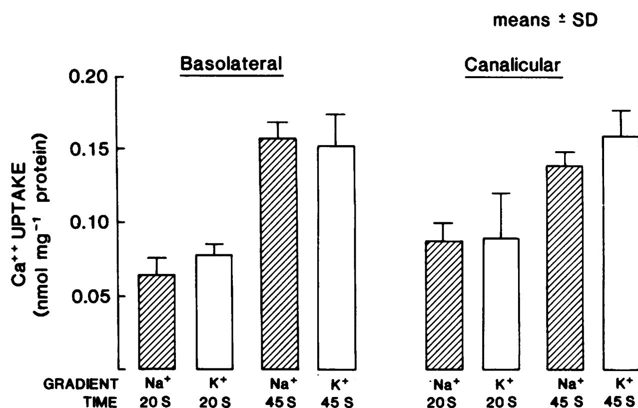

Figure 9. Effects of an outwardly directed Na^+ gradient on basolateral and canalicular Ca^{2+} uptake. Vesicles were preloaded with solutions containing 63 mM Na^+ or K^+, 175 mM sucrose and 10 mM Hepes/KOH, pH 7.4. Uptake of ^{45}Ca was then assayed in a medium containing (final concentrations) 199 mM sucrose, 1 mM Mg^{2+}, 0.5 mM EGTA, unlabeled Ca^{2+} to yield a free concentration of 10^{-8} M and either 3.1 mM Na^+ and 48 mM K^+ (for Na^+-loaded vesicles) or 51 mM K^+ (for K^+-loaded vesicles). Depicted are the mean uptakes at 20 and 45 s for Na^+-loaded and K^+-loaded vesicles.

several important characteristics. One major dissimilarity between ATP-dependent Ca^{2+} transport by basolateral and canalicular vesicles pertains to the dependence of transport on Mg^{2+}. While ATP-dependent basolateral transport required Mg^{2+} for maximal activity, canalicular transport was Mg^{2+}-independent.

Basolateral and canalicular transport also differed significantly with respect to the effects of pH. Transport by vesicles from either domain was virtually absent at pH 6.8. However, while canalicular activity was stimulated fourfold at pH 8.0 compared to pH 7.4, basolateral transport was unchanged at the more alkaline pH. The stimulation of canalicular ATP-dependent Ca^{2+} transport at the higher pH may be important in vivo due to the alkalinity of bile which is presumably established by the recently described canalicular Cl^-/HCO_3^- exchanger (14).

Although the kinetic parameters for the two membrane domains were of the same order of magnitude, both the K_m and V_{max} were significantly lower for basolateral than for canalicular transport. Moreover, at Ca^{2+} concentrations greater than 10^{-7} M, stimulation of canalicular transport by ATP was no longer detectable. In contrast, basolateral transport activity maintained a constant plateau value over this concentration range. Although the values for the basolateral and canalicular K_m (1.4 and $4.8 \cdot 10^{-8}$ M, respectively) agree closely with several previously reported values for the K_m for ATP-dependent Ca^{2+} uptake (2, 3, 15) or (Ca^{2+}-Mg^{2+})-ATPase activity (5, 15) in mixed liver plasma membrane fractions, they are approximately an order of magnitude below current estimates of the cytosolic free Ca^{2+} concentration. As it is unlikely that hepatocyte Ca^{2+} transport is maximally driven at physiological concentrations of free Ca^{2+}, this disparity might be explained by the absence of regulatory influences normally present in the intact cell. For example, in hepatic membrane fractions, both an activator protein and a glucagon-sensitive inhibitor protein (5, 15–18) have been identified which modulate Ca^{2+} pump activity. Conceivably, these and other as yet unidentified mechanisms regulating the kinetics of Ca^{2+} transport may not be operative in the highly purified vesicle preparations used in the present study.

The absence of canalicular ATP-dependent Ca^{2+} transport at free Ca^{2+} concentrations greater than 10^{-7} M is unlikely to be due to a detection artifact secondary to high background (ATP independent) uptake. Since the V_{max} for canalicular uptake (9.0 nmol mg^{-1} protein min^{-1}) is ~ 64% of ATP-independent uptake at the highest Ca^{2+} concentrations (Fig. 7), the plateau in ATP-dependent uptake would be readily detectable. A more likely explanation for the absence of ATP-dependent uptake at high Ca^{2+} concentrations is substrate inhibition. Possible mechanisms (19) for such inhibition include: (*a*) reaction of the substrate with an inactive form of the transporter ("dead-end" inhibitor); (*b*) alteration of the order in which substrates combine with the carrier; and (*c*) substrate binding to an allosteric site. Definitive identification of the precise mechanisms responsible for substrate inhibition awaits further detailed kinetic studies. However, the present findings may explain the failure to detect ATP-dependent canalicular transport in a just published study (20) in which a relatively high Ca^{2+} concentration (50 μM) was used.

While ATP stimulated both basolateral and canalicular transport considerably more than the other nucleotides tested, canalicular transport was somewhat less specific, especially with respect to UTP and GTP. Conceivably, a small degree of contamination of these nucleotides with ATP could explain their stimulation of Ca^{2+} transport. However, studies with low ATP concentrations (data not shown) suggest that even if the degree of contamination were 10%, it could not fully account for the observed stimulation. Finally, basolateral ATP-dependent Ca^{2+} transport was severalfold more sensitive to vanadate inhibition than canalicular transport (IC_{50} 130 and 500 μM, respectively).

The presence of distinctly different ATP-dependent Ca^{2+} transport activities in vesicles isolated from the two surface membrane domains is not likely to be due to varying degrees of contamination with membranes derived from subcellular organelles. Both the basolateral and canalicular vesicle preparations used in this study have been extensively characterized and validated (7, 8) and neither is enriched in marker enzyme activities for mitochondria or endoplasmic reticulum. Moreover, azide or oligomycin, known inhibitors of mitochondrial Ca^{2+} pumps, had no effect on basolateral or canalicular transport. Similarly, oxalate or low pH (6.8), conditions that stimulate microsomal pumps, did not enhance transport by vesicles from either domain. As shown, both basolateral and canalicular ATP-dependent Ca^{2+} transport were actually inactivated at pH 6.8.

The distinctive characteristics of basolateral and canalicular transport also suggest that small degrees of cross-contamination of the preparations by membranes from the contralateral surface cannot account for the finding of Ca^{2+} pump activity on both membrane domains. In addition, the unique features of the two transporters are not due to differences in the sidedness of the membrane preparations used since they were similar in this regard (72 and 75% right side-out for basolateral and canalicular vesicles, respectively). Since the vesicles are relatively impermeable to ATP, ATP-dependent Ca^{2+} uptake is thought to be limited to inside-out vesicles (21). Thus, membrane preparations must have similar degrees of sidedness if valid comparisons of transport characteristics are to be

made. Further, since neither the basolateral nor the canalicular vesicle preparation scheme employs Mg^{2+}-containing buffers, the observed lack of Mg^{2+}-dependence of canalicular ATP-dependent Ca^{2+} transport is unlikely to be due to residual Mg^{2+} contamination from preparation buffers.

Several properties of canalicular Mg^{2+}-independent, ATP-dependent Ca^{2+} transport are similar to those reported for Mg^{2+}-independent, Ca^{2+}-dependent ATPases in renal basolateral membranes (22, 23), cardiac sarcolemma (24), Ehrlich ascites cell membranes (25), gastric smooth muscle membranes (26), and mouse liver membranes (27). In general, in these preparations, activity was relatively insensitive to vanadate (23–25), not stimulated by calmodulin (22–24, 26, 27) and not fully specific for ATP (22, 24). Although the relative contributions of Mg^{2+}-independent and -dependent ATPases to overall calcium pumping in these cells are not known at present (22, 23, 26), the finding that renal basolateral enzyme activity is increased by vitamin D administration (23) may suggest a physiological role for this enzyme.

Recently, the issue of whether hepatic (Ca^{2+}-Mg^{2+})-ATPase is the biochemical equivalent of the liver ATP-dependent Ca^{2+} pump has been the subject of controversy (4, 15, 28). While one laboratory has reported significant differences between the purified enzyme preparation and Ca^{2+} pump activity reconstituted into artificial liposomes with respect to kinetics, vanadate sensitivity, Mg^{2+} requirements, and physical properties (28), another has found that the purified enzyme also mediates ATP-dependent Ca^{2+} pump activity when incorporated into soybean phospholipid vesicles (15). Both of these studies used mixed plasma membrane preparations, which may have contained different proportions of basolateral and canalicular membranes. One may speculate that their seemingly disparate results may have been secondary to differences in the relative activities of the two domain specific ATP-dependent Ca^{2+} transporters described herein. It is conceivable that (Ca^{2+}-Mg^{2+})-ATPase may be identical to only one of the domain specific Ca^{2+} pumps and not to the other. Alternatively, different forms of the enzyme may be present on different membrane domains. Future studies of the localization of (Ca^{2+}-Mg^{2+})-ATPase to the basolateral and/or canalicular membranes will be of interest in this regard.

Neither basolateral nor canalicular ATP-dependent Ca^{2+} transport showed sensitivity to calmodulin or the calmodulin antagonist calmidazolium. These findings are in agreement with those of previous studies showing a lack of calmodulin responsiveness for (Ca^{2+}-Mg^{2+})-ATPase or ATP-dependent Ca^{2+} transport in mixed liver plasma membrane fractions (3, 5, 6, 28).

In the present study, no evidence for liver plasma membrane Na^+/Ca^{2+} exchange was found despite the use of three separate experimental protocols to detect this transport process. Further, this transport activity was not found in freshly isolated vesicles (data not shown), thereby precluding the possibility that Na^+/Ca^{2+} exchange activity was lost during freezing. These results are in contrast to those of a recent report (29), which did find evidence for a Na^+-dependent Ca^{2+} flux in liver plasma membranes. However, two aspects of the latter study, raise questions as to the validity of the conclusions reached. First, in the liver plasma membrane vesicle preparation used, ATP-dependent Ca^{2+} transport was optimal at pH 6.8. As discussed above, at this pH, microsomal ATP-dependent Ca^{2+} transport is maximal (12, 13) and as shown here, transport by highly purified basolateral and canalicular vesicles is virtually abolished. Therefore, contamination by subcellular organelles may have contributed to their findings. Second, the authors found no effect of external Na^+ on the initial velocity of Ca^{2+} efflux. However, Na^+/Ca^{2+} exchange activity by cardiac sarcolemmal vesicles is stimulated by proteolysis (30), alkaline pH (31), and redox modification (32). Conceivably, the absence of critical modulating conditions may have prevented the detection of exchange activity in liver vesicles. Nevertheless, the absence of this transporter in liver membranes is consistent with the view that the high affinity, low capacity ATP-dependent Ca^{2+} transporter predominates in nonexcitable cells and the low affinity, high capacity Na^+/Ca^{2+} exchanger predominates in excitable cells (1).

In summary, the present studies provide evidence for functionally distinct ATP-dependent Ca^{2+} transport mechanisms on the basolateral and canalicular membranes of the hepatocyte. Since no evidence was found for Na^+/Ca^{2+} exchange, these mechanisms are apparently the sole means by which the liver cell extrudes calcium against a steep electrochemical gradient. Recognition of these two domain-specific processes may aid future studies of hepatocyte calcium homeostasis.

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