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H^+- and K^+-Dependence of Ca^{2+} Uptake in Lung Lamellar Bodies

S.J. Wadsworth, A. Chander
Department of Pediatrics, Division of Neonatology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107 USA

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Abstract. Lung lamellar bodies maintain an acidic interior by an energy-dependent process. The acidic pH may affect the packaging of surfactant phospholipids, processing of surfactant proteins, or surfactant protein A-dependent lipid aggregation. The electron-probe microanalysis of lamellar body elemental composition has previously suggested that lamellar bodies contain high levels of calcium some of which may be in ionic form. In this study, we investigated the Ca^{2+} uptake characteristics in isolated lung lamellar bodies. The uptake of Ca^{2+} was measured by monitoring changes in the fluorescence of Fluo-3, a Ca^{2+} indicator dye. The uptake of Ca^{2+} in lamellar bodies was ATP-dependent and increased with increasing concentrations of Ca^{2+}. At 100 nM Ca^{2+}, the uptake was almost completely inhibited by bafilomycin A1, a selective inhibitor of vacuolar type H^+-ATPase, or by NH_4Cl, which raises the lamellar body pH, suggesting that the pH gradient regulates the uptake. The uptake of Ca^{2+} increased as the Ca^{2+} concentration was increased, but the relative contribution of bafilomycin A1-sensitive uptake decreased. At 700 nM, it comprised only 20% of the total uptake. These results suggest the presence of additional mechanism(s) for uptake at higher Ca^{2+} concentrations. At 700 nM Ca^{2+}, the rate and extent of uptake were lower in the absence of K^+ than in the presence of K^+. The inhibitors of Ca^{2+}-activated K^+-channels, tetraethylammonium, Penitrem A, and 4-aminopyridine, also inhibited the K^+-dependent Ca^{2+} uptake at 700 nM Ca^{2+}. Thus the uptake of Ca^{2+} in isolated lung lamellar bodies appears to be regulated by two mechanisms, (i) the H^+-gradient and (ii) the K^+ transport across the lamellar body membrane. We speculate that lamellar bodies accumulate Ca^{2+} and contribute to regulation of cytosolic Ca^{2+} in type II cells under resting and stimulated conditions.

Correspondence to: A. Chander

Key words: pH gradient — Ca^{2+}/H^+-exchange — Ca^{2+}-activated K^+ channels — ATP-dependent Ca^{2+} uptake — Vacuolar type H^+-ATPase

The lung epithelial type II cells contain lamellar bodies, which are specialized acidic organelles that release their contents during secretion of lung surfactant [8, 31]. We have previously shown that a vacuolar type ATPase (V-ATPase), which is present in the lamellar body membrane, is responsible for generating and maintaining the proton electrochemical gradient [7, 12, 41]. In addition, we have also identified the presence of Na^+/H^+ exchange(r) activity by functional analysis and that of $Na^+/K^+/Cl^-$ cotransporter by both functional and immunochemical analyses [41]. These two transporters appear to modify the proton chemical (pH) as well as the electrical gradients across the lamellar body membrane. The physiological role of the proton electrochemical gradient of lamellar bodies is not clear, but various investigators have suggested that the acidic pH may be important for packaging of phosphatidylcholine [13] and phosphatidylglycerol [9], processing of surfactant proteins B [40] and C [3], and surfactant protein A-dependent aggregation and organization of lipids in the lamellar bodies [32].

Electron-probe microanalysis of elemental composition has indicated that lamellar bodies contain high levels of calcium, some of which may be in ionic form [21]. Although these investigators did not measure the Ca^{2+} uptake by lamellar bodies, their findings suggested that the lamellar bodies may take up calcium in either ionic or in protein conjugate form. The simultaneous analysis of other elements (Na, K, and Cl) led these investigators to further suggest a relationship between calcium and other electrolytes in lung lamellar bodies. Recently, the study of organelle uptake of Ca^{2+} has received increasing attention because of its possible role in the regulation of cytosolic Ca^{2+}. In a variety of cell types, the stimulation of cells with several agents rapidly increases the levels of cytosolic Ca^{2+} from approximately 100 nM to as high as

1 μM or higher [reviewed in 26]. This is followed by a decline in the level of cytosolic Ca^{2+} probably because of Ca^{2+} uptake by certain intracellular organelles. Several distinct processes appear to regulate the Ca^{2+} uptake by these organelles. The mitochondria appear to regulate Ca^{2+} uptake by Ca^{2+}-ATPases, Ca^{2+}/H^+ exchange(r)s, and Ca^{2+} uniporter(s), which are present in the mitochondrial membrane [4, 20, 28]. The uptake of Ca^{2+} appears to be of physiologic significance since it is coupled to increased ATP synthesis in the mitochondria [20]. A Ca^{2+}-ATPase activity is also found in the membranes of intracellular organelles such as the endoplasmic and sarcoplasmic reticulum [29, 33, 37, 44], secretory granules [15, 27] and renal tubular endosomes [39]. At least two mechanisms for the Ca^{2+} transport appear to function in these organelles. A vanadate-sensitive electrogenic Ca^{2+}-ATPase, which is present in the endo- and sarcoplasmic reticulum in most cell types [18, 33, 37, 39], pumps Ca^{2+} into the organelle and appears to require H^+ efflux as a counter-ion in order to maintain electroneutral transport [37]. A second mechanism involves Ca^{2+}/H^+-exchange(r), which is independent of the Ca^{2+}-ATPase but is secondary to active transport driven by the organelle V-ATPase [39]. The role of Ca^{2+} transport and subsequent storage by organelles is not clear but may be to regulate the levels of cytosolic Ca^{2+}, which is an important intracellular signal for several physiologic processes. In pancreatic zymogen granules, an increase in vesicle pH inhibits the secretagogue-induced Ca^{2+} spikes suggesting a role for organelle H^+-dependent Ca^{2+} transport in the regulation of cytosolic Ca^{2+} [38].

The Ca^{2+} uptake characteristics and the mechanisms that regulate such uptake in lung lamellar bodies are unknown. A knowledge of these mechanisms would help understand the storage and secretory functions of lamellar bodies. In this study, we characterized the Ca^{2+} uptake and its regulation in isolated lamellar bodies. We postulated that the acidic pH of lung lamellar bodies regulates the Ca^{2+} uptake in a manner similar to that seen in other organelles. We also investigated the influence of other ions on Ca^{2+} uptake since several ions regulate the acidic pH and the membrane potential in several organelle including lamellar bodies [4, 16, 41]. The uptake of Ca^{2+} was followed by measuring changes in the fluorescence of a Ca^{2+}-binding dye, Fluo-3. This dye shows minimal fluorescence in the absence of Ca^{2+}. The binding of Ca^{2+} enhances its fluorescence severalfold in direct proportion to Ca^{2+} concentration in the sub μM to low μM range. Removal of Ca^{2+} from the solution, e.g., by sequestration into an impermeable compartment or chelation with EGTA, results in a decrease in the fluorescence. Thus, in our assay system, a decrease (or increase) in Fluo-3 fluorescence provides an indirect measure of Ca^{2+} uptake in (or release from) the lamellar bodies. We have used the initial rate of decline in Fluo-3 fluorescence and the maximum decrease in the fluorescence as representing the initial rate of Ca^{2+} uptake and the maximum uptake of Ca^{2+} in lung lamellar bodies. Our studies suggest that two separate mechanisms contribute to the regulation of Ca^{2+} uptake in lamellar bodies, one involving Ca^{2+}/H^+ exchange and the other involving Ca^{2+}/K^+ exchange. The latter has not been described in other organelles and thus is a novel finding of this study.

Materials and Methods

Fluo-3 ammonium salt (membrane impermeant), bafilomycin A1, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 4-aminopyridine (4-AP), tetraethylammonium (TEA), vanadate-free ATP, vanadate and all other fine chemicals were purchased from Sigma Chemical (St. Louis, MO). Penitrem A and glyburide were purchased from BioMol (Plymouth Meeting, PA).

Buffer Composition and Inhibitors

The uptake buffers were prepared as described in a previous study [41]. Stock solutions of 500 mM Na-isethionate and 100 mM HCl and 60 mM Tris, pH 7.0 were appropriately diluted to provide indicated concentrations of Na^+, or Cl^-. We maintained the calculated osmolarity of the uptake buffers between 300 and 340 mosM. For preparation of Na^+- or K^+-free uptake buffers, we added appropriate amounts of 0.25 M sucrose to maintain the calculated osmolarity in the specified range. The Ca^{2+} uptake was routinely measured in 10 mM Hepes-Tris buffer (pH, 7.0) containing 130 mM K-gluconate, 20 mM Na-isethionate, 10 mM Cl^-, 10 mM ATP, 10 mM $MgSO_4$ and indicated concentrations of Ca^{2+}, which were buffered with 1 mM EGTA. A 2 mM stock solution of Ca^{2+}-gluconate was appropriately diluted to provide 100–700 nM free Ca^{2+} [23] in which Ca^{2+}-chelation by 10 mM ATP, 1 mM EGTA, and 10 mM $MgSO_4$ were taken into account.

Inhibitors used in this study include bafilomycin A1, a V-ATPase inhibitor [5], NH_4Cl, an agent that dissipates the proton gradient and raises lamellar body pH [41], CCCP, a protonophore, vanadate, an inhibitor of phosphorylating (P)-type ATPases [19], glyburide, an inhibitor of K_{ATP} channel [22], as well as Penitrem A, 4-aminopyridine and tetraethylammonium, all inhibitors of the Ca^{2+}-activated K^+ channels [2, 17, 24, 42, 43, 45]. Lamellar bodies were preincubated for 5 min at 37°C with these inhibitors, before measuring the Ca^{2+} uptake. In experiments evaluating the effects of CCCP, this protonophore was added after the maximum decrease in the fluorescence (representing the maximum uptake of Ca^{2+}) had been obtained. The stock solutions for bafilomycin A1 and glyburide were prepared in DMSO and added to the buffer at a 1,000-fold dilution so that the DMSO concentration in the assay buffer was 0.1%. The stock solution for CCCP in ethanol was also added in a 1,000-fold dilution so that the assay buffer contained 0.1% ethanol.

Isolation of Lamellar Bodies

Lung lamellar bodies were isolated as described previously [11, 41]. Isolated lamellar bodies were resuspended in 0.25 M sucrose in 10 mM HEPES-Tris buffer, pH 7.0, and were either used immediately or stored on ice. The lamellar bodies remained stable on ice for up to 3 hr as determined by comparing the rate and extent of uptake under similar conditions at the beginning and end of the 3 hr period.

Fluo-3 Studies

The Ca^{2+} uptake by lamellar bodies was studied at 37°C by monitoring fluorescence of a Ca^{2+} indicator, Fluo-3 (excitation = 490 nm, emission = 530 nm). The fluorescence was followed in a fluorescence spectrometer (Aminco Bowman Spectrometer, series 2, Milton Roy, Rochester, NY). In all experiments, the instrument settings (slit width, band pass, and voltage) were maintained constant to minimize inter-experiment variations in Fluo-3 fluorescence intensity. A fixed volume (15 μl) of lamellar body suspension (3.75–15.0 μg protein) was added to 0.5 ml of buffer (without or with ATP and/or without or with K^+) containing 100 to 700 nM Ca^{2+} buffered with 1 mM EGTA, and 1 μM Fluo-3 (ammonium salt). The membrane impermeant form of Fluo-3 remains outside the lamellar body so that a decrease in its fluorescence reflects Ca^{2+} removal by lamellar bodies and provides an indirect measurement of Ca^{2+} uptake. The fluorescence data were acquired at 0.1 sec intervals. All fluorescence data were corrected for dilution (3%) caused by the addition of lamellar body suspension. The Ca^{2+} uptake was recorded as arbitrary fluorescence units (F.U.). The Ca^{2+} uptake rate (as F.U./sec) was calculated from the initial linear part of the fluorescence trace. The maximum uptake (as F.U.) was calculated from maximum change in fluorescence intensity. All fluorescence data were later transformed into pmoles Ca^{2+} based on the slope of Fluo-3 fluorescence calibration curve as a function of Ca^{2+} concentration.

Analytical

Lung homogenates and lamellar body preparations were assayed for marker enzymes for other subcellular compartments. Cytochrome-c oxidase, NADPH-cytochrome c reductase, and Na^+/K^+-ATPase were assayed as described previously [41]. Compared to lung homogenate, the lamellar bodies showed more than 20-fold lower activity for the mitochondrial marker enzyme, cytochrome c oxidase (lung homogenate; 52 ± 15, lamellar bodies, 2.4 ± 1.5 nmole ferrocytochrome c oxidized/min/mg protein, mean ± SE, $n = 3$), and almost 7-fold lower activity of the microsomal marker enzyme, NADPH cytochrome c reductase (lung homogenate, 19.2 ± 1.7; lamellar bodies, 2.8 ± 1.6 nmole cytochrome c reduced/min/mg protein, $n = 3$). For all studies described in this report, n represents the number of lamellar body preparations. We also analyzed two preparations of lung lamellar bodies for the Na^+/K^+-ATPase activity. We could not detect any strophanthidin-sensitive Na^+/K^+-ATPase activity (a marker for plasma membrane) in the lamellar bodies. The activity (μmole NADH oxidized/min/mg protein) was 0.19 ± 0.01 in the absence and 0.23 ± 0.03 in the presence of 0.5 mM strophanthidin (mean ± range, $n = 2$). Thus, the isolated lamellar bodies showed minimal to no contamination with the mitochondria, microsomes, or the plasma membrane. The lung homogenate and lamellar body samples were analyzed for protein content using protein-binding dye reagent (Bio-Rad Laboratories, Richmond, VA) and bovine-γ-globulin as the standard [6].

Statistical Analyses

Results were analyzed by Student's t test for experiments unpaired or paired with respect to lamellar body preparations as indicated. For experiments with multiple groups, results were compared by one way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. Differences were considered significant at $P < 0.05$.

Results

The Lamellar Body Preparation

We have previously carried out extensive ultrastructural and biochemical characterization of our lamellar body preparation employed in this study [7, 10–12, 34, 41]. At the ultrastructural level, the isolated lamellar bodies appear homogeneous and the individual lamellar bodies appear to be enclosed in an intact limiting membrane [7, 11, 12]. This preparation also shows lack of Golgi stacks and mitochondria. The biochemical characterization showed that this preparation contained no detectable activities of the marker enzymes for the Golgi and plasma membrane, while those for the microsomes, mitochondria, and the lysosomes were decreased approximately 7- to 20-fold when compared with the lung homogenate [7, 12, 41, this study]. Thus our lamellar body preparation appears nominally free of contamination with these subcellular compartments.

Concentration-Dependence of Ca^{2+} Uptake

For all uptake studies reported here, we used four concentrations of Ca^{2+} (100, 300, 500, and 700 nM), which are within the range of intracellular Ca^{2+} levels in resting and in agonist-stimulated type II cells [30, 35], to which the lamellar bodies may be exposed *in situ.* In initial studies, we measured the uptake at 100 nM Ca^{2+}, which is the approximate cytosolic Ca^{2+} in a resting type II cell [35] and is similar to that reported for other cells. The effect of lamellar body protein concentration on Ca^{2+} uptake was evaluated at the lowest (100 nM) and at the highest (700 nM) Ca^{2+} concentration employed in this study. At both concentrations, the uptake of Ca^{2+} increased with increasing amounts of lamellar body protein (Fig. 1*A* and *B*). Because of the rapid rate of uptake, the abscissa was expanded to determine the linear part of decline in the fluorescence intensity (Fig. 1*C*). The rate of uptake was then calculated as fluorescence change per unit time. The rate of uptake and the maximum uptake also increased as the Ca^{2+} concentration was increased from 100 to 700 nM Ca^{2+} at a constant protein concentration (Fig. 2*A* and *B*).

ATP-Dependent Ca^{2+} Uptake

The above experiments were conducted in buffer containing 10 mM ATP. Removal of ATP showed that the uptake of Ca^{2+} was ATP-dependent (Table 1). At 700 nM Ca^{2+} also, the rate and extent of uptake were lower in the absence of ATP than in the presence of ATP.

Coupling of pH Gradient to Ca^{2+} Uptake

To understand the role of lamellar body pH, we investigated the effects of bafilomycin A1, a selective inhibitor of the V-ATPase [5], on Ca^{2+} uptake. We have previously shown that V-ATPase is the proton 'pump' in lamellar bodies [41]. At 100 nM Ca^{2+}, bafilomycin A1 (10 nM)

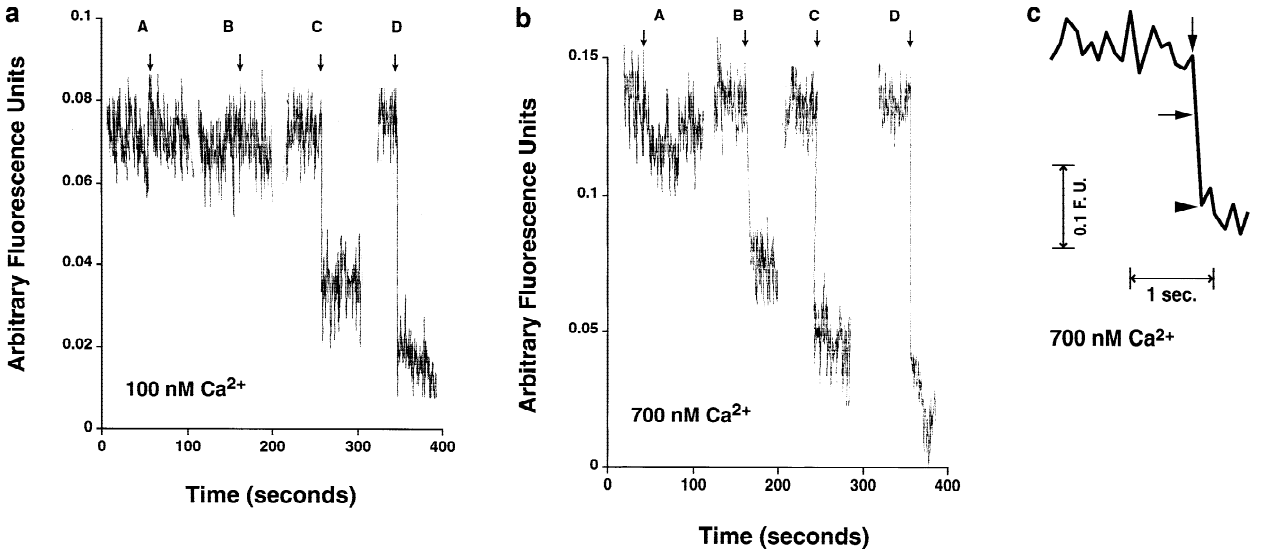

Fig. 1. Fluo-3 fluorescence reflecting the uptake of Ca^{2+} in lung lamellar bodies. The fluorescence (exc. = 490 nm; em. = 530 nm) of 1 μM Fluo-3 in assay buffer (0.5 ml) was monitored at 37°C before and after addition (down arrow) of a fixed volume (15 μl) of lamellar body suspension containing indicated amounts of protein. (*A*) 3.75 μg; (*B*) 5.0 μg; (*C*) 10 μg; and (*D*) 15 μg protein. (*a*) Uptake at 100 nM Ca^{2+}. (*b*) Uptake at 700 nM Ca^{2+}. Note that the two panels are drawn to different scales of fluorescence. (*c*) Illustration of rapid rate of Ca^{2+} uptake in lamellar bodies. The uptake was measured at 700 nM Ca^{2+} using 10 μg protein. The data (arbitrary fluorescence units, F.U.) were recorded at 0.1 sec intervals. The abscissa was then expanded to determine the initial linear part of the decline for the calculation of uptake rate. The down arrow indicates the time of lamellar bodies addition; the fluorescence change because of dilution is indicated by the horizontal arrow; the fluorescence change between the horizontal arrow and the arrowhead represents the decline in fluorescence during the initial linear rate of Ca^{2+} uptake. Results are from one representative experiment.

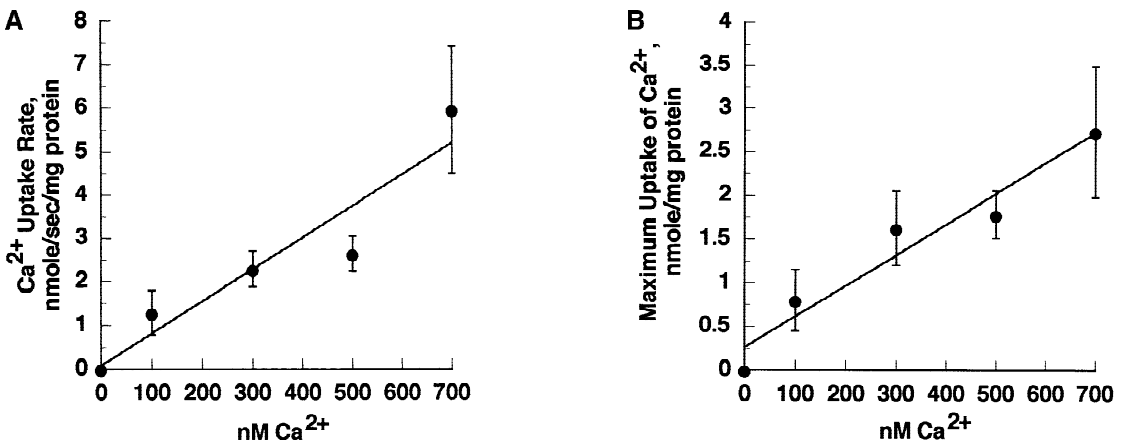

Fig. 2. Ca^{2+}-dependence of Ca^{2+} uptake in lamellar bodies. The uptake of Ca^{2+} was measured by following changes in Fluo-3 fluorescence as described in Fig. 1. The initial rate of uptake was calculated from the initial rate of decline in Fluo-3 fluorescence and the maximum uptake was calculated from the steady-state decrease in the fluorescence. The results are mean ± SE of experiments in 7 preparations of lamellar bodies. The initial rate of uptake (*A*) and the maximum uptake (*B*) are plotted against Ca^{2+} concentrations in the uptake buffer. Lines were drawn after linear regression analysis ((*A*) $y = 7.3441^{-3}\,x + 8.1293^{-2}$, $r = 0.95$; (*B*) $y = 3.5099^{-3}\,x + 2.664^{-1}$, $r = 0.97$).

inhibited the rate of uptake (Fig. 3*A*) and the maximum uptake (Fig. 3*B*) of Ca^{2+} in lamellar bodies. As the Ca^{2+} concentration was increased to 700 nM, the total uptake increased but the relative contribution of bafilomycin A1-inhibitable uptake decreased steadily. At 700 nM Ca^{2+}, the rate and extent of bafilomycin A1-sensitive uptake were only about 20% of the total uptake (Fig. 3*A* and *B*). These results suggest that most of the uptake at 100 nM Ca^{2+} is dependent on the pH gradient driven by the V-ATPase. As the Ca^{2+} concentration increases, the uptake appears to be regulated by a second transport process in conjunction with the V-ATPase. In another

Table 1. ATP-dependence of Ca^{2+} uptake in lung lamellar bodies

	Initial rate of Ca^{2+} uptake (nmole/sec/mg protein)		Maximum Ca^{2+} uptake (nmole/mg protein)	
	10 mM ATP	0 mM ATP	10 mM ATP	0 mM ATP
Ca^{2+} in uptake buffer				
100 nM (10)	1.57 ± 0.46	0.18 ± 0.13*	0.92 ± 0.27	0.16 ± 0.13*
300 nM (7)	1.97 ± 0.45	0.97 ± 0.65	1.33 ± 0.58	0.81 ± 0.69
500 nM (7)	2.94 ± 0.51	1.66 ± 0.57	1.76 ± 0.40	1.06 ± 0.55
700 nM (8)	4.46 ± 0.65	2.30 ± 0.35*	2.04 ± 0.47	1.05 ± 0.17*

Results are mean ± SE of experiments in lamellar body preparations indicated in parentheses. *$P < 0.05$ when compared with that in the presence of 10 mM ATP by Student's *t* test paired with respect to lamellar body preparation.

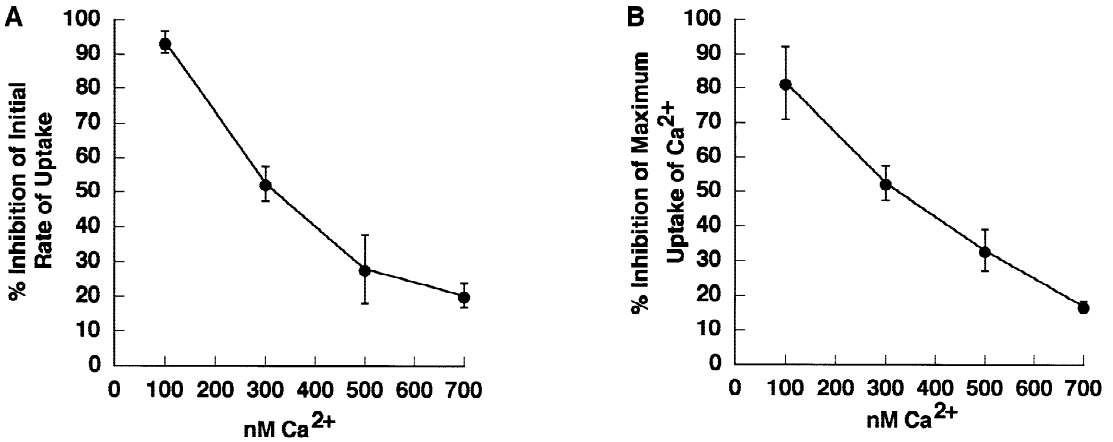

Fig. 3. Bafilomycin A1 inhibition of Ca^{2+} uptake in lamellar bodies. Bafilomycin A1 inhibition of the initial rate of Ca^{2+} uptake (*left panel*) and the maximum uptake of Ca^{2+} (*right panel*). Lamellar bodies (5–10 μg protein) were preincubated for 5 min at 37°C with 10 nM bafilomycin A1 before addition to the uptake buffer containing Fluo-3 and indicated concentrations of Ca^{2+}. The results are expressed as the inhibition of each parameter when compared with uptake in the absence of bafilomycin A1. The results are mean ± range of experiments in two preparations of lamellar bodies.

approach, we used a weak base, NH_4Cl, to dissipate the pH gradient across the lamellar body membrane. Weak bases are accumulated in acidic organelles where they become protonated and increase the organelle pH. At 100 nM Ca^{2+}, the rate and extent of Ca^{2+} uptake were lower in lamellar bodies that were preincubated for 5 min with 10 mM NH_4Cl (Table 2), suggesting that most of the Ca^{2+} uptake at this concentration was dependent on the pH gradient. At 700 nM Ca^{2+}, NH_4Cl had no significant effect on Ca^{2+} uptake. These results support the conclusion that the uptake of Ca^{2+} is almost entirely pH gradient-dependent at low Ca^{2+} concentration. At high concentrations of Ca^{2+}, an additional mechanism appears to be active in Ca^{2+} uptake in lung lamellar bodies.

We also evaluated the effects of vanadate (10 μM) on Ca^{2+} uptake at 100 and 700 nM Ca^{2+}. Vanadate is a nonspecific inhibitor of P-type ATPases including Na^+/K^+-ATPase, H^+/K^+-ATPase, and the Ca^{2+}-ATPases. At both concentrations of Ca^{2+}, vanadate did not affect either the rate or the maximum uptake of Ca^{2+} (*not shown*).

Effects of Na^+ and K^+ on Ca^{2+} Uptake

To evaluate the effects of Na^+, the Ca^{2+} uptake in Na^+-containing or Na^+-free buffer was compared. The rate and the extent of Ca^{2+} uptake were similar in the absence or presence of Na^+ at 100 or 700 nM Ca^{2+} (*data not shown*). Similarly, the effect of K^+ was first evaluated at 100 and 700 nM Ca^{2+} by comparing the Ca^{2+} uptake in K^+-free and K^+-containing buffers. Compared to the uptake in K^+-containing buffer, the rate and the maximum uptake in K^+-free buffer were not different at 100 nM Ca^{2+} (Table 3). At 700 nM, however, the rate and maximum uptake were decreased in the absence of K^+. Subsequent studies showed that the presence of K^+ also

Table 2. Effects of NH_4Cl on Ca^{2+} uptake in lung lamellar bodies

	Initial rate of Ca^{2+} uptake (nmole/sec/mg protein)		Maximum Ca^{2+} uptake (nmole/mg protein)	
	− NH_4Cl	+ NH_4Cl	− NH_4Cl	+ NH_4Cl
Ca^{2+} in uptake buffer				
100 nM	1.40 ± 0.40	0.07 ± 0.05*	0.98 ± 0.50	0.20 ± 0.05*
700 nM	5.20 ± 1.92	3.64 ± 1.55	2.46 ± 0.96	1.82 ± 0.78

The uptake of Ca^{2+} was measured at 100 and 700 nM Ca^{2+} and in the absence or presence of 10 mM NH_4Cl. Lamellar bodies were preincubated for 5 min at 37°C with NH_4Cl before addition to the uptake buffer containing Fluo-3 and indicated concentrations of Ca^{2+}. Results are mean ± SE of experiments in 4 preparations of lamellar bodies. *$P < 0.05$ by Student's *t* test (paired with respect to lamellar body preparation) when compared with uptake in the absence of NH_4Cl.

Table 3. K^+ dependence of Ca^{2+} uptake in lung lamellar bodies

	Initial rate of Ca^{2+} uptake (nmole/sec/mg protein)		Maximum Ca^{2+} uptake (nmole/mg protein)	
	+ K^+	− K^+	+ K^+	− K^+
Ca^{2+} in uptake buffer				
100 nM	1.63 ± 0.66	0.74 ± 0.37	1.04 ± 0.46	0.41 ± 0.28
300 nM	2.15 ± 0.59	0.48 ± 0.32*	1.54 ± 0.79	0.48 ± 0.32*
500 nM	2.85 ± 0.76	0.53 ± 0.27*	2.09 ± 0.33	0.53 ± 0.27*
700 nM	5.50 ± 1.71	1.12 ± 0.95*	2.32 ± 0.85	0.81 ± 0.46*

The Ca^{2+} uptake was measured in buffer containing indicated concentrations of Ca^{2+} 10 mM ATP, and in the absence or presence of 130 mM K^+. Results are mean ± SE of experiments in 5 separate preparations of lamellar bodies. *$P < 0.05$ by Student's *t* test (paired with respect to lamellar body preparation) when compared to uptake in K^+ containing buffer.

facilitated the uptake of Ca^{2+} at 300 and 500 nM Ca^{2+}. These results suggest that a K^+-dependent transport process, in conjunction with H^+-dependent Ca^{2+} uptake, regulates Ca^{2+} uptake at 300 nM or higher Ca^{2+} concentrations.

Effects of K^+ Channel Blockers on Ca^{2+} Uptake

Next, we tested several inhibitors of K^+ channels to determine if the K^+-dependent Ca^{2+} uptake was linked to K^+ transport. These inhibitors, TEA, 4-AP, and Penitrem A, have been shown to inhibit Ca^{2+}-activated K^+ channels in a variety of cell types [2, 17, 24, 42, 43, 45]. The Ca^{2+} uptake (the rate and maximum uptake) at 700 nM Ca^{2+} was inhibited by TEA in a dose-dependent manner (Fig. 4). The other two inhibitors, 4-AP (10 μM) and Penitrem A (10 nM), also blocked K^+-dependent uptake of Ca^{2+} (the rate and maximum uptake) at 700 nM but not at 100 nM Ca^{2+} (Tables 4 and 5). By contrast, glyburide (50 nM to 5 μM), an inhibitor of K_{ATP} channels [22] did not affect the K^+-dependent Ca^{2+} uptake (*not shown*). These results suggest that the K^+-dependent uptake of Ca^{2+} in lamellar bodies is regulated by K^+ transport, which occurs by Ca^{2+}-activated K^+ channels. This uptake appears to respond to pH changes or H^+ transport since CCCP, an uncoupler of oxidative phosphorylation in mitochondria and a protonophore, decreased the uptake of Ca^{2+} in K^+-containing buffer but not in the K^+-free buffer (Fig. 5). At 700 nM Ca^{2+} and in the presence of K^+, the uptake of Ca^{2+} was 1.17 ± 0.23 and 0.58 ± 0.11 nmole/mg protein ($n = 4$, $P < 0.05$), before and after the addition of 10 μM CCCP, respectively. In the absence of K^+, the corresponding uptake was 0.69 ± 0.28 and 0.81 ± 0.31 nmole/mg protein ($n = 3$; $P > 0.05$).

Discussion

In previous studies, we have demonstrated that lamellar bodies maintain an acidic pH by a bafilomycin A1-sensitive V-ATPase, which is electrogenic in nature [12, 41]. Furthermore, different ions regulate the electrical and chemical gradients by affecting the Na^+/H^+ exchange and $Na^+/K^+/Cl^-$-cotransport processes that are present in the lamellar body membrane [41]. Electron-probe microanalysis of elemental composition suggests that lamellar bodies contain high calcium and that some

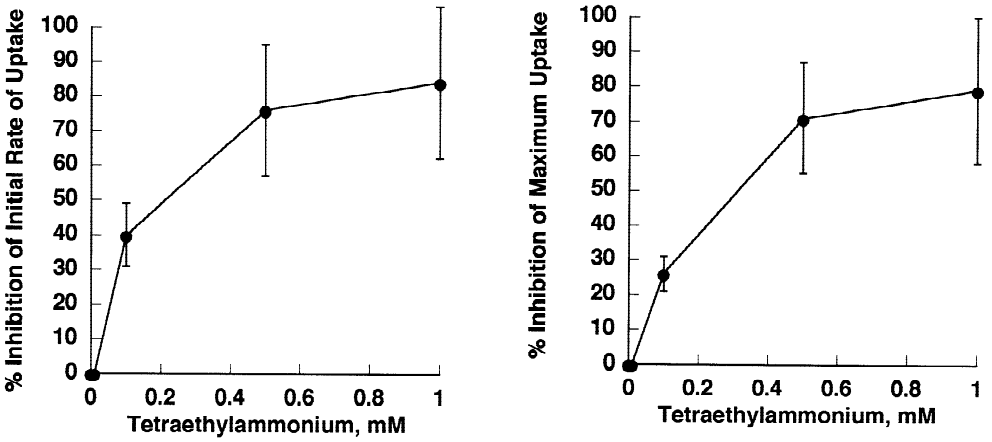

Fig. 4. Concentration-dependence of tetraethylammonium (TEA) inhibition of K^+-dependent Ca^{2+} uptake in lamellar bodies. The uptake was measured at 700 nM Ca^{2+}. Lamellar bodies (5–10 μg protein) were preincubated for 5 min at 37°C with indicated concentration of TEA before addition to the Fluo-3 solution in uptake buffer. The results are expressed as percentage inhibition of the uptake rate (*left panel*) and maximum uptake (*right panel*) when compared with those in the absence of TEA and are mean ± SE of experiments in three preparations of lamellar bodies.

Table 4. Effects of 4-aminopyridine on Ca^{2+} uptake in lung lamellar bodies

	Initial rate of Ca^{2+} uptake (nmole/sec/mg protein)		Maximum Ca^{2+} uptake (nmole Ca^{2+}/mg protein)	
	− 4-AP	+ 4-AP	− 4-AP	+ 4-AP
Ca^{2+} in uptake buffer				
100 nM (4)	1.70 ± 0.28	2.08 ± 0.40	1.24 ± 0.32	1.00 ± 0.22
700 nM (3)	5.32 ± 0.57	0.54 ± 0.45*	2.10 ± 0.49	0.19 ± 0.13*

Lamellar bodies were incubated for 5 min at 37°C without or with 10 μM 4-aminopyridine (4-AP) before measuring Ca^{2+} uptake in buffer containing indicated concentrations of Ca^{2+}. Results are mean ± SE of experiments in lamellar body preparations indicated in parentheses. *$P < 0.05$ by paired Student's *t* test when compared with uptake in the absence of 4-AP.

Table 5. Effects of Penitrem A on Ca^{2+} uptake in lung lamellar bodies

	Initial rate of Ca^{2+} uptake (nmole/sec/mg protein)		Maximum Ca^{2+} uptake (nmole/mg protein)	
	− Penitrem A	+ Penitrem A	− Penitrem A	+ Penitrem A
Ca^{2+} in uptake buffer				
100 nM (3)	1.14 ± 0.55	0.94 ± 0.73	1.14 ± 0.55	1.00 ± 0.69
700 nM (4)	5.06 ± 0.56	1.25 ± 0.65*	3.07 ± 0.28	1.25 ± 0.61*

Lamellar bodies were incubated for 5 min at 37°C without or with 10 nM Penitrem A before measuring Ca^{2+} uptake in buffer containing 10 mM ATP, 130 mM K^+, and indicated concentrations of Ca^{2+}. Results are mean ± SE of experiments in lamellar body preparations indicated in parentheses. *$P < 0.05$ by paired Student's *t* test when compared with uptake in the absence of Penitrem A.

of this calcium may be in ionic form [21]. However, the uptake of Ca^{2+} and the mechanisms that regulate such uptake in lamellar bodies have not been investigated. This is the first study to demonstrate that lamellar bodies take up Ca^{2+} at a rapid rate and that the presence of ATP increases such uptake. At 100 nM Ca^{2+}, the intralamellar body Ca^{2+} levels were approximately 0.9 nmole Ca^{2+}/mg protein (Table 1). We have previously shown that the water space of lamellar bodies is approximately 3 μl/mg protein [12]. Thus, the calculated lamellar body Ca^{2+} would be approximately 0.3 mM. At 700 nM Ca^{2+}, the Ca^{2+} uptake in lamellar bodies was approximately 2 nmole/mg protein, which translates into approximately 0.7 mM Ca^{2+}. Thus, the lamellar bodies appear to take up

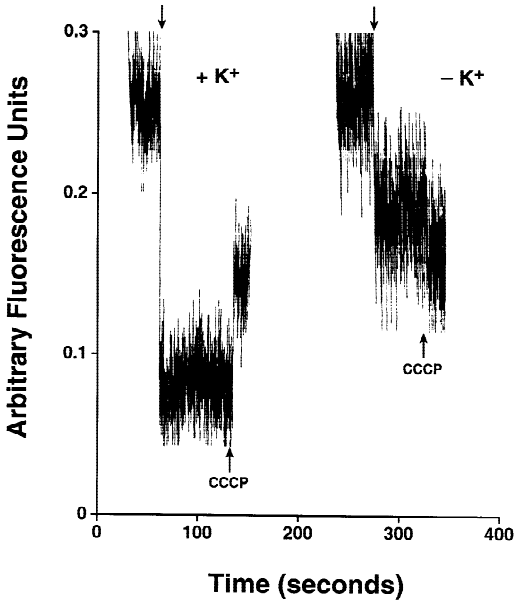

Fig. 5. Effects of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) on K^+-dependent uptake of Ca^{2+} in lamellar bodies. The uptake was measured at 700 nM Ca^{2+}. The Fluo-3 fluorescence in K^+-containing (left) or K^+-free buffer (right) was monitored after addition of lamellar bodies (*down arrow*). After achieving a steady-state maximum decrease in the fluorescence, CCCP (10 μM) was added (*up arrow*). The decrease in fluorescence could be partially reversed in K^+-containing but not in K^+-free buffer. These results are from a representative experiment.

Ca^{2+} against the concentration gradient. Because the Ca^{2+} concentrations employed here are well within the physiologic range in resting and stimulated cells, it is likely that the lamellar bodies in situ may take up Ca^{2+} by mechanisms discussed below.

Our results show that two mechanisms, one H^+-dependent and one K^+-dependent, appear to regulate the Ca^{2+} uptake in lamellar bodies. The relative contributions of these mechanisms depend on the Ca^{2+} concentration to which the lamellar bodies are exposed. Previous studies have described the voltage- and H^+-dependence of Ca^{2+} uptake in cells and in several organelles like microsomes and mitochondria [1, 4, 15, 20, 27, 28]. Our study presents new findings that the Ca^{2+} uptake in lung lamellar bodies may be dependent on K^+-transport. This finding of K^+-transport-dependent Ca^{2+} uptake may also have a bearing on other organelles, which are postulated to sequester cytosolic Ca^{2+}. The ATP-dependence of Ca^{2+} uptake at all concentrations of Ca^{2+} agrees with the ATP-requiring characteristics of the V-ATPase and the Ca^{2+}-activated K^+-channels. While ATP is hydrolyzed to 'pump' protons, the presence of ATP activates the Ca^{2+}-activated K^+-channels. Recent patch-clamp studies in adrenal chromaffin cells have indicated that ATP (30 μM to 10 mM) increases the open probability of these channels [14]. Thus the ATP requirement for activation of these channels is in the physiologic range.

In other organelles, the Ca^{2+}-ATPase and the Ca^{2+}/H^+ exchange appear to regulate the Ca^{2+} uptake [20, 26, 33, 37, 39]. The Ca^{2+}-ATPase is a P-type ATPase, which is sensitive to vanadate. Since the uptake in lamellar bodies was insensitive to vanadate, we suggest that a Ca^{2+}-ATPase does not facilitate the uptake of Ca^{2+} in lamellar bodies. Bafilomycin A1, a selective inhibitor of V-ATPase, however, inhibited the uptake suggesting that the pH gradient across the lamellar body membrane is required for Ca^{2+} uptake. Since the relative contribution of bafilomycin A1-sensitive uptake towards total uptake declined as the external Ca^{2+} concentration was increased, we suggest that a second mechanism may regulate the uptake at high Ca^{2+} concentrations. In agreement with this concept, a weak base, NH_4Cl, which dissipates the pH gradient across the lamellar body membrane, also decreased the uptake at low Ca^{2+} concentrations. At high Ca^{2+} concentrations, the uptake was not sensitive to treatment with NH_4Cl. Thus, the pH gradient-dependent uptake appears to be the primary mechanism of uptake at low Ca^{2+} concentration. These results also suggest that a secondary active transport driven by the V-ATPase mediates Ca^{2+} entry in exchange for lamellar body H^+. One such transport is by a Ca^{2+}/H^+ exchange(r), which has been described in other acidic organelles [27]. The relative contribution of this exchanger to calcium transport, however, decreases as the Ca^{2+} concentration increases from 100 to 700 nM suggesting that a second transport process, the K^+-dependent Ca^{2+} uptake, becomes active.

A new finding of this study is the K^+-dependence of Ca^{2+} uptake in lamellar bodies. We have shown the K^+-dependence of Ca^{2+} uptake by two separate approaches. First, our studies showed that the Ca^{2+} uptake was greater in the presence of K^+ than in the absence of K^+ (Table 3) and, second, the higher uptake of Ca^{2+} in the presence of K^+ can be blocked with inhibitors of Ca^{2+}-activated K^+ channels (Fig. 4, Tables 4 and 5). These observations lead us to suggest that a novel mechanism, which has not been described in any other system, may link K^+ transport with Ca^{2+} uptake in lamellar bodies. The precise mechanisms for coupling of K^+ transport or exchange with Ca^{2+}, however, are not known. A likely possibility is that the efflux of K^+ through the Ca^{2+}-activated K^+-channels may promote Ca^{2+} influx leading to a K^+/Ca^{2+} exchange. The stoichiometry of this exchange cannot be determined from our studies. The presence of Cl^- may also affect the kinetics of Ca^{2+} uptake. The V-ATPase, which is normally coupled to Cl^- transport in lamellar bodies, is functional at all Ca^{2+} concentrations (Fig. 3). The Cl^- in the lamellar bodies can indirectly facilitate some of the Ca^{2+} uptake by reducing the positive charge against which Ca^{2+} must en-

ter. A cotransport of Cl^-, if any, can directly facilitate Ca^{2+} uptake but would also affect the stoichiometry of exchange process. In this model of K^+/Ca^{2+}-exchange, the external (cytosolic) Ca^{2+} appears to be a limiting factor, since the K^+-dependent Ca^{2+}-uptake increases with increasing Ca^{2+} concentrations.

A schematic of Ca^{2+} uptake and its relationships with the H^+ and K^+ transport in lamellar bodies is shown in Fig. 6. At low Ca^{2+} concentration, the ATP-generated pH gradient facilitates the uptake of Ca^{2+} in exchange for H^+ efflux. At higher Ca^{2+} concentrations, the Ca^{2+} in lamellar bodies activates the Ca^{2+}-activated K^+ channels prompting an efflux of K^+, which facilitates further uptake of Ca^{2+} either in exchange for K^+ or through another transport process. An inhibition of K^+ efflux with TEA, 4-AP, or Penitrem A prevents the K^+-dependent uptake of Ca^{2+}. These channels are sensitive to a pH change as indicated by reversal of Ca^{2+} uptake by CCCP (Fig. 5).

We have also considered alternate mechanisms for K^+-dependent Ca^{2+} uptake. In our previous study we have demonstrated that K^+ rapidly equilibrates across the lamellar body membrane and is primarily responsible for determining the magnitude of the lamellar body membrane potential. One mechanism of K^+ effect could be that the lamellar body K^+ can exchange with the external H^+ through an H^+/K^+-exchange, which is followed by $Ca^{2+}{}_{out}/H^+{}_{in}$ exchange. In the plasma membrane, such exchange is driven by H^+/K^+-ATPase [25], a P-type ATPase. In the mitochondrial uptake of Ca^{2+}, an H^+/K^+-exchange followed by subsequent efflux of H^+ with Ca^{2+} has been invoked [4]. Two observations argue against such mechanism(s) in the lamellar bodies. First, our previous study showed that the proton 'pump' activity and the pH gradient in lamellar bodies were unaffected by K^+ [41], which would rule out the $K^+{}_{out}/H^+{}_{in}$-exchange and, second, the K^+-dependent Ca^{2+} uptake in lamellar bodies was insensitive to vanadate, an inhibitor of P-type ATPases. The uptake of Ca^{2+} would also be against the electrical gradient since the presence of K^+ would render the lamellar bodies more electropositive and would exclude positively charged ions including Ca^{2+}.

Our studies with inhibitors of Ca^{2+}-activated K^+-channels have provided pharmacologic evidence for the presence of these channels in the lamellar bodies. These inhibitors have been previously used to inhibit K^+ fluxes in several systems suggesting that they inhibit K^+ transport irrespective of the membrane system to which they are applied. Although not tested before in the lamellar bodies, these inhibitors appear to inhibit K^+ transport in lamellar bodies because their effects were similar to those observed with omitting K^+. The Ca^{2+}-activated K^+-channels in lamellar bodies, however, appear to be somewhat different from those in the plasma membrane. The Ca^{2+}-activated K^+-channels in the plasma membrane are sensitive to changes in external pH. A decrease in external pH decreases the open probability of the Ca^{2+}-activated K^+ channel in fetal rat hippocampal neurons [15, 18]. In the lamellar bodies, these channels may show increased open probability in the acidic environment since the addition of CCCP increases the lamellar body pH and decreases the K^+-dependent Ca^{2+} uptake (Fig. 5).

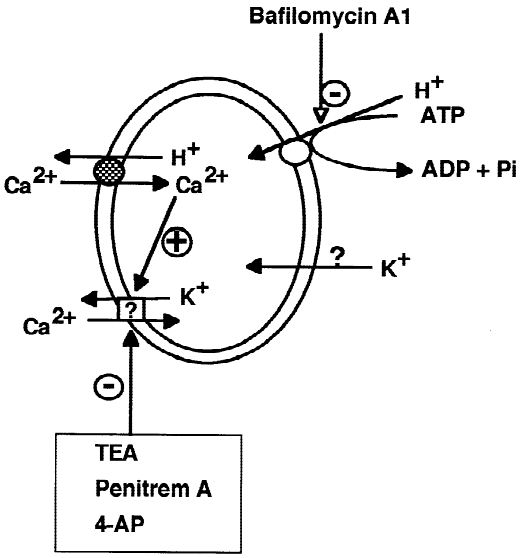

Fig. 6. A schematic of ion transports in the regulation of Ca^{2+} uptake in lamellar bodies. At low Ca^{2+}, the lamellar bodies take up Ca^{2+} by a pH-gradient-dependent mechanism in which $Ca^{2+}{}_{out}$ is exchanged for $H^+{}_{in}$. This process is inhibited by bafilomycin A1 because of inhibition of V-ATPase. At high Ca^{2+}, the lamellar bodies take up Ca^{2+} in a K^+-dependent manner, which is inhibited by inhibitors of Ca^{2+}-activated K^+-channels. Our postulation is that the initial Ca^{2+} influx can activate the K^+ channels in the lamellar body membrane, which causes efflux of K^+ and subsequent uptake of Ca^{2+}. These channels are sensitive to a pH change as indicated by reversal of Ca^{2+} uptake by CCCP, and can be activated by ATP. The mechanisms for K^+ influx or exchange with Ca^{2+} are not known and are, therefore, indicated by question marks.

Given that the Ca^{2+} concentrations as well as the ATP requirement for Ca^{2+} uptake used in this study are physiologic, it can be assumed that the lamellar bodies *in situ* would readily take up Ca^{2+} as observed in vitro. In other words, the pH gradient-dependent uptake would be the primary system for Ca^{2+} uptake in resting type II cells. A second system will become active in response to an increase in cytosolic Ca^{2+}. Thus this second system can sequester Ca^{2+} and contribute to the regulation of cytosolic Ca^{2+} in agonist-stimulated type II cells. A similar role in the regulation of cytosolic Ca^{2+} has been suggested previously for the mitochondria, the endoplasmic reticulum, or other subcellular compartments [20, 26]. While the Ca^{2+} uptake in mitochondria is associated with increased synthesis of ATP and that in the ER is for refilling of these stores, the function of lamellar body Ca^{2+} is unknown. It is not known if the lamellar bodies

maintain more than one pool of Ca^{2+}. One of these could be a steady-state pool that is maintained in resting cells, while the other may be a transient pool that forms during cell stimulation. Although there is no evidence, one can speculate that the Ca^{2+} from the latter may be released to facilitate cellular functions involving lamellar bodies. Since lamellar bodies are secretory organelles that release their contents after membrane fusion during exocytosis, a Ca^{2+} requiring step, we speculate that the stored Ca^{2+} may be released at the site of exocytosis to facilitate membrane fusion. Our previous studies have suggested a role for Ca^{2+} in synexin-mediated fusion between lamellar body and plasma membrane [10, 34], which occurs prior to exocytosis and secretion of surfactant into the alveolar space.

In summary, this study characterizes the uptake of Ca^{2+} in lung lamellar bodies. The uptake is rapid, ATP-dependent, and is dependent on the Ca^{2+} concentrations. Two distinct mechanisms appear to regulate the Ca^{2+} uptake in lamellar bodies. The first one is dependent on the pH gradient across the lamellar body membrane and the second is dependent on K^+ transport through the Ca^{2+}-activated K^+-channel, as evidenced by use of pharmacologic agents. The latter is a novel finding for organelle uptake of Ca^{2+}. Since this study used Ca^{2+} concentrations, which are in the physiologic range for cytosolic Ca^{2+} in the resting and agonist-stimulated type II cells, we speculate that the lamellar bodies can sequester Ca^{2+} and contribute to the regulation of cytosolic Ca^{2+} in alveolar type II cells.

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