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Vanadate-Sensitive Microsomal ATPases and Microsomal $^{45}Ca^{2+}$ Uptake in Tracheal Epithelial Cells[1]

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Cytosolic free Ca^{2+} plays important roles in the regulation of physiological processes in tracheal epithelial cells and is probably regulated by many ion-transporting ATPases in these cells. Therefore, the effect of vanadate was investigated to characterize microsomal ion-transporting ATPases. Dose response experiments showed that vanadate had a biphasic effect on the microsomal ATPase activity: a decrease at the vanadate concentration below 100 μM, and a steep decrease at the concentration above 100 μM. The dose response data were fitted to two sigmoidal functions, corresponding to a low-affinity vanadate-sensitive (LAVS) ATPase and a high-affinity vanadate-sensitive (HAVS) ATPase. In $^{45}Ca^{2+}$ uptake experiments, both LAVS and HAVS ATPases mediated microsomal $^{45}Ca^{2+}$ uptake. The LAVS ATPase was selectively sensitive to thapsigargin in both ATPase activity and $^{45}Ca^{2+}$ uptake, suggesting that it is an ER/SR-type intracellular Ca^{2+}-ATPase. Although the HAVS ATPase mediated one-fourth of microsomal $^{45}Ca^{2+}$ uptake, its activity was not sensitive to thapsigargin. These results indicate that the activities of these two vanadate-sensitive ATPases are mediated by different enzymes, since thapsigargin only blocks the activity of LAVS ATPase. In conclusion, there are two types of vanadate-sensitive microsomal ATPases, and these ATPases mediate microsomal $^{45}Ca^{2+}$ uptake in airway epithelial cells.

Key words: $^{45}Ca^{2+}$ uptake, microsomal ATPase, thapsigargin, tracheal epithelium, vanadate.

Epithelial cells in the airway have ciliary activity and play major roles in transepithelial secretion and mucociliary clearance. These physiological roles are essential functions for fundamental pulmonary defense. Many physiological processes of these cells are thought to be regulated by cytosolic free Ca^{2+}. An increase in cytosolic free Ca^{2+} correlates with a rise in ciliary beating in these cells (*1, 2*). However, the detailed relationship between cytosolic free Ca^{2+} and ciliary activity is not well established.

When a tracheal epithelial cell was stimulated, the concentration of cytosolic free Ca^{2+} was rapidly increased, forming an intracellular Ca^{2+} wave, and the Ca^{2+} wave initiated an increase in ciliary beating frequency (*3*). The release of Ca^{2+} from an $InsP_3$-sensitive Ca^{2+} store has been demonstrated to mediate the intracellular Ca^{2+} wave in cultured epithelial cells (*4*). In addition to the $InsP_3$-sensitive Ca^{2+} store, a caffeine-sensitive Ca^{2+} store (*5, 6*) and a thapsigargin-insensitive Ca^{2+} store (*7*) have been reported in these cells. Ca^{2+} release from these stores may also contribute to the increase in cytosolic Ca^{2+}. A voltage-sensitive Ca^{2+}-conducting channel has been identified and demonstrated to be sensitive to dihydropyridine compounds (*8*). This channel may be another source of Ca^{2+} influx in the plasma membrane of airway epithelial cells. After a rapid rise in cytosolic free Ca^{2+}, the cytosolic Ca^{2+} decreases to the resting concentration. The sequestration of cytosolic Ca^{2+} could be mediated by Ca^{2+}-ATPases and Ca^{2+}-translocating enzymes located on the membranes of all the above Ca^{2+} sources.

Since the characteristics of ion-transporting ATPases, including various Ca^{2+}-ATPases, had not been studied in detail, microsomal vesicles were prepared from the epithelial cells of porcine airway, and microsomal ATPases were characterized in order to understand the regulation of cytosolic ion concentration. When the effects of various inhibitors were examined, we found that there were two types of vanadate-sensitive microsomal ATPases, a low-affinity one and a high-affinity one, and both ATPases were able to mediate microsomal $^{45}Ca^{2+}$ uptakes.

MATERIALS AND METHODS

Materials—Porcine tracheas of freshly killed pigs were generously supplied by a local slaughterhouse in Cheongju, Korea. $^{45}CaCl_2$ was purchased from DuPont-NEN Research Products (Boston, MA, USA). All other chemicals and enzymes were obtained from Sigma Chemical Co. (St.

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Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); NADH, nicotinamide adenine dinucleotide; HAVS, high-affinity vanadate-sensitive; LAVS, low-affinity vanadate-sensitive; TG, thapsigargin.

Louis, MO, USA).

Preparation of Microsomes from the Epithelial Cells of Porcine Trachea—Mucosa was dissected from its cartilaginous backing after the trachea had been slit longitudinally (*9*). The mucosa was ground in a food processor in a solution containing 130 mM NaCl, 5 mM KCl, 25 mM Hepes (pH 7.2), 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM glucose. The homogenate was filtered through three layers of cheesecloth. The filtrate was centrifuged for 30 min at 9,000 rpm (12,530×*g*) to remove mitochondrial fractions and cell debris. The supernatant was centrifuged twice for 1 h at 23,000 rpm (95,000×*g*) in a type SW-28 rotor (Beckman Instruments, INC., Palo Alto, CA, USA). The pellet containing membrane vesicles was resuspended in a buffer containing 0.3 M sucrose, 100 mM KCl, and 5 mM Na-Pipes (pH 6.8), frozen in liquid nitrogen, and stored at −80°C before use. The concentration of microsomal proteins was determined by the Lowry method (*10*).

Measurement of Microsomal $^{45}Ca^{2+}$ Uptake—Microsomal $^{45}Ca^{2+}$ uptake was measured by a modified method of Ghosh *et al.* (*11*). Briefly, $^{45}Ca^{2+}$ uptake was performed in an uptake medium containing 1.5 μM $^{45}CaCl_2$, 50 μM $CaCl_2$, 120 mM KCl, 30 mM Hepes (pH 7.4), 1 mM $MgCl_2$, and 10 mM KCN. The uptake was initiated by the addition of 0.5 mM ATP. The microsomes were transferred onto a filter paper (Whatman GF/B) and washed three times with a small amount of washing solution containing 120 mM KCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 100 μg/ml heparin, and 30 mM Hepes (pH 6.5). The radioactivity remaining in the microsomes on the filter paper was measured by liquid scintillation counting.

Measurement of Microsomal ATPase Activity—ATPase activity of microsomal vesicles was measured by an enzyme-coupled assay (*12*). Briefly, the activity of microsomal ATPases was monitored in a physiological solution containing 120 mM KCl, 30 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 0.5 mM Na-ATP, 0.4 mM NADH, 50 μM $CaCl_2$, 2 mM phosphoenolpyruvate, 1 IU/ml of pyruvate kinase, and 1 IU/ml of lactate dehydrogenase. The rate of ATP hydrolysis by microsomal ATPase was quantitatively coupled to the oxidation of NADH in the reaction solution, resulting in an absorbance decrease at 340 nm. Decrease in absorbance was continuously monitored by use of a spectrophotometer (U-2000, Hitachi, Tokyo), and the activity was calculated from the slope of the decrease in absorbance, representing the rate of ATP hydrolysis. Microsomes containing ~35 μg microsomal proteins were used for each experiment.

RESULTS

Effects of Inhibitors on Microsomal ATPase Activity—In order to characterize microsomal ATPases prepared from tracheal epithelial cells, the effects of two selective inhibitors on their activities were investigated. The rate of decrease in absorbance of NADH at 340 nm in the enzyme-coupled assay is shown in Fig. 1A. The total activity of microsomal ATPases was 300±11 nmol/min/mg protein (Fig. 1B, CON). Vanadate (1 mM), an inhibitor of P-type ion-motive ATPases (*13*), decreased the ATPase activity to 186±9 nmol/min/mg protein. In the presence of 10 μM thapsigargin, a specific antagonist of sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER) Ca^{2+}-ATPases (*14*), the activity of microsomal ATPase was reduced to 141±17 nmol/min/mg protein. After the simultaneous addition of both thapsigargin and vanadate, the activity was 90±13 nmol/min/mg protein, showing a partial additive effect.

Effect of Vanadate on Microsomal ATPases—Since vanadate specifically inhibits P-type ATPases by binding to the phosphorylation site of ATPases located in cytoplasmic and ER membranes, the dose response of vanadate was investigated to characterize microsomal ATPases (Fig. 2). When the concentration of vanadate was increased from 1 nM to 100 μM, the microsomal ATPase activity first decreased by ~20% of its total activity and then reached a plateau phase. At concentrations of vanadate above 100 μM, the ATPase activity rapidly decreased and was completely inhibited by 5 mM vanadate.

The data were fitted to two sigmoidal functions, which were calculated by a computer program and are plotted as a solid line in Fig. 2. The data-fitting analysis indicates that there are two types of vanadate-induced inhibition which correspond to a low-affinity vanadate-sensitive (LAVS) ATPase and a high-affinity vanadate-sensitive (HAVS) ATPase. Analysis of the dose response revealed that the apparent K_i values of vanadate were 1.3 mM and 280 nM for LAVS and HAVS ATPases, respectively.

As shown in Fig. 1, thapsigargin inhibited more than 50% of total microsomal ATPase activity, implying that thapsigargin-sensitive ATPase is the one of major ATPases in the microsomes. When the dose response of vanadate was

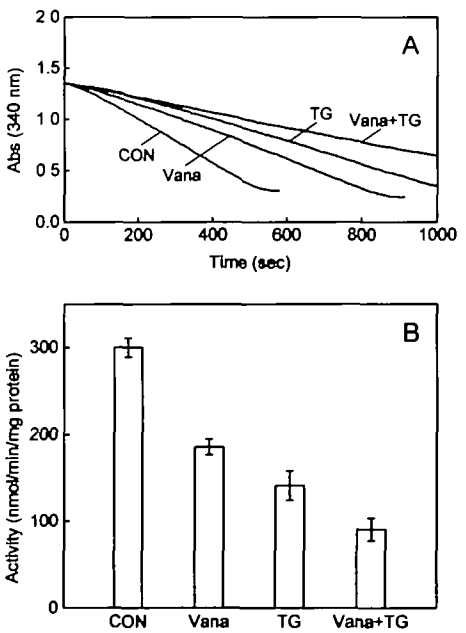

Fig. 1. **Effects of selective inhibitors on the activity of microsomal ATPases.** The activity of microsomal ATPase was measured using an enzyme-coupled system as described under MATERIALS AND METHODS. (A) The activity of microsomal ATPase was coupled to the decrease in absorbance of NADH at 340 nm, which was continuously monitored in the presence of vanadate (Vana, 1 mM), thapsigargin (TG, 10 μM), and both (Vana+TG). (B) The activity of microsomal ATPase was calculated from the slope of the decrease in absorbance. Concentrations of vanadate (Vana) and thapsigargin (TG) were 1 mM and 10 μM, respectively. Values are means±SD (n=4-8).

investigated in the presence of 100 μM thapsigargin, the ATPase activities that were resistant to 100 μM thapsigargin were also fitted to two sigmoidal functions, corresponding to the activities of LAVS and HAVS ATPases (Fig. 3A, filled circles). However, the activity of LAVS ATPase was markedly inhibited by 100 μM thapsigargin. Therefore, thapsigargin seems to selectively inhibit the LAVS ATPase. This became apparent when the thapsigargin-sensitive activity was calculated from the data shown in Fig. 3A by subtracting the thapsigargin-resistant activity (●) from the control activity (■) at each concentration of vanadate. In its dose response to vanadate, the thapsigargin-sensitive activity markedly decreased at the concentrations of vanadate above 100 μM (Fig. 3B). The thapsigargin-sensitive activity remained at ~70% of the control level at concentrations of vanadate up to 100 μM and it decreased to zero at 5 mM.

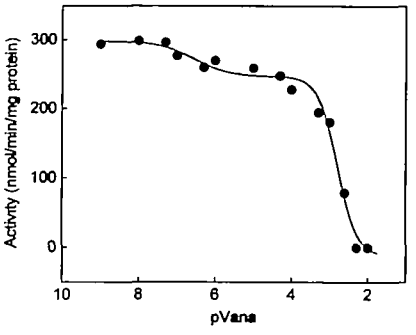

Fig. 2. **Dose-dependent effects of vanadate on the activity of microsomal ATPases.** The microsomal ATPase activities were measured in the presence of vanadate at various concentrations from 1 nM to 5 mM. The data were fitted to two sigmoidal functions, which are represented by the solid line. The negative value of the logarithmic concentration of vanadate is expressed as pVana. The data represent four experiments.

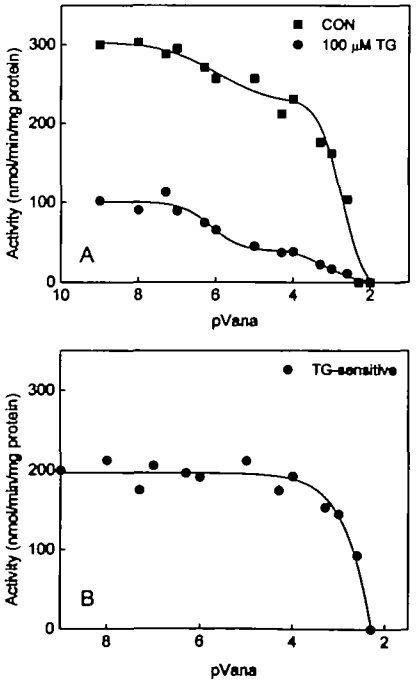

Fig. 3. **Inhibition of low-affinity vanadate-sensitive ATPase by thapsigargin.** (A) Dose response to vanadate of microsomal ATPase activities. Vanadate-resistant activities were measured in the absence (■) and presence (●) of 100 μM thapsigargin. The concentration of vanadate was increased from 1 nM to 5 mM. (B) Dose response to vanadate of thapsigargin-sensitive ATPase activity. The thapsigargin-sensitive activity was calculated from the data shown in Fig. 3A by subtracting the activity in the presence of thapsigargin (●) from that in the absence of thapsigargin (■) at each concentration of vanadate.

Effect of Thapsigargin on Microsomal ATPases—To examine whether thapsigargin inhibits the activity of LAVS ATPase as suggested above, the dose response to thapsigargin was measured in the presence of 100 μM vanadate, a sufficient concentration to block the activity of HAVS ATPase. When the concentration of thapsigargin was increased from 1 nM to 100 μM, the microsomal ATPase activity rapidly decreased at the concentrations above 1 μM in the absence of vanadate (Fig. 4A, CON). In the presence of 100 μM vanadate, the pattern of decrease in the activity was similar to that of the control (Fig. 4A, Vana). The apparent K_i values of thapsigargin were close to 10 μM in both cases.

At each concentration of thapsigargin used in Fig. 4A, the

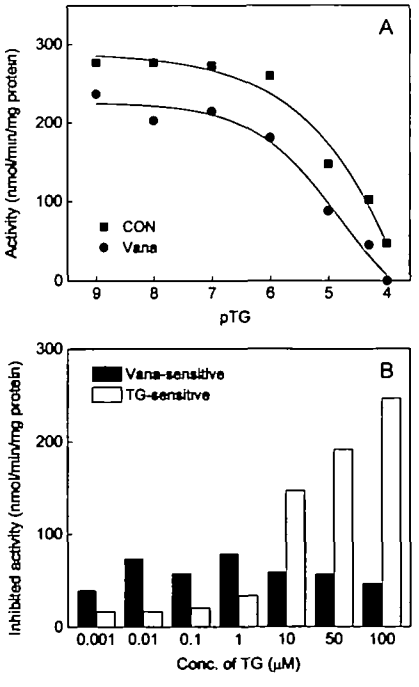

Fig. 4. **Dose response to thapsigargin of microsomal ATPase activities.** (A) Dose responses to thapsigargin in the absence (■) and in the presence (●) of 100 μM vanadate. The concentration of thapsigargin was increased from 1 nM to 100 μM and expressed as the negative value of logarithmic concentration of thapsigargin (pTG). (B) Activities of thapsigargin-sensitive and vanadate-sensitive ATPases. The activities of thapsigargin-sensitive ATPases (open bar) were calculated from the data shown in Fig. 4A by subtracting the activities in the presence of various concentrations of thapsigargin from the control activity in the absence of thapsigargin. Similarly, the activities of 100 μM vanadate-sensitive ATPases (filled bar) were calculated from the data shown in Fig. 4A by subtracting the activities in the presence of 100 μM vanadate (●) from the activities in the presence of the corresponding concentration of thapsigargin (■).

vanadate-sensitive activity and the thapsigargin-sensitive activity were calculated as shown in Fig. 4B. The HAVS ATPase activity inhibited by 100 μM vanadate was 40-70 nmol/min/mg protein over the whole range of thapsigargin concentration, implying that the activity of HAVS ATPase is not sensitive to thapsigargin up to 100 μM. However, the activity inhibited by thapsigargin was greatly increased at concentrations of thapsigargin above 1 μM. This indicates that the activity of LAVS ATPase is very sensitive to thapsigargin at concentrations above 1 μM.

$^{45}Ca^{2+}$ Uptake Mediated by Microsomal ATPases—We next examined whether microsomal ATPase activity is correlated with microsomal $^{45}Ca^{2+}$ uptake. Active uptake was initiated by the addition of 0.5 mM ATP and measured by a filtration method under various conditions (Fig. 5A). In the control experiment (no inhibitor), active uptake reached near maximal level after 7 min. The inhibitors of microsomal ATPase, vanadate and thapsigargin, decreased the maximal uptake level. When microsomal $^{45}Ca^{2+}$ uptake was measured in the absence of ATP, passive uptake was one-third of the control uptake. Specific uptake was calculated after 7 min in each condition (Fig. 5B). The microsomal $^{45}Ca^{2+}$ uptake was 17.13±0.89 pmol/μg protein in the control experiment, and it was decreased to 13.12±0.55 pmol/μg protein by 100 μM vanadate. Thapsigargin (100 μM) inhibited the microsomal $^{45}Ca^{2+}$ uptake further, to 8.50±0.63 pmol/μg protein. In the presence of both vanadate and thapsigargin, the microsomal $^{45}Ca^{2+}$ uptake was 6.80±0.36 pmol/μg protein, showing a partial additive effect of these drugs. In the absence of ATP, passive $^{45}Ca^{2+}$ uptake was measured at 5.30±0.28 pmol/μg protein.

Inhibitory effects of 100 μM vanadate and 100 μM thapsigargin were additive on the activities of microsomal ATPases (Fig. 6). Vanadate and thapsigargin at the concentrations of 100 μM inhibited the activity of microsomal ATPase by 25% and by 80%, respectively. The residual activity was negligible after the inhibition of ATPase activities by both inhibitors. When the activity of HAVS ATPase was blocked by 100 μM vanadate, the activity of microsomal ATPase was decreased by 25% (Fig. 6) and the microsomal $^{45}Ca^{2+}$ uptake was inhibited by ~20% (Fig. 5). In the presence of 100 μM thapsigargin, more than 80% of microsomal ATPase activity was inhibited (Fig. 6) and the microsomal $^{45}Ca^{2+}$ uptake decreased to 50% (Fig. 5). However, after subtracting the radioactivity of passive uptake, which was obtained in the absence of ATP, the thapsigargin-induced inhibition of microsomal $^{45}Ca^{2+}$ uptake was ~70%. Although the amount of microsomal $^{45}Ca^{2+}$ taken up may not be directly proportional to the cumulated activity of the microsomal ATPase for 7 min, these results qualitatively show a positive correlation between the microsomal $^{45}Ca^{2+}$ uptake and the activity of microsomal ATPases.

Dose Responses to Vanadate and Thapsigargin of Microsomal $^{45}Ca^{2+}$ Uptake—It appears that the two types of vanadate-sensitive microsomal ATPases correlate with microsomal $^{45}Ca^{2+}$ uptake, since the effects of 100 μM vanadate and 100 μM thapsigargin on microsomal $^{45}Ca^{2+}$ uptake appear to be additive. To confirm this, the dose responses to vanadate and thapsigargin of the microsomal $^{45}Ca^{2+}$ uptake were obtained. The microsomal $^{45}Ca^{2+}$ uptake was gradually decreased by increasing the concentration of vanadate (Fig. 7A). We were not clearly able to observe the presence of two steps of inhibition of microsomal $^{45}Ca^{2+}$ uptake as shown above for the ATPase activity. This is probably because, in this method, we are measuring the cumulated microsomal $^{45}Ca^{2+}$ rather than the rate of microsomal $^{45}Ca^{2+}$ uptake. A similar inhibitory effect of thapsigargin on microsomal $^{45}Ca^{2+}$ uptake was observed (data not shown). When the dose response of microsomal $^{45}Ca^{2+}$ uptake mediated by LAVS ATPase was measured by

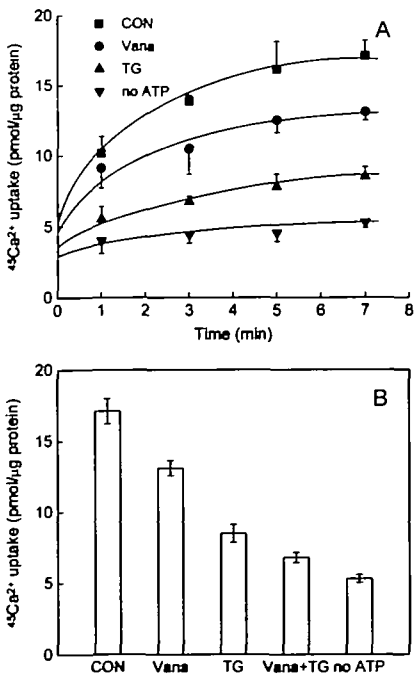

Fig. 5. **Vanadate- and thapsigargin-induced inhibition of microsomal $^{45}Ca^{2+}$ uptake.** (A) Microsomal $^{45}Ca^{2+}$ uptake was measured by a filtration method in the uptake medium after incubation for 1, 3, 5, and 7 min. The uptake was measured in the presence of 100 μM vanadate (Vana), 100 μM thapsigargin (TG), or both inhibitors (Vana+TG). Passive $^{45}Ca^{2+}$ uptake was measured in the absence of ATP (no ATP). (B) Microsomal $^{45}Ca^{2+}$ uptake under the above conditions after incubation for 7 min. Values are means±SD (n=5).

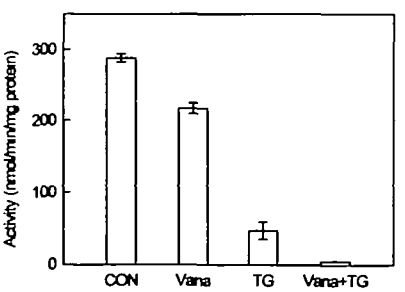

Fig. 6. **Effects of thapsigargin and vanadate on the activity of microsomal ATPase.** The activity of microsomal ATPase was measured by an enzyme-coupled assay. The activity of microsomal ATPase was calculated from the slope of the decrease in absorbance and it was 288±6 nmol/min/mg protein in control condition (CON). In the presence of 100 μM vanadate (Vana), the activity was 217±7.5 nmol/min/mg protein and 100 μM thapsigargin (TG) decreased the activity to 47±12 nmol/min/mg protein. The activity decreased to near zero, 5±0.5 nmol/min/mg protein, in the presence of both vanadate and thapsigargin. Values are means±SD (n=5).

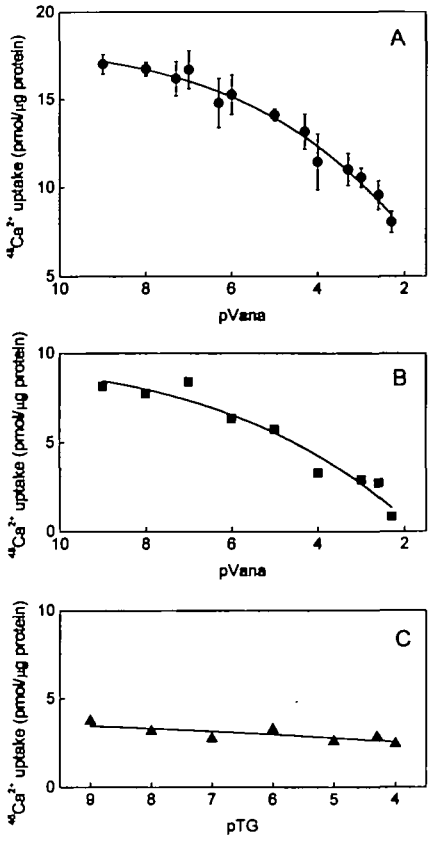

Fig. 7. **Dose responses to vanadate and thapsigargin of microsomal $^{45}Ca^{2+}$ uptake.** (A) Microsomal $^{45}Ca^{2+}$ uptake was measured at various concentrations of vanadate after incubation for 7 min. Values are means ± SD (n=3-5). (B) Dose response to vanadate of microsomal $^{45}Ca^{2+}$ uptake mediated by LAVS ATPase. The LAVS ATPase-mediated microsomal $^{45}Ca^{2+}$ uptake was measured by subtracting microsomal $^{45}Ca^{2+}$ uptake in the presence of 100 μM thapsigargin from the control uptake at each concentration of vanadate. (C) Dose response to thapsigargin of the microsomal $^{45}Ca^{2+}$ uptake mediated by HAVS ATPase. The HAVS ATPase-mediated microsomal $^{45}Ca^{2+}$ uptake was calculated by subtracting microsomal $^{45}Ca^{2+}$ uptake in the presence of 100 μM vanadate from the control uptake at each concentration of thapsigargin.

subtracting microsomal $^{45}Ca^{2+}$ uptake in the presence of 100 μM thapsigargin from the control uptake at each concentration of vanadate, the microsomal $^{45}Ca^{2+}$ uptake was gradually decreased by increasing the concentration of vanadate and completely inhibited by 5 mM vanadate (Fig. 7B). However, when the dose response of microsomal $^{45}Ca^{2+}$ uptake mediated by HAVS ATPase was measured by subtracting microsomal $^{45}Ca^{2+}$ uptake in the presence of 100 μM vanadate from the control uptake at each concentration of thapsigargin, no significant dependence of microsomal $^{45}Ca^{2+}$ uptake on thapsigargin was observed (Fig. 7C). These results demonstrate again that two types of vanadate-sensitive ATPases mediate microsomal $^{45}Ca^{2+}$ uptake, and only the uptake by LAVS ATPase is sensitive to thapsigargin.

Effect of Ionophore on the Microsomal $^{45}Ca^{2+}$ Uptake—The above results suggest that microsomal $^{45}Ca^{2+}$ uptake is mediated by both types of vanadate-sensitive microsomal ATPases. However, it is also possible that it is mediated by ion-coupled Ca^{2+} transporters rather than by ATP-hydrolyzing primary Ca^{2+} transporters. To investigate the presence of monovalent cation-coupled secondary Ca^{2+} transporters, the effect of gramicidin, an ionophore for monovalent cations, was measured on the microsomal $^{45}Ca^{2+}$ uptake (Fig. 8). The microsomal $^{45}Ca^{2+}$ uptake mediated by the two types of vanadate-sensitive ATPases seem to be independent of monovalent cations. The microsomal $^{45}Ca^{2+}$ uptake was 17.36±0.88 pmol/μg protein in the control experiment (CON). When 5 μg/ml gramicidin was added to dissipate the electrochemical gradients of H^+ and monovalent cations which could be formed by any H^+ or monovalent cation-transporting ATPases, if present, the microsomal $^{45}Ca^{2+}$ uptake was 15.32±1.80 pmol/μg protein (Gram). In the presence of thapsigargin (100 μM), the microsomal $^{45}Ca^{2+}$ uptake decreased to 8.50±0.35 pmol/μg protein. Microsomal $^{45}Ca^{2+}$ uptake in the presence of both thapsigargin and gramicidin was 9.35±1.30 pmol/μg protein. These results indicate that gramicidin has no significant effect on the microsomal $^{45}Ca^{2+}$ uptake, and the microsomal $^{45}Ca^{2+}$ uptake mediated by either HAVS ATPase or LAVS ATPase is not likely to be associated with H^+ or monovalent cations.

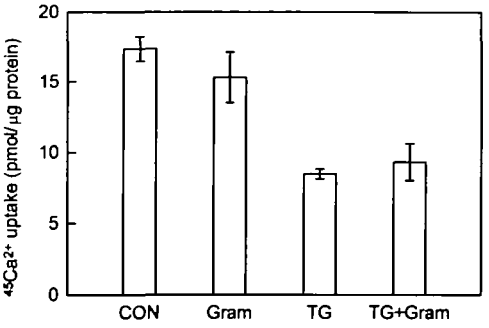

Fig. 8. **Effect of gramicidin on the microsomal $^{45}Ca^{2+}$ uptake.** Microsomal $^{45}Ca^{2+}$ uptake under various conditions was measured by a filtration method after incubation for 7 min. Each experiment was done with microsomes containing ~40 μg proteins. Uptake was measured in the presence of 5 μg/ml gramicidin (Gram), 100 μM thapsigargin (TG), or both drugs (TG + Gram). Values are means ± SD (n=5).

DISCUSSION

Cytosolic free Ca^{2+} plays critical roles in many physiological processes in airway epithelial cells (*3, 7*). Therefore, it is important to understand the mechanism which regulates cytosolic free Ca^{2+} concentration. Ca^{2+}-ATPases, active Ca^{2+} transporters, are known to play a major role in the regulation of cytosolic Ca^{2+} concentration; however, the properties of Ca^{2+}-ATPases have not been well studied in airway epithelial cells. In the present study, we identified two types of vanadate-sensitive microsomal ATPases in porcine airway epithelial cells. One-fifth of microsomal ATPase activity was mediated by the HAVS ATPase and four-fifths by the LAVS ATPase. Both HAVS and LAVS ATPases mediated microsomal $^{45}Ca^{2+}$ uptake, and this uptake seemed to be independent of monovalent cations. Our results suggest that these vanadate-sensitive ATPases are somehow related to Ca^{2+} transport processes in these cells.

In the dose response to vanadate, the activity of thapsigargin-sensitive ATPase rapidly decreased at concentrations of vanadate above 100 μM (Fig. 3B), indicating that the LAVS ATPase is thapsigargin-sensitive. These results imply that the LAVS ATPase is an ER/SR-type Ca^{2+}-ATPase. The apparent K_i values of LAVS ATPase for vanadate and thapsigargin were 1.3 mM and 10 μM, respectively. On the other hand, the activity of HAVS ATPase seemed to be thapsigargin-insensitive with an apparent K_i value for vanadate of 280 nM. Although the HAVS ATPase mediates microsomal $^{45}Ca^{2+}$ uptake, it is not clear whether HAVS ATPase has a primary Ca^{2+}-ATPase activity. The Ca^{2+}-ATPase in SR has been suggested to have two vanadate-binding sites with different affinities (*13*). This may not be the case for the vanadate-sensitive ATPases in tracheal epithelial cells. The activities of LAVS and HAVS ATPases in these cells are probably mediated by two types of microsomal ATPases, since the inhibitory effect of thapsigargin appears only on the LAVS ATPase.

Although most cells in the tracheal epithelial layer are ciliated, other types of cells, goblet cells and submucosal cells, are also present (*15*). Which of these cell types and what cellular locations the vanadate-sensitive microsomal ATPases occupy is unknown. However, the activities of Ca^{2+}-ATPases are essential for Ca^{2+} transport in four possible intracellular locations in airway epithelial cells: the $InsP_3$-sensitive Ca^{2+} store (*4*), the caffeine-sensitive Ca^{2+} store (*5*), the thapsigargin-insensitive Ca^{2+} store (*7*), and the plasma membrane. Thapsigargin is often used to deplete the intracellular Ca^{2+} stores by inhibiting ER-type Ca^{2+}-ATPase in ciliated epithelial cells (*7, 16*). Therefore, the thapsigargin-sensitive LAVS ATPase is probably located on the membranes of intracellular Ca^{2+} stores. The vanadate-sensitivity as well as thapsigargin-sensitivity of LAVS ATPase also supports this idea. The activity of LAVS ATPase was completely inhibited at millimolar concentrations of vanadate. The low sensitivity of LAVS ATPase to vanadate appeared similar to those of intracellular ER-type Ca^{2+}-ATPases reported from neurons (*17*), skeletal muscles (*17, 18*), and pancreatic acinar cells (*19*).

The HAVS ATPase showed high sensitivity to vanadate with a K_i value of 280 nM (Fig. 2). In contrast, thapsigargin did not decrease the activity of HAVS ATPase up to 100 μM. This result may indicate that the HAVS ATPase is located on the plasma membrane, since the Ca^{2+}-ATPase in the plasma membrane is thapsigargin-insensitive and has a K_i value for vanadate of $\sim$1 μM in other cells (*20, 21*). However, it is possible that the HAVS ATPase occupies the membrane of a thapsigargin-insensitive Ca^{2+} store. It has been reported that the Ca^{2+} release from thapsigargin-insensitive stores induces an increase in ciliary activity in airway epithelial cells (*7*).

We also examined whether microsomal ATPase activity is correlated with microsomal $^{45}Ca^{2+}$ uptake. The inhibitors of microsomal ATPase, vanadate and thapsigargin, decreased microsomal $^{45}Ca^{2+}$ uptake, and the inhibitory effects of vanadate and thapsigargin were additive (Fig. 5). These results demonstrate that two types of vanadate-sensitive microsomal ATPases correlate with microsomal $^{45}Ca^{2+}$ uptake. In order to characterize ATPase-mediated microsomal $^{45}Ca^{2+}$ uptake, the dose responses to vanadate and thapsigargin were measured. The microsomal $^{45}Ca^{2+}$ uptake gradually decreased at concentrations of vanadate below 1 μM and steeply decreased at higher concentrations (Fig. 7A). However, no clear two-step inhibition of microsomal $^{45}Ca^{2+}$ uptake by vanadate was observed. Nevertheless, the presence of two types of microsomal $^{45}Ca^{2+}$ uptake was demonstrated by isolating 100 μM vanadate-sensitive and 100 μM thapsigargin-sensitive $^{45}Ca^{2+}$ uptakes. The microsomal $^{45}Ca^{2+}$ uptake mediated by LAVS ATPase was sensitive to 100 μM thapsigargin and was completely inhibited by 5 mM vanadate (Fig. 7B). However, the microsomal $^{45}Ca^{2+}$ uptake mediated by HAVS ATPase was inhibited by 100 μM vanadate but was not sensitive to thapsigargin (Fig. 7C). These results imply that there are two types of vanadate-sensitive $^{45}Ca^{2+}$-translocating ATPases in tracheal epithelial cells, of which only LAVS ATPase is inhibited by thapsigargin.

The majority of microsomes used in this study seem to be tightly sealed vesicles, since we have shown previously a saturation in microsomal $^{45}Ca^{2+}$ uptake and spontaneous release of luminal Ca^{2+} by $InsP_3$, caffeine, and Ca^{2+} ionophore A23187 (*5*). The $^{45}Ca^{2+}$ uptake experiment shown in Fig. 5 demonstrates a significant amount of $^{45}Ca^{2+}$ accumulation in microsomes. However, the orientation of some vesicles might be reversed during the procedure of microsomal preparation (*22*). The formation of inside-out vesicles may make it possible to measure the activity of ATPases in the plasma membrane.

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