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ATP-driven Ca^{2+}/H^+ antiport in acid vesicles from *Dictyostelium*

(vacuolar ATPase/Ca-ATPase/acidification/differentiation/slime mold)

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ABSTRACT **Amoebae of the cellular slime mold *Dictyostelium discoideum* possess an extensive and dynamic endomembrane system that includes many types of acidic vacuoles. A light membrane fraction from *Dictyostelium*, rich in vacuolar-type H^+-ATPase, has been described [Padh, H., Lavasa, M. & Steck, T. L. (1989) *J. Cell Biol.* 108, 865–874]. Here, we show that this "acidosomal" fraction also contains a high-affinity vanadate-sensitive Ca^{2+} uptake activity that is stimulated by the pH gradient formed by the H^+-ATPase. We attribute this Ca^{2+} uptake to the presence of a H^+-countertransporting Ca^{2+}-ATPase, pumping Ca^{2+} into an acidic compartment.**

Cytoplasmic Ca^{2+} is a ubiquitous regulator of cellular activities in higher eukaryotes and plays an essential role as a second messenger in signal transduction. Accordingly, cells have evolved a variety of molecular devices (channels, pumps, and transporters) for regulating influx and efflux of Ca^{2+} across the plasma membrane and between intracellular stores to adjust its concentration in the cytoplasm (1).

To understand the role of calcium in the choice of cell differentiation pathway in the cellular slime mold *Dictyostelium discoideum*, we have investigated the mechanisms of cellular calcium homeostasis in this organism. A model (2) for the effects of weak bases on cell-type specification in *D. discoideum* that suggests the involvement of acidic intracellular compartments in the control of cytoplasmic [Ca^{2+}] led us to seek a Ca^{2+}/H^+ antiport in membranes from *D. discoideum*. Padh *et al.* (3, 4) had described a light membrane fraction from *D. discoideum* that is rich in vacuolar-type H^+-ATPase and contains vesicles that are capable of internal acidification in the presence of ATP. Here, in the same subcellular membrane fraction, we demonstrate high-affinity uptake of Ca^{2+} from the medium. This uptake is completely inhibited by vanadate and is partially dependent on the proton gradient formed by the action of the H^+-ATPase. It appears to be mediated by a H^+-translocating Ca^{2+}-ATPase.

MATERIALS AND METHODS

Isolation of Membranes. *D. discoideum* Ax-3 was grown in shaking suspension in Ashax (5) medium, to a density of 8–10 $\times$ 10^5 cells per ml. Cells were washed in pH 8.5 buffer (100 mM sucrose/5 mM glycine·NaOH/1 mM dithiothreitol/50 μM phenylmethylsulfonyl fluoride) and lysed by a single pass through a Nuclepore filter (5-μm pores) (6). Light endomembrane vesicles were isolated by successive centrifugation in 45% and 12% (wt/wt) sucrose in pH 8.5 buffer, largely by the procedure of Padh *et al.* (4). Membrane pellets were resuspended in a small volume of buffer and kept on ice until used. In some experiments, the 12–45% fraction was applied to continuous sucrose gradients (30 ml, 25–45% sucrose in pH 8.5 buffer, over a 1.5-ml cushion of 53% sucrose). Gradients were centrifuged for 3–4 h at 25,000 rpm in a Beckman Sw28 rotor; 1.5-ml fractions were collected, starting from the 53–45% interface, and kept on ice until used.

Protein was assayed by a modification of the Lowry method (7), using bovine serum albumin as a standard.

ATPase Activity. Rates of hydrolysis of ATP at 25°C and pH 7.3 (100 mM sucrose/100 mM KCl/5 mM $MgCl_2$/20 mM Hepes·NaOH) were estimated by analysis of liberated P_i as the phosphomolybdate complex (8) or were measured in a continuous spectrophotometric assay by the enzyme-linked oxidation of NADH (9). In either case, the reaction was started by addition of an aliquot of an ATP solution to an incubation mixture containing 15–60 μg of protein in 1.2 ml. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole, bafilomycin A_1, or Na_3VO_4 were added to the incubation mixture as concentrated solutions in ethanol or buffer, 3–5 min prior to addition of ATP. The volume of ethanol added never exceeded 1% of the total.

Proton Pump Activity. Acidification of membrane vesicles was monitored at 25°C, by concentrative quenching of fluorescence (excitation at 480 nm and emission at 530 nm) of the weak base acridine orange using a Shimadzu RF5001-PC spectrofluorimeter. Activity was quantified as the maximum (negative) slope of a plot of fluorescence against time, by using a computer program written for that purpose. The reaction was started by addition of ATP (final concentration, 2 mM) to an incubation mixture typically containing 30–100 μg of protein in 2 ml of buffer (100 mM sucrose/100 mM KCl/5 mM $MgCl_2$/2 μM acridine orange/5 mM Hepes·NaOH, pH 7.3). In some experiments, an ATP-regenerating system (creatine phosphate plus creatine kinase, see below) was included. Inhibitors were preincubated with the membranes, as described above.

Uptake of ^{45}Ca. Uptake and retention of $^{45}Ca^{2+}$ was estimated by rapid filtration and scintillation counting. Membranes (0.2–1.6 mg of protein) were preincubated for 5 min at 25°C with 0.5 ml of buffer (100 mM sucrose/100 mM KCl/5 mM $MgCl_2$/0.6 mM $CaCl_2$/0.55 mM EGTA/5 mM Hepes·NaOH, pH 7.3) containing 4–5 μCi of $^{45}Ca^{2+}$ (1 Ci = 37 GBq). Final specific radioactivity of $^{45}Ca^{2+}$ was normally in the range 10–12 Ci/mol. Inhibitors were added as concentrated solutions in buffer or ethanol at the start of the preincubation period. Calcium uptake was initiated by addition of an aliquot of ATP, also containing creatine phosphate and 100 units of creatine kinase, to give final concentrations of 2 mM ATP, 9 mM creatine phosphate, and 7.5 μM free Ca^{2+} (see below). After 5 min at 25°C, triplicate aliquots were filtered rapidly on Millipore GVWP filters (pore size, 0.22 μm) and washed with 7–10 ml of ice-cold buffer (100 mM sucrose/100 mM KCl/5 mM $MgCl_2$/5 mM $CaCl_2$/5 mM Hepes·NaOH, pH 7.3). Filters were immersed in 5 ml of scintillation fluid [toluene/methoxyethanol, 5:1 (vol/vol), containing 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-

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oxadiazole] at 0.5 mg/ml and radioactivity was measured at 80% efficiency using the wide channel of a Beckman LS1701 liquid scintillation counter. In some experiments, concentrations of $CaCl_2$ and EGTA were varied to achieve a range of final free Ca^{2+} concentrations that was calculated from the final total concentrations of Ca^{2+}, Mg^{2+}, H^+, EGTA, ATP, and creatine phosphate, as described in ref. 10.

Acid Phosphatase. Membranes (8–20 μg of protein) were incubated at room temperature in 250 μl of 100 mM glycine·HCl (pH 3) containing 0.2% Triton X-100 and 10 mM *para*-nitrophenyl phosphate. The reaction was stopped after 20 min by addition of 750 μl of 0.2 M Na_3PO_4 (pH 12), and liberated *para*-nitrophenol was estimated from its absorbance at 410 nm.

Materials. Bafilomycin A_1 was a gift from K. Altendorf (University of Osnabrück, Osnabrück, F.R.G.). Creatine phosphate, creatine kinase, pyruvate kinase, lactate dehydrogenase, NADH, and vanadate-free ATP were obtained from Boehringer Mannheim; EGTA and $CaCO_3$ were from Fluka (MicroSelect grade); and acridine orange was from Aldrich. $^{45}CaCl_2$ was obtained from Amersham. All other reagents were from BDH (AnalaR grade) or from Sigma.

Experiments were repeated on two to six separate occasions, and representative data are shown in the figures. Where data have been combined from several experiments, variability is indicated by mean ± SEM for the number of preparations stated.

RESULTS

Padh *et al.* (4) characterized the ATPase activity of the light membrane (acidosome) fraction from *Dictyostelium* as predominantly due to a vacuolar-type ATPase on the basis of inhibitor sensitivities. In agreement with their result, we find (data not shown) that up to 85% of the ATPase activity in the 12–45% sucrose fraction (hereafter referred to as the acidosomal fraction) can be abolished by preincubation with 25 μM 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. In addition, a similar degree of inhibition is seen with micromolar concentrations of bafilomycin A_1 (11), a relatively specific antibiotic inhibitor of vacuolar-type ATPases. The remainder of the activity can be inhibited by 0.1 mM vanadate.

Addition of ATP to a lightly buffered acidosomal suspension in the presence of the fluorescent weak base acridine orange results in rapid quenching of fluorescence, as acridine orange is entrapped and accumulated in its protonated form inside acidified vesicles (Fig. 1). The maximum rate of quenching is linear with protein concentration up to the highest concentration tested (data not shown). Fluorescence quenching is prevented by preincubation with bafilomycin and is rapidly reversed on addition of the electroneutral K^+/H^+ exchanger nigericin (data not shown).

Active uptake of calcium can be demonstrated directly by incubating membranes with $^{45}Ca^{2+}$, buffered with EGTA to 7.5 μM free Ca^{2+}. Uptake is rapid and occurs over a time course similar to that of vesicle acidification (Fig. 1). The level of uptake varied considerably between preparations, but the mean, 15.7 ± 5.7 nmol of Ca^{2+} per mg of membrane protein (±SEM, six preparations), is comparable with that seen, for example, with preparations of endoplasmic reticulum from liver (12).

Nigericin (10 μM) abolished 54 ± 11% (mean ± SEM, n = 6) of ATP-dependent calcium uptake in acidosomal preparations, and 5 μM bafilomycin A_1 was found to eliminate the same proportion of calcium uptake. Inhibition of proton pump activity and of the nigericin-sensitive component of calcium uptake show very similar concentration dependencies (Fig. 2), indicating that nigericin reduces calcium uptake by collapsing the pH gradient generated by the bafilomycin-sensitive proton pump. These results are consistent with vesicular calcium uptake being in part mediated by a Ca^{2+}/H^+ antiporter mechanism, with the motive force for calcium transport being provided by the pH gradient generated by the vacuolar H^+-ATPase. However, 100 μM sodium orthovanadate almost totally (94 ± 6%; mean ± SEM; n = 6) abolishes calcium uptake. Vanadate ion, an inhibitor of P type ATPases such as the Ca^{2+}-ATPases of sarco- or endoplasmic reticulum, does not inhibit ATP-driven proton pumping in acidosomal preparations (data not shown) and would not be expected to inhibit a Ca^{2+}/H^+ antiporter. Essentially complete inhibition of calcium uptake by vanadate indicates that all of the uptake involves an enzyme with a phosphorylated intermediate, and the data, therefore, are more compatible with the operation of a H^+-countertransporting Ca^{2+}-ATPase (ATP-driven Ca^{2+}/H^+ exchanger) located in an acidic compartment. If such an enzyme were to pump Ca^{2+} into the acidic compartment, it could use the energy stored in

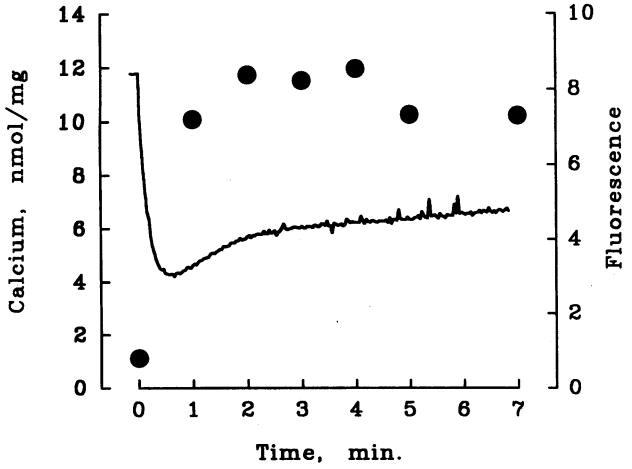

FIG. 1. Time courses of acidification and calcium accumulation by acidosomal membranes. ATP was added at 0 min, to a membrane suspension containing 0.96 mg of protein per ml. Solid circles, uptake of $^{45}Ca^{2+}$; trace, quenching of acridine orange fluorescence.

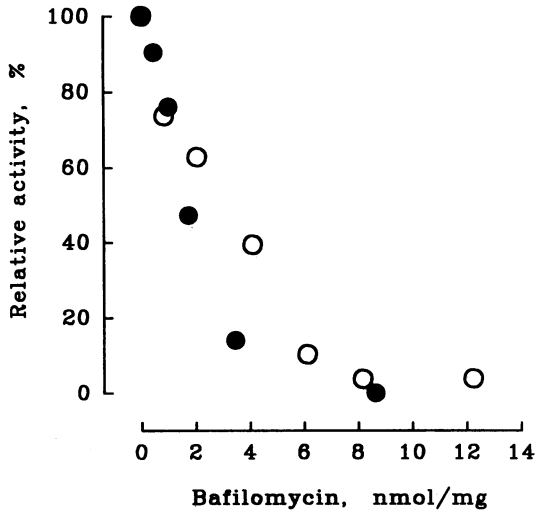

FIG. 2. Inhibition of acidification and of uncoupler-sensitive calcium uptake by bafilomycin A_1. ○, Rate of fluorescence quenching relative to the rate in the absence of the inhibitor; ●, uptake of $^{45}Ca^{2+}$ in 5 min relative to the maximum nigericin-sensitive uptake (5.5 nmol/mg, out of a total ATP-dependent uptake of 9.0 nmol/mg). Protein concentrations were 0.58 mg/ml and 0.14 mg/ml, respectively, for the ^{45}Ca and fluorescence experiments, and the concentration of bafilomycin was varied between 0.1 and 5 μM.

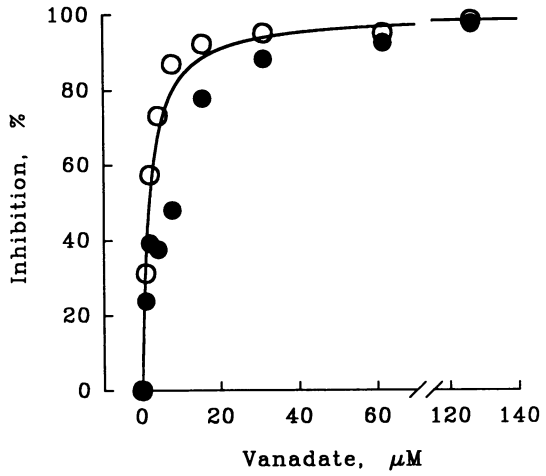

FIG. 3. Sensitivity of acidosomal calcium uptake to vanadate. Inhibition of ATP-dependent uptake of $^{45}Ca^{2+}$ in the absence (○, 100% = 24.6 nmol/mg) or presence (●, 100% = 7.5 nmol/mg) of 8 μM nigericin. The line is drawn assuming an inhibitory binding constant of 2 μM.

the transmembrane pH gradient to assist active accumulation of Ca^{2+} (13).

Vanadate is a potent inhibitor; the concentration dependence of vanadate inhibition of calcium uptake in the presence or absence of nigericin is shown in Fig. 3. Fig. 4 shows the dependence of calcium uptake on free calcium concentration; the apparent affinity of the uptake system for calcium, 0.28 μM, is high enough to be physiologically relevant. In the presence of 80 μM vanadate, only background levels of calcium uptake, probably reflecting binding to acidic phospholipids, are observed.

To determine whether calcium uptake activity was consistently associated with proton pumping activity, the acidosomal fraction was subjected to further fractionation on sucrose density gradients. Fig. 5 shows that proton pump activity and calcium uptake codistribute; both correlate well with the presence of bafilomycin-sensitive (i.e., vacuolar) H^+-ATPase. The calcium uptake in the central fractions of the gradient was blocked >40% by 10 μM nigericin, and the proton-pumping activity was completely sensitive to bafilomycin (data not shown). Fig. 5 also shows that both the calcium-uptake and proton-pumping activities run underneath a broad peak of acid phosphatase activity, which we take to be an endosomal marker.

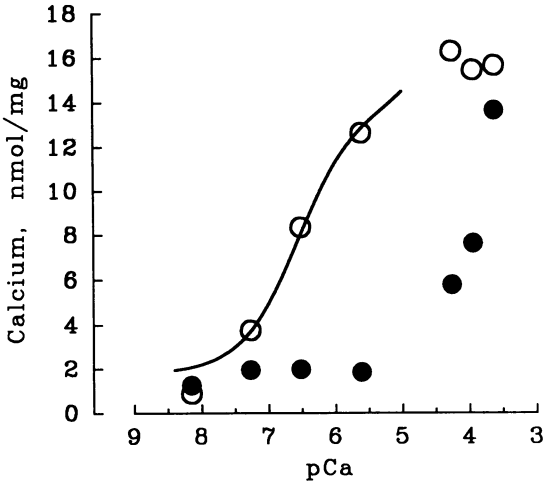

FIG. 4. Calcium dependence of calcium uptake by acidosomal membranes. Accumulation of $^{45}Ca^{2+}$ in the absence (○) or presence (●) of 80 μM sodium orthovanadate. The line is a simulation. Binding constant for activation, 0.28 μM Ca^{2+}; Hill coefficient, 1; maximum uptake, 12 nmol/mg, corrected for background (vanadate-insensitive) binding of Ca^{2+} (K_d, 0.49 mM; capacity, 50 nmol/mg).

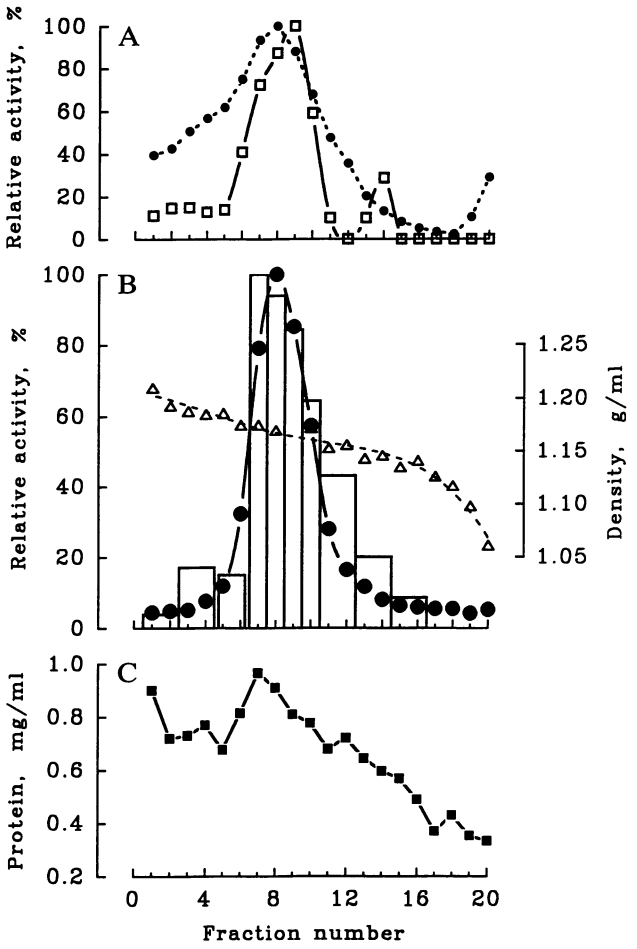

FIG. 5. Sucrose gradient fractionation of acidosomal membranes. (*B*) ●, Rate of acidification (fluorescence quenching); histogram, ATP-dependent uptake of $^{45}Ca^{2+}$ (calcium uptake in the peak fraction is 7.9 nmol/mg, of which 45% is sensitive to nigericin and 97% is sensitive to 0.1 mM vanadate); △, densities of gradient fractions. (*A*) □, ATPase activity sensitive to 4 μM bafilomycin A_1 (the activity of the peak fraction is 0.28 μmol/min/mg, 57% of the total activity in that fraction); ●, acid phosphatase activity (the activity of the peak fraction corresponds to 0.37 μmol/min/mg). (*C*) Distribution of protein.

To investigate the contribution of the H^+-countertransporting Ca^{2+}-ATPase to overall cellular calcium homeostasis, we have examined the inhibitor sensitivity of calcium sequestration by cell lysates. The results in Fig. 6 show that, at a free Ca^{2+} concentration of 7.5 μM, total uptake (bar A) is partially blocked by the mitochondrial inhibitors oligomycin, antimycin, and azide (bar C), as would be expected; further inhibition is seen when the mitochondrial inhibitors are supplemented with the uncoupler nigericin (bar D). The nonmitochondrial component of calcium uptake can be completely blocked by vanadate (bars E–K), with the same concentration dependence as is found for calcium uptake by the acidosomal fraction (Fig. 6 *Inset*), indicating that the same or a very similar calcium pump is involved and, therefore, that the H^+-countertransporting Ca^{2+}-ATPase may make a major contribution to calcium homeostasis *in vivo*.

DISCUSSION

We have demonstrated an association between "acidosomal" proton-pumping activity and high-affinity vanadate-

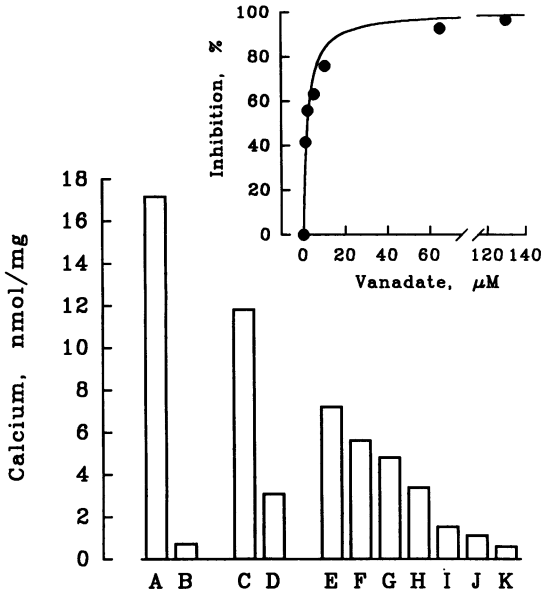

FIG. 6. Uptake of calcium by cell lysate. Bars: A, no inhibitors; B, no ATP; C, plus mitochondrial inhibitors (oligomycin at 6 μg/ml, antimycin at 6 μg/ml, and 0.1 mM azide); D, plus mitochondrial inhibitors and 10 μM nigericin; E–K, plus mitochondrial inhibitors and 1, 2, 5, 10, 65, 130, or 640 μM vanadate, respectively. (*Inset*) Inhibition of nonmitochondrial calcium uptake by vanadate (data from bars C and E–K). The line is drawn assuming an inhibitory binding constant of 2 μM.

sensitive calcium uptake activity. The vanadate sensitivity of the calcium uptake leads us to believe that a P-type ATPase, presumably a Ca-ATPase, is responsible. This sensitivity to vanadate argues against the involvement of the type of Ca^{2+}/H^+ antiporter believed to exist in the vacuoles of fungi and higher plants, the activity of which is insensitive to vanadate (14, 15). The partial inhibition of calcium uptake by the K^+/H^+ exchanger nigericin and by the vacuolar H^+-ATPase inhibitor bafilomycin A_1 suggests that the inside-acid pH gradient due to the vacuolar proton pump facilitates calcium uptake. An alternative explanation (namely, direct inhibition to the same extent) of the vanadate-sensitive calcium uptake by these two unrelated agents seems unlikely; nigericin is not known to have direct inhibitory effects on ATPase activities, and although bafilomycin A_1 has been reported to inhibit the Ca-ATPase of skeletal muscle sarcoplasmic reticulum (11), it did so only at concentrations orders of magnitude higher than those demonstrated here for inhibition of proton pump activity (Fig. 2).

There is a growing body of evidence that calcium-pumping ATPases act as Ca^{2+}/H^+ exchangers (16). It has been proposed that rat parotid (17) and pancreatic (18) acinar cells possess an acidified calcium store filled by the action of a Ca^{2+}/H^+ exchanger, which may be an ATPase. Miller *et al.* (13) have presented evidence that the plasma membrane of *Neurospora* possesses an ATP-driven Ca^{2+}/H^+ exchanger that is stimulated by the inwardly directed proton gradient due to the plasma membrane proton pump and advance a convincing thermodynamic argument that this interaction is important for the maintenance of low cytosolic $[Ca^{2+}]$ in that organism. In *Dictyostelium* and possibly in other cell types where there is evidence for protonophore-sensitive calcium sequestration (17–22), if such an enzyme pumps calcium ions into an acidic compartment, then the cell could use the energy stored in the proton gradient to augment that available from ATP (13). The efficiency of calcium sequestration into acidic intracellular stores would then depend on the magnitude of the transmembrane pH gradient and should be reduced by exposure to weak bases such as ammonia. Although the presence of multiple calcium pools has been proposed in the case of—e.g., rat pancreatic acinar cells (18)—the similar vanadate dependence of total and nigericin-resistant calcium uptake by the acidosomal fractions (Fig. 3) lead us to interpret our data in terms of a single class of calcium uptake system. We cannot on present data exclude the possibility of coexisting calcium pools with similar sensitivities to vanadate, only one of which is sensitive to nigericin; however, even in such a case, the nigericin-sensitive pool would have to account for at least half the observed uptake.

The calcium uptake activity demonstrated in detergent-permeabilized *Dictyostelium* cells (23) displays similar calcium dependence and vanadate sensitivity and is almost certainly the same as that studied by us. However, Milne and Coukell (23) did not detect any protonophore-sensitive nonmitochondrial calcium uptake. We do not know the reason for this difference, but the calcium uptake activity of their system was considerably lower than that of our homogenates. There is also evidence for high-affinity calcium uptake into an inositol trisphosphate-sensitive pool in *Dictyostelium* (24).

Acidosomes (25) are specialized organelles, so far identified only in protozoa, that appear to be responsible for acidifying the endocytic circuit by reversible interactions with incoming endosomes (26). The H^+-countertransporting Ca^{2+}-ATPase involved in calcium sequestration may reside in the acidosomes themselves or in a communicating, possibly endosomal, compartment that cosediments with the acidosomes under our experimental conditions (Fig. 5). It appears to play a major role in overall cellular calcium homeostasis since a calcium sequestering activity with similar inhibitor sensitivities is responsible for most or all of the nonmitochondrial calcium uptake in whole cell lysates (Fig. 6).

The model of cell-type specification proposed by Gross *et al.* (2) requires that calcium be sequestered into an acidic compartment and to that extent, therefore, it is supported by our results. In this model, the choice of pathway of developmental gene expression is dependent upon differences in the elevation of cytoplasmic $[Ca^{2+}]$ in response to cAMP; an increase of $[Ca^{2+}]$ by stimulation with cAMP would direct a cell to the prespore pathway, whereas active calcium uptake into acid vesicles would restrict elevation of cytoplasmic $[Ca^{2+}]$ and, possibly in conjunction with the actions of the stalk-cell-inducing factor DIF-1, induce the cell to enter the prestalk pathway. Other well-documented effects of ammonia on growth and development in *Dictyostelium* (27–30) may also be explicable in terms of elevation of cytoplasmic $[Ca^{2+}]$.

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