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Specific Increase in the Number of Vanadium-Containing Blood Cells by Some Ionophores and Inhibitors of Proton-ATPases in the Ascidian, *Ascidia sydneiensis samea*

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ABSTRACT—Ascidians are known to accumulate high levels of vanadium. Vanadium-accumulating blood cells (vanadocytes) have one large and highly acidic vacuole. Recently, it was found unexpectedly that the number of vanadocytes increased rapidly and significantly when ascidians were immersed in 10 mM or 20 mM NH_4Cl solution for 20 hr to neutralize vacuole content. Suspecting that changes in intra-organellar pH and in levels of ATP caused by the treatment might be involved, we examined whether or not several reagents that perturb either acidic pH or ATP synthesis affected the increase in the number of vanadocytes. SF6847 (a proton conductor), nigericin, monensin, valinomycin (ionophores), 2,4-dinitrophenol (an uncoupler), bafilomycin A_1 (a V-ATPase inhibitor), oligomycin and NaN_3 (F-ATPase inhibitors) all increased the number of vanadocytes by about three- to five-fold over that of control. However, treatment with NaCl, KCl, LiCl, $CaCl_2$, TJ24373, sporeamycin (macrolide antibiotics), ouabain and Na_3VO_4 (P-ATPase inhibitors) had no effect on the increase. These results suggest that neutralization of intra-organellar pH triggers an increase in the number of vanadocytes. Vanadocytes that increased in number in the coelomic fluid after treatment were revealed by immunohistochemical study, to have originated in the connective tissues around the alimentary canal.

INTRODUCTION

Ascidians belonging to the family Ascidiidae are known to accumulate high levels of vanadium corresponding to 10^5 to 10^7 times that in seawater. Among the approximately ten types of blood cells (coelomic cells), signet ring cells are designated vanadocytes that purport vanadium storage (Michibata *et al.*, 1987, 1991). Each vanadocyte has one large vacuole, the content of which is highly acidic; around pH 1.9 to 4.2 (Michibata *et al.*, 1991). Recently, we found that this acidity is maintained by vacuolar H^+-ATPases (V-ATPases) (Uyama *et al.*, 1994). In our previous paper, when vanadium-rich ascidians, *Ascidia sydneiensis samea*, were immersed in seawater that contained 10 mM or 20 mM NH_4Cl to neutralize the vacuole content, it was found unexpectedly that the number of vanadocytes in the coelomic fluid increased rapidly and specifically (Hayashi *et al.*, 1996).

The present experiment was, therefore, planned in which ascidians were treated with several reagents that perturb either acidic pH or ATP synthesis, expecting that changes in intra-organellar pH and in levels of ATP might be involved in the mechanism of the increase in vanadocyte number.

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MATERIALS AND METHODS

Treatment with reagents

Ascidians, *Ascidia sydneiensis samea*, were collected at Otsuchi Marine Research Center, Ocean Research Institute, the University of Tokyo, Otsuchi, Iwate Prefecture and at Asamushi Marine Biological Station, Tohoku University, Asamushi, Aomori Prefecture, Japan. The ascidians were maintained in an aquarium that contained circulating natural seawater at 20°C. For the experiment, they were immersed individually in 50 ml of filtered seawater with or without several kinds of reagents for 18 to 20 hr at 20°C. The reagents tested were chloride salts (10 mM NaCl, 10 mM or 50 mM KCl, 10 mM LiCl, 5 mM $CaCl_2$, 10 mM NH_4Cl), ammonium sulfate (5 mM $(NH_4)_2SO_4$), a proton conductor (1 μM SF6847 (3,5-di-*t*-butyl-4-hydroxybenzilidenemalononitrile)), ionophores (5 μg/ml nigericin plus 50 mM KCl, 5 μg/ml monensin, 5 μM valinomycin plus 10 mM KCl), macrolide antibiotics (2 μM bafilomycin A_1, 10 μg/ml TJ24373, 10 μg/ml sporeamycin), inhibitors of mitochondrial ATP synthetase (F-ATPase) (5 μM oligomycin, 1 mM NaN_3), an uncoupler (1 mM 2,4-dinitrophenol), and inhibitors of P-type ATPases (1 mM ouabain, 1 mM Na_3VO_4). After the treatment, the tunic was removed and the coelomic fluid was drawn by cardiac puncture into 2 ml of ice cold artificial seawater (ASW) containing 460 mM NaCl, 9 mM KCl, 33 mM Na_2SO_4, 6 mM $NaHCO_3$ and 5 mM HEPES (N-2-

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hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.0, to avoid clotting.

Cell count

An aliquot of 100 μl of coelomic fluid containing coelomic cells suspended in ASW was used to count the number of each type of coelomic cell with a hemocytometer.

Measurement of vanadium content

The vanadium content in the coelomic cells was measured by flameless atomic absorption spectrophotometry. The coelomic fluid was centrifuged at 400 × g for 10 min at 4°C. The resultant pellet was suspended in 10 ml of ASW containing 200 mM sucrose and 20 mM MOPS (3-(N-Morpholino)propanesulfonic acid) -Tris (2-Amino-2-hydroxymethyl-1,3-propanediol) at pH 8.0. Then, the suspension was centrifuged at 300 × g for 10 min at 4°C to remove giant cells having no vanadium. The cells obtained were resuspended in ASW and centrifuged twice at 400 × g for 10 min at 4°C. The pellet was resuspended in 500 μl of ASW, and 100 μl of the suspended solution was used to count the cell number as described above. The remaining cell suspension was diluted appropriately with 0.1 N HNO_3 (super special grade; Wako Pure Chemical Indust. Ltd., Japan), and 10 μl of this solution was loaded onto the flameless atomic absorption spectrophotometer (Seiko Instruments Inc., Nagano, Japan). The absorption line was 318.4 nm.

Measurement of the concentration of ATP

ATP concentrations were measured utilizing bioluminescence as described (Strehler and Totter, 1954). The coelomic fluid was adjusted to pH 7.0 with Tris, and then boiled immediately for 5 min. Next the samples (40 μl) were mixed with 50 μl of 200 mM HEPES-NaOH (pH 7.75), 5 μl of 200 mM $MgSO_4$, 5 μl of 1 mg/ml luciferase, and 100 μl of 1 mM D-luciferin. After fifteen seconds, photoluminescence was measured for 1 min by an Aloka BLR-101C bioluminescence reader (Aloka CO., LTD., Tokyo, Japan). ATP concentrations were expressed as nmols/mg protein.

Protein assay

Protein concentrations were measured by the method of Bradford (Bradford, 1976) using a Bio-Rad protein assay kit (Nippon Bio-Rad Laboratories, Inc., Tokyo, Japan), with bovine serum albumin as the standard.

Statistical analysis

Data were statistically analyzed by Student's *t*-test.

Immunohistochemical staining

The specimens were fixed with 100% methanol for 20 min and then 100% ethanol for 20 min at -20°C. Then, the specimens were embedded in a polyester wax and sliced with a microtome at a thickness of 6 μm. After removal of the polyester waxes with 100% ethanol, specimens were treated with the monoclonal antibody S4D5 which interacts specifically with vanadocytes (Uyama *et al.*, 1991). After extensive washing, the immunoreactivity was visualized with a Histofine SAB-PO (M) immunohistochemical staining kit (Nichirei Inc., Tokyo, Japan) according to the manufacture's instructions.

RESULTS

Dissipation of a transmembrane proton gradient (Δ pH)

After treatment with 10 mM NH_4Cl for 20 hr, the size of the population of vanadocytes in coelomic fluid was observed to increase to about three times that of control. No such increase in any of the other cell types occurred (Fig. 1), confirming the results described previously (Hayashi *et al.*, 1996). Treatment with chloride salts (10 mM NaCl, 10 mM or 50 mM KCl, 10 mM LiCl and 5 mM $CaCl_2$) had no effect. Thus, it is apparent that NH_4^+ is responsible for the increase in the number of vanadocytes. The fact that treatment with 5 mM $(NH_4)_2SO_4$ also resulted in an increase in the population of vanadocytes (Fig. 1) supports this.

NH_4^+ accumulated in ascidian tissues was observed to abolish the pH gradient in vacuoles of vanadocytes, as described later. Therefore, neutralization of the acidic compartment might cause the increase in the number of vanadocytes. The next experiment was designed to examine whether or not the dissipation of intracellular Δ pH causes an increase in the population of vanadocytes. After treatment with 1 μM SF6847, a kind of proton conductor, the number of vanadocytes increased about four times that of control (Fig. 1). Nigericin and monensin are known to translocate H^+ into K^+, and H^+ into Na^+, respectively, across the membrane systems (Pressman, 1976). Treatment with 5 μM nigericin plus 50 mM KCl caused an increase in cell number, as did 5 μM monensin (Fig. 1). Treatment with 50 mM KCl alone had no effect. These results clearly indicated that dissipation of intracellular Δ pH awakes an increase in the number of vanadocytes.

A macrolide antibiotic, bafilomycin A_1, a specific inhibitor of V-ATPase (Bowman *et al.*, 1988), increased the number of vanadocytes as reported previously (Hayashi *et al.*, 1996) (Fig. 1). TJ24373 and sporeamycin, macrolide antibiotics but not inhibitors of V-ATPase, had no effect. It is, therefore, apparent that specific inhibition of V-ATPase by bafilomycin A_1 causes an increase in cell number.

Inhibition of ATP synthesis

Ion-pumping ATPases are classified into three groups, V-, F- and P-types (Pedersen and Carafoli, 1987). To examine whether F- and P-ATPases are involved in the increase in vanadocyte numbers, ascidians were treated with some inhibitors of these ATPases. Treatments with 1 mM ouabain and 1 mM Na_3VO_4, specific inhibitors of P-ATPase (Pedersen and Carafoli, 1987), caused no increase in cell number but treatments with 5 μM oligomycin and 1 mM NaN_3, known inhibitors of F-ATPase (Futai and Kanazawa, 1980), increased the number of vanadocytes (Fig. 1).

The main functions of V-ATPase and F-ATPase are to expend and to synthesize ATP, respectively, differing from the function of P-ATPase which is to form a phospho-enzyme intermediate. These results, therefore, suggest that inhibition of ATP synthesis might be involved in the increase in the number of vanadocytes. To examine this possibility, ascidians were treated with uncoupler or potassium ionophore, 2,4-dinitrophenol and valinomycin, known to inhibit ATP synthesis without inhibition of F-ATPases. Consequently, treatment with 1 mM 2,4-dinitrophenol and 5 μM valinomycin plus 10 mM KCl resulted in an increase in the number of vanadocytes (Fig. 1).

The results obtained by a series of the above experiments suggest that a decrease in ATP levels causes the increase in

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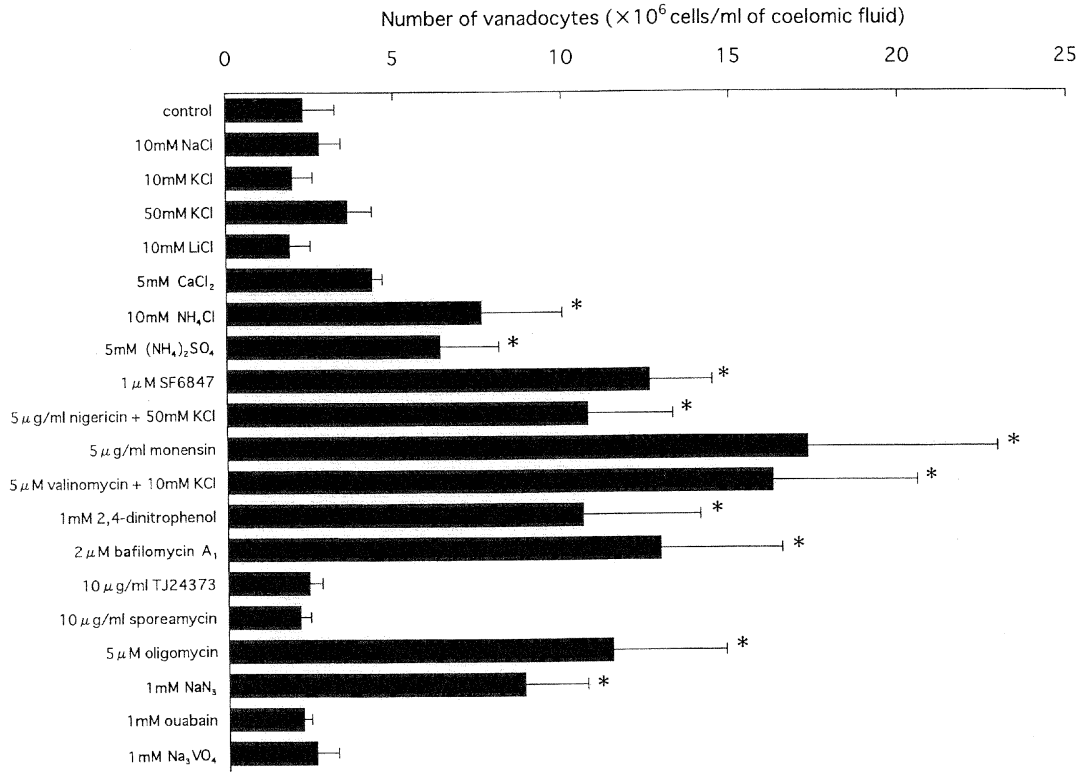

Fig. 1. Changes in the number of vanadocytes. The ascidian, *Ascidia sydneiensis samea*, was immersed in seawater with or without reagents for 20 hr, and the number of vanadocytes counted. The number per one ml of coelomic fluid was calculated. Data represent the average of three trials ± S.E. * indicates statistically significant difference from the control ($P < 0.01$). The number of vanadocytes was increased to about 3 to 5 times that of control by treatment with NH_4Cl, ionophores or the inhibitors of V- and F-ATPases.

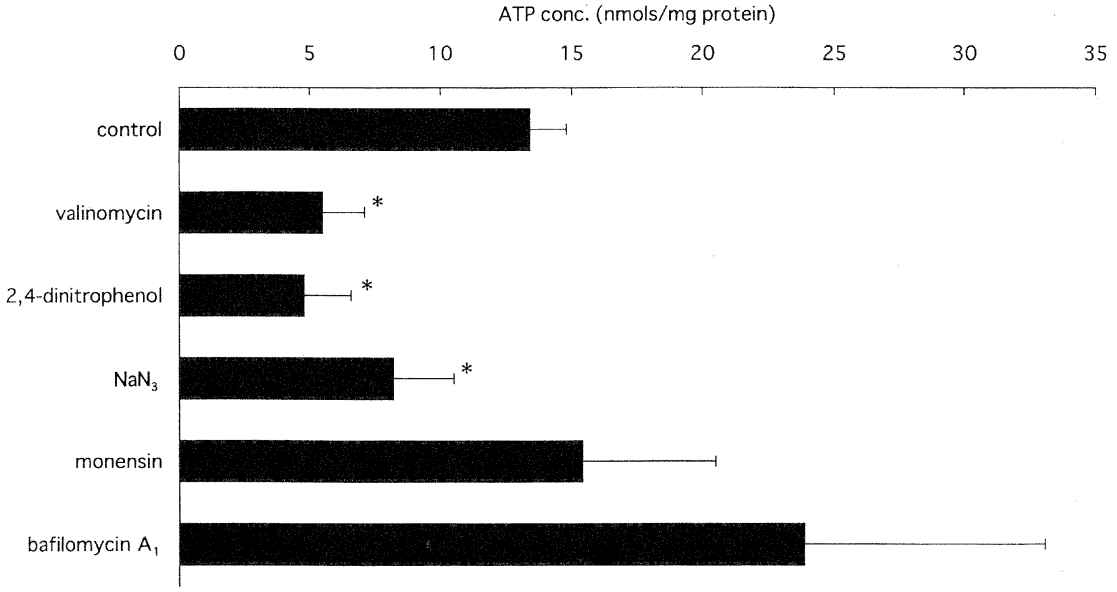

Fig. 2. Levels of ATP in the coelomic fluid. Levels of ATP in the coelomic fluid were determined after treatment with or without reagents. Treatment with valinomycin, NaN_3 or 2,4-dinitrophenol decreased significantly ATP levels but treatment with monensin or bafilomycin A_1 had no such effect. * indicates statistically significant difference from the control ($P < 0.01$).

the number of vanadocytes. As shown in Fig. 2, ATP levels in coelomic fluid decreased by treatment with valinomycin, NaN_3 or 2,4-dinitrophenol but not with monensin or bafilomycin A_1.

Vanadium contents in vanadocytes

No significant differences were observed in levels of vanadium contained in vanadocytes between, before, and after

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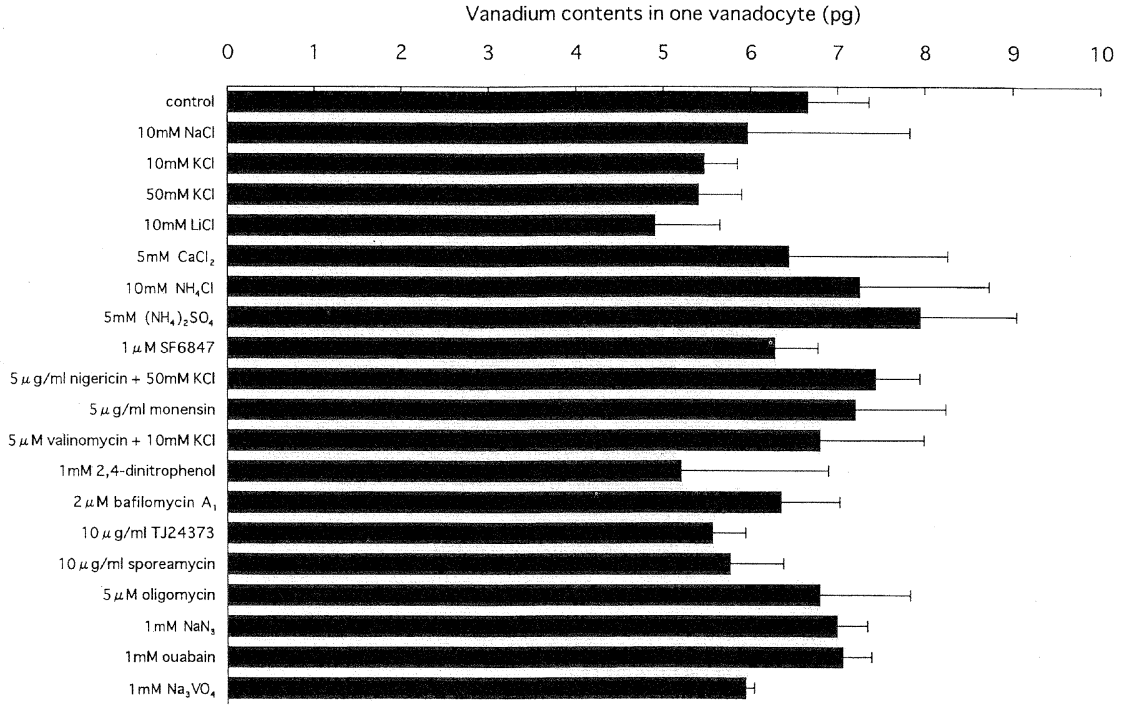

Fig. 3. Contents of vanadium in vanadocyte. The vanadium content in vanadocytes was determined after treatment with or without reagents. No significant differences were observed between control and treated groups. The vanadium content per vanadocyte was calculated. Data represents the average of three trials ± S.E.

treatments, as shown in Fig. 3. The vanadium content per vanadocyte was estimated to be 5 to 7 pg.

Source of vanadocytes

To identify the source of the vanadocytes that react to increase in their number in coelomic fluid by treatment with the above reagents, several tissues, the branchial sac, the peribranchial epithelium and the connective tissues around the alimentary canal, were stained immunohistologically with a monoclonal antibody, S4D5, specific to vanadocytes. In the non-treated animals, numerous vanadocytes were found in the connective tissues around the alimentary canal, as reported previously (Kaneko *et al.*, 1995), but few vanadocytes were observed in other tissues. After treatment with 10 mM NH_4Cl or 2 μM bafilomycin A_1, however, vanadocytes were observed to decrease in number in the same tissues (Fig. 4). This result revealed clearly that those vanadocytes reacted with the reagents were reserved in the connective tissue around the digestive alimentary canal.

DISCUSSION

We have previously reported that the number of vanadocytes increased markedly when *Ascidia sydneiensis samea* was immersed in seawater containing NH_4Cl for 20 hr (Hayashi *et al.*, 1996). Vanadocytes are known to have an ability to accumulate high levels of both vanadium and sulfate in their vacuoles under extremely low pH conditions (Kanamori and Michibata, 1994; Michibata *et al.*, 1991). Therefore, it is well worth examining how this treatment increases vanadocyte numbers.

In the present study, it was revealed that ionophores and inhibitors of V-ATPase are able to cause rapid increases in the size of the vanadocyte population, as shown in Fig. 1. The ionophores, SF6847, nigericin, and monensin, are known to increase the permeability of H^+ across the membrane and to dissipate intracellular Δ pH. Bafilomycin A_1, a specific inhibitor of V-ATPases (Bowman *et al.*, 1988) is also known to dissipate intracellular Δ pH. Therefore, dissipation of the intracellular Δ pH might correlate with the increase in the number of vanadocytes. In fact, other macrolide antibiotics, such as TJ24373 and sporeamycin, that do not dissipate intracellular Δ pH were ineffective.

Furthermore, inhibitors of F-ATPases, uncouplers and potassium ionophores, also caused increases in the number of vanadocytes. F-ATPases are known to act in ATP formation. Next we examined whether ATP levels in ascidian coelomic fluid decrease after treatment with inhibitors of F-ATPase and whether such a decrease would trigger an increase in the number of vanadocytes. ATP levels in the coelomic fluid decreased following treatment with valinomycin, NaN_3 or 2,4-dinitrophenol but not after monensin or bafilomycin A_1 treatment (Fig. 2). Thus, not all reagents able to increase the size of the vanadocyte population decreased ATP levels.

However, it became clear that dissipation of intracellular Δ pH could have triggered the rapid increase in the number of vanadocytes. Monensin and bafilomycin A_1, known to increase the permeability of H^+ across the membrane and to dissipate

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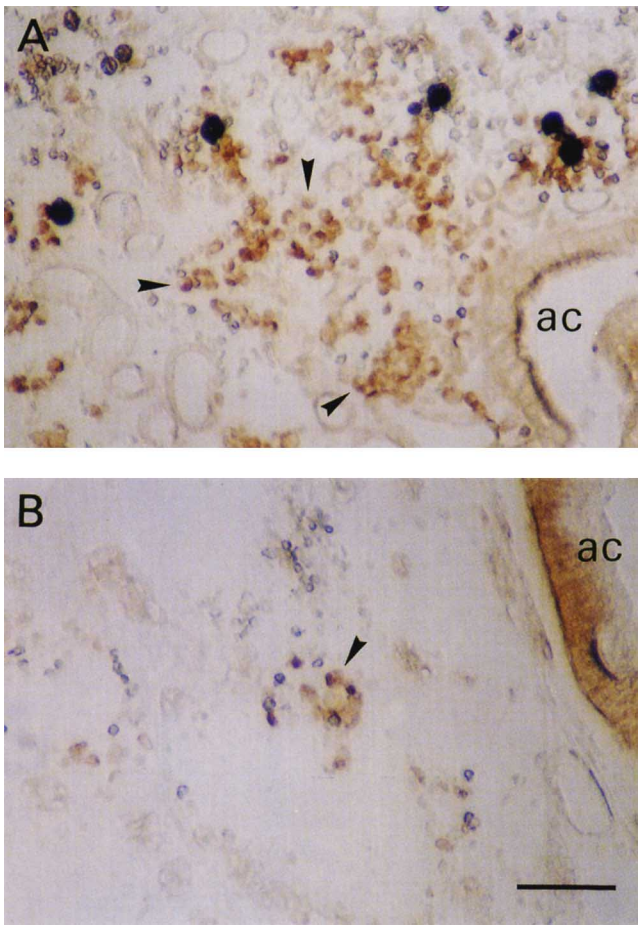

Fig. 4. Immunological detection of vanadocytes in the connective tissues around the alimentary canal. Immunohistological staining with a monoclonal antibody, S4D5, specific to vanadocytes revealed clearly that the number of vanadocytes embedded in the connective tissues decreased after treatment with 10 mM NH_4Cl or 2 μM bafilomycin A_1. No such phenomenon was observed in the other tissues examined. Therefore, the connective tissues around the alimentary canal are the source of these vanadocytes. In photograph **A**, a sample without treatment, a lot of immunoreactive cells are present. However, few immunoreactive cells appear in photograph **B**, a sample treated with 10 mM NH_4Cl. Arrowhead indicates the immunoreactive cells colored with brown color. ac, alimentary canal. Scale bar indicates 50 μm.

intracellular Δ pH, did not decrease the level of ATP but did cause an increase in the number of vanadocytes. Valinomycin, NaN_3 and 2,4-dinitrophenol, known to be inhibitors of F-ATPases, decreased the level of ATP with a subsequent dissipation of intracellular Δ pH. In other words, dissipation of intracellular Δ pH appears to trigger a rapid increase in the number of vanadocytes, although the cascade remains to be determined.

Which tissue is the source of the vanadocytes that increased rapidly in number? Although hematogenic tissues were reported to locate in the pharyngeal wall and around the alimentary canal (Ermak, 1976; Kalk, 1963), recently, we found that a lot of vanadocytes and precursors of vanadocyte were present in the connective tissues around the alimentary canal in *A. sydneiensis samea* (Kaneko *et al.*, 1995). As shown in Fig. 4, immunohistological staining revealed clearly that the number of vanadocytes embedded in the connective tissues decreased after the treatment with 10 mM NH_4Cl or 2 μM bafilomycin A_1. No such phenomenon was observed in the other tissues examined. Therefore, those vanadocytes that increased in number in the coelomic fluid after treatment must have originated in the connective tissues. However, the rapid increase in the number of vanadocytes did not result in a change in the vanadium content in the vanadocytes, as shown in Fig. 3. This can be explained as follows: The vanadocytes that reacted with the reagents had matured in the connective tissues and contained high levels of vanadium as high as those of circulating vanadocytes.

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