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VANADATE STIMULATES Na^+/H^+ EXCHANGE ACTIVITY IN A431 CELLS

Dan Cassel, Ying-Xin Zhuang, and Luis Glaser

Department of Biological Chemistry
Division of Biology and Biomedical Sciences
Washington University School of Medicine
St. Louis, Missouri 63110

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SUMMARY: Recent studies have established that polypeptide growth factors cause an elevation of the cytoplasmic pH (pH_i) in cultured mammalian cells by stimulating Na^+/H^+ exchange. We show that vanadate, previously found to act as a mitogen for a number of cells, reversibly activates Na^+/H^+ exchange at micromolar concentrations in A431 cells, leading to a large increase of pH_i. The stimulation of Na^+/H^+ exchange by vanadate is not due to inhibition of the Na^+/K^+ ATPase and is unrelated to possible effects of vanadate on cAMP levels. Elevation of pH_i by vanadate and by epidermal growth factor (EGF) both display similar kinetics, and both EGF and vanadate stimulate the rate of pH_i recovery following an acute acid load, suggesting that vanadate stimulates Na^+/H^+ exchange by a mechanism similar to that of polypeptide growth factor stimulation. Thus, stimulation of Na^+/H^+ exchange may be a common property not only of polypeptide growth factors but also of other, chemically unrelated mitogens.

Among the earliest events following addition of mitogenic polypeptides and hormones to cultured animal cells is the stimulation of an amiloride-sensitive Na^+ influx apparently representing activation of an Na^+/H^+ antiport (1-7). Recent development of techniques for intracellular pH measurement in cells grown in monolayer cultures resulted in the demonstration that activation of Na^+/H^+ exchange by growth factor leads to cytoplasmic alkalinization (8-11). Such alkalinization can have diverse effects on cellular function and could be involved in mitogenic signaling. If activation of Na^+/H^+ exchange is an important component of mitogenic stimulation of cells, then it should be activated by a diverse group of mitogenic compounds. Vanadate at micromolecular concentrations has been found to elicit a mitogenic response in quiescent cultures of human fibroblasts (12), mouse mammary gland (13) and the 3T3 and

Abbreviations used are: EGF, epidermal growth factor; PDGF, platelet derived growth factor; pH_i, intracellular pH.

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3T6 cell lines (14). We investigated the effect of vanadate on the previously characterized (10) Na^+/H^+ exchange activity in A431 cells and show that vanadate stimulates Na^+/H^+ exchange in a manner similar to the stimulation by polypeptide growth factors.

MATERIALS AND METHODS

Sodium orthovanadate was purchased from Sigma Chemical Co. $^{22}Na^+$ uptake assays were carried out on nearly confluent cultures of A431 cells at pH 7.2 in the presence of 1 mM ouabain as previously described (7). Fluorimetric measurements of pH_i in cells that have been loaded with dimethylfluorescein coupled to dextran (7,11) were carried out at 37°C in a physiological salt solution consisting of 130 mM NaCl, 5.4 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1 mM Pi, 25 mM glucose, 25 mM 3-(N-morpholino)-2-hydroxypropane sulfonic acid (MOPSO buffer), pH 6.8.

RESULTS AND DISCUSSION

Na^+/H^+ exchange in A431 cells can be activated by epidermal growth factor (EGF)[1], leading to an increased $^{22}Na^+$ influx and cytoplasmic alkalinization (7,10). As shown in Fig. 1a, addition of 100 μM vanadate to A431 cells rapidly stimulates $^{22}Na^+$ uptake. The rate of vanadate-dependent uptake is linear for at least 16 min and is similar to that obtained with EGF. Addition of both vanadate and EGF results in a partially additive response. Stimula-

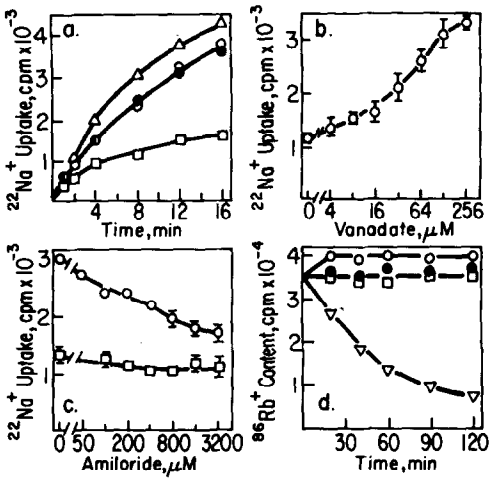


Fig. 1. (a-c) Effect of vanadate on $^{22}Na^+$ uptake in A431 cells. (a) Time course; (b,c) effect of different vanadate or amiloride concentrations on $^{22}Na^+$ uptake measured after 12 min. (d) shows that vanadate and EGF have little effect on $^{86}Rb^+$ efflux from cells preequilibrated with the tracer, while ouabain causes a decrease in $^{86}Rb^+$ content due to inhibition of the Na^+/K^+ ATPase. Cells were preincubated for 2 h in physiological salt solution containing 10^6 cpm/ml $^{86}Rb^+$. At zero time the solution was exchanged with an identical one containing various additions and incubation continued for the times specified. Symbols in a-d: ([]) no addition; (○) 0.1 mM vanadate; (●) 0.2 μg/ml EGF. (△), 0.1 mM vanadate + 0.2 μg/ml EGF; (▽), 1 mM ouabain.

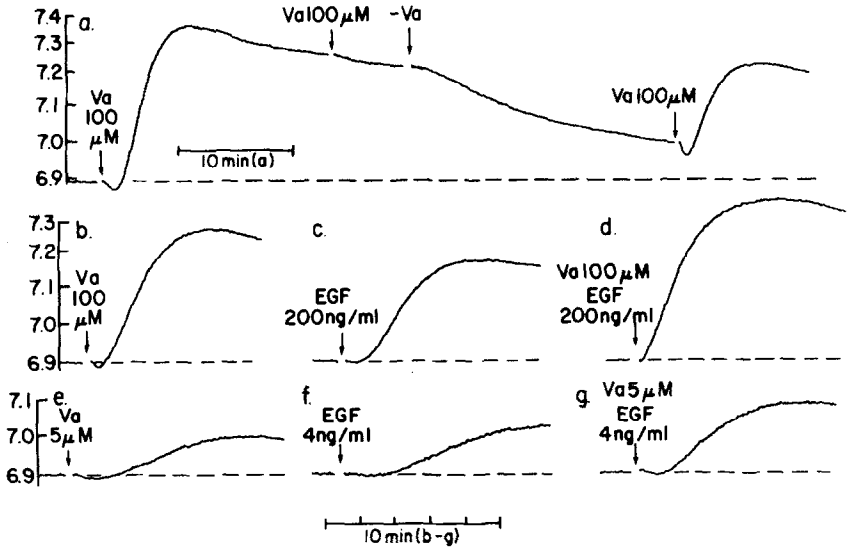


Fig. 2. Effect of vanadate and EGF on pH_i. pH_i values are based on initial pH_i of 6.9 ± 0.13 at pH_o = 6.8 as determined by calibration with ouabain (10).

tion of $^{22}Na^+$ uptake by vanadate is saturable with half maximal effect at ~40 μM (Fig. 1b). Amiloride inhibits vanadate-dependent $^{22}Na^+$ uptake with a concentration dependence (IC_{50} ~200 μM) similar to that previously found for EGF-dependent uptake (7) (Fig. 1c). Although vanadate is known as a potent inhibitor of the Na^+/K^+ ATPase in purified preparations (15), stimulation of $^{22}Na^+$ uptake by vanadate is not due to an inhibition of the ATPase since the ATPase is inhibited by maximally inhibitory (1 mM) ouabain concentration added to eliminate $^{22}Na^+$ extrusion during the uptake assay. Moreover, vanadate does not inhibit the Na^+/K^+ ATPase in A431 cells as judged by its inability to enhance $^{86}Rb^+$ efflux from cells preequilibrated with this K^+ tracer; rather a slight increase in $^{86}Rb^+$ content was observed (Fig. 1d). A lack of inhibition of the ATPase in intact cells was previously observed in rat adipocytes (16) and cultured heart cells (17), probably reflecting a reduction of vanadate ion to a noninhibitory form (18,19).

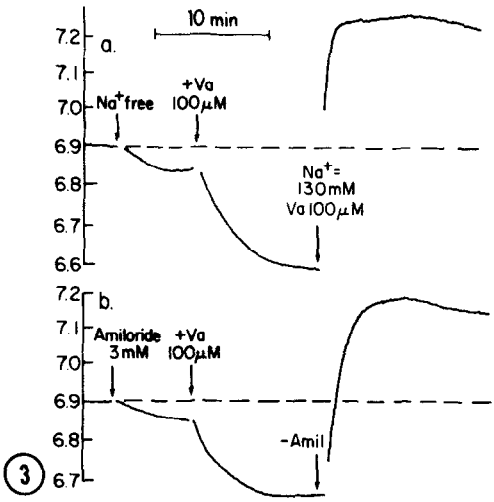
Addition of 0.1 mM vanadate to A431 cells causes a large elevation of pH_i (Fig. 2, trace a). pH_i elevation follows a lag period of 1-2 min during which a slight acidification is usually observed. Subsequently pH_i rises rapidly, reaches a maximum of ΔpH_i = 0.4-0.5 within 10 min and then declines

slightly. Removal of vanadate results in a decline of pH_i. Readdition of vanadate elicits a new pH_i elevation response, indicating that the effect of vanadate is reversible. pH_i elevation by vanadate is half maximal at 15-30 μM (not shown), a concentration similar though somewhat lower than the vanadate concentration required for half maximal stimulation of $^{22}Na^+$ uptake (Fig. 1b). Traces b-g in Fig. 2 show a comparison between the effects of vanadate and EGF on pH_i. pH_i elevation by both EGF and vanadate displays similar kinetics with respect to the lag period and the time at which pH_i reaches a maximal level. At maximally effective concentrations (b-d), pH_i elevation elicited by vanadate is somewhat larger than that elicited by EGF. Simultaneous addition of vanadate and EGF (trace d) results in a partially additive response and considerably shortens the initial lag period. When submaximal concentrations of EGF and vanadate are used, simultaneous addition of these reagents elevates pH_i in an additive manner (traces e-g).

Another known effect of vanadate is the activation of adenylate cyclase (20) and the elevation of cAMP levels in certain tissues (21,22). While the effect of vanadate on cAMP levels in A431 cells has not been determined, cAMP elevating reagents such as cholera toxin and forskolin do not elicit pH_i elevation in these cells (not shown), indicating that pH_i elevation by vanadate is not mediated through cAMP.

As shown in Fig. 3, vanadate-dependent pH_i elevation is inhibited in Na^+-free medium or upon addition of amiloride. Under both conditions addition of vanadate leads to initial intracellular acidification. pH_i rapidly increases upon readdition of Na^+ or removal of amiloride. Unlike vanadate, EGF does not promote an acidification of A431 cells in the absence of Na^+ or in the presence of amiloride (not shown). However, stimulation of intracellular acidification upon inhibition of Na^+/H^+ exchange was previously observed with PDGF in NR6 cells (11). The mechanism and significance of this acidification remain obscure.

Rothenberg *et al.* (10) have shown that EGF enhances the rate of recovery from an acute acid load in A431 cells due to the stimulation of Na^+/H^+ ex-



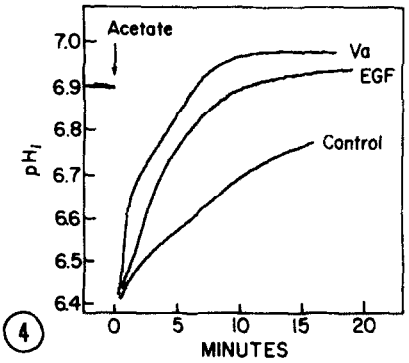


Fig. 3. Na^+ dependency (a) and amiloride sensitivity (b) of pH_i elevation by 0.1 mM vanadate. Note the rapid initial acidification which is promoted by vanadate under both conditions. Na^+-free medium contained physiological salt solution with an isoosmotic replacement of NaCl with tetramethylammonium chloride.

Fig. 4. Effect of 0.1 mM vanadate and 0.2 μg/ml EGF on pH_i recovery following an acute acid load upon exposure of cells to 25 mM acetate in physiological salt solution pH 6.8.

change. As shown in Fig. 4, the recovery of pH_i in A431 cells following an exposure to acetate buffer is also strongly enhanced by vanadate. Similar results were obtained using the weak acid 5,5-dimethyloxazolidine-2,4-dione (DMO) (not shown). Moolenaar et al. (9) have similarly observed that the apparent rate of recovery of pH_i following acid loading of fibroblasts is enhanced by serum. The findings that following accute acidification the rate of alkalinization at any given intracellular pH is enhanced by the polypeptide mitogens as well as by vanadate suggest that the activation of the Na^+/H^+ antiporter can take place over a broad range of pH_i. It is thus likely that mitogens increase the V_{max} of the antiporter rather than its affinity for protons.

The observed similarity between the effects of vanadate and growth factors on pH_i under different conditions, including a characteristic lag which precedes alkalinization of the cytoplasm, suggests that they both activate Na^+/H^+ exchange by similar mechanisms. The lag period which precedes pH_i elevation elicited by vanadate and growth factors suggests that the activation

of Na^+/H^+ exchange may be secondary to other events which are elicited by these agents. It is noteworthy that EGF and PDGF stimulate phosphorylation of tyrosine residues on proteins (23-25) while vanadate is a potent inhibitor of phosphotyrosine phosphatase in plasma membrane preparations (26). Whether tyrosine phosphorylation is involved in the activation of Na^+/H^+ exchange remains to be determined.

Although neither vanadate nor EGF is mitogenic for A431 cells, we found in preliminary studies that in NR6 cells vanadate elicits a mitogenic response and activates Na^+/H^+ exchange, leading to cytoplasmic alkalinization. The vanadate concentration required for maximal stimulation of Na^+/H^+ exchange in A431 or NR6 cells is considerably higher than the optimal mitogenic concentration for which values ranging from 5 μM (12,13) to 20-50 μM (14) have been reported. However, the mitogenic effect of vanadate is probably restricted due to a growth inhibitory effect at supraoptimal vanadate concentrations (12-14). The fact that chemically unrelated mitogens such as vanadate and polypeptide growth factors stimulate Na^+/H^+ exchange provides a support for the notion that activation of Na^+/H^+ exchange is a common property of mitogenic factors. Whether activation of Na^+/H^+ exchange is necessary for the mitogenic response or whether it is a secondary result of the events leading to this response remains to be established.

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