Vanadium Stimulates the (Na^+,K^+) Pump in Friend Erythroleukemia Cells and Blocks Erythropoiesis

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ABSTRACT Friend murine erythroleukemia cells underwent apparently normal erythropoiesis when treated with dimethyl sulfoxide. One of the earliest events associated with this induction was a decrease in ouabain sensitive $^{86}Rb^+$ uptake, an assay of the plasma membrane Na,K(ATPase). Ammonium vanadate (10 μM) blocked differentiation of these cells without affecting cell viability. Vanadium was taken up by Friend cells and prevented the dimethyl sulfoxide-induced decrease in ouabain sensitive $^{86}Rb^+$ uptake. Vanadate reactivated $^{86}Rb^+$ transport previously inhibited by dimethyl sulfoxide treatment but had no affect on $^{86}Rb^+$ transport in untreated cells. These results suggest an essential role for the (Na,K)ATPase in cell differentiation.

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Friend murine erythroleukemia cells grow indefinitely in suspension but may be induced to terminally differentiate by a wide variety of drugs, including dimethyl sulfoxide (DMSO[1]). One of the earliest events associated with induction is a decrease in ouabain sensitive $^{86}Rb^+$ uptake, an assay of the plasma membrane (Na,K)ATPase (1, 2). This event appears to be the rate limiting step in commitment since pretreatment of cells with ouabain prior to adding DMSO accelerates commitment to erythropoiesis by eliminating a characteristic lag time (2). In some cells lines, ouabain alone will induce erythropoiesis (3).

To further characterize the role of the (Na,K)ATPase in Friend cell differentiation we have investigated the effects of vanadate on this system. Vanadate is a potent (Na,K)ATPase inhibitor in vitro (4) but has been reported to activate this enzyme in certain hormone sensitive tissues (5). Here we show that addition of 10–20 μM ammonium vanadate to Friend cell cultures completely blocks differentiation. We also show that vanadate is taken up by the cells and reverses the DMSO induced inhibition of ouabain sensitive $^{86}Rb^+$ uptake. We suggest that intracellular vanadium blocks several events essential for terminal differentiation, one of which is a decrease in the plasma membrane (Na,K) pump activity.

MATERIALS AND METHODS

The Friend murine erythroleukemia cells (cell line 745-PL-4 obtained from David Housman, Massachusetts Institute of Technology) were maintained at 1–40 × 10^4 cells/ml to insure logarithmic growth in α medium (Gibco Laboratories, Grand Island, NY) and supplemented with 13% fetal calf serum (M. A. Bioproducts, Walkersville, MD). Commitment of Friend cells to terminal differentiation was assayed in plasma clot culture (6). In this technique, the cells are scored as committed to erythropoiesis if they form small colonies (<32 cells) and stain orange with benzidine. Cell viability was measured by the exclusion of trypan blue.

Ouabain inhibitable $^{86}Rb^+$ (New England Nuclear, Boston, MA) uptake was used to assay the pumping activity of the (Na,K)ATPase as previously described (2). In this assay, suspended cells in ^{86}Rb-labeled assay medium were centrifuged through dinonyl-phthalate/silicone oil (1:1) to remove extracellular radioactivity. The rate of $^{86}Rb^+$ uptake was assumed to roughly approximate K^+ uptake. The uptake of [^{48}V]vanadate was measured using the same procedure described above. [^{48}V]vanadyl chloride, obtained from Amersham Corp. (Arlington Heights, IL) was converted to vanadate by dilution into 100 mM NH_4VO_3 at pH 8.0 and incubated at 70°C for 2 h. The data were normalized to the volume of cell water estimated by the difference between the internally trapped ^3H_2O and the externally trapped [^{14}C]sucrose in the pellet in parallel experiments.

Externally trapped [^{48}V]vanadate was determined by the amount of radioactivity in the pellets of cells that were cooled to 4°C immediately before [^{48}V]-vanadate addition and centrifuged immediately afterward.

RESULTS

Inhibition of DMSO induced Friend cell differentiation by vanadate is demonstrated in Fig. 1. When 20 μm ammonium vanadate was added with the DMSO, 2% of the cells commit in 72 h compared with 95% committed in the absence of vanadate. 10 μM of ammonium vanadate was equally effective and cell growth was slightly stimulated under these conditions. 20 μM ammonium chloride had no effect on viability or commitment. Fig. 1 also demonstrates that when vanadate was added 3–30 h after the DMSO addition it still inhibited commitment although it did not block differentiation of cells that were committed prior to vanadate addition. Fig. 2*A* shows that vanadate also prevented DMSO from inhibiting ouabain sensitive $^{86}Rb^+$ uptake. Friend cells grown in 1.5% DMSO for 10–20 h showed a 25–50% reduction in ouabain sensitive $^{86}Rb^+$ uptake (1, 2). When the growth medium was

[1] *Abbreviation used in this paper:* DMSO, dimethyl sulfoxide.

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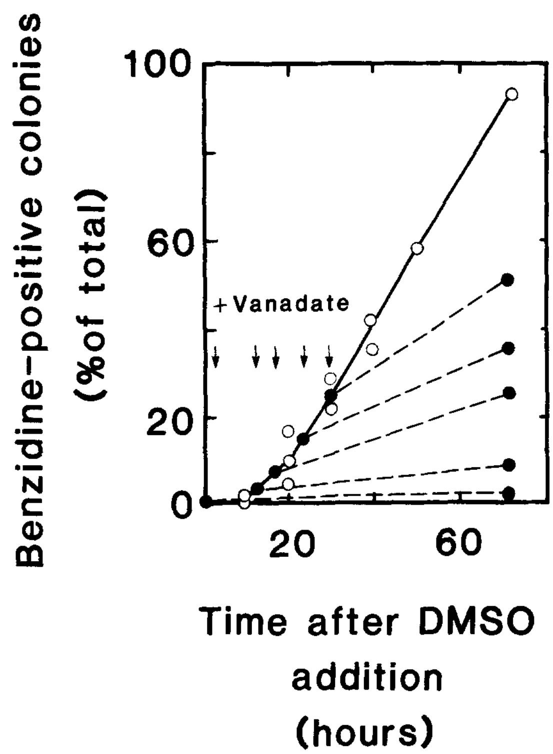

FIGURE 1 Vanadate blocks commitment of Friend cells to erythroid differentiation. At time zero, DMSO (1.5%) was added to Friend cells cultured in suspension with α-medium plus 13% fetal calf serum. In some cultures (●) 20 μM of vanadate was added either at time zero, 3, 12, 15, 24, or 30 h after the DMSO addition as indicated by the arrows. In a control culture, only DMSO was added (○). At the times indicated by the circles on the graph, a fraction of cells from each culture was plated in a plasma clot culture (6) in the absence of DMSO or ammonium vanadate. After 96 h in plasma culture, the colonies were scored for commitment. Greater than 95% of the cells in all cultures were viable and in the absence of vanadate, 92% of the cells produced erythroid colonies after 72 h in DMSO.

supplemented with 15 μM ammonium vanadate, the rate of $^{86}Rb^+$ uptake returned almost to that of control cells. Interestingly, 15 μM ammonium vanadate had no effect on $^{86}Rb^+$ uptake of cells not exposed to DMSO. The ouabain resistant $^{86}Rb^+$ uptake was unaffected by DMSO or vanadate.

The time course for DMSO inhibition of $^{86}Rb^+$ uptake and vanadate reversal of this inhibition are shown in Fig. 2, *B* and *C*. As previously shown by Mager and Bernstein (1), DMSO treatment inhibits the $^{86}Rb^+$ uptake rate between 6 and 12 h after addition to the cells. Vanadate reduced the magnitude of this inhibition when added along with the DMSO (Fig. 2*B*). When vanadate was added after full inhibition of $^{86}Rb^+$ uptake had occurred (i.e., 20 h after DMSO), there was a slow increase in the rate of $^{86}Rb^+$ uptake reaching 90% of the rate of untreated cells after 4–7 h (Fig. 2*C*).

The data in Fig. 2*D* suggest that the slow reversal of the $^{86}Rb^+$ uptake rate is due to a slow rate of vanadium entry into Friend cells. After 5 h in 15 μM vanadate, the DMSO treated cells have ~30 μmol of vanadium per liter of cell water. Untreated cells concentrated vanadate somewhat faster but the vanadium did not affect $^{86}Rb^+$ uptake (Fig. 2, *A*–*C*).

The concentration dependence of the vanadium effects on $^{86}Rb^+$ uptake, hemoglobin synthesis, and cell growth are shown in Fig. 3. A maximal effect on $^{86}Rb^+$ uptake and differentiation (as assayed by hemoglobin production) were observed at 25 μM ammonium vanadate. At higher concentrations of vanadate, cell viability and $^{86}Rb^+$ uptake were significantly reduced (data not presented). The 50% maximum effects on both hemoglobin production and $^{86}Rb^+$ uptake were observed at 5 μM vanadate.

DISCUSSION

We have shown that low concentrations of ammonium vanadate added to Friend cell cultures prevented DMSO-induced differentiation and reversed the DMSO inhibition of the plasma membrane (Na,K) pump. The vanadium stimulation of $^{86}Rb^+$ uptake seems to be a specific reversal of the DMSO-induced inhibition since (*a*) vanadium had no effect on $^{86}Rb^+$ uptake in control cells even though it was taken up faster in these cells and (*b*) the stimulated uptake was always less than the uptake rate in control cells.

A number of effects of vanadium on whole cells and enzymes have been recently reviewed (7–9). Of particular interest to the present study is a previous report that vanadate mimics insulin in stimulating $^{86}Rb^+$ uptake in cultured heart muscle cells but not nonmuscle cells (5). The authors argued that vanadium is acting from the inside of the cell although they did not determine intracellular vanadium concentrations or monitor vanadium uptake. The results presented here also suggest that vanadium is affecting transport in Friend cells from the cytoplasmic side of the membrane. There is a lag of several hours before a maximum stimulation occurred (Fig. 2) and the stimulation persisted after extracellular vanadate was removed (data not presented). Although vanadate is an extremely potent inhibitor of the (Na,K)ATPase, we have previously shown that cytoplasmic vanadate is reduced to vanadyl (VO^{2+}) which is relatively ineffective as a (Na,K)ATPase inhibitor. (4, 10–12). It is not clear why vanadium stimulates the (Na,K)ATPase in whole cells. (Purified (Na,K)ATPase is not stimulated by vanadyl ions (11) nor is it inhibited by 1–2% DMSO; unpublished results of L. Cantley).

The considerations above imply that the effects on $^{86}Rb^+$ transport and differentiation are mediated by an enzyme that is affected by relatively low concentrations of vanadyl ions. Since vanadyl is a potent inhibitor of a variety of phosphatases ($K_I \simeq 10^{-7}$ M; 13–15) changes in protein phosphorylation are likely to occur in vanadate treated cells. We have recently shown that the (Na,K)ATPase is phosphorylated by a plasma membrane bound protein kinase in Friend cells (16). We are now investigating the possibility that DMSO and vanadate effect phosphorylation of the (Na,K)ATPase in vivo.

Finally, it is interesting to compare the effects of vanadate on Friend cells with the effects of several other agents that block differentiation without affecting cell growth. We have previously shown that amiloride prevents Friend cell differentiation by either directly or indirectly blocking an essential Ca^{2+} influx (2, 17). However, amiloride did not prevent the DMSO-induced decrease in $^{86}Rb^+$ uptake, which suggests that the Ca^{2+} flux change follows the decrease in the (Na,K) pump activity. We have also investigated the effects of phorbol esters and dexamethasone and found that these agents act at a step in commitment later than the changes in (Na,K) pump activity and Ca^{2+} flux (Macara, I-G. and L. Cantley, submitted manuscript). The recent report that the phorbol ester receptor is a Ca^{2+}-dependent kinase (18) further supports the role of protein kinases in signaling differentiation.

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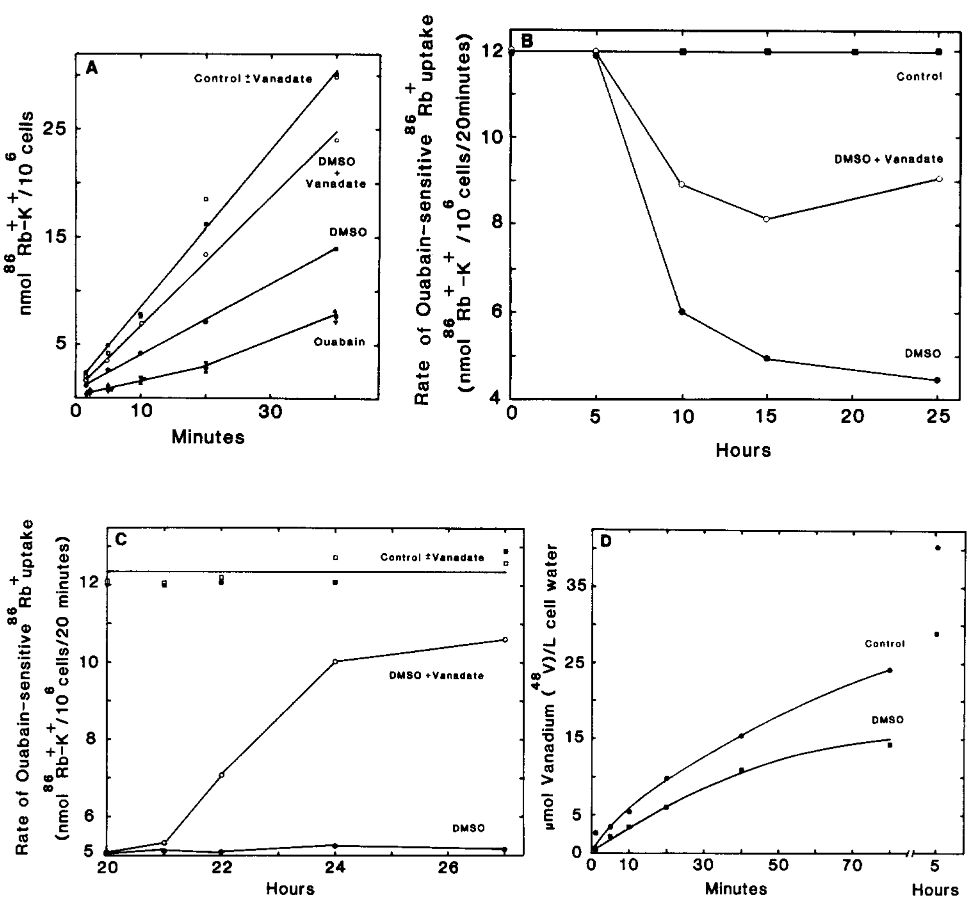

FIGURE 2 Vanadate reverses the DMSO inhibition of ouabain sensitive $^{86}Rb^+$ uptake. (*A*) Friend cells were grown in fetal calf serum supplemented α-medium as in Fig. 1 for 24 h in the presence (○,●) or absence (□,■) of 1.5% DMSO and in the presence (○,□) or absence (●,■) of 15 µM ammonium vanadate for the last 5 h. The cells were then harvested and resuspended to 5 × 10^6 cells/ml in α-medium (23°) supplemented with 5 mM HEPES (pH 7.4) and the same concentration of DMSO and vanadate as the original culture. At time zero, $^{86}Rb^+$ was added to all cultures and 1 mM ouabain was added to some (△,▽,▲,▼). Periodically, 200 µl of cells were extracted and centrifuged through 800-ml label-free assay medium and 300 µl of dinonyl phthalate/silicone oil (1:1) and the pellets counted for radioactivity. Assuming $^{86}Rb^+$ tracer uptake roughly approximates K^+ uptake, the data are plotted as nanomoles of K^+ uptake per 10^6 cells. The ouabain insensitive $^{86}Rb^+$ uptake was nearly identical for all cultures: ▼, control; ▲, DMSO; ▽, vanadate; △ DMSO plus vanadate. Cell viability was >95% in all cultures. (*B*) Friend cells were grown in the presence (●,○) or absence (■) of 1.5% DMSO and the presence (○) or absence (●,■) of 15 µM ammonium vanadate at 37° in 13% fetal calf serum supplemented α-medium. At the indicated times, the cells were concentrated and assayed for $^{86}Rb^+$ uptake as in *A*. The ouabain insensitive uptake (~2.5 nmol/10^6 cells/20 min in all cases) was subtracted away. (*C*) Friend cells were grown in the presence (●,○) or absence, (■,□) of 1.5% DMSO for 20 h at 37° in fetal calf serum supplemented α-medium. At 20 h, 15 µM of ammonium vanadate were added to two of the cultures (○,□) and growth was continued at 37°. At the times indicated, the cells were concentrated and ouabain sensitive $^{86}Rb^+$ uptake was determined as in *B*. (*D*) Friend cells were grown in the presence (■) or absence (●) of 1.5% DMSO for 20 h at 37° as above. The cells were then concentrated to 2 × 10^6 cells/ml in 5 mM HEPES (pH 7.4) supplemented α medium at 37° with or without 1.5% DMSO. At time zero, 15 µM of [^{48}V]ammonium vanadate was added to each culture and at the indicated times, 200 µl of cells were centrifuged through oil as above and the pellets counted for radioactivity. The data were normalized to the cell water determined to be 0.58 µl for control and 0.45 µl for DMSO-treated cells. Externally trapped [^{48}V]vanadate (~10 nmol/liter cell water) was subtracted away.

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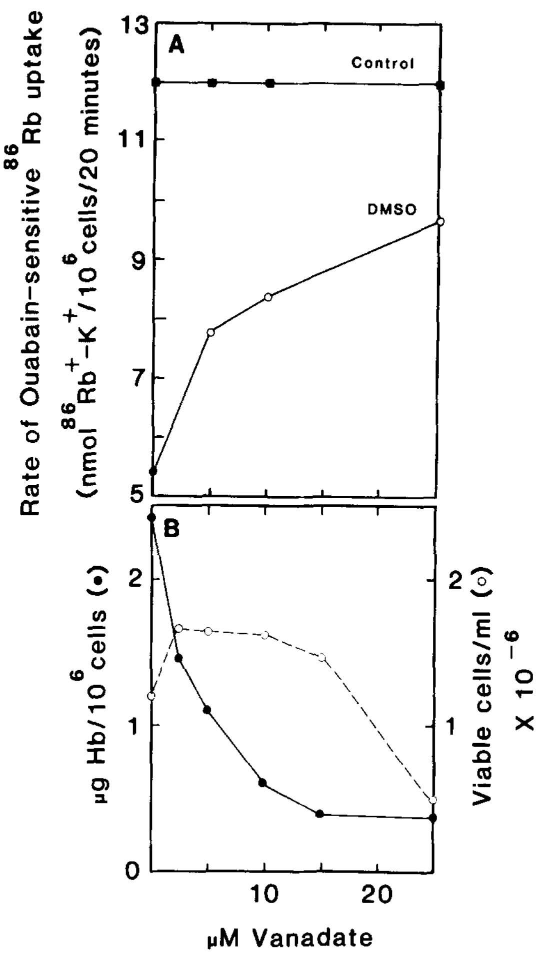

FIGURE 3 The concentration dependence of vanadate effects on Friend cells. (*A*) Friend cells were grown in the presence (○) or absence (●) of 1.5% DMSO for 20 h as in Fig. 2. Varying amounts of ammonium vanadate were then added to the cultures and growth was continued for five more hours. The cells were then concentrated, and assayed for ouabain sensitive $^{86}Rb^+$ uptake as in Fig. 2. (*B*) Friend cells were grown in fetal calf serum supplemented α-medium for 72 h in the presence of varying amounts of ammonium vanadate. The cells were then assayed for cell number, viability and hemoglobin content. 25 μM of NH_4Cl had no effect on viability, proliferation, or differentiation (data not shown). Hemoglobin content was measured according to the method of Tsiftsoglou et al. (19).

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