Okadaic Acid Inhibition of KCl Cotransport

Evidence That Protein Dephosphorylation Is Necessary for Activation of Transport by Either Cell Swelling or N-Ethylmaleimide

MICHAEL L. JENNINGS and RICHARD K. SCHULZ

From the Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

ABSTRACT   The mechanism of activation of KCl cotransport has been examined in rabbit red blood cells. Previous work has provided evidence that a net dephosphorylation is required for activation of transport by cell swelling. In the present study okadaic acid, an inhibitor of protein phosphatases, was used to test this idea in more detail. We find that okadaic acid strongly inhibits swelling-stimulated KCl cotransport. The IC_{50} for okadaic acid is ~40 nM, consistent with the involvement of type 1 protein phosphatase in transport activation. N-Ethylmaleimide (NEM) is well known to activate KCl cotransport in cells of normal volume. Okadaic acid, added before NEM, inhibits the activation of transport by NEM, indicating that a dephosphorylation is necessary for the NEM effect. Okadaic acid added after NEM inhibits transport only very slightly. After a brief exposure to NEM and rapid removal of unreacted NEM, KCl cotransport activates with a time delay that is similar to that for swelling activation. Okadaic acid causes a slight increase in the delay time. These findings are all consistent with the idea that NEM activates transport not by a direct action on the transport protein but by altering a phosphorylation–dephosphorylation cycle. The simplest hypothesis that is consistent with the data is that both cell swelling and NEM cause inhibition of a protein kinase. Kinase inhibition causes net dephosphorylation of some key substrate (not necessarily the transport protein); dephosphorylation of this substrate, probably by type 1 protein phosphatase, causes transport activation.

INTRODUCTION

Red blood cells of several species exhibit a K^+-Cl^- cotransport flux that can be activated by either cell swelling or chemical modification with N-ethylmaleimide (NEM) (see Lauf, 1983a, 1984, 1985; Brugnara et al., 1985, 1989; McManus et al.,

Address reprint requests to Dr. Michael L. Jennings, Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77550.

1985; Canessa et al., 1986; Kaji, 1986; Dunham, 1989). The physiological function of the system appears to be cell volume regulation; a slight increase in cell volume stimulates a net loss of K^+, Cl^-, and water, which tends to return cell volume to normal. A major unanswered question regarding volume-sensitive KCl cotransport is the mechanism by which cell swelling activates the flux. Using the rabbit red cell as an experimental system, we have previously presented evidence that a phosphorylation–dephosphorylation cycle is important in the regulation of the KCl cotransporter (Jennings and Al-Rohil, 1990). Specifically, the kinetics of activation of the flux by a step change in cell volume are consistent with a simple two-state model in which the transporter is in either a resting (R) or activated (A) state. The transitions between the two states are characterized by unimolecular rate constants k_{12} and k_{21}:

$$R \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A$$
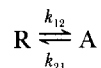
According to the model, cell swelling activates transport by increasing the ratio k_{12}/k_{21}. From measurements of the rates of activation and inactivation of the flux after step changes in cell volume, we concluded that cell swelling activates transport by lowering k_{21} rather than raising k_{12}. We cannot rule out a slight dependence of k_{12} on cell volume, but certainly k_{21} is more volume dependent than k_{12}. Although the data fit the model quite well, the model itself is probably an oversimplification; the rate constants k_{12} and k_{21} are lumped parameters that may each reflect the rates of two or more processes. The model is presented as a minimal mechanism designed to serve as a framework for quantitative study of volume-sensitive transport.

The phosphatase inhibitors fluoride, orthovanadate, and inorganic phosphate all cause inhibition of the swelling-stimulated flux (Coker and O'Neill, 1989; Jennings and Al-Rohil, 1989, 1990). The kinetics of activation of the flux by cell swelling in the presence of phosphatase inhibitor suggest that these agents cause a decrease in k_{12} (Jennings and Al-Rohil, 1990). If a phosphatase activity is associated with k_{12}, then it is reasonable to postulate that a kinase activity is an important determinant of the reverse rate constant k_{21}. Our current hypothesis, then, is that cell swelling activates transport by way of a swelling-inhibited kinase. There is little information available regarding the identity of either the phosphatase or the kinase. Kim et al. (1989) have shown that cyclic AMP stimulates KCl cotransport in pig red cells, but it is doubtful that cell swelling activates cotransport by way of an increase in intracellular cAMP, because pig red cells lack a functional adenylate cyclase system.

The evidence for the involvement of a phosphatase in the activation of transport is based on the effects of agents (orthovanadate, fluoride, inorganic phosphate) that are rather nonspecific and were used at relatively high concentrations. To examine further the possible role of a phosphatase in the activation of KCl cotransport, we have used okadaic acid, a C_{38} fatty acid derivative first isolated from marine sponges (Tachibana et al., 1981). Okadaic acid is a very potent inhibitor of some (but not all) protein phosphatases (see Cohen et al., 1990). We show in this paper that okadaic acid, at submicromolar concentrations, inhibits swelling-activated KCl cotransport in rabbit red cells. The concentration dependence of the effect is consistent with the

involvement of type 1 protein phosphatase (Cohen et al., 1990) in the activation of KCl cotransport by cell swelling.

It is well established that NEM treatment of LK sheep red cells in an isotonic medium activates the same KCl cotransport system that is activated by cell swelling (see Dunham and Ellory, 1981; Lauf, 1983*a*, 1984, 1985; Logue et al., 1983). The relationship between the swelling and NEM activation pathways is not clear. Interpretation of the NEM effect is complicated by the fact that KCl cotransport is influenced by at least two sulfhydryl groups with different chemical properties (Logue et al., 1983; Lauf, 1987). Al-Rohil (1988) showed that, although fluoride strongly inhibits swelling-stimulated transport, it has no significant effect on the flux in cells that were pretreated with a maximal dose of NEM. The original interpretation of this effect was that NEM exerts its effect directly on the transport protein rather than on the regulatory machinery. In fact, most discussions of NEM effects on transport have postulated that the effects are a consequence of the modification of a sulfhydryl group on the transport protein (Lauf, 1985, 1987).

The evidence for the involvement of a kinase/phosphatase cycle opens another possibility for the mechanism of NEM activation of transport. NEM may not act on the transport protein itself but rather could alter either k_{12} or k_{21} by activating or inhibiting the enzymes that control these rate constants. We have used the more specific phosphatase inhibitor okadaic acid to examine the relationship between NEM action and the proposed phosphorylation–dephosphorylation cycle. The results indicate that a dephosphorylation, probably by type 1 protein phosphatase, is required for the NEM effect. We interpret the data as evidence that NEM activates the flux not by a direct effect on the transport protein itself, but by inhibiting a protein kinase. This interpretation is consistent with the known inhibitory effects of NEM on several kinases.

MATERIALS AND METHODS

Materials

Okadaic acid was purchased from Moana Bioproducts, Honolulu, HI (lot #105). It is supplied as a 100 μg/ml (127 μM) solution in N,N-dimethylformamide (DMF). DMF, NEM, cyclic AMP, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{86}Rb]RbCl was purchased from DuPont NEN (Boston, MA) or Amersham Corp. (Arlington Heights, IL). All other reagents, salts, and buffers were from either Sigma Chemical Co. or Fisher Scientific Co. (Pittsburgh, PA).

Rabbit Red Blood Cells

Rabbit blood was drawn into heparin or EDTA by cardiac puncture from New Zealand white rabbits that were killed to obtain not only blood but also several other tissues for use in other laboratories. The animals had been given a terminal injection of sodium pentobarbital before cardiac puncture. All animal procedures were performed according to the current guidelines set by the NIH and the American Physiological Society.

Most experiments were performed on blood that was either freshly drawn or stored at 4°C for 1 d. Blood that had been stored for as long as 3 d was used for some experiments, with no major differences in the characteristics of the flux except that the flux in isotonic medium may be slightly higher in stored blood. The least-dense 25–35% of the population of rabbit red cells

was prepared as described previously (Al-Rohil and Jennings, 1989), without removal of reticulocytes. If the blood had been stored overnight or longer, the cells were then incubated 60–90 min at 37°C in HEPES-buffered physiological saline (150 mM NaCl, 5 mM KCl, 1 mM Na-phosphate, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.5, at 23°C) to try to establish a reproducible steady state before the flux measurements.

Influx Experiments

The standard "isotonic" flux medium (medium A) consisted of 155 mM NaCl, 5 mM KCl, 10 mM HEPES-Na, pH 7.4, and 10^{-4} M ouabain. The medium was intentionally slightly hypertonic to minimize the basal flux. Cells were suspended in this medium at a hematocrit of 3–5%. In some experiments (see figure legends) the cells were preincubated as long as 30 min in the flux medium before addition of $^{86}Rb^+$, but in most experiments the $^{86}Rb^+$ (0.5–2 μCi/ml) was added after a temperature equilibration period of ~5 min. The influx of $^{86}Rb^+$ was measured at 37°C as described previously (Al-Rohil and Jennings, 1989; Jennings and Al-Rohil, 1990). In the plots of intracellular tracer vs. time, "1 ml cells" represents the number of cells that occupy 1 ml in an isotonic medium. The sequences of addition of 5 mM KCl (for hypotonic swelling), okadaic acid, NEM, and cysteine are described in the figure legends. All suspensions in a given experiment contained DMF at the same final concentration (at most 0.6% and usually 0.1–0.3%); DMF at these concentrations has no effect on the ouabain-insensitive $^{86}Rb^+$ flux in these cells, in agreement with the results of Starke (1989) with duck red cells. When indicated, influx data were fit to the two-state model (see Introduction) using commercially available software (NFIT; Island Products, Galveston, TX) as described previously (Jennings and Al-Rohil, 1990).

RESULTS

Okadaic Acid Inhibits Swelling-stimulated Transport

Fig. 1 shows the effects of okadaic acid on the ouabain-insensitive $^{86}Rb^+$ influx into young rabbit red cells. As in previous work, we routinely measured influx rather than efflux because initial influx data are more accurate. The least-dense third of the population of cells was used in all flux experiments to maximize the magnitude of the swelling-stimulated flux (see Brugnara and Tosteson, 1987; Canessa et al., 1987; O'Neill, 1989). Cells were incubated 10 min at 3% hematocrit in isotonic medium with or without okadaic acid before addition of $^{86}Rb^+$. The initial influx was measured for 17 min, and then the suspensions were made hypotonic (210 mosmol/kg H_2O) by adding 5 mM KCl/$^{86}Rb^+$. As observed previously in several laboratories (Kregenow, 1971; Dunham, 1989; Kim et al., 1989; Starke, 1989; Jennings and Al-Rohil, 1990), a step increase in cell volume caused transport activation after a time lag of 5–10 min. In the presence of okadaic acid the swelling-stimulated flux was strongly inhibited. Okadaic acid also significantly inhibited the flux in isotonic medium. (In isotonic medium the KCl cotransporter appears to be slightly activated in these cells [see Al-Rohil and Jennings, 1989]; therefore, any agent that inhibits the swelling-stimulated flux would be expected to inhibit the basal flux).

Time Course of Okadaic Acid Effect

In the experiment in Fig. 1, okadaic acid was added 10 min before the flux measurement began and 27 min before the cells were swollen. For more detailed

studies it is desirable to estimate the time course of the effects of okadaic acid on the swelling-stimulated flux (Fig. 2). Four identical suspensions were preincubated 20 min in isotonic medium before the addition of $^{86}Rb^+$ simultaneously with cell swelling at $t = 0$. In one suspension no okadaic acid was present, and the flux activated with the usual time lag. Addition of okadaic acid 1 min before swelling caused significant inhibition of the flux, but the inhibition was not fully developed until ~20 min after addition of the agent. There were only very slight differences between cells preincubated 10 or 20 min with okadaic acid.

Effect of Okadaic Acid on the Time Lag for Transport Activation

Fig. 3 shows the effect of 135 nM okadaic acid on the time course of activation of KCl cotransport after cell swelling. Cells were preincubated in isotonic medium with or without okadaic acid for 30 min. Cell swelling and addition of $^{86}Rb^+$ both took place

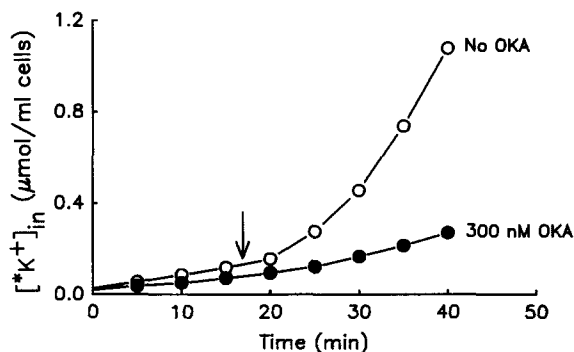
FIGURE 1. Effect of okadaic acid on the swelling-stimulated influx of $^{86}Rb^+$ into rabbit red blood cells. Cells were incubated at 3% hematocrit, 37°C, in medium A: 155 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4, and 10^{-4} M ouabain. At $t = 0$ $^{86}Rb^+$ was added and the accumulation of intracellular radioactivity was determined at the indicated times. At the arrow, the media were made hypotonic (210 mosmol/kg H_2O) by adding 5 mM KCl and $^{86}Rb^+$ in the proportions necessary to keep the extracellular specific activity constant. *Open circles,* no okadaic acid; *filled circles,* okadaic acid added 10 min before $^{86}Rb^+$. The 5 mM KCl that was added at the arrow contained no DMF or okadaic acid. Therefore, the okadaic acid concentration was 30% lower after swelling than before. The final concentration of okadaic acid was 300 nM. Both suspensions contained 0.9% DMF before swelling and 0.6% after swelling.

at $t = 0$. The influx data fit the two-state model reasonably well, and, as found previously for the other phosphatase inhibitors (Jennings and Al-Rohil, 1990), okadaic acid appeared to prolong the time lag in addition to lowering the steady-state flux (see Discussion).

Okadaic Acid Concentration Dependence

From the above data, it appears that, at least at a concentration of 135 nM, a preincubation of 30 min is sufficient time for okadaic acid to exert its full effect on swelling-stimulated transport. We used a preincubation of 30 ± 10 min before swelling (210 mosmol/kg H_2O) to generate a rough dose–response curve, for the purpose of comparing the concentrations necessary for transport inhibition with the concentrations that inhibit various protein phosphatases.

The results of several experiments are summarized in Fig. 4. There is considerable scatter, but the data can be fit reasonably well by a hyperbolic curve describing a single inhibitory site with a dissociation constant of 40 nM. The range of concentrations over which transport is inhibited is similar to the concentrations that inhibit type 1 protein phosphatases (see Cohen et al., 1990). However, we are not measuring phosphatase activity directly, so 40 nM may not be the actual dissociation constant for okadaic acid binding to the target phosphatase.

The dose–response curve in Fig. 4 could be misleading if so much okadaic acid is bound by the cells that the free concentration (the relevant parameter) is significantly less than the total. For example, a 1% suspension of red cells binds about half the phloretin in a 50-μM solution (LeFevre and Marshall, 1959). To determine whether cell binding affects the free concentration of okadaic acid, an experiment was performed in which the same amount of okadaic acid was added to suspensions of 1.5, 3, and 6% hematocrit. The degree of inhibition of the swelling-stimulated flux
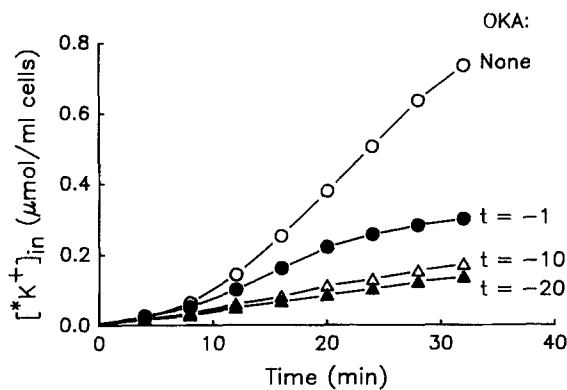

FIGURE 2. Time course of the effect of okadaic acid on swelling-stimulated $^{86}Rb^+$ influx in rabbit red cells. Cells were incubated at 37°C in isotonic medium A for 20 min before the beginning of the influx. Cell swelling was simultaneous with the addition of $^{86}Rb^+$ at $t = 0$. The $^{86}Rb^+$ was added with 5 mM KCl to lower the osmolality to 210 mosmol/kg H_2O. Okadaic acid (*OKA*) was present at a final concentration of of 135 nM. The okadaic acid was added 1, 10, or 20 min before cell swelling, as indicated.

(after a 20-min preincubation) was 80–82% at all three hematocrits, indicating that cell binding does not cause a major decrease in the free concentration in the range of okadaic acid concentrations and hematocrits used here. Therefore, the half-maximal inhibitory concentration of ~40 nM represents the free concentration.

Relation between Effects of Okadaic Acid and NEM

As described in the Introduction, KCl cotransport can be activated by treatment with NEM as well as by cell swelling. We have used okadaic acid to investigate the role of a kinase or phosphatase in the mechanism of NEM action. Fig. 5 depicts an experiment in which NEM, okadaic acid, and $^{86}Rb^+$ were added in various orders. If NEM was added 20 min before $^{86}Rb^+$, the influx was large and constant over a 30-min period, as expected. The addition of 150 nM okadaic acid with $^{86}Rb^+$ at $t = 0$ has only a slight effect on the influx into the cells that had been pretreated for 20 min with NEM; there is slight inhibition at late time points, but the effect is not large. The lack

of effect (or very slow effect) of phosphatase inhibition *after* NEM activation is in agreement with the finding of Al-Rohil (1988) that fluoride added after a maximal dose of NEM has little effect.

Although okadaic acid has little effect when added after NEM, it has a major effect when added *before* NEM. Fig. 5 (right) shows that NEM, added 1 min before ^{86}Rb$^+$, causes activation of transport after a slight time lag that is examined further below. If 150 nM okadaic acid was present for 20 min before the addition of NEM, the flux is much smaller. That is, pretreatment with okadaic acid inhibits NEM activation of transport. The concentration of NEM is > 1,000 times that of okadaic acid, and it is therefore not likely that okadaic acid somehow inactivates NEM chemically. Instead, the prevention (or delay) of the NEM effect by okadaic acid indicates that activation of the flux by NEM requires the action of an okadaic acid–sensitive phosphatase.
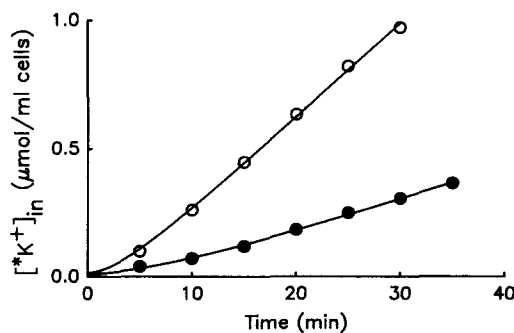

FIGURE 3. Effect of okadaic acid on the time course of activation of KCl cotransport by cell swelling. Cells were incubated in isotonic medium A in the presence (*filled circles*) or absence (*open circles*) of okadaic acid for 30 min at 37°C. Cell swelling and ^{86}Rb$^+$ addition were simultaneous at $t = 0$. Final osmolality was 210 mosmol/kg H$_2$O. The final okadaic acid concentration was 135 nM. The curves through the data are least-squares fits to the two-state model (Jennings and Al-Rohil, 1990), with delay times of 3.4 min (control) and 8.6 min (okadaic acid). In a separate experiment the data appeared to be quite similar to those in this experiment, but the effect of okadaic acid on the delay time was smaller. See Discussion for consideration of the statistical significance of changes in delay times.

Time Lag for NEM Activation

The data in Fig. 5 suggest that, after NEM addition to the flux medium, the influx of ^{86}Rb$^+$ activates with a time lag of $\sim$3–5 min. The time lag in the activation of the flux by NEM could be caused by the finite rate of irreversible reaction of NEM with sulfhydryl groups. Alternatively, the time lag could be a consequence of events that take place *after* the NEM covalent reaction is complete. To distinguish between these possibilities, a method was devised to treat cells with a pulse of NEM for 2 min, followed by quenching of the reaction with a scavenging agent, cysteine.

NEM is a small, uncharged, nonpolar compound; it should penetrate the bilayer portion of the membrane very rapidly. The permeability coefficient of the human red cell membrane to succinimide is $\sim$2 $\times$ 10^{-5} cm/s (Deuticke, 1977). This permeability corresponds to a half-time of uptake (and efflux) of $\sim$2 s. The NEM permeability coefficient should be even larger than that of succinimide because the hydrophobic ethyl group replaces the imide hydrogen. Accordingly, it should be possible to scavenge unreacted NEM with extracellular cysteine, because unreacted intracellular NEM should diffuse out of the cells and react rapidly with cysteine. The half-time of

the reaction between cysteine and NEM at neutral pH and 1 mM concentrations is <1 s (Gorin et al., 1966).

Logue et al. (1983) and Lauf (1987) have measured the time course of NEM activation of KCl cotransport in sheep red cells for the purpose of characterizing the rates of NEM reaction with transport-related sulfhydryl groups. An important difference between the present work and that of Logue et al. (1983) and Lauf (1987) is that we did not wash the cells after NEM treatment and before the flux measurement. Any time lag in transport activation after the completion of the NEM reaction would have occurred during the washes in the earlier work. In our studies the washes were omitted for the express purpose of trying to detect such time lags.

Fig. 6 shows a representative NEM pulse experiment. In one suspension (open symbols), 3 mM cysteine was added 1 min before 2 mM NEM. Cysteine addition
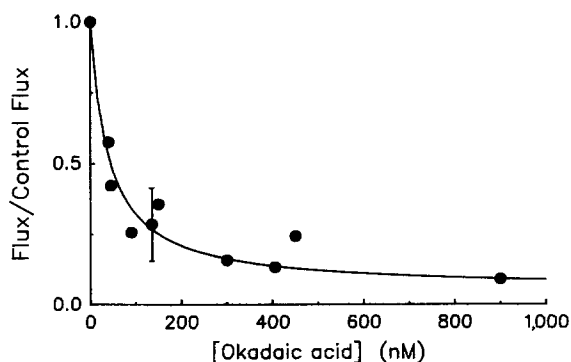

FIGURE 4. Concentration dependence of the inhibition by okadaic acid of the swelling-stimulated $^{86}Rb^+$ influx in rabbit red cells. Cells were incubated 30 ± 10 min with okadaic acid in isotonic medium, pH 7.4, at 37°C before swelling to a final osmolality of 210 mosmol/kg H_2O. The steady-state flux was estimated from the slope of the influx data between 15 and 40 min after swelling. Data are plotted as relative flux (each flux divided by a control from the same cell preparation) vs. the final okadaic acid concentration in nanomolar. The curve through the data represents that expected for a single inhibitory site of dissociation constant 40 nM, and an okadaic acid–insensitive flux equal to 5% of the control flux. The curve is not intended as a rigorous fit of the data, because there is too much scatter for a best fit to be meaningful. The curve is intended to illustrate that the data follow a hyperbolic relationship reasonably well, with half-maximal inhibition at an okadaic acid concentration of between 20 and 70 nM.

before NEM totally prevented transport activation; the time course of accumulation of $^{86}Rb^+$ was unchanged by the additions. Therefore, the scavenging reaction is rapid and complete, in agreement with the finding of Lauf (1987) that NEM reaction with intact cells can be inactivated rapidly. Also, neither cysteine nor the product of cysteine reacting with NEM appears to have any effect on the flux over the time and concentration ranges used here.

The solid symbols in Fig. 6 show the influx into cells that were treated with NEM for 2 min before the cysteine quench. The influx activated with a time delay that was longer than the 2 min of the exposure. That is, the flux continued to increase for a few minutes after the NEM reaction was complete. The time course of transport activation by swelling and NEM were compared in a single preparation of cells in the experiment in Fig. 7. Four identical suspensions were initially in 310 mosM medium.

Two of the suspensions were made hypotonic at $t = 2$ min (Fig. 7, right), and the flux activated with a time lag of 9 min, which is essentially identical to that previously published (Jennings and Al-Rohil, 1990). The other two suspensions (Fig. 7, left) were treated with NEM at $t = 7$ min, followed by cysteine quench 2 min later. The steady-state flux after NEM treatment is similar to that in the swollen cells, but the time lag (6 min from the beginning of the NEM pulse) is slightly smaller. Because of the difficulties in determining accurate time lags, it is not clear whether the NEM time lag is significantly different from that for cell swelling.

The effect of okadaic acid on the time lag for NEM activation was examined using the pulse-quench method. Fig. 8 shows an experiment in which okadaic acid was present for 15 min before NEM addition. Okadaic acid lowers the steady-state flux and also appears to prolong the time lag for transport activation (see Discussion).
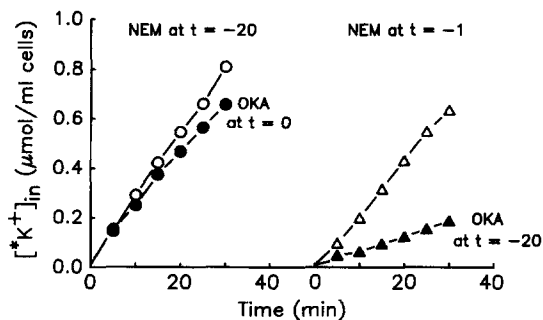

FIGURE 5. Effect of okadaic acid on the $^{86}Rb^+$ influx stimulated by treatment with NEM. Cells were incubated at 4% hematocrit in medium A at 37°C. In the influxes shown at left, 2 mM NEM (0.2% DMF) was added 20 min before $^{86}Rb^+$. In one of the suspensions (*filled circles*), 150 nM okadaic acid was added with $^{86}Rb^+$ at $t = 0$. The data on the right are from the same cell preparation, same solutions, and hematocrit, but 2 mM NEM was added 1 min before $^{86}Rb^+$. In one of the suspensions (*filled triangles*), 150 nM okadaic acid was present for 20 min before NEM.

Evidence against the Involvement of Protein Kinases A and C

The above data indicate that a net dephosphorylation is necessary for activation of KCl cotransport by either cell swelling or NEM. The relatively long time lags for activation are consistent with the idea that transport activation involves the inhibition of a kinase rather than the stimulation of a phosphatase (see Jennings and Al-Rohil, 1990). If so, then which kinase is it? We have only very limited information on this important question. As mentioned in the Introduction, cyclic AMP stimulates KCl cotransport in pig red cells (Kim et al., 1989). In rabbit red cells we find a qualitatively similar but smaller effect (Fig. 9). A 90-min incubation with 1 mM cyclic AMP before the start of the flux caused a 25% increase in the ouabain-insensitive $^{86}Rb^+$ influx in hypotonic medium. The large effect of cell volume on the influx persists in cells preincubated with cAMP.

In the same experiment parallel suspensions of cells were incubated in isotonic physiological saline with [^{32}P] inorganic phosphate for metabolic incorporation of ^{32}P into ATP and phosphoproteins. An SDS-polyacrylamide gel (not shown) of membrane protein from these cells revealed that incubation of intact cells with cyclic AMP stimulated the phosphorylation of a polypeptide that migrates slightly more slowly

than actin, as expected from results obtained with human red cell membranes (see Fairbanks et al., 1983). Thus, sufficient cAMP entered the cells to stimulate the cAMP-dependent protein kinase, under conditions in which the volume dependence of transport was only slightly affected. We believe, therefore, that cyclic AMP–mediated phosphorylations may have modulatory effects on KCl cotransport but are not involved in the primary phosphorylation–dephosphorylation that controls transport activation by cell volume.

A similar experiment was performed with 1 μM PMA. PMA has no effect on the flux in isotonic medium, in fair agreement with the results of Kim et al. (1989), who found a slight acceleration by PMA of the flux in pig red cells. Cell swelling (210 mosmol/kg H_2O) caused a large increase in the influx in either the presence or absence of PMA (not shown). A parallel ^{32}P phosphorylation experiment (not shown) with the same cell preparation showed a phorbol ester–stimulated phosphorylation of band 4.1, as expected from the literature (Ling and Sapirstein, 1984). Although each
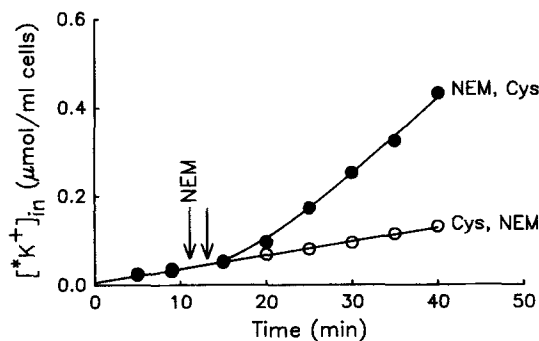

FIGURE 6. Time lag for the stimulation of $^{86}Rb^+$ influx after a short exposure to NEM. Cells were incubated at 4% hematocrit in medium A at 37°C, and $^{86}Rb^+$ was added at $t = 0$. For both sets of data, NEM was added at $t = 11$ min (*first arrow*) from a freshly prepared 1 M stock in DMF. Final NEM concentration was 2.5 mM (0.25% DMF). 2 min later (*second arrow*), cysteine was added from a 200 mM stock to a final concentration of 4 mM (*filled circles*). The open circles represent a parallel influx in which cysteine was added 1 min before rather than 2 min after NEM. The curve drawn through the filled circles represent an exponential approach to the new steady state (two state model), with a time lag of 10 min from the first arrow.

of the above transport–phosphorylation experiments was done only once, we feel that it is unlikely that either the A or C kinase has a major role in the regulation of volume-sensitive KCl cotransport in rabbit red cells.

DISCUSSION

The large effect of submicromolar concentrations of okadaic acid on swelling-stimulated KCl cotransport is strong evidence that a net dephosphorylation is necessary for the activation of KCl cotransport by swelling. Okadaic acid is reasonably specific for certain serine–threonine protein phosphatases; it has no effect on tyrosine phosphatases, alkaline and acid phosphatase, inositol trisphosphatase, or any of eight different protein kinases (see Cohen et al., 1990). Four different (serine–threonine) protein phosphatases are known to exist in mammalian cells (see

Cohen, 1989). The concentration range in which okadaic acid inhibits KCl cotransport (IC_{50} = 40 nM) is similar to the range (IC_{50} = 10–15 nM) in which type 1 protein phosphatase (PP1) is inhibited (Cohen et al., 1990). In contrast, protein phosphatase 2A is strongly inhibited by 1 nM okadaic acid, and protein phosphatase 2C is inhibited only by concentrations that are much higher than those that inhibit KCl cotransport. The other protein phosphatase (2B) is dependent on Ca^{2+}/calmodulin and is unlikely to have a role in red cell KCl cotransport, because Ca^{2+} removal or calmodulin antagonists have little effect on the swelling-stimulated flux (Brugnara et al., 1985). PP1 is known to be present in human red cells and is inhibited by fluoride and inorganic phosphate (Keiner et al., 1987) in the same concentration range as those that inhibit swelling-activated KCl cotransport (Al-Rohil, 1988; Jennings and Al-Rohil, 1990).

Interpretation of the dose–response curve for okadaic acid is complicated by the time dependence of the effect. The inhibition appears to be fully developed after an
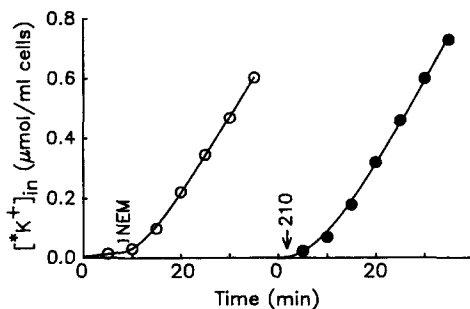

FIGURE 7. Time lag for the stimulation of $^{86}Rb^+$ influx after a short exposure to NEM (*left*) or hypotonic swelling (*right*). Cells were incubated at 4% hematocrit in medium A at 37°C; $^{86}Rb^+$ was added at t = 0. For the open circles, NEM was added at t = 7 min from a freshly prepared 1 M stock to a final concentration of 2.5 mM (0.25% DMF), followed by 4 mM cysteine 2 min later. The curve through the data represents the two-state model, with a time lag of 6 min from the beginning of the NEM exposure. The data on the right are from the same preparation of cells, in which the cells were hypotonically swollen (210 mosmol/kg H_2O) at t = 2 min. The curve through the data represents the two-state model with a time lag of 9 min. For both sets of data, duplicate fluxes were performed. Variations between the duplicates were smaller than the symbols.

exposure of ~25 min (Fig. 2), but there could be slower effects that escape detection. Moreover, we do not know the extent of reversibility of the okadaic acid effect. Pretreatment with 250 nM for 20 min, followed by two rapid washes, gives as much inhibition of the swelling-stimulated flux as does 200 nM okadaic acid in the flux medium (one experiment, not shown). The lack of rapid reversibility could be a consequence of slow release of the inhibitor from the phosphatase or perhaps secondary effects related to mutual interactions among kinases and phosphatases. We know too little about the system to rigorously interpret the data in Fig. 4, and the best that can be said at present is that PP1 is the leading candidate for the target of okadaic acid as an inhibitor of KCl cotransport.

Quantitative Interpretation of Time Lag Data

Table I summarizes some rough numerical estimates of the forward and reverse rate constants for transport activation and inactivation under various conditions. The

estimates should be considered orders of magnitude; the purpose of the table is to illustrate the theoretical effect of changes in each rate constant on the flux and on the relaxation time for approach to the final steady state. The entries are consistent with the kinetic data shown here and previously (Jennings and Al-Rohil, 1990). If okadaic acid lowers the forward rate constant k_{12} and has no effect on k_{21}, then the percent inhibition of transport is necessarily smaller than the percent inhibition of k_{12}, because the steady-state flux is proportional to $k_{12}/(k_{12} + k_{21})$. In fact, the experimental data are consistent with the idea that k_{12} is inhibited ~90% by 135 nM okadaic acid, in close agreement with the IC_{50} of 10–15 nM for inhibition of PP1.

According to the estimated rate constants in Table I, a 90% inhibition of k_{12} should increase the time lag for swelling (210 mosmol/kg) activation by less than a factor of two. Experimentally, phosphatase inhibitors cause a reasonably consistent increase in the time lag (Fig. 10), as expected if phosphatase inhibitors cause a decrease in k_{12}. However, quantitative estimates of time lags are subject to considerable uncertainty; slight errors, especially in late time points, have large effects on the estimated time
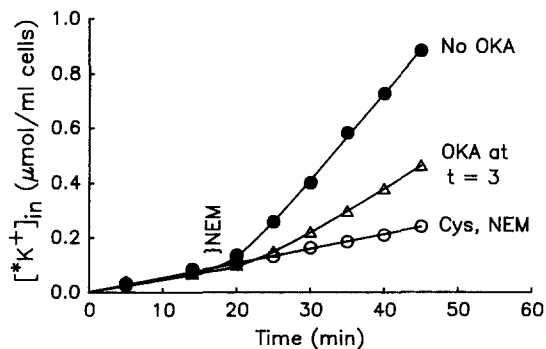

FIGURE 8. Effect of okadaic acid on the time course of NEM activation of $^{86}Rb^+$ influx. Cells were incubated at 4% hematocrit in medium A at 37°C; $^{86}Rb^+$ was added at $t = 0$. Filled circles and open triangles, a 2-min pulse of 2.5 mM NEM was given at $t = 16$–18 min. 150 nM okadaic acid was added at $t = 3$ min (open triangles). The curves through the data correspond to delay times of 4.7 min (filled circles) and 7.8 min (open triangles) from the beginning of the NEM exposure. Open circles, control influx with no okadaic acid present, and cysteine added 1 min before NEM.

delays. For example, a 5% increase in the intracellular $^{86}Rb^+$ in the last time point in the data in Fig. 3 would cause a 30% increase in the estimated time delay. Therefore, small effects on time lags should be viewed with caution. The data, however, are completely consistent with the idea that k_{12} is directly proportional to a phosphatase activity that is inhibited half-maximally by 10–15 nM okadaic acid.

Evidence that NEM Activates Transport by Inhibiting a Kinase

This work provides new information on the mechanism of NEM activation of transport. Okadaic acid, added before NEM, inhibits transport activation by NEM. This finding suggests that NEM does not activate transport by modifying a sulfhydryl group on the transport protein itself, but rather acts by modifying a kinase or phosphatase. The data do not completely rule out the possibility of a direct effect on the transport protein. For example, NEM could react directly with the transporter, but the transporter may be activated only if subsequently dephosphorylated. Although this mechanism has not been disproven, we believe that it is more likely that

NEM activates transport by inhibiting a protein kinase and thereby causing net dephosphorylation of some critical substrate.

Our current hypothesis, then, is that the state of phosphorylation of some key protein, not necessarily the transport protein itself, has a major effect on transport activity; when the protein is dephosphorylated, transport is activated. This substrate is phosphorylated by a kinase of unknown identity, but probably not the A or C kinase (see above). The kinase, designated for the present purposes as the "V kinase," is inhibited by cell swelling (by an unknown mechanism) and NEM (by modification of a sulfhydryl group). In the presence of a tonic phosphatase activity, the kinase inhibition causes a net dephosphorylation of the key substrate, and the net dephosphorylation results in activation of transport. If the phosphatase is inhibited by okadaic acid before cell swelling or NEM treatment, then both the steady-state flux and the rate of approach to the steady state are reduced.
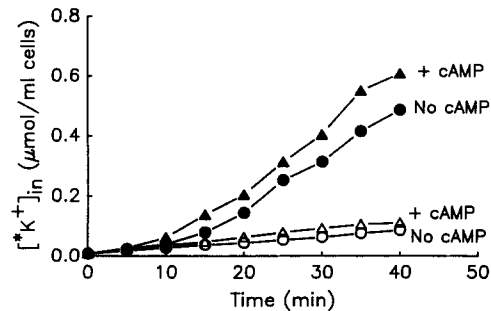

FIGURE 9. Effect of cyclic AMP on the ouabain-insensitive $^{86}Rb^+$ influx from isotonic (*open symbols*) or hypotonic medium (210 mosmol/kg H_2O; *filled symbols*). Cells were preincubated in HEPES-buffered physiological saline with glucose for 90 min in the presence or absence of 1 mM cAMP. Cells were then centrifuged and resuspended at $t = 0$ in isotonic or hypotonic flux medium.

Simple Model Cannot Explain Effects of Metabolic Depletion

Our model cannot explain all aspects of the data in the literature on swelling- and NEM-activated transport in red cells. For example, in LK sheep (Lauf, 1983*b*), human (Lauf et al., 1985), and rabbit (Al-Rohil, 1988) red cells, depletion of ATP (to ~0.1 mM) does not prevent cell swelling from activating the KCl cotransport flux. However, the same metabolic depletion strongly inhibits NEM activation. If both swelling and NEM acted by inhibiting the same kinase (which presumably can still work at an ATP concentration of 0.1 mM), then metabolic depletion would have the same effect on each. Therefore, even though swelling and NEM may inhibit the same kinase, there must be additional effects that are not common to both interventions.

As described above, the okadaic acid dose–response relationship indicates that PP1 is the most likely candidate for the dephosphorylation of the substrate (i.e., the transport protein or modulatory protein) that causes transport activation. PP1, at least in some cells, is activated by a phosphorylation mediated by a kinase known as F_A (Cohen, 1989; Tung and Reed, 1989). Thus, the ATP dependence of KCl cotransport that has been observed in red cell ghosts (Dunham and Logue, 1986; Brugnara et al., 1988; Sachs, 1988) could be a consequence of the requirement for kinase activation of PP1. Kinase activation of PP1 still does not explain the differential effects of moderate ATP depletion on swelling- vs. NEM-stimulated transport.

NEM Could Affect Both Forward and Reverse Rate Constants

It is worth mentioning that NEM inhibits PP1 in some cells (see Ballou and Fischer, 1986). The detailed characteristics of this inhibition are not known, and it is not known if red cell PP1 is inhibited by NEM. However, if our hypothesis regarding the action of NEM and okadaic acid on KCl cotransport is correct, then NEM must inhibit PP1 less than it inhibits the "V kinase." Otherwise NEM would not cause the net dephosphorylation we believe is necessary for transport activation. NEM inhibition of both the kinase and phosphatase could explain the fact that okadaic acid, added after NEM, has so little effect (or such a slow effect) on the KCl cotransport flux.

Inhibition by NEM of both the forward and reverse rate constants could be somewhat analogous to the fixation of the dog red cell Na^+-H^+ exchanger into a volume-insensitive state by glutaraldehyde (Parker, 1984) or N-phenylmaleimide (Parker and Glosson, 1987). If the agent inhibits both the forward and reverse rate constants equally and completely, then the transporter is frozen in the state (activated or inactivated) that it was in during the treatment. However, if the agent inhibits one

TABLE I

Numerical Estimates of the Forward and Reverse Rate Constants k_{12} and k_{21}

Condition	k_{12}	k_{21}	Relative flux, $k_{12}/(k_{12} + k_{21})$	Time lag
	min^{-1}	min^{-1}		min
Normal volume	0.05	0.95	0.05	1.0
210 mosmol/kg H_2O	0.05	0.05	0.5	10
210 mosmol/kg H_2O 135 nM okadaic acid	0.005	0.05	0.1	18

rate constant more than the other, transport could be forced into the activated or inactivated state, depending on which rate constant is inhibited more completely.

The fact that NEM could affect both the forward and reverse rate constants greatly complicates the interpretation of the time lag for NEM activation of transport. For example, NEM could inhibit the reverse rate constant k_{21} very rapidly but inhibit the forward rate constant more slowly, perhaps by an indirect mechanism (e.g., inhibition of a kinase that activates PP1). Accordingly, the time course of activation of transport by NEM is a function of the initial phosphatase activity as well as time course of inactivation of the phosphatase. It could therefore be a coincidence that the time courses for NEM activation and swelling activation of transport are so similar.

Relation to Other Recent Studies on NEM Activation of Transport

The idea that NEM activates transport by altering the rate constants of a phosphorylation–dephosphorylation cycle could help explain two recent interesting findings regarding NEM-activated K^+ transport in human red cells. Berkowitz (1990) showed that the presence of various agents, including some (e.g., benzthiazide) that have no

effect on KCl cotransport itself, can strongly enhance the activation of cotransport by NEM. Enhancement of the NEM effect could be a consequence of benzthiazide protecting the phosphatase from inhibition by NEM or somehow enhancing the inhibition of the kinase by NEM. This suggestion does not really explain the effect, of course, but it offers a set of possibilities that do not require the interaction of benzthiazide with the transporter itself.

Sheerin et al. (1989) recently found that the NEM-activated K^+ flux in human red cells slowly declines over a period of hours after NEM treatment. Such a decline would not be expected if NEM activated the protein directly. However, if the activation depends on a phosphorylation–dephosphorylation cycle that is altered by NEM, then very slow changes after the NEM treatment could take place, possibly mediated by kinases or phosphatases that are different from those that are important
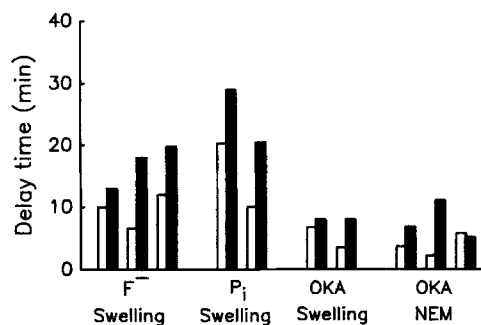

FIGURE 10.   Effect of fluoride, inorganic phosphate, or okadaic acid on the delay times for swelling- or NEM-activated ouabain-insensitive $^{86}Rb^+$ influx in rabbit red cells. Each pair of bars represents a single preparation of cells; *open bars* represent control cells and *filled bars* represent cells in the presence of phosphatase inhibitor. The data were fit to the two-state model as described previously (Jennings and Al-Rohil, 1990). Fluoride concentration was 2 mM extracellular (1.4 mM intracellular). Inorganic phosphate concentration was 10 mM extracellular (~6 mM intracellular). Okadaic acid concentration was 135 nM for cell swelling and 150 nM for the first two NEM experiments. For the last NEM experiment (far right) the okadaic acid concentration was 60 nM.

in untreated cells. None of our experiments were carried out for long enough times for comparison between our results and those of Sheerin et al. (1989).

NEM-activated Proteins Are Rare

The mechanism for the action of NEM proposed here has the attraction of explaining the NEM effect without requiring a direct activation of a protein by such an unphysiological agent. NEM is well known to inhibit the functions of many proteins, but direct activation of protein function by NEM is not common. One example is the hemin-controlled translation inhibitor, which itself is a protein kinase (see Suzuki et al., 1985). Thus, kinase activation by NEM is possible, but there are many more protein kinases that are inhibited by NEM (Bechtel et al., 1977; Buhrow et al., 1982; Wilden et al., 1986; Inoue et al., 1988). To our knowledge, no transport protein has been shown to be activated directly by NEM.

The fact that direct activation of proteins by NEM is so much less common than inhibition does not prove anything about KCl cotransport, but we feel it is worth mentioning because the focus of most discussion of NEM effects on cotransport has

been on direct effects of the agent (Lauf, 1985, 1987; Al-Rohil and Jennings, 1989). We believe that an indirect effect, through the inhibition of a protein kinase, is the most reasonable explanation of our date and is also consistent with the known effects of NEM on proteins.

Cytoplasm as Volume Sensor

We recently showed (Jennings and Schulz, 1990) that changes in cell shape have only minor effects on volume-sensitive KCl cotransport in rabbit red cells, suggesting that cytoplasmic factors are of major importance in determining the activity of the transporter. The present evidence for a phosphorylation–dephosphorylation cycle is completely consistent with this idea. The actual mechanism by which cell swelling may inhibit a kinase is of course not known. If the kinase has a soluble activator and/or the phosphatase has a soluble inhibitor (which are known to exist for PP1 in some cells [Cohen, 1989]), then dilution of the cytoplasm would inhibit the kinase but only have minor effects on the phosphatase.

One possible soluble mediator is Mg^{2+}. Lowering the cytoplasmic free Mg^{2+} concentration is known to activate KCl cotransport (Lauf, 1985; Brugnara et al., 1988; Dunham, 1989). Mg^{2+} depletion could possibly activate transport by inhibiting the kinase that we believe is inhibited by NEM and cell swelling. However, Mg^{2+} depletion appears to eliminate the time lag for swelling activation (Dunham, 1989); the effect of low Mg^{2+} on the time lag indicates an increase in k_{12} rather than a decrease in k_{21}. An increase in k_{12} by low Mg^{2+} is not in agreement with the idea that k_{12} is related to PP1 activity, because Mg^{2+} is an activator of purified PP1 (Kiener et al., 1987). Thus, the effects of Mg^{2+} are not yet understood in the context of the proposed phosphorylation–dephosphorylation mechanism. A systematic study of the effects of Mg^{2+} on activation and inactivation of transport and on various kinase and phosphatase activities under physiological conditions is required before a concrete mechanism for Mg^{2+} action can be proposed.

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