Stimulation of membrane serine-threonine phosphatase in erythrocytes by hydrogen peroxide and staurosporine

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Bize, Isabel, Patricia Muñoz, Mitzy Canessa, and Philip B. Dunham. Stimulation of membrane serine-threonine phosphatase in erythrocytes by hydrogen peroxide and staurosporine. Am. J. Physiol. 274 (Cell Physiol. 43): C440–C446, 1998.—Indirect evidence has suggested that K-Cl cotransport in human and sheep erythrocytes is activated physiologically by a serine-threonine phosphatase. It is activated experimentally by H2O2 and by staurosporine, a kinase inhibitor. Activation by H2O2 and staurosporine is inhibited by serine-threonine phosphatase inhibitors, suggesting that the activators stimulate the phosphatase. The present study shows that sheep and human erythrocytes contain membrane-associated as well as cytosolic serine-threonine phosphatases, assayed from the dephosphorylation of 32P-labeled glycogen phosphorylase. In cells from both species, the relatively low sensitivity of the membrane enzyme to okadaic acid suggests it is type 1 protein phosphatase. The cytosolic phosphatase was much more sensitive to okadaic acid. Membrane-associated phosphatase was stimulated by both H2O2 and staurosporine. The results support earlier conclusions that the membrane-associated type 1 phosphatase identified here is regulated by phosphorylation and oxidation. The results are consistent with the phosphatase, or a portion of it, being responsible for activating K-Cl cotransport.

METHODS

Cells. Red blood cells from LK sheep were obtained as reported before (4) from sheep maintained at the Vinzant Family Farms (Borodino, NY). Human red blood cells were obtained at the University of Chile from three healthy adult volunteers after informed consent. Cells were washed by centrifugation in solutions described previously (3). For treatment of intact cells, cells were incubated for 30 min at 37°C, 10% hematocrit, with the desired agents. Control cells were incubated in parallel.

Preparation and treatment of membranes. Red blood cells were lysed in 15 volumes of lysis buffer containing 10 mM trishydroxymethylaminomethane (Tris)-HCl (pH 7.4), 0.1% 2-mercaptoethanol, and 1 µM phenylmethylsulfonyl fluoride. For human cells, lysis buffer also contained 10 µg/ml of leupeptin, and, for sheep cells, the buffer also contained 25 µg/ml each of leupeptin and aprotinin. Cell lysates were centrifuged (30,000 g for 20 min), and the membrane pellet was washed by centrifugation in lysis buffer. The first supernatant was used as the cytosolic fraction. The membrane and cytosolic fractions were stored frozen (−20°C) until use, usually within 1 wk.

One hundred microliters of previously frozen membrane suspensions (obtained from 100 µl of untreated cells) containing 2–3 mg/ml of protein were mixed with 100 µl of lysis buffer containing increasing concentrations of H2O2 or staurosporine, as desired. In some experiments, the membranes were suspended in 100 µl of cytosol diluted ~15-fold. The mixtures were incubated for 10 min at 30°C and centrifuged in a Microfuge for 3 min, and the pellets were resuspended in lysis buffer. For treatment of human membranes with H2O2, 0.5% Triton X-100 was added to the lysis buffer before centrifugation. Phosphatase activity was determined on the resuspended pellets.
Phosphatase activity. Phosphatase activity was determined using 32P-labeled glycogen phosphorylase a as substrate as previously described (11). Briefly, 32P-labeled glycogen phosphorylase a was prepared by reacting [γ-32P]ATP with purified glycogen phosphorylase b in the presence of purified phosphorylase kinase. The reaction was allowed to go to completion, and the excess [32P]ATP was removed by precipitation of the phosphorylase with ammonium sulfate and washing of the pellet. The ammonium sulfate was removed, and the labeled phosphorylase a was stored until use at 4°C at a concentration of 3 mg/ml. The phosphatase assay reaction media contained 16.7 mM Tris·HCl, 13.3 mM imidazole (final pH of 7.4), 5 mM caffeine, 0.1 mM EDTA, 0.4% 2-mercaptoethanol, 0.67 mg/ml bovine serum albumin, and 1 mg/ml of the 32P-labeled substrate. Protein concentrations in the red cell fractions to be assayed were 50–100 µg/ml for the membranes and 100–250 for the cytosol. Triton X-100 was added to all samples before the phosphatase assay was performed (final concentration, 0.5% vol/vol). The reaction mixture was incubated for 15 min at 30°C. The fraction of substrate dephosphorylated never exceeded 25%, and the reaction was linear with time. Phosphatase activity is expressed as units per milligram protein, in which 1 unit (U) equals 1 nmol of phosphate released per minute. Concentrations giving half-maximal inhibition (IC50) by okadaic acid were estimated graphically. Protein concentrations were determined using the Lowry assay (28). The phosphatase assay measures serine-threonine phosphatase activity because the substrate used is specific for serine-threonine phosphatases.

Statistics. The results were analyzed for differences between means using Student’s t-test when the data presented a single normal distribution or with a signed rank test when standardized skewness and kurtosis values were not within the range expected for data from a normal distribution (Statgraphics).

Materials. Okadaic acid was obtained from LC Laboratories or from Sigma Chemical and dissolved in either ethanol or dimethyl sulfoxide at 100 µM. Calyculin was obtained from LC Laboratories and dissolved in ethanol at 100 µM. Both stock solutions were stored at −20°C. [γ-32P]ATP was obtained from Dupont New England Nuclear. Purified glycogen phosphorylase b and phosphorylase kinase were obtained from Gibco BRL (Gaithersburg, MD). All other chemicals were obtained from Sigma.

RESULTS

Erythrocyte cytosolic and membrane-associated serine-threonine phosphatase activity. Human and sheep red cell cytosols and membranes contain significant phosphorylase phosphatase activity (Fig. 1, Table 1). In human and sheep red blood cells, the membrane-associated activity per milligram protein exceeds cytosolic activity. About 98% of the cell protein is in the cytosol, and the membrane fraction contains ~5% of the total phosphatase activity. Cytosolic phosphatase activity in sheep red blood cells is higher than in human red blood cells (0.8 vs. 0.3 U/mg protein). Membrane-associated phosphatase is also higher in sheep than in human red blood cells (3.1 vs. 0.9 U/mg protein). Phosphatase activity in membrane fractions obtained from sheep red blood cells ranged from 0.2 to 10.5 U/mg protein. We cannot explain the large range except to say that the few very high and low values (>8 and <0.5 U/mg) were all obtained early in the project. There was also a large range of values for membrane-
associated phosphatase activities from human red blood cells (0.3 to 3.6 U/mg protein). After separation of the cytosolic and membrane-associated components by centrifugation at 30,000 g, 80-100% of the total lysate phosphatase activity was recovered.

In an effort to characterize the properties of the cytosolic and membrane-associated serine-threonine phosphatases, we examined the effect of the serine-threonine phosphatase inhibitor okadaic acid on the activity of cytosol and membrane fractions. Figure 1 shows that the serine-threonine phosphatases of the cytosol and membrane in human and sheep red blood cells can be distinguished by large differences in sensitivity to okadaic acid. In human red blood cells, the IC_{50} of phosphatase by okadaic acid was ~0.23 nM in the cytosol and 27 nM for the membranes (Fig. 1A and Table 1). In sheep red blood cells (Fig. 1B), the IC_{50} for okadaic acid was ~0.03 nM in the cytosol and 16 nM for the membranes. The results of preparations from sheep cells are means of three experiments. In two of the experiments, there was a suggestion of two components in terms of sensitivity to okadaic acid of the membrane preparation, with ~30% of the phosphatase activity having an IC_{50} of 0.1 nM or less. In the curve for the mean of these three experiments, there is a hint of this high-affinity component, but it is difficult to prove. So we present these data as though there is a single component but recognize that there may be two.

Thus, in cells from both species, serine-threonine phosphatase of the membrane was much less sensitive to inhibition by okadaic acid than was the cytosolic phosphatase. The IC_{50} values for the membrane-associated phosphatases from the two species were similar. The high IC_{50} values are consistent with most of the membrane-associated phosphatases being PP-1 (10). In contrast, the IC_{50} values for cytosolic fractions from red blood cells of the two species differed by nearly an order of magnitude. The explanation for this is unclear; because the focus of this study is the membrane-associated enzyme, we have not explored this point.

The sensitivity to calyculin A and okadaic acid was compared for membrane-associated phosphatase from sheep red blood cells with the two inhibitors at 1 nM (Fig. 2). The membrane-associated phosphatase was considerably less sensitive to okadaic acid than to calyculin, consistent with at least a large fraction of the membrane-associated activity being PP-1 (10).

Stimulation of membrane-associated phosphatase activity by H_{2}O_{2}. Two protocols were used to study the effect of H_{2}O_{2}; pretreatment of intact cells and treatment of isolated membranes. Because K-Cl cotransport activity is maximally stimulated in intact sheep red blood cells by pretreatment with 5 mM H_{2}O_{2} (4), phosphatase activity was determined in preparations of membranes from sheep red blood cells treated in this manner. Phosphatase activities in membrane fractions obtained from H_{2}O_{2}-treated cells were stimulated by 26 ± 9% compared with preparations from untreated cells (Fig. 3), and the stimulation was statistically significant (P = 0.014, paired t-test, 2-tailed, n = 19). In contrast, the phosphatase activities in cytosolic fractions were unaffected by pretreatment of the cells with H_{2}O_{2}. In membrane fractions from human red blood cells treated with 5 mM H_{2}O_{2}, there was also a stimulation of membrane-associated phosphatase activity (results not shown). The stimulation was less than in sheep red cell membranes, 8 ± 2%, but was statistically significant (P = 0.006, n = 13, signed rank test).

Stimulation by H_{2}O_{2} of phosphatase activity in membranes isolated from untreated cells. We also tested if direct treatment of isolated membranes with H_{2}O_{2} could stimulate membrane-associated phosphatase activity. Isolated red cell membrane preparations do not contain catalase or glutathione peroxidase, two cytosolic enzymes that scavenge H_{2}O_{2} (8). Therefore, much lower concentrations of H_{2}O_{2} were used in these experiments than with intact cells. H_{2}O_{2} treatment (50–100 µM) did stimulate phosphatase activity in the isolated membrane suspension of sheep cells (Fig. 4).
H₂O₂ AND STAUROSPORINE STIMULATE A PHOSPHATASE

Stimulation of membrane-associated phosphatase activity by staurosporine. Stimulation of sheep red cell K-Cl cotransport activity by 5 µM staurosporine is abolished by subsequent treatment of the cells with calyculin, suggesting that staurosporine may stimulate a serine-threonine phosphatase involved in activation of K-Cl cotransport (4). Therefore, we examined phosphatase activity of cytosol and membrane fractions from cells pretreated with 5 µM staurosporine. In preparations from sheep red blood cells, staurosporine pretreatment stimulated phosphatase activity in the membrane fraction but not in the cytosol (Fig. 5), just like the effect of H₂O₂ pretreatment of sheep cells. The stimulation of membrane phosphatase by staurosporine was 34 ± 8%, which was statistically significant (P = 0.002, paired t-test, n = 13). Also shown in Fig. 5 is the effect of pretreatment of human red blood cells with 5 µM staurosporine. Phosphatase activity in membranes from these cells was enhanced by 16 ± 5%, also statistically significant (P = 0.03, paired t-test, n = 14).

We also measured the phosphatase activity of membranes treated directly with staurosporine (not membranes from pretreated cells). Membranes were incubated with increasing concentrations of staurosporine, up to 5 µM. No stimulation by staurosporine was observed in these experiments (n = 2 in sheep cells, n = 1 in human cells) (results not shown). This result is in contrast to that with H₂O₂, which stimulated phosphatase after treatment of isolated membranes. Because lack of stimulation by staurosporine in this preparation might be due to the loss of soluble components that mediate the effect of staurosporine, we treated membranes with increasing concentrations of staurosporine in the presence of diluted cytosol. No stimulation by staurosporine was observed (results not shown), indicating that the response requires cellular integrity or that a soluble mediator becomes too dilute during lysis.

Inhibition of H₂O₂- and staurosporine-stimulated phosphatase by okadaic acid. We determined the effects of varying concentrations of okadaic acid on the phosphatase activity of membranes from sheep red blood cells pretreated with H₂O₂ or staurosporine. The results are shown in Fig. 6 along with phosphatase activity of membranes from untreated cells. The IC₅₀ for inhibition of the phosphatase in control membranes was ~30 nM, in the range of the IC₅₀ in Fig. 1B. IC₅₀ values were similar or slightly lower for the activity stimulated by H₂O₂ and staurosporine than that for membranes from untreated cells. Therefore, these agents stimulate a phosphatase activity that is inhibited by okadaic acid with a sensitivity similar or identical to that for untreated preparations.

Fig. 4. Effect of H₂O₂ on phosphatase activity of membranes (from sheep and human cells) treated with H₂O₂. After incubation with various H₂O₂ concentrations (10 min at 30°C), suspensions were centrifuged in a Microfuge and pellets were resuspended in lysis buffer containing 0.5% Triton X-100. Percent of total protein recovered in the pellet was unchanged by treatment with H₂O₂. Results are means of 4 experiments on sheep red blood cells and 2 experiments on human red blood cells.

Fig. 5. Effect of staurosporine (stauro; 5 µM) on phosphatase activity of fractions from sheep and human red blood cells. Phosphatase activities are shown for cytosol (n = 3) and membrane fractions (n = 13) obtained from control and staurosporine-treated sheep red blood cells and in membrane fractions from control and staurosporine-treated human red blood cells (n = 14). Stimulation by staurosporine was statistically significant in sheep red cell membranes (P = 0.002) and in human cell membranes (P = 0.03, paired t-tests).

DISCUSSION

We found serine-threonine protein phosphatase activity in human and sheep red blood cells, both in the cytosol and associated with the membranes. The conclusion that the phosphatases are serine-threonine phos-
phosphatases is based on the substrate used in the assays and on their sensitivity to okadaic acid (Fig. 1, Table 1); the membrane-associated activity from sheep red blood cells is also inhibited by calyculin A (Fig. 2). These two inhibitors are specific for the serine-threonine phosphatases PP-1 and PP-2A. On the basis of their very different sensitivities to okadaic acid, we conclude that the cytosolic phosphatase is mainly PP-2A and the membrane-associated phosphatase is mainly PP-1. The IC$_{50}$ values for the membrane-associated phosphatase in cells from the two species were 27 and 16 nM (Table 1), in the range of IC$_{50}$ values for inhibition of PP-1 from various sources by okadaic acid (12–75 nM; Refs. 10 and 12). The comparison of sensitivities to calyculin A and okadaic acid for the membrane-associated phosphatase from sheep red blood cells (Fig. 2) was consistent with at least a large fraction of the membrane-associated activity being PP-1, since this phosphatase activity was considerably less sensitive to okadaic acid than to calyculin (10).

The IC$_{50}$ values for cytosolic phosphatase in cells from the two species were 0.23 and 0.03 nM, in or near the range of IC$_{50}$ for inhibition of PP-2A by okadaic acid (0.1–2 nM; Refs. 13 and 20). The locations in the cells of the two types of phosphatases are consistent with earlier reports: PP-1 is generally bound to particles or membranes, whereas PP-2A is usually cytosolic (29).

There have been previous reports of at least two distinct serine-threonine phosphatases in whole red cell lysates (24, 35). Using a variety of substrates, Kiener et al. (24) isolated a type 1 phosphatase from human red blood cells and Usui et al. (35) described a type 2A phosphatase, also in human red blood cells. Neither of these reports included studies of isolated red cell membranes or experiments using okadaic acid. Studies using phosphorylated casein as a substrate did not detect serine-threonine phosphatases associated with human red cell membranes (6). The present report is the first demonstration of serine-threonine phosphatase associated with red cell membranes.

The results of this study show that H$_2$O$_2$ and staurosporine, agents that stimulate K-Cl cotransport in sheep red blood cells (3, 4), also stimulate membrane-associated serine-threonine phosphatase in red blood cells from humans and sheep. H$_2$O$_2$ stimulates membrane-associated phosphatase activity when intact cells are pretreated with 5 mM H$_2$O$_2$ (Fig. 3) or isolated membranes are treated with 50 µM H$_2$O$_2$ (Fig. 4). In sheep red cell membranes, phosphatase activity stimulated by H$_2$O$_2$ is almost entirely inhibited by okadaic acid (Fig. 6). The IC$_{50}$ for inhibition of the H$_2$O$_2$-stimulated phosphatase activities, 10–15 nM (Fig. 6), is in the range of IC$_{50}$ values for inhibition of PP-1 by okadaic acid (10, 12).

The mechanism by which the mild oxidant H$_2$O$_2$ stimulates phosphorylase phosphatase activity is unknown. Stimulation can occur in the absence of cytosolic components (Fig. 4); therefore, a direct effect of H$_2$O$_2$ on the phosphatase is likely. The activities of the catalytic subunits of types 1 and 2A phosphatases are regulated by differential association with regulatory and targeting subunits (5, 29). A possible mechanism for stimulation by H$_2$O$_2$ of the phosphatase is oxidation of sulfhydryl groups involved in maintaining the structure of the holoenzyme. Oxidation could lead to the release of inhibitory subunits known to be associated with phosphatases (29). For a recent discussion of the mechanism of regulation of serine-threonine phosphatase, see Barford (1).

Serine-threonine phosphatase activity of the membrane fraction was also stimulated in cells pretreated with staurosporine (Fig. 5). In contrast to the result with H$_2$O$_2$, incubation of isolated membranes with staurosporine did not result in stimulation of phosphatase activity. Because pretreatment of intact cells is necessary for the stimulation by staurosporine, the effect is indirect and may be exerted on a cytosolic agent regulating the phosphatase. The results support an earlier conclusion that staurosporine stimulates phosphatase activity indirectly by inhibition of a kinase that in turn inhibits the phosphatase (3). The addition of diluted cytosol to isolated membranes did not reconstitute phosphatase stimulation by staurosporine, suggesting that the kinase or other target of staurosporine is too dilute or that the response requires cellular integrity. Similar to the phosphatase activity stimulated by H$_2$O$_2$, the staurosporine-stimulated activity is inhibited by okadaic acid with an IC$_{50}$ consistent with the stimulated phosphatase being PP-1 (Fig. 6). The stimulation of a membrane-associated phosphatase activity when intact cells are treated with staurosporine indicates the physiological relevance of a staurosporine-sensitive kinase in the regulation of a serine-threonine
reactivated K-Cl cotransport (25). Ghosts made from calyculin-treated red blood cells from a study in which PP-1 incorporated into resealed membrane-associated enzyme to okadaic acid (present that it is PP-1 (33), as does the sensitivity of the sensitivity of K-Cl cotransport to okadaic acid suggests cotransport is a membrane-associated enzyme (4). The phosphatase involved in regulation of K-Cl cotransport studied in this work is the one that regulates K-Cl cotransport. There were earlier proposals that the phosphatase involved in regulation of K-Cl cotransport is a membrane-associated enzyme (4). The sensitivity of K-Cl cotransport to okadaic acid suggests that is PP-1 (33), as does the sensitivity of the membrane-associated enzyme to okadaic acid (present study). Other evidence supporting this conclusion comes from a study in which PP-1 incorporated into resealed ghosts made from calyculin-treated red blood cells reactivated K-Cl cotransport (25).

The stimulation of cotransport by both H2O2 and staurosporine was severalfold, whereas the stimulation of membrane-associated phosphatase activity by these agents was 25% or less. The explanation for this is not clear, and there are a number of possibilities. The simplest explanation is that there is more than one phosphatase associated with the membrane, as suggested by the okadaic sensitivity curve, and one of these phosphatases is involved in regulation of K-Cl cotransport and is activated by H2O2 and staurosporine. On the other hand, it is possible that all of the membrane-associated phosphatase dephosphorylates K-Cl cotransporters and some other explanation accounts for the discrepancy noted.

Several enzymes produce H2O2 as a normal by-product of their activity (14), stressing the importance of oxidative processes in cellular functions. Moreover, evidence from plant and animal cells suggests that H2O2 may act as a second messenger (19, 34). For example, in mouse fibroblasts, H2O2 rapidly induces the binding of iron regulatory protein to iron-responsive elements. This induction is blocked by okadaic acid, suggesting the presence of a protein phosphatase in a transduction pathway induced by oxidative stress (31). Furthermore, recent data suggest a role for reactive oxygen species in apoptosis (16, 30, 36) and in cellular aging (27, 36). Our data on stimulation of K-Cl cotransport by treatment with H2O2, which leads to cell shrinkage, is consistent with reports that oxidizing agents can cause red cell dehydration (18). Because aging red blood cells lose water (32), oxidants may participate in cell aging and initiate apoptosis in cells in general by stimulating a membrane-associated phosphatase that in turn activates K-Cl cotransport and the associated water loss.

In summary, this study demonstrates a membrane-associated serine-threonine phosphatase, largely PP-1, in sheep and human red blood cells. This phosphatase is stimulated by H2O2 and staurosporine, two agents that activate K-Cl cotransport in sheep red blood cells. Therefore, the membrane-associated phosphatase activity, or a portion of it, may be the phosphatase that regulates cotransport.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-33640, Fondecyt 1960879 and 1940339 from Conicyt, Chile, and Fundación Andes.

Received 16 June 1997; accepted in final form 31 October 1997.

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