Hypotonicity Stimulates Phosphatidylcholine Hydrolysis and Generates Diacylglycerol in Erythrocytes*

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Exposure of skate erythrocytes to hypotonic medium stimulates a rapid increase in levels of 1,2-diacylglycerol. Other treatments which produce cell swelling such as replacement of a portion of medium NaCl with the permeant solutes ethylene glycol or ammonium chloride also stimulate increases in diacylglycerol. Whereas the reduction of medium osmolarity to 460 mosm (from 940) stimulated a persistent diacylglycerol increase, the increase after reduction to 660 mosm was transient, peaking at 2.5 min and then slowly declining. This decline could be prevented by preincubation with the diacylglycerol kinase inhibitor R59022. To investigate the source of the increased diacylglycerol, the rate of incorporation of [32P]PO4 into each major phospholipid was measured. Reduction of osmolarity to 660 mosm stimulated the incorporation of phosphate into phosphatidylcholine markedly, with a smaller increase observed into phosphatidylinositol. To demonstrate phosphatidylcholine hydrolysis, erythrocytes were prelabeled with [32P]PO4. Subsequent exposure to hypotonic (660 mosm) medium stimulated a decrease in radioactivity in phosphatidylcholine and a large increase in radioactivity in phosphatidic acid. When stimulated in the presence of ethanol, [32P]PO4-labeled phosphatidylethanol was formed, suggesting activation of phospholipase D. In addition, the initial formation of 32PO4-labeled phosphatidic acid was not sensitive to inhibition of diacylglycerol kinase, supporting the role of direct activation of phospholipase D. These results indicate that hypotonicity and the accompanying cell swelling induce cell membrane phospholipid turnover, predominantly phosphatidylcholine, and production of the protein kinase C activator, diacylglycerol, which appears to occur via activation of phospholipase D.

Exposure of little skate (Raja erinacea) erythrocytes to hypotonic medium stimulates influx of water, increasing cell volume followed by a regulatory volume decrease (1-4). The regulatory volume decrease is due to efflux of cellular osmoles, including the potassium and the $\beta$-amino acid taurine, accompanied by cell water. If a portion of medium NaCl is replaced by the permeant solute ethylene glycol, a rapid and persistent volume increase is observed (3). When a portion of medium NaCl is replaced by NH4Cl, cell volume increases, but the increase is not rapid and is of smaller magnitude than with either ethylene glycol-containing medium or hypotonic shock (3). The stimulation of taurine efflux by hypotonicity can be mimicked by the addition of either phorbol ester or the divergent ionophore A23187 in standard (940 mosm) medium (1).

Reduction of osmolarity from 940 to 660 mosm stimulates both the generation of inositol 1-phosphate and the rate of incorporation of exogenous arachidonic acid into 1,2-diacylglycerol, suggesting generation of diacylglycerol (2). Thus a possible mechanism responsible for the stimulation of potassium and taurine efflux may be DAG generation from membrane phospholipids and activation of protein kinase C.

Increased phospholipid metabolism has been demonstrated as the signal transduction mechanism of many neurohumoral agents (5-7) and as a response to hypotonic exposure (8). Not only can membrane phosphatidylinositols be hydrolyzed by stimuli (8-11 as limited examples), but also phosphatidylethanolamine (12) and phosphatidylcholine (13-23). In addition, although not neurohumoral, calcium ionophores and the protein kinase C activators phorbol esters are known to stimulate phosphatidylcholine metabolism in a number of cell systems (19, 20, 22, 23). Both synthetic and degradative processes for phosphatidylcholine can be stimulated (18). Relevant to the generation of diacylglycerol, it has recently been demonstrated that phosphatidylcholine "specific" phospholipase D activity followed by a phosphatidic acid hydrolase, rather than direct activation of phospholipase C activity, appears to be the major pathway for diacylglycerol formation in dimethyl sulfoxide-differentiated HL-60 granulocytes (17, 20) as well as human neutrophils (19, 21, 23) stimulated with chemotactic peptide.

The following study was undertaken to determine whether conditions that induce swelling of skate erythrocytes might stimulate phospholipid turnover and diacylglycerol production. Using a sensitive mass quantitative technique to measure cell DAG with recombinant DAG kinase (24), we determined whether the increased incorporation of $[^{14}C]$arachidonic acid into DAG found previously (2) is accompanied by an increase in concentration of DAG. As a measure of phospholipid turnover, rates of incorporation of phosphate into membrane phospholipid were assayed to determine the phospholipid source of the stimulated levels of DAG. To directly determine whether hydrolysis occurred, cells were prelabeled with [32P]PO4 and decreases in radioactivity in phospholipids deter-

The abbreviations used are: DAG, 1,2-diacylglycerol; EIM, elastomembranch incubation medium; R59022, 6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperadiny1]ethyl]-7-methyl-5H-thiazolo[3,2-alpyrimidin-5-one.

* This work was supported by Grant DCB8801370 (to L. G.) from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Hypotonicity Stimulates Phosphatidylcholine Hydrolysis

Isolation of State Erythrocytes and Measurement of Diacylglycerol—Little skates were caught in Frenchman's Bay, ME or Woods Hole, MA and kept in 15 °C running seawater. Blood was removed from the tail vein into a heparinized syringe. Cells were pelleted (400 × g for 5 min at room temperature) and the plasma and buffy coat removed. Mature, nucleated erythrocytes accounted for greater than 97% of the cells in the final preparation. Of the other cells present, no granulocytes were observed. The other 3% of the cells resembled mammalian lymphocytes. Erythrocytes were resuspended in 5 volumes of Elasmobranch incubation medium (EIM) (composition in millimoles/liter: 300 NaCl; 5.2 KCl; 2.7 MgSO4; 5 CaCl2; 370 urea; 15 Tris, pH 7.4; 940 mosm). After washing, cells were resuspended at 20% hematocrit in EIM and kept at 15 °C. At designated times, cells were diluted 1:10 into urea, osmolalities of 340, 460 mosm (as above except only 200 mM NaCl and 270 mM urea), and 460 mosm (EIM as above except 150 mM NaCl and 170 mM urea). For experiments with ethylene glycol EIM or NH4Cl EIM, 200 mM ethylene glycol or 150 mM NH4Cl replaced the equivalent osmotic strength of NaCl. When appropriate, erythrocytes were pretreated with the DAG kinase inhibitor R59022 for 10 min prior to stimulation. The samples were added and pipetted into 1000 μl of chloroform:methanol:concentrated HCl (100:200:4, by volume). After all samples were taken, 150 μl of 2 M KCl and 150 μl of chloroform were added. Phases were separated by centrifugation (12,500 × g for 3 min) and the organic layer removed and washed with 1 ml of methanol:water:concentrated HCl (100:100:1, by volume). The organic phase was removed and dried at 45 °C in a vacuum oven. The lipid residue was resuspended in 20 μl of a chloroform solution containing: 5 mM calcium chloride and 17.5% (w/v) docetylglucopyranoside. The samples were left to solubilize for 4 h. Diacylglycerol was measured in the samples by modifications of DAG kinase assays (22, 23). Briefly, the lipids were added to the assay mixture (final composition in millimoles/liter: 50 MES, pH 6.8; 5 MgCl2; 1 dithiothreitol; 0.5 ATP (44,000 dpm/nmol [γ-32P]ATP); and 25 units/ml DAG kinase.) Assays were carried out at 25 °C for 60 min. Standard curves of 1,2-diacylglycerol (purified from a mixture of 1,2 and 1,3 isomers by thin layer chromatography and mass quantified by ester analysis (26)) were generated for each experiment. Mass amounts used for standards ranged from 25 to 1000 pmol. Over this range, using the enzyme concentrations above, quantitative conversion of 1,2-diacylglycerol to [32P]phosphatidic acid occurred. The addition of HCl in the extraction procedure did not cause significant acyl migration over the period used (15 min). Samples were always analyzed as rapidly as possible and analysis of DAG performed on the same day. Omitting HCl from the extraction protocol and including NaCl had no effect on diacyl-DAG standards measured by the assay (data not shown). To determine that drying the samples in a vacuum oven did not affect the recovery of DAG due to oxidation, 100 ng of diolyl-DAG and sn-1-stearoyl-2-arachidonoyl-DAG were extracted and DAG measured concurrently. In six separate determinations, the recovery of the unsaturated form of DAG was greater than 96% (96.4 ± 1.6%). Thus, even if oxidation occurs the oxidized form is apparently still measured by the assay.

DAG kinase assays were repeated by the addition of 1000 μl of chloroform:methanol:concentrated HCl and lipids extracted and washed as described above. Initially, samples were dried under N2, resuspended in 25 μl of chloroform:methanol (2:1, v/v), and applied to Silica Gel G thin layer chromatography plates. Plates were developed in a solvent system of chloroform:methanol:acetic acid:H2O (50:20:15:10, by volume). Three radioactive spots were observed. The major spot of radioactivity co-migrated with phosphatidic acid (Rf = 0.58) and smaller amounts of radioactivity in ceramide (Rf = 0.37) and minor third labeled lipid (Rf = 0.25; less than 2% of total radioactivity) which could be lysocephatidic acid. The percentage of labeled ceramide was 15.5 ± 0.8% of the total labeled radioactivity in the lipid extracts of the erythrocytes. The amount of labeled ceramide did not change with stimulation and was subtracted in each case. In subsequent experiments, the chloroform phase of the extracted DAG kinase assay was counted by Cerenkov spectrometry with an efficiency of 38%. In each experiment, aliquots were saved for determination of total membrane lipid phosphorus. Lipid phosphorus was measured by a modification of the method of Ames and Dubin (27). Cellular levels of DAG were expressed as picomoles of DAG/mmol of lipid phosphorus.

Labeling of Cell Phospholipids with [32P]PO4—Erythrocytes were obtained and washed as above. Cells were resuspended at 20% hematocrit in 940 mosm EIM. After thermal equilibration, approximately 5 min, [32P]PO4 was added (10 μCi/μl). Samples (200 μl) were removed and lipids extracted as described above. After 10 min, aliquots were removed and added to either 940 or 660 mosm EIM, and incubated for 10 min. Samples were removed at varying times afterward and lipids extracted. After washing, the organic phase was separated into halves and each dried under N2. The samples were resuspended in chloroform:methanol (2:1, v/v) and applied to Silica Gel G TLC plates. One set of plates was pretreated with 2 mM EDTA and 1% oxalic acid and air-dried overnight. The TLC plates were used only for the separation of polyphosphoinositides (phosphatidylinositol 4-phosphate and 4,5-bisphosphate). These plates were developed in a solvent system of chloroform:methanol:NH4OH:H2O (45:35:6:4.3, by volume). The other set of TLC plates was used to separate phosphatidylethanolamine, serine, inositol, and choline. These plates were developed in a solvent system of chloroform:methanol:acetic acid:H2O (50:30:5:2, by volume). Labeled phospholipids were localized by autoradiography with Kodak XRP film (Rochester, NY). Labeled lipids were scraped from the plates, lipids eluted from a toluene-based scintillation fluid (Budget Solve, RPI, Mt. Prospect, IL), and radioactivity quantified by liquid scintillation spectrometry.

In experiments to determine phospholipid hydrolysis, erythrocytes were resuspended at 20% hematocrit in 940 mosm EIM and 100 μCi/ml [32P]PO4, added after 4 h, one-half were diluted 1:10-fold (to 2% hematocrit) in 940 mosm EIM and the other half diluted to 2% hematocrit in 660 mosm EIM with or without 0.5% (v/v) ethanol. When appropriate, cells were treated with 10 μM R59022, a diacylglycerol kinase inhibitor, 10 min prior to exposure to hypotonicity, and the inhibitor was included in the hypotonic buffer. Samples were removed at varying times after dilution and phospholipids extracted as described above. The phospholipids were analyzed in the TLC solvent system described above for one-half the distance of the plate. The plates were then dried and run in the same direction in a solvent system consisting of the organic phase of a mixture of 2,2,4-trimethylpentane:ethyl acetate:acetic acid:water (110:50:20:100, by volume). This additional development resolved phosphatidic acid from phosphatidylethanolamine. When the samples were analyzed for only labeled phosphatic acid and phosphatidylethanolamine, the TLC plates were developed only in the second solvent system. Labeled lipids were visualized by autoradiography, scraped, and counted as described above.

Materials—[γ-32P]ATP and [32P]PO4 were obtained from Du Pont-New England Nuclear; cardiolipin and 1,2-diacylglycerol from Serday Research Laboratory (London, Ontario); DAG kinase from Lipidex (Madison, WI); octyglycophosphorylase from Calbiochem; R59022 from Janssen Research Foundation (Beerse, Belgium). All solvents and salts were of reagent grade or better and were obtained from either Sigma or Fisher.

RESULTS

DAG Levels in Resting and Osmotically Stimulated Erythrocytes—When placed in a hypotonic medium, skatc erythrocytes rapidly generate DAG (Fig. 1A). DAG elevation occurs in medium reduced to either one-half (460 mosm) or two-thirds (660 mosm). Stimulation of DAG levels is rapid (significant at 40 s) and persistent, lasting over 1 h after stimulation. The maximal elevations stimulated by either change in osmolality are not statistically different; however, the rise stimulated by reducing osmolality to 660 mosm does not persist as long as the elevation stimulated by the further reduction of osmolality to 460 mosm. To determine whether endogenous DAG kinase activity may be responsible for this decline, cells were preincubated with a DAG kinase inhibitor R59022 (28) and then exposed to reduced (660 mosm) osmolar. Pretreatment with the DAG kinase inhibitor poten-
Hypotonicity Stimulates Phosphatidylcholine Hydrolysis

FIG. 1. Time courses of elevation of DAG in skate erythrocytes stimulated by changes in medium osmolarity (A); reduction of osmolarity to 660 mosm in cells pretreated with the DAG kinase inhibitor R59022 (B), and media with NaCl partially replaced by ethylene glycol or NH₄Cl (C). Values are means for six experiments. Standard errors were always less than 10% and generally less than 5% of the mean. *, p < 0.05; +, p < 0.01 compared with paired control (940 mosm) by Student's t test. A, ▲ 660 mosm; □, 460 mosm; △, 990 mosm. B, ▲ 660 mosm/R59022; △, control. C, ▲, ethylene glycol; △, NH₄Cl; □, control.

tiated (to a small degree) the elevation of cell DAG and also prevented subsequent metabolism back to basal levels (Fig. 1B).

To determine whether other media alterations which also cause cell swelling stimulate DAG formation, erythrocytes were transferred to media where a portion of the NaCl was replaced with either ethylene glycol or NH₄Cl. Both of these agents have been shown to stimulate cell swelling in skate erythrocytes, but to differing degrees and with different kinetics (3). Ethylene glycol penetrates the cell rapidly and stimulates a rapid, large increase in cell volume which maintained for at least 1 h. NH₄Cl does not stimulate cell swelling until 5-15 min after exposure to the salt, and swelling occurs more slowly. Exposure of skate erythrocytes to medium with ethylene glycol stimulates a rapid and persistent increase of DAG, whereas the stimulation of DAG by NH₄Cl is slower in onset and smaller in magnitude (Fig. 1C). For the alterations above it appears that the time course of elevation of DAG correlates with the time of onset and speed of cell swelling.

Phospholipid Source of DAG—To determine whether phospholipid turnover may be responsible as a source for the DAG, the rate of incorporation of [32P]PO₄ was measured into all phospholipid classes following transfer of erythrocytes to 660 mosm EIM. As shown in Fig. 2, the rate of incorporation was stimulated in phosphatidylethanolamine and to a smaller degree in phosphatidylinositol. Stimulation of skate erythrocytes stimulates the formation of inositol 1-monophosphate without the generation of inositol 1,4-bisphosphate or inositol 1,4,5-trisphosphate (2), a finding which is supported by lack of stimulation in the rate of 32P04 incorporation into phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. The turnover of phospholipid suggests that phosphatidylcholine and to some extent phosphatidylinositol may be sources of the DAG formed after osmotic stress and cell swelling in these cells.

Hydrolysis of Phosphatidylcholine and Generation of Phosphatic Acid and Phosphatidylethanol—To directly demonstrate that hydrolysis of phosphatidylcholine occurred, we initially attempted to label cells with [3H]choline. Incorporation of the label was minimal over 4 h; therefore, the erythrocytes were prelabeled with [32P]PO₄. After labeling for 4 h at 20% hematocrit in 940 mosm EIM, cells were diluted 10-fold into either 940 or 660 mosm EIM. Samples were removed at varying times, lipids extracted and analyzed for the major phospholipids as well as phosphatidic acid as described under “Experimental Procedures.” Exposure to hypotonicity stimulated a rapid decrease in label in phosphatidylcholine (Fig. 3A). The decrease in phosphatidylcholine was paralleled by

FIG. 2. Time courses of incorporation of [32P]PO₄ into membrane phospholipids following reduction of osmolarity to 660 mosm. Erythrocytes were washed and resuspended at 20% hematocrit in 940 mosm EIM. After thermal equilibration, [32P]PO₄ was added (10 μCi/ml) and incubations continued for an additional 10 min before dilution to 2% hematocrit in either 940 or 660 mosm EIM. Prior to dilution, 20-μl samples were removed; subsequently, 200-μl samples were removed and lipids analyzed as described under “Experimental Procedures.” Data are means of four experiments. Standard errors were always less than 5% of the mean. *, p < 0.05; +, p < 0.01 compared with 940 mosm EIM by paired Student's t test. PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PIP, phosphatidylinositol monophosphate; PIP2, phosphatidylinositol bisphosphate.
an increase in the label in phosphatidic acid (Fig. 3B). The increase in phosphatidic acid accounted for a majority (>90%) of the early decrease of phosphatidylycholine but did not account for the decrease at later times (>5 min), perhaps due to metabolism of the phosphatidic acid.

To determine if phospholipase D was involved in the hydrolysis of phosphatidylycholine, ethanol was included in the incubation reactions. It has been demonstrated by a number of investigators that in the phospholipase D-mediated hydrolysis of phospholipids in the presence of ethanol trans-phosphatidylation can occur, resulting in the formation of phosphatidylethanol (17, 19, 22). Formation of phosphatidylethanol can only occur via this pathway (22) and not via phospholipase C followed by modification of the resulting diacylglycerol. When $^{32}$P-preciliated erythrocytes were exposed to hypotonic medium in the presence of 0.5% ethanol, both $^{32}$P-labeled phosphatidic acid and phosphatidylethanol were formed (Fig. 4). Thus it appears likely that the diacylglycerol formed results from the actions of phospholipase D followed by a phosphohydrolase (21). As further evidence that the labeled phosphatidic acid formed was generated by phospholipase D-mediated hydrolysis, cells were exposed to hypotonicity in the presence of the diacylglycerol kinase inhibitor R59022. If a phospholipase C were involved followed by phosphorylation, then R59022 should greatly decrease the amount of labeled phosphatidic acid formed. Incubation with 10 μM R59022, however, did not significantly alter the amount of labeled phosphatidic acid formed (Fig. 5), suggesting that a phospholipase C/diacylglycerol kinase pathway was not involved.

**DISCUSSION**

A number of changes in the environment of the erythrocyte may stimulate cell swelling and subsequent responses to return back to a preselected, set volume, the regulatory volume decrease (1-4). Skate erythrocytes take up water and swell rapidly after exposure to hypotonicity or to permeant solutes. They react to this change by increased efflux of two major cell osmolytes, potassium and the β-amino acid taurine (1, 4). The mechanism(s) which mediate this stimulation of osmolyte efflux are not completely understood. Within 1 min after exposure to hypotonicity, the formation of inositol 1-monophosphate was stimulated.

**Fig. 3.** Time course of $^{32}$P incorporation into phosphatidylcholine (top) and phosphatidic acid (bottom). Erythrocytes were prelabeled for 4 h in 940 mosm EIM with 100 μCi/ml $^{32}$PO$_4$ at 20% hematocrit. Triplicate 20-μl samples were removed and cells were then diluted to 2% hematocrit with either 940 or 660 mosmol EIM. Triplicate 200-μl samples were removed at varying times and lipids analyzed as described under "Experimental Procedures." Data are means ± S.E. of four experiments. *, p < 0.05; +, p < 0.01; §, p < 0.001 compared with 940 mosmol EIM by analysis of variance.

**Fig. 4.** Time course of $^{32}$PO$_4$-labeled phosphatidylethanol (PEthanol) and phosphatidic acid (PA) formation. Erythrocytes were prelabeled and stimulated as described under "Experimental Procedures" except that 0.5% ethanol (v/v) was included in the final incubation. Duplicate 200-μl samples were removed at varying times and samples analyzed for phosphatidylethanol and phosphatidic acid. Data are means ± S.E. of four experiments. *, p < 0.05; +, p < 0.01; §, p < 0.001 compared with 940 mosmol EIM by analysis of variance.

**Fig. 5.** Time course of generation of phosphatidic acid in the presence and absence of the diacylglycerol kinase inhibitor R59022. Erythrocytes were prelabeled with $^{32}$PO$_4$ as described under "Experimental Procedures" except that 10 μM R59022 was added during the last 10 min. Duplicate 200-μl samples were taken at varying times and analyzed for $^{32}$PO$_4$-labeled phosphatidic acid (PA) as described under "Experimental Procedures." Data are means ± S.E. of four experiments. *, p < 0.05; +, p < 0.01; §, p < 0.001 compared with 940 mosmol EIM by analysis of variance.
phospholipase D is involved, we used the specific phospholipase D-mediated transphosphatidylation reaction. Phosphatidylethanolamine or phosphatidylserine. Thus, phosphatidylcholine appears to be the major source of DAG. This reaction has been best characterized in the phospholipase D-mediated transphosphatidylation reaction. Phosphatidylcholine turnover might mediate the effects of hypotonicity on these cells. To demonstrate that phosphatidylcholine breakdown occurred, cells prelabeled with [32P]Pi were exposed to hypotonicity and a decrease in the radioactivity in phosphatidylcholine was in fact observed (Fig. 3). Although not shown, phosphatidylinositol showed a small (<0.5%) decrease in radioactivity and no changes were observed in phosphatidylethanolamine or phosphatidylycerine. Thus, phosphatidylcholine appears to be the major source of the DAG generated after hypotonic shock.

The generation of DAG from phosphatidylcholine could occur by one of the mechanisms illustrated in the schematic representation (Fig. 1). The latter pathway appears to be the major source of DAG generated in human neutrophils after stimulation with the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (21, 23). Phospholipase D activities have been shown to be stimulated by a variety of neurohumoral and other agents (16, 18-20). The rapid generation of phosphatidic acid in [32P]Pi prelabelled cells (maximal at 1 min) appears to precede the changes in DAG (maximal at 2 min). To demonstrate that phospholipase D is involved, we used the specific phospholipase D-mediated transphosphatidylation reaction. Phospholipase D can mediate the generation of phosphatidylethanol. This reaction has been best characterized in the phospholipase D-mediated breakdown of phosphatidylcholine in neutrophils and HL-60 granulocytes (17, 20-22). Phosphatidylethanol can only be formed by this reaction (23). Thus phosphatidylethanol formation is strong evidence that phospholipase D mediates the hydrolysis of a membrane phospholipid. A rapid increase in phosphatidylethanol was observed in erythrocytes exposed to hypotonicity in the presence of ethanol. Thus it appears that the phosphatidic acid formed is generated directly from phosphatidylcholine by the action of phospholipase D. Subsequently, a phosphohydrolase would convert the phosphatidic acid to diacylglycerol. We have demonstrated that the early rise of phosphatidic acid formed does not result from the action of a diacylglycerol kinase (Fig. 5). Using the diacylglycerol kinase inhibitor R59022 we did not observe significant decrease in the phosphatidic acid formed. This result suggests that phospholipase C activity followed by diacylglycerol kinase does not account for the phosphatidic acid formed. Along with the formation of phosphatidylethanol, the data strongly indicate a role for phospholipase D in DAG formation.

In summary, the present results suggest that cell swelling stimulates DAG formation, probably via phospholipase D which prefers phosphatidylcholine as its substrate. The generation of DAG is required to activate protein kinase C. The activated kinase may have phosphoprotein targets which are the transporters responsible for stimulated efflux of potassium and taurine. Although not fully characterized, these transporters may be: 1) a KCl cotransporter (for K') which is well characterized to mediate the volume decrease in erythrocytes of many species (4, 30, 31) or possibly Na+/H+ or K+/H+ exchangers which are believed to mediate the regulatory volume decrease in *Amphiuma* red blood cells (32); and 2) the anion transporter band III which appears to be responsible for the stimulated efflux of taurine (33). Whether these transport activities are regulated by protein kinase C in skat erythrocytes is currently unknown.

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