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Erythropoietin Stimulates Tyrosine Phosphorylation and Taurine Transport in Skate Erythrocytes

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ABSTRACT Taurine, a β amino acid, is a primary osmolyte in nucleated skate erythrocytes and is involved in the regulation of cell volume. Growth factors may be involved in the regulation of cell volume which occurs during cell division. Erythropoietin (EPO) is the primary growth factor controlling erythropoiesis. To investigate its mechanism of action, we used nucleated skate erythrocytes. EPO stimulates Na^+-independent uptake of taurine in a concentration-dependent manner. The uptake was inhibited by the tyrosine kinase inhibitor genistein. Concomitantly, EPO stimulates tyrosine phosphorylation of a number of proteins, particularly ones of molecular masses 145, 120, 100, 80, 65, and 35 kDa. Using specific antibodies, the 145 kDa protein is identified as phospholipase C γ-1 (PLC γ-1) and the 100 kDa protein as the skate homolog of the anion exchanger band 3. Since PLC γ-1 is activated, turnover of membrane lipids was determined. EPO increased 1,2-diacylglycerol formation from phosphatidylinositols (phosphatidylinositol-4-monophosphate and 4,5-biphosphate) during an early phase and later preferentially from phosphatidylcholine. The early hydrolysis of phosphoinositides was confirmed measuring generation of inositol-1,4,5-trisphosphate, demonstrating an activation of PLC γ-1 activity. To determine if phospholipase D (PLD) stimulation also occurred, ethanol was included in the reactions. Phosphatidylethanol, synthesized by PLD-mediated transphosphatidylation, appeared at times longer than 5 min, suggesting delayed activation of PLD. These results demonstrate that EPO, via simulation of tyrosine phosphorylation, stimulates taurine transport in skate erythrocytes.

Cells in an anisotonic (hypertonic or hypotonic) media attempt to return to their original "set" volume by increasing the rate of uptake or release of solutes with entrained water to or from their environment. The solutes used in these processes generally fall into two classes of compounds: 1) organic osmolytes (amino acids, polyols, and methylamines) and 2) inorganic ions (K and Cl). Recently, Tilly et al. ('93) reported that EGF acting along or in conjunction with hypotonicity activated ion channels via protein tyrosine phosphorylation in the human intestinal 407 cell line. One cannot say for certain that tyrosine phosphorylation is the final event; however, some step in the activation cascade involves tyrosine phosphorylation. Phosphorylation/dephosphorylation activities may alter the activity of membrane transport activities involved in cell volume regulation such as the Na-K-2Cl cotransporter (Pewitt et al., '90; Vigne et al., '94), the erythrocyte KCl cotransporter (Jennings and Schultz, '91), and sodium-hydrogen exchange activity (L'Allemain et al., '91; Sardet et al., '91).

EPO is the primary regulator of growth and differentiation of erythroid progenitor cells (Koury and Bondurant, '88). Therefore, we tested the effects of EPO on the transport of taurine, an organic osmolyte important in cell volume regulation, and on cell signaling processes in the skate erythrocyte. Although EPO is believed to act predomi-

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Abbreviations: BS^3, bis(sulfosuccinimidyl)suberate; DAG, diacylglycerol; EGF, epidermal growth factor; EPO, erythropoietin; H_2DIDS, 4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid; IPB, immunoprecipitation buffer; IP_3, inositol-1,4,5-trisphosphate; MAP, mitogen-activated protein; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PCA, perchloric acid; PEth, phosphatidylethanol; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-monophosphate; PIP_2, phosphatidylinositol-4,5-biphosphate; PLC, phospholipase C; PLD, phospholipase D; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidenedifluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

nately on mature progenitors, the precise state(s) of development at which EPO acts have not been unequivocally identified. The receptor for EPO has been cloned from multiple species and closely resembles many other cytokine receptors (D'Andrea et al., '89; Jones et al., '90). One unique feature which distinguishes this receptor from other growth factor receptors is the lack of endogenous tyrosine kinase activity as predicted by consensus sequences (Bazan, '89). EPO, however, stimulates the tyrosine phosphorylation of a large number of proteins including its own receptor, RasGAP (Torti et al., '92), Raf-1 (Carroll et al., '91), PLC γ-1 (Ren et al., '94), and a number of others (e.g., see Miura et al., '91; Komatsu et al., '92; Damen et al., '92; Yoshimura and Lodish, '92). Thus EPO appears to activate a receptor-associated and/or activated tyrosine kinase).

In the present studies, we demonstrate that EPO stimulates Na^+-independent taurine transport in skate erythrocytes. Na^+-independent taurine transport is known to play an important role in cell volume regulation in skate erythrocytes (Goldstein and Davis, '94). EPO's effect on taurine uptake is inhibited by the tyrosine kinase inhibitor genistein, and EPO stimulates tyrosine phosphorylation of skate erythrocyte proteins including PLC γ-1 and band 3. The activation of PLC γ-1 is rapid and results in hydrolysis of inositol phospholipids, yielding the water soluble IP_3 and 1,2-DAG. EPO-stimulated increase in DAG is derived, however, from both PIs and PC. At later times, preferential hydrolysis of PC occurs through a PLD pathway to yield DAG.

EXPERIMENTAL PROCEDURES

Isolation of skate erythrocytes and Na^+-independent taurine uptake

Little skates (*Raja erinacea*) were caught by trawl nets in Frenchman's Bay, ME, or off Cape Cod, MA. Blood was drawn from a tail vessel into a heparinized syringe, pelleted at 500*g* for 2 min, plasma and buffy coat removed, and washed an additional time in isotonic (940 mosmol/l) elasmobranch incubation medium (940 EIM, composition in mmol/l:300 NaCl; 5.2 KCl; 2.7 $MgSO_4$; 5.0 $CaCl_2$; 15 Tris, pH 7.5; and 470 urea).

Taurine uptake medium contained 1 μCi/ml [^{3}H]-taurine; 200 μl of cells at 20% hematocrit in 940 LiCl-EIM (LiCl replaced NaCl) was added to 800 μl uptake medium (940 LiCl-EIM with or without EPO and/or inhibitors). Li-EIM was used so that taurine uptake via a sodium-dependent system was not activated. In some experiments, hypotonic EIM was used to determine additivity of the effects of EPO and hypotonicity. For these experiments, osmolarity was reduced to 660 mosmol/l (decreasing LiCl to 200 and urea to 250) or 460 mosmol/l (LiCl reduced to 100 and urea to 250). Aliquots of incubation medium (800 μl) were removed at varying times and immediately mixed with 2.4 ml 940 Li-EIM. The cells were pelleted by centrifugation and washed 3× with 940 Li-EIM. The cell pellet was resuspended in 1 ml of 7% PCA, vortexed, and centrifuged; 400 μl of the PCA extract was removed and radioactivity determined in a liquid scintillation counter.

Phosphorylation studies

Erythrocytes were washed and resuspended at 20% hematocrit in 940 EIM. Cells were diluted 1:2 in 940 EIM with or without 50 U/ml EPO. At varying times, cells were pelleted, washed in 940 EIM with 2 mM orthovanadate, and resuspended in 500 μl IPB (concentrations in mmol/l: 20 HEPES, pH 7.4; 150 NaCl; 2 EDTA, 5 Na-pyrophosphate; 1 PMSF; 2 Na-orthovanadate; 2 NaF; 0.5% v/v NP-40; 0.5% v/v Triton X-100; 10 μg/ml each of leupeptin and soybean trypsin inhibitor; and 1 μg/ml each of aprotinin and pepstatin). After 60 min on ice, insoluble material was pelleted by centrifugation (12,000*g* for 10 min at 4°C). Antiphosphotyrosine antibody 4G10 linked to protein A Sepharose was then added (2 μg antibody equivalent per tube) and left to incubate at 4°C overnight, rotating gently. Samples were pelleted, beads washed with IPB, and then proteins eluted with 1× Laemmli sample buffer at 65°C for 20 min. Beads were quickly pelleted and samples were resolved on 10% SDS-PAGE. The resolved proteins were immediately transferred to a PVDF membrane in 1× Towbin's buffer with 15% methanol at 400 mA for at least 4 hr. Membranes were then blocked overnight with 5% w/v non-fat dry milk in PBS with 0.2% v/v NP-40 (5% Blotto). To visualize specific proteins, blots were then incubated with various antibodies: 1) monoclonal 4G10 to determine phosphotyrosine containing proteins (1 μg antibody/ml); 2) mixed monoclonals to PLC γ-1 (2 μg/ml); and 3) polyclonal anti-human band 3 (a generous gift of P. Low, Purdue University) at 1:100 dilution. Blots were incubated with one of these antibodies for 60 min, washed 5× with 5% Blotto and 2× with 1% Blotto (1% w/v milk), and then incubated with horseradish peroxidase-conjugated secondary antibodies (donkey anti-rabbit IgG at 1:2,500 or goat anti-mouse IgG at

1:5,000) in 1% Blotto for 60 min. Blots were then washed with 1% Blotto 5× and 2× in PBS with NP-40. Detection was performed using chemiluminescent system (Renaissance, New England Nuclear, Boston, MA).

Measurement of DAG

Cells were resuspended at 20% hematocrit in 940 EIM and kept at 15°C. At designated times, cells were diluted 1:10 into 940 EIM with or without 50 U/ml EPO, or 460 EIM (EIM as above except 100 mM NaCl and 250 urea). Samples (200 µl) were removed and pipetted into 1 ml of chloroform:methanol:2 M KCl (100:200:4 by volume). After all samples were taken, phases were separated by the addition of 150 µl each chloroform and 2 M KCl, vortexed, and centrifuged (12,000*g* for 3 min). The chloroform phase was removed, washed with 1 ml methanol:water:2 M KCl (100:90:1 by volume), phases were separated by centrifugation, and the chloroform phase was removed and dried at 45°C in a vacuum oven. The lipid residue was resuspended in 20 µl of solubilization solution (5 mM cardiolipin and 7.5% w/v octyl-D-glucoside). Samples were left to solubilize for 6 hr at room temperature. DAG was measured at previously described (Musch and Goldstein, '90). Briefly, lipids were added to the reaction mixture (final concentrations in mmol/l: 50 morpholino-ethane sulfonic acid, pH 6.8; 5 $MgCl_2$; 1 dithiothreitol; 0.5 ATP (44,000 dpm/nmol [γ^{32}P]ATP), and 25 U/ml DAG kinase). Assays were carried out at 22°C for 60 min and reactions terminated by addition of 1 ml chloroform:methanol:2 M KCl. Phases were separated and washed as described above. The chloroform was dried in scintillation vials, 4 ml of scintillation fluid added, and radioactivity determined by liquid scintillation spectroscopy. In each experiment, a standard curve of 1,2-DAG was run. Standards (25–1,000 pmol) were resuspended in 20 µl solubilization buffer and assayed above. We have previously shown using thin layer chromatography (TLC) that approximately 16% of the radioactive products in unstimulated cells is phosphorylated ceramide. This value was determined and subtracted from all of the counts. For all experiments, triplicate aliquots were saved for measurement of lipid phosphorus.

Labeling of cellular phospholipids with [$^{32}PO_4$]

Erythrocytes were washed and resuspended at 20% hematocrit in 940 EIM with 100 µCi/ml [$^{32}PO_4$]. If appropriate, cells were treated for the last 10 min with the DAG kinase inhibitor R59022. After 4 hr, cells were diluted 1:10 into 940 EIM with or without EPO (50 U/ml) and 200 µl samples removed at varying times. In some experiments, 0.5% v/v ethanol was included in the dilution medium to determine the formation of PEth by PLD activity. Samples (200 µl) were removed at varying times and lipids extracted as described. The samples were resuspended in chloroform:methanol (2:1 v/v) and applied to silica gel G TLC plates. One set of plates was dipped in a solution of 2 mM EDTA and 1% oxalic acid and air dried overnight. These TLC plates were only used for the analysis of polyphosphoinositides (PIP and PIP_2). These plates were developed in a solvent system of chloroform:methanol:NH_4OH:water (45:35:6.4:3.6 by volume). The other set of TLC plates was developed for half the distance in a system of chloroform:methanol:acetic acid:water (50:30:8:2 by volume), air dried, and then in the same direction in a system using the organic phase of 2,2,4-trimethylpentane:ethyl acetate:acetic acid:water (110:50:20:100 by volume). This two-solvent system was used so that PA and PEth could be resolved from the major phospholipids. Samples were visualized by autoradiography, scraped, and counted after addition of scintillation fluid.

Measurement of IP_3

Cells were resuspended at 20% hematocrit and kept at 15°C. Cells were diluted 1:10 into 940 EIM with or without EPO (50 U/ml) and 200 µl samples removed at varying times and reactions stopped by addition of 200 µl 14% PCA. Samples were kept on ice 15 min, proteins pelleted at 12,000*g* for 3 min, and the supernatants removed and neutralized to pH 7.0 with 10 N KOH. Aliquots of the samples were assayed for IP_3 using a competitive binding assay. Protein in the PCA-precipitated pellet was measured following solubilization in 1 N NaOH by the bicinchoninic acid procedure.

Oligomerization of band 3

To determine if EPO caused oligomers to band 3 to form, cells were washed and resuspended at 20% hematocrit and diluted 1:2 into 940 EIM with or without 50 U/ml EPO or 460 EIM containing 0.5 µM [^{3}H]-H_2DIDS. Samples (1 ml) were incubated at 15°C for 30 min and plasma membranes isolated (Musch et al., '94b). Peripheral membrane proteins were stripped by incubation with 0.1 N NaOH, neutralized by three washes with 5 mM Na-phosphate, pH 8.0, to return pH to pH 7.5.

Membranes were resuspended in 10 mM HEPES, pH 7.4, with 0.1 mM PMSF, 10 μg/ml each of leupeptin and soybean trypsin inhibitor, and 1 μg/ml each of aprotinin, pepstatin, and tosylphenylchloromethyl ketone. Protein was measured by the bicinchoninic acid procedure; 200 μg of plasma membrane protein was used for crosslinking. The homobifunctional crosslinker BS^3 (5 mM) was added and crosslinking was allowed to proceed for 60 min at room temperature. Reactions were stopped by addition of 0.1 volume 1 M Tris, pH 8.0, and then by addition of 0.5 volume 3× Laemmli sample buffer. Samples were heated to 55°C for 10 min and resolved on 5% SDS-PAGE and gel slices cut. Gel slices were dissolved in scintillation fluid with 4% Soluene and radioactivity was counted.

Materials

Monoclonal antibodies 4G10 and mixed monoclonal anti-PLC γ-1, anti-$p56/53^{lyn}$, anti-$p59^{fyn}$, anti-$p56^{lck}$, anti-$pp60^{src}$, and anti-$p72^{syk}$ antibodies were purchased from UBI (Lake Placid, NY); [^{3}H]-H_2DIDS was from Hospital for Sick Children (Toronto, Ontario, Canada); [γ^{32}P]-ATP, $H_3{}^{32}PO_4$, [^{3}H]-taurine, Reflection film, and Renaissance Western Blot Chemiluminescent Kit were from New England Nuclear (Boston, MA); EPOGEN was a generous gift of AMGEN (Thousand Oaks, CA); orosomucoid was a gift of Dr. Eugene Goldwasser, University of Chicago; protein-A Sepharose was obtained from Pharmacia (Piscataway, NJ); Immobilon PVDF membrane was from Millipore (Medford, MA); horseradish peroxidase-conjugated donkey anti-rabbit IgG and goat anti-mouse IgG were from Jackson Laboratories (Jackson, PA); BS^3 was from Pierce (Rockford, IL); DAG kinase assay kit, IP_3 assay kit, and NP-40 were from Amersham (Arlington Heights, IL; Cleveland, OH); silica gel G plates were from Brinkman (Westbury, NY); octyl-D-glucoside was from Novabiochem (La Jolla, CA); cardiolipin, 1,2-DAG and phospholipids were from Avanti Polar Lipids (Birmingham, AL); and R59022 was from Janssen Life Sciences (Belgium). All other reagents were of the highest grade available and purchased from GIBCO/BRL (Gaithersburg, MD), Fisher (Springfield, NJ), or Sigma (St. Louis, MO).

RESULTS

EPO stimulates taurine uptake via a genistein-inhibitable pathway

Under isoosmotic conditions, Na^+-independent taurine uptake into skate erythrocytes averages 0.66 nmol/10 min/g•RBC (Fig. 1A). When the cells are stimulated with EPO under isoosmotic conditions, taurine uptake increases approximately 60% (Fig. 1A). The effect of EPO is concentration dependent with stimulation observed at concentrations as low as 1 U/ml and the effect is maximal at 25 U/ml (Fig. 1B). EPO stimulation of taurine uptake is inhibited by pretreatment (10 min) with the tyrosine kinase inhibitor genistein (50 μM; Fig. 1C). Pretreatment with genistein does not inhibit a comparable stimulation of uptake by medium made hypotonic to 660 mosmol/l (Fig. 1C). The effect of EPO is additive to that of hypotonicity. At all osmolarities tested (940, 660, 460), EPO enhanced taurine uptake (data not shown).

The EPO preparation used in these experiments (EPOGEN, AMGEN) contains 150 mM Na^+. Although this results in a concentration of Na^+ in the incubation medium of only about 2 mM (vs. Na^+ $k_{1/2}$ for activating uptake of 80 mM), we tested the effects of EPO in the total absence of Na^+ by using an EPO preparation dialyzed (against HEPES-buffered LiCl) free of Na^+. The dialyzed preparation stimulated taurine uptake similar to the undialyzed preparation (data not shown). To determine whether the effect of EPO was specific, another heavily glycosylated protein, orosomucoid (alpha-1 acid glycoprotein), was tested. The carbohydrate portion of both EPO and orosomucoid is approximately 35% and the composition is quite similar. Using equivalent amounts of EPO and orosomucoid (1.25 μg/ml, which is equivalent in mass to 50 U/ml EPO), stimulation of taurine uptake was readily observed with EPO but not orosomucoid, suggesting that the effect on taurine uptake was specific. Two other important growth factors were also tested. Neither EGF nor insulin-like growth factor II had any effect on taurine uptake (data not shown).

Effect of EPO on tyrosine phosphorylation

Since the effect of EPO on taurine uptake was inhibited by genistein, we tested whether protein tyrosine phosphorylation occurred after EPO stimulation. To determine tyrosine phosphorylation, cells were stimulated with EPO (50 U/ml) and patterns of phosphotyrosine containing proteins determined following immunoprecipitation using a specific antiphosphotyrosine monoclonal antibody (4G10). Under control conditions, a number of proteins had phosphotyrosines, most notably proteins between 80 and 150 kDa (Fig. 2). Upon stimulation with EPO, the tyrosine phosphorylation of these proteins, as well as other pro-

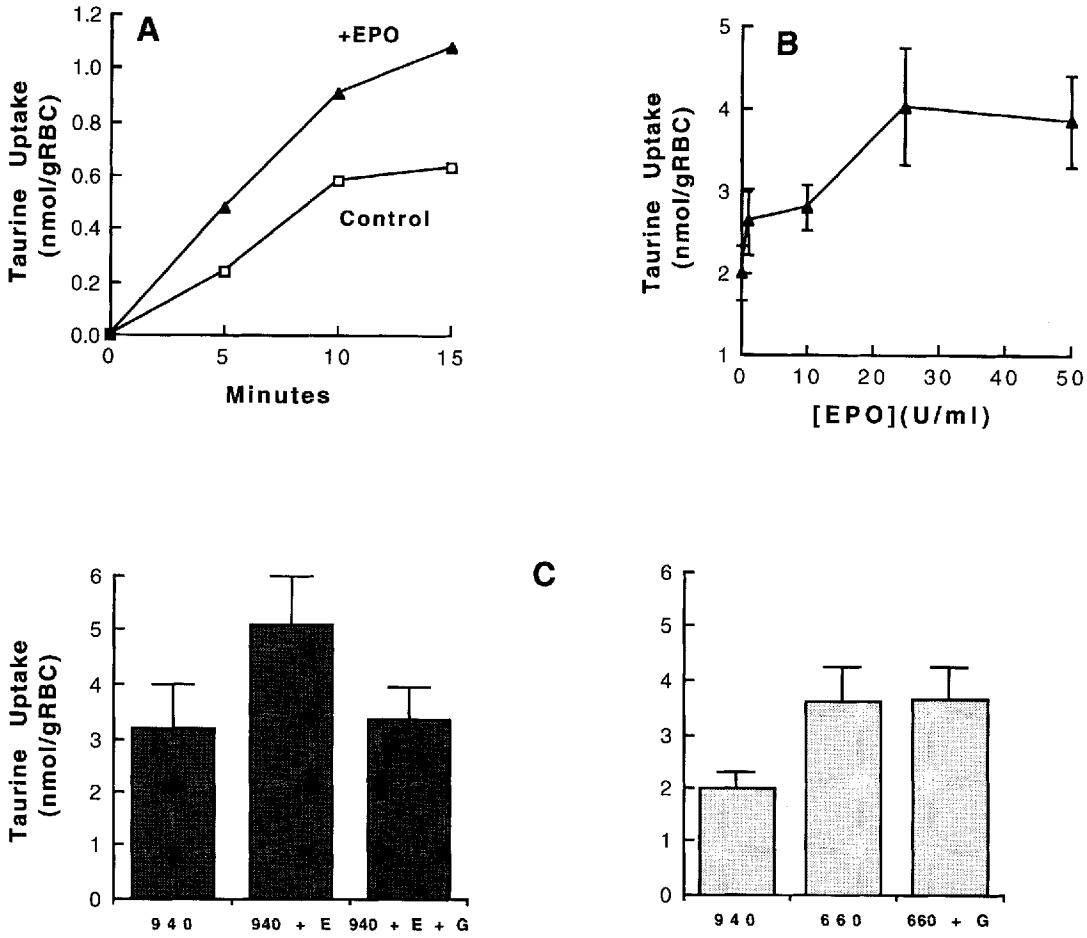

Fig. 1. **A**: Effect of EPO (50 U/ml) on Na^+-independent taurine uptake. Erythrocytes were washed, resuspended in 940 Li-EIM (940 mosmol/l Li-substituted elasmobranch incubation medium), and then uptake was measured in the presence and absence of EPO. Uptake was terminated at 15 min as described in Experimental Procedures. Data are means ± SE for three experiments. **B**: Concentration dependence of EPO effect on taurine uptake. Taurine uptakes were performed as described in Experimental Procedures. Uptakes were measured over 15 min. Values are means ± SE for three experiments. **C**: Inhibition of EPO (50 U/ml) stimulated taurine uptake by genistein. Cells were pretreated with 50 μM genistein (G), tyrosine kinase inhibitor, for 10 min prior to measuring taurine uptake in 940 Li-EIM. Uptakes were measured over the next 15 min in either 940 Li-EIM ± EPO (E) or EPO ± genistein, or 660 Li-EIM ± genistein.

teins of different molecular weights, increased as determined by antiphosphotyrosine immunoprecipitation. The most predominant proteins whose levels increased were the 150, 120, 100, and 45 kDa, whereas proteins which did not appear to have phosphotyrosine prior to stimulation appeared at molecular weights of 80, 65, and 32. With longer exposures of the Western blots, additional phosphoproteins, which demonstrated smaller changes in tyrosine phosphorylation could be observed. Stimulation of protein tyrosine phosphorylation was a rapid event; some proteins increased maximally by 1–2 min (150 and 45 kDa proteins), whereas others (100 and 32 kDa proteins) increased less rapidly. The fold stimulations of the proteins varied. As representatives, the range of stimulation for the 150 kDa protein was 15–28 (mean of 21 ± 10 for 3 experiments) and for the 100 kDa protein the range was 3–10 (mean of 6 ± 2.5 for 3 experiments).

To determine the possible identity of these proteins, some of the proteins known to be tyrosine phosphorylated after EPO were studied using specific antibodies. Since the PLC γ-1 has been shown to be heavily tyrosine phosphorylated in EPO-dependent UT7A cells (Ren et al., '94), we determined whether the amount of PLC γ-1 increased in the phosphotyrosine immunoprecipitates of skate erythrocytes using a set of mixed monoclonal anti-PLC γ-1 antibodies to probe the Western

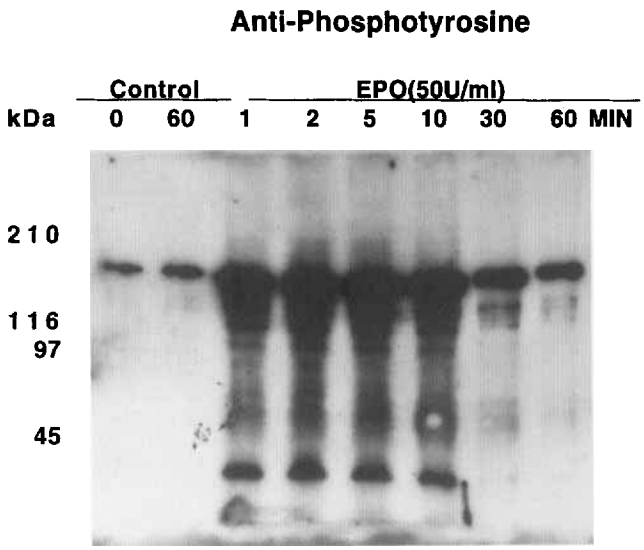

Fig. 2. Western blot of phosphotyrosine containing proteins stimulated by EPO (50 U/ml). Cells were washed, resuspended at 20% hematocrit, and diluted 1:2 in 940 EIM with or without EPO at varying times. Immunoprecipitations, gel analysis, and visualization were performed as described in Experimental Procedures. Image shown is representative of those performed on three different skates.

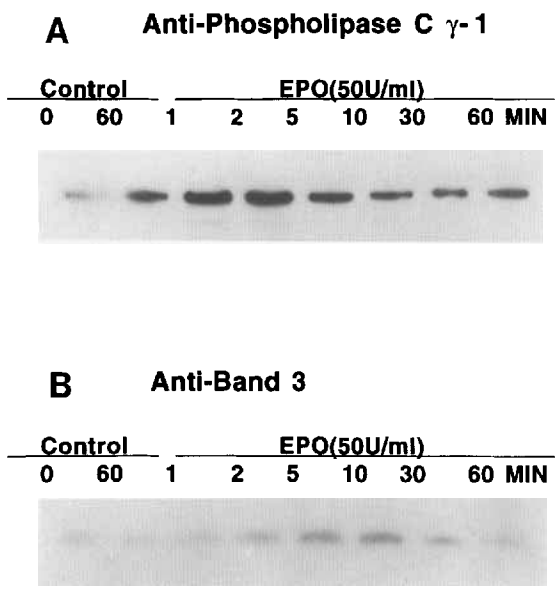

Fig. 3. Effect of EPO on tyrosine phosphorylation of PLC γ-1 (**A**) and band 3 (**B**). Samples were isolated and immunoprecipitated as described in Experimental Procedures. Immunoprecipitation was performed using 4G10 antiphosphotyrosine antibody. Blots were developed with (A) mixed monoclonal anti-PLC γ-1 and (B) anti-human band 3 polyclonal serum. Images shown are representative of results obtained from three different skates.

blot. As shown in Figure 3A, using blots generated following immunoprecipitation with 4G10 antiphosphotyrosine, a major phosphoprotein could be identified with the anti-PLC γ-1 antibodies. Using this method, the levels were found to increase 15 ± 6-fold for 3 experiments. Similarly, the identity of the phosphoprotein near 100 kDa was confirmed used a specific polyclonal antiserum against human band 3 (Fig. 3B). Using the anti-band 3 antiserum, the fold increase over control at the maximal time point (5 min) was 5 ± 2.

We have attempted to use a number of specific tyrosine kinase antibodies to find which kinases are activated during EPO stimulation. Using specific antibodies, we have found kinases homologous to $p72^{syk}$, $pp60^{src}$, and $p56^{lck}$ (but no protein reactive with $p59^{fyn}$ or $p56/53^{lyn}$) in skate erythrocytes (Musch et al., unpublished results). The three kinases have slightly different molecular weights which may relate to species differences. However, using an immune complex assay their activities do not appear to change after EPO.

Hydrolysis of PI and PC

Since EPO stimulated the tyrosine phosphorylation of PLC γ-1, we determined whether cell DAG levels would increase as well. Additionally, since we have previously demonstrated that volume expansion stimulates PLD activity in skate erythrocytes, we determined whether EPO might stimulate PLD. Cells were stimulated with EPO under isoosmotic conditions and lipids extracted for mass measurements of DAG using recombinant DAG kinase. As shown in Figure 4, EPO (50 U/ml) caused a rapid and prolonged elevation of DAG. To compare these DAG levels with those induced by volume expansion due to hypotonic stress, cells were exposed to 460 EIM and a sample taken at a previously determined maximal time point (Musch and Goldstein, '90). Hypotonic exposure caused a greater elevation (compared to EPO stimulation) in DAG. At 2 min the levels reached 1.77 ± 0.14 pmol/nmol cell phospholipid (compared with the zero time control of 1.13 ± 0.08).

Since DAG can be generated from multiple sources, we looked for the phospholipid pool from which the DAG was derived. Cells were labeled with [$^{32}PO_4$] and then stimulated with EPO (50 U/ml). Although we analyzed all of the major cell phospholipids, only those for which we observed changes are shown (Fig. 5). Early decreases could be noted in the polyphosphoinositides (PIP and PIP_2) and a smaller (not statistically significant) change was observed in PI itself (Fig. 5). These

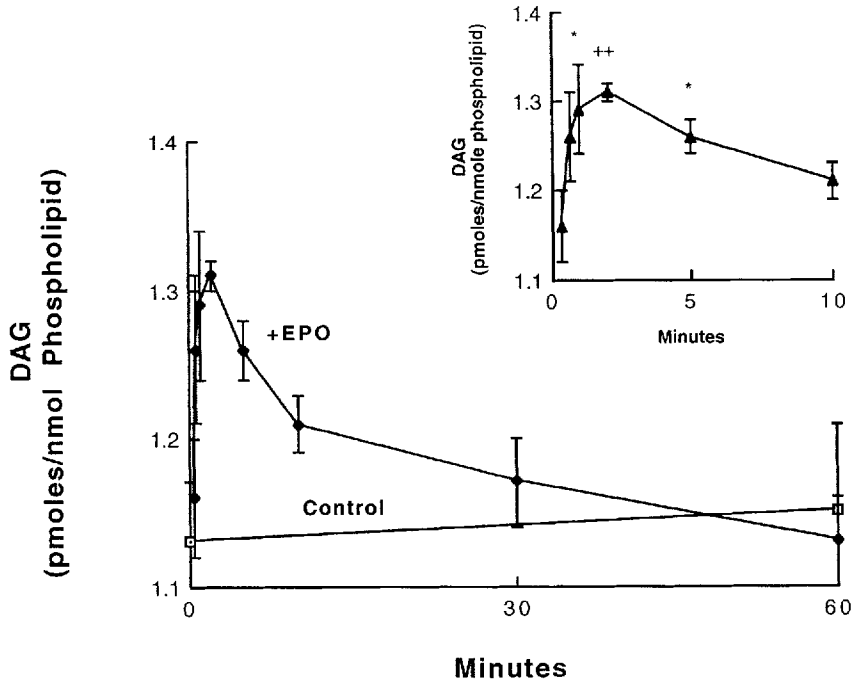

Fig. 4. Effect of EPO on DAG content. Cells were washed and resuspended at 20% hematocrit. Cells were diluted 1:10 into 940 EIM with or without EPO. Samples were removed at varying times and stopped. Lipids were extracted and DAG measured as described in Experimental Procedures. Values are means ± SE for three experiments, each point performed in triplicate in each experiment. $^{*}P < 0.05$; $^{++}P < 0.001$ compared with zero time control by ANOVA.

changes strongly support an activation of PLC γ-1. After the first 5 min the inositol phosphate levels were no different from controls. However, significant decreases were observed in PC, suggesting a possible activation of PLD.

As additional evidence for PLD activation, we investigated the formation of labeled PA in these cells. To determine whether a PLC γ-1 or D was involved, we used the DAG kinase inhibitor R59022. This agent should only inhibit PA formed from PLC γ-1, but not from D activation. As shown in Figure 6A, PA rose rapidly after EPO. Whereas the early increase in PA was inhibited by R59022, the latter phase was less so, supporting an early activation of PLC γ-1 followed by activation of PLD. To unequivocally demonstrate activation of PLD, ethanol was included to promote the formation of PEth by transphosphatidylation by PLD. As shown in Figure 6B, a delayed increase in [^{32}P]-labeled PEth occurred, demonstrating the activation of PLD.

As further support for the sequential activation of these phospholipases, we measured the formation of IP_3 which occurs solely from PLC γ-1 activation. As shown in Figure 7, IP_3 levels increased rapidly after EPO, the maximal increase occurring after 2 min. The cells were not treated with lithium to inhibit phosphodiesterase activity, possibly accounting for the transient nature of the response. These results, along with those demonstrating formation of PEth, strongly support the sequential activation of PLC γ-1 and then D.

Oligomerization of band 3

We have recently shown that hypotonic volume expansion of skate erythrocytes stimulates the formation of dimeric and tetrameric forms of the anion exchanger protein band 3 (Musch et al., '94a). To test whether EPO also causes oligomerization, band 3 was labeled with the irreversible binding agent [^{3}H]-H_2DIDS in isoosmotic medium with or without EPO (50 U/ml). As a control, an aliquot of cells was labeled in hypotonic medium which stimulates an increase in H_2DIDS binding and which promotes oligmerization. To observe the formation of oligomeric forms, plasma membranes were isolated and proteins crosslinked using the homobifunctional crosslinker BS^3. As shown in

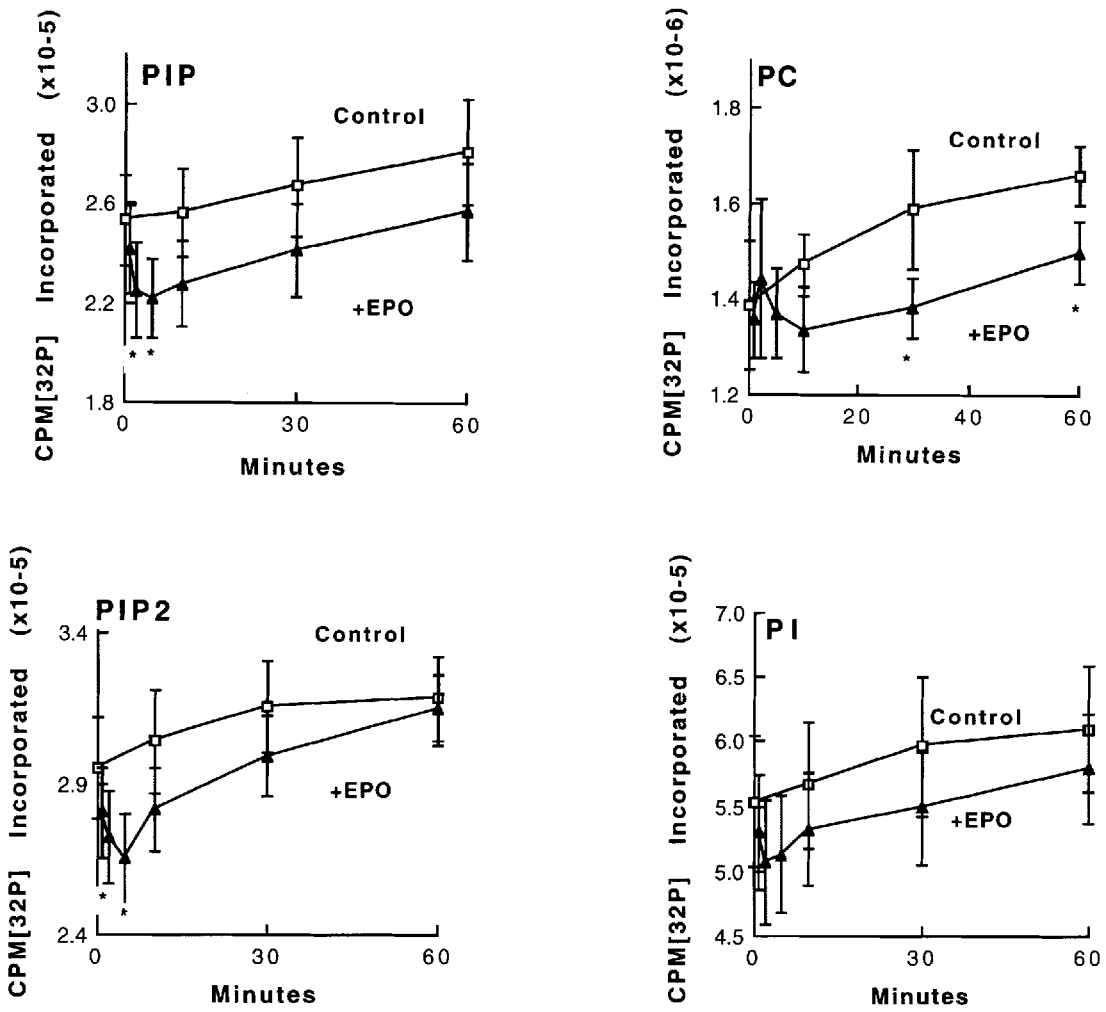

Fig. 5. Hydrolysis of [^{32}P]-labeled phospholipids stimulated by EPO. Cells were prelabeled for 4 hr with [^{32}P]-orthophosphate in 940 EIM and diluted 1:10 into EIM with or without EPO (50 U/ml). Samples were taken at varying times, stopped, and lipids extracted as described in Experimental Procedures. Samples were analyzed by TLC as described, lipids visualized by autoradiography, and major phospholipids scraped off and radioactivity quantified. Values are means ± SE for three experiments, each point done in duplicate in each experiment. *$P < 0.05$ compared with zero time control by ANOVA.

Figure 8, hypotonic exposure causes an increase in [^{3}H]-H_2DIDS binding and a shift in distribution toward oligomeric forms. EPO caused a small increase in binding which was not significant and only a small shift toward oligmeric forms was observed.

DISCUSSION

In the present studies we demonstrate that the erythroid growth factor EPO stimulates Na^+-independent taurine uptake in skate erythrocytes. This effect is inhibited by the tyrosine kinase inhibitor genistein. In addition, EPO stimulates tyrosine phosphorylation on a number of proteins, predominantly ones of molecular masses 150, 120, 100, 80, 45, and 32 kDa. The 150 kDa protein was identified as PLC γ-1 using specific monoclonal antibodies. A tyrosine phosphoprotein near 100 kDa was determined to be skate band 3 using a specific anti-human band 3 polyclonal serum. EPO stimulated the generation of DAG from membrane polyphosphoinositides as well as from PC. Direct evidence for stimulation of PLC γ-1 and D was obtained by measuring the stimulation of cell IP_3 levels and the generation of PEth. Differences in the mechanism of stimulation of taurine transport by hypotonic volume expansion and EPO were the effect of genistein on the former and

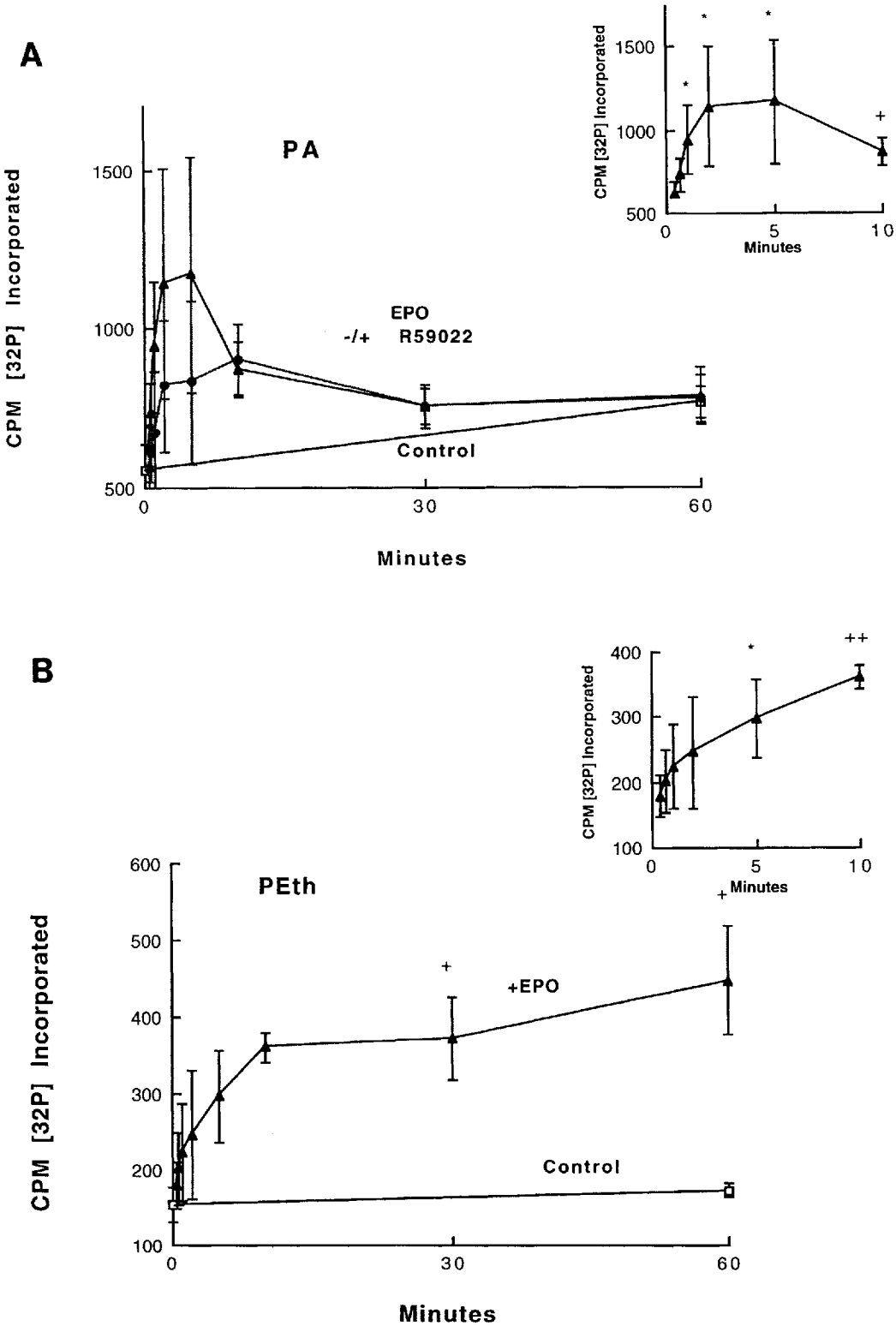

Fig. 6. Formation of [^{32}P]-labeled PA (**A**) in the presence and absence of the DAG kinase inhibitor R59022 and the formation of [^{32}P]-labeled PEth (**B**). Cells were labeled for 4 hr with [$^{32}PO_4$] and if appropriate, treated for the last 10 min with 10 μM R59022. Cells were diluted 1:10 into medium with or without EPO. Ethanol (0.5% v/v) was included to measure formation of PEth. Lipids were analyzed by TLC, and PA and PEth localized by autoradiography, scaped from the plates, and counted. Values are means ± SE for three experiments, each point done in duplicate in each experiment. $^{*}P < 0.05$; $^{+}P < 0.01$, $^{++}P < 0.001$ compared with zero time control by ANOVA.

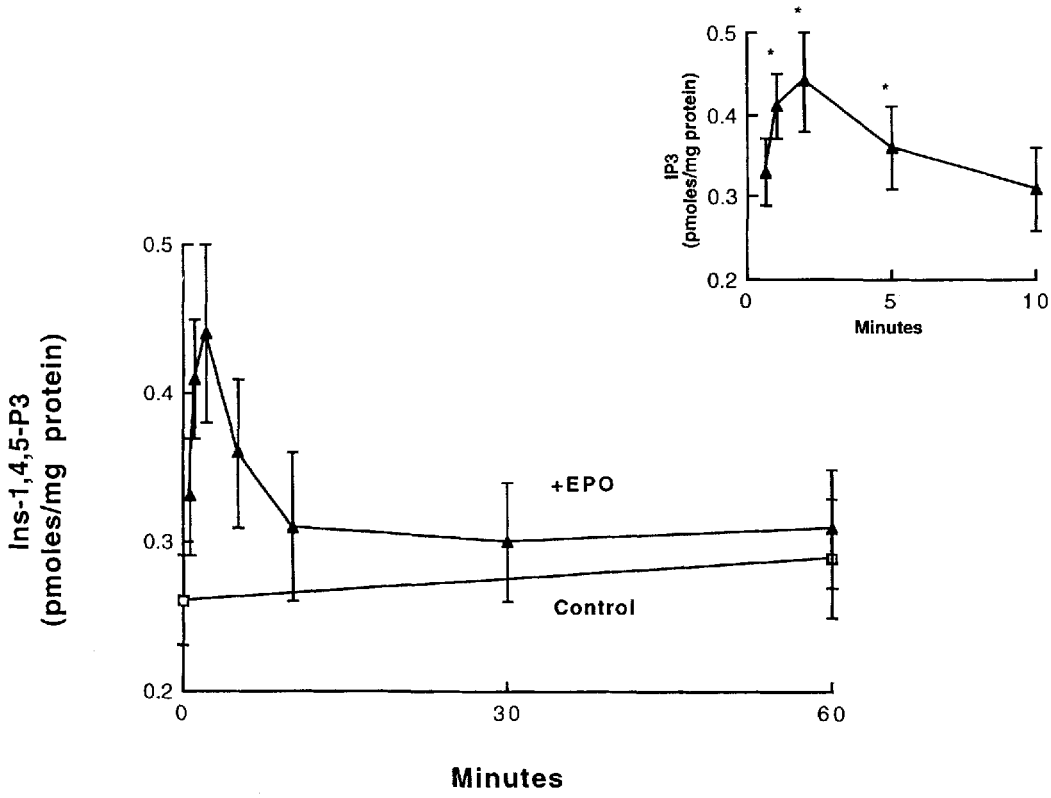

Fig. 7. Stimulation of IP_3 formation by EPO. Cells at 20% hematocrit were diluted 1:10 into 940 EIM with or without EPO (50 U/ml). Samples were removed at varying times and stopped by addition of PCA. IP_3 was measured in the supernatant by a specific competitive protein binding assay. Values are means ± SE for three experiments, each point done in duplicate in each experiment. $^*P < 0.05$ compared with zero time control by ANOVA.

small effect of EPO on oligomerization of band 3 compared to hypotonicity. Thus multiple mechanisms may be involved in the regulation of taurine transport by skate cells.

Our results are similar to those of Tilly et al., ('93) investigating the efflux of Cl and K which often occurs in mammalian cells during the regulatory volume decrease after volume expansion. Using the INT407 cells they observed that tyrosine phosphorylation played a pivotal role in the regulation of ion (K and Cl) channel activity. These investigators found increased phosphorylation of major phosphoproteins of 125, 75, and 70 kDa, and a number of minor phosphoproteins increased as well during hypotonic stress. In this cell line, the growth factor and well-characterized tyrosine kinase activator EGF also stimulated efflux of K and Cl, but only in the presence of the phosphatase inhibitor vanadate. Without vanadate present we observed the effect of EPO and therefore chose not to confound our results with this additional agent. It should be noted that vanadate by itself did not stimulate taurine uptake in skate erythrocytes, but it did potentiate the effect of EPO (data not shown).

The number of kinases which could be involved in EPO stimulation of the cells is large. Erythrocytes contain large numbers of tyrosine kinases and which one(s) are involved as second messengers that stimulate taurine transport is (are) unknown. Additionally, EPO generates DAG which would activate protein kinase C, a known activator of taurine transport (Leite and Goldstein, '87). It is also possible that other serine/threonine kinases may also play a role in the regulation of taurine transport. In the INT407 cells, EGF stimulated MAP kinase activity (as well as a number of other kinases) (Tilley et al., '93). EPO has been shown to stimulate one of the MAP kinases (Miura et al., '91), and a number of MAP-like kinases (jnk and hog) have been determined to be involved in osmosensing (Maeda, '94; Galcheva-Gargova et al., '94; Han et al., '94). Whether these are present in elasmobranchs and how they are regulated are unknown.

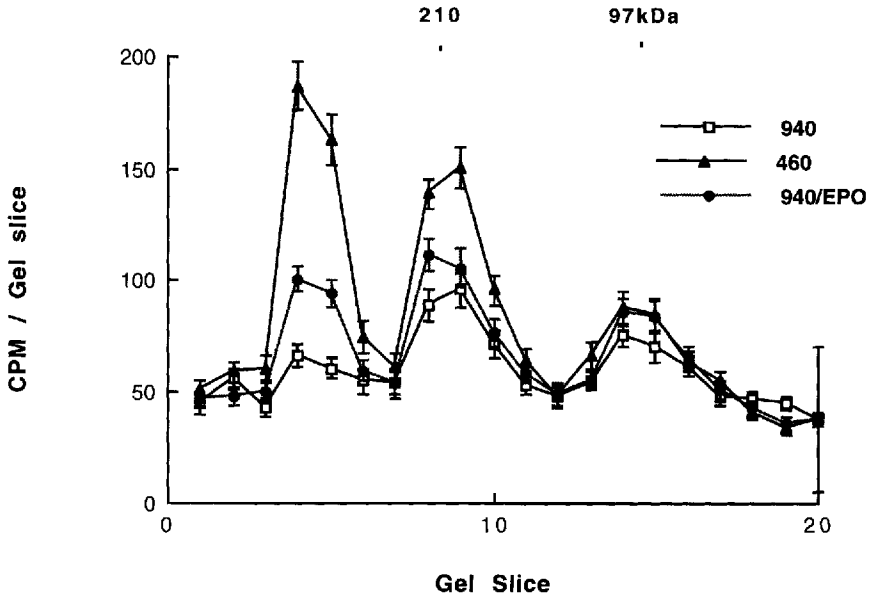

Fig. 8. Effect of EPO on band 3 oligomerization. Erythrocytes were incubated with 0.5 μM [^{3}H]-H_2DIDS for 30 min in 940 EIM with or without EPO and 460 EIM. Plasma membranes were isolated and reacted with the crosslinker BS^3. Samples were resolved on SDS-PAGE, gel slices cut, and radioactivity quantified. Values are means ± SE for three experiments.

In conclusion, under isoosmotic conditions, EPO stimulates taurine transport in skate erythrocytes via its stimulation of tyrosine kinase activities. EPO stimulates PLC γ-1 activity markedly, resulting in the generation of IP_3 and DAG. The DAG may be important in the effects of EPO by activating protein kinase C, a known modulator of taurine transport in these cells. EPO also stimulates a delayed increase in PLD activity which may be useful to prolong its effect. The precise mechanism of EPO stimulation will require a great deal of additional work.

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