HYPOTONICITY STIMULATES DIACYLGLYCEROL FORMATION VIA PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLINOSITOL HYDROLYSIS IN SKATE (RAJA ERINACEA) ERYTHROCYTES

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Treatment of skate erythrocytes with phorbol ester, a specific activator of protein kinase C, stimulates taurine efflux in isotonic (940mosmol/L) Elasmobranch Ringer solution (Leite and Goldstein, J.Exp.Zool. 242:95, 1987). Taurine efflux can also be stimulated by the divalent ionophore A23187, reduction of medium osmolarity (to 660 or 460 mosmol/L), or replacement of medium NaCl by the permeant solutes NH_4Cl or ethylene glycol. The mechanism(s) of action of hypotonic shock or the substituted media is unknown.

Since phorbol esters are structural analogs of 1,2-diacylglycerols (DAG) and are thought to act by mimicking DAG, treatments which increase DAG levels may stimulate taurine efflux. Indeed, an early event in response of skate RBC to either hypotonicity or ethylene glycol medium is increased incorporation of [^{14}C]-arachidonic acid into DAG, which is an indirect measure of increased cell DAG turnover and content (mass). Hypotonicity also stimulates the release of inositol-1-monophosphate (but not inositol-1,4,5-trisphosphate; McConnell and Goldstein, Am.J.Physiol. 255:R982, 1988), suggesting that hypotonicity does not stimulate the phospholipase C which specifically hydrolyzes polyphosphoinositides such as phosphatidylinositol diphosphate. DAG may be formed, however, by phospholipase C hydrolysis of other phospholipids and has been demonstrated to be generated from phosphatidyl choline and ethanolamine.

In this study, skate erythrocyte DAG mass levels were measured to confirm that incorporation of [^{14}C]-arachidonate correlated with increased DAG levels. A specific and sensitive assay using recombinant E. coli DAG kinase was used. When assayed with necessary cofactors and gamma [^{32}P]-ATP, cell DAG is quantitatively converted to [^{32}P]-phosphatidic acid. Only minor interferences are observed with other phosphorylated lipids, e.g. ceramides. To measure DAG levels after treatment of the RBC with the stimuli described above, skate erythrocytes were washed and resuspended at 20% hematocrit in 940mosmol/L medium at 15°C. After thermal equilibration (5 min) samples were diluted to 2% crit into media of varying osmolarities (940, 660, and 460 mosmol/L) or into substituted media (NH_4Cl or ethylene glycol substituted for 200mM NaCl) and at varying times samples were removed and reactions stopped by addition of chloroform:methanol:HCl (100:200:4 by vol). Lipids were extracted, washed and resuspended in detergent containing buffer for DAG kinase assay as described by Preiss et al., J.Biol.Chem. 261:8597, 1986. Aliquots were also analyzed for total cellular phospholipid.

Decreasing medium osmolarity to 460 mosmol/L stimulated a rapid (less that 1 min) and sustained elevation of DAG. DAG was maximally elevated (17% over paired control) at 2.5 min and remained elevated for 60 min. Reduction of medium osmolarity to only 660 mosmol/L stimulated a transient increase in DAG levels. Maximal stimulation was observed at 2.5 min. when DAG was increased 17% over paired control. The DAG level then decreased, the elevation being only 11% at 60 min.

To determine whether endogenous erythrocyte DAG kinase activity was important in regulating stimulated DAG levels, erythrocytes were pretreated

for 10 min with the specific DAG kinase inhibitor R59022. Treatment with R59022 resulted in a more rapid and larger elevation of DAG levels that was sustained for 1 hr. At 2.5 min, DAG was elevated 36% and remained elevated for 60 min following hypotonic treatment. Thus cellular DAG kinase activity may play a role in the regulation of DAG levels after hypotonic stimulation.

Replacement of medium NaCl with NH_4Cl stimulated only a 5% increase in DAG levels at 2.5 min, which correlates with the low taurine efflux induced by NH_4Cl. However the DAG levels rose with time to a maximal increase of 21% at 30 min. Ethylene glycol replacement stimulated a rapid and persistent increase in DAG. DAG increased 19% at 20 sec and 33% at 30 min.

To investigate the phospholipid source of DAG, turnover of membrane phospholipids was measured by rates of incorporation of [^{32}P] after stimulation with 660 mosmol/L medium. Of the major phospholipids, phosphatidyl choline showed the largest increase. Incorporation was stimulated 45% by 10 min and increased 64% over control at 60 min after stimulation. Of the other major phospholipids, only phosphatidyl inositol showed a significant increase, the stimulation over control being 23% at 60 min.

The above studies suggest that stimuli which cause cell swelling stimulate turnover of membrane phospholipids, in particular PC and PI. DAG is formed which may stimulate protein kinase C. Phosphorylation of a membrane transporter for taurine by this kinase may increase taurine efflux, a critical event in the regulatory volume decrease which enables the cells to return to a normal volume.

Supported by NSF grant DCB-0883170