DETERMINATION OF THE STOICHIOMETRY OF STIMULATORY PHOSPHORYLATION ON THE SHARK (SQUALUS ACANTHIAS) RECTAL GLAND Na-K-Cl COTRANSPORTER.

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The activation of the shark rectal gland Na-K-Cl cotransporter is tightly correlated with its phosphorylation state (Lytle and Forbush. J. Biol. Chem. 267:25438, 1992). In order to examine this relationship further, we establish here the stoichiometry of phosphate incorporation per Na-K-Cl cotransport protein under conditions that should maximally activate the cotransporter.

A suspension of shark rectal gland tubules was incubated with $^{32}P_i$ for 40 minutes to incorporate ^{32}P into the cellular ATP pool. Under resting conditions there is minimal activation of cotransporter and minimal incorporation of ^{32}P into Na-K-Cl cotransport protein (*ibid.*). The tubules were then exposed to forskolin and calyculin A for 15 min, a combination that maximally activates the cotransporter and results in maximal cotransporter phosphorylation. The majority of the tubules were solubilized with Triton X-100 in the presence of phosphatase inhibitors. A fraction of the tubules was extracted with cold 5% TCA to produce a protein-free cell extract. Na-K-Cl cotransport protein was immunopurified from the solubilized preparation using J4 antibody (*ibid.*). The TCA extracts were used for cellular [γ-^{32}P]ATP analysis.

To determine specific activity of ^{32}P in the γ position of ATP, total ATP in the TCA extract was measured using a luciferin-luciferase assay (Sigma) and the amount of γ-^{32}P in that ATP was determined as follows: Aliquots of the TCA samples were treated with hexokinase, an enzyme that transfers the γ $^{32}PO_4$ of ATP to glucose. Control and treated samples were subjected to thin layer chromatography to resolve ATP and ADP from other labeled nucleotides (See Fig. 1). These spots were quantified by phosphor image analysis. The total ^{32}P incorporation into ATP was determined by dividing pmole ATP loaded on the plate by the counts calculated from the counts in the $ATP_{control}$ spot. The fraction of label in the γ position of ATP was calculated from the change in ATP and ADP spots after hexokinase treatment.

To determine the specific activity of $^{32}PO_4$ incorporation into the cotransporter protein, slices of gels containing immunopurified cotransporter were counted for Cerenkov radiation and assayed for protein content by total amino acid analysis. Based on results from 3 independent experiments, we determined a stoichiometry of 2.4$\pm$ 0.6 (SEM) mol PO_4 / mol Na-K-Cl cotransporter protein.

The finding that multiple phosphates are incorporated per cotransporter molecule during activation of transport is consistent with our previous identification of three threonine

phosphoacceptors (T184, T189, T1114; *op cit.* Lytle and Forbush; Behnke and Forbush. Bull. MDIBL. 36:44, 1997) and evidence for at least one serine phosphoacceptor (*op cit.* Lytle and Forbush). Our current stoichiometry determination is a lower limit on the level of maximal phosphorylation, due to the possibility of incomplete phosphorylation at the phosphoacceptor sites during the stimulation period and the possibility that some ^{32}P loss occurred during sample preparation.

Importantly, a stoichiometric relationship greater than 1.0 is permissive for the hypothesis that phosphorylation is a required for Na-K-Cl cotransporter activation. In combination with previous work demonstrating a correlation of activation with phosphorylation as well as appropriate effects of phosphatase and kinase inhibitors *(op cit.*, Lytle and Forbush), this result provides strong evidence that phosphorylation is the mechanism for Na-K-Cl cotransporter activation in the shark rectal gland.

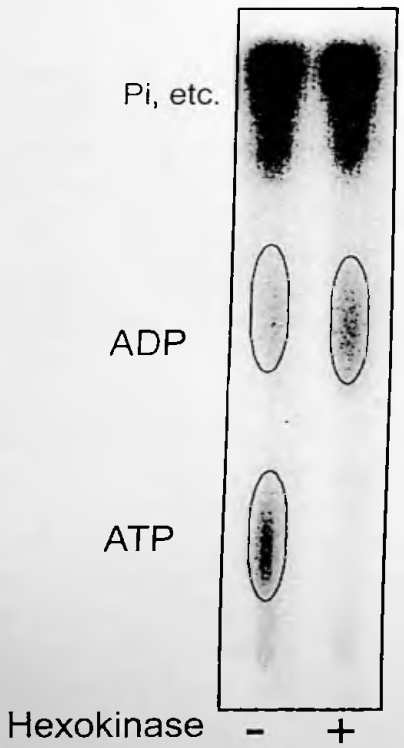

Figure 1: *Quantification of label incorporation into ATP from cellular extracts.* Cellular extracts, treated or not treated with hexokinase, were spotted onto PEI cellulose and run for 45 min in 0.43M KH_2PO_4 buffer. Storage phosphor screens were laid over dried plates for 16 hrs then scanned. Counts in the circled spots were quantified using ImageQuant software.

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