Effects of arsenic, an inhibitor of CFTR function, on intracellular signaling in *Fundulus*

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The euryhaline teleost *Fundulus heteroclitus* (killifish) is able to adapt to rapid changes in environmental salinity and therefore represents an excellent model for studies on the regulation of salt transport. Marshall, et al.[1] reported that adaptation to seawater was accompanied by a transient increase in plasma cortisol levels, a sustained increase in CFTR chloride ion transporter expression, and increased Cl^- secretion by opercular epithelia. Stanton and colleagues[2] (and unpublished results) found that killifish exposed to non-toxic doses of arsenic (5 μmol/kg) for 24h exhibited significantly reduced CFTR-mediated opercular Cl^- secretion, reduced CFTR gene expression and were unable to adapt to seawater. Since arsenic inhibits the transcription factor activity but not the expression of the glucocorticoid receptor[3], the Stanton lab is investigating the hypothesis that arsenic blocks adaptation to seawater by inhibiting a cortisol-induced glucocorticoid receptor-mediated increase in CFTR expression. Here we have undertaken a pilot project to identify signal transduction pathways in killifish that are affected by exposure to arsenic and thus may warrant further investigation for involvement in both the regulation of CFTR expression and adaptation to seawater. Our working hypothesis is that signal transduction pathways potentially involved in regulating CFTR function will be inhibited or stimulated by arsenic treatment.

Commercially available polyclonal and monoclonal antibodies (Upstate Biotechnology, Lake Placid, NY; Cell Signaling Technology, Beverly, MA; B-D Biosciences, San Jose, CA; Santa Cruz Biotechnology, Santa Cruz, CA) raised against phosphopeptides corresponding to regions of activated mammalian signaling kinases were tested for immunoreactivity in western blots on killifish liver and gill lysates. CFTR is expressed in gill but not in liver. This approach depends upon a structural conservation of epitopes between orthologous mammalian and killifish proteins that is sufficient for antibody cross-reactivity, and it is subject to the caveat that immunological cross-reactivity alone does not definitively establish protein identity. The tissues tested were from killifish maintained in seawater that were either untreated or injected with arsenic at 5 μmol/kg. Bound antibodies were detected with an Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE).

The signaling intermediates chosen for initial study were enzymes in the phosphatidylinositol 3'-kinase (PI3-kinase) signal transduction cascade. PI3-kinase signaling is known to be involved in endosome-mediated intracellular translocation of proteins, and the movement of CFTR between endosomes and the plasma membrane is thought to be an important mechanism in regulating CFTR function. In addition, a downstream mediator of PI3-kinase signaling, serum and glucocorticoid-regulated kinase (SGK)[4], has been reported in recent years to be involved in regulating the activities of several ion channels and transporters including epithelial Na^+ channels (ENaC)[5,6], K^+ channels[7], Na^+/H^+ exchanger isoform 3 (NHE3)[8], and the NKCC2/BSC1 Na-K-2Cl co-transporter[9]. Human SGK-1 expression has been reported to stimulate CFTR-mediated currents in *Xenopus* oocytes[10]. We also probed liver and gill lysates with antibodies to phosphorylated mitogen-activated protein kinase (MAPK)/extracellularly regulated kinase (ERK). MAPK/ERK is activated in response to a number of

growth factors that bind receptors with intrinsic tyrosine kinase activity, and cell proliferation is invariably accompanied by activation of MAPK/ERK.

As expected, some of the antibodies to activated mammalian signaling enzymes reacted weakly or not at all with killifish liver or gill lysates. Antibodies to phosphorylated PI3-kinase reacted weakly with both control and arsenic-treated liver and gill lysates (data not shown). However, no signals were detected with antibodies to phosphorylated forms of phophatidylinositol-dependent kinase (PDK), Akt/PKB, or p70 S6 kinase, which are downstream mediators of PI3-kinase signaling. By contrast, an immunoreactive band consistent with phosphorylated SGK, an enzyme that is activated by both PI3-kinase-dependent and -independent mechanisms, was detected in both liver and gill (Fig. 1). The low level of phospho-SGK detected in untreated gill lysate (lane 3) was decreased in the arsenic-treated gill lysate (lane 4), but phospho-SGK was increased in lysates of arsenic-treated (lane 2) versus untreated (lane 1) liver. A band consistent with phosphorylated MAPK/ERK was also detected in liver and gill lysates (Fig. 1), and its expression in both tissues decreased in response to arsenic treatment (lanes 2 and 4). These results suggest that activation of SGK and MAPK/ERK is affected by a 24h exposure to a non-cytotoxic dose of arsenic. The apparent decrease in the levels of activated SGK and MAPK/ERK in arsenic-treated gill tissue may indicate roles for these enzymes in regulating CFTR function or in adaptation to increased salinity. For example, a reduction in SGK activity may lead to increased endocytosis and degradation of CFTR resulting in the observed inhibition of CFTR expression by arsenic.

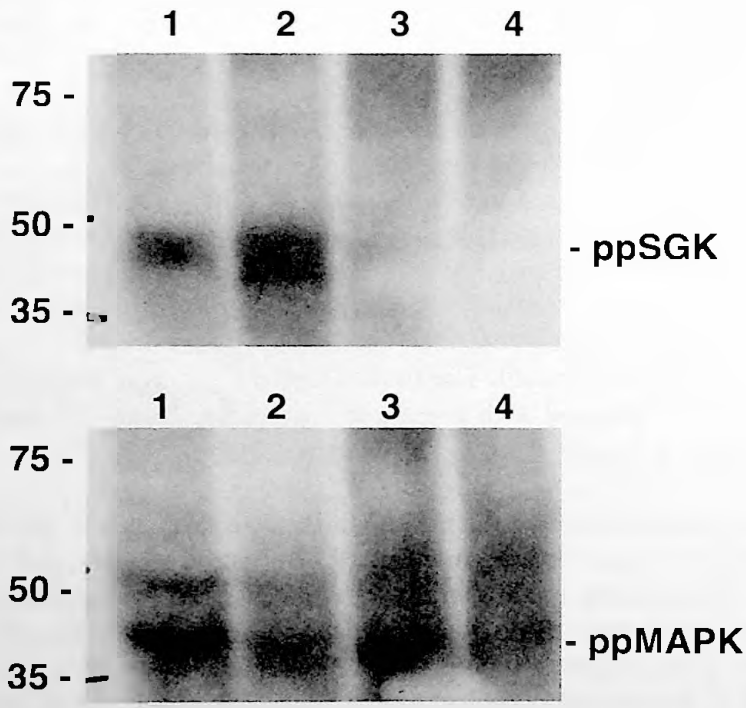

Fig. 1. Western blots of liver and gill lysates reacted with antibodies to mammalian signaling enzymes. Antibodies to synthetic phosphopeptides from serum and glucocorticoid-inducible kinase (ppSGK), and mitogen-activated protein kinase (ppMAPK) were reacted with killifish liver (1,2) and gill (3,4) lysates. The tissues were from seawater-adapted fish that were untreated (1,3) or treated with arsenic (2,4) for 24h.

The killifish is an excellent model organism for studying adaptation to changes in environmental salinity. Since adaptation to increased salinity requires an upregulation of CFTR Cl^- channel activity, studies of environmental factors that affect adaptation to seawater are directly relevant to the human disease cystic fibrosis, in which CFTR function has been compromised. Stanton and colleagues have shown that non-toxic doses of arsenic affect both CFTR gene expression and CFTR function in killifish opercula resulting in a reduced ability to adapt to seawater. Here we identify two intracellular signaling enzymes that appear to be activated to a lesser degree in gill tissue from arsenic-treated versus untreated killifish. Based on these results, SGK and MAPK/ERK warrant further investigation for involvement in the direct or indirect regulation of CFTR function in model fish and in mammals. Screening antibodies to activated signaling enzymes against gill or opercular tissue lysates from untreated and arsenic-treated killifish may be a convenient method of identifying signaling effector molecules involved in CFTR function.

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