GENISTEIN, A PROTEIN TYROSINE KINASE INHIBITOR, STIMULATES CATECHOLAMINE-DEPENDENT Na/H EXCHANGE IN RED CELLS OF THE ATLANTIC MACKEREL (*SCOMBER SCOMBRUS*)

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Na/H exchange, a ubiquitous constituent of eukaryotic cell membranes, so far has five isoforms that have been cloned and sequenced (Noel and Pouyssegur, Am. J. Physiol. 268: C283-C293, 1995). The only cAMP-dependent one (ß-NHE) is found in fish red cells where adrenergic regulation of Na/H exchange is critical for oxygen uptake at the gill under hypoxic conditions (Fievet and Motais, Adv. in Comp. and Environ. Physiol. 8: 79-104, 1991). ß-NHE can only be activated by ß-adrenergic agonists epinephrine, norepinephrine, isoproterenol) or their surrogates (cAMP, forskolin, etc.). Most of the work on the intracellular signaling pathway has been done on red cells from Rainbow trout (*Oncorhynchus mykiss*). Red cells of Atlantic mackerel (*Scomber scombrus*) also possess a catecholamine-dependent, amiloride-sensitive Na/H exchange similar to that of the trout (McManus and Starke, Bull. MDIBL 31: 147-149, 1992).

Compared to the other isoforms, ß-NHE is unique in being unresponsive to decreases in either cell volume or pH. Moreover, ß-adrenergic activation is transient. Within minutes of *in vitro* exposure to an agonist, desensitization is initiated, inactivating Na/H exchange and rendering the system resistant to further stimulation for 4-5 hours (Garcia-Romeu et al., J. Gen. Physiol. 91: 529-548, 1988). The intracellular signaling sequence that follows binding of the agonist to its receptor is activation of adenylate cyclase, generation of cAMP, activation of protein kinase A (PKA), phosphorylation of the Na/H transporter (and/or an associated regulatory protein), and finally dephosphorylation by protein phosphatase (Motais et al., Progress in Cell Research, 1: 179-193, 1990). Experimentally, phorbol esters have also been shown to activate, suggesting the presence of a phosphorylation site for protein kinase C (PKC) (Motais et al., Comp. Biochem. Physiol. 102A: 597-602, 1992). Desensitization is thought to involve an arrestin type of mechanism (Jahns et al. Biochem. J. 316: 497-506, 1996). In the following report, we propose that protein tyrosine kinase (PTK) may also play a regulatory role in this pathway.

Genistein, an isoflavone isolated from the soybean, *Genista tridentata*, or from the culture broth of Pseudomonas (Akiyama and Ogawara, Methods in Enzymology, 201:362-370, 1991), strongly inhibits PTK, but has little or no effect on other protein kinases, such as PKA, PKC or serine-threonine kinase. This agent competitively inhibits ATP binding to PTK, but not to the other kinases. In cell membrane transport systems, genistein has a remarkable variety of effects, some stimulatory and some inhibitory. For example, it inhibits insulin-dependent Na transport in A6 cells (Matsumoto et al. Am. J. Physiol. 264:C246-250, 1993), stimulates chloride secretion in primary culture monolayers of shark rectal gland tubular cells (Lehrich and Forrest, Am. J. Physiol. 269: F594-F600, 1995), inhibits brush-border Na/H exchange in rabbit ileum (Donowitz et al. Am. J. Physiol. 266: G647-G656, 1994), activates CFTR channels in NIH 3T3 cells (Illek et al. Am. J. Physiol. 268: C886-893, 1995), inhibits [Na–K–2Cl] cotransport in human red cells (Zaidi and Kaji, J. Gen. Physiol. 108:20a, 1996), and inhibits activation of Na/H exchange by arginine vasopressin in human platelets (Aharonovits et al. Biochim. Biophys. Acta 1112: 181-186, 1992). In those cases where Na/H exchange is affected, it is not known at present which particular isoforms are involved. In light of these reports, we were interested in observing the effect of this compound on Na/H exchange in mackerel red cells.

Adult mackerel were captured by hook and line in the waters around Mount Desert Island. To avoid the effects of capture stress on red cell ion content (Starke and McManus, Bull. MDIBL 28: 17-19, 1989) each fish was immediately bled from the caudal vein using a heparinized syringe. Blood from several fish was pooled, and the cells washed 3 times, then stored overnight at 4°C in an isotonic buffer to stabilize their ion contents. The buffer consisted of (mM): NaCl (167), KCl (2.5), $CaCl_2$ (0.75), $MgCl_2$ (1.0), glucose (5.0), K_2HPO_4 (1.0), and HEPES (10), pH 7.65 @ 15°C. The following morning, cells were washed two more times and resuspended in the same buffer for incubation in air at 15°C. Since the use of radioisotopes in this setting was not feasible, Na/H exchange had to be estimated from the difference in cell Na levels in the presence of ouabain (0.1 mM) with and without addition of amiloride (4 mM). Thus, the amiloride-sensitive net Na uptake in the presence of an inhibited Na pump was assumed to be a reasonable estimate of Na/H exchange activity. The representative ß-adrenergic agonist used in these experiments was isoproterenol (1 µM). At the end of a 30 minute incubation period, cells were separated from the medium by centrifugation in specially fabricated nylon tubes and cell water content assayed gravimetrically on an aliquot of packed cells as previously described (Schmidt and McManus, J. Gen. Physiol. 70: 59-79, 1977). Another weighed aliquot of cells from a parallel tube was extracted with perchloric acid (3.6%) and the supernatant fluid diluted appropriately in 4 mM CsCl. Cell Na was determined on a Perkin Elmer Model 460 atomic absorption spectrophotometer in emission mode. Osmolality of plasma and the incubation media was monitored with a Wescor vapor-pressure osmometer (5100B). The gravimetric data and the results of the flame analysis were entered into an Excel spreadsheet programmed to correct for extracellular trapping and calculate concentration of Na per liter of cell water and kilogram of dry cell solids.

Genistein stimulated Na/H exchange, but only after activation of the transporter by catecholamine or one of its surrogates. When genistein was added to cells incubated in the absence of an activating system, it had no effect. When concentration of inhibitor was varied between 5 and 50 µM and the genistein-stimulated component plotted according to the method of Eadie (Science, 116: 688, 1952), half-maximum stimulation occurred at 5 µM. The response of different batches of cells to both catecholamine and genistein was variable, but uptake was always stimulated over the level produced by the hormone. The average percent increase was 40.9 ± 24.0 % (n = 18). Genistin, an inactive analog of genistein, had no effect.

Others (e.g. Illek et al., Am. J. Physiol. 268: C886-893, 1995; Lehrich and Forrest, Am. J. Physiol. 269: F594-F600, 1995) have shown in different systems that genistein by itself does not increase intracellular cAMP. If, as appears likely, that is also true in mackerel red cells, then the effect of this compound on catecholamine-activated cells does not involve inhibition of phosphodiesterase or activation of PKA.

In systems of this sort, inhibition of protein phosphatase is often a useful approach for invoking an experimental response. This technique has been used in the trout red cell (Guizouarn et al., J. Biol Chem. 268: 8632-8639, 1993; Guizouarn et al., Am. J. Physiol. 268: C434-C441, 1995). Two protein phosphatase inhibitors, okadaic acid (OA) and calyculin A (ClA), were investigated. OA by itself did not activate Na/H exchange, but ClA did. However, they both fully inhibited the subsequent desensitization phenomenon. The effect of ClA on Na/H exchange was independent and additive to that of the cAMP-dependent pathway. To reconcile these observations, two phosphorylation sites were hypothesized, only one of which is affected by OA inhibition of protein phosphatase, but both of which are sensitive to ClA (Guizouarn et al., Am. J. Physiol. 268: C434-C441, 1995). The model therefore assigned different roles to these sites in activation and desensitization, respectively. Moreover, the Na/H exchange stimulated by ClA exhibited significantly different kinetic and regulatory characteristics, so it was suggested that ClA turns on a separate pool of transporters, perhaps those previously sequestered by desensitization. In experiments on mackerel red cells, we confirmed the effect of both inhibitors on desensitization

and ClA activation of the transporter in the absence of agonist. As in the trout system, the effect of ClA was additive to that of the catecholamine. Although genistein stimulated hormonally activated Na/H exchange as usual , it had no effect when added to cells in which Na/H exchange was activated by ClA in the absence of hormone, nor did it further stimulate uptake in the presence of both ClA and catecholamine. Like the phosphatase inhibitors, genistein also blocked desensitization. It is not yet apparent how these results can be reconciled with the previous model (Guizouarn et al. Am. J. Physiol. 268: C434-C441, 1995). Nevertheless, it is clear that in addition to phosphorylation sites controlled by PKA and PKC, there may also be PTK sites in the regulatory pathway of ß-NHE.

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