Signal transduction mechanisms involved in the regulation of NHE1 from *Pleuronectes Americanus* red blood cells by osmotic shrinkage, β-adrenergic stimuli, and calyculin A.

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In most mammalian cells studied, the ubiquitous plasma membrane Na^+/H^+ exchanger, NHE1, is activated by cell shrinkage, and plays a major role in the regulatory volume increase (RVI) process after osmotic shrinkage[1]. In red blood cells (RBCs) from many teleosts, however, NHE1 is relatively or completely insensitive to osmotic cell shrinkage at physiological O_2 pressure[3]. We recently cloned a novel, amiloride-, EIPA-, and HOE 694-insensitive NHE1 homolog, paNHE1, from the RBCs of the winter flounder, *Pleuronectes Americanus*[5]. The paNHE1 was found to be potently activated at physiological P_{O2} by three different stimuli: osmotic shrinkage, β-adrenergic stimuli (isoproterenol, IP) and other increases in cellular cAMP level, and exposure to Calyculin A (CLA), a marine toxin which potently inhibits Ser/Thr protein phosphatases PP1 and PP2A[5]. Osmotic shrinkage, IP, and CLA were at least partially additive in their effect on paNHE1, suggesting that the signaling pathways involved are not identical[5]. The signaling mechanisms controlling NHE1 function are incompletely understood, and a particularly controversial subject is the role(s) of protein phosphorylation. The aim of the present study was to gain insight in these mechanisms by analysis of the signaling pathways involved in paNHE1 activation by osmotic shrinkage, IP, and CLA. The long term goal of our studies is to exploit the regulatory and structural differences between paNHE1 and the human NHE1 in a comparative approach to understanding the structural basis of NHE1 regulation.

Net Na^+, K^+, Cl^-, and water fluxes were measured in the flounder RBCs as previously described[2,5]. Osmotic shrinkage, IP, and CLA all elicited a rapid, substantial net Na^+ influx, previously shown to reflect NHE1 activation[4]. Supporting this notion, the Na^+ fluxes elicited by all three stimuli were bumetanide-insensitive (10 μM; n=2-4; data not shown), and in Western blots of crude RBC membrane fractions, there was no detectable labeling with the NKCC1 antibody T4[a] (n=2, not shown), but robust labeling of a ~100 kDa band by several NHE1 antibodies[4].

The changes in RBC Na^+ content over time after maximal stimulation by osmotic challenge (2.0 isotonic osmolarity), IP (10 μM), CLA (100 nM), or combinations thereof, are shown in Fig. 1A. Pairwise, the stimuli were at least partially additive in their effect on paNHE1. Simultaneous exposure to all three stimuli elicited an increase in Na^+ content that was no greater than that induced by IP + CLA[b]. In swollen cells, exposure to CLA failed to activate paNHE1, while IP-mediated paNHE1 activation, although much smaller, was essentially unaffected by osmotic swelling (Fig. 1B). This is consistent with the interpretation that PP1 and/or PP2A inhibit paNHE1 activity by dephosphorylating substrate(s) of a volume-sensitive kinase, the activity of which is essentially zero in osmotically swollen cells, intermediate in isotonic cells, and greatest in shrunken cells. The possible involvement of changes in cAMP level and protein kinase A (PKA) activity in paNHE1 activation was investigated. The cAMP level in the RBCs was rapidly and substantially increased by exposure to IP, but unaffected by CLA or osmotic shrinkage (Fig. 1C). Osmotic shrinkage markedly delayed the IP-induced cAMP

[a] The T4 antibody was developed against a fragment of the C-terminus of human NKCC1, and labels NKCC1 in as diverse species as human, dog, duck, and shark[4].
[b] An inwardly directed driving force for Na^+ influx via NHE1 exists for all conditions shown, at least until t=30 min.

increase, while CLA had no effect (Fig. 1C). The PKA inhibitor H89 (10 μM) partially inhibited IP-mediated paNHE1 activation, while shrinkage- and CLA-mediated activation was unaffected by H89 (CLA, RVI n=2; IP, n=3). Calphostin C (5 μM), and ML-7 (10 μM), inhibitors of, respectively, protein kinase C (PKC) and myosin light chain kinase (MLCK), had no effect on paNHE1 activation by either shrinkage, IP, or CLA (n=2-4 for each treatment). KT5828 (10 μM), inhibitor of protein kinase G (PKG), had no effect on paNHE1 activation by shrinkage or IP (IP, n=2-3; not tested for CLA). The C-terminal cytoplasmic region of paNHE1 exhibits several Ser/Thr kinase consensus sites, including sites for PKA and PKC[5], essentially all with Ser as the phosphorylatable residue. Ser phosphorylation of paNHE1, as evaluated using Ser-phosphospecific antibodies, was increased after IP- and apparently also CLA-treatment, but appeared unaffected by osmotic shrinkage (Fig. 1D).

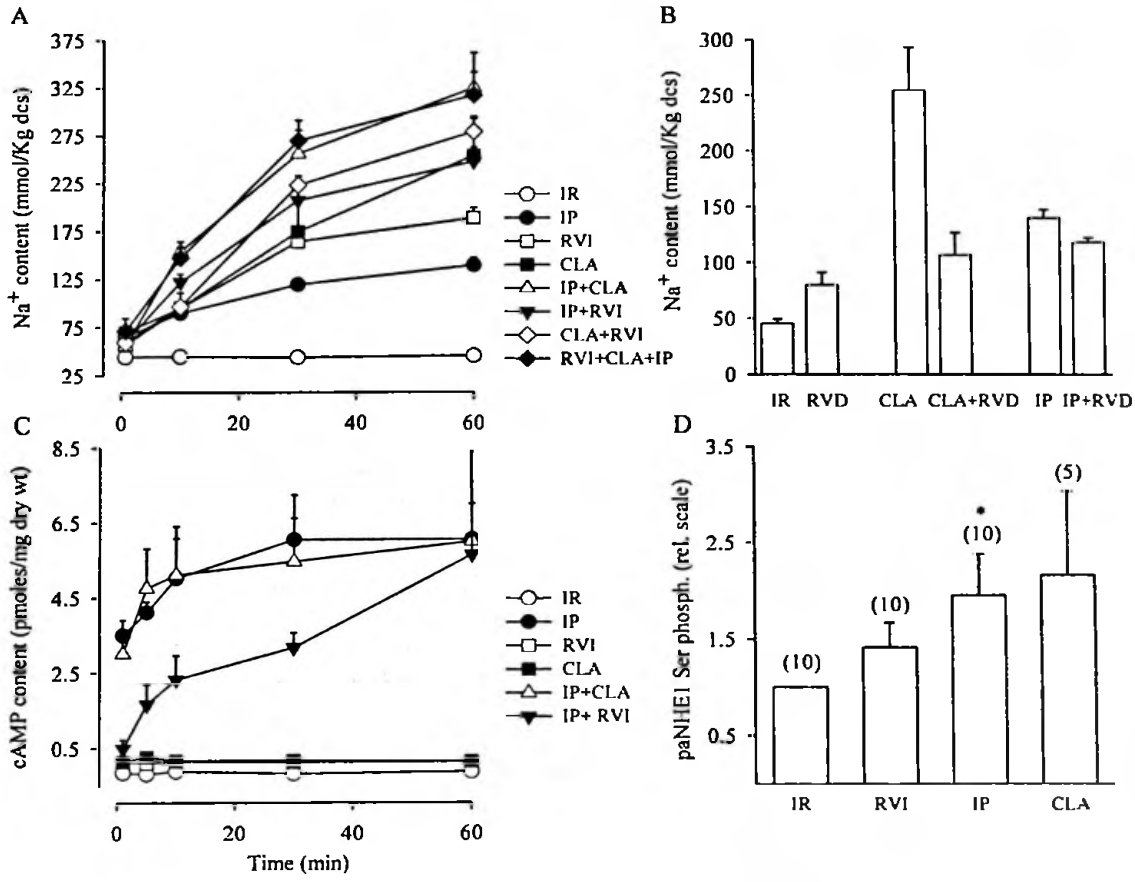

Figure 1. A. Net Na^+ influx in flounder RBCs in isotonic medium (IR), and after osmotic shrinkage (RVI, 2.0 isotonic osmolarity), 10 μM isoproterenol (IP), 100 nM Calyculin A (CLA), or combinations. B. Net Na^+ influx at t=60 min after stimulation in IR, and after hypotonic swelling (RVD, 0.5 isotonic osmolarity), CLA, IP, or combinations. C. RBC cAMP level in IR, and after IP, CLA (500 nM), RVI, or combinations (^{3}HcAMP competition assay, Amersham). D. Ser phosphorylation of paNHE1 t=20 min after stimulation, in IR, and after RVI, IP, or CLA (paNHE1 immunoprecipitation, Western blotting with phospho-Ser antibody, membrane stripping, reprobing with paNHE1 antibody, and normalization to paNHE1 signal).

Based on these observations, we propose the following working model: β-adrenergic stimuli activate adenylate cyclase, resulting in an increase in cellular cAMP, activation of PKA, and paNHE1 activation and concomitant increase in paNHE1 Ser phosphorylation. The incomplete inhibition by H89 suggests the involvement of both PKA-dependent and –independent signaling events in the cAMP-dependent paNHE1 activation. The non-PKA component remains to be investigated. Possible

candidates are ERK1/2, which are PKA-independent, downstream effectors of cAMP. Osmotic shrinkage activates a volume-sensitive kinase, which is neither PKA, PKC, MLCK, or PKG. This kinase activates paNHE1 via a chain of events which has yet to be elucidated, but which does not appear to involve direct Ser phosphorylation of paNHE1. The PP1/PP2A inhibitor CLA activates paNHE1 by preventing dephosphorylation of the substrate(s) of a volume-sensitive kinase (since CLA had essentially no effect on paNHE1 activity in swollen cells, Fig. 1B). In addition, CLA treatment appears to increase direct Ser phosphorylation of the paNHE1 protein. Notably, PP1 and PP2A exhibit broad substrate specificity, and the CLA-mediated effects on paNHE1 could therefore involve the reduced dephosphorylation of many possible players, from paNHE1 to upstream kinases that are themselves regulated by phosphorylation/dephosphorylation events. The fact that shrinkage- and CLA-mediated paNHE1 activation is only partially additive may reflect that shrinkage both activates a volume-sensitive kinase, and inhibits the corresponding phosphatase. The lack of additional paNHE1 activation in cells treated with all three stimuli together, compared to cells treated with only IP + CLA, could reflect shrinkage-induced attenuation of the IP-mediated cAMP-response (Fig. 1C). Thus, osmotic shrinkage and CLA activate paNHE1 by only partially different pathways. Activation by PKA appears to occur by a pathway distinct from these, but interactions between the events elicited by all three stimuli were noted. The findings strongly indicate that protein phosphorylation processes, directly or indirectly, play important roles in paNHE1 activation by all three stimuli. However, whether there is a causal relationship between the direct paNHE1 phosphorylation and –activation remains to be investigated.

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