THE EFFECT OF A NUMBER OF MARINE TOXINS ON DEPHOSPHORYLATION OF *SQUALUS ACANTHIAS* Na-K-Cl COTRANSPORTER (sNKCC1)

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Many aquatic toxins are known to be potent inhibitors of serine and threonine phosphatases including protein phosphatase 1 and 2a (PP1 and PP2a). We have examined the potency of a number of these toxins in preventing the dephosphorylation of the Na-K-Cl cotransporter (NKCC1) from spiny dogfish (*Squalus acanthias*) rectal gland. The problem is of physiological and toxicological importance, since it is clear that phosphorylation and dephosphorylation of residues in the N-terminus of NKCC1 is the mechanism regulating activation of the cotransporter, and since NKCC1 activation is a necessary component of activation of intestinal fluid secretion in diarrheal toxicity.

In these experiments we examined the effect of aquatic toxins microcystin-LR, nodularin, calyculin A, and okadaic acid, as well as tautomycin, cantharidin and endothall. All of these compounds are known to inhibit serine and threonine proteases with varying degrees of potency (see Figure 2, below). Microcystin-LR and nodularin are related cyclic heptapeptides isolated from cyanobacteria of genera Microcystis, Nodularia, Anabaena, and Oscillatoria, which grow worldwide in fresh and brackish waters and represent a significant health problem for wild animals, livestock, and humans. Calyculin A and okadaic acid are isolated from marine sponges, where they are probably synthesized by symbiotic microorganisms; okadaic acid is found in various dinoflagellates of the genus Prorocentrum and is one of the agents involved in diarrhretic shellfish poisoning. Tautomycin, structurally similar to okadaic acid, is a polyketide antibiotic produced by the soil bacterium Streptomyces verticillatus. Cantharidin is a toxin from the blister beetle, and endothall is a similar compound used as a herbicide.

Previous work has implicated PP1 in the dephosphorylation of NKCC1 (C. Lytle and B. Forbush, Am. J. Physiol., 270:C437-C448, 1996), but such studies have utilized a narrow range of inhibitors in intact cell systems. With the cell membrane in place, it is difficult to assess the relative potency of various compounds, since their membrane permeability is generally unknown – $K_{0.5}$'s are generally much higher in intact cells than *in vitro*. Thus in the present work we examined the dephosphorylation of NKCC1 by cytoplasmic phosphatases following solubilization of rectal gland tubule cells with Triton X-100. The assay utilizes anti-P-NKCC, a sensitive and specific antibody that recognizes NKCC1 only when it is phosphorylated on regulatory residues T184 and T189 (Flemmer et al; Bull. MDIBL 38:80-2, 1999). The procedures are very similar to those described by Giménez et al (this volume), except that the initial cytocrit was 0.8%, and the "stop" solution in the current experiments contained 20% EtOH, 0.1% SDS, 0.2M glycine/Tris, 0.2 μM calyculin A, pH8.3.

Figure 1A illustrates typical dephosphorylation time courses measured with anti-P-NKCC, showing the effectiveness of calyculin A and microcystin-LR in inhibiting dephosphorylation. Rate constants were obtained from exponential fits of such data (lines in figure 1) at each of seven inhibitor concentrations. An example of the concentration dependence of the rate is presented in figure 1B. It is clear in this example that microcystin-LR is a more potent inhibitor

of NKCC dephosphorylation compared to calyculin A. The rate data were in turn fit by a model of inhibitor binding at a single site (lines in figure 2) in order to obtain inhibitory constants.

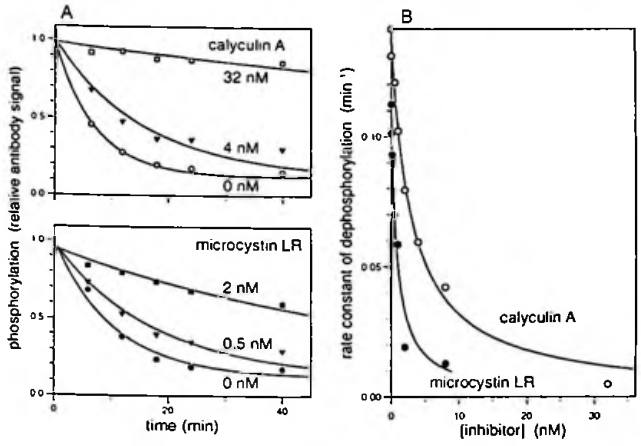

Figure 1. Dephosphorylation of solubilized NKCC1 and inhibition by calyculin A and microcystin-LR. A. Decrease in phosphorylation determined with anti-P-NKCC. B. Dephosphorylation rate as a function of inhibitor concentration, replotted from fits of the data in panels A and additional data.

Figure 2 is a summary of our experiments, which compares inhibition constants obtained as described above with ranges of published values for inhibition of PP1 and PP2a by the various toxins. Microcystin, nodularin, and calyculin A were all found to be potent inhibitors of the dephosphorylation of NKCC1, which demonstrates that either PP1 or PP2a is probably the phosphatase that is involved. However because of the great overlap in the inhibitory ranges, these agents are of limited value to distinguish between the two phosphatases. On the other hand, tautomycin, okadaic acid, cantharidin and endothall discriminate well between phosphatases, the first being a more potent inhibitor of PP1 than of PP2, and the latter three being more effective inhibitors of PP2. It is seen that for each of these, inhibition of NKCC1 dephosphorylation follows the pattern of PP1 inhibition, providing fairly conclusive evidence that PP1 is the phosphatase that acts on NKCC1. This result is consistent with and predicted by our recent finding that an RVNFD sequence in the N-terminus of NKCC1 provides a site for the direct binding of PP1 to the cotransporter (Behnke et al. FASEB J. 13, A397, 1999).

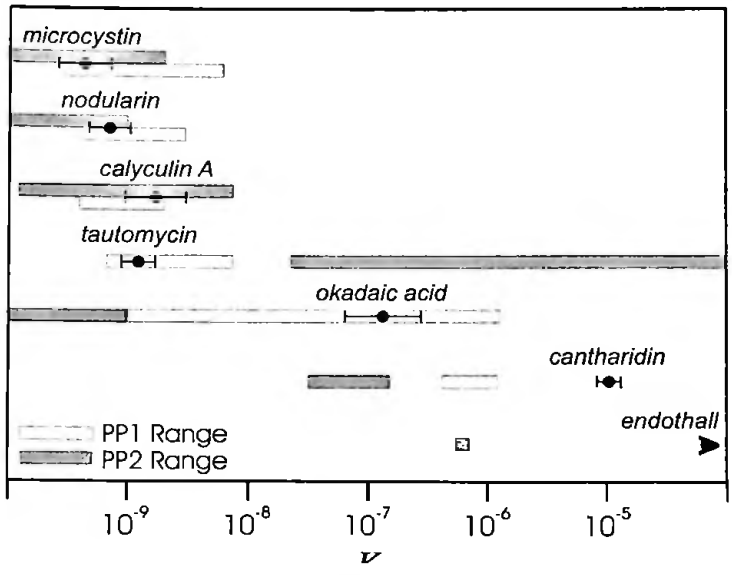

Figure 2. $K_{0.5}$ values for inhibition of NKCC1 dephosphorylation. Results are from 3-5 experiments for each inhibitor and are presented as mean ± SE.

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