Inhibition of adenylyl cyclase by somatostatin reduces chloride secretion in CNP stimulated cultured rectal gland epithelial cells of the spiny dogfish shark, *Squalus acanthias*

Daniel J. Pfau[1,4,5], Montana T. Morris[3,5], Hugo R. de Jonge[2,5] and John N. Forrest, Jr.[4,5]
[1] Fordham University, Bronx, NY 10458
[2] Erasmus University Medical Center, Rotterdam, Netherlands
[3] Stanford University, Palo Alto, CA 94304
[4] Yale University School of Medicine, New Haven, CT 06510
[5] Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672

C-type natriuretic peptide (CNP) stimulates chloride secretion in the shark rectal gland by inducing production of the intracellular messenger cyclic GMP (cGMP). Our lab sought to better understand the role that cGMP plays in transducing the intracellular signal responsible for the exit of chloride through the cystic fibrosis transmembrane conductance regulator (CFTR) and how the activity of cGMP is coordinated with another intracellular messenger: cyclic AMP (cAMP). Short circuit current experiments were performed on cultured monolayers of primary rectal gland epithelial cells in which the adenylyl cyclase inhibiting drug somatostatin was used to suppress levels of cAMP in CNP stimulated epithelial cells. Our results indicate that cGMP alone cannot account for the CNP stimulation of chloride secretion in the rectal gland.

C-type natriuretic peptide (CNP), a hormone released from the heart of the dogfish shark, strongly activates chloride secretion through CFTR channels in the osmoregulatory shark rectal gland (SRG)[4]. CNP binds to its surface receptor NPR-B and elicits this response by inducing production of the intracellular messenger cyclic GMP (cGMP)[1]. Cyclic GMP and cyclic AMP (cAMP) levels in the SRG are regulated by the specific isozyme type III phosphodiesterase (PDE3)[3]. Previous studies in our lab have indicated that cGMP acts as a competitive inhibitor of PDE3, preventing the hydrolysis of the intracellular messenger cAMP and thus activating the cAMP-PKA-CFTR pathway responsible for chloride secretion[2,5]. Thus, as CNP increases cytosolic cGMP levels, cAMP levels simultaneously rise and chloride secretion results. Here we sought to exclude the possibility of direct cGMP cross activation of PKA, a cAMP-independent mechanism that would bypass the early steps in the cAMP signaling pathway.

To investigate cGMP's potential role as a cross activator of PKA, short circuit current measurements of chloride secretion (expressed as microAmperes/cm^2) were performed across filter-grown monolayers of SRG epithelial cells in Ussing chambers. To this aim, primary rectal gland cell cultures were prepared[6] and were grown on Corning-Costar Transwell collagen I/III coated 0.4 μm pore PFTE membrane inserts (6.5 mm in diameter). Cells were allowed to grow for approximately two weeks in cell culture medium. Confluent monolayers were placed in an Ussing chamber (EM-LVSYS-4 Physiologic Instruments, San Diego) and voltage clamped.

Figure 1 shows a representative experiment of 3 that were performed. Once a baseline current was established chloride flux was stimulated by the addition of CNP (0.05 μM) to the serosal compartment. After approximately 20 min, somatostatin (0.1 μM) (known to inhibit adenylyl cyclase but not guanylyl cyclase through a G_i-coupled receptor) was added to the serosal side of the monolayer followed by amrinone (50 μM), a selective inhibitor of PDE3. Subsequently the adenylyl cyclase activator forskolin (10 μM) was added to the serosal compartment, followed by the NKCC inhibitor bumetanide.

The results show that Cl^- secretion induced by CNP is almost fully inhibited by somatostatin, indicating that cGMP-activated Cl^- secretion in the SRG is dependent on *basal* cAMP generation by adenylyl cyclase, in line with our model of CNP-cGMP-PDE3-cAMP-PKA-CFTR signaling. As predicted, the subsequent blockade of cAMP and cGMP hydrolysis by amrinone, mimicking the effect of CNP/cGMP, was unable to overcome the inhibitory effect of somatostatin. In contrast, hyperactivation of adenylyl cyclase by forskolin apparently counteracted adenylyl cyclase inhibition by somatostatin effectively, resulting in full activation of CFTR by the cAMP-PKA-CFTR pathway.

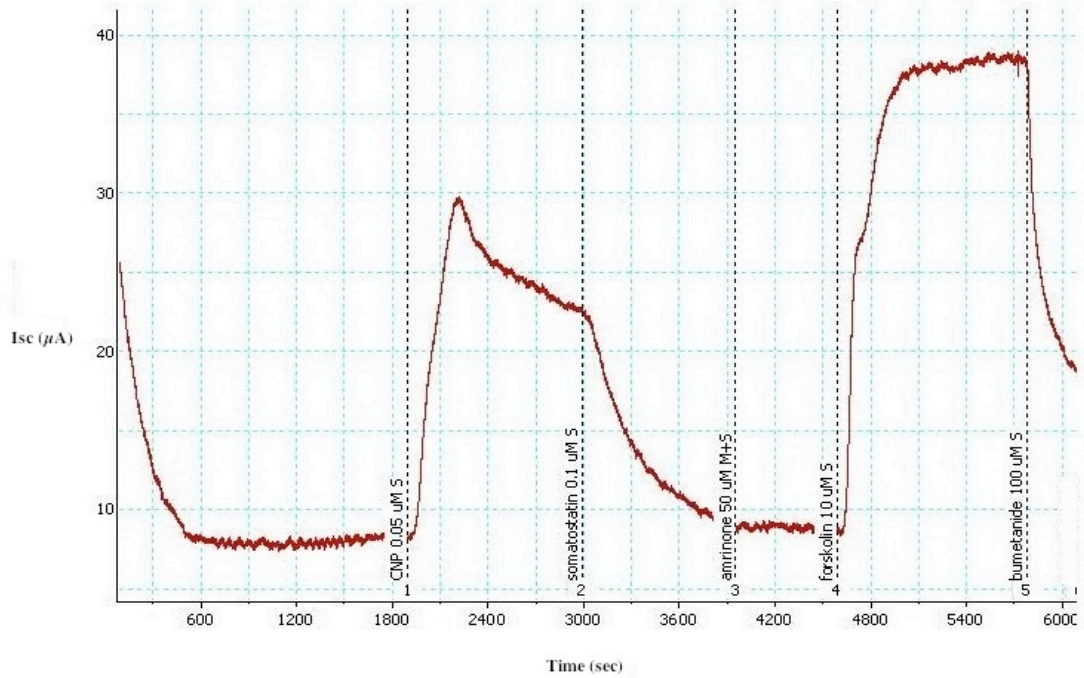

Figure 1. Treatment of cultured monolayer of SRG epithelial cells with somatostatin (0.1 μM) after stimulation with CNP (0.05 μM) caused a drop in current to almost base line levels that could not be stimulated further by the PDE3 inhibitor amrinone but was effectively re-activated by forskolin.

From these results we conclude (1) CNP/cGMP-activated Cl^- secretion by SRG epithelial cells is dependent on basal cAMP formation and cAMP signaling; (2) cGMP alone fails to activate CFTR through a cAMP-independent mechanism, *e.g.,* cGMP-activation of a specific cGMP-dependent protein kinase or cAMP-cross activation of PKA.

This work was supported by NIH grants DK 34208, NIEHS 5 P30 ES03828 (Dr. Forrest) and an NSF grant DBI-0453391.

1. **Aller SG, Lombardo ID, Bhanot S, Forrest JN Jr**. Cloning, characterization, and functional expression of a CNP receptor regulating CFTR in the shark rectal gland. *Am. J. Physiol*. 276:C442-9, 1999.
2. **Kelley CA, Kufner A, Epstein W, Melita A, Hart M, Tilly BC, de Jonge HR, Forrest JN Jr.** Stimulation of chloride secretion by CNP is mediated by Cyclic GMP inhibition of phosphodiesterase III in the rectal gland of the spiny dogfish, *Squalus acanthias*: Evidence from *in vitro* perfusion studies. *Bull. Mt. Desert Isl. Bio. Lab* 48:31-34, 2009.
3. **Kelley MH, Melita A, Edelstein H, Kelley, CA, Epstein WS, Vosburgh B, Kufner AE, Burks KL, de Jonge HR, Forrest, JN Jr**. Effects of type specific phosphodiesterase inhibitors on chloride secretion in the perfused rectal gland of the dogfish shark (*Squalus acanthias*). *Bull. Mt. Desert Isl. Bio. Lab*. 48: 21-22, 2010.
4. **Schofield JP, Jones DS, Forrest JN Jr**. Identification of C-type natriuretic peptide in heart of spiny dogfish shark *(Squalus acanthias). Am. J. Physiol*. 261: F734-F739, 1991.
5. **Tilly BC, Hogema BM, Kelley CA, Forrest JN Jr, De Jonge HR.** Cyclic GMP inhibition of phosphodiesterase III mediates C-type natriuretic peptide (CNP) stimulation of chloride secretion in the rectal gland of the spiny dog fish (*Squalus acanthias*). *Bull. Mt. Desert Isl. Bio. Lab*. 48: 27-30, 2009.
6. **Valentich JD, Forrest JN Jr.** Cl^- secretion by cultured shark rectal gland cells. I. Transepithelial transport. *Am. J. Physiol*. 260: C813-823, 1991.