ANP, CNP and sildenafil activate CFTR expressed in *Xenopus laevis* oocytes by direct activation of PKA

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The natriuretic peptides atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) have been studied extensively for their roles in maintaining body fluid homeostasis. We investigated the pathway that is employed for activation of chloride conduction by these peptides when the shark cystic fibrosis transmembrane conductance regulator (sCFTR) chloride channel is expressed in *Xenopus* oocytes. Using ANP, CNP, permeant non-hydrolysable cyclic nucleotides as well as several phosphodiesterase (PDE) inhibitors, we find that ANP, CNP and sildenafil activate chloride conductance in the oocyte by cyclic GMP (cGMP) cross activation of protein kinase A (PKA) which then phosphorylates sCFTR.

Natriuretic peptides have been studied extensively for their roles in maintaining body fluid homeostasis[6,7,13]. In mammals, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are cardiac peptides that exert renal hemodynamic and tubular actions resulting in diuresis and natriuresis. In the shark heart, C-type natriuretic peptide (CNP), the most recently discovered natriuretic peptide, activates chloride secretion in the shark rectal gland[9]. Membrane bound receptor guanlyl cyclases are essential in the signaling pathway of these three natriuretic peptides[3].

In elasmobranchs, CNP is the only natriuretic peptide hormone synthesized in the heart[9,10] and circulates in plasma at concentrations exceeding those in mammals[11]. Sharks have an extra-renal osmoregulatory organ, the rectal gland, which is highly specialized for hormone regulated chloride secretion through CFTR[1,2,5,8]. CNP is a potent activator of chloride secretion in the shark rectal gland and is many fold more potent than ANP[9]. This lab recently discovered that CNP acts in the shark rectal gland by increasing cGMP and subsequently inhibiting phosphodiesterase type 3 (PDE3), thus increasing cAMP levels that lead to CFTR activation[4,12]. In this work we studied the pathway by which the natriuretic peptides ANP and CNP act on sCFTR expressed in Xenopus oocytes. The results indicate a pathway different from that employed in the rectal gland to activate sCFTR.

Expression vectors for sCFTR and the CNP receptor (NPR-B) were grown in 150 ml cultures of TOP10 E. coli (Invitrogen, Carlsbad, CA), and the full length construct was sequenced to confirm the integrity of the CFTR open reading frames (ORFs). 12μg CFTR DNA was linearized with XhoI and purified by PCR purification (Qiagen, Alameda, CA). Capped cDNA was synthesized using T7 RNA polymerase and *in vitro* transcription following the instructions of T7 *in vitro* transcription system (Ambion, Austin, TX). The reaction products were precipitated using lithium-chloride precipitation and measured with the Agilent Bioanalyzer system (Agilent, Santa Clara, CA).

Mature female *Xenopus laevis* (Xenopus I, Dexter, Michigan) were anesthetized with tricaine and several ovarian lobules were removed through a sterile abdominal incision per a protocol approved by the MDIBL and Yale University IACUC. Ovarian lobules of mature female *Xenopus laevis* (Xenopus I, Dexter, Michigan) were obtained and stage V and VI oocytes were selected for two electrode voltage clamping (TEVC) as described previously[4,12]. After 12h the oocytes were injected with 10ng sCFTR cRNA/50nl or an equivalent volume of water and then stored for 1-2 days in MBS at 18°C. Electrophysiological recordings were performed 1-2 days after injection of the cRNA. Electrodes, pulled on a micropipette puller (Sutter Instruments, Novata, CA), were filled with 3 M KCl and had input resistances between 0.6 and 1.3 MΩ. During TEVC, oocytes were clamped at a holding potential of -30 mV and current-voltage (I-V) curves were obtained by taking ramps from -120 to +60 mV at a rate of 100mV/s with the use of a two electrode voltage clamp (TEV-200, Dagan Instruments, Foster City, CA). Data were analyzed with pCLAMP software (Axon Instruments). Results are expressed as micro Siemens (μS) ± SEM. Statistical significance was determined by Student's t test.

After recording the baseline conductance, the oocytes were perfused with ANP (Fig 1). Conductance at baseline was 5.6 ± 1.2 μS and increased after 500 nM of ANP to 58 ± 5.5 μS ($P < 0.001$). This conductance was inhibited by 55.8 % to 25.6 ± 1.7 μS after addition of 50 μM of the PKA inhibitor H89 ($P < 0.01$), consistent

with cross activation of PKA. In oocytes expressing both the CNP receptor and sCFTR, CNP also stimulated conductance from 4 μS to 119 μS and was significantly inhibited by H89 (P<0.001).

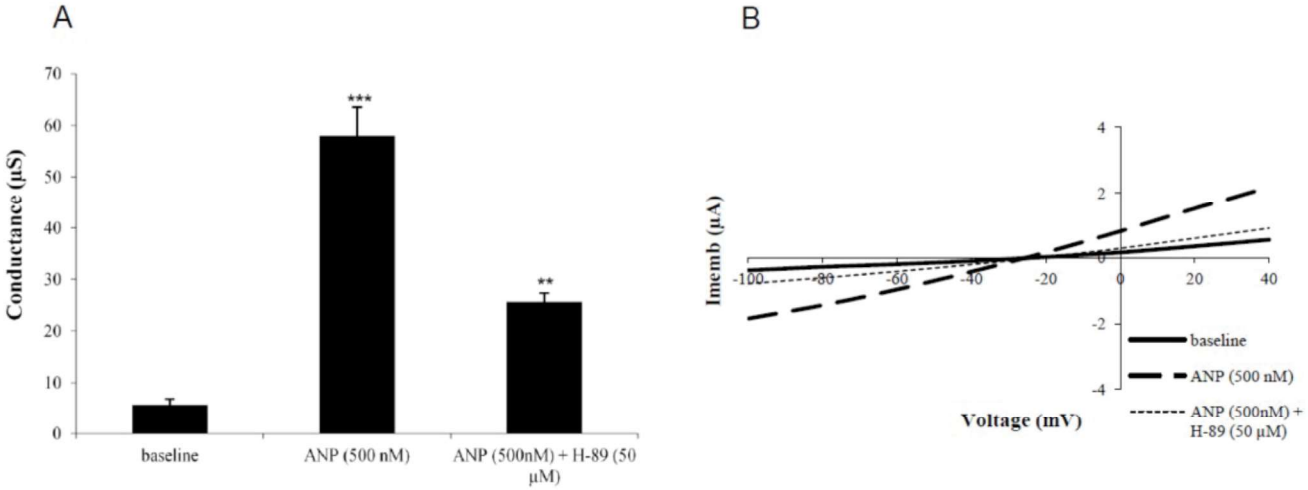

Figure 1. Stimulation of chloride conductance by ANP. Panel A: Summary of mean conductance at baseline (n=3), after stimulation with 500 nM ANP (n = 3) and inhibition with 50 μM H-89 in the presence of ANP (n = 3). Panel B: I-V plot of a representative experiment.

We next examined the effects of various cyclic nucleotides and PDE inhibitors on chloride conductance (Fig 2). 8-bromo cyclic AMP significantly increased conductance, as did 8-bromo cyclic GMP, consistent with cross activation by the cyclic GMP agonist. In contrast, 8-pCPT-cGMP, the direct activator of G kinase II, had no effect. Responses to several type specific PDE inhibitors, including EHNA (type 2 PDE inhibitor) and Rolipram (type 4 PDE inhibitor) were not increased above baseline. The type specific inhibitors Amrinone (PDE3), Vinpocetine (PDE1) and Sildenafil (PDE5) significantly increased chloride conductance, suggesting these PDE isoforms are present in the oocyte.

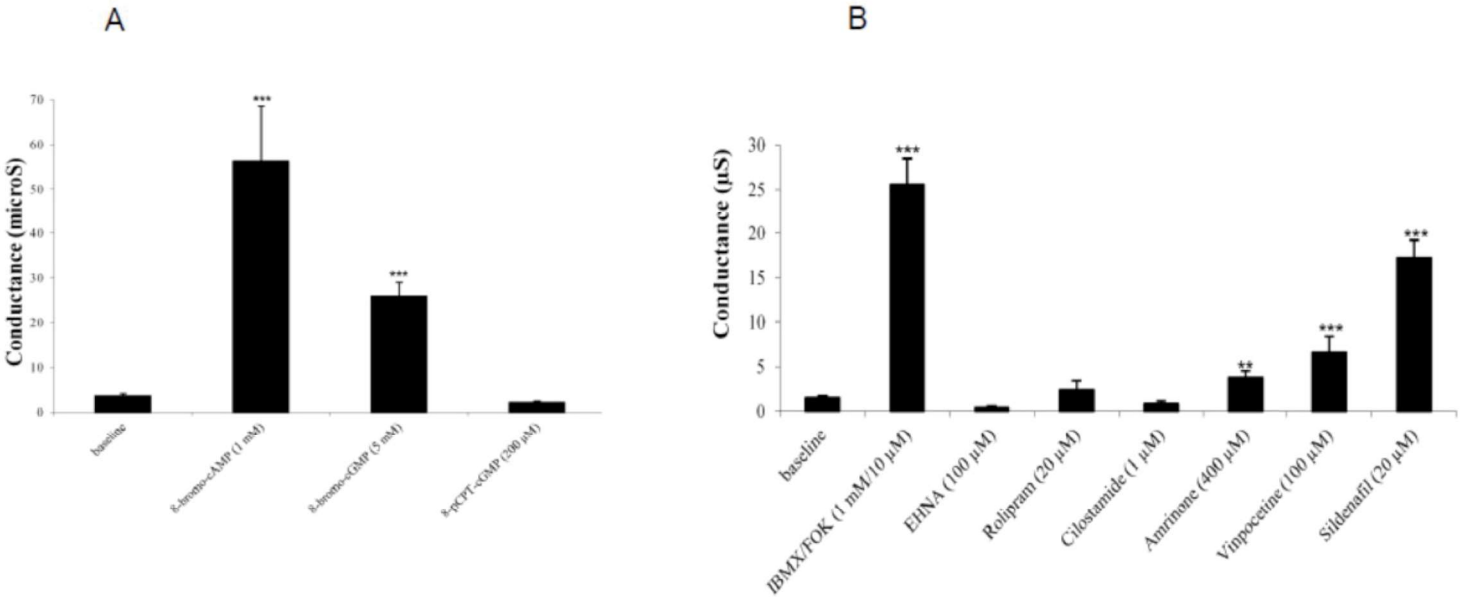

Figure 2. Response of sCFTR current to cyclic nucleotides and type specific PDE inhibitors. Panel A. Conductances at baseline and after addition of 1 mM 8-bromo-cAMP (n = 6), 5 mM 8-bromo-cGMP (n = 4) and 200 μM 8-pCPT-cGMP (n = 3). Panel B: Conductances after addition of 100 μM EHNA (n = 4), 20 μM rolipram (n = 5), 1 μM cilostamide (n = 3), 400 μM amrinone (n = 5) and 100 μM vinpocetine (n=3) and 20 μM sildenafil (n = 15).

Figure 3 illustrates that the response to the conductance increase with sildenafil is blocked both by H89 and the specific PKA inhibitor PKI. In summary, these experiments indicate that in *Xenopus* oocytes, the effect of ANP and CNP to stimulate sCFTR conductance is by cross activation of PKA. Consistent with this conclusion are our findings that: 1) both the response to ANP and CNP is inhibited by low concentrations of H-89 that binds to PKA with high affinity to inhibit this protein kinase; 2) a non-hydrolysable cGMP analog (8-bromo-cGMP) also increases CFTR conductance, whereas 3) 8-pCPT-cGMP, a direct activator of G kinase II had no effect.

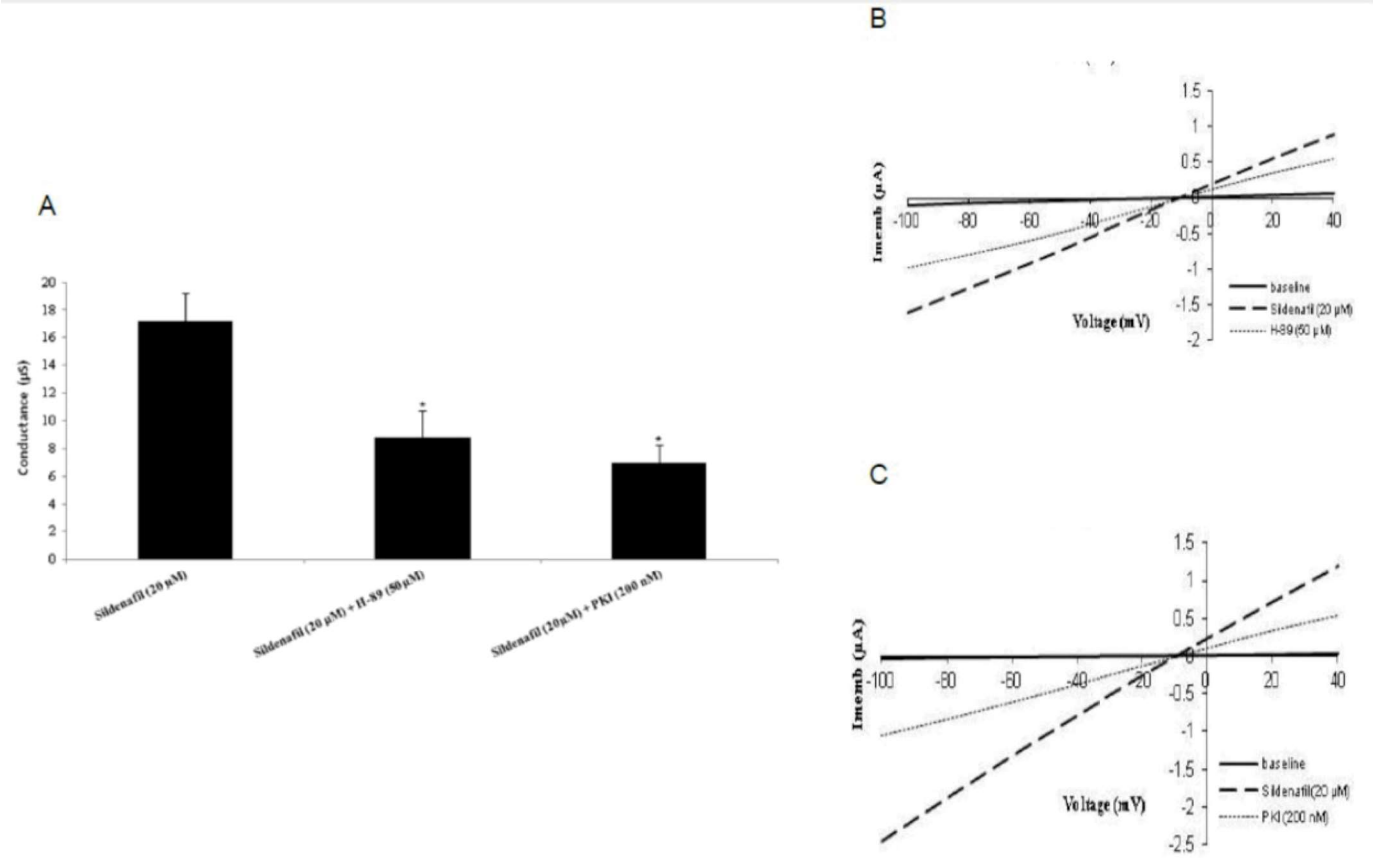

Figure 3. Panel A: Stimulation of chloride conductance by Sildenafil and inhibition with 50 μM H-89 (n = 11) and 200 nM PKI (n = 4). Panels B and C: I-V plots of representative experiments.

Sildenafil, an inhibitor of type 5 PDE that degrades cGMP, also stimulated chloride conductance, and the effect was inhibited by two PKA inhibitors, H-89 and PKI (Fig 3, Panels B and C). We conclude that in the *Xenopus* oocyte, PKA activation is the primary pathway for activating CFTR by ANP, by CNP, by sildenafil and possibly other PDE inhibitors.

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