CHARACTERIZATION OF A cAMP-MEDIATED Cl^- CURRENT IN PRIMARY RECTAL GLAND CULTURES OF THE SPINY DOGFISH (SQUALUS ACANTHIAS)

Daniel C. Devor[1], William K. Suggs[2], John N. Forrest[2], and Raymond A. Frizzell[1]
[1]Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, AL 35294-0005
[2]Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06510

The rectal gland of the spiny dogfish secretes a NaCl rich fluid in response to increases in cellular cAMP (Greger et al., Pflugers Arch. 402: 376-384, 1984; Stoff et al., J. Exp. Zool. 199: 443-448, 1977). This secretion has been shown to be due to the activation of an apical membrane Cl^- channel causing Cl^- to flow out of the cell down its electrochemical gradient. Cl^- flowing out of the cell establishes a lumen negative transepithelial potential difference with Na^+ then passively moving through the paracellular spaces resulting in NaCl secretion (Greger et al., Pflugers Arch. 402: 376-384, 1984). A dogfish homolog of the human cystic fibrosis transmembrane conductance regulator (CFTR) has recently been identified and shown to be 72% homologous with human CFTR at the protein level (Marshall et al., J. Biol. Chem. 266: 22749-22754, 1991). This similarity between DFTR and CFTR suggests that the apical membrane Cl^- channel responsible for Cl^- secretion in the dogfish rectal gland may possess biophysical and pharmacological characteristics similar to those ascribed to CFTR. We previously demonstrated, using whole-cell patch-clamp techniques, that increased cellular cAMP activates a current with similar biophysical characteristics to that of CFTR (Devor, et al., Bull. MDIBL 32: 45-47, 1993), i.e., linear current-voltage (I-V) relationship with no time or voltage dependence (Drumm et al., Cell. 62: 1227-1233, 1990). We further demonstrated that cAMP-mediated stimulation activated a small linear Cl^- channel with a conductance of 4-6 pS during cell-attached recording (Devor et al., Bull. MDIBL 32: 45-47, 1993). Finally, heterologous expression of DFTR in Xenopus oocytes or Sf9 insect cells has recently been shown to result in the expression of a low conductance (<4 pS) Cl^- channel (Hanrahan et al., Bull. MDIBL 32: 48-49, 1993). These results suggest that DFTR encodes a protein which functions as a Cl^- channel with similar characteristics to CFTR. In the present study we have further characterized the whole-cell and single channel properties of this Cl^- channel with regard to its ion selectivity and blocker pharmacology using primary cultures of dogfish rectal gland (Valentich and Forrest, Am. J. Physiol. 260: C813-C823, 1991).

Figure 1A shows the response of one cell to stimulation by forskolin (10 μM) and 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate (cpt-cAMP; 400 μM) during whole-cell patch-clamp recording. cAMP stimulated an inward current (downward deflection) when the cell was voltage-clamped to -60 mV in symmetric NMDG-Cl (N-Methyl-D-Glucamine) solutions, which returned to baseline upon removal of agonist. This result is consistent with the activation of a Cl^- conductance in these cells. The I-V relationship is nearly linear (Fig. 1C) consistent with our previous findings (Devor et al., Bull. MDIBL 32: 45-47, 1993). Similar results were obtained in 9 additional cells with an average increase in current of 32±7 (SEM) fold between -100 and +80 mV (n=10).

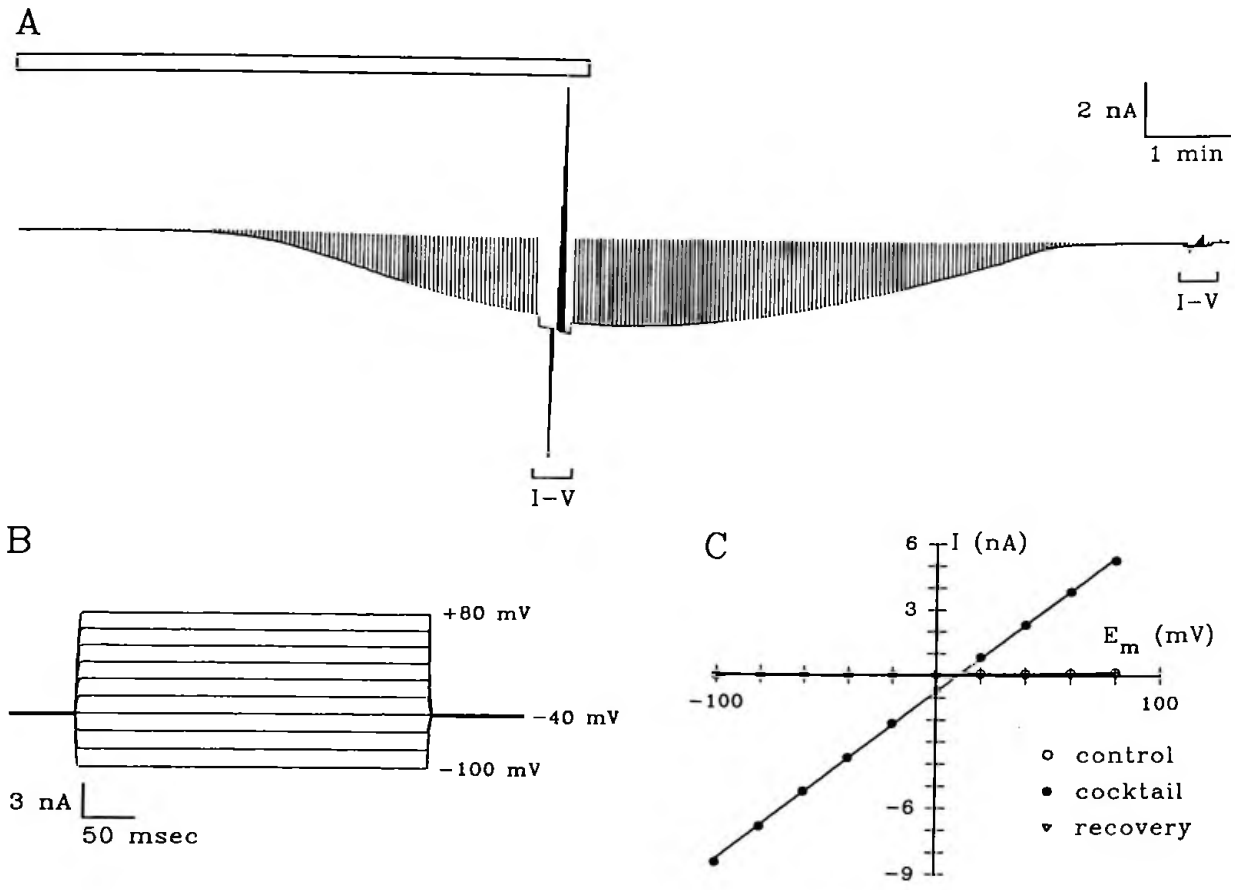

Figure 1. A. Whole-cell current response to forskolin and cAMP (open bar). The cell was voltage-clamped to -60 mV and pulsed to 0 mV for 100 msec at 3 sec intervals. The pulse protocol for determining the I-V relationship was run at the indicated times. B. Overlay of cell currents during the I-V pulse protocol after stimulation (from panel A). C. I-V relations for control, stimulated (cocktail), and recovery from stimulation (see Fig. for key). The pipette solution contained (in mM) 275 NMDG-Cl, 350 urea, 3 $MgCl_2$, 1 EGTA (free Ca^{2+}=100 nM), and 20 HEPES. The bath solution contained (in mM) 275 NMDG-Cl, 350 urea, 3 $MgCl_2$, 3 $CaCl_2$, 5 glucose, and 20 HEPES. The pH of both solutions was adjusted to 7.4 with NaOH.

During excised, inside-out patch-clamp recording in symmetric NMDG-Cl we observe a small linear Cl^- channel with an average conductance of 6.0±0.2 pS (n=6; data not shown). This is consistent with the cAMP-activated single channel Cl^- conductance we previously described during cell-attached recording (Devor et al., Bull. MDIBL 32: 45-47, 1993). To demonstrate this Cl^- channel underlies the whole-cell responses observed during cAMP stimulation we characterized the Cl^- to I^- selectivity of this channel at both the whole-cell and single channel level. Figure 2A shows single channel currents recorded at +100 or -100 mV in an inside-out patch using either the standard NMDG-Cl bath (left panel) or one in which 235 mM NMDG-Cl was replaced by NMDG-I (right panel). Both outward (+100 mV) and inward (-100 mV) currents were inhibited by I^- with clear inward events rarely observed. Similar results were observed in 2 additional patches. At +100 mV the average single channel current

amplitude was reduced 50% from 0.54±0.01 pA to 0.27±0.00 pA (n=3). This inhibition of the single channel events by I^- made it impossible to reliably construct an I-V relationship in order to determine the Cl^- to I^- selectivity at the single channel level. Figure 2B shows the I-V relationships for one whole-cell recording during control and stimulated (cocktail) conditions, followed by replacement of 235 mEq Cl^- with I^- (Iodide). I^- inhibited both the inward and outward current and caused a shift in the reversal potential to a more positive potential. In two cells the total current was reduced by 64%, and the average change in reversal potential was +7 mV indicating a Cl^-:I^- selectivity of 1.2:1. This large reduction in current amplitude with only a modest shift in reversal potential suggests that I^- is blocking the flow of Cl^- through the channel as previoulsy described for CFTR (Tabcharani et al., Biophys. J. 62: 1-4, 1992).

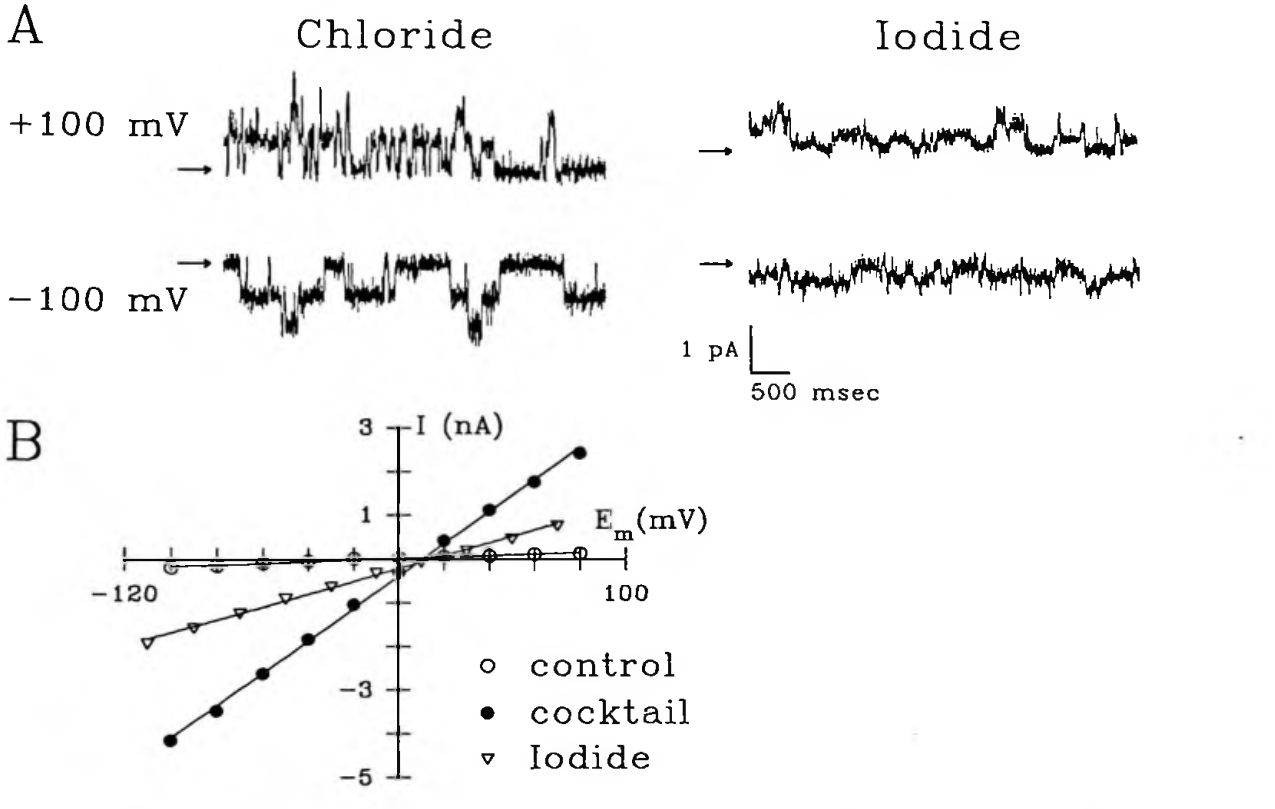

Figure 2. A. Single channel currents recorded in the excised, inside-out configuration at either +100 or -100 mV as indicated. Currents were recorded in either symmetric 275 mM NMDG-Cl (Chloride) or after substituting 235 mEq I^- for Cl^- (Iodide). The arrows indicate the closed state of the channel. B. I-V relationships for one whole-cell recording during control and stimulated (cocktail) conditions, followed by substitution of 235 mM NMDG-Cl with NMDG-I in the bath (see Fig. for key).

The ATP-dependent K^+ channel blocker, glibenclamide has recently been shown to block CFTR at both the whole-cell (Sheppard and Welsh, J. Gen. Physiol. 100: 573-591, 1992) and single channel (DeRoos et al., Ped. Pulmonology, suppl. 9: 213, 1993) level. Therefore, we determined whether glibenclamide (300 μM) would inhibit the Cl^- channel present in SRG. Fig. 3A shows the response of one cell to forskolin (10 μM) and cpt-cAMP (400 μM) and its

inhibition by glibenclamide. cAMP stimulated a large inward Cl^- current (I-V relationships not shown) which was completely inhibited by glibenclamide (open bar) in an apparently irreversible fashion. Similar results were obtained in two additional cells. This inability to reverse the effects of glibenclamide is consistent with the results of Sheppard and Welsh (J. Gen. Physiol. 100: 573-591, 1992). The effect of glibenclamide (300 μM) on single channel currents recorded in the excised, inside-out configuration is shown in Fig. 3B. Glibenclamide caused a rapid inhibition of the channel by an apparent decrease in the open time of the channel. Glibenclamide reduced the P_o from 0.38±0.02 to 0.10±0.04 (n=3), while not reducing single channel current amplitude (0.50±0.05 vs. 0.47±0.07) indicative of an open channel blocker. Similarly, in 4 outside-out recordings glibenclamide in the bath inhibited the channel, reducing P_o from 0.38±0.07 to 0.15±0.05. The effects of glibenclamide were reversible during single channel recording. The results are consistent with those previously reported for CFTR (DeRoos et al., Ped. Pulmonology, suppl. 9: 213, 1993).

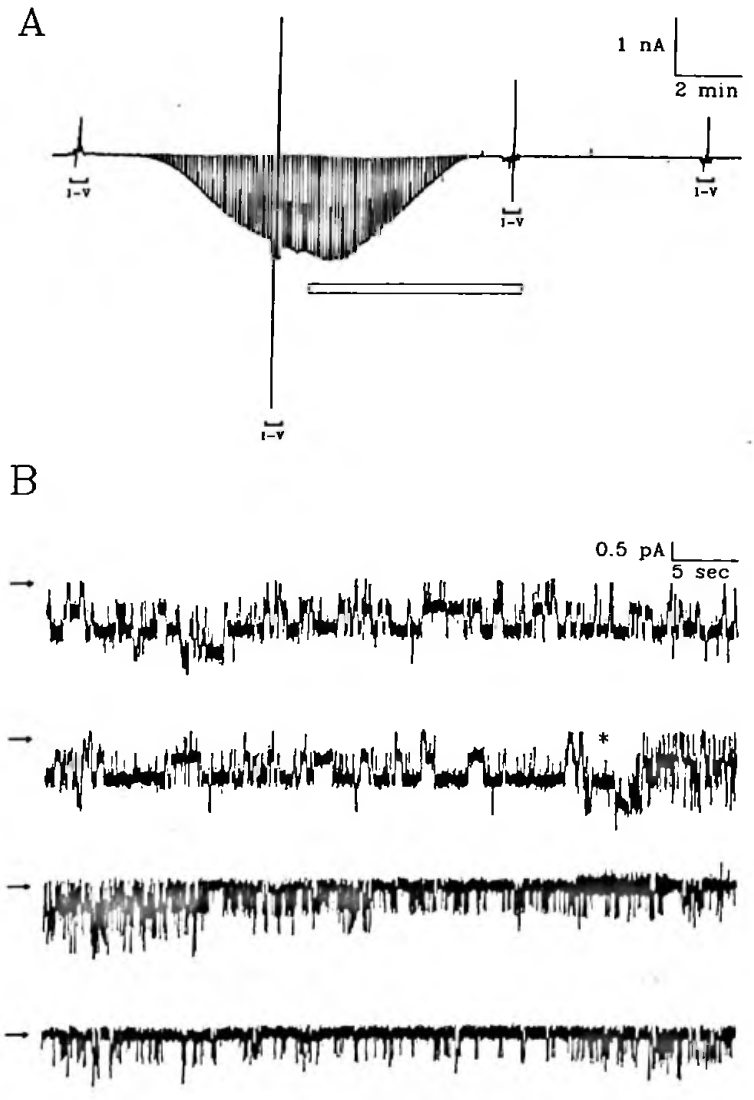

Figure 3. A. Whole-cell current response to 10 μM forskolin and 400 μM cpt-cAMP (present throughout recording), and inhibition by glibenclamide (300 μM; open bar). I-V relationships were run at the indicated times although the data are not shown. B. Effect of glibenclamide (300 μM) on excised, inside-out single channel currents. Glibenclamide was added at the asterisk. Data are from a continuous recording. The patch was held at -100 mV (inside negative). The arrows indicate the closed state of the channel.

These results further suggest that increased cellular cAMP activates a Cl^- channel with biophysical and pharmacological characteristics similar to human CFTR. (Supported by NIH DK38518, DK08810, DK34208, and AHA 92011310).