Bertrand et al., http://www.jgp.org/cgi/content/full/jgp.200810097/DC1

Characterization of the HEK 293 expression system

The HEK 293 expression system was used for many of these studies, and as a generic human cell model it offers many advantages, including ease of transfection and patch clamping. Several endogenous ion channels have been identified in this system, however, and although their contribution is typically small relative to the overexpressed ion channel, their presence could impact results. Of note for these experiments, endogenous Cl^- channels (Zhu et al., 1998) and H^+ channels (Decoursey, 2003) have been identified. The Cl^- channels exhibited voltage activation and outward rectification, being maximally active at positive potentials. Identified H^+ channels were voltage, pH, and temperature sensitive, requiring depolarization, 37°C, and a driving force for H^+ extrusion for activity. Their contribution to current measurements is minimal, however, in the range of 0.2 to 1 pA/pF. HEK 293 cells also endogenously express several K^+ channels (Jiang et al., 2002). The impact of these channels was minimized in these studies through the use of NMDG-Cl solutions.

We examined the behavior of endogenous channels in HEK 293 to confirm they had minimal impact on our measured currents. The majority of control (GFP alone) or CFTR-expressing cells had minimal current on break-in at our standard holding potential of −40 mV (Fig. S1). The I/V curves of 67% of cells demonstrated a sigmoidal response, with a small current at positive holding potentials on break-in that disappeared within 60 s (Fig. S1 A). The transient behavior of this positive current matches

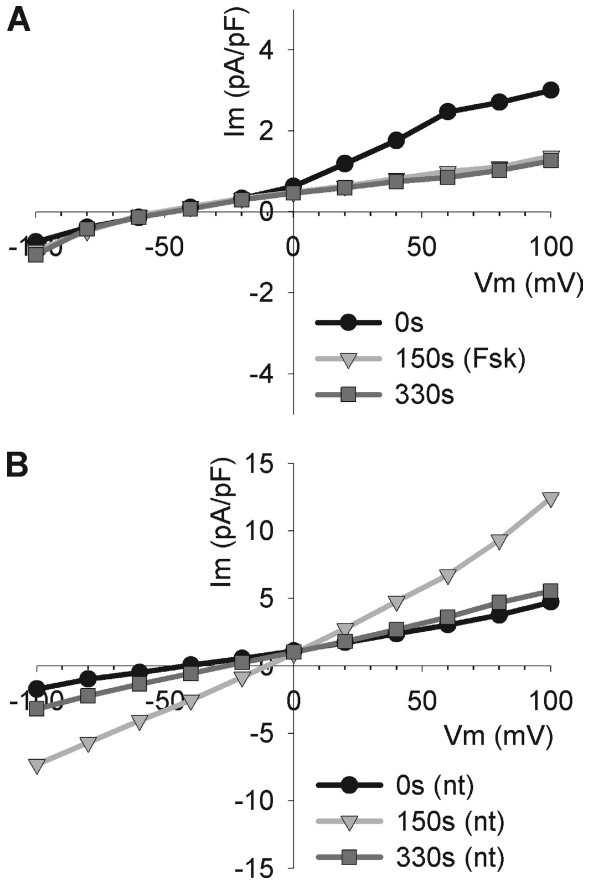

Figure S1. Endogenous conductance properties of HEK 293 cells. Two types of conductance were observed on break-in, and both rapidly ran down. (A) 67% of control or CFTR-expressing cells (basal conditions) exhibited a small, sigmoidal I/V relation with a reversal potential of −40 mV. (B) The remaining 33% exhibited a small, outward-rectified I/V relation. In <5% (shown), the current briefly increased before rundown. nt, no treatment.

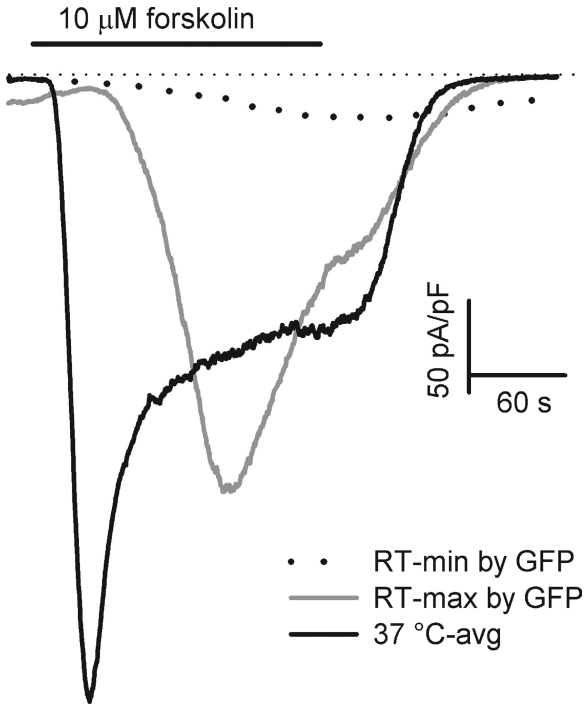

Figure S2. CFTR currents are temperature sensitive in HEK 293 cells. Experiments performed at either 22°C (RT, dotted and gray trace) or 37°C (black trace) demonstrate significant differences in forskolin-stimulated response. The RT min and max responses correlated with GFP fluorescence intensity and represent the extremes of six experiments. Both peak current and the kinetics of response are affected by temperature. $V_H = -40$ mV.

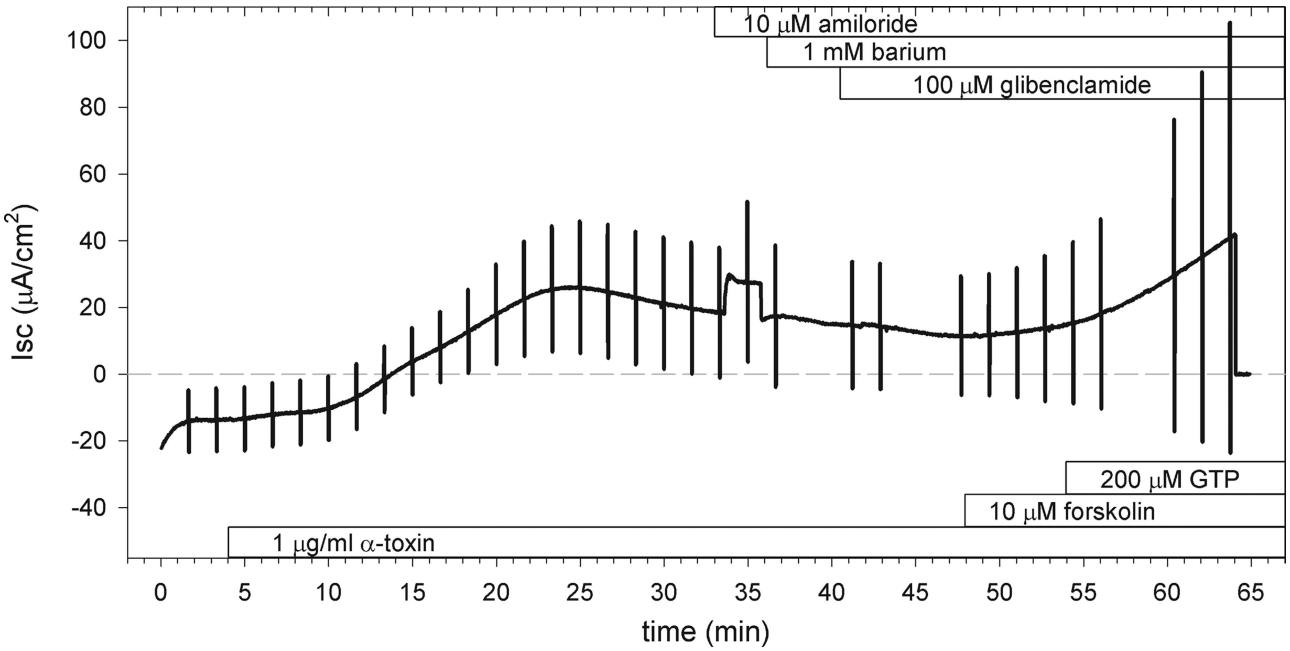

Figure S3. HBE monolayers permeabilized with α-toxin failed to respond to forskolin without ATP in the basolateral bath solution. The lack of forskolin response is not due to pretreatment with glibenclamide, as both HEK 293 cells and HBE monolayers expressing wtCFTR demonstrated that glibenclamide inhibited ≤52% of the forskolin-stimulated current (Figs. 7 A and 10 A in Results). Even with a small dose of α-toxin (1 μg/ml), monolayer resistance abruptly drops after a 1-h exposure. A similar experiment including basolateral ATP is shown in Fig. 10 A and exhibits the required stability.

the description of H^+ channel behavior in HEK 293 cells (Decoursey, 2003), and it was unaffected by forskolin. We recorded a small current in 33% of cells on break-in (either GFP alone or CFTR) that demonstrated an outward-rectified I/V curve (Fig. S1 B). In fewer than 5% of these cases (shown in Fig. S1 B), this current transiently increased, and then recovered over the course of several minutes. Overall, the magnitudes of endogenous currents were substantially less than those produced by expression of the SLC26A9 ± CFTR constructs.

Recorded forskolin-stimulated CFTR currents (see Fig. 6 A in Results) were greater than previously published data obtained at room temperature (Lee et al., 1999; Ko et al., 2004) and included an initial overshoot with recovery to a plateau. We repeated our experiments at room temperature and recorded lower peak and plateau currents with slower activation and recovery after washout (Fig. S2), in line with these previous studies. Because of the temperature sensitivity of many cellular functions potentially contributing to CFTR activity (i.e., phosphorylation, ATP hydrolysis, etc.), we performed all of our experiments at 37°C.

Nystatin and α-toxin permeabilization of HBE monolayers

We first tested the use of nystatin to permeabilize the basolateral membrane, a method used successfully in isolating cation transport in HBE monolayers (Devor and Pilewski, 1999). HBE monolayers permeabilized with 250 μM nystatin (the maximum concentration we were able to solubilize in 0.1% DMSO) did not generate the absorptive Isc expected with a mucosal to serosal chloride gradient (E_{Cl} = −13 mV; Isc remained secretory), and forskolin-stimulated currents remained sensitive to basolateral application of bumetanide. The permeability of nystatin to anions can be as low as 25% of its permeability to cations (Lewis et al., 1977), however, and we concluded that the basolateral permeabilization obtained with nystatin was insufficient to eliminate the basolateral membrane resistance to transcellular chloride fluxes, and thus would not electrically isolate I/V relations to those of the apical membrane alone.

The use of α-toxin to assess CFTR function through permeabilization of the basolateral membrane has been established in T84 cells (Ostedgaard et al., 1992; Hallows et al., 2003) and human sweat duct (Reddy and Quinton, 2003). We started with the relatively low dose of 5 μg/ml and found that, although the basolateral membrane was successfully permeabilized in <15 min, monolayer integrity rapidly deteriorated within 15 min of the start of an experiment, as indicated by transepithelial resistance (R_T) dropping to the level of the fluid resistance. This may have been due to the toxin traversing the cell to permeabilize the apical membrane, or through degradation of the tight junctions. Extracellular calcium was maintained at normal levels at all times in the apical bath, although the basolateral calcium levels were nominally zero (no added calcium, but no added EGTA).

Next, we decreased the dose of α-toxin to 1 μg/ml, lower than that tested previously with T84 monolayers but recommended in a review of pore-forming toxins (Bhakdi et al., 1993). We were able to successfully permeabilize the basolateral membrane within 15 min; however, after the addition of cation channel blockers and glibenclamide, there was no response to forskolin (Fig. S3). Suspecting intracellular depletion of ATP, we revised the protocol to include both 100 μM ouabain, to inhibit the NaK-ATPase and thus limit ATP consumption, and added 1 mM Na-ATP to the basolateral solution to maintain intracellular nucleotide stores. Both were added after initial evidence of α-toxin permeabilization, as identified by the rapid drop in current. Neither addition changed the shape of the permeabilization response, indicating also that addition of ATP to the basolateral solution did not activate purinergic receptors (compare Fig. S3 with Fig. 10 A in Results).

Actions of other chloride channel blockers

The alternate CFTR inhibitor CFI-172 did not significantly inhibit SLC26A9 currents across permeabilized HBE after 3 min of application (currents remained at 78.6 ± 12.7% of basal; $n = 3$); however, we also found that CFI-172 did not inhibit wtCFTR currents within a similar time frame in whole cell patch clamp experiments on HEK 293 cells (not depicted). We did not test longer applications of the drug given the rapid and differential actions of GlyH-101 and glibenclamide on SLC26A9 (Fig. 4 A) and CFTR currents (Fig. 7 A). Dorwart et al. (2007) reported that the calcium-activated chloride conductance (CaCC) inhibitor, niflumic acid (NA), inhibited SLC26A9 with an EC_{50} of 0.4 mM, and that DIDS had no effect. We found that 250 μM NA blocked 61% of the constitutive SLC26A9 current, similar to GlyH-101; unfortunately, this dose of NA also abolished the response of wtCFTR to forskolin (from a peak current of −214 pA/pF [Fig. 6 B] to −24 pA/pF), as had been noted previously (Scott-ward et al., 2004). Because this dose of NA greatly exceeds the inhibition constant for NA on CaCC (17 μM) (White and Aylwin, 1990), and is not specific for SLC26A9, we did not pursue it further. We also found that DIDS did not have an effect on the chloride currents of SLC26A9 (not depicted), in agreement with Dorwart et al. (2007).

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