Inositol   PIP$_2$ PIP$_3$ Fluorescein-PIP$_2$-HG
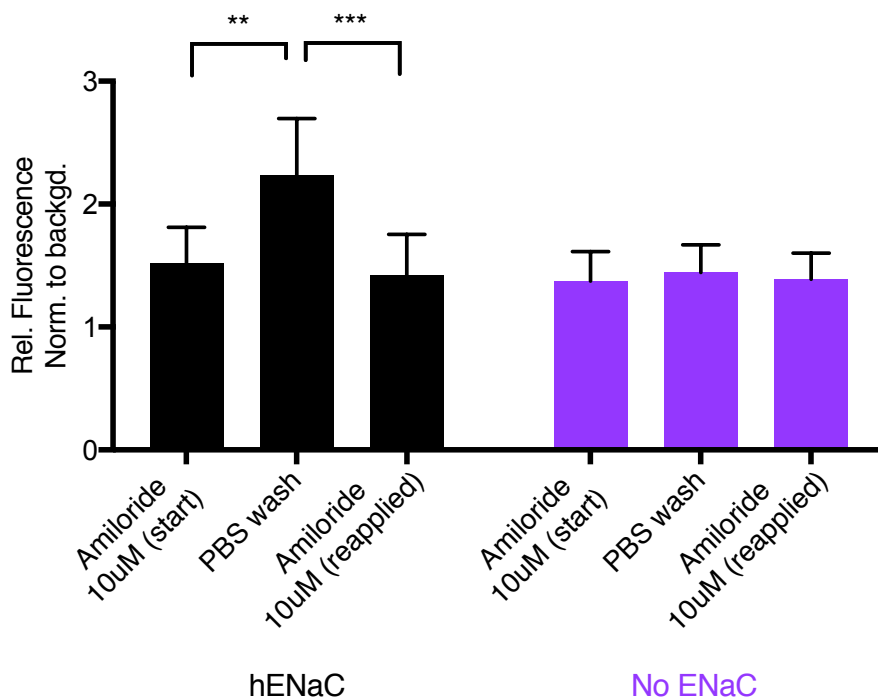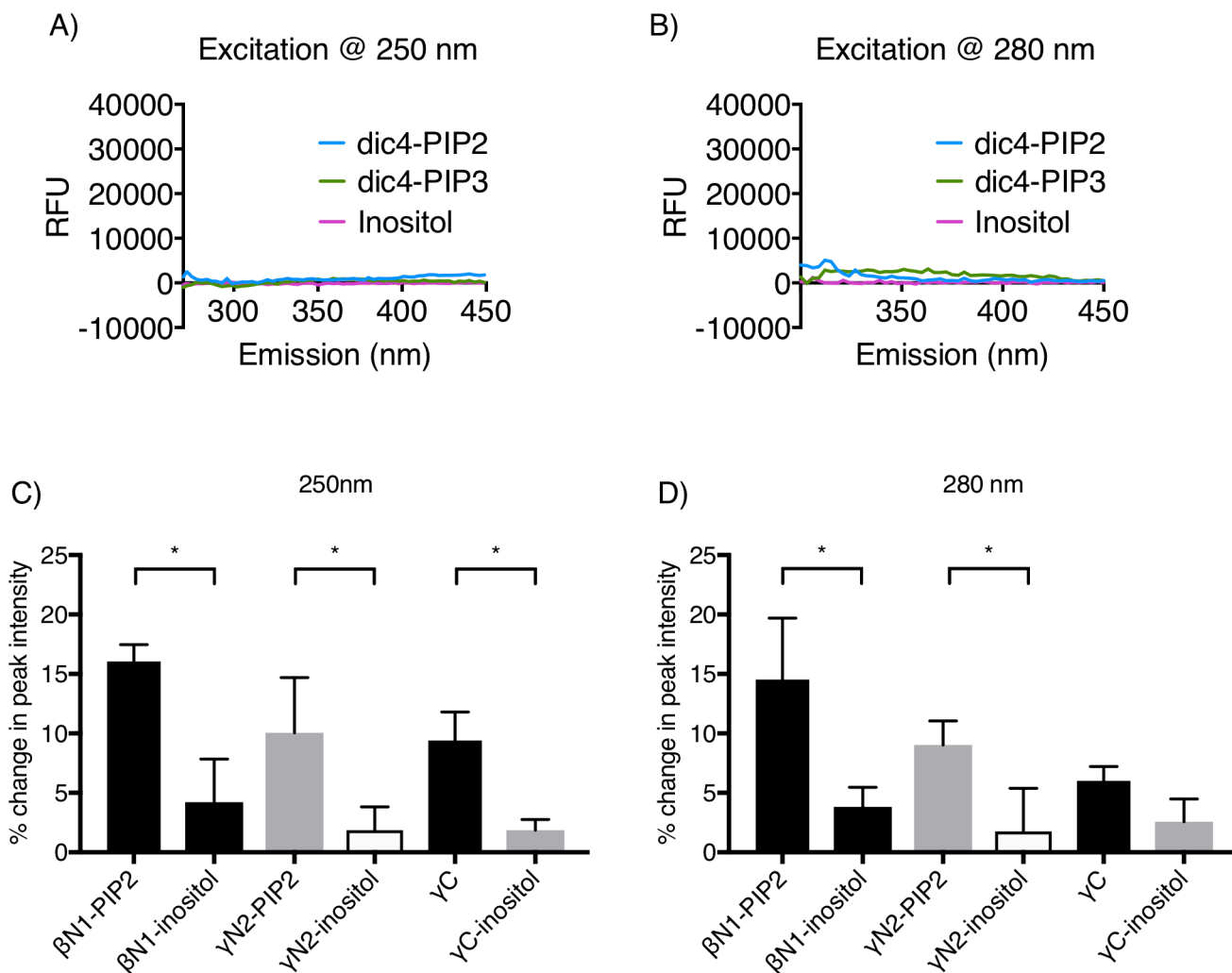
Supplemental Figure S1. Chemical structure for the PIP$_2$ analogs used in this study. Number indicates the carbon position within the inositol ring. Each ring has hydroxyl groups and phosphoryl groups. The latter are indicated by orange boxes at positions C1, C3, C4, and C5. Inositol, left, a binding control, has no phosphoryl groups. The glycerol-fatty acid groups, indicated by the blue lines labeled "FA", and fluorescein, indicated by the green box, are attached to the C1 phosphoryl group of PIP$_2$, PIP$_3$ and the labeled headgroup of PIP$_2$, Fluoroscein-PIP$_2$-HG. Schematics are not to scale. Inositol and the unlabeled PIP$_{2/3}$ analogs were used in SSIF spectroscopy experiments, and Fluoroscein-PIP$_2$-HG was used for MST experiments.

Supplemental Figure S2. **Fluorescence imaging data of CoroNa Green levels.** Relative fluorescence of cells containing CoroNa Green in the presence (black bars) and absence (purple bars) of eCFP-ENaC. Data represent cells that were first imaged in 10 μM amiloride, followed by PBS without amiloride and 10 μM amiloride reapplied to the cells. Data represent n=10-12 cells for each condition, ±SD. Significance determined by two-way ANOVA followed by Dunnett's t-test compared to 10 μM PBS + hENaC ; **p<0.005; *** p<0.0001.

A)

Excitation @ 250 nm

B)

Excitation @ 280 nm

C)

D)

Supplemental Figure S3. **Steady state intrinsic fluorescence data for phosphoinositides.** Emission spectra in response to excitation at 250 nm **(A)** and 280 nm **(B)** for 10 μM diC4-PIP$_2$ (cyan), diC4-PIP$_3$ (green) and inositol (pink). **C, D.** A comparison of the changes in peak intensities of βN1, γN2 and γC peptides in response to diC4-PIP$_2$ with changes after addition of the binding control, 10 μM inositol. No significant difference was noted between peaks of peptide and peptide + inositol. Data are expressed as the mean % change in peak ±SD and represent n=3, where *, $p < 0.05$.

Template alignment │ Template 3D Structure │ PDBe

1. **6AVE_B** Acid-sensing ion channel 1; Ion channel, ASIC, ASIC1a, Sodium; HET: NAG;{Gallus gallus}; Related PDB entries: 6AVE_C 6AVE_A

   Probability: 92.52%, E-value: 0.0086, Score: 41.07, Aligned cols: 33, Identities: 15%, Similarity: 0.253,

```
Q ss_pred                    CCHHHHHHHHhcCCCCCCCeeEEee--CCcccccC
Q Q_8704195      20          YTYKELLVWYCDNTNTHGPKRIICE--GPKKKAMW   52 (52)
Q Consensus      20          ~S~~EL~~~fC~NTt~HG~~Riv~s--n~lKt~~W   52 (52)
                             .+.++++..||.+|++||-.+|+.+  ...++++|
T Consensus      13          ~~~~~~~~~~F~~~tsihGl~~i~~~~~~~~~ri~W   47 (527)
T 6AVE_B         13          SGQPVSIQAFASSSTLHGISHIFSYERLSLKRVVW   47 (527)
T ss_dssp                    --------------------------CHHHHHH
T ss_pred                    cCCCchHHHHHHcCccccHHHHhcCCCchHHHHHH
```

Template alignment │ Template 3D Structure │ PDBe

2. **5TTT_A** Degenerin mec-4; Structural Genomics, PSI-Biology, Northeast Structural; NMR {Caenorhabditis elegans}; Related PDB entries: 2K2B_A

   Probability: 75.61%, E-value: 2.1, Score: 28.1, Aligned cols: 21, Identities: 24%, Similarity: 0.311,

```
Q ss_pred                    CHHHHHHHHhcCCCCCCCeeE
Q Q_8704195      21          TYKELLVWYCDNTNTHGPKRI   41 (52)
Q Consensus      21          S~~EL~~~fC~NTt~HG~~Ri   41 (52)
                             +++.++.=||..|+.||-.+|
T Consensus      79          ri~whlKeFC~KTSsHGIPmI   99 (111)
T 5TTT_A         79          RLAWHFKEFCYKTSAHGIPMI   99 (111)
T ss_dssp                    HHCCCCCCCCCCCCCCSCCSS
T ss_pred                    hHHHHHHHHHHhchhcCCCcc
```

Supplemental Figure S4. HHPred analysis of β-ENaC. HHPred analysis indicates that the amino terminus of β-ENaC is similar to that of ASIC and Mec-4. Positions with a red H indicate the regions with helical propensity, suggesting that the amino terminus of β-ENaC may be helical.

Table S1

Table S1: Peptide sequences of proposed PIP$_2$ binding sites of ENaC.

Peptide name	Pochynyuk et al 2007 corresponding site:	Amino Acid Sequence
βN1	βND	MHVKKYLLKGLHRLQKGPGYTYKELLVWY
βN1mutant	βND	MHVEEYLLKGLHELQEGPGYTYKELLVWY
βN2	β1D	TNTHGPKRIICEGPKKKAMW
βC	β2D	KLVALAKSLRQRRAQASYAG
γN1	γNS	MAPGEKIKAKIKKNLPVTGPQAPTIKELMRW
γN2	γ1D	TNTHGCRRIVVSRGRLRRLLW
γN2mutant	γ1D	TNTHGCEEIVVSRGELEELLW
γC	γ2D	ARRQWQKAKEWWAWKQA
γCmutant	γ2D	AEEQWQEAEEWWAWKQA