Supplementary Information for

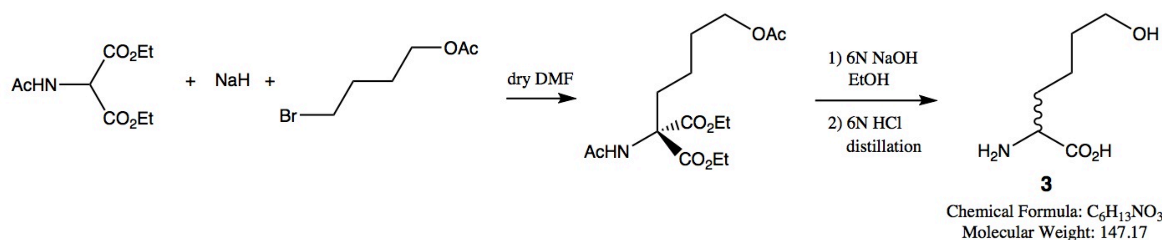
Contributions of Counter-Charge in a Potassium Channel Voltage-Sensor

Stephan A. Pless, Jason D. Galpin, Ana P. Niciforovic and Christopher A. Ahern

Supplementary Methods
Supplementary Results

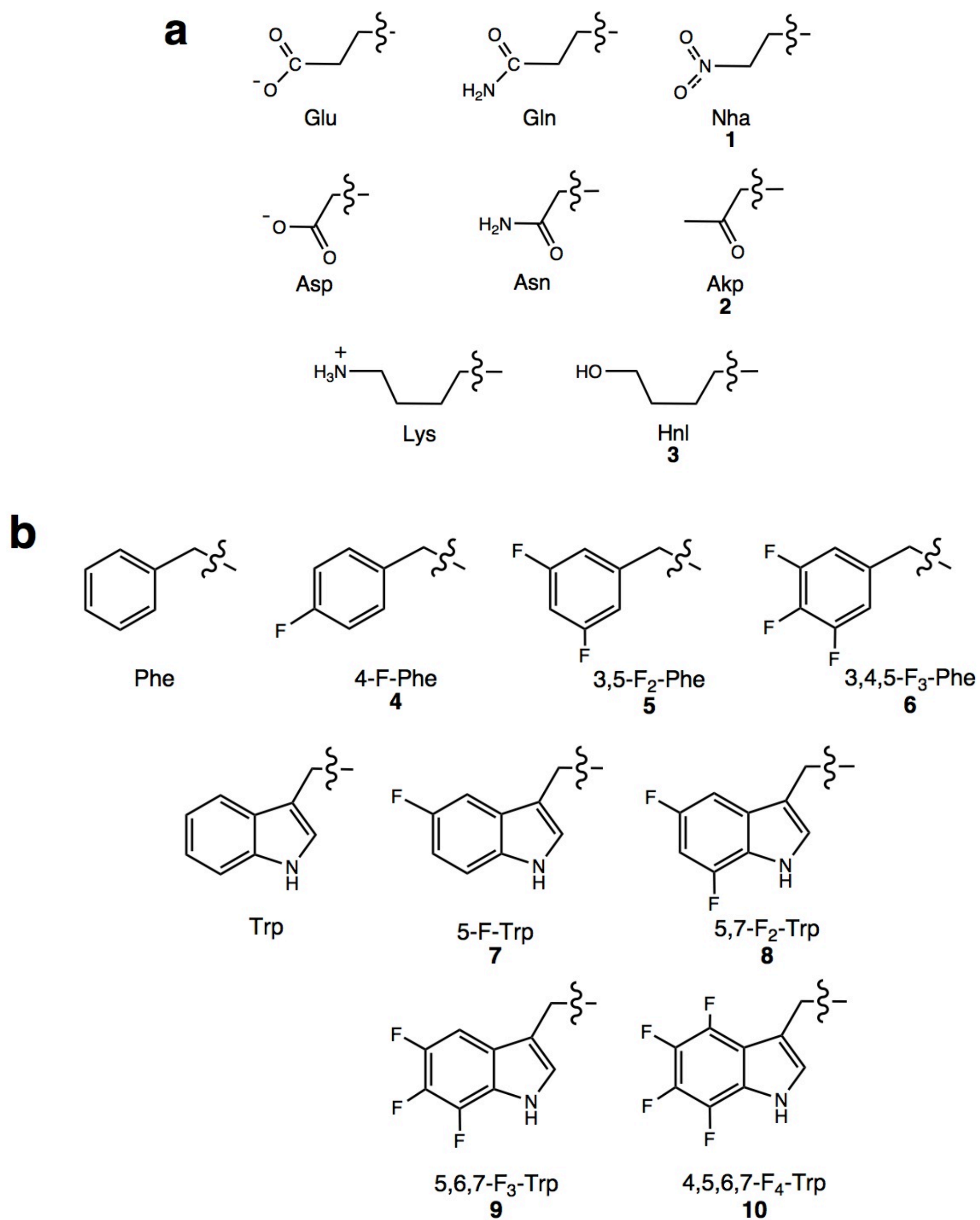
Supplementary Methods

Synthesis of 6-hydroxy norleucine (Hnl)[1]. A stirred suspension of sodium hydride (1.01 g, 25.3 mmol, 60% suspension in oil) in dry DMF (5 mL) was cooled to 0°C under dry nitrogen in an ice bath. A solution of diethyl acetomidomalonate (5 g, 23 mmol) in dry DMF (12 mL) was slowly added over a period of 30 minutes while keeping the temperature around 10°C or lower. The mixture was then allowed to warm to room temperature and stirred 1 hour, afterwhich 4-bromobutyl acetate (4.7 g, 24.2 mmol) was added via syringe. The mixture was stirred at 60°C overnight. The following day the slurry was cooled to room temperature and ethanol (0.5 mL) and glacial acetic acid (50 uL) were added to quench the reaction.

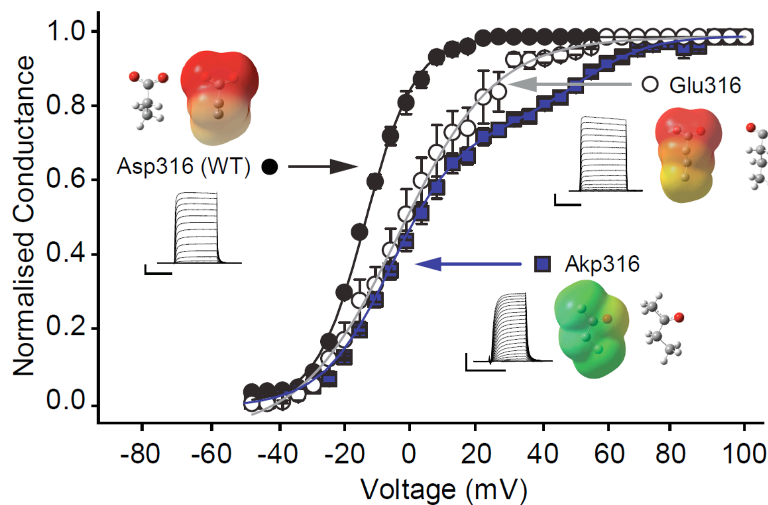


After 15 minutes of stirring the mixture was poured into a 10% solution of lithium chloride (25 mL) and extracted twice with ethyl acetate, which were combined and backwashed with 10% lithium chloride (3 x 30 mL) dried over sodium sulfate and evaporated to give the intermediate (6.6 g, 87%). This material was dissolved in absolute ethanol (3 mL) in a flask equipped with a condenser and sodium hydroxide (6 N, 16 mL) was added and heated to 70°C for 5 hours. The reaction was cooled to room temperature as hydrochloric acid (6 N, 13 mL) was very slowly added to adjust the pH to 1.3, and a still head was added to the condensor, which was configured for a short-path distillation. Subsequently the ethanol was distilled off as the temperature was increased to 90°C and held for 10 hours. The volume of ethanol collected was as expected for loss of two equivalents from the deprotection, plus the 3 mL starting solvent, 5.5 mL. The crude mixture was stripped and concentrated from toluene (2 x 10 mL), triturated with absolute ethanol (10 mL) filtered and rinsed with additional absolute ethanol (5 mL). The filtrate was concentrated to give a viscous oil which contained residual ethanol and toluene. A small amount of the product 6-hydroxy norleucine (**3**) was recrystallized from hot ethyl acetate/ethanol and stored at room temperature as a flakey white solid (100 mg, 3%). Mass calculated for C6H13NO3: 147.2, found: 148.4 (M+1).

Supplementary Results

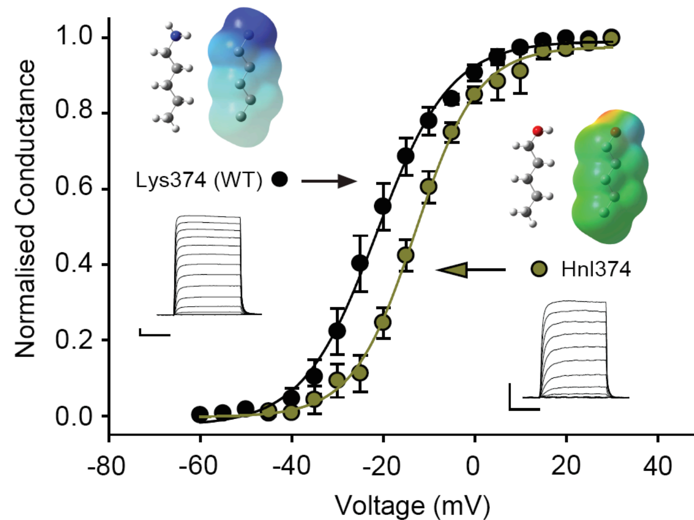


Supplementary Figure 1. Structures of the natural and unnatural amino acids used in this study. (a) Natural and unnatural derivatives of glutamic acid, aspartic acid and lysine (b) Fluorinated derivatives of phenylalanine and tryptophan.

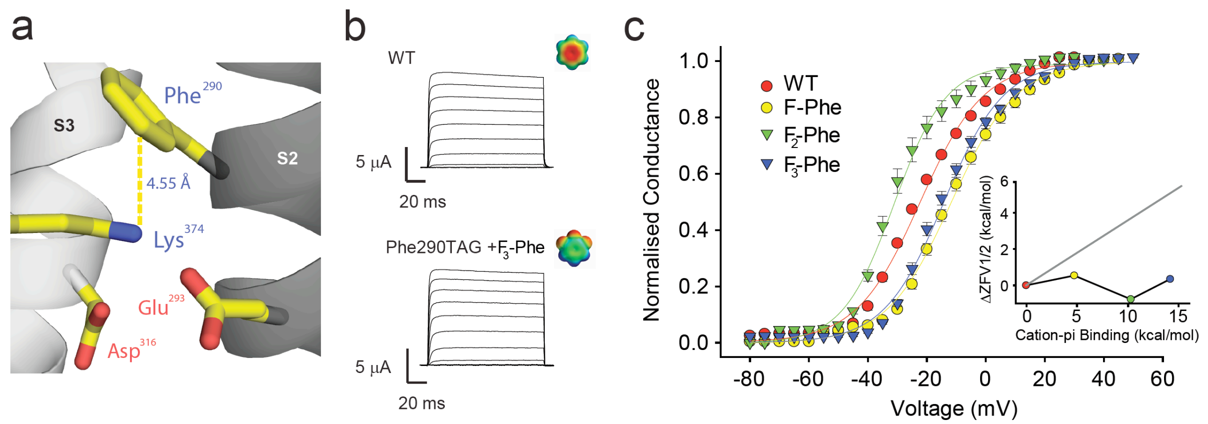


Supplementary Figure 2. Effects of glutamic acid and a keto-analog of aspartic acid in position 316. Introduction of glutamic acid in position 316 led to a modest but not statistically significant ($p > 0.05$) right-shift in the GV compared WT ($V_{1/2}$ = -11.7 ± 5.3 mV, n = 6). Together with our findings from Nha316 this suggests that a negative charge alone in position 316 is not necessary to support for normal channel function. Although the observed right-shift with Glu316 suggests that the length of the side chain in position 316 may be important, the WT-like behavior of Nha316 clearly demonstrates that this is only true for charged, but not neutral side-chains. The more severe phenotype of the longer and negatively charged Glu316 (compared to Asp316 or Nha316) could be due to the closer proximity of a negative charge in position 316 to the positive charges of S4. This possibility would also explain why the homologous side-chains to *Shaker* Asp316 are invariably Asp side chains in other voltage-gated proteins, while position 293 can tolerate both Glu and Asp in different proteins (Fig. 1).

We also introduced the neutral keto analog of aspartic acid, 2-amino-4-ketopentanoic acid (Akp, Supplementary Figure S1) [2] at position 316. This substitution led to a marked reduction in macroscopic currents and produced channels with a GV relationship that contained two distinct components: the first, comprising around 75% of the total signal, only showed a minor right-shift compared to WT ($V_{1/2}$ = -10.0 ± 1.5 mV), while the second was shifted to very depolarized potentials ($V_{1/2}$ = 50.0 ± 3.0 mV) (n = 9). We reasoned that the second, smaller component could possibly be due to residual steric clashing of the Akp methyl group hydrogens and other residues in the area (potentially hydrogens from S4 charges). This is consistent with the results obtained from Asn at position 316, the amino group of which is a much stronger hydrogen bond donor than the keto group of Akp, and consequently leads to a right-shifted GV with a single component. Insets show currents, ESP maps (red = -100 kcal, green = 0 kcal, blue = +100 kcal) and energy-minimized structures of amino acids used at position 316. Scale bars: 2 μA for current, 50 ms for time.



Supplementary Figure 3. Incorporation of a neutral lysine analog at position 374.
Our data support the notion that Asp316 and Glu293 do not participate in an electrostatic interaction that stabilizes a particular state of the channel, such as that predicted with Lys374. If this was true, then neutralizing Lys374 should also have a negligible effect on channel function. As no naturally occurring neutral lysine analog is available, we generated 6-hydroxy norleucine (Hnl), in which the ε amino group is replaced by an ε hydroxy group (Supplementary Fig. S1). Incorporating this isosteric neutral lysine analog at position 374 only led to a small right-shift in the conductance-voltage relationship ($V_{1/2}$ = -13.6 ± 1.1 mV for Hnl374 (n = 5) vs. $V_{1/2}$ = -22.3 ± 1.1 mV for Lys374 (WT) (n = 4); note that both constructs carried no serine substitutions at positions 301 and 308 to maximize expression). Consistent with the observations from neutralized side-chains at positions 293 and 316, the data do not support the role for a functionally important electrostatic interaction including Lys374 in the channel open state. Insets show currents, ESP maps (red = -100 kcal / mol, green = 0 kcal / mol, blue = +100 kcal / mol) and energy-minimized structures of amino acids used at position 374. Scale bars: 1 μA for current, 50 ms for time.



Supplementary Figure 4. The electronegative surface potential of Phe290 does not contribute to a cation-pi interaction (a) Model of the gating charge transfer center highlighting Phe290, Lys374, Asp316 and Glu293 (PDB 2R9R); (b) Current traces recorded from WT channels (upper panel) and channels with 3,4,5-F_3-Phe incorporated at position 290 (lower panel); voltage pulses from -80 to +30 mV in 10 mV increments. Insets show ESPs for Phe and 3,4,5-F_3-Phe; scale for ESPs: red = -25 kcal / mol, green = 0 kcal / mol, blue = +25 kcal / mol. (c) GVs for fluorinated Phe derivatives at position 290. The cation-pi plot in the inset clearly shows the lack of a trend for fluorinated Phe derivatives at position 290 (black line). For comparison, the fit derived from fluorinated Trp derivatives at position 290 is shown in grey (reproduced from Fig. 5).

	Amino Acid	$V_{1/2}$ (mV)	Z	$ZFV_{1/2}$ (kcal/mol)	ΔZFV1/2 (kcal/mol)	n
Phe290TAG (WT)	Trp	-60.06 ± 0.83	6.17 ± 0.36	-8.51 ± 0.47	-	5
	5-F-Trp	-57.13 ± 1.08	5.99 ± 0.36	-7.89 ± 0.57	0.6167	5
	5,7-F_2-Trp	-51.69 ± 0.54	5.16 ± 0.43	-6.15 ± 0.53	2.365	5
	5,6,7-F_3-Trp	-39.74 ± 1.00	4.41 ± 0.43	-4.29 ± 0.39	4.2182	5
	4,5,6,7-F_4-Trp	-38.01 ± 1.10	3.86 ± 0.54	-3.41 ± 0.55	5.1037	7
Arg374, Phe290TAG	Trp	30.02 ± 1.05	1.69 ± 0.13	1.16 ± 0.10	-	6
	5-F-Trp	34.72 ± 2.53	1.76 ± 0.25	1.37 ± 0.13	0.2097	4
	5,7-F_2-Trp	37.88 ± 1.10	1.84 ± 0.24	1.59 ± 0.18	0.4312	4
	5,6,7-F_3-Trp	52.44 ± 0.92	2.13 ± 0.27	2.57 ± 0.33	1.4109	6
	4,5,6,7-F_4-Trp	68.72 ± 0.59	2.35 ± 0.13	3.51 ± 0.08	2.3462	4

Supplementary Table 1: Effect of fluorination for different Trp derivatives introduced at position 290 on the WT or the Arg374 background. Values for $V_{1/2}$ and Z were obtained by fitting the data to a two-state Boltzmann function; the free energy between open and closed states is shown as $ZFV_{1/2}$ or as $\Delta ZFV_{1/2}$ for comparison with the non-fluorinated parent Trp.

Supplementary References

1. Robl, J. A. *et al. J Med Chem* **39**, 494-502 (1996).
2. Cashin, A. L., Torrice, M. M., McMenimen, K. A., Lester, H. A. & Dougherty, D. A. *Biochemistry* **46**, 630-639 (2007).