Supplementary Material

Chernysh *et al.* Sodium-dependent Inactivation of Sodium-Calcium Exchange in Transfected Chinese Hamster Ovary Cells

Effect of Carbachol on PIP2 levels in M1-transfected CHO cells. Figure S-1 shows images of CHO cells stably transfected to express the human M1 receptor; the cells were kindly provided by Dr. Donald Hilgemann, UT Southwestern Medical Center, Dallas, TX. We transfected the cells to express the canine WT NCX, but NCX activity was low and so they were not used in activity measurements. The cells had then been transfected to transiently express a fusion protein between GFP and the plekstrin homology (PH) domain of phospholipase Cδ1 (PLCδ1PH-GFP). The cDNA for the PLCδ1PH-GFP fusion protein was kindly provided by Dr. T. Balla (NICHD, NIH, Bethesda, MD). The PH domain binds tightly to PIP2 and so the distribution of the probe mirrors the distribution of PIP2.

The upper row of images shows the distribution of the fusion protein before and 30 or 120 s after the addition of 100 μM carbachol. Before carbachol addition, the fluorescence intensity was highly enriched in the plasma membrane, as expected. Following carbachol addition, fusion protein fluorescence became more evenly distributed through the cytosol, consistent with the hydrolysis of a large fraction of the membrane PIP2 (26, 27). The lower row of images shows the distribution of PLCδ1PH-GFP fusion protein fluorescence of a cell treated with 100 μM ATP. The fluorescence remained highly localized to the plasma membrane following ATP addition; when carbachol was added to the cell 3 min after ATP, fluorescence again became delocalized within 30 s. ATP interacts with purinergic receptors in CHO cells and induces the release

of Ca from internal stores, presumably by hydrolyzing enough PIP2 to effect Ca release by the inositol-3,4,5-trisphosphate receptor in the ER. However, the overall decline in PIP2 is too small to be detectable with the fusion protein, either because ATP is a weaker agonist than carbachol or because the purinergic receptors desensitize rapidly. The results support the comparison of ATP vs carbachol in discerning the effects of PIP2 depletion on NCX activity (Figs. 3 and 4 in main report).

Effect of carbachol in non-transfected CHO cells. The data in Fig. S-2A show that carbachol by itself did not induce Ca^{2+} release in WT NCX cells, consistent with the absence of an endogenous M1 receptor. Addition of carbachol + Tg induced a slow release of Ca^{2+} which reflected the action of Tg in inhibiting SERCA and bringing about a gradual release of Ca^{2+}. The subsequent initiation of reverse-mode NCX activity by application of 0.1 mM $CaCl_2$ in K-PSS induced a rapid uptake of Ca^{2+}. In Fig. S-2B, the data from Fig. 3 of the main report for the cells responding to carbachol are reproduced (filled circles), along with the data for the cells from the same cover slips that did not respond to carbachol (open circles). The non-responding cells behaved essentially the same as the cells shown in panel A, which had not been subjected to the transfection procedure. Note that the rate of Ca^{2+} uptake by the non-responding cells was greater than for the responding cells. This is because NCX is allosterically activated by the increased $[Ca^{2+}]_i$ in these cells, due to the slow release of Ca^{2+} from internal stores elicited by Tg. These experiments, by analogy to those shown in Fig. 3, were conducted at room temperature.

Figures S-2C and S-2D show the corresponding data for gramicidin-treated cells incubated in Na-PSS at 37^o, assayed under the conditions depicted in Fig. 4 of the main

report. Again, cells that had not been subjected to the M1 transfection procedure did not respond to carbachol (panel C) and, after transfection, the non-responding cells (open circles) behaved similarly (panel D).

PIP2 depletion and NCX activity. Figure S-3 compares the values for v_{max} (panel A), K_h (panel B) and the time-to-v_{max} (panel C) for the data shown in Figure 3 of the main report. For the WT and K229Q cells, the v_{max} values after carbachol treatment were 2.0-fold ($p<0.005$) and 1.5-fold ($p = 0.012$) greater than after ATP (panel A). There was no significant difference in the K_h values (panel B). The time-to-v_{max} values tended to be reduced after carbachol, but this was significant only for the K229Q cells ($p<0.005$).

Allosteric Ca activation of NCX at pH 6.4. CHO cells expressing the WT, K229Q, F223E and Δ(241-680) exchangers were treated with ATP/Tg and gramicidin in 10/130 Na/K-PSS as described in Methods. Four minutes prior to beginning recordings, the medium was changed to 10/130 Na/K-PSS buffered to either pH 6.4 or 6.9 with MES/Tris or Mops/Tris. Two minutes prior to beginning the experiment, the cells were treated additionally with 10 μM nigericin and 10 μM monensin; the combination of nigericin and monensin ensured that the cytosolic pH would equilibrate with the external pH within 60 s. Transport was initiated by applying 5 ml of 0.1 mM $CaCl_2$ in the same media used for the pre-equilibration. Ca uptake data are shown in Fig. 5 of the main report. Analysis of the results in terms of v_{max} and K_h was carried out as described in Methods.

Figure S-4 compares the values for v_{max} (panel A), K_h (panel B) and the time-to-v_{max} (panel C) for the data obtained at pH 7.4 and pH 6.4 for the WT exchanger and various mutants listed. At pH 7.4, the v_{max} value for the cells expressing F223E was 56%

greater than for the WT exchanger ($p<0.01$). This result differs from the data in Figure 1 of the main report, where the v_{max} values at room temperature for the WT, K229Q and F223E exchangers were not significantly different. Perhaps the higher temperature (37^{o} C), or the use of monensin and nigericin in the present experiments accounts for the difference in the two sets of results.

At pH 6.4, the v_{max} value for cells expressing the K229Q exchanger was 51% greater than for the WT exchanger ($p<0.03$). There were no significant differences among the WT, K229Q or F223E exchangers in K_h values at pH 7.4 or at pH 6.4. The K_h values for the WT and F223E exchangers at pH 6.4 were significantly greater than at pH 7.4 ($p<0.002$); for the K229Q cells, the K_h value at pH 6.4 (290 ± 40 nM) was not significantly higher than that at pH 7.4 (184 ± 18 nM) ($p = 0.53$), perhaps because of the variability of the results obtained at pH 6.4. The time-to-v_{max} values, i.e. the time elapsed from the addition of Ca until v_{max} was attained, were much higher at pH 6.4 than at pH 7.4; at the lower pH, the time-to-v_{max} for the K229Q expressing cells (71 ± 15 s) was significantly lower than for the WT (129 ± 13 s)($p < 0.03$).

Figure S-5 compares the v_{max} values for the Ca uptake data in 140 mM Na (100 mM Na for F223E) at pH 7.4, 6.9 and 6.4 for cells expressing the WT exchanger or the mutants listed (see also Fig. 6 in main report). The v_{max} for the K229Q cells was nearly 3-fold higher than for the WT cells ($p<0.003$), suggesting that under these experimental conditions, the WT cells may have shown some inactivation. The value for the Δ(241-680) cells was nearly 40% higher than for the WT cells ($p\sim0.03$) whereas the v_{max} for the F223E mutant was only 33% of the value for the WT cells ($p\sim0.002$). At pH 6.9 and 6.4, all v_{max} values were significantly different from those at pH 7.4 (p values ranging from

10^{-5} to 0.03) with the exception of the value at pH 6.9 for the cells expressing the K229Q exchanger. Note that no data were obtained at pH 6.9 for the cells expressing the Δ(241-680) mutant.

FIGURE LEGENDS – SUPPLEMENTARY MATERIAL

Figure S-1. PIP2 depletion induced by carbachol in CHO cells expressing the M1 receptor. CHO cells stably expressing the human M1 receptor were transiently transfected to express the PLCδ1PH-GFP fusion protein. The upper row of images shows fusion protein fluorescence before and 30 or 120 s after the addition of 100 μM carbachol. The lower row of figures shows the distribution of PLCδ1PH-GFP fluorescence in a cell before and after the addition of 100 μM ATP. Three minutes after ATP addition, 100 μM carbachol was added and the final image was taken 30 s later. The traces below the figures show the fluorescence intensity profiles along the lines indicated in the left hand panel; the intensity profiles were obtained using the ImageJ program available from NIH (http://rsb.info.nih.gov/ij/download.html).

Figure S-2. Lack of effect of carbachol in WT NCX cells. A. Cells expressing the WT NCX were incubated in Na-PSS + 0.3 mM EGTA. Carbachol (100 μM) was applied at 30 s – note that no $[Ca^{2+}]_i$ transient was elicited. At 90 s, 100 μM carbachol and 2 μM Tg were applied. In this case there was a gradual rise in $[Ca^{2+}]_i$ due to inhibition of SERCA by Tg; a rise in $[Ca^{2+}]_i$ with similar kinetics was seen when Tg alone was applied (data not shown). At 150 s, NCX activity was initiated by applying 0.1 mM $CaCl_2$ in K-PSS. The data points are the mean values (+SEM bars) obtained with 3 cover slips. B. Cells expressing the WT NCX were transfected to express the M1 receptor as described in Methods. At 30 s, 100 μM carbachol + 2 μM Tg was applied and at 120 s, 0.1 mM $CaCl_2$ was applied in K-PSS. Data from cells showing a rapid release of Ca^{2+} are depicted by the filled circles; the data from the remaining cells, which show only a slow Ca^{2+} release

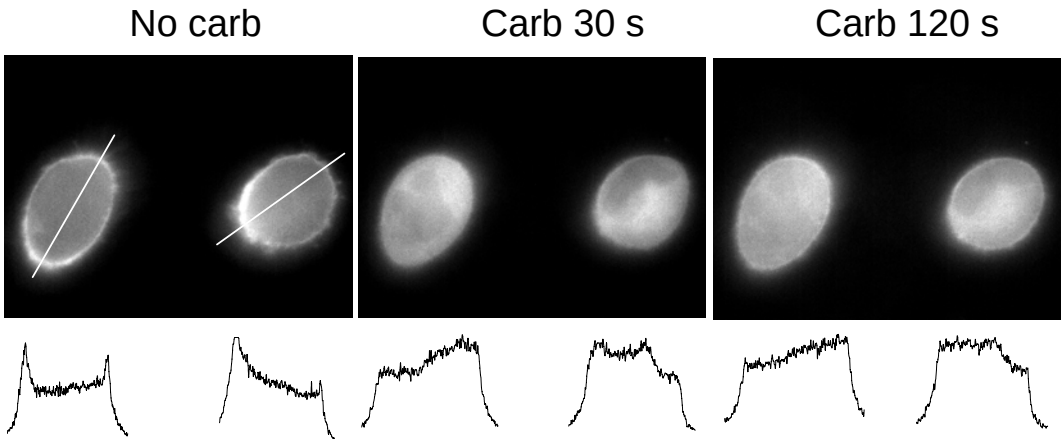
due to Tg, are depicted by the open circles (n = 7). The experiments shown in panels A and B were conducted at room temperature. C. Cells expressing the WT NCX were preincubated for 8 min with 1 μg/ml gramicidin in Na-PSS + 0.3 mM EGTA. Carbachol was added at 30 s, followed by carbachol + Tg at 90 s and 0.1 mM $CaCl_2$ in Na-PSS at 180 s. As in panel A, carbachol by itself did not elicit release of Ca^{2+} from internal stores. D. Cells expressing the WT NCX were transfected to express the M1 receptor as described in Methods. The cells were preincubated for 8 min in Na-PSS containing gramicidin. Carbachol + Tg was applied at 30 s, followed by 0.1 mM $CaCl_2$ in Na-PSS at 120 s. Data form cells showing rapid Ca^{2+} release in response to carbachol/Tg are depicted by the filled circles (reproduced from Fig. 4A); data from the non-responding cells are shown by open circles. The experiments in panels C and D were conducted at 37^{o}.

Figure S-3. Cells were transiently transfected with cDNA for the human M1 receptor as described in Methods. The cells were incubated in Na-PSS + 0.3 mM EGTA and then treated with either 100 μM ATP or 100 μM carbachol. For the carbachol-treated cells, only cells that exhibited a rapid $[Ca]_i$ transient in response to carbachol were analyzed (cf. Figure S-2 above). Ninety seconds after adding ATP/carbachol, NCX activity was initiated by applying 0.1 mM $CaCl_2$ in K-PSS; the Ca^{2+} uptake data are shown in Fig. 3 of the main report. The data were analyzed to determine v_{max} (panel A), K_h for allosteric Ca^{2+} activation (panel B) and the time required for Ca^{2+} uptake to achieve v_{max} (time to v_{max}; panel C) as described in methods. * p~0.01 or less.

Figure S-4. V_{max}, K_h and time-to-ν_{max} values for Ca uptake at pH 7.4 and 6.4 in 10/130 Na/K-PSS. NCX activity was initiated by applying 0.1 mM Ca in 10/130 Na/K-PSS to cells pre-equilbrated in 10/130 Na/K-PSS + 0.3 mM EGTA with gramicidin, monensin and nigericin, as described in the main report. Time courses of Ca uptake are presented in Fig. 5 of the main report. Determination of ν_{max}, K_h and time-to-ν_{max} was carried out as described in Methods. A. V_{max} values for cells expressing WT, K229Q, F223E and Δ(241-680) exchangers. B. K_h values for cells expressing WT, K229Q and F223E exchangers. C. Time-to-ν_{max}, i.e. time elapsed from application of Ca until ν_{max} attained, for WT, K229Q and F223E exchangers. See text for statistical tests.

Figure S-5. V_{max} values for Ca uptake at pH 7.4, 6.9 and 6.4 in high [Na]. Cells were pre-equilbrated with gramicidin + monensin + nigericin in Na-PSS (WT, K229Q, Δ(241-680)) or 100/40 Na/K-PSS (F223E), pH 7.4, 6.9 or 6.4, and exchange activity was initiated by applying 0.1 Ca in the same medium used for pre-equilibration. Time courses of Ca uptake are presented in Fig. 6 of the main report. Determination of ν_{max} was carried out as described in Methods. See text for statistical tests.

Experiment 1 - carbachol



Experiment 2 – ATP, then carbachol

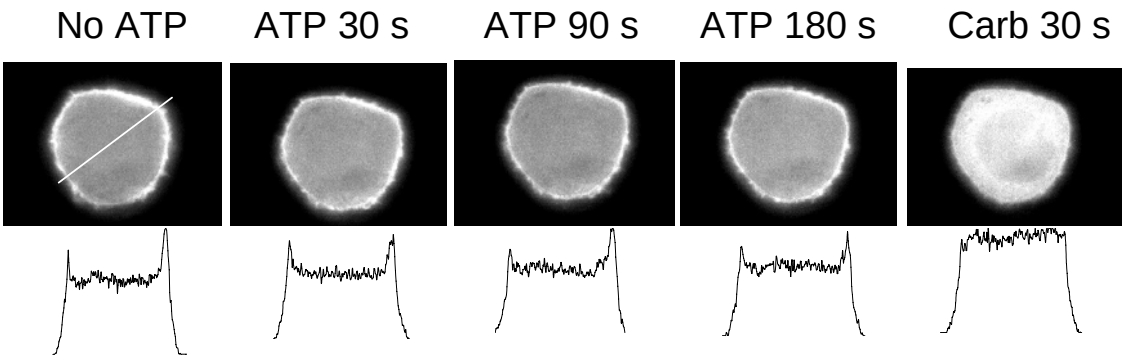


Figure S-1

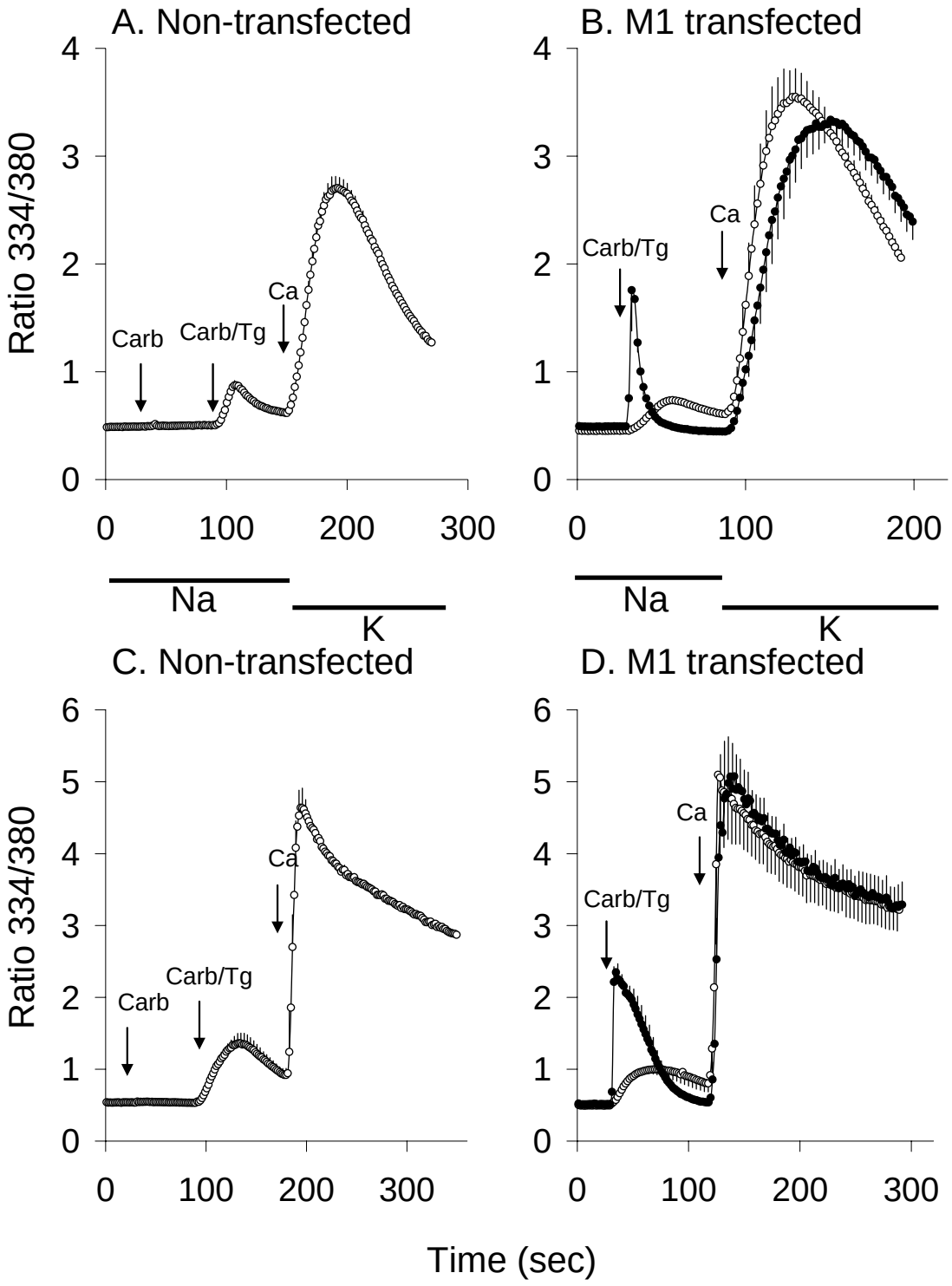

A. Non-transfected
B. M1 transfected
C. Non-transfected
D. M1 transfected
Ratio 334/380
Carb
Carb/Tg
Ca
Na
K
Time (sec)

Figure S-2

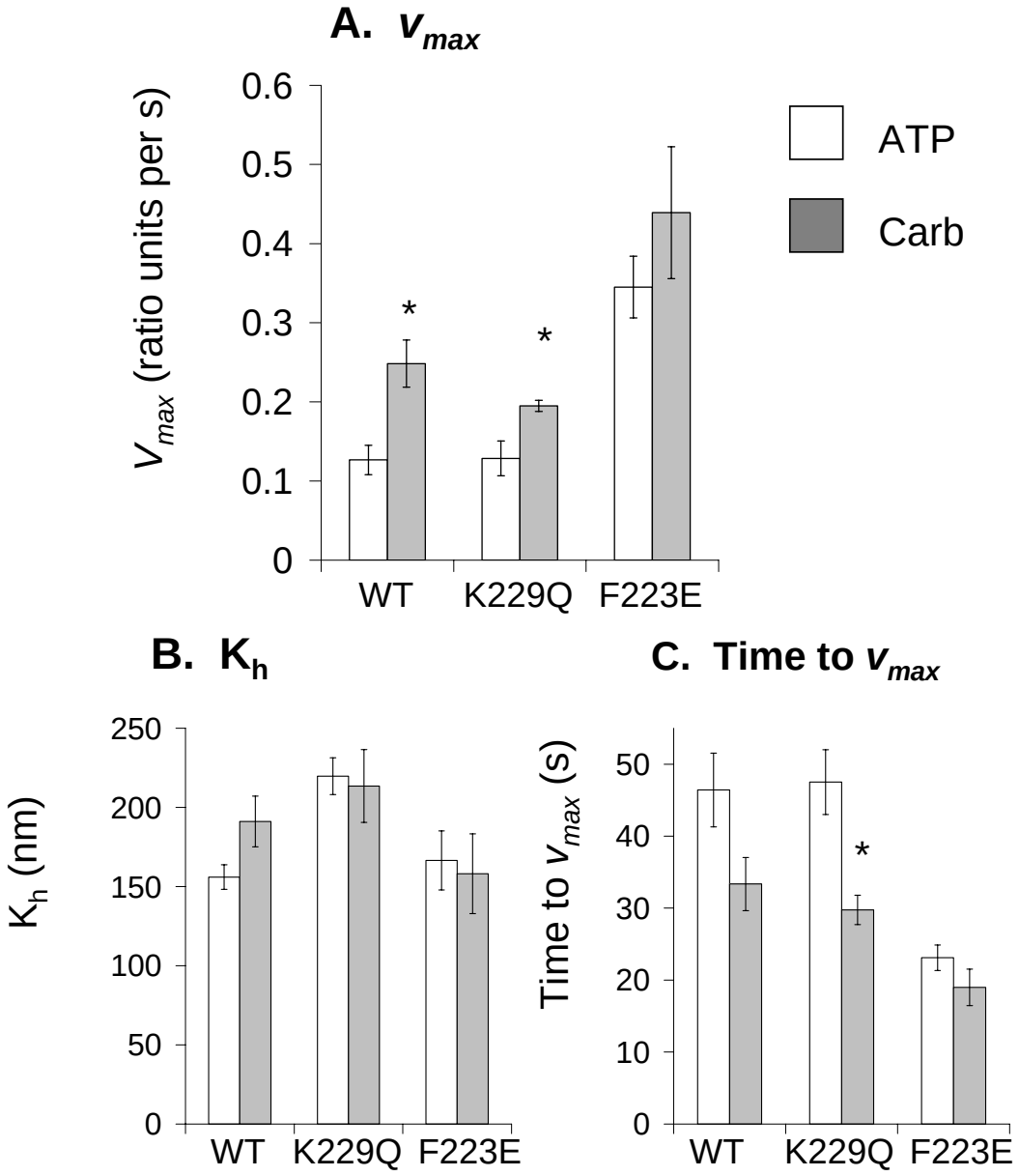

A. v_{max}
V_{max} (ratio units per s)
0.6
0.5
0.4
0.3
0.2
0.1
0
ATP
Carb
*
*
WT
K229Q
F223E
B. K_h
K_h (nm)
250
200
150
100
50
0
WT
K229Q
F223E
C. Time to v_{max}
Time to v_{max} (s)
50
40
30
20
10
0
*
WT
K229Q
F223E

Figure S-3

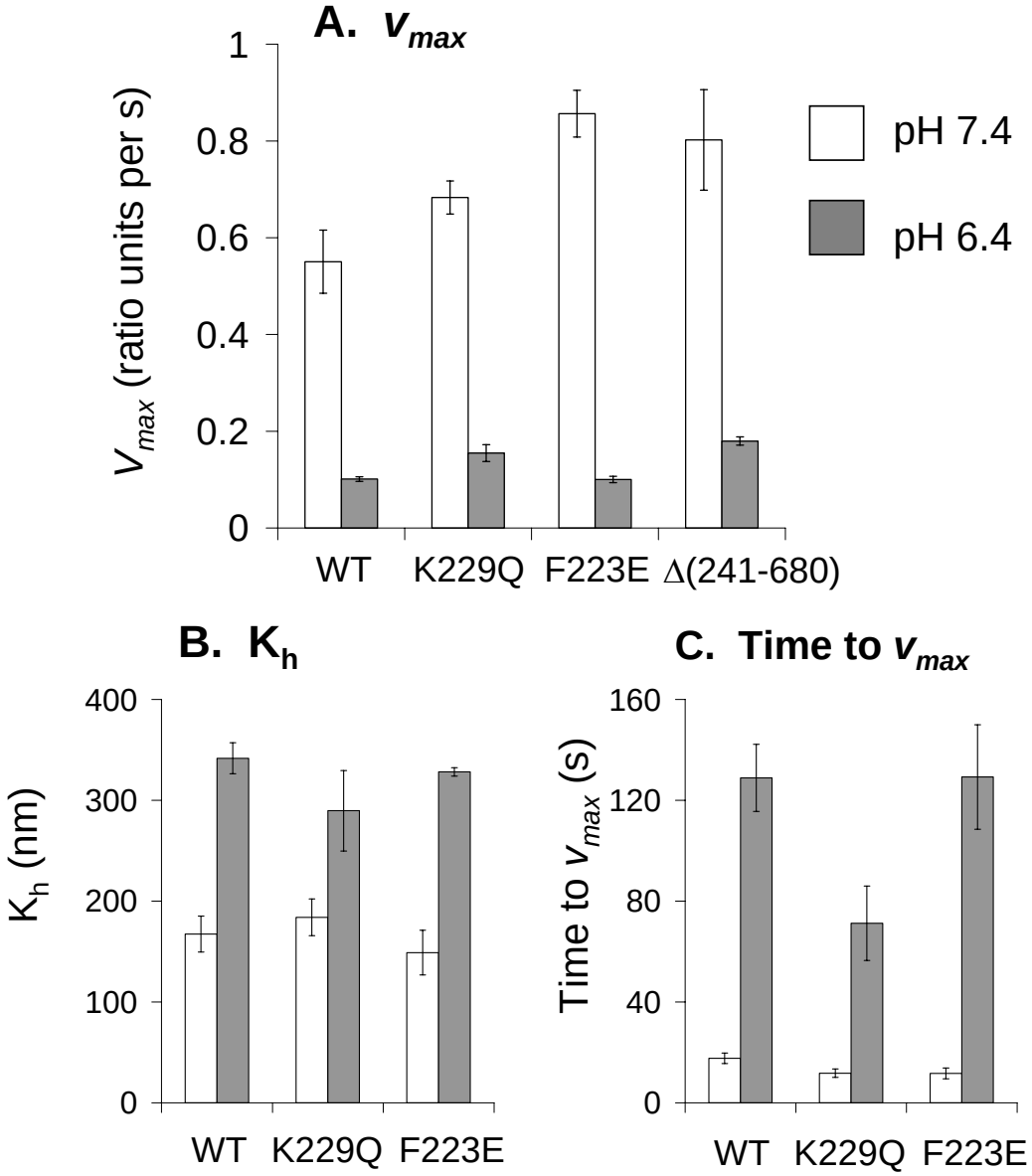

A. v_{max}
V_{max} (ratio units per s)
1
0.8
0.6
0.4
0.2
0
pH 7.4
pH 6.4
WT
K229Q
F223E
Δ(241-680)
B. K_h
K_h (nm)
400
300
200
100
0
WT
K229Q
F223E
C. Time to v_{max}
Time to v_{max} (s)
160
120
80
40
0
WT
K229Q
F223E

Figure S-4

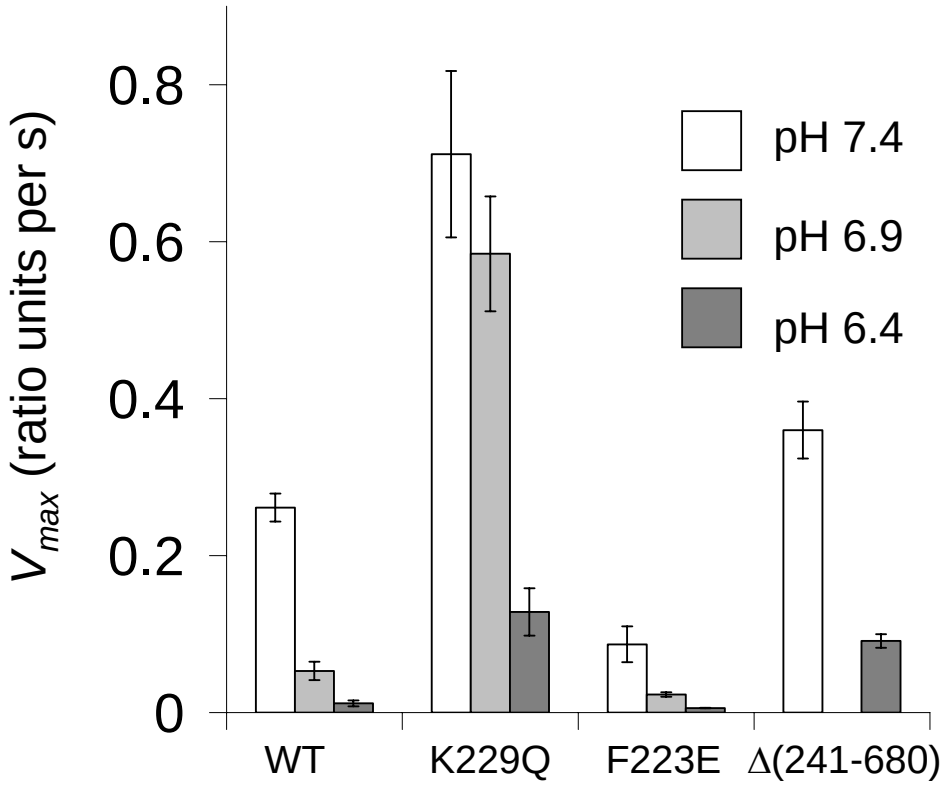

0.8
0.6
0.4
0.2
0
V_{max} (ratio units per s)
pH 7.4
pH 6.9
pH 6.4
WT
K229Q
F223E
Δ(241-680)

Figure S-5