PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP)/PAC$_1$HOP1 RECEPTOR ACTIVATION COORDINATES MULTIPLE NEUROTROPHIC SIGNALING PATHWAYS: AKT ACTIVATION THROUGH PI3Kγ AND VESICLE ENDOCYTOSIS FOR NEURONAL SURVIVAL

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Running head: PACAP Neurotrophic signaling

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Fig. 1. Diagram of PAC$_1$HOP1-GFP receptor plasmid expression construct. The hPAC1R cDNA insert, with the position of oligonucleotide primers and restriction sites used to clone into pAcGFP-N1 plasmid is shown. The predicted amino acid sequence for the hPAC1 receptor sequence (black) is followed by a three amino acid bridge (red) and in-frame green fluoresence protein open reading frame (GFP-ORF; purple). The position of the extra 84 bp HOP1 cassette in the region encoding the third intracellular loop in is also shown. Below is the expanded multiple cloning site (MCS) sequence and vector diagram for the parental plasmid pAcGFP1-N1.

Supplemental Fig. 2. PACAP potently activates ERK1/2 phosphorylation, activity and nuclear translocation in sympathetic neurons. A) Primary sympathetic cultures were treated with different concentrations of PACAP peptides for 15 minutes before extraction for phosphorylated ERK1/2 by Western blot analyses. B) Changes in ERK1/2 activation were noted at subnanomolar peptide concentrations which were accentuated in kinase activity assays using Elk1 as substrate as described in Methods. The upper band for Elk1 represented protein hyperphosphorylation which increased with increasing PACAP concentrations. C) For inhibitor studies, all cultures were pretreated with inhibitors (15 minutes) before PACAP addition for another 15 minutes. By Western analysis, the PACAP-mediated ERK1/2 phosphorylation was abrogated completely by MEK inhibitor PD08059, severely blocked by PKA inhibitor H89 and attenuated by the PI3K inhibitor LY294002 (all at 25 μM concentration). A similar pattern of inhibition was observed in kinase assays with Elk1. Each lane represents an individual culture; data representative of 3 - 4 independent experiments. D, E) Sympathetic cultures were treated with 100 nM PACAP27 for 15 minutes before fixation and immunocytochemical processing for phosphorylated ERK immunoreactivity. PACAP induced phosphorylated ERK nuclear translocation consistent with activated ERK transcriptional responses.

Supplemental Fig. 3. PACAP/PAC$_1$HOP1 receptor signaling increases sympathetic Akt phosphorylation. Samples from sympathetic NGF-deprived cultures were treated with PACAP27 in time-course studies as described in Figure 2, and prepared for quantitative Western analyses (see Methods). All data are normalized to total Akt levels in the individual samples and expressed as fold increase in Akt phosphorylation compared to NGF-deprived control cultures. PACAP27 stimulated Akt phosphorylation after 2 h of treatment. Data represent mean of 3 experiments ± SEM; *, significantly different from control (-NGF) at $p < 0.05$.

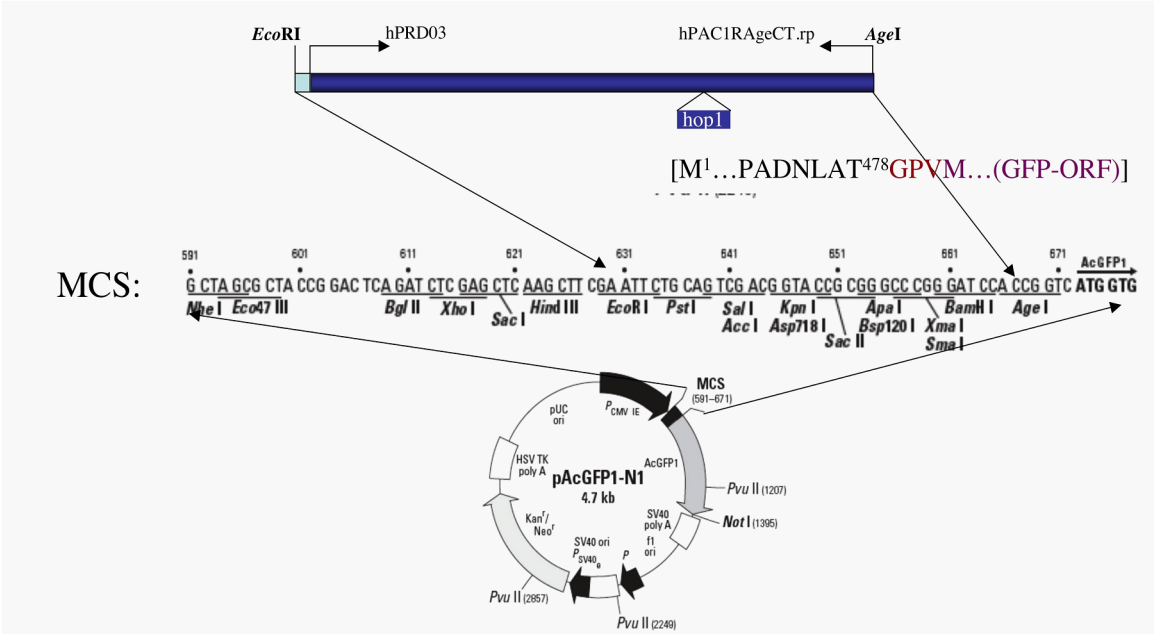
Supplemental Fig. 4. Quantitative Western analyses of signaling inhibitors on PACAP-stimulated Akt activation. NGF-deprived cultures were pretreated with the indicated inhibitors for 15 minutes before PACAP addition for 2 hours, as described in Fig. 3B. Data were normalized to total Akt levels and represent mean of 4 experiments ± SEM. *, significantly different from control (-NGF); +, significantly different from PACAP-stimulated levels, at $p < 0.05$.

Supplemental Fig. 5. PACAP augments Akt phosphorylation in NGF-supplemented sympathetic cultures. Primary sympathetic neuronal cultures were maintained in complete serum-free defined medium containing 50 ng/ml NGF. Parallel culture wells were treated by direct 100 nM PACAP27 addition without growth factor removal. After 4 h PACAP exposure, the cultures were harvested for phosphorylated Akt measurements by quantitative Western analysis. Despite high Akt phosphorylation levels from NGF/TrkA signaling, PACAP was able to further augment sympathetic neuronal Akt activation. Data were normalized to total Akt levels in the samples and represent mean of 3 experiments ± SEM; *, significantly different from control NGF cultures at $p < 0.05$.

Supplemental Fig. 6. PACAP/PAC_1 receptor-stimulated Akt activation is blocked by Gαq and PI3Kγ mechanisms. Stable AtT-20/PAC_1HOP1 cells were treated with 100 nM PACAP27 for 4 h, in the presence or absence of PI3Kγ inhibitor AS252424 (10 μM), Gαq siRNA or PI3Kγ siRNA as described in Methods, and prepared for quantitative Western analyses. Data were normalized to total Akt levels in the samples and represent mean of 3- 4 experiments ± SEM; *, significantly different from control untreated cultures at $p < 0.05$.

Supplemental Figure 7. Intracellular localization of PAC1HOP1 receptor, Gβ and Akt in sympathetic neurons. Cultured primary sympathetic neurons were transfected with the PAC_1HOP1-GFP construct by biolistic gene transfer. After 24 - 48 h in defined serum-free medium, PAC_1HOP1-GFP protein expression was localized predominantly to plasma membrane on the cell soma (A); after 100 nM PACAP stimulation (P27, 4 h), most of the PAC_1HOP1 receptor was internalized from the cell surface and localized to vesicular structures (D). Under basal conditions, Gβ immunoreactivity, identified using a mouse monoclonal antibody and visualized with Cy3 (red), was restricted to the plasma membrane of sympathetic neurons. While Gβ immunoreactivity could be found in vesicles after the same 4 h PACAP stimulation, a significant fraction remained on the cell surface (B). Similarly, using a rabbit monoclonal antibody, phosphorylated Akt immunoreactivity (Cy3, red) was restricted to cytoplasmic vesicles after PACAP treatment (C and E), and could be identified with internalized PAC_1HOP1 receptors (green) in colocalization studies (F). The comparable distribution patterns for PAC_1HOP1 receptor, G protein and phosphorylated Akt suggest vesicular interactions for signaling. Scale bar = 10 μm

Supplemental Figure 8. Endocytosis pathway inhibitors have no effects on untreated control sympathetic neurons. NGF-deprived sympathetic neuronal cultures were treated with the indicated reagents as described in Figure 6 to inhibit endocytosis. The drugs alone have no effect on basal neuronal Akt phosphorylation levels; PACAP-stimulated Akt activation is shown to illustrate response magnitude in the same culture preparation. Quantitative Western data were normalized to total culture Akt levels and expressed as fold change from untreated control. Data represent mean of duplicate culture wells ± range.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Nhe I Eco47 III Bgl II Xho I Sac I Hind III EcoR I Pst I Sal I Acc I Kpn I Asp718 I Sac II Apa I Bsp120 I BamH I Xma I Sma I Age I
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P CMV IE
HSV TK poly A
pAcGFP1-N1
4.7 kb
AcGFP1
Pvu II (1207)
Not I (1395)
SV40 poly A
f1 ori
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P SV40
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Pvu II (2857)
Pvu II (2249)

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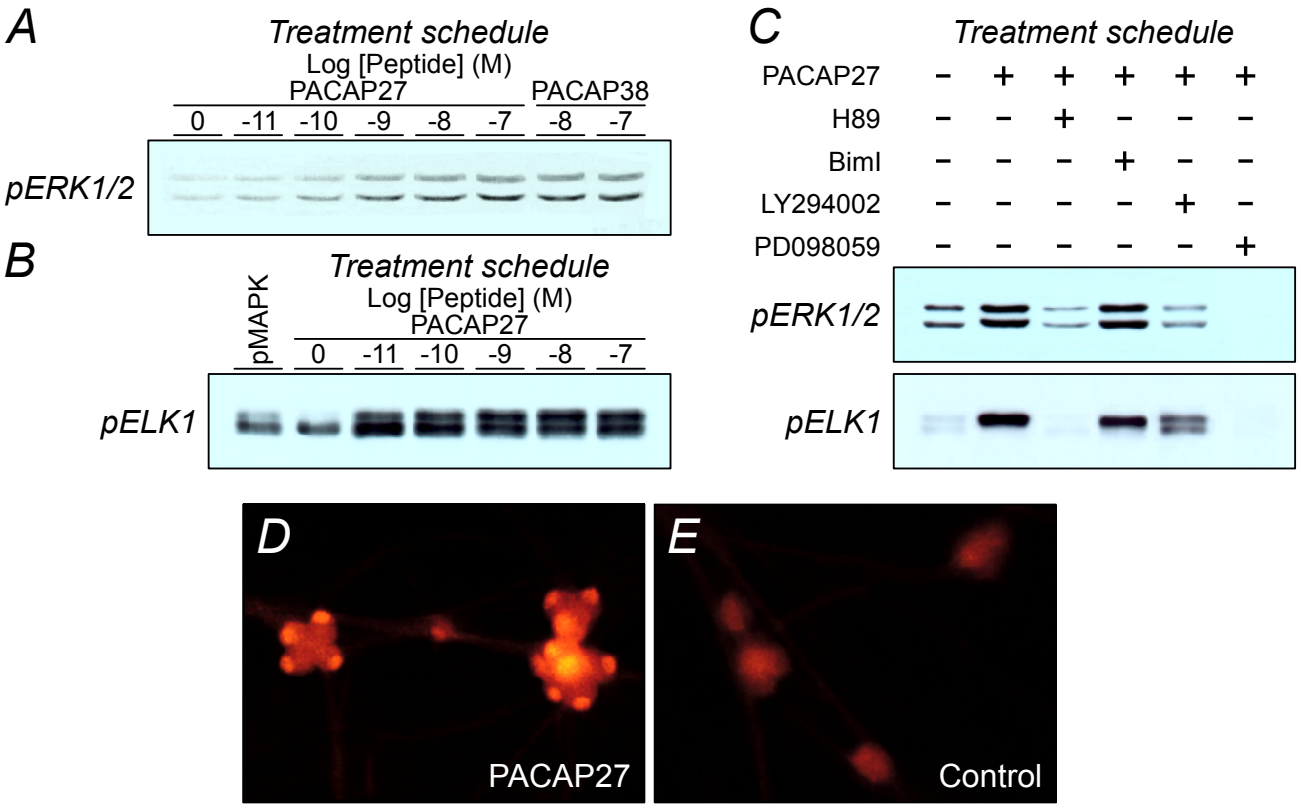
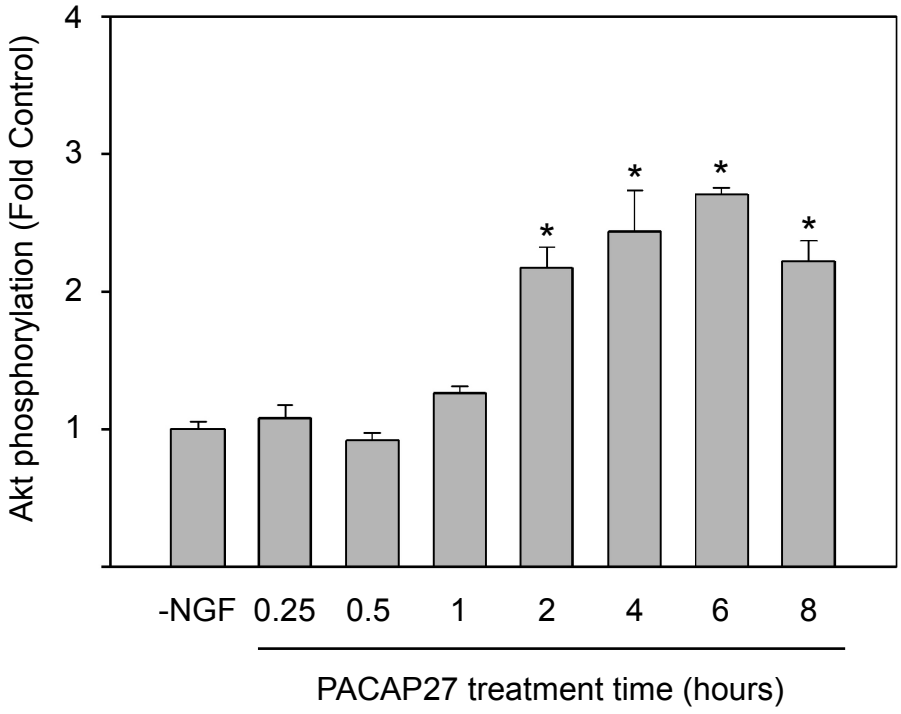
Suppl. Figure 2




Akt phosphorylation (Fold Control)
4
3
2
1
*
*
*
*
-NGF
0.25
0.5
1
2
4
6
8
PACAP27 treatment time (hours)

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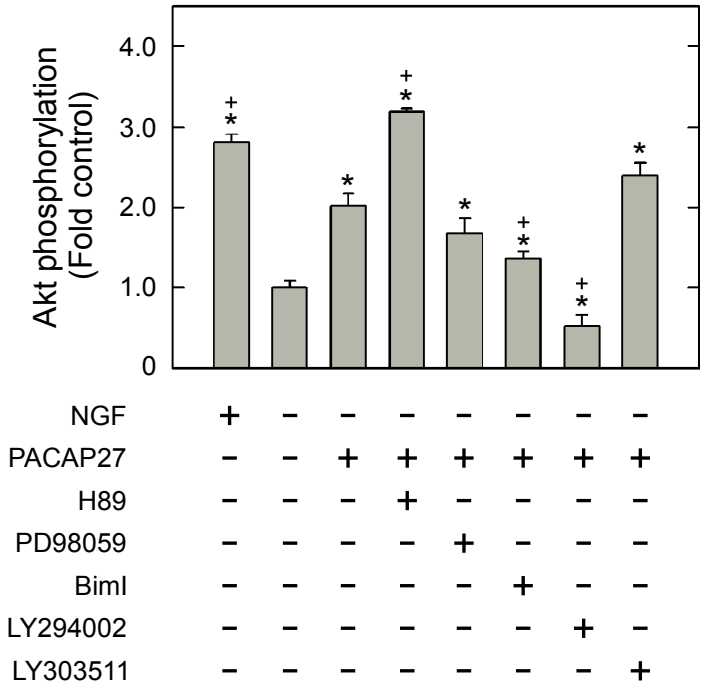
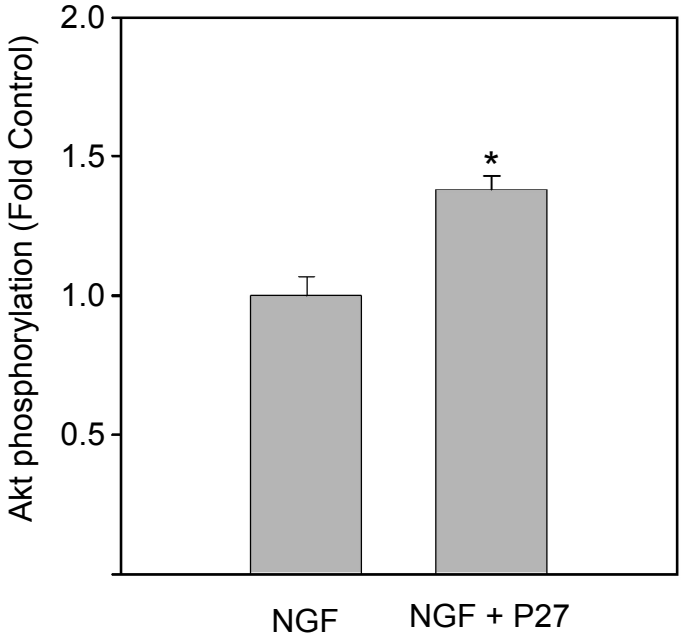
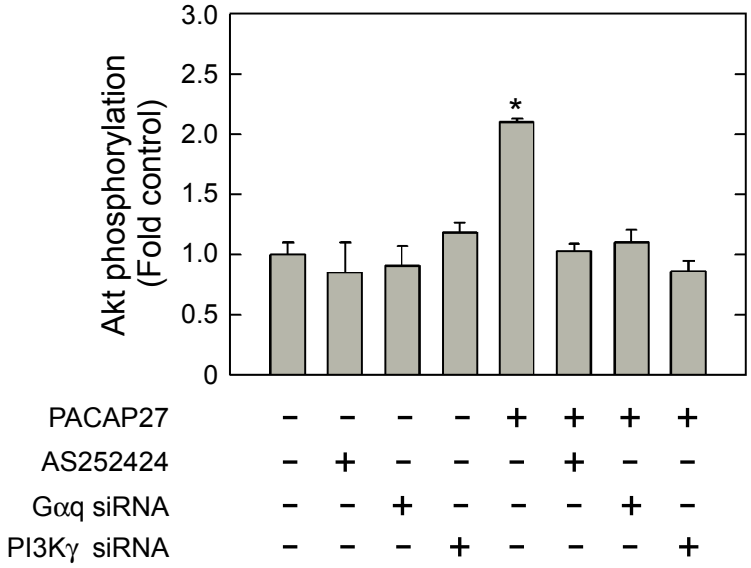
Suppl. Figure 4




2.0
1.5
1.0
0.5
Akt phosphorylation (Fold Control)
*
NGF
NGF + P27


Akt phosphorylation
(Fold control)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
PACAP27 − − − − + + + +
AS252424 − + − − − + − −
Gαq siRNA − − + − − − + −
PI3Kγ siRNA − − − + − − − +

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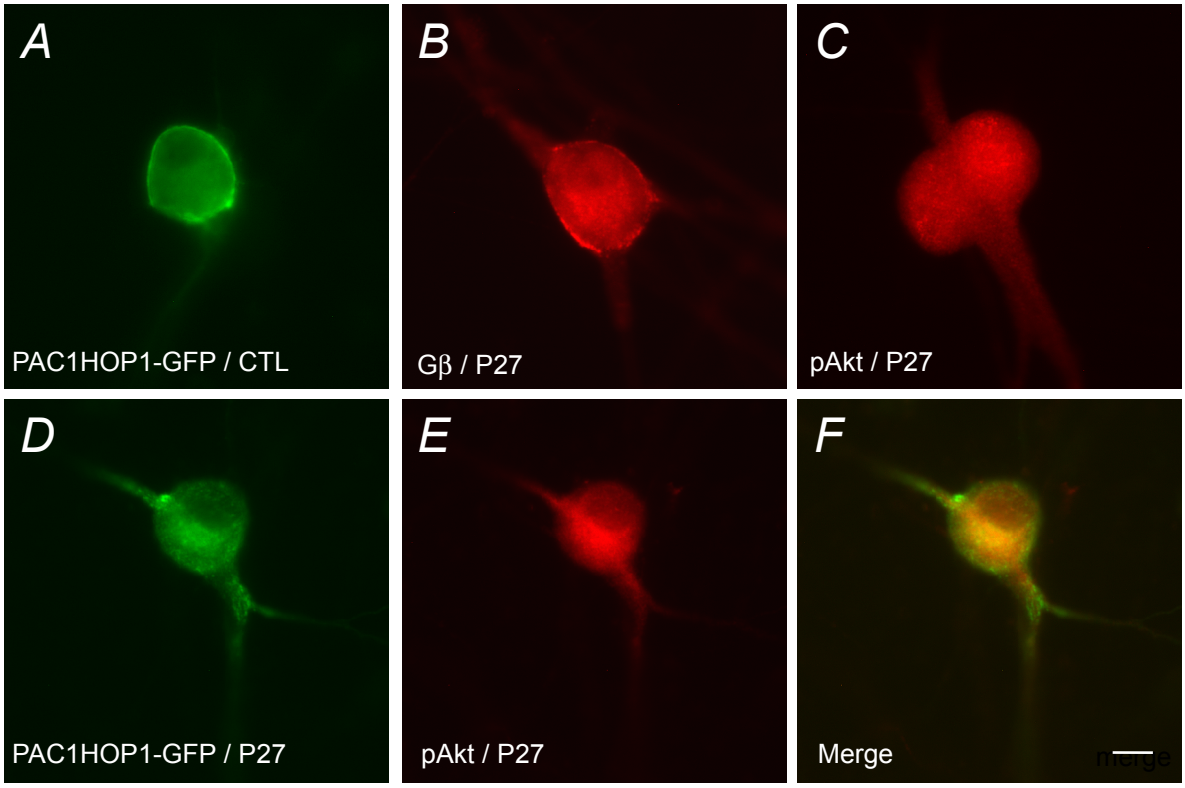
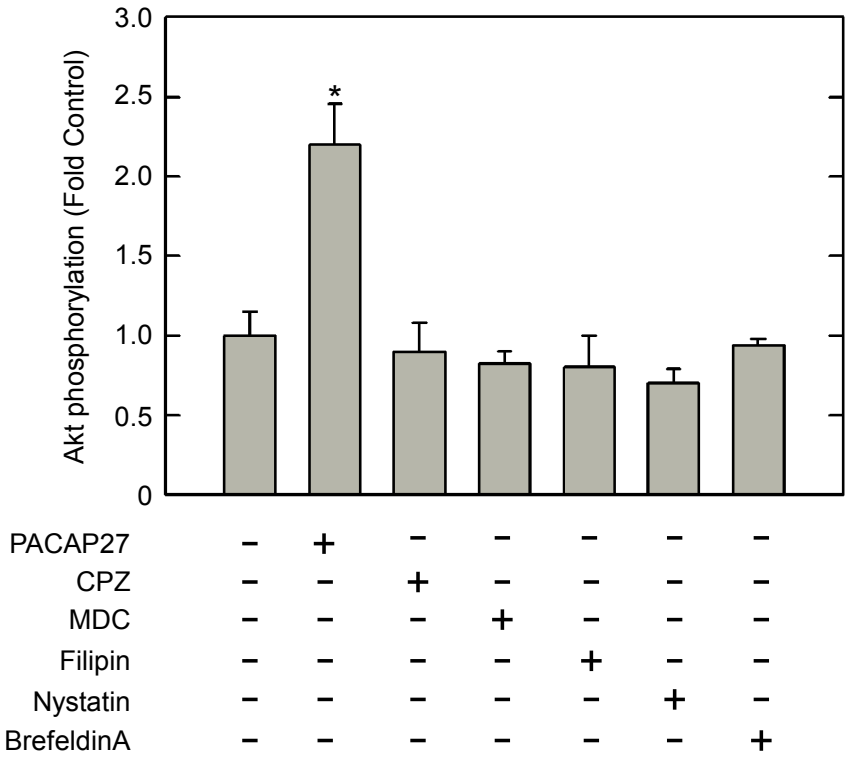
Suppl. Figure 7




Akt phosphorylation (Fold Control)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
PACAP27 − + − − − − −
CPZ − − + − − − −
MDC − − − + − − −
Filipin − − − − + − −
Nystatin − − − − − + −
BrefeldinA − − − − − − +