SUPPLEMENTAL MATERIALS AND METHODS

Western Blot analysis

To detect endogenous PACAP, membrane fractions were prepared as previously described with minor modifications (47). Briefly, LβT2 cells were cultured to near-confluence, rinsed twice in cold calcium- and magnesium-free Dulbecco's PBS, harvested and homogenized in ice-cold 10 mM Tris-Cl (pH 8.0 at 0°C) containing 0.32 M sucrose, 1 mM EDTA, 1 mM β-mercaptoethanol, and 1X Complete, EDTA-Free Protease Inhibitor Cocktail (PIC, Roche Diagnostics, Indianapolis, IN) and centrifuged at 2,000 x g for 4 min at 4°C. The supernatant was then centrifuged at 55,000 x g for 60 min at 4° C. Pelleted membrane fractions were resuspended in 50 mM Tris-Cl (pH 8.0 at 0°C) containing 1% SDS, 1 mM EDTA, 1 mM β–mercaptoethanol, and 1X PIC. Protein concentrations were determined using the Pierce Coomassie Plus Protein Assay Reagent Kit (Rockford, IL). 20 μg LβT2 membrane protein was diluted with 2X Novex Tricine SDS Sample Buffer and 10X NuPAGE Sample Reducing Agent. As controls, synthetic PACAP27 and PACAP38 peptides (Phoenix Pharmaceuticals, Burlingame, CA and Calbiochem, San Diego, CA, respectively) were prepared in a similar manner. Samples were heated to 85°C for 2 min, electrophoresed on a 16% Novex Tricine minigel (Invitrogen, Carlsbad, CA) using Novex Tricine SDS Running Buffer (Invitrogen), and transferred onto 0.45 μm Immobilon-P membrane (Millipore, Billerica, MA) using Novex Tris-Glycine Transfer Buffer (Invitrogen) containing 20% methanol. The membrane was rinsed in distilled H_2O and dried overnight to covalently bind peptides to the membrane. After re-wetting, the membrane was blocked in 5% Blotting Grade Non-Fat Dry Milk (Bio-Rad Laboratories, Hercules, CA) diluted in SuperBlock Buffer (Pierce) and probed with a 1:5000 dilution of rabbit anti-PACAP27 (P/N T-4465; Peninsula Labs, King of Prussia, PA) diluted in 1% dry milk/TBST (TBS plus 0.1% Tween 20), followed by addition of a 1:50,000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (GE Healthcare, Piscataway, NJ). The membrane was washed sequentially in TBST, High Salt Wash (0.1 mM Tris-Cl pH 7.4, 0.5 M NaCl, 0.2% SDS) and TBST, 15 min each. The bound secondary antibody was visualized using the SuperSignal West Femto Substrate kit (Pierce).

EMSA

LβT2 cells were grown to 40-50% confluence in 100 mm culture dishes. On the day of harvest, cells were cultured in fresh DMEM containing 10% FBS and 100 nM GnRH or vehicle control for additional culture periods, as indicated. Cells were harvested by scraping in cold PBS containing 0.6 mM EDTA, pelleted, and nuclear proteins extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) supplemented with 1 μg/ml aprotinin, 1 μg/ml pepstatin, 10 mM sodium fluoride and 0.5 mM phenylmethylsulfonylfluoride (PMSF) according to the manufacturer's recommendations. Total protein concentration of the extracts was determined by Pierce BCA Protein Assay using bovine serum albumin as a standard. Double-stranded oligonucleotide probes were created by T4 polynucleotide kinase (Invitrogen) end-labeling with [γ-^{32}P]-ATP (GE Healthcare) and purified over a Quick Spin G-25 Sephadex Column (Roche Applied Science; Indianapolis, IN). 5 μg nuclear protein was incubated with 60,000 cpm of probe in DNA-binding buffer containing 100 mM HEPES (pH 7.9), 300 mM KCl, 25 mM $MgCl_2$, 5 mg/ml BSA, 10 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM dithiothreitol, 0.1 mg/ml salmon sperm DNA and 25% v/v glycerol for 20 minutes at room temperature prior to resolution electrophoresis on a 5% nondenaturing PAGE gel in 0.5X Tris-borate-EDTA buffer. Oligonucleotides used for probes can be found in Supplemental Table 1.

Chromatin immunoprecipitation (ChIP) assay

35 x 10^6 LβT2 cells were cultured overnight in 145 mm culture dishes and treated for indicated times with 100 nM GnRH or H_2O control. Chromatin and associated nuclear proteins were cross-linked in 1% formaldehyde for 10 min and the reaction quenched by addition of 0.125 M glycine

for 5 min. Cells were washed 2X in cold PBS, harvested in 5 ml cold PBS supplemented with 1X PIC, pelleted, resuspended in 2 ml lysis buffer (50 mM Tris-Cl pH 8.0 at 0°C, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 1X PIC) and incubated 10 min on ice. Samples were then homogenized with 20 strokes in a glass-Teflon homogenizer on ice, centrifuged at 2000 x g for 5 min at 4°C. Cell pellets were resuspended in 2 ml sonication buffer (50 mM Tris-Cl pH 8.0 at 0°C, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.05% SDS and 1X PIC) and sonicated on ice with a 3 mm probe for 10 10-second bursts at a setting of 2.5 on a Heat Systems–Ultrasonics, Inc. (Farmingdale, NY), Model W-375 Sonicator Cell Disruptor. Insoluble material was pelleted by centrifugation at 13,000 x g for 10 min at 4°C. Samples were precleared with Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) for 2 hours and 5% of each lysate was reserved at -80°C as input chromatin. Each pre-cleared lysate was split into equal aliquots and IP was performed at 4°C overnight in the presence of 2 μg anti-JunB (sc-46) or normal control rabbit IgG (Santa Cruz Biotechnology) followed by addition of Protein A/G PLUS-Agarose beads for 1 hour. Beads were pelleted and washed sequentially in Low Salt Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), High Salt Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl Wash Buffer (0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris-HCl, pH 8.1) and twice with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Complexes were eluted by shaking 2 x 30 min at room temperature in 120 μl freshly made Elution Buffer (TE pH 8.0 plus 1% SDS). NaCl was added to samples to a final 0.3 M and cross-linking was reversed at 65° C overnight. Eluted chromatin was purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and analyzed in triplicate by qPCR. Relative abundances of co-immunoprecipitated PACAP promoter fragments from GnRH-treated or H_2O control-treated cells were measured using by SYBR Green-based qPCR in 15 to 25 μl reaction volumes in the presence of 450 nM primers. Primer sets are listed in Supplemental Table 1. Relative proportions of co-immunoprecipitated promoter fragments in the various treatment groups were calculated using the Comparative C_T Method where the C_T (threshold cycle) value for each sample was determined and a ΔC_T value for each sample was defined as $\Delta C_T = C_{T\,(Eluate)} - C_{T\,(Input)}$. $\Delta\Delta C_T$ was then defined as $\Delta C_{T\,(Time\ X)} - \Delta C_{T\,(Time\ Zero)}$. The fold difference relative to the "Time Zero Control" was then calculated as $2^{-\Delta\Delta C_T}$.