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Supplemental Data

Dynamic Regulation of cAMP

Synthesis through Anchored

PKA-Adenylyl Cyclase V/VI Complexes

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Supplemental experimental procedures:

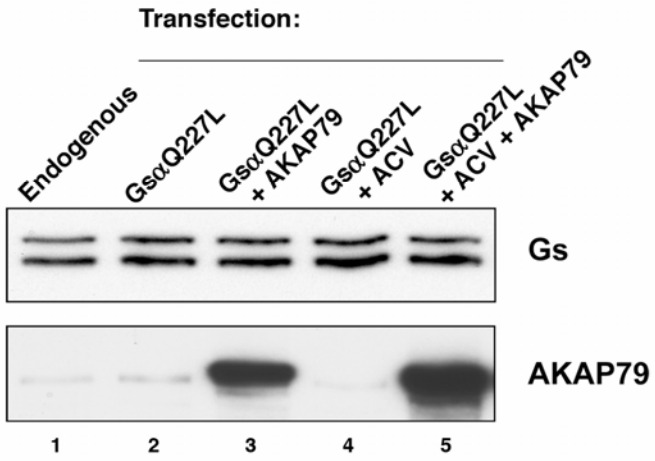
Tissue extract preparations and immunoprecipitations. Adult rat brains were homogenized in 10 ml HSE buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM AEBSF, 1 mM Benzamidine). Following centrifugation at 38,000 X g for 30 min, the pellets were resuspended in either 10 ml RIPA buffer (150 mM NaCl, 10 mM Tris [pH 7.2], 0.1%SDS, 1.0% Triton X-100, 1% Deoxycholate, 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM AEBSF, 1 mM Benzamidine) or 10 ml IP buffer (10 mM phosphate, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, with 1% Triton X-100) and centrifuged at 38,000 x g for 30 min at 4°C.

For immunoprecipitation of adenylyl cyclase-AKAP complexes, 500 μg extract was incubated with 4 μg antibody and 40 μl protein A sepharose beads (50% slurry) overnight at 4ºC. Samples were washed twice in IP buffer and twice with RIPA buffer. Bound proteins were eluted with SDS-sample buffer, separated by SDS-PAGE on a 7% gel and transferred to nitrocellulose membrane. Bound proteins were visualized by enhanced chemiluminescence (ECL; Pierce) and captured on autoradiography film (Kodak Biomax film).

Forskolin-agarose pulldowns. Tissue extracts were prepared as described above. Extracts (500 μg) were incubated overnight at 4ºC with 30 μl forskolin agarose (Sigma). Following washing, the bound proteins were eluted by incubation at 80°C for 5 min in Laemmli sample buffer. After cooling to RT, 5 μl of 300 mM NEM (Sigma) was added for 10 min. The samples were run on a 7% SDS-PAGE gel, transferred to nitrocellulose and analyzed by Western blot or RII overlay.

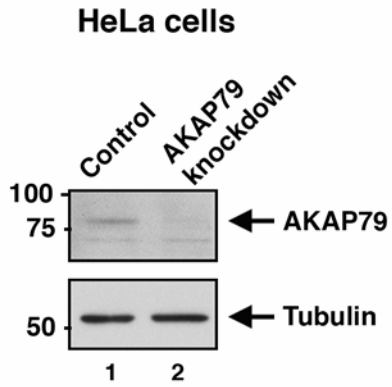
cAMP changes monitored by Ca^{2+} influx CNG channels. Knockdown of AKAP79 in HEK293 cells was performed as previously described (Hoshi et al., 2005). Two days later cells were infected with adenovirus encoding the α subunit of rat olfactory CNG channel with mutations Cys460Trp and Glu583Met. The following day the CNG channel expressing cells were enriched for AKAP79 deficient cells (Hoshi et al., 2005) and cells were treated with the phosphodiesterase inhibitor (IBMX 100 μM). After loading with Fura-2, the cells were washed and then imaged using a CoolSnap CCD-camera (Photometrics) and monochromator system (Cairn Research) attached to a Nikon TMD microscope (40X objective). Emission images (D510/80M) at 340nm and 380nm excitation were collected at 1Hz using MetaFluor software (Universal Imaging). Data were plotted as 340/380nm ratio change relative to the pre-stimulus fluorescence ratio (F340/380) or presented as pseudocolor images of Fura-2 ratio intensity.

Supplementary figure 1



Supplementary information 1: Endogenous and ectopically expressed Gs and AKAP79. Total cell lysate (25 µg) from HEK293 cells transfected with the indicated constructs was loaded in each lane. Rabbit anti-Gαs/olf (1:500; Santa Cruz Biotechnology) was used for the detection of endogenous Gs and over-expressed GsαQ227L (long form of Gs). Mouse anti-AKAP79 (1:400; Transduction Laboratories) was used for the detection of endogenous and overexpressed AKAP79.

Supplementary figure 2



Supplementary information 2: RNA interference of AKAP79 knocks down endogenous levels of AKAP79 in HeLa cells. HeLa cells were transfected with pSAKAP79i, CpJPA-CD4 and pEGFP-N1 using lipofectamin 2000. After three days, anti-CD4-coupled magnetic beads were used to enrich for transfected cells as described previously. About 60% of recovered cell were GFP positive. Cells were then lysed and analyzed with immunoblotting for AKAP79 and tubulin.