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STC1 induction by PACAP is mediated through cAMP and ERK1/2 but not PKA in cultured cortical neurons

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Abstract

The neuroprotective actions of PACAP (pituitary adenylate cyclase-activating polypeptide) in vitro and in vivo suggest that activation of its cognate G protein-coupled receptor PAC1 or downstream signaling molecules, and thus activation of PACAP target genes, could be of therapeutic benefit. Here we show, that cultured rat cortical neurons predominantly expressed the PAC1hop and null variants, activation of which resulted in elevation of the two second messengers cAMP and Ca^{2+} and expression of the putative neuroprotectant stanniocalcin 1 (STC1). PACAP signaling to the STC1 gene proceeded through the extracellular signal-regulated kinases 1 and 2 (ERK1/2), but not through the cAMP dependent protein kinase (PKA), and was mimicked by the adenylate cyclase activator forskolin. PACAP- and forskolin-mediated activation of ERK1/2 occurred through cAMP, but not PKA. These results suggest that STC1 gene induction proceeds through cAMP and ERK1/2, independently of PKA, the canonical cAMP effector. In contrast, PACAP signaling to the BDNF gene proceeded through PKA, suggesting that two different neuroprotective cAMP pathways co-exist in differentiated cortical neurons. The selective activation of a potentially neuroprotective cAMP dependent pathway different from the canonical cAMP pathway used in many physiological processes, such as memory storage, has implications for pharmacological activation of neuroprotection in vivo.

Keywords

PACAP; cAMP; PKA; ERK; STC1 gene induction; signaling

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP), occurring as two C-terminally α-amidated forms (PACAP-27 and PACAP-38), is a member of the vasoactive intestinal polypeptide (VIP)-secretin-growth hormone releasing hormone (GHRH)-glucagon superfamily (Miyata et al., 1989; Miyata et al., 1990). PACAP is found throughout the central nervous system (CNS), with high levels in brain structures such as the hypothalamus, from which it was isolated, the cerebral cortex, amygdala, nucleus accumbens, hippocampus, cerebellum and substantia nigra (nerve terminals) (Arimura et al., 1991; Ghatei et al., 1993; Hannibal, 2002). In the peripheral nervous system (PNS), PACAP is found in sensory neurons, sympathetic preganglionic neurons and parasympathetic pre- and

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postganglionic neurons (Sundler et al., 1996; Hamelink et al., 2002b). PACAP-38 is the predominant form expressed.

In the developing nervous system, PACAP functions as a neurotrophic factor promoting cell survival and neurite outgrowth in a variety of cell types, including cerebellar granule cells (Gonzalez et al., 1997; Vaudry et al., 2000), cortical neuroblasts (Lu et al., 1997) and dorsal root ganglion neurons (Lioudyno et al., 1998). PACAP also promotes survival and regeneration in the mature nervous system and inhibits apoptotic cell death under paraphysiological and pathophysiological conditions, such as stroke (Waschek, 2002; Brenneman, 2007). In vivo, endogenous and exogenous PACAP reduce infarct volume and ameliorate neurological defects after middle cerebral artery occlusion (MCAO) (Reglodi et al., 2000; Reglodi et al., 2002; Tamas et al., 2002; Chen et al., 2006; Ohtaki et al., 2006). PACAP is also protective in other neurodegenerative conditions, e.g., in association with Parkinson's (Reglodi et al., 2004; Reglodi et al., 2006) and Alzheimer's disease (Kojro et al., 2006; Rat et al., 2011). In cell culture, PACAP promotes the survival of cultured cortical neurons under hypoxic/ischemic and excitotoxic conditions (Morio et al., 1996; Pellegri et al., 1998; Said et al., 1998; Frechilla et al., 2001; Shintani et al., 2005; Nowak et al., 2007; Stumm et al., 2007). Multiple other cell types are protected by PACAP under a variety of conditions that promote apoptosis. Specifically, PACAP ameliorates cell death in hippocampal cultures exposed to the HIV envelope protein gp120 (Arimura et al., 1994) and in cerebellar granule cells incubated in low-potassium or serum-free medium, or exposed to ethanol or oxidative stress (Cavallaro et al., 1996; Kienlen Campard et al., 1997; Villalba et al., 1997; Vaudry et al., 2002a; Vaudry et al., 2002b; Tabuchi et al., 2003). Serum and NGF withdrawal-induced cell death in differentiated PC12 cells (Tanaka et al., 1997) and primary sympathetic neurons (May et al., 2010) is reduced by PACAP. PACAP also rescues motoneurons (Tomimatsu et al., 2008), retinal neurons (Shoge et al., 1999; Endo et al., 2011) and PC12 cells (Onoue et al., 2002b) from glutamate-induced excitotoxicity.

PACAP mediates its effects through activation of three G protein-coupled receptors (GPCRs): VPAC1, VPAC2 and PAC1. VPAC1 and VPAC2 bind PACAP and its related peptide VIP with similar affinities, whereas PAC1 is the PACAP-preferring receptor and the predominant receptor in the nervous system (Ishihara et al., 1992; Lutz et al., 1993; Pisegna et al., 1993; Harmar et al., 1998). As members of the class B receptor subfamily, all PACAP receptors regulate intracellular concentrations of cyclic adenosine 3′5′-monophosphate (cAMP) by coupling to adenylate cyclases (ACs) through the stimulatory G protein Gαs, and depending on receptor subtype can also couple to phospholipase Cβ (PLCβ) (Harmar, 2001). PAC1 occurs in several splice variants, which are generated through alternative splicing within the N-terminal extracellular domain and the C-terminal end of the third intracellular loop (ic3), a region critical for G protein coupling. N-terminal splicing generates 21 or 57 amino acid deletions, affecting ligand binding and the relative potencies of the ligands in second messenger stimulation (Pantaloni et al., 1996; Dautzenberg et al., 1999). The embryonic brain expresses high levels of receptors containing a short N-terminus lacking 21 amino acids, whereas the receptor with a full-length N-terminus is the predominant form in the adult brain. Ic3 variants result from hip or hop insertions, consisting of 28 amino acids, respectively. Both cassettes can also be included together to give rise to hiphop. The alternative use of two contiguous consensus splice acceptor sites at the 5′-end of the hop cassette generates hop1 and hop2. PAC1null and hop1 are the major receptor variants in the brain, and couple to Gαs and Gαq proteins to increase intracellular cAMP generation through AC activation and inositol phosphate production, activating IP_3 receptor-mediated Ca^{2+} mobilization through PLCβ activation, respectively (Spengler et al., 1993; Pisegna et al., 1996; Zhou et al., 2000; Lutz et al., 2006; Holighaus et al., 2011).

PACAP's neuroprotective effects during glutamate-induced excitotoxicity in cortical neurons (Morio et al., 1996; Said et al., 1998) and during K^+ and serum deprivation and hydrogen peroxide exposure of cerebellar granule neurons (Cavallaro et al., 1996; Vaudry et al., 2002a) are mimicked by intracellular cAMP production, suggesting that PACAP mediates its effects via a cAMP dependent signaling pathway. Activation of PKA (Kienlen Campard et al., 1997; Tanaka et al., 1997; Vaudry et al., 1998; Shoge et al., 1999; Tomimatsu et al., 2008; Baxter et al., 2011) and ERK1/2 (Villalba et al., 1997; Vaudry et al., 2002a; Pugh et al., 2006; May et al., 2010) have been implicated in mediating PACAP's neuroprotective effects in various cell types under diverse conditions promoting apoptosis. PACAP-mediated neuritogenesis in PC12 cells can occur via PKA dependent (Hernandez et al., 1995) and PKA independent but cAMP and ERK1/2 dependent (Ravni et al., 2008) signaling pathways.

It has been suggested that PACAP mediates its neuroprotective effects via direct and indirect mechanisms (Dejda et al., 2011). Activation of PKA (Tanaka et al., 1997; Shoge et al., 1999; Stetler et al., 2010) and ERK1/2 (Villalba et al., 1997; Pugh et al., 2006; Stumm et al., 2007; May et al., 2010), inhibition of caspase-3 (Onoue et al., 2002a; Vaudry et al., 2002a; Dejda et al., 2008), and transcriptional regulation of proteins involved in cell death and survival (Grumolato et al., 2003) play important roles in PACAP-mediated prevention of neuronal apoptosis induced by various insults. Induction of the neurotrophic factor BDNF by PACAP suppresses cell death in cortical neurons exposed to excitotoxicity (Frechilla et al., 2001). Indirectly, PACAP may mediate neuroprotection via modulation of glial cells, such as astroglia (Stumm et al., 2007) and microglia (Delgado et al., 2003; Armstrong et al., 2008) to provide neurotrophic support and control of the inflammatory response, respectively. PACAP-induced astroglial IL-6 release (Gottschall et al., 1994) has been implicated in PACAP's neuroprotective effects during ischemia in vivo (Ohtaki et al., 2006).

We identified stanniocalcin 1 (STC1) as a PACAP-regulated gene in chromaffin cells of the adrenal medulla (Ait-Ali et al., 2010) and confirmed its induction by PACAP in neural cells of the CNS (Holighaus et al., 2011). STC (previously called teleocalcin) was identified in bony fish, where it is secreted from the corpuscules of Stannius in response to increased extracellular Ca^{2+} levels, regulating Ca^{2+} and phosphate homeostasis and protecting against toxic hypercalcemia (Wagner et al., 1986; Wagner et al., 1989; Lu et al., 1994). STC is highly conserved through vertebrate evolution and exists as a homodimeric glycoprotein (Chang et al., 1995; Chang et al., 1996). A second STC protein with 30% sequence homology to STC1 has been identified (Chang et al., 1998; Luo et al., 2005). Mammalian STC2 has been implicated in cell survival during endoplasmic reticulum (ER) stress (Ito et al., 2004) and has recently been shown to regulate Ca^{2+} homeostasis through modulation of store-operated Ca^{2+} entry (Zeiger et al., 2011). In mammals, STC1 and STC2 are expressed in many tissues, in contrast to their localized glandular expression in fish, suggesting an autocrine/paracrine rather than an endocrine function, extending beyond the regulation of mineral metabolism. STC1 is upregulated during neuronal differentiation (Zhang et al., 1998) and in neurons of the ischemic penumbra (i.e., ischemic but still viable cerebral tissue), presumably supporting neuronal survival (Zhang et al., 2000). In cell culture, STC1 increases cell resistance to hypoxic and hypercalcemic insult (Zhang et al., 2000). Induction of STC1 after neuronal and myocardial hypoxic preconditioning, conferring resistance to further ischemic damage, is dependent on IL-6 (Westberg et al., 2007a; Westberg et al., 2007b). Moreover, transgenic mouse models of STC1 and STC2 indicate a role in the regulation of fertility and postnatal growth (Filvaroff et al., 2002; Varghese et al., 2002; Chang et al., 2005; Chang et al., 2008).

The biological significance of mammalian STC proteins, however, still remains elusive. Given its induction by the neuroprotective peptide PACAP in neuroendocrine and neural

cells and its proposed neuroprotective effects, lead us to investigate its regulation in primary CNS neurons. Elucidation of how the STC gene is regulated will give important information towards understanding its function in vivo. Here we show that the neuropeptide PACAP regulates STC1 gene induction in primary cultures of rat cortical neurons. Signaling to the STC1 gene proceeds through cAMP and ERK1/2 but is independent of PKA, suggesting that STC1 induction through non-canonical cAMP-dependent signaling is implicated in PACAP's neurotrophic and neuroprotective effects during development and brain injury.

Materials and Methods

Materials

PACAP-38 was purchased from Phoenix Pharmaceuticals (Mountain View, CA). Forskolin, 2′5′-dideoxyadenosine, H89 and U0126 were obtained from Calbiochem (San Diego, CA). All cell culture media and supplements were obtained from Invitrogen (Carlsbad, CA) unless otherwise specified.

Preparation and culture of primary rat cortical neurons

Rat cortical neurons were prepared from cortices of embryos from 18-day-pregnant Wistar rats. Pregnant animals were killed by exposure to CO_2 and embryos were extracted. After extracting the embryonic brains, the cortices were dissected and placed in Neurobasal medium supplemented with 1X B27, 500 μM L-glutamine and 25 μM L-glutamate (complete Neurobasal medium). Cortices were triturated in complete Neurobasal medium containing 50 μg/ml DNase and 0.25% Trypsin. After a 15-min-incubation, 30% horse serum was added and cells were triturated again. Cells were washed twice with complete Neurobasal medium and resuspended in 1X DMEM High Glucose (4.5 g/L D-Glucose) supplemented with 10% FBS and 2 mM L-glutamine. Following filtration, cells were plated at a density of 125,000 cells/0.95 cm^2 growth area on poly-L-lysine-coated (PLL; 0.1 mg/ml) plates. For neuronal viability, it was crucial to incubate the dishes with PLL solution for at least 6 h. After one day in culture, the medium was changed to complete Neurobasal medium. Cells were kept under 95% air and 5% CO_2 and used after 8–13 days in culture.

Analysis of PACAP receptor mRNAs by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Rat cortical neurons were grown in 6-well plates and harvested for RNA extraction using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Genomic DNA was removed by digestion with RNase-free DNase I (Roche Applied Science, Mannheim, Germany). 1 μg RNA was reverse-transcribed with the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was PCR-amplified with gene-specific primers for PACAP receptors (PAC1 F, 5′-GGC CCC GTG GTT GGC TCT ATA ATG G-3′; PAC1 R, 5′-GAG AGA AGG CGA ATA CTG TG-3′; PAC1hop R, 5′-AGA GTA ATG GTG GAT AGT TCT GAC A-3′; PAC1hip R, 5′-TGG GGA CTC TCA GTC TTA AA-3′; VPAC1 F, 5′-GCA GCA ACA GAC CAA GTT CTA C-3′; VPAC1 R, 5′-TGA ACA GGC TCA AGA TAG CCA T-3′; VPAC2 F, 5′-AAG CAA AAA CTG CAC TAG TGA-3′; VPAC2 R, 5′-GCC CAA GGT ATA AAT GGC CTT CA-3′) and GAPDH (F, 5′-GTT ACC AGG GCT GCC TTC TC-3′; R, 5′-GGG TTT CCC GTT GAT GAC C-3′), and Ampli Taq Gold DNA Polymerase (Applied Biosystems, Carlsbad, CA) (5 min at 95°C, followed by 35 cycles at 95°C, 55°C and 72°C for 30 sec, respectively and a final incubation at 72°C for 7 min) as previously described (Holighaus et al., 2011). Amplification products were electrophoresed on an ethidium bromide-stained 1.8% agarose/TAE gel.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Rat cortical neurons were treated with 100 nM PACAP-38 or 25 μM forskolin in the presence or absence of various pharmacological inhibitors. cDNA samples were prepared as described above and qRT-PCR-amplified by using 200 nM gene-specific primers and iQ SYBR Green Supermix on an iCycler iQ Real Time PCR System (Bio-Rad, Carlsbad, CA). cDNA levels of the gene of interest were normalized to GAPDH levels. The following primers were used: STC1 F, 5′-CTA CTT TCC AGA GGA TGA TCG C-3′; STC1 R, 5′-ACT TCA GTG ATG GCT TCC GG-3′; BDNF F, 5′-TCA TAC TTC GGT TGC ATG AAG G-3′; BDNF R, 5′-AGA CCT CTC GAA CCT GCC C-3′; GAPDH, see above.

Measurement of intracellular cyclic AMP generation

Rat cortical neurons were grown in 48-well plates and stimulated with 25 μM forskolin or 100 nM PACAP-38 in the presence or absence of various concentrations of the adenylate cyclase inhibitor 2′5′-dideoxyadenosine in medium containing 500 μM of the non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). After a 20-min-incubation at 37°C, intracellular cAMP levels were measured with the cAMP Biotrak Enzymeimmunoassay (EIA) System (Amersham Biosciences) using the non-acetylation EIA procedure with the provided lysis reagent according to the manufacturer's instructions.

Measurement of intracellular calcium concentrations ($[Ca^{2+}]_i$) in single cells

Rat cortical neurons were grown on 1.5-cm-diameter glass cover slips (Assistent, Sondheim/Rhoen, Germany) coated with 0.5 mg/ml poly-L-lysine (in 12-well plates). $[Ca^{2+}]_i$ were measured as previously described (Holighaus et al., 2011). Briefly, cells were loaded with 4 μM fura-2 AM (Molecular Probes, Eugene, OR) in Krebs-Ringer buffer (KRB: 20 mM HEPES, 125 mM NaCl, 5.5 mM glucose, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgSO_4$ and 1 mM $CaCl_2$; pH 7.3) for 22 min under gentle agitation and washed with KRB for an additional 22 min. Cover slips were mounted onto a custom-built perfusion chamber and placed on an inverted Olympus IX70 microscope. Cells were perfused with KRB in the presence or absence of drugs at a flow rate of 800 μl/min. Ca^{2+}-free measurements were carried out in KRB without $CaCl_2$ supplemented with 100 μM EGTA. $[Ca^{2+}]_i$ were measured using the 340/380 excitation ratio (R 340/380) and an emission wavelength of 510 nm. Images were captured every 2 sec and analyzed with the software MetaFluor (Molecular Devices).

Immunoblotting of phosphorylated and total p44/42 MAPK

Immunoblotting was performed as previously described (Holighaus et al., 2011) according to the protocol of Cell Signaling Technology (Beverly, MA) using the NuPAGE electrophoresis system (Invitrogen, Carlsbad, CA). Briefly, cells were treated with 100 nM PACAP-38 or 25 μM forskolin in the presence or absence of various pharmacological inhibitors (in 12-well plates). After the indicated incubation time, cells were lysed in 100 μl of freshly prepared 1X lysis buffer (NuPAGE LDS sample buffer, NuPAGE reducing agent, Roche cocktail inhibitor tablet and Thermo Scientific Halt phosphatase inhibitor cocktail) and sonicated for 10–15 sec to reduce sample viscosity. Samples were heat-treated for 5 min at 95°C and micro-centrifuged. 15 μl of each sample was subjected to SDS-PAGE (120 V for 1.5 h) on 4–12% Novex Bis-Tris Gels. Separated proteins were electrotransferred to a 0.45 μm nitrocellulose membrane (30 V for 1.5 h). The membrane was blocked and successively incubated with rabbit polyclonal antibody specific for phosphorylated p44/42 MAP Kinase and total p44/42 MAP Kinase (1:1000 dilution; Cell Signaling Technology, Beverly, MA). After incubating the membrane with HRP-conjugated anti-rabbit secondary antibody (1:3000 dilution), immunoreactive bands were detected with the Super Signal West Pico Chemiluminescence Substrate (Thermo Fisher Scientific, Rockford, IL). To remove the

bound phospho-p44/42 MAP Kinase antibody, the membrane was incubated in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Rockford, IL) before incubating the same blot with total p44/42 MAP Kinase antibody.

Statistics

Statistical analysis was carried out in Prism 4 (GraphPad Software, La Jolla, CA) by unpaired t-test or one-way ANOVA with Dunnett's Multiple Comparison Test. Significance was set at $p < 0.05$.

Results

Characteristics of PACAP receptor expression and second messenger production in rat cortical neurons

To determine which PACAP receptors are expressed in primary cultures of rat cortical neurons, an analysis of VPAC/PAC receptor-mRNAs was performed by RT-PCR. To differentiate between the different PAC1 splice variants of the third intracellular loop (ic3), a single PAC1 forward primer (PAC1 F) was used in combination with three different PAC1 reverse primers (PAC1 R, PAC1hop R, PAC1hip R). PAC1 F matches the beginning of transmembrane region five (TM 5) corresponding to bases 1018 to 1037 of the rat PAC1null sequence Z23279. PAC1 R matches the end of TM 6 corresponding to bases 1185 to 1204 of the rat PAC1null sequence Z23279, generating a 187- or 271-bp fragment, depending on whether or not a hip- or hop-insert is present. PAC1hop R matches the end of the hop cassette corresponding to bases 1193–1217 of the rat PAC1hop1 sequence Z23274, generating a 200-bp fragment. PAC1hip R matches the beginning of the hip cassette corresponding to bases 1140–1159 of the rat PAC1hip sequence Z23273 and generates a 142-bp fragment. Cortical neurons predominantly expressed the PAC1hop and null receptor variants. A low expression of PAC1hip receptors and a very low expression of VPAC1 and VPAC2 receptors was also detected, suggesting that PACAP's action in cortical neurons is mainly mediated by the PAC1hop and null receptor variants with a minor contribution of PAC1hip and VPACs (Fig. 1B).

PACAP-mediated second messenger production was next examined in cortical neurons. Treatment of neuronal cultures with 100 nM PACAP resulted in a robust increase of intracellular cAMP. The amount generated was approximately 60% of the amount generated by supramaximal stimulation of adenylate cyclases (ACs) with 25 μM forskolin. PACAP- and forskolin-induced stimulation was 8.6 ± 2.3 and 16.5 ± 4.2 fold of control, respectively. The AC inhibitor 2′5′-dideoxyadenosine (ddAd) reduced PACAP-stimulated cAMP levels in a concentration-dependent manner. At a concentration of 600 μM, ddAd completely blocked cAMP production (Fig. 1C).

A robust Ca^{2+} response was also obtained by stimulation with 100 nM PACAP as determined by single cell Ca^{2+} imaging experiments. The Ca^{2+} response consisted of a rapid elevation of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) followed by a persisting plateau. To differentiate between Ca^{2+} influx from the extracellular and Ca^{2+} release from intracellular stores (i.e., Ca^{2+} mobilization), cells were stimulated with PACAP in Ca^{2+}-free buffer. PACAP induced a rapid and temporally restricted rise in intracellular Ca^{2+}, representing Ca^{2+} release from intracellular stores. Upon reintroduction of Ca^{2+}, Ca^{2+} entered the cell leading to prolonged Ca^{2+} influx. 25% of all cells showed no response or a very weak response to PACAP and were excluded from the graph (Fig. 1D).

These data suggest that the majority of rat cortical neurons are capable of engaging combinatorial signaling through cAMP and Ca^{2+}, which is in agreement with a predominant expression of PAC1hop and null receptors. Cyclic AMP accumulation through AC

activation presumably occurs through activation of Gs, whereas Ca^{2+} mobilization is most likely Gq-mediated, proceeding through PLCβ activation, DAG and IP_3 generation, and IP_3 receptor-mediated Ca^{2+} release from intracellular stores.

PACAP and forskolin activate the MAPK ERK1/2 in cortical neurons independently of PKA

The importance of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) pathway in mediating PACAP's neurotrophic effects has been extensively studied in PC12 cells. Here, the activation of ERK was characterized in primary cultures of rat cortical neurons by phosphorylation of the p44/42 MAPK (ERK1/2). Exposure of neurons to 100 nM PACAP or 25 μM of the AC activator forskolin resulted in a rapid and prolonged activation of ERK1/2 (Fig. 2A). ERK activation was blocked by 600 μM of the AC inhibitor 2′5′-dideoxyadenosine (ddAd). 300 μM ddAd, however, failed to block PACAP-mediated ERK activation, indicating that very low levels of cAMP are sufficient to activate ERK (see Fig. 1C for effects of ddAd on PACAP-stimulated cAMP generation). H89, an inhibitor of the cAMP dependent protein kinase (PKA), neither blocked PACAP- nor forskolin-induced ERK activation (Fig. 2B and C), suggesting that ERK activation in rat cortical neurons proceeds through cAMP but not PKA. The MEK1/2 inhibitor U0126 completely blocked the activation of ERK, as expected. No treatment affected total ERK levels.

PACAP and forskolin activate STC1 gene induction through ERK1/2 but not PKA in cortical neurons

In a microarray analysis we identified stanniocalcin 1 (STC1) up-regulated upon 6 h of PACAP treatment in PC12-G cells expressing physiological levels of the PAC1hop receptor and in primary bovine chromaffin cells (BCCs) (Ait-Ali et al., 2010). In the present study the induction of STC1 was examined in neurons of the central nervous system, where STC1 could be a mediator of PACAP's neuroprotective effects in e.g., stroke. Primary rat cortical neurons responded to 100 nM PACAP with an induction of STC1 mRNA after 3 and 6 h as measured by qRT-PCR (Fig. 3A). STC1 induction after 6 h (8.9 ± 3.1 fold of control) was blocked by the MEK1/2 inhibitor U0126, whereas the PKA inhibitor H89 was without effect (Fig. 3B). Moreover, the AC activator forskolin also induced STC1 gene transcription (6.3 ± 1.6 fold of control) via the same signaling pathway used by PACAP; i.e., induction was blocked by U0126 but not blocked by H89 (Fig. 3C), suggesting that signaling to the STC1 gene in cortical neurons proceeds through cAMP and ERK1/2 but not through PKA.

PACAP activates BDNF gene induction through PKA in cortical neurons

Neurotrophic factors like brain-derived neurotrophic factor (BDNF) are important mediators of neuronal survival after brain injury. Exposure of cortical neurons to 100 nM PACAP induced BDNF mRNA significantly after 3 h (Fig. 4A). This induction was, unlike the induction of STC1, blocked by the PKA inhibitor H89 (Fig. 4B), suggesting that two potentially neuroprotective pathways co-exist in cortical neurons, one being PKA dependent, and another being PKA independent, but cAMP and ERK1/2 dependent.

Discussion

PACAP's neuroprotective effects in the central nervous system (CNS) in vivo, specifically after middle cerebral artery occlusion (MCAO) producing cortical infarction (Reglodi et al., 2000; Reglodi et al., 2002; Tamas et al., 2002; Chen et al., 2006; Ohtaki et al., 2006) suggests that activation of PAC1 and respective signal transduction pathways mediate this effect. We found that cultures of rat cortical neurons predominantly expressed the PAC1null and hop receptor variants and low levels of PAC1hip, VPAC1 and VPAC2 receptors, in agreement with previously published reports showing a predominant expression of

PAC1hop and null in the human and rat brain and neuronal cells of various brain regions including the cerebral cortex (Cavallaro et al., 1996; Pisegna et al., 1996; Nogi et al., 1997; Zhou et al., 2000; Lutz et al., 2006). PACAP-stimulation of cortical cultures triggered a robust intracellular cAMP production that was ~60% of the maximal cAMP production generated by direct supramaximal stimulation of ACs with 25 μM forskolin, suggesting that PAC1 receptors in differentiated cortical neurons are efficiently coupled to ACs, in agreement with a high endogenous expression level of PAC1hop and null receptors in these cells. PACAP also stimulated an intracellular Ca^{2+} response, consisting of a robust initial burst of Ca^{2+} mobilization, presumably Ca^{2+} release from IP_3-sensitive intracellular stores, followed by prolonged Ca^{2+} influx, in agreement with previously published results (Grimaldi et al., 1999). Only ~75% of all cells responded to PACAP with a robust increase in $[Ca^{2+}]_i$, whereas the remaining 25% showed only a weak or no response. This indicates that a fraction of the cell population expresses no PACAP receptors or expresses PACAP receptors that either fail to couple to Ca^{2+} or couple to Ca^{2+} inefficiently, such as the PAC1hip or PAC1null receptor variant, respectively (Holighaus et al., 2011). It has been shown that PACAP fails to stimulate an intracellular Ca^{2+} response in cortical precursors endogenously expressing the PAC1null receptor, whereas ectopic expression of PAC1hop confers a PACAP-mediated rise in $[Ca^{2+}]_i$ (Lu et al., 1998; Nicot et al., 2001).

In differentiated neurons, the MAPK/ERK pathway has been implicated in cell survival, e.g., following excitotoxic or hypoxic/ischemic injury (Hetman et al., 1999; Gonzalez-Zulueta et al., 2000; Irving et al., 2000; Elliott-Hunt et al., 2002; Jin et al., 2002; Hetman et al., 2004). Moreover, the importance of ERK in mediating PACAP's neuroprotective effects has been shown in cerebellar granule cells during K^+ deprivation (Villalba et al., 1997), and in parasympathetic (Pugh et al., 2006) and sympathetic neurons during growth factor withdrawal (May et al., 2010). Here, we show that PACAP activated ERK1/2 in a rapid and sustained fashion. Activation was blocked by AC but not PKA inhibition, therefore proceeding through a non-canonical cAMP pathway (i.e., independently of PKA). High concentrations of the AC inhibitor ddAd were required to block ERK activation in cortical neurons, suggesting that very low concentrations of cAMP are sufficient to signal to ERK in these cells. PACAP-mediated ERK activation was mimicked by the AC activator forskolin, which was also not blocked by inhibition of PKA with H89. These results suggest that cAMP is required and sufficient to engage a non-canonical (PKA independent) signaling pathway in cortical neurons to activate ERK.

Whether PACAP induces neuroprotective genes through ERK activation in primary CNS neurons, potentially mediating PACAP's neuroprotective effects during brain injury has not been previously delineated. The present study shows that PACAP induces the potentially neuroprotective gene stanniocalcin 1 (STC1), but not the related STC2 (not shown), in rat cortical neurons. STC1 is therefore a candidate target gene for mediating PACAP's neurotrophic and neuroprotective effects in the CNS. PACAP-mediated induction of STC1 was blocked by the MEK1/2 inhibitor U0126. The PKA inhibitor H89 failed to block STC1 induction by PACAP. The AC activator forskolin also induced STC1, also via ERK independently of PKA. These results indicate a cAMP and ERK dependent but PKA independent signaling pathway to the STC1 gene, in agreement with a cAMP dependent and PKA independent activation of ERK.

PACAP-mediated non-canonical cAMP dependent signaling to ERK and STC1 gene induction was shown before by our laboratory in PAC1hop-expressing neuroblastoma x glioma NG108-15 cells (Holighaus et al., 2011). Moreover, activation of this signaling pathway by PACAP in pheochromocytoma PC12 (Ravni et al., 2008), neuroblastoma SH-SY5Y (Monaghan et al., 2008) and primary chromaffin cells (Hamelink et al., 2002a) is implicated in neuronal differentiation and gene induction through activation of ERK.

Activation of non-canonical cAMP signaling and induction of STC1 in primary cortical neurons, as demonstrated by the present study, may be involved in PACAP's cAMP dependent neuroprotective effects during excitotoxicity (Morio et al., 1996; Said et al., 1998; Shintani et al., 2005) and hypoxia/ischemia (Stumm et al., 2007) in neuronal cell culture and during stroke in vivo (Chen et al., 2006).

PACAP also activated the canonical cAMP/PKA pathway to induce the neurotrophin BDNF, in agreement with previously published results (Pellegri et al., 1998). Activation of PKA has been shown to be involved in mediating PACAP's survival-promoting effects, e.g., during growth factor withdrawal in PC12 cells (Tanaka et al., 1997) and primary sympathetic neurons (May et al., 2010), during glutamate-induced excitotoxicity in cultured retinal neurons (Shoge et al., 1999) and PC12 cells (Onoue et al., 2002b), and in cerebellar granule neurons incubated in low-potassium medium (Kienlen Campard et al., 1997; Villalba et al., 1997). Moreover, BDNF has been implicated in mediating PACAP's neuroprotective effects during excitotoxicity (Frechilla et al., 2001). In addition to PACAP's stimulatory effects on PKA and the MAP kinases ERK1/2, negative modulation of the MAP kinases p38 and Jun N-terminal kinases (JNK) have also been implicated in the neuroprotective mechanisms of PACAP during ischemia. Activation of neuronal STAT3 by IL-6 is another putative neuroprotective pathway, which is indirectly activated by PACAP (Ohtaki et al., 2008), suggesting that several PACAP-activated neuroprotective pathways exist in the CNS. PACAP does not induce IL-6 in cultured cortical neurons, as measured after 1, 3 and 6 h by RT-PCR, whereas it induces IL-6 in PAC1hop-expressing NG108-15 cells most prominently after 3 h (not shown). This suggests that PACAP induces IL-6 in glial cells, most likely in astrocytes, to protect neurons from apoptosis (Tatsuno et al., 1996; Shioda et al., 2006).

In conclusion, we provide evidence for the existence of non-canonical cAMP signaling in primary CNS neurons, mediating activation of ERK and induction of the putative neuroprotectant STC1. PACAP activates both canonical (to e.g., induce BDNF) and non-canonical cAMP signaling. However, the selective pharmacological activation of a cAMP pathway not involved in physiological processes such as learning and memory might reduce the detrimental side effects of drugs targeting the cAMP-PKA pathway (Kuo et al., 1969; Kandel, 2001; Arnsten et al., 2005).

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Abbreviations

PACAP	pituitary adenylate cyclase-activating polypeptide
PAC1	PACAP type 1 receptor
ic3	third intracellular loop
STC1	stanniocalcin 1
BDNF	brain-derived neurotrophic factor
GPCR	G protein-coupled receptor
AC	adenylate cyclase
PKA	protein kinase A

ERK	extracellular signal-regulated kinase
MEK	MAPK/ERK kinase
CNS	central nervous system
F	forward primer
R	reverse primer

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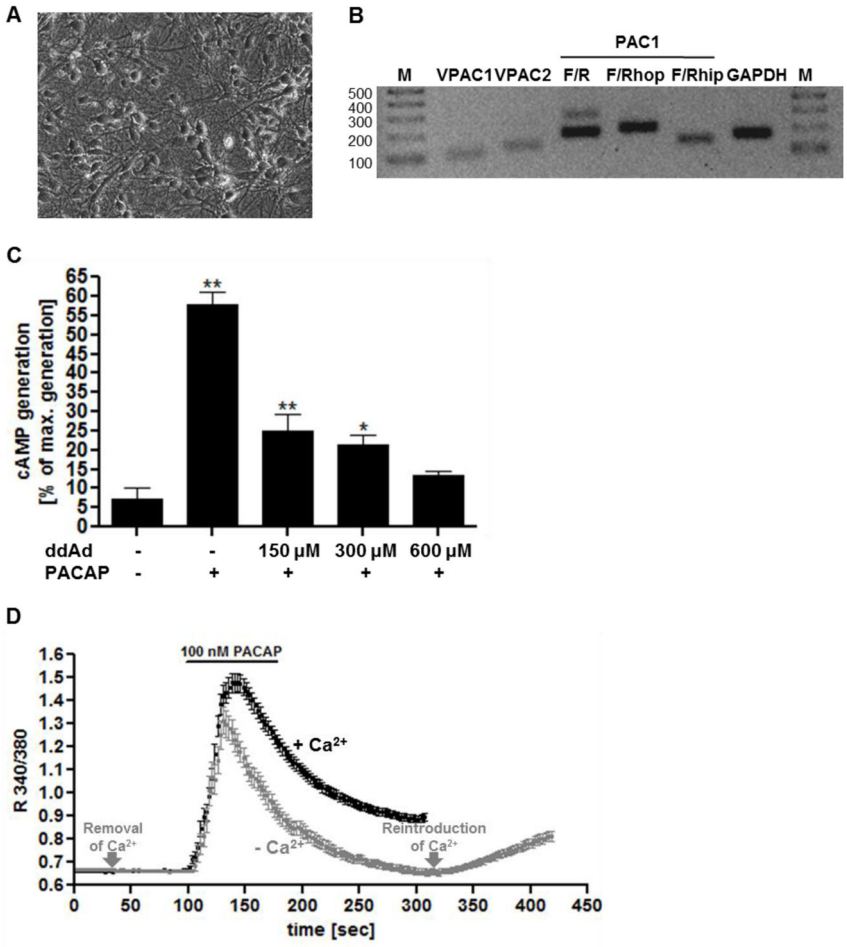
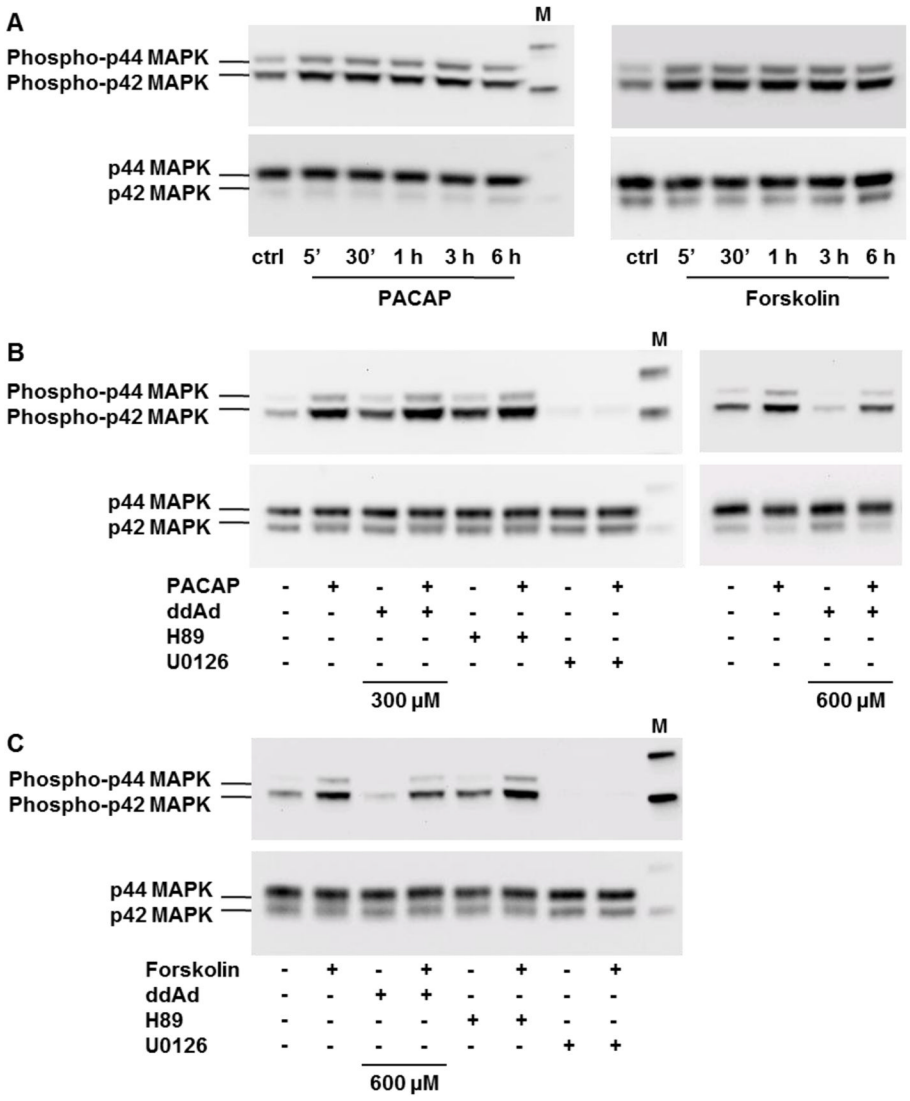


Fig. 1. Rat cortical neurons predominantly express the PAC1null and hop variants (B). Stimulation with PACAP induces intracellular cyclic AMP generation (C) and Ca^{2+} mobilization and influx (D)

(A) Photomicrograph of rat cortical neurons and (B) RT-PCR analysis of endogenous VPAC1, VPAC2 and PAC1 receptors. Total RNA was reverse-transcribed and PCR-amplified (35 cycles) with different primer pairs for rat VPAC1, VPAC2 and PAC1. To differentiate between PAC1 splice variants of the third intracellular loop different primer pairs within the transmembrane region 5 and 6 were used. The same forward primer (F) was used with different reverse primers (R): F/R generates a 187- or 271-bp fragment, depending on whether or not a hip- or hop-insert is present; the hop-specific primer pair F/hopR generates a 200-bp fragment; the hip-specific primer pair F/hipR generates a 142-bp fragment. Primers for VPAC1 generate a 107-bp fragment and for VPAC2 a 133-bp fragment. GAPDH product size is 168-bp. M: 100-bp DNA ladder. (C) Cells were treated with 100 nM PACAP-38 for 20 min with or without pre-treatment with 150, 300 or 600 µM 2′5′-dideoxyadenosine (ddAd) for 30 min and intracellular cAMP generation was measured. Plot represents the grand mean ± SEM of three independent experiments performed in triplicates. Values are expressed as percentage of maximal intracellular cAMP generation, which was determined by stimulation with 25 µM forskolin. ** $P < 0.01$, * $P < 0.05$ versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test. (D) Cells were loaded with 4 µM fura-2 AM and stimulated with 100 nM PACAP-38 for 75 sec in Ca^{2+}-containing (black line) or Ca^{2+}-free (gray line) Krebs-Ringer buffer. Intracellular Ca^{2+} concentrations were measured in single cells using the 340/380 excitation ratio (R 340/380)

and an emission wavelength of 510 nm. Images were captured every 2 sec. Plots represent the average ± SEM of four (− Ca^{2+}) or 11 (+ Ca^{2+}) independent experiments. Each experiment represents 6–22 cells (− Ca^{2+}: n=59, + Ca^{2+}: n=155). ~25% of all cells show no or a very weak response to PACAP and are excluded from this graph.



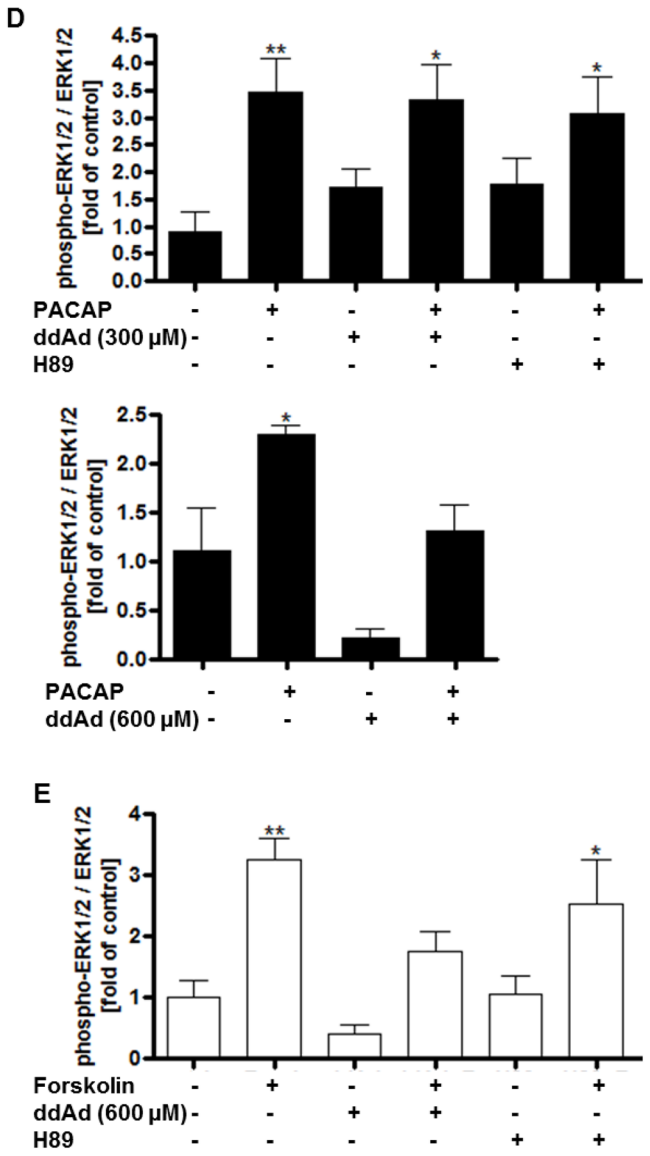


Fig. 2. PACAP and forskolin activation of the p44/42 MAPK (ERK1/2) is rapid and prolonged in rat cortical neurons (A). Activation is blocked by the AC inhibitor 2′5′-dideoxyadenosine but not blocked by the PKA inhibitor H89 (B+C)

Cells were stimulated with 100 nM PACAP-38 or 25 μM forskolin in the presence or absence of the AC inhibitor 2′5′-dideoxyadenosine (ddAd, 300 and 600 μM), the PKA inhibitor H89 (10 μM) or the MEK1/2 inhibitor U0126 (10 μM) (30 min pre-treatment with inhibitors). Whole cell extracts were harvested after 5 min, 30 min, 1, 3 or 6 h (A) or after 5 min (B+C). ERK activation by PACAP and forskolin is rapid and prolonged (A) and unaffected by H89, but blocked by 600 μM ddAd and U0126 (B+C). No treatment affects total ERK1/2 levels. M: 40 and 50 kDa band. (D+F) Quantification of B+C. Values are expressed as the ratio of phospho-ERK divided by total ERK and represent the mean ± SEM of three to four independent experiments performed in singlicates. The control value represents the mean of the fold of average control, which was determined by calculating the p-ERK/ERK mean of the control values and dividing each pERK/ERK control value by this mean. U0126 is not shown in the plots. ** $P < 0.01$, * $P < 0.05$ versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test.

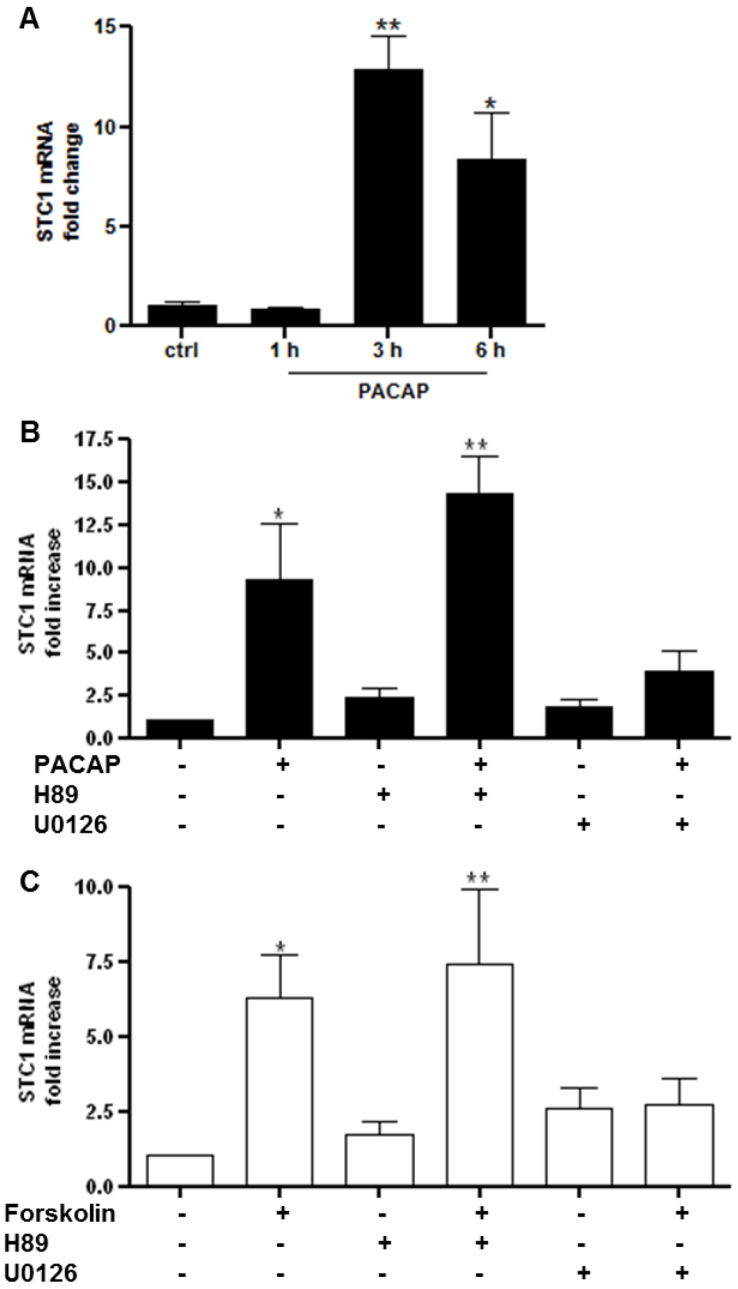


Fig. 3. Induction of STC1 mRNA by PACAP or forskolin after 6 h is blocked by the MEK1/2 inhibitor U0126 but not blocked by the PKA inhibitor H89 in rat cortical neurons

Cells were stimulated with 100 nM PACAP-38 (black bars) or 25 μM forskolin (white bars) in the presence or absence of the PKA inhibitor H89 (10 μM) or the MEK1/2 inhibitor U0126 (10 μM) (30 min pre-treatment with inhibitors). Cells were lysed after 1, 3 or 6 h (A) or after 6 h (B+C). Total RNA was extracted and reverse-transcribed. Transcript levels were measured by qRT-PCR. Values in A represent the mean ± SEM of one experiment performed in triplicates. Experiment was repeated once with similar results. In B and C, values represent the grand mean ± SEM of five to nine independent experiments performed in triplicates. Values are expressed as fold change of STC1 versus GAPDH mRNA. * P <

0.05, ** P < 0.01 versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test.

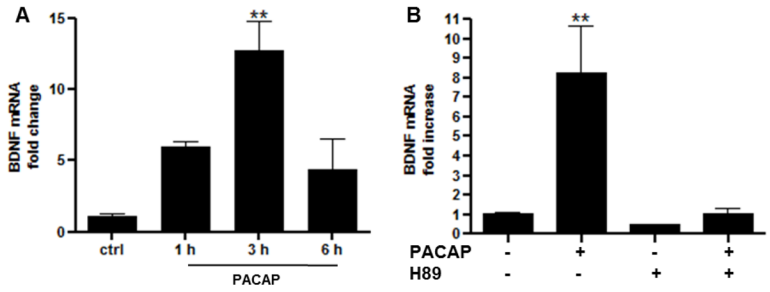


Fig. 4. Induction of BDNF mRNA by PACAP is blocked by the PKA inhibitor H89 in rat cortical neurons

Cells were stimulated with 100 nM PACAP-38 in the presence or absence of the PKA inhibitor H89 (10 μM, 30 min pre-treatment). Cells were lysed after 1, 3 or 6 h (A) or after 3 h (B). Total RNA was extracted and reverse-transcribed. Transcript levels were measured by qRT-PCR. Values in A represent the mean ± SEM of one experiment performed in triplicates. Experiment was repeated once with similar results. In B, values represent the grand mean ± SEM of four independent experiments performed in triplicates. Values are expressed as fold change of BDNF versus GAPDH mRNA. ** $P < 0.01$ versus control; one-way ANOVA, with Dunnett's Multiple Comparison Test.