Neuroprotective signalling mechanisms in the mammalian brain

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Neuronal AKAP150 coordinates PKA and Epac2 mediated PKB/Akt phosphorylation

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Abstract

In diverse neuronal processes ranging from neuronal survival to synaptic plasticity, cyclic adenosine monophosphate (cAMP)-dependent signaling is tightly connected with the protein kinase B (PKB)/Akt pathway but the precise nature of this connection remains unknown. In the current study we investigated the effect of two mainstream pathways initiated by cAMP, cAMP-dependent protein kinase (PKA) and exchange proteins directly activated by cAMP (Epac1 and Epac2) on PKB/Akt phosphorylation in primary cortical neurons and HT-4 cells. We demonstrate that PKA activation leads to a reduction of PKB/Akt phosphorylation, whereas activation of Epac has the opposite effect. This effect of Epac on PKB/Akt phosphorylation was mediated by Rap activation. The increase in PKB/Akt phosphorylation after Epac activation could be blocked by pretreatment with Epac2 siRNA and, to a somewhat smaller extent, by Epac1 siRNA. PKA, PKB/Akt and Epac were all shown to establish complexes with neuronal A-kinase anchoring protein 150 (AKAP150). Interestingly, activation of Epac increased phosphorylation of PKB/Akt complexed to AKAP150. From experiments using PKA-binding deficient AKAP150 and peptides disrupting PKA anchoring to AKAPs, we conclude that AKAP150 acts as a key regulator in the two cAMP pathways to control PKB/Akt phosphorylation.
3.1 Introduction

Cyclic adenosine monophosphate (cAMP) is one of the most common and versatile intracellular signaling compounds and has been implicated in the downstream transfer of molecular messages upon stimulation by a large number of hormones, neurotransmitters, prostaglandins and odorants. It functions as a universal and highly modulated second messenger for events underlying a wide variety of cellular processes such as central metabolic events, cardiac and smooth muscle contraction, secretory processes, ion channel conductance, learning and memory, cell growth and differentiation, apoptosis and inflammatory responses (Beavo and Brunton, 2002). Although originally cAMP-dependent protein kinase (PKA) was thought to be the major if not the sole effector of cAMP, meanwhile other targets have been identified, in particular, exchange factors directly activated by cAMP (Epac) proteins. To date, there are two variants of Epac known, Epac1 and Epac2, and both were initially characterized as exchange factors for the small GTPases Rap1 and Rap2 (Bos, 2006). Epac1 is ubiquitously distributed with predominant expression in the thyroid, kidney, ovary, skeletal muscle, and specific brain regions, whereas Epac2 is mainly expressed in the brain and adrenal gland (de Rooij et al., 1998; Kawasaki et al., 1998). The available information on the functional role of Epac in neurons is still rather limited. It was shown that Epac enhances neurotransmitter release in glutamatergic synapses of the rat brain calyx of Held (Sakaba and Neher, 2003) and in the crayfish neuromuscular junction (Zhong and Zucker, 2005). In dorsal root ganglion neurons, Epac mediates the translocation and activation of protein kinase C (PKC) leading to the establishment of inflammatory pain (Hucho et al., 2005) and in cerebellar granule cells Epac can modulate neuronal excitability (Ster et al., 2007).

Since PKA and Epac are co-expressed in many tissues, an increase in intracellular cAMP levels can lead to the activation of both cAMP targets, thus specificity and coordination of cAMP signaling is urgently required. Members of the A-kinase anchoring protein (AKAP) family were shown to play a pivotal role in the intracellular targeting and compartmentalization of cAMP signaling pathways (Taskén and Aandahl, 2004; Wong and Scott, 2004). AKAPs represent a group of more than 50 identified functionally related proteins. Although they share few primary structure similarities, they all have the ability to bind the PKA regulatory subunit (Herberg et al., 2000). Besides a binding site for PKA many AKAPs contain distinct binding sites for other signaling enzymes such as phosphatases (Colledge and Scott, 1999), phosphodiesterases (Dodge et al., 2001) and other protein kinases such as PKC (Klauck et al., 1996; Nauert et al., 1997; Takahashi et al., 1999).

Intriguingly, recent studies identified a signal transduction complex formed by muscle AKAP (mAKAP) at the nuclear envelope of striated myocytes, which contains both PKA and Epac1 (Dodge-Kafka et al., 2005). Upon association with both PKA and Epac1, mAKAP serves as a coordinator of two cAMP effector pathways to regulate cellular processes. Hitherto, mAKAP is the only AKAP reported to associate with both PKA and Epac1. However, both PKA anchored to neuronal AKAP79/150
and Rap GTPases have been shown to be involved in AMPA receptor trafficking during synaptic plasticity (Snyder et al., 2005; Zhu et al., 2002). Thus, AKAP79/150 might also serve to integrate cAMP signals to coordinate distinct signaling properties in neuronal cells.

Elevation of cAMP was shown to influence neuronal survival and axonal outgrowth via multiple signaling cascades including protein kinase B (PKB)/Akt signaling (Cui and So, 2004). PKB/Akt activated in response to elevated cAMP has also been reported to be of importance for learning and memory (Lin et al., 2001). Since the alteration of PKB/Akt activity is associated with several diseases, including Alzheimer’s disease (Griffin et al., 2005), novel insights into the molecular mechanisms of the regulation of PKB/Akt signaling are essential for successfully developing new therapeutics. So far, the precise mechanism of how PKA and Epac regulate PKB/Akt activity in neurons remained unknown.

Therefore, we investigated herein the molecular mechanisms by which PKA and Epac affect the PKB/Akt signaling in primary cortical neurons and HT-4 cells, in particular we focused on the role of AKAP150 in this neuronal response.

3.2 Materials and Methods

Cell cultures and toxin treatment. Primary cortical neurons were isolated from embryonic brains (E15-16) of C57BL/6J mice (Harlan, Horst, The Netherlands). The meninges were removed and the cells were separated by mechanical dissociation. Cortical neurons were plated in a density of $2 \times 10^6$ cells/well on 2 µg/ml poly-D-lysine (PDL, Sigma-Aldrich, St. Louis, MO, USA) coated 6 well plates. Neurobasal medium with B27-supplement (Invitrogen, Carlsbad, CA, USA), 0.5 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA), 50 units/ml penicillin/streptomycin (Invitrogen) and 2.5 µg/ml amphotericin B (Sigma-Aldrich) was used as a culture medium. After 48 h cells were treated with 10 µM cytosine arabinoside (Sigma-Aldrich) for another 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged and, after 6 days of in vitro culture, primary cortical neurons were used for experiments. Clostridium difficile Toxin B-1470 (strain 82) was purchased from tgcBiomics (Mainz, Germany) and applied on neurons for 3 h, at a concentration of 300 pg/ml (Schmidt et al., 1998; Reineke et al., 2007).

Acquisition of primary cultures was under the regulation of the Ethical Committee for the use of experimental animals of the University of Groningen, The Netherlands (DEC 4048). The mouse hippocampal-derived HT-4 cell line was grown in RPMI 1640 (Invitrogen) optimal medium containing 10% (v/v) fetal calf serum (Invitrogen) and 50 units/ml penicillin/streptomycin. HEK293FT cells were cultured in D-MEM (Invitrogen) full medium containing 10% (v/v) fetal calf serum, 50 units/ml penicillin/streptomycin, 1% (v/v) non essential amino acids (Invitrogen) and 4 µg/ml geneticin (Invitrogen).
Drug treatment. Cells were incubated for the indicated time period with 50 µM forskolin, 50 µM H89, 50 µM cell-permeable Ht31 (InCELLect® AKAP St-Ht31 inhibitor peptide (Promega, Madison, WI, USA)), 50 µM of the cell permeable Ht31 inactive control peptide (InCELLect® St-Ht31P (Promega)), 5 or 20 µM stearated superAKAP-IS (kindly provided by B. Penke, University of Szeged, Hungary), 1, 5 or 20 µM 5.6 dichlorobenzi-midazole-riboside-3',5'-cyclic monophosphoro-thioate Sp-isomer (Sp-5,6-DCl-cBIMPS), or 5, 10, 50 or 100 µM 8-(4-chlorophenylthio)-2'-O-methyl cyclic AMP (8-pCPT-2Me-cAMP (BioLog, Lifescience Institute, Bremen, Germany)). The final concentration of dimethyl sulfoxide in all experiments was less than 0.5% (v/v). Dimethyl sulfoxide solvent itself had no effect on PKB/Akt phosphorylation (data not shown).

Protein analysis. Primary cortical neurons or HT-4 cells, which were grown in a 6 well plate, were washed twice with ice cold phosphate-buffered saline (PBS) and subsequently lysed by the addition of 0.15 ml lysis buffer 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and complete mini protease inhibitor cocktail tablet (Roche Diagnostics, Basel, Switzerland). The samples were centrifuged at 9 000g for 10 min at 4 °C and the supernatant boiled for 5 min in Laemmli’s sample buffer. Twenty µg of total protein was separated by 10% SDS-polyacrylamide gel electrophoresis. After transfer to a PVDF transfer membrane (Millipore Corporation, Billerica, MA, USA) proteins were linked with primary antibodies overnight at 4 °C. Primary antibodies used were a rabbit polyclonal antibody specific for total PKB/Akt (9272, Cell Signaling, Danvers, MA, USA, 1:2 000 dilution), p-Akt (Ser473, 9271, Cell Signaling, 1:2 000 dilution), C-terminal AKAP150 C20 (sc-6445, Santa Cruz, CA, USA, 1:2 000 dilution), N-terminal AKAP150 antibody N-19 (sc-6446, Santa Cruz, CA, USA, 1:1 000 dilution), Epac1 (1:10 000 dilution for Western blot), Epac2 (1:10 000 Western blot), PKA RIIβ (610625, BD Biosciences, Franklin Lakes, NJ, USA, 1:3 000 dilution), PKBα/Akt1 (5919, Abcam, Cambridge, UK, 1:2 000 dilution), PKBβ/Akt2 (5920, Abcam, 1:2 000 dilution), PKBγ/Akt3 (5922, Abcam, 1:2 000 dilution) and a monoclonal mouse antibody specific for actin as internal standard (MP Biomedicals, Irvine, CA, USA, 1:100 000 dilution). Phosphorylation of PKB/Akt was evaluated as the ratio of p-Akt Ser473/ total Akt. Actin was used as an internal control to correct for variations in protein content. The blots were incubated with alkaline phosphatase-conjugated goat anti-mouse (Applied Biosystems, Bedford, MA, USA, 1:10 000 dilution) or alkaline phosphatase-conjugated goat anti-rabbit (Applied Biosystems, Bedford, MA, USA, 1:10 000 dilution). Proteins were detected with an enhanced chemoluminescence detection system (Nitroblock II and CDP-Star, Applied Biosystems, Bedford, MA, USA) according to the manufacturer’s instructions (Applied Biosystems, Bedford, MA, USA). Integrated optical densities (IOD) were measured by the Leica DFC 320 Image Analysis System (Leica, Cambridge, UK) and densitometric analysis was evaluated by the Leica Qwin program. Quantification of IOD was performed only
in images in which saturation of the signal had not occurred. IOD measurements were corrected for background intensity.

**Rap activation assay.** GTP-loading of Rap1 and Rap2 was performed by glutathione S-transferase fusion of the Rap1 and Rap2-binding domain of RalGDS (GST-RalDGS). Cells were grown to 70% confluence, then incubated in minimal serum medium (1% (v/v)) for 24 h. Cells were incubated with 50 µM forskolin or 50 µM 8-pCPT-2Me-cAMP for 10 min. Activation of Rap was terminated by washing twice with cold PBS. Cells were lysed with 10% (v/v) glycerin, 1% (v/v) Nonidet-P40, 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2 mM MgCl2, 0.2 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride and complete mini protease inhibitor cocktail tablet (Roche Diagnostics). Following a short centrifugation at 3 000g for 5 min, the supernatants were incubated with 15 µg of GST-RalGDS at 4 °C for 2 h with gentle agitation. The beads were washed 3 times with lysis buffer and then treated with 30 µl of SDS-sample buffer. 20 µl of protein sample was separated by 10% (v/v) SDS-polyacrylamide gel electrophoresis and probed with specific Rap1 (Santa Cruz, 1:5 000 dilution) and Rap2 (Santa Cruz, 1:5 000 dilution) antibodies.

**Confocal microscopy.** Cortical neurons were grown on PDL-coated coverslips for 6 days. Subsequently, they were fixed with 4% paraformaldehyde in PBS (pH 7.4) at 4 °C. Neurons were permeabilized with 0.2% TritonX-100/PBS and blocked with 10% (v/v) normal donkey serum (NDS) and 2% (v/v) bovine serum albumin (BSA) in PBS for one hour at room temperature. Slices were incubated overnight at 4 °C with antibodies raised against total PKB/Akt (1:1 000 dilution), C-terminal AKAP150 (1:750 dilution), Epac1 (1:750 dilution), Epac2 (1:750 dilution) or PKA (1:750 dilution). The next day, cells were incubated with secondary antibodies (Alexa 488, 555 and 633, Invitrogen, 1:500 dilution). For mounting we used PreLong Gold (Invitrogen). The confocal images were analyzed with a Leica TCS SP2 confocal microscope using an Ar/ArKr laser (488 nm) or a HeNe laser (543 and 633 nm). To avoid overlap in emission spectra we used sequential scanning by frame.

**Immunoprecipitation.** Lysed cell homogenates containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and complete mini protease inhibitor cocktail tablet (Roche) were used for AKAP150, Epac1, Epac2, PKA, PKB/Akt and uncoupled IgG immunoprecipitation. Per sample 100 µl of Dynabeads protein A (Dynal Biotech, Oslo, Norway) was washed twice with Na-phosphate buffer (0.1 M, pH 8.1). Ten µg of antibody was incubated with the beads for 10 min. Afterwards, the beads were washed three times with Na-phosphate buffer (0.1 M, pH 8.1) and twice with triethanolamine (0.2 M). IgGs were crosslinked with dimethyl pimelimidate (20 mM in 0.2 M triethanolamine) for 30 min. The beads were washed for 15 min with Tris (50 mM, pH 7.5) and three times with PBS. Unbound IgG was removed by washing
twice for 30 min with Na-citrate (0.1 M, pH 2-3). 500 μg of protein sample was incubated for 1 h with the beads. Bound proteins were eluted with Na-citrate (0.1 M, pH 2-3). pH of the eluted sample was equilibrated by 0.5 M Na-phosphate buffer with pH 8.1. All the steps of the immunoprecipitation procedure were performed at room temperature. The immunoprecipitated sample was stored at −80 °C until use for protein analysis.

siRNA experiments. siRNA probes targeted to either Epac1 or Epac2 were purchased from Dharmacon (Dharmacon, Inc. Lafayette, CO, USA). The target sequences for the mouse-specific Epac1 siRNAs mixture were as follows: sense: GA-ACGUAUCUCUCAGACCU (D-057800-01), sense: CGUGGUACAUUAUCUGGAAU (D-057800-02), sense: GGUCAAUUCUGCCGGUAAU (D-057800-03), sense: CGGCAACGGUUGGAAAAUU (D-057800-04) and for the Epac2 siRNA mixture: sense: GUACGGCAAAUUAUGUGAUU (D-057784-01), sense: CAAGUGUACGUGUGGAAC (D-057784-02), sense: ACGACGAGCUCCUUCAUAUU (D-057784-03), sense: GGACGUGAAGCUAAAU (D-057784-04). A scrambled siRNA probe was used as a control in all siRNA transfection experiments. Primary cortical neurons were transfected with siRNAs using the Amaxa nucleofector technology (Amaxa, Cologne, Germany). Cells were cultured for 4 days after nucleofection, treated, lysed and subsequently analyzed for protein content.

HT-4 cell lines expressing wild-type AKAP150 or AKAP150ΔPKA. For the generation of HT-4 cell lines expressing wild-type AKAP150 or AKAP150ΔPKA, the lentiviral expression system from Invitrogen was used. In short, AKAP150 or AKAP150ΔPKA (∆706-729) cDNA was cloned into the destination vector pLenti6/UBC/V5DEST. HEK293FT cells were transfected with the destination vector and an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG using calcium precipitation. Viral particles were harvested 40 h post-transfection and added to HT-4 cells for infection. Blasticidin was used for the selection of infected cells according to the manufacturer’s recommendations.

Statistical analysis. Results shown represent mean ±S.E.M. Statistical analysis was performed by Statistical Package for the Social Science (SPSS) program. The One-Sample T-Test was used to check whether the mean of a single variable differs from their appropriate control samples. These tests were followed by Univariate Analysis of Variance and one-way ANOVA LSD post-hoc program. P values of < 0.05 were considered to be significant.
3.3 Results

3.3.1 Opposing effects of PKA and Epac on PKB/Akt phosphorylation in primary cortical neurons.

PKB/Akt is activated through phosphorylation of the Ser$^{473}$ and Thr$^{308}$ sites (Lawlor and Alessi, 2001; Franke et al., 1997). In this study we focussed on phosphorylation of the Ser$^{473}$ site but preliminary data showed that phosphorylation at the Thr$^{308}$ site is regulated in a similar way (data not shown). Initially, we examined the temporal kinetics of the adenylate cyclase activator, forskolin, on PKB/Akt phosphorylation. Forskolin reduced PKB/Akt phosphorylation at the Ser$^{473}$ site in a time-dependent manner (Fig. 3.1(a)). Since 10 min of forskolin treatment already resulted in a strong decrease of PKB/Akt phosphorylation, this time-point was taken to investigate the concentration-dependent effect of the specific PKA activator Sp-5,6-DCI-cBIMPS on PKB/Akt phosphorylation. Sp-5,6-DCI-cBIMPS treatment for 10 min resulted in decreased PKB/Akt phosphorylation similar to forskolin treatment (Fig. 3.1(b)). In contrast, inhibition of PKA by H89, increased PKB/Akt phosphorylation in a time-dependent manner (Fig. 3.1(c)).

To investigate the effect of Epac activation on PKB/Akt phosphorylation, we first checked for the endogenous expression of Epac1 and Epac2 in primary cortical neurons. Both Epac1 and Epac2 were found to be expressed in primary cortical neurons (Fig. 3.2(a)). Treatment with forskolin or 8-pCPT-2Me-cAMP led to increased GTP-loading of Rap1 and Rap2, the main downstream effectors of Epac (Fig. 3.2(b) and (c)). Interestingly, PKB/Akt phosphorylation was increased by 8-pCPT-2Me-cAMP in a time- and concentration-dependent manner (Fig. 3.2(d) and (e)). Under the same experimental conditions, the highest concentration of 8-pCPT-2Me-cAMP used (100 µM) did not induce phosphorylation of the PKA specific effector CREB (data not shown). To validate whether Rap activation is an intermediate step in Epac-induced PKB/Akt phosphorylation we treated neurons with toxin B-1470, which is known to inactivate the main Epac effector Rap (Schmidt et al., 1998). 8-pCPT-2Me-cAMP-induced PKB/Akt phosphorylation was completely blocked by toxin B-1470 treatment (Fig. 3.2(f)). Toxin B-1470 treatment reduced PKB/Akt phosphorylation even under unstimulated conditions (Fig. 3.2(f)).

The role of Epac1 and Epac2 in Epac-induced phosphorylation of PKB/Akt was studied using specific siRNA probes. Epac1 and Epac2 siRNA probes reduced Epac1 and Epac2 expression by 42.1% and 53.2%, respectively, when compared to control samples. Scrambled siRNA did not significantly affect Epac1 and Epac2 expression (Fig. 3.3(a) and (b)). Pretreatment with Epac1 partially attenuated the 8-pCPT-2Me-cAMP-induced increase in PKB/Akt phosphorylation, whereas pretreatment with Epac2 siRNA completely blocked the effect of 8-pCPT-2Me-cAMP on PKB/Akt phosphorylation (Fig. 3.3(c)).

3.3.2 Epac and PKB/Akt are complexed to AKAP150.

Using confocal microscopy we assessed the subcellular localization of AKAP150,
3.3. Results

Figure 3.1: PKA activation negatively regulates PKB/Akt phosphorylation in cortical neurons. Forskolin (a) and H89 (c) were applied for the indicated time periods on cortical neurons. (b) A specific PKA activator Sp-5,6-DCI-cBIMPS (BIMPS) was applied in different concentrations for 10 min. Representative immunoblots are shown below the quantified data of PKB/Akt phosphorylation at the Ser\(^{473}\) site (α p473-Akt) analyzed from three separate experiments. The bars indicate the mean ± S.E.M. of IOD of protein bands corresponding to α p473-Akt as percentage of total PKB/Akt (depicted as total Akt) (Univariate Analysis of Variance and LSD test, \(p < 0.05\) versus non-treated neurons).
Figure 3.2: Epac activator 8-pCPT-2Me-cAMP increases PKB/Akt phosphorylation in cortical neurons. (a) Representative immunoblots of Epac1 and Epac2 expression in cortical neurons. (b) & (c). Neurons were treated with forskolin or 100 µM 8-pCPT-2Me-cAMP for Rap1 (b) and Rap2 (c) pull-down assays. Untreated cells served as controls. GTP-bound Rap1 (α GTP-Rap1) and GTP-bound Rap2 (α GTP-Rap2) pulled down by a GST immunoprecipitation and total Rap1/Rap2 were detected by Western blot. (d) & (e). 8-pCPT-2Me-cAMP was applied for the indicated concentrations (d) and time periods (e) on cortical neurons and PKB/Akt phosphorylation assessed. (f) Neurons were treated with Toxin B-1470 for 3 h. Where indicated, this treatment was followed by an incubation of 100 µM 8-pCPT-2Me-cAMP for 10 min. Representative immunoblots are shown below the quantified data analyzed from at least three separate experiments (mean ± SEM, LSD test, p < 0.05 versus indicated neurons).
3.3. Results

Figure 3.3: Silencing of Epac1 and Epac2 decreases PKB/Akt phosphorylation. Primary cortical neurons were transfected with specific Epac1 siRNA (a), Epac2 siRNA (b) or with scrambled siRNA. After 4 days, where indicated, neurons were treated with 100 µM 8-pCPT-2Me-cAMP for 10 min. (c) Cortical lysates were analyzed for PKB/Akt phosphorylation at the Ser473 site (α p473-Akt). Cortical neurons electroporated without siRNA served as additional controls to scramble siRNA (LSD test, n = 3, p < 0.05 versus indicated neurons).

PKA, PKB/Akt, Epac1 and Epac2 in primary cortical neurons (Fig. 3.4). Besides PKA, AKAP150 appeared to be co-localized with PKB/Akt, Epac1 and Epac2 (Fig. 3.4). Interestingly, AKAP150 immunoreactivity showed more overlap with Epac2 than Epac1. Co-immunoprecipitation studies confirmed that PKA, PKB/Akt and Epac2 indeed bind to AKAP150, whereas Epac1 could not be detected (Fig. 3.5(a)). Moreover, we found all three mammalian isoforms of PKB/Akt: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (Fayard et al., 2005) to be complexed to AKAP150 (Fig. 3.5(a)). Experiments with control-IgG resulted in no detectable bands for AKAP150, PKA, PKB/Akt or Epac2 proteins (data not shown).

Next, we assessed whether Epac activation led to phosphorylation of PKB/Akt bound to AKAP150. Cortical neurons were treated with 8-pCPT-2Me-cAMP, followed by immunoprecipitation of AKAP150-bound PKB/Akt with an antibody directed against AKAP150. Activation of Epac by 8-pCPT-2Me-cAMP increased phosphorylation of PKB/Akt bound to AKAP150 already at early time points (Fig. 3.5(b)).
Figure 3.4: AKAP150 co-localizes with PKA, PKB/Akt and Epac1 and Epac2 in cultured cortical neurons. Cultured cortical neurons were fixed, permeabilized and stained with anti-AKAP150 antibody coupled to Alexa 633, anti-PKA, anti-Epac1/2 antibodies coupled to Alexa 488 and anti-PKB/Akt antibody coupled to Alexa 555 as indicated in the respective panels. Images were collected from stained neurons by confocal microscopy and merged in the respective color image. In the colocalization panels, the overlap between AKAP150 and PKA, PKB/Akt, Epac1 and Epac2 is shown. Scale bar, 10 µm.

3.3.3 AKAP150 balances PKA and Epac activation to regulate PKB/Akt phosphorylation.

To investigate the role of AKAP150 in the regulation of PKB/Akt phosphorylation in more detail, we generated HT-4 cells overexpressing wild-type AKAP150 or
Figure 3.5: PKA, Epac2 and PKB/Akt bind AKAP150. (a) Co-immunoprecipitation studies were performed using antibodies directed against PKB/Akt and AKAP150, respectively. Pulled down fractions (IP) were tested in Western blot (WB) using antibodies for the detection of AKAP150, PKA, total PKB/Akt (presented as total Akt), PKBo/Akt1, PKBβ/Akt2, PKBγ/Akt3, Epac1 and Epac2. As a negative control we used uncoupled rabbit IgG antibody. The first row represents 10-20 µg of total input from primary cortical neurons lysates as a positive control. Interrupted membranes are due to differences in exposure time to detect the protein of interest. (b) The AKAP150 complex was precipitated from untreated control neurons and neurons treated with 100 µM 8-pCPT-2Me-cAMP for the indicated time periods. Afterwards PKB/Akt phosphorylation was detected by Western blot. Representative immunoblots are shown below the quantified data of p473-Akt expression. The bar graphs represent the ratio between IOD of p473-Akt versus AKAP150 expression (LSD test, n = 3, p < 0.05 versus non-treated neurons).

AKAP150 lacking the PKA-binding domain (AKAP150ΔPKA). Although endogenous AKAP150 could not be detected in unmodified HT-4 cells, AKAP150 could be detected in HT-4 cells overexpressing AKAP150 or AKAP150ΔPKA, using an AKAP150 antibody directed against the N-terminal part of the protein (Fig. 3.6(a)). The finding that AKAP150ΔPKA is not detectable with an C-terminal AKAP150 antibody confirms that this mutated protein lacks the PKA binding site, since this binding site is located at the C-terminal end of the protein (Fig. 3.6(a)). Deletion of the PKA binding site in AKAP150ΔPKA was supported by co-immunoprecipitation experiments. AKAP150 was bound to PKA in HT-4 cells transfected with wild-type AKAP150, but PKA binding was significantly reduced in HT-4 cells expressing AKAP150ΔPKA (Fig. 3.6(a)).

HT-4 cells also expressed Epac1 and Epac2 (Fig. 3.6(b)). Similar to our findings in primary cortical neurons (Fig. 3.2(c)), forskolin and 8-pCPT-2Me-cAMP treatment
led to Rap1 activation in HT-4 cells (Fig. 3.6(c)). Importantly, HT-4 cells transfected with AKAP150ΔPKA showed significantly higher basal Rap1 activation than HT-4 cells expressing wild-type AKAP150 (Fig. 3.6(d)). These findings were paralleled by enhanced basal PKB/Akt phosphorylation in HT-4 cells expressing AKAP150ΔPKA compared to phosphorylation levels in HT-4 cells overexpressing wild-type AKAP150 (Fig. 3.6(e)). Interestingly, overexpression of wild-type AKAP150 in HT-4 cells already led to increased PKB/Akt phosphorylation, when compared to PKB/Akt phosphorylation levels in unmodified HT-4 cells (Fig. 3.6(e)).

Further evidence for a coordinative function of AKAP150 in PKB/Akt signaling came from studies with cortical neurons that were treated with cell-permeable Ht31, a peptide known to disrupt the binding of PKA to AKAPs (Vijayaraghavan et al., 1997). Similar to PKA inhibition by H89, treatment of the cells with Ht31 significantly increased PKB/Akt phosphorylation (Fig. 3.7(a)), whereas its control peptide did not alter this neuronal response (Fig. 3.7(b)). Since AKAP150 was found to be mainly bound to PKA regulatory subunit RII (Hausken et al., 1996), we also treated neurons with cell permeable superAKAP-IS. Whereas Ht31 has the potential to disrupt RII but also some PKA-RII anchoring (Herberg et al., 2000), superAKAP-IS is a peptide that is 10 000-fold more selective for the RII isoform relative to RI (Gold et al., 2006). SuperAKAP-IS treatment also led to a significant increase in PKB/Akt phosphorylation (Fig. 3.7(c)). Finally, treatment of cortical neurons with Ht31 led to increased activation of the Epac effector Rap1 when compared to untreated neurons (Fig. 3.7(d)). This suggests that inhibition of PKA-binding to AKAP150 redirects cAMP signaling in cortical neurons to Epac-mediated Rap1 activation.

To investigate whether inhibition of PKA to AKAPs indeed promotes Epac-induced phosphorylation of PKB/Akt bound to AKAP150, cortical neurons were treated with superAKAP-IS, followed by immunoprecipitation of the AKAP150 complex with an antibody directed against AKAP150. As expected, treatment with superAKAP-IS reduced the amount of PKA bound to AKAP150 in comparison to untreated neurons (Fig. 3.8). Most importantly, disrupting PKA binding to AKAP150 increased the amount of Epac2 bound to AKAP150 and enhanced PKB/Akt phosphorylation bound to AKAP150 (Fig. 3.8).

### 3.4 Discussion

cAMP is a ubiquitous mediator of intracellular signaling events. For many years, it was thought to act principally through stimulation of PKA. However, the discovery of Epac proteins as additional cAMP effectors dramatically altered this cAMP-PKA dogma and opened up new opportunities to further dissect the divergent roles that cAMP exerts. It is now generally assumed that AKAPs play a central role in intracellular targeting and compartmentalization of diverse cAMP-dependent signaling events (Taskén and Aandahl, 2004; Wong and Scott, 2004).

Recent studies indicated that cAMP-dependent signaling is closely interwoven
3.4. Discussion

Figure 3.6: AKAP150 coordinates PKA/Epac signaling in neuronal HT-4 cells. (a) AKAP150 protein expression was checked in unmodified HT-4 cells and HT-4 cells overexpressing wild-type AKAP150 or AKAP150ΔPKA using antibodies against the C-terminal or N-terminal end of the protein. Co-immunoprecipitation studies were performed using the N-terminal AKAP150 antibody. (b) Expression of Epac1 and Epac2 in HT-4 cells. (c) HT-4 cells treated with forskolin or 100 µM 8-pCPT-2Me-cAMP were used for a Rap1 pull-down assay. Untreated cells served as controls. GTP-bound Rap1 (α GTP-Rap1) pulled down by a GST immunoprecipitation and total Rap1 were detected by Western blot. (d) Rap1 expression analyzed from unmodified HT-4 cells and HT-4 cells overexpressing wild-type AKAP150 or AKAP150ΔPKA (T test, n=3, p < 0.05 versus HT-4 cells overexpressing wild-type AKAP150). Representative immunoblots are shown at the lower part of the quantified data. (e) Quantification and representative immunoblots of PKB/Akt phosphorylation in unmodified HT-4 cells and HT-4 cells overexpressing wild-type AKAP150 or AKAP150ΔPKA (T test, n = 3, p < 0.01 versus unmodified HT-4 cells).
Figure 3.7: Disrupting PKA binding to AKAPs increases PKB/Akt phosphorylation.
(a) Cortical neurons were incubated with Ht31 for the indicated time periods. Representative immunoblots are shown below the quantified data of PKB/Akt phosphorylation at the Ser\textsuperscript{473} site (\(\alpha\) p\textsubscript{473}-Akt) analyzed from three separate experiments (LSD test, p < 0.05 versus non-treated neurons). (b) Cortical neurons were incubated with Ht31 control peptide for the indicated time periods. Representative immunoblots are shown below the quantified data of PKB/Akt phosphorylation at the Ser\textsuperscript{473} site (\(\alpha\) p\textsubscript{473}-Akt) analyzed from three separate experiments (T test, p < 0.05 versus non-treated neurons). (c) Cortical neurons were incubated with 5 or 20 M of superAKAP-IS for 10 minutes. Representative immunoblots are shown below the quantified data of PKB/Akt phosphorylation at the Ser\textsuperscript{473} site (\(\alpha\) p\textsubscript{473}-Akt) (LSD test, n = 3, p < 0.05 versus non-treated neurons). (d) Non-treated neurons or Ht31-treated cortical neurons were analyzed for Rap1 activation. GTP-bound Rap1 (\(\alpha\) GTP-Rap1) and total Rap1 were detected by Western blot (LSD test, n=3, p < 0.05 versus non-treated neurons).
with the phosphatidylinositol 3-kinase (PI3K)/PKB/Akt pathway (Cui and So, 2004). PKB/Akt is of tremendous importance for several neuronal key signaling events, including cell differentiation, proliferation and survival (Kim et al., 2004). We reported before that PKB/Akt is able to activate NF-κB and therefore to induce anti-apoptotic signals in primary cortical neurons (Marchetti et al., 2004; Dolga et al., 2008) and that in neuronal tissue PKB/Akt is instrumental in neuroprotective signaling upon ischemia/reperfusion (Fontaine et al., 2002). Neuronal survival and axonal regeneration mediated by PI3K-dependent PKB/Akt signaling were shown to be induced by elevated cAMP levels (Cui and So, 2004). Interestingly, PI3K-dependent PKB/Akt signaling activated in response to elevated cAMP is also required for the expression of long term potentiation (LTP) in the hippocampal CA1 region (Lin et al., 2001; Sanna et al., 2002), and has been even implicated in memory consolidation in fear conditioning (Sweatt, 2001). In contrast, the classical counter regulator of PKB/Akt signaling, the phosphatase PTEN, seems to be important for the induction of long term depression (Wang et al., 2006). Most of the observed effects are thought to be mediated by PKA; however, a role of Epac can in some cases not be excluded. For example, glutamatergic NMDA receptor functions are modulated in a cAMP and PI3K-dependent manner through PKB/Akt in cerebellar granule cells. Based on the findings that four different PKA inhibitors did not affect cAMP-dependent NMDA receptor functions, Epac has been implicated to play a role in this scenario (Llansola et al., 2004; Sánchez-Pérez et al., 2006). Furthermore, an upregulation of cAMP signaling pathways in parallel to increased activation of PKB/Akt (Griffin et al., 2005) has also been associated with the pathophysiology of Alzheimer’s disease (Martínez et al., 1999). Interestingly, it was also reported that the expression levels of both Epac1 and Epac2 are altered in brain regions that are predominantly affected in Alzheimer’s disease (McPhee et al., 2005). Thus, several lines of evidence point to concerted mechanisms in neuronal cellular actions, involving cAMP-driven effector proteins and PKB/Akt signaling but at present the precise role that PKA and Epac play in these processes remains unclear.

Here we report for the first time the role of the two cAMP-regulated effector proteins PKA and Epac on PKB/Akt phosphorylation in cortical neurons and hippocampal neuron derived HT-4 cells. An interconnectivity of these two cAMP-dependent effector proteins and PKB/Akt signaling has been reported before in non-neuronal cells (Mei et al., 2002). We demonstrate that the signaling properties of PKB/Akt are profoundly altered in a reciprocal fashion upon activation of PKA and Epac, respectively. After treatment of the cells with the adenylate cyclase activator, forskolin, and the specific PKA activator, Sp-5,6-DCI-cBIMPS, we found a strong inhibition of PKB/Akt phosphorylation, whereas inhibition of PKA yielded the opposite effect. Interestingly, activation of Epac mimicked the effect of PKA inhibition on the PKB/Akt response, an effect being paralleled by activation of Rap proteins. Forskolin is expected to activate both PKA and Epac, and therefore such a scenario is expected to result in a rather neutral effect on PKB/Akt phosphorylation. The results pre-
Figure 3.8: SuperAKAP-IS increases the phosphorylation of PKB/Akt bound to AKAP150. The AKAP150 complex was precipitated with the C-terminal AKAP150 antibody from homogenates of non-treated control neurons and neurons treated with 20 µM superAKAP-IS for 10 min. Afterwards AKAP150, PKB/Akt, p473-Akt and PKA were detected by Western blotting. (a) Representative immunoblots. Interrupted membranes are due to differences in exposure time to detect the protein of interest. (b) Quantification of AKAP150, PKA, p473-Akt/Akt expression in untreated- and superAKAP-IS-treated neurons. Precipitation with control IgG did not show any bands (LSD test, n = 2, p < 0.05 versus indicated neurons).

Presented, however, point strongly to an inhibitory effect of forskolin via PKA activation towards PKB/Akt activation in cortical neurons and HT-4 cells. The molecular mechanisms underlying the distinct effects of PKA and Epac on PKB/Akt phosphorylation are currently under investigation.

Significant support for a coordinative role of AKAPs in the integration of the two distinct cAMP effector pathways, PKA and Epac, came from studies in neonatal rat cardiomyocytes. A cAMP-responsive multiprotein complex, maintained by mAKAP including PKA and Epac1, was recently identified and reported to be functional in sensing and transmitting local cAMP fluctuations (Dodge-Kafka et al., 2005). To date, only mAKAP was reported to associate with both PKA and Epac. However, AKAP150 might also represent a suitable candidate to coordinate distinct cAMP-driven signaling routes in neuronal cells. AKAP150 was found to be widely expressed throughout the brain especially in those brain areas that are known to be involved in learning and memory (Ostroveanu et al., 2007). We and others recently found support for an important role of this anchoring kinase in learning and memory (Moita et al. (2002); Nijholt et al. (2007); Nijholt et al. (2008)). This multi-enzyme signaling
complex was also reported to coordinate changes in synaptic structure and receptor signaling functions underlying synaptic plasticity (Dell’Acqua et al., 2006). It was shown that synaptic anchoring of PKA through association with AKAP150 is important in the regulation of AMPA receptor surface expression and synaptic plasticity (Snyder et al., 2005). Similarly Rap GTPases also have been shown to be involved in AMPA receptor trafficking during synaptic plasticity (Zhu et al. (2002); Zhu et al. (2005)).

The main conclusion from the present study is that neuronal AKAP150 specifically interacts with PKA, PKB/Akt and Epac. As such, we define a novel cAMP-responsive multiprotein complex in neuronal cells, maintained by AKAP150 that includes PKA and Epac, and that finally determines the neuronal PKB/Akt response. Several lines of evidence support this notion. First, the effect of PKA inhibition on Epac-mediated Rap activation and PKB/Akt phosphorylation were mimicked upon expression of the PKA-binding deficient AKAP150 mutant and by the AKAP inhibitory peptides Ht31 and superAKAP-IS. Under conditions where PKA is not able to bind AKAP150, Epac2 binding to AKAP150 is enhanced and Rap activated. This may lead to the observed increase in phosphorylation of PKB/Akt bound to AKAP150. Second, the association of both PKA and Epac to AKAP150 clearly indicates that the two cAMP-dependent signaling pathways are physically linked to the AKAP150 complex. Finally, the immunoprecipitation studies clearly showed that PKB/Akt bound to AKAP150 is rapidly phosphorylated upon Epac activation.

Interestingly, co-immunoprecipitation experiments showed that Epac2 is bound to AKAP150, whereas Epac1 could not be detected. Similar, the results from the confocal colocalization studies also pointed towards a stronger overlap between AKAP150 and Epac2. Pretreatment with Epac2 siRNA also completely blocked the Epac-induced PKB/Akt phosphorylation, whereas the effect of Epac1 siRNA pretreatment was weaker. Thus, it is tempting to attribute neuronal PKB/Akt activation mainly to Epac2. Indeed, as shown herein Epac driven phosphorylation of PKB/Akt was diminished upon silencing of Epac2. However, silencing of Epac1 also blocked Epac-induced PKB/Akt phosphorylation. Therefore, a role of Epac1 in Epac-induced PKB/Akt phosphorylation cannot be completely ruled out. The low detection level of Epac1 in the co-immunoprecipitation and confocal experiments may merely be the effect of lower Epac1 antibody affinity. Until now, at least to our knowledge, Epac2 has been specifically attributed to the regulation of insulin secretion in pancreatic β-cells (Bos, 2006). Association of mAKAP with Epac1 modulates neonatal rat cardiomyocyte responses upon inhibition of extracellular signal-regulated kinase signaling (Dodge-Kafka et al., 2005). The data reported herein indicate that association of AKAP150 with Epac2 modulates neuronal responses upon stimulation of PKB/Akt signaling.
3.5 Conclusion

We report here on a novel molecular link between neuronal AKAP150, PKA, PKB/Akt and Epac, and provide evidence that these two cAMP-dependent effectors exert a reciprocal effect on neuronal PKB/Akt signaling. By modulation of PKB/Akt signaling, known to regulate many early and late neuronal functions, ranging from cell differentiation, proliferation, and survival to an essential role in learning and memory processes (Brazil et al., 2004), cAMP-regulated and AKAP150-coordinated PKA and Epac most likely control essential neuronal functions.

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