PACAP-38 induces neuronal differentiation of human SH-SY5Y neuroblastoma cells via cAMP-mediated activation of ERK and p38 MAP kinases

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Abstract

The intracellular signaling pathways mediating the neurotrophic actions of pituitary adenylate cyclase-activating polypeptide (PACAP) were investigated in human neuroblastoma SH-SY5Y cells. Previously, we showed that SH-SY5Y cells express the PAC1 and VIP/PACAP receptor type 2 (VPAC2) receptors, and that the robust cAMP production in response to PACAP and vasoactive intestinal peptide (VIP) was mediated by PAC1 receptors (Lutz et al. 2006). Here, we investigated the ability of PACAP-38 to differentiate SH-SY5Y cells by measuring morphological changes and the expression of neuronal markers. PACAP-38 caused a concentration-dependent increase in the number of neurite-bearing cells and an up-regulation in the expression of the neuronal proteins Bcl-2, growth-associated protein-43 (GAP-43) and choline acetyltransferase: VIP was less effective than PACAP-38 and the VPAC2 receptor-specific agonist, Ro 25-1553, had no effect. The effects of PACAP-38 and VIP were blocked by the PAC1 receptor antagonist, PACAP6-38. As observed with PACAP-38, the adenylyl cyclase activator, forskolin, also induced an increase in the number of neurite-bearing cells and an up-regulation in the expression of Bcl-2 and GAP-43.

PACAP-induced differentiation was prevented by the adenylyl cyclase inhibitor, 2',5'-dideoxyadenosine (DDA), but not the protein kinase A (PKA) inhibitor, H89, or by siRNA-mediated knock-down of the PKA catalytic subunit. PACAP-38 and forskolin stimulated the activation of extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAP; p38 MAP kinase) and c-Jun N-terminal kinase (JNK). PACAP-induced neuritogenesis was blocked by the MEK1 inhibitor PD98059 and partially by the p38 MAP kinase inhibitor SB203580. Activation of exchange protein directly activated by cAMP (Epac) partially mimicked the effects of PACAP-38, and led to the phosphorylation of ERK but not p38 MAP kinase. These results provide evidence that the neurotrophic effects of PACAP-38 on human SH-SY5Y neuroblastoma cells are mediated by the PAC1 receptor through a cAMP-dependent but PKA-independent mechanism, and furthermore suggest that this involves Epac-dependent activation of ERK as well as activation of the p38 MAP kinase signaling pathway.

Keywords: cAMP-dependent, Epac, neuritogenesis, PAC1 receptor, PACAP, VIP.

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PACAP is at the VPAC receptors (Laburthe et al. 1999; Langer et al. 2000; Ono et al. 2002; Farkas et al. 2004; Tamas et al. 2006). PACAP expression is up-regulated at sites of neuronal injury (Zhang et al. 1995, 1996; Moller et al. 1997; Boeshore et al. 2004) where it may help prevent cell death and promote neuronal regeneration.

The actions of PACAP are mediated through three G protein-coupled receptors (GPCRs), the VPAC1, VPAC2, and PAC1 receptors (Zhou et al. 2002), which are members of the Group II secretin receptor family (Harmar 2001). PACAP and the structurally related neuropeptide, vasoactive intestinal peptide (VIP), have similar potencies (Harrington et al. 1999; Inoue et al. 2000; Onoe et al. 2002; Farkas et al. 2004; Tamas et al. 2006). PACAP and PAC1 receptors (Zhou et al. 2002), however, the signal transduction mechanisms underlying this were not determined. We have shown previously that SH-SY5Y cells express PAC1 and VPAC2 receptors and that PAC1 receptors mediate the activation of cAMP production in these cells by PACAP and VIP (Lutz et al. 2006). Here we have investigated PAC1 receptor-mediated activation of cAMP production in promoting the differentiation of SH-SY5Y cells by PACAP.

Materials and methods

Materials

Tissue culture media were obtained from Sigma–Aldrich (Poole, UK), and animal sera from Biowest Ltd. (Ringmer, UK). The SH-SY5Y cell line was obtained from ECACC (Salisbury, UK). Peptides, all-trans retinoic acid, phorbol-12-myristate-13-acetate (PMA) and enzyme inhibitors were supplied by Merck Biosciences (UK) Ltd. (Nottingham, UK), except for PD98059 which was obtained from Promega (Southampton, UK). The selective VPAC2 receptor agonist, Ro 25-1553, was obtained from Dr. Patrick Robberecht, Université Libre de Bruxelles, Brussels, Belgium. The exchange protein directly activated by cAMP (Epac) activator, 8-(4-chlorophenylthio)-2’O-methyladenosine 3’,5’-cyclic monophosphate sodium salt was obtained from Sigma–Aldrich. Antibodies against Bcl-2, ERK, phosphoERK, GAP-43, tyrosine hydroxylase (TH) and GAPDH were obtained from Santa Cruz Biotechnology (Autogen Bioclear UK Ltd., Calne, UK), all other antibodies were obtained from Biosource (Paisley, UK). Standard laboratory chemicals of Analytical grade were obtained from Sigma–Aldrich or BDH Chemicals Ltd. (Poole, UK).

Cell culture

SH-SY5Y cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air and cultured in a 1 : 1 mixture of Ham’s F12 and Eagle’s minimum essential medium supplemented with non-essential amino acids, 1-glutamine, sodium pyruvate, 100 U/mL each of penicillin and streptomycin, and 5% fetal bovine serum (FBS). Medium was changed every fourth day. All the cells used in this study were used at a low passage number (<20).

cAMP assay

The production of cAMP was measured by the β-galactosidase enzyme fragment complementation-based HitHunter cAMP XS+ chemiluminescence assay (DiscoveRx, Birmingham, UK) according to the manufacturer’s instructions. Briefly, SH-SY5Y cells at ~80% confluency in 75 cm2 flasks were quiesced for 2 h in OptiMEM (Invitrogen Ltd., Paisley, UK) before being scraped into phosphate-buffered saline and counted. Cells were centrifugated at 400 g for 5 mins and resuspended in MEM containing 0.25% bovine serum albumin (BSA; Sigma–Aldrich) and 500 μmol/L 3-isobutyl-1-methylxanthine for 15 min. Cells were assayed in suspension by putting 104 cells per well into a 96-well plate before stimulating with varying concentrations of ligand. The assay was stopped after 15 min by lysing cells and luminescence was detected using a LUMistar Galaxy plate reader (BMG Labtech Ltd., Aylesbury, Bucks, UK). In addition, SH-SY5Y cells plated into 24-well plates were pre-labeled overnight with 1[3H]-adenine and 1[3H]-adenosine and increased expression of markers of neuronal differentiation including Bel-2 and GAP-43 (Leli et al. 1992; Hanada et al. 1993; Encinas et al. 1999; Feng and Porter 1999; Jamsa et al. 2004; Pan et al. 2005). Recently, it has been shown that PACAP induces neurite outgrowths and increased expression of neuronal cytoskeletal proteins in SH-SY5Y cells (Héraud et al. 2004), however, the signal transduction mechanisms underlying this were not determined. We have shown previously that SH-SY5Y cells express PAC1 and VPAC2 receptors and that PAC1 receptors mediate the activation of cAMP production in these cells by PACAP and VIP (Lutz et al. 2006). Here we have investigated PAC1 receptor-mediated activation of cAMP production in promoting the differentiation of SH-SY5Y cells by PACAP.

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MAP kinase phosphorylation assay

SH-SYSY cells were seeded into 12-well plates at a density of 3 × 10^4 cells per well and cultured for 48 h, after which the medium was replaced with low-serum medium containing 0.2% FBS and incubated overnight. Medium was replaced with fresh low-serum medium for 2 h before agonists were added, inhibitors were added 45 min before adding agonists. Stimulation was halted by placing plates on ice and cells harvested by preparing whole cell extracts on ice after aspirating the medium and adding lysis buffer (250 mmol/L Tris, pH 6.8, 14 mmol/L sodium pyrophosphate, 20 mmol/L ethylenediaminetetraacetic acid (EDTA), 35 mmol/L sodium dodecyl sulphate as described previously (McLees et al. 1995). Cells scraped into lysis buffer were homogenized by passing through a 21G needle and protein concentration quantified using a standard Coomassie-based Bradford assay.

Differentiation of SH-SYSY cells

SH-SYSY cells were plated into 12-well plates at a density of 10^5 cells per well and cultured for 48 h. Before starting treatments, the cells were incubated for 16–24 h in low-serum medium containing 0.2% FBS, then photographed under a 40x objective lens using a Nikon Coolpix 4500 camera attached to an Olympus CK40 microscope (Microscopes Scales & Servicing, Glasgow, UK). Medium was replaced with fresh low-serum medium and treatments added. Where indicated, inhibitors were added 45 min before the treatment. Micrographs were taken 48 h after the initial treatment and medium-containing treatments were renewed; this was repeated every 48 h for up to 8 days. Cells were harvested as described above.

Cell morphology analysis

Cell morphology analysis was carried out on captured micrographs of treated cells. For each treatment, the total number of cells and total number of cells with neurites longer than the length of the cell body were manually assessed from at least six micrographs in a blind count. The ratio between the number of neurite-bearing cells and total number of cells was calculated for each treatment, and the fold change over the control was determined. Each experiment was repeated a minimum of three times and variance between datasets determined by ANOVA analysis using GraphPad Prism (GraphPad Software, Inc., San Diego CA, USA). The data are expressed as mean ± standard error (SE). One- and two-way ANOVA were carried out as appropriate to identify significant differences between treatments.

Results

PACAP-38 induces neuritogenesis and the expression of neuronal marker proteins in SH-SYSY cells

Cells maintained in medium containing 0.2% FBS (low serum medium) were treated with different concentrations of PACAP-38 (0.1 nmol/L to 1 μmol/L) and examined for changes in cell morphology and expression of neuronal differentiation-associated proteins. The number of neurite-bearing cells increased within 2 days of treatment: those treated with 10 nmol/L to 1 μmol/L PACAP-38 displayed a bipolar cell body with long, thin, neurite-like processes by day 4 (Fig. 1a). Cells treated with 10 μmol/L RA also developed long, thin, neurite extensions (Fig. 1a), as shown in previous studies (Pålhm et al. 1994), whereas control cells treated with dimethylformamide (vehicle) or maintained in low serum medium alone did not. Since SH-SYSY cells express PAC1 receptor splice variants that are responsive to VIP, as well as the VIP/PACAP-responsive VPAC2 receptors (Lutz et al. 2006), the effects of VIP and the VPAC2 receptor-specific agonist, Ro 25-1553, were tested. VIP was also able to induce the development of neurite-like processes, but required 100-fold higher concentrations than PACAP-38.
and only the highest concentration of Ro 25-1553 (10 μmol/L) elicited the development of neurites in a small proportion of cells. Figure 1b shows the increase in neurite-bearing cells on day 4 in response to PACAP-38, VIP, and Ro 25-1553. A concentration-dependent increase was observed, with 100 nmol/L PACAP-38 eliciting the greatest increase (5.6 ± 0.5-fold), compared to 1 μmol/L VIP (3.2 ± 0.4-fold) and 10 μmol/L Ro 25-1553 (2.1 ± 0.3-fold). The number of neurite-bearing cells increased by 8.3 ± 1.3-fold (p < 0.001) following 4 day treatment with 10 μmol/L RA. The effects of various concentrations of PACAP-38, VIP, and Ro 25-1553 on the expression of Bcl-2 and GAP-43 were assessed by western blotting (Fig. 1c). Protein loading for each sample was checked by blotting for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Low concentrations of PACAP-38 were found to increase the expression of Bcl-2 and GAP-43 (0.1 nmol/L and 1 nmol/L, respectively). In comparison, much higher concentrations of VIP were needed to induce expression of these two markers of neuronal differentiation (approximately 10 nmol/L and 1 μmol/L, respectively, Fig. 1c), whereas Ro 25-1553 was even less potent. In addition, the ability of PACAP-38 to affect the neurotransmitter phenotype was determined by examining the expression of choline acetyltransferase (ChAT), a marker for cholinergic neurones, and tyrosine hydroxylase (TH), a marker for noradrenergic neurones. SH-SY5Y cells normally express TH, and the levels of TH were not significantly affected by the 4-day treatment of cells with the varying concentrations of PACAP-38 used in this study (Fig. 1c). However, treatment of SH-SY5Y cells with 1 nmol/L PACAP-38 was sufficient to induce expression of ChAT, the level of expression increasing in a concentration-dependent manner. (Fig. 1c).

PACAP6-38 inhibits PACAP-38-induced neuronal differentiation of SH-SY5Y cells
The PAC1 receptor antagonist PACAP6-38 was used in order to determine if PAC1 receptors are involved in mediating the
differentiating effects of PACAP-38 on SH-SY5Y cells. This inhibitor has also been shown to act at VPAC2 receptors (Dickinson et al. 1997; Moro et al. 1999), however, since Ro 25-1553 did not have a significant effect on neuritogenesis or the expression of Bcl-2 and GAP-43, it seems unlikely that the VPAC2 receptor has a significant role in mediating PACAP-38-induced neuronal differentiation of SH-SY5Y cells. The ability of PACAP6-38 to inhibit PACAP-38-, VIP- and Ro 25-1553-stimulated cAMP production was tested in SH-SY5Y cells. Fig. 2a shows that the inhibitor had no effect on basal levels of cAMP, but significantly inhibited 100 nmol/L PACAP-38- and 1 µmol/L VIP-stimulated cAMP production by 81 ± 25% and 85 ± 26%, respectively. Likewise, 4-day treatment with 10 µmol/L PACAP6-38 did not significantly alter the basal number of neurite-bearing cells whereas the 100 nmol/L PACAP-38- or 1 µmol/L VIP-stimulated increase in the number of neurite-bearing cells was significantly reduced, by 78 ± 23% and 77 ± 22%, respectively, following co-treatment with 10 µmol/L PACAP6-38 (Fig. 2b). PACAP-38-induced expression of Bcl-2 and GAP-43 was also inhibited by 10 µmol/L PACAP6-38 (Fig. 2c). Thus, it appears likely that this is mediated principally through the PAC1 receptor.

Production of cAMP is required for PACAP-38-induced neuronal differentiation of SH-SY5Y cells

Treatment of SH-SY5Y cells with cAMP analogues has been shown to induce differentiation (Sánchez et al. 2004). Since PACAP elicits a robust activation of cAMP production in SH-SY5Y cells through the PAC1 receptor (Lutz et al. 2006), the AC inhibitor, DDA, was used in this study to determine if PACAP-38-induced differentiation of SH-SY5Y cells was cAMP-dependent. In SH-SY5Y cells 300 µmol/L DDA inhibited 100 nmol/L PACAP-38-stimulated cAMP production to 25 ± 19% of the PACAP-38-stimulated control (n = 3), DDA was calculated to have an IC50 of 208 ± 0.3 nmol/L (n = 3; data not shown). The AC activator, forskolin, was used for comparison to PACAP-38. Forskolin elicits cAMP production with an EC50 of 5.8 ± 0.2 µmol/L and Emax of 12 ± 1-fold of basal control in SH-SY5Y cells (data not shown). Cells were then maintained in: low serum medium; 300 µmol/L DDA; 100 nmol/L PACAP-38; 300 µmol/L DDA and 100 nmol/L PACAP-38; 10 µmol/L forskolin. Changes in cell morphology and expression of Bcl-2 and GAP-43 were examined after 4 days of treatment. As shown in Fig. 3a, treatment with DDA alone had no effect compared to control cells but inhibits the PACAP-38-induced increase in neurite-bearing cells by approximately 76 ± 36%. Furthermore, DDA had no effect on basal levels of Bcl-2 and GAP-43 expression compared to control cells, but caused a near complete inhibition of their induction by 100 nmol/L PACAP-38 (Fig. 3b). Four day treatment of SH-SY5Y cells maintained in low serum medium with 10 µmol/L forskolin caused an
increase in the number of neurite-bearing cells to 6.8 ± 1.0-fold of basal control (Fig. 3a) and induced the expression of Bcl-2 and GAP-43 (Fig. 3b).

**Protein kinase A is not required for PACAP-38-induced neuritogenesis**

As protein kinase A (PKA) is one of the major intracellular effectors activated by elevated levels of cAMP, we set out to determine if PKA is required for PACAP-38-mediated differentiation of SH-SY5Y cells. The transcription factor cAMP response element binding protein (CREB) is a downstream target of PKA and was used in this study as a measure of PACAP-38-mediated PKA activation and the effectiveness of PKA inhibitors. Concentrations of PACAP-38 as low as 0.1 nmol/L elicited an increase in pCREB over basal levels (Fig. 4a, upper left panel). For cells treated with 100 nmol/L PACAP-38, pCREB levels were elevated within 5 min and remained elevated for at least 60 min (right panel, Fig. 4a). PACAP-38-stimulated CREB phosphorylation was inhibited by treating cells with the PKA inhibitor H89 at concentrations of 1, 10, and 100 μmol/L (Fig. 4b). Interestingly, treatment of cells with 10 μmol/L H89 in low serum medium for 4 days elicited a significant increase in the number of neurite-bearing cells to 3.5 ± 0.3-fold of basal control and augmented the 100 nmol/L PACAP-38-induced increase (Fig. 4b, right panel), although this augmentation was not significant compared to PACAP-38 alone. The PACAP-38-induced expression of Bcl-2 was partially inhibited by 10 μmol/L H89, however, that of GAP-43 was not affected (Fig. 4b, lower left panel).

Although H89 is widely used as a specific PKA inhibitor, it has been shown to potently inhibit other kinases including Rho kinase/ROCK-II (Davies et al. 2000). Inhibition of this kinase rather than PKA by H89 has been shown to lead to the formation of neurite-like extensions in NG108-15 cells (Leemhuis et al. 2002). In order to explore further the role of PKA in PACAP-38-induced neurite extensions in SH-SY5Y cells, siRNA was used to knock-down expression of the catalytic subunit. The effectiveness and specificity of this is shown in the top panel of Fig. 4c, where expression of the catalytic subunit of PKA is decreased by treatment with PKAc siRNA, but not with the scrambled siRNA sequence (ss). Expression of the closely related protein kinase B (second panel, Fig. 4c) is not affected by either treatment. Down-regulation of PKAc by siRNA effectively inhibits the increase in pCREB levels in cells treated with 100 nmol/L PACAP-38 (third panel, Fig. 4c), but only partially reduces PACAP-38-induced Bcl-2 expression and does not affect that of GAP-43 (bottom panels, Fig. 4c), as we have observed following H89 treatment (Fig. 4b). However, down-regulation of PKAc had no significant effect on the formation of neurite extensions compared to controls, and did not augment 100 nmol/L PACAP-38-induced increase in neurite-bearing cells compared to control cells (graph, Fig. 4c), as found with H89. Taken together, these results suggest that PKA activity is required for PACAP-38-induced expression of Bcl-2, but not for GAP-43 or for PACAP-38-induced neuritogenesis. Furthermore, they suggest that H89 may be inhibiting another protein kinase in addition to PKA in SH-SY5Y cells, as observed in NG108-15 cells (Leemhuis et al. 2002). The identity of this kinase remains to be established.

**PACAP-38-induced neuronal differentiation of SH-SY5Y cells is dependent upon ERK and p38 MAP kinase activity**

The MAP kinase signaling pathways have a well-documented role in cell proliferation and differentiation. Here, activation of the MAP kinase signaling pathways by...
PACAP-38 treatment of SH-SY5Y cells was examined. Cells maintained in low serum medium were stimulated for 15 min with a concentration range of 0.1 nmol/L to 10 nmol/L PACAP-38, and phosphorylation of ERK 1 and 2, p38 MAP kinase and JNK were assessed by western blotting using specific antibodies (Fig. 5). PACAP-38 stimulated phosphorylation of ERK (pERK), p38 MAP kinase (p-p38 MAP kinase) and JNK (pJNK) in a concentration-dependent manner (Fig. 5a), with EC50 values of 0.7 nmol/L, 1.5 nmol/L and 37.7 nmol/L, respectively. The pERK and p-p38 MAP kinase levels reached a maximum at 10–100 nmol/L PACAP-38, a similar con-
centration range required to obtain a maximum PACAP-differentiated phenotype. The increase in pJNK was more modest and required 10-fold greater concentration of PACAP-38 to reach a maximal level, compared to the other MAP kinases. Cells treated with 100 nmol/L PACAP-38 displayed prolonged phosphorylation of ERK and p38 MAP kinase (Fig. 5b) with pERK becoming localized in the nucleus after 30 min of stimulation (Fig. 5c). This is an event believed to switch ERK activity from that mediating proliferation to that inducing differentiation (Traverse et al. 1992; Ebisuya et al. 2005).

Although the levels of pERK declined almost back to basal levels over the 2 h time course of the experiment, the levels of p-p38 MAP kinase remained elevated.

Fig. 5 PACAP-38-mediated activation of ERK, p38 MAP kinase and JNK. SH-SY5Y cells were cultured in low serum medium for 24 h and incubated with increasing concentrations of PACAP-38 for 15 min (a) or with 100 nmol/L PACAP-38 for up to 2 h (b). Western blot analysis of whole cell extracts was carried out using antibodies specific for ERK, pERK, p-p38 MAP kinase and pJNK. The increase in phosphorylation levels was determined by densitometry as described in Materials and methods and is graphically represented in the lower section of the figure. Standard error bars are shown (n = 5). (c) The cells were incubated with a pERK-specific antibody and its respective fluorescent dye-conjugated secondary antibody (green) following treatment with 100 nmol/L PACAP-38 for 30 min. Cells were also stained with DAPI, the DNA-binding fluorescent stain (blue). Each micrograph is representative of three independent experiments.

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(Fig. 5b). JNK phosphorylation appeared weak and declined quickly in comparison.

Because of the strength and duration of PACAP-elicted phosphorylation of ERK and p38 MAP kinase, the involvement of these kinase pathways in PACAP-38-induced cell differentiation was examined. SH-SY5Y cells were maintained in low serum medium for 4 days with 10 μmol/L PD98059 (an inhibitor of MEK1), with 10 μmol/L SB203580 (an inhibitor of p38 MAP kinase) or in low serum medium alone as the control, with or without 100 nmol/L PACAP-38. Changes in cell morphology and expression of Bcl-2 and GAP-43 were determined. As shown in Fig. 6a, inhibition of the ERK pathway caused a significant reduction in the number of neurite-bearing cells induced by PACAP-38, from 4.6 ± 0.6-fold to 1.4 ± 0.4-fold of basal control, an inhibition of approximately 85% of the stimulated response. Inhibition of p38 MAP kinase pathway caused a more modest reduction in the number of neurite-bearing cells to 2.8 ± 0.4-fold of the basal control, an inhibition of approximately 58% of the stimulated response. The PACAP-induced increase in Bcl-2 levels was inhibited by SB203580 and not by PD98059 whereas the converse was true for GAP-43 (Fig. 6b).

The relationship between AC activation and MAP kinase activation was explored by incubating cells with 300 μmol/L DDA or low serum medium alone and stimulating with 100 nmol/L PACAP-38. The effects on the levels of phosphorylated MAP kinase following treatment with 10 μmol/L forskolin were examined as well. As shown in Fig. 6c, DDA inhibited the PACAP-38-stimulated increase in pERK and p-p38 MAP kinase. Forskolin, like PACAP-38, stimulates an increase in pERK and p-p38 MAP kinase. This was not prevented by inhibiting PKA, as neither H89 (Fig. 6d) nor siRNA-mediated knock-down of the catalytic subunit of PKA prevented the PACAP-38-induced increase in pERK and p-p38 MAP kinase levels (Fig. 6e).

The Epac activator 8-CPT-Me-cAMP stimulates ERK but not p38 MAP kinase activity

The PACAP-38-stimulated activation of ERK and p38 MAP kinase is cAMP-dependent but PKA-independent in SH-SY5Y cells. The guanine nucleotide exchange factor, Epac, is also known to mediate cAMP-dependent activation of MAP kinase in certain cell types, including PC6 cells (Shi et al. 2006). We have examined this possibility here. Figure 7a shows the effect of the specific Epac activator, 8-CPT-Me-cAMP, PACAP-38 and forskolin on pERK and p-p38 MAP kinase levels. 8-CPT-Me-cAMP stimulated an increase in pERK but not p-p38 MAP kinase. The time course of this activation of ERK was examined and
compared to that of 100 nmol/L PACAP-38 (Fig. 7b), cells treated with 100 μmol/L 8-CPT-Me-cAMP had similar levels of pERK to those treated with PACAP-38 within 30 min, but the levels declined more slowly than the PACAP-38-stimulated levels. However, 100 μmol/L 8-CPT-Me-cAMP elicited an increase in the number of neurite-bearing cells to 2.2 ± 0.9-fold of basal control compared to 5.8 ± 1.3-fold following treatment with 100 nmol/L PACAP-38 (Fig. 7c). Expression of GAP-43 but not Bcl-2 was up-regulated by 100 μmol/L 8-CPT-Me-cAMP (Fig. 7d).

Discussion

Human SH-SY5Y cells differentiate into a more neuronal phenotype when treated with the differentiating factors RA, PMA, or brain-derived neurotrophic factor (Leli et al. 1992; Jamsa et al. 2004; Pan et al. 2005) and we have used these cells to explore the mechanisms involved in the neurotrophic actions of PACAP. This is the first study, to our knowledge, to examine the signaling pathways underlying PACAP-38-stimulated neuritogenesis in human cells. PACAP treatment of SH-SY5Y cells elicited a large increase in the number of neurite-bearing cells, corresponding to the formation of neurons during development and to their regeneration after injury. A number of studies have now identified PACAP as having a role in increasing axon growth and preventing cell death following: facial injury (Suarez et al. 2006); spinal cord injury (Kim et al. 2000); traumatic brain injury (Farkas et al. 2004); ischemia (Chen et al. 2006). The formation of growing axons is accompanied by an increase in expression of the growth cone protein, GAP-43 (Meiri et al. 1986): in SH-SY5Y cells, differentiating factors, such as RA and PMA have been shown to stimulate the expression of GAP-43 (Feng and Porter 1999). The anti-apoptotic protein Bcl-2 is also up-regulated following differentiation of SH-SY5Y cells (Feng and Porter 1999; Raguenes et al. 1999). In this study, we have shown that PACAP treatment causes a clear up-regulation of GAP-43 and Bcl-2 in SH-SY5Y cells, over the
that culturing neuroblastoma cells, including SH-SY5Y cells, differing cell culturing conditions used during the differentiation processes that mediate a more compartmentalized cAMP pathway. Furthermore, the pac1 receptor can also couple to the activation of phospholipase C and phosphodiesterases D in addition to AC activation (McCulloch et al. 2001; Lutz et al. 2006), and the activation of these additional pathways may modify the cAMP signal and/or the resulting cellular effects evoked by PACAP. We are currently investigating this possibility. Maintaining cells for a long period of time in medium containing a constant amount of a stable cAMP analogue such as db-cAMP bypasses the GPCR/Gs/AC regulatory control mechanisms. Furthermore, the possible long-term global increase in intracellular cAMP levels may actually alter, or even prevent, the activation of pathways that mediate a more compartmentalized cAMP.

Indeed, when global cellular levels of cAMP were raised, this was blocked. Furthermore, the cAMP signal is often integrated with that of other signals, particularly Ca²⁺, that also regulate the activity of ACs and phosphodiesterases (Zaccolo and Pozzan 2003). The pac1 receptor can also couple to the activation of phospholipase C and phosphodiesterases D in addition to AC activation (McCulloch et al. 2001; Lutz et al. 2006), and the activation of these additional pathways may modify the cAMP signal and/or the resulting cellular effects evoked by PACAP. We are currently investigating this possibility. Maintaining cells for a long period of time in medium containing a constant amount of a stable cAMP analogue such as db-cAMP bypasses the GPCR/Gs/AC regulatory control mechanisms. Furthermore, the possible long-term global increase in intracellular cAMP levels may actually alter, or even prevent, the activation of pathways that mediate a more compartmentalized cAMP.
signal, such as may occur through GPCR-mediated activation of AC and presumably the subsequent regulation of the signal through the activation of phosphodiesterases, protein kinases and phosphatases that may be tethered or recruited into nearby signaling complexes. The role of cAMP compartmentalization in neuronal differentiation remains to be determined.

It is clear from studies of rat PC12 and related phaeochromocytoma cell lines that there are certain parallels in the mechanisms mediating the neurotrophic actions of PACAP on these cells [see review by Ravni et al. (2006)] and those which we have observed in SH-SY5Y cells. A role for Epac in the cAMP-mediated differentiation of neuronal cells has recently been described by Kiermayer et al. (2005), whose study in PC12 cells described a cAMP-mediated, PKA-independent activation of Epac causing a sustained activation of ERK activity resulting in the differentiation of neuronal cells. Shi et al. (2006) described distinct Epac/Rit/p38 and Epac/Rap/ERK pathways contributing to PACAP-38-stimulated neuritogenesis in another rat phaeochromocytoma cell line, PC6 cells. However, in the human cells, the specific activator of Epac, 8-CPT-Me-cAMP, caused a sustained activation of ERK, a clear increase in GAP-43 expression and a modest increase in neuritogenesis without influencing p38 MAP kinase activation (Fig. 7). PACAP-38 stimulated p38 MAP kinase through an AC-dependent and PKA-independent mechanism that does not appear to be Epac-mediated. The PKA-dependent activation of p38 MAP kinase has been described (Delghandi et al. 2005), in this case, however, PKA inhibition does not influence p38 MAP kinase phosphorylation or neuritogenesis. However, CREB phosphorylation and Bcl-2 expression are sensitive to PACAP-mediated neurodifferentiation of human SH-SY5Y cells | 85

Fig. 8 Proposed model for the cAMP-dependent signaling events involved in the neurotrophic actions of PACAP-38 on SH-SY5Y cells. PACAP-38 binding to the PAC1 receptor, which couples to the activation of AC, evokes an increase in the intracellular levels of cAMP (Lutz et al. 2006). This in turn leads to the activation of PKA-dependent and PKA-independent mechanisms that are involved in the induction of expression of neuronal proteins and in neuritogenesis, respectively. The PKA-dependent mechanism involves PKA phosphorylation of the transcription factor CREB, initiating its activation and translocation to the nucleus where it may be involved in the up-regulated expression of proteins involved in neuronal differentiation, including Bcl-2. The PKA-independent mechanisms involve activation of MEK and ERK as well as p38 MAP kinase that are required for neuritogenesis. The MEK/ERK activation possibly occurs through one or a combination of different mechanisms. The neuronal MAP kinase signaling cascade involves B-Raf as the first component (Dugan et al. 1999) and it has been shown that the 95 kDa B-Raf kinase is expressed in SH-SY5Y cells (Stephens et al. 1992). However, it is still unclear how cAMP activates B-Raf (Dumaz and Marais 2005). This may occur through activation of the cAMP-dependent Rap 1 GEF, Epac, or through another mechanism that may be potentiated by Epac (Lin et al. 2003) but may or may not involve Rap1 (Dumaz and Marais 2005). An additional possibility is the cAMP-mediated activation of PP2A (Feschenko et al. 2002) which directly induce B-Raf activation (Strack 2002). Activated ERK is required for neuritogenesis as well as being involved in the up-regulated expression of GAP-43, possibly through translocation to the nucleus. cAMP mediates the activation of p38 MAP kinase through a non-canonical pathway that may be similar to that observed in Th2 cells (Chen et al. 2000) and in cardiac fibroblasts (Yin et al. 2006), and which also may influence PP2A (Boudreau et al. 2004). Activated p38 MAP kinase in turn is involved in the increased expression of Bcl-2, possibly through phosphorylating CREB (Yin et al. 2006) and/or other factors, as well as in neuritogenesis. The arrows indicate direct interactions, the dotted arrows translocation, and the dot dash arrows the possibility of one or a number of intermediary steps that have not been worked out.

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These processes. The importance of cAMP, Epac, ERK, and p38 MAP kinase in ERK and the resultant neuritogenesis. These results provide exchange factor, Epac, was implicated in the activation of cells with PACAP. The cAMP-dependent guanine nucleotide were shown to be essential to fully differentiate SH-SY5Y mechanisms. Activation of AC, ERK and p38 MAP kinase of p38 MAP kinase pathways takes place in response to PACAP-38 treatment and that both these pathways contribute to PACAP-38-stimulated neuritogenesis in human neuroblastoma cells. Interestingly, the cAMP-dependent but PKA-independent mechanism involved in the activation of ERK in PC12 cells has not been entirely elucidated and there is the distinct possibility that there may be additional novel cAMP-regulated components involved in this (Gerdin and Eiden 2007) as appears to be the case in SH-SY5Y cells. One possibility that we are investigating is the cAMP-mediated activation of protein phosphatase 2A (Feschenko et al. 2004) as appears to be the case in SH-SY5Y cells. One possibility that we are investigating is the cAMP-mediated activation of protein phosphatase 2A (Feschenko et al. 2004) may induce B-Raf activation (Strack 2002) as well as p38 MAP kinase activation (Boudreaux et al. 2004). A proposed model of the signaling pathways involved in mediating the neurotrophic actions of PACAP in SH-SY5Y cells is shown in Fig. 8.

In summary, we have shown that PACAP-38-mediated differentiation of SH-SY5Y cells is cAMP-dependent and involves both PKA-dependent and PKA-independent mechanisms. The increase in cAMP levels led to phosphorylation of ERK, p38 MAP kinase and JNK via a PKA-independent mechanism. Activation of AC, ERK and p38 MAP kinase were shown to be essential to fully differentiate SH-SY5Y cells with PACAP. The cAMP-dependent guanine nucleotide exchange factor, Epac, was implicated in the activation of ERK and the resultant neuritogenesis. These results provide further evidence of the neurotrophic actions of PACAP and provide insight into how PACAP may function during neuronal development and regeneration, highlighting the importance of cAMP, Epac, ERK, and p38 MAP kinase in these processes.

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