Sustained activation of extracellular signal-regulated kinase 1/2 (ERK1/2) is critical for initiating differentiation of the PC12 cell to a sympathetic-like neurone. The neuropeptide, pituitary adenyl cyclase-activating polypeptide (PACAP), has been demonstrated to cause cells to adopt a neuronal phenotype, although the mechanism of this activity is unclear. PACAP through its type I receptor stimulates a biphasic activation of ERK1/2; a >10-fold increase within 5 min, followed by a >5-fold increase that is sustained for ~60 min. An equivalent stimulation is seen in PC12 cells expressing a dominant negative Ras mutant. However, the mitogen-activated kinase/ERK kinase (MEK1/2) inhibitor PD98059 blocked both PACAP-induced stimulation of ERK1/2 activity and neurite outgrowth. Thus, the activation signal from the PACAP type I receptor on the ERK1/2 cascade pathway is received downstream of Ras, either at Raf or MEK. Down-regulation of protein kinase C or its inhibition by calphostin C blocked the ability of PACAP to stimulate ERK1/2. We conclude that activation of PACAP type I receptor activates protein kinase C, which then activates the ERK1/2 cascade in a Ras-independent manner at either Raf or MEK1/2.

The two forms of pituitary adenyl cyclase-activating polypeptide (PACAP27 and -38) are neuropeptides of the secretin/glucagon/vasoactive intestinal peptide/growth hormone-releasing hormone family. They share the same 27 amino-terminal amino acids and arise from a precursor peptide by post-translational processing (1). Two receptor subtypes have been identified for PACAP; both are G protein-coupled receptors. The PACAP type I receptor (found in hypothalamus, brain stem, pituitary, adrenal gland, and testes) is specific for PACAP, having a Kd of 0.5–2.0 nM. There are several splice variants that demonstrate different abilities to activate adenyl cyclase and phospholipase C (2). The type II receptor does not discriminate between PACAP and VIP and is only positively coupled to adenyl cyclase. PACAP potently stimulates neuritogenesis of neuroblastoma cells (3), neonatal chromaffin cells (4), corticosterone cells (5), and PC12 cells (6–8).

The MAP kinases ERK1/2 are thought to be key players in the control of gene transcription events that lead to proliferation or differentiation in PC12 cells in response to epidermal growth factor or NGF, respectively. It has been suggested that the determinant of the nature of the response of PC12 cells (proliferation or differentiation) correlates with the duration of ERK1/2 activation and its translocation to the nucleus (9).

ERK1/2 activation following agonist stimulation of G protein-coupled receptors has been reported (reviewed in Ref. 10), although various effectors are employed to couple these receptors to the MAP kinase cascade. In light of the neurite-stimulatory role of PACAP38 in pituitary neural cells and neuroblastoma cell lines, we have investigated the relationship of PACAP38 stimulation to ERK1/2 activation and compared this to that for NGF. In this study, we have demonstrated that PACAP38 activates ERK1/2 for prolonged times and that this activation is independent of Ras but dependent on MEK1/2.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture reagents were purchased from Life Technologies, Inc. [γ-32P]ATP (10 mCi/ml), p42/p44 MAP kinase enzyme assay kit, and the ECL detection kit were from Amersham Corp.; PD98059 and phospho-specific ERK1/2 antibodies were from New England Biolabs; H89 was from Alexis Corp.; calphostin C and PMA were from Calbiochem; nitrocellulose was from Schleicher & Schuell; nerve growth factor (2.5S) was from Serotec; PACAP38 was from Peninsula Laboratories; BSA Patho-o-cyte 4 was from ICN-flow (High Wycombe); PC12asn17-W7 cells were from Glaxo-Wellcome (Stevenage); and all other reagents were purchased from Sigma.

**Generation of a Dominant Inhibitory p21PC12 Cell Subline**—A dominant negative mutant form of human c-Ha-ras gene was obtained from Feig and Cooper (11), where the serine residue at position 17 was converted to asparagine. This was ligated into a HindIII-BamHI restriction site of the pRSV, and the resultant plasmid, pRSVp21, was sequenced to confirm the integrity of the construct. This was then transfected with the plasmid p21' (gift from Dr. Martin Page, Wellcome Research Laboratories), which encodes a neomycin resistance gene into PC12 cells. Following selection with G418, clones were screened for expression of p21'. Clone PC12asn17-W7 was chosen because of its high expression of p21' upon Western blotting, the
homogeneous appearance of this cell subline population, and the failure to respond morphologically to high concentrations of NGF. For a more detailed description of this work, see Ref. 12.

**Cell Culture**—PC12 cells were grown on polystyrene tissue culture dishes, coated with air-dried rat tail collagen at 37 °C in 6.5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) fetal calf serum, 2 mM glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin.

PC12saas.17-W7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with a SATO mix (13) and (1% (v/v) BSA Patho-ocyte 4, 2% (v/v) fetal calf serum, 2 mM glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin before commencement of the experiment.

**Measurement of ERK1/2 Activity**—The BIOTRAK® MAP kinase enzyme assay kit (Amersham) was used to measure ERK1/2 activity. After stimulation for the indicated time, the cells were washed in ice-cold PBS and then lysed in 750 µl of lysis buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM diethiothreitol, and 1 mM orthovanadate with 10 µg/ml each chymostatin, leupeptin, antipain, and 1 µg/ml pepsin A added just prior to use. Samples were then centrifuged for 20 min at 25,000 × g at 4 °C (Beckman, Avanti J25). To determine ERK1/2 activity, 15 µl of supernatant and 10 µl of peptide buffer were mixed, and the reaction was started by the addition of (γ-32P]ATP and incubation at 30 °C for 30 min. The reaction was stopped by the addition of 10 µl of stop buffer. 30 µl of sample was pipetted into the center of a filter disc. To remove nonspecifically bound γ-32P]ATP, discs were washed twice (5 min/wash) in 75 mM orthophosphoric acid followed by one final wash in distilled H2O. Phosphorylation was quantitated by scintillation counting of 32P. Protein concentration of the samples was determined by a Bradford assay (14) using the Bio-Rad protein assay reagent and BSA as a standard.

**Western Blot Analysis**—After treatments the cells were washed and harvested into ice-cold PBS and then centrifuged for 5 min at 12,000 rpm at 4 °C. Pellets were solubilized in sample buffer (3% SDS, 62.5 mM Tris-HCl (pH 6.8), and 10% glycerol). Sample protein concentration was determined by a Lowry assay (15). 0.1% (w/v) bromphenol blue and 0.05% (v/v) β-mercaptoethanol were added to the samples, which were boiled for 5 min and then loaded (100 µg/lane) on a 10% SDS-polyacrylamide gel along with phosphorylated and nonphosphorylated ERK2 control proteins (16). After electrophoresis, the proteins were transferred to nitrocellulose membrane (0.2 µm pore size) as described (17). The protein blots were processed for ERK1/2 immunoreactivity as follows: (a) a 5-min wash in Tris-buffered saline (TBS; 100 mM Tris-Cl, pH 7.5, 150 mM NaCl) followed by a 3-h incubation at room temperature in TBS, 5% nonfat dry milk, 0.1% Tween-20; (b) overnight incubation at 4 °C in TBS, 5% BSA, 0.1% Tween-20 including a 1:1000 dilution of phospho-specific MAPK antibody; (c) washing with three changes of TBS, 0.1% Tween-20 leaving the final wash for 30 min; (d) incubation with a 1:2500 dilution of horseradish peroxidase-conjugated anti-rabbit antibody in TBS, 0.1% Tween-20 for 90 min at 4 °C; (e) washing with three changes of TBS, 0.1% Tween-20, leaving the final wash for 30 min. Finally, proteins were detected with the ECL system.

**Preparing Cells for Photography**—PC12 cells were seeded at 0.6 × 105 cells/60-mm plate and precoated with poly-c-lysine followed by air-dried rat tail collagen. Treatments were made as indicated, and cells were incubated at 37 °C, 6.5% CO2 for 48 h. Cells were then washed with PBS and then fixed by exposure to 2% (v/v) glutaraldehyde/PBS for 20 min, followed by two washes in PBS. Fixed cells were then photographed under a phase-contrast microscope.

**RESULTS**

**PACAP Type 1 Receptor Activation Increases ERK1/2 Activity**—Fig. 1A demonstrates that 5 nM PACAP38 results in a >10-fold increase in ERK1/2 activity in PC12 cells within 5 min. This is followed by a lower, more sustained activation of ERK1/2, lasting at least 60 min. Increasing the concentration of PACAP38 to 500 nM did not significantly increase ERK1/2 activity over that observed for 5 nM PACAP38 (data not shown). Thus, 5 nM PACAP38 appears to elicit the maximal response of ERK1/2 to PACAP38 in these cells. These data are consistent with the proposal that PACAP is acting through its high affinity receptor, type I (Kd = 0.5–2.0 nM). Although higher concentrations of PACAP38 did not increase the activity of ERK1/2 activity, other agents (NGF) resulted in considerably higher activation levels, indicating that the response to 5 nM PACAP38 was maximal only for this stimulus. Thus, 50 ng/ml NGF consistently increased ERK1/2 activity 3-fold more than PACAP38 (Fig. 1B). The response to NGF was also relatively fast, occurring within the first 5 min, and sustained.

**PACAP Stimulation of ERK1/2 Is Ras-independent**—To ex-
amine the involvement of p21ras in the PACAP-induced stimulation of ERK1/2 activity, a dominant negative Ras (PC12asn17-W7) cell line was used. Consistent with the known requirement of NGF for Ras, the addition of 50 ng/ml NGF to these cells failed to elicit any response of ERK1/2 activation (Fig. 2A). These data clearly demonstrate that the dominant negative form of Ras is able to inhibit the classical NGF-stimulated ERK1/2 cascade. The addition of 5 nM PACAP38 to these cells, however, resulted in an identical response of ERK1/2 activation compared with wild type PC12 cells (Fig. 2B). These data suggest that PACAP38 activation of ERK1/2 is independent of Ras.

To confirm this finding, protein extracts of the cells were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting analysis to detect the presence of nonphosphorylated and phosphorylated ERK1/2. Nonphosphorylated ERK1/2 could be detected in all extracts regardless of prior treatment of the cells (Fig. 2C, upper blot). In PC12 cells, treatment with either 50 ng/ml NGF for 10 min or 5 nM PACAP38 for 5 min resulted in the appearance of phosphorylated ERK1/2. In contrast, phosphorylated ERK1/2 could only be detected following treatment of PC12asn17-W7 cells with PACAP38. Consistent with the measurement of ERK1/2 activity in this cell line, treatment of these cells with NGF did not generate phosphorylated ERK1/2 (Fig. 2C, lower blot).

These data indicate that PACAP38, unlike NGF, activates ERK1/2 in a Ras-independent manner.

MEK1/2 Activation Is Essential for PACAP-induced Activation of ERK1/2—To examine the mechanism of PACAP38 activation of ERK1/2, the effect of an inhibitor of MEK was examined. MEK is the target of activated Raf and is the dual specificity kinase required to phosphorylate and thereby activate ERK1/2. PD98059 (18) inhibits both the activation and phosphorylation of MEK1 (IC50 = 5–10 μM) and MEK 2 (IC50 = 50 μM). ERK1/2 activity in response to a 5-min stimulation with 5 nM PACAP38 was reduced to 22.2 ± 2.2% (S.E.) and 4.6% ± 3.7% (S.E.) by a preincubation for 30 min with 10 or 50 μM PD98059, respectively (Fig. 3A). Taken together, these data indicate that PACAP38 activates ERK1/2 in a Ras-independent, MEK-dependent manner.

Studies on the morphological response to PACAP38 in the presence of 50 μM PD98059 were carried out. Fig. 3B shows that PD98059 inhibited the ability of PACAP38 to induce neurite outgrowth in PC12 cells. Thus, the concentration of PD98059 that inhibits the activation of ERK1/2 by PACAP38 also abolishes the morphological response of these cells to PACAP38.

Which Second Messenger(s) Is Stimulating the MAP Kinase Pathway?—PACAP type I receptors are positively coupled to adenylyl cyclase and phospholipase C (2, 3, 6). There are conflicting reports on the ability of cAMP to stimulate ERK1 or -2 activity in PC12 cells. (19–21). The difference between these
studies may relate to the very small changes observed. In our hands, neither the cAMP analogue dibutyryl cAMP nor the adenyl cyclase activator forskolin had any activation effect on ERK1/2 activity (Fig. 4). Furthermore, blocking PKA activation by preincubating cells with H89 (10 μM, 30 min) did not inhibit the activation of ERK1/2 by PACAP38. Indeed, H89 preincubation resulted in a slight increase in PACAP38 stimulation of ERK1/2 activity (Fig. 5). From these data, we can conclude that cAMP elevation via adenyl cyclase activation, and the subsequent PKA activation contribute minimally in this PACAP38-induced stimulation of ERK1/2 activity.

As previously mentioned, PACAP type I receptors are also positively coupled to phospholipase C (2), which through production of diacylglycerol will activate PKC. To investigate the role of PKC in ERK1/2 stimulation, cells were treated with the PKC inhibitor 1 μM calphostin C, for 30 min prior to PACAP38 addition. Fig. 5 shows that 1 μM calphostin C significantly inhibits the PACAP38-induced peak stimulation of ERK1/2 activity (100%) to 16.1 ± 10% (S.E.). Consistent with this, down-regulation of PKC activity by overnight incubation with 1

![Figure 3](image-url)
Effects of PACAP on the MAP Kinase Pathway in PC12 Cells

Sustained activation of the ERK1/2 pathway has been suggested to be sufficient to cause differentiation of PC12 cells into a neuronal phenotype (22, 23). Work by Traverse et al. (9) in PC12 cells, suggests that a sustained activation is required for nuclear translocation of ERK1/2 and that this translocation is required to initiate neuronal differentiation through activation of transcription events. Here we report that stimulation of the PACAP type I receptor increases ERK1/2 activity 10-fold within 5 min. This initial peak is followed by a lower, more sustained activity up to 60 min of >5-fold above basal levels. This pattern of the response of ERK1/2 to PACAP38 was similar to that observed for NOF. The MEK1/2 inhibitor PD98059 has previously been reported to block the NGF-induced differentiation of PC12 cells (24). We find this inhibitor to be equally effective in blocking the PACAP38 stimulation of ERK1/2 and neurite outgrowth in PC12 cells. These data provide further evidence that the activation of ERK1/2 is sufficient for PC12 cell differentiation and, furthermore, strongly suggest that PACAP38-induced neurite outgrowth is mediated via the activation of ERK1/2.

The lack of effect of cAMP on ERK1/2 activation we report here is consistent with our previous observation that the PKA inhibitor H89 does not block PACAP38-induced neurite outgrowth (7). In addition to the ubiquitous Raf-1, PC12 cells contain the B-Raf isoform, which is specific for neuronal and PC12 cells (25, 26). Work by Peraldi et al. (27) demonstrates that although B-Raf is capable of being activated by NGF and cAMP, cAMP cotreatment with these agents returns B-Raf activity to basal levels. The mechanism of inhibition is thought to arise via the phosphorylation of B-Raf by PKA, causing a reduced p21<sup>B-Raf</sup> affinity, similar to that shown in fibroblasts by Wu et al. (28). Thus, the enhancement of PACAP38-stimulated ERK1/2 activity seen in the presence of H89 could be explained by removal of the inhibitory contribution from PKA-phosphorylated B-Raf.

Here we demonstrate that the PACAP type I receptor, which is positively coupled to phosphoinositide hydrolysis, stimulates ERK1/2 activity in a Ras-independent manner. The magnitude of activation is equivalent for the wild type PC12 and PC12asn17-W7 cells, suggesting that a single Ras-independent pathway is activated. Furthermore, the PACAP38 stimulation of ERK1/2 activity is sensitive to PKC inhibition. From the literature, the activation of ERK1/2 by the pertussis-insensitive, G<sub>i</sub>/11-coupled receptors has been reported in both Ras-dependent (29, 30) and Ras-independent manners (32, 33). Also, activation of phospholipase C by expression of phospholipase C<sub>b2</sub> or an activated Ga<sub>q</sub> mutant has been shown to stimulate ERK1/2 by Faure et al. (34). Phorbol esters have been reported to potently activate ERK in various cell types, and PKC-mediated phosphorylation and activation of Raf is a strong contender for the mechanism of activation. For example, PKC<sub>M</sub> has been shown to activate c-Raf (31), and recently Ueda et al. (33) have demonstrated a Ras-independent, Raf-dependent, PKC<sub>M</sub>-mediated activation of ERK. In COS7 and Chinese hamster ovary cells, increases in ERK activity by G<sub>B</sub>-coupled receptors occurs independently of p21<sup>B-Raf</sup> activation but is blocked by expression of dominant negative Raf or PKC depletion (32). Thus, the data presented here are consistent with a Ras-independent, PKC-dependent activation of ERK1/2, and as discussed here, similar effects have already been reported for other cell types.

With respect to determining which component of the ERK signaling pathway receives the activation signal from the PACAP receptor, these data indicate that the recipient is downstream of Ras, either at Raf or MEK1/2, and further work is ongoing to determine the exact component of the ERK1/2 pathway that is the recipient of the stimulatory signal from the activated PACAP receptor.

Acknowledgments—We thank Terri McShane for expert technical assistance throughout and Jill Brown and Grace Ballantyne for assistance with preliminary experiments. We are also grateful to Glaxo-
Wellcome for providing the dominant negative p21ras PC12 cell line, PC12asn17-W7.

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