Journal of Cellular Biochemistry Supplement 36:89–98 (2001)

Mitogenic Signaling by Cyclic Adenosine Monophosphate in Chromaffin Cells Involves Phosphatidylinositol 3-Kinase Activation

James F. Powers,[1]* Suniti Misra,[2] Kimberly Schelling,[1] Lyuba Varticovski,[2,3] and Arthur S. Tischler[1]

[1]Department of Pathology, Tufts University School of Medicine, Boston, Massachussetts
[2]Departments of Pathology and Physiology, Department of Medicine, Tufts University School of Medicine, Boston, Massachussetts
[3]Department of Medicine, St. Elizabeth's Medical Center, Boston , Massachussetts

Abstract Increase of intracellular cyclic adenosine monophosphate by the permeant cyclic adenosine monophosphate analog, 8-(4-chlorophenylthio)-adenosine 3′:5′- cyclic monophosphate, is mitogenic for normal adult rat chromaffin cells. The mitogenic effect is blocked by the phosphatidylinositol 3-kinase inhibitor, LY294002, and is associated with accumulation of phosphorylated Akt and p70S6 kinase, suggesting that cyclic adenosine monophosphate activates Type I phosphatidylinositol 3-kinase. The mechanism of activation was examined in PC12 pheochromocytoma cells, which are neoplastic chromaffin cells that exhibit many of the biochemical characteristics of their normal counterparts. Incubation of PC12 cells with 8-(4-chlorophenylthio)-adenosine 3′:5′- cyclic monophosphate led to a significant increase in total phosphatidylinositol 3-kinase activity that was sensitive to low concentrations of LY294002. The increase was maximal at 1 h and returned to basal levels within six hours. Immunoprecipitation studies showed no increase in phosphatidylinositol 3-kinase activity in anti-phosphotyrosine immune complexes from PC12 cells stimulated by 8-(4-chlorophenylthio)-adenosine 3′:5′- cyclic monophosphate, in contrast to cells stimulated by nerve growth factor. Instead, activity was demonstrated in association with p110γ and p85. These findings suggest that cyclic adenosine monophosphate causes activation of Types IA and IB phosphatidylinositol 3-kinase by a novel mechanism in chromaffin and pheochromocytoma cells. That activation may contribute to chromaffin cell proliferation and to the development and progression of pheochromocytomas. J. Cell. Biochem. Suppl. 36:89–98, 2001.

Key words: adrenal medulla; PC12 cell; proliferation; Akt; MAP kinases

The adrenal medulla contains catecholamine-producing neuroendocrine cells known as chromaffin cells. These cells give rise to tumors known as pheochromocytomas. Although rare in most species, pheochromocytomas are common in rats and their frequency is increased by prolonged exposure to a variety of non-genotoxic agents [Tischler et al., 1989]. We have previously hypothesized that these agents induce pheochromocytomas indirectly by stimulating chromaffin cell proliferation, thereby providing a setting that permits DNA damage leading to autonomous proliferation [Tischler et al., 1989]. Studies of chromaffin cell proliferation thus have the dual objective of characterizing normal mitogenic signaling in these cells and determining how signaling goes awry in neoplastic transformation.

Chromaffin cell proliferation in rats appears to be under the control of neural signals and is markedly reduced by adrenal denervation [Tischler et al., 1991]. Substantial evidence suggests that the mitogenic effect of innervation is mediated, in part, by cyclic adenosine monophosphate (cAMP). The peptide neurotransmitter, pituitary adenylate cyclase-activating polypeptide (PACAP), is present in nerve fibers that innervate a subset of chromaffin cells in vivo [Holgert et al., 1996] and we have pre-

Grant sponsor: NIH to AST; Grant number(s): CA48017, NS37685; Grant sponsor: NIH to LV; Grant number(s): CA53094, DK54785.

*Correspondence to: Dr. James F. Powers, Department of Pathology, Box 802 New England Medical Center, 750 Washington Street, Boston, MA 02111.
E-mail: jpowers1@lifespan.org

Received 19 July 2000; Accepted 29 November 2000

This article published online in Wiley InterScience, March 19, 2001.

viously demonstrated that PACAP, forskolin, and cholera toxin, which activate adenylate cyclase, are mitogenic for normal adult rat chromaffin cells in vitro [Tischler et al., 1994, 1995].

Normal chromaffin cells from the rat adrenal medulla also proliferate in vitro in response to peptide growth factors that activate tyrosine kinase receptors. The prototype for these is nerve growth factor (NGF) [Tischler et al., 1993]. Lack of additivity or mutual inhibition between the two classes of mitogens, e.g. NGF and cAMP, suggest that cross-communication between mitogenic signaling pathways may exist at several levels [Tischler et al., 1994].

NGF activates the same major signaling pathways in normal chromaffin cells [Powers et al., 1999a] and PC12 cells [Kaplan and Stephens, 1994], a cell line derived from a rat pheochromocytoma [Greene and Tischler, 1976]. PC12 cells are therefore very useful for biochemical studies of signaling intermediates that can not be performed with the small numbers of chromaffin cells obtainable from primary cultures. Paradoxically, however, NGF is antimitogenic and induces neuronal differentiation of PC12 cells [Greene and Tischler, 1976], suggesting that the cellular consequences of particular signaling events may be profoundly altered as a result of neoplastic transformation.

Previous studies of proliferation of chromaffin cells have demonstrated a critical role for activation of phosphatidylinositol 3-kinase (PI 3-kinase) in mitogenic signaling by NGF. NGF-stimulated chromaffin cell proliferation is abolished by LY294002, an inhibitor of Type l PI 3-kinase [Powers et al., 1999a] and is greatly diminished by rapamycin, which acts downstream of PI 3-kinase and blocks activation of p70S6 kinase [Powers et al., 1999b]. The present Investigation sought to determine whether PI 3-kinase is also involved in mitogenic signaling by cAMP in normal chromaffin cells and to evaluate the mechanism of its activation using PC12 cells as a biochemical model.

METHODS

Cell Cultures

Adrenal medullary cells from 6–9 month old female retired breeder F344 rats were dissociated in collagenase followed by trypsin, enriched for chromaffin cells by three cycles of plating and differential detachment [Lillien and Claude, 1985] and plated on 35 mm collagen-coated dishes as previously described [Tischler et al., 1992, 1993]. PC12 cells were from original stocks maintained in our own laboratory as described by Greene and Tischler [1976]. Cultures were maintained in RPMI 1640 medium with 10% horse serum and 5% fetal bovine serum. The serum concentration was reduced to 1% horse serum and 0.5% fetal bovine serum overnight prior to experiments involving PI 3-kinase assays and immunoblots. Animal use was under a protocol approved by the Tufts-New England Medical Center Department of Laboratory Animal Medicine. All efforts were made to minimize animal suffering and to utilize the minimum number of animals required to produce reliable scientific data.

Bromodeoxyuridine-Labeling

Proliferative responses of chromaffin cells to the permeant cAMP analog, 8-(4-chlorophenylthio)-adenosine 3′:5′- cyclic monophosphate (cpt-cAMP) were studied by labeling with BrdU [Gratzner, 1982], followed by fixation and immunohistochemical staining for BrdU and tyrosine hydroxylase to discriminate labeled and unlabeled chromaffin cells from other cell types as previously described [Tischler et al., 1992]. At least 500 consecutively scanned chromaffin cells were counted and scored for the presence or absence of label for each data point, and all experiments were repeated on at least three occasions. In some experiments, the PI 3-kinase inhibitor LY294002 [Yao and Cooper, 1995] (10 μM, CalBiochem, LaJolla CA) was added to the culture medium.

Protein Extraction and Immunoblotting

Immunoblots of proteins extracted from chromaffin cells and PC12 cells were utilized to evaluate phosphorylation of Akt and p70S6 kinase, which serve as downstream readouts of PI 3-kinase activation [Duronio et al., 1998]. Phosphorylation of ERKs 1 and 2, which serves as a readout for activation of the ras-mediated MAP kinase cascade [Kaplan and Stephens, 1994] and was anticipated to be independent of PI 3-kinase, was assessed in parallel on the same blots.

Cells in control cultures or cultures incubated for varied intervals with cpt-cAMP (Sigma Chemical Co., St Louis, MO) were rinsed twice

with Hank's balanced salt solution and lysed in TBS Lyse (0.02M Tris pH 8.0, 137 mM sodium chloride, 1%(v/v) Nonidet P-40, 10% (v/v) glycerol, 100 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM sodium vanadate, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 2 mM phenylmethylsulfonyl fluoride). Extracts were incubated at 4°C for 15 min, centrifuged to remove insoluble material (16,000*g*, 5 min, 4°C) and stored at −80°C until used. Proteins were separated in 10% or 7.5 % polyacrylamide gels and transferred to nylon membranes (Nytran N, Schleicher and Schuell, Keene, NH). Phosphorylation of Akt and ERKs was assessed by probing with anti-sera against phosphorylated-Akt (serine 473)(New England Biolabs, Beverly, MA) or phosphorylated-ERKs 1 and 2 (Promega, Madison, WI). Phosphorylation of p70S6 kinase was evaluated by electrophoretic mobility shift assay [Chung et al., 1992], which proved technically superior to the use of anti-phosphoprotein antibodies for that purpose (antibody from Santa Cruz Biotechnology, Santa Cruz, CA). Blots were stripped and re-probed with an antibody against total ERKs 1 and 2 (Santa Cruz) as a loading control. Primary antibodies (all at 1:2000 dilutions) were followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (Schleicher and Schuell, Keene, NH, 1:5000 dilution) and bands were detected by chemiluminescence with CDP-star (Tropix, Bedford, MA) as the alkaline phosphatase substrate. Pre-stained protein standards (Bio-Rad) were used as molecular weight markers.

Phosphatidylinositol 3-Kinase Assay

Direct measurements of PI 3-kinase activation were performed on PC12 cells and effects of cpt-cAMP were compared to those of NGF (recombinant human NGF, Alomone Labs, Jerusalem, Israel), which is known to be a potent PI 3-kinase activator [Kaplan and Stephens, 1994].

Total PI 3-kinase activity in whole cell lysates was determined after dilution 1,000-fold to remove detergent as previously described [Susa et al., 1992; Misra et al., 1999]. Activity associated with P-Tyr, p85 and p110γ was assayed in anti-P-Tyr, anti-p85, and anti-p110γ immune complexes as previously described [Varticovski et al., 1991]. Briefly, protein complexes on protein A-Sepharose beads were sequentially washed with lysis buffer (50 mM HEPES, pH 7.5, 10% glycerol, 1% Nonidet-P40, 0.5 mM EDTA, 5 mM Na_3VO_4), PBS, TNE (10 mM Tris, pH 7.5; 0.5 mM EDTA) and PBS. Reaction mixture contained sonicated standard phospholipid mixtures (equal amounts of PS, PI, and PIP_2) at a final concentration of 0.2 mg/ml in 50 ml reaction. After addition of ATP to a final concentration of 150 mM and 15 mCi of (γ^{32}P)-ATP (4 mCi/nMol, Dupont, NEN), the reaction was carried out at 37°C for 20 min and terminated by addition of Methanol:1N HCl (1:1). Phospholipids were extracted twice with in same volume of chloroform, separated by TLC and visualized by autoradiography. Enzymatic activity was expressed as pmoles of phosphate incorporated into PI 3,4,5-P_3 and presented as fold increase compared with activity from untreated cells. In addition to assays of kinase activity, in some experiments immunoblots of proteins immunoprecipitated with anti-P-Tyr or anti-p110γ were probed with anti-p85, and p85 immunoprecipitates were probed for p110 α, β, and γ. In some experiments, cells were pre-treated with the tyrosine kinase inhibitor, genistein [Akiyama and Ogawara, 1991] (370 μM, Sigma Chemical Co.) to determine whether activation of PI 3-kinase by cpt-cAMP occurs independently of tyrosine phosphorylation.

RESULTS

Mitogenic and Biochemical Responses of Chromaffin Cells to Cyclic Adenosine Monophosphate are Blocked by Phosphatidylinositol 3-Kinase Inhibitors

Robust mitogenic effects were elicited in chromaffin cells by increasing intracellular cAMP directly with cpt-cAMP, as previously reported for indirect increases achieved with forskolin or cholera toxin [Tischler et al., 1995]. Preliminary dose-response curves demonstrated half-maximal and maximal mitogenicity with 200 μM and 300 μM cpt-cAMP, respectively, with a plateau to at least 500 μM. A concentration of 300 μM was therefore employed for all subsequent studies. Mitogenesis by cpt-cAMP was abolished by LY294002 (Table I), similarly to mitogenesis mediated by NGF [Powers et al., 1999a]. These results suggested that the mitogenic response to cAMP involves activation of PI 3-kinase. However, in many models, including neuronal cells [Blair et al., 1999; Vaillant et al., 1999], PI 3-kinase

TABLE I. Inhibition of cAMP-Mediated Chromaffin Cell Proliferation by LY294002*

Additive	BrdU-Labeled Chromaffin Cells(%±S.E)	n	*P*
No Add	0.95±0.65	4	
Ly2940002 (10 μM)	0.25±0.29	4	
cpt-cAMP (300μM)	22.1±6.6	4	<0.001 (vs. No Add)
LY294002 + cpt-cAMP	2.1±0.5	4	<0.001 (vs. cpt-cAMP)

*Cultures were maintained for three days with BrdU and the indicated additives. Values represent % ±S.E. for approximately 500 consecutively scanned chromaffin cells counted and scored for each condition in four independent experiments. *P*-values were derived from Student *t*-tests. Differences with $P < 0.05$ were considered significant. Mitogenicity of cpt-cAMP was comparable to that of an optimal mitogenic concentration of forskolin (5μM), which was also comparably inhibited by LY294002 (not shown)

inhibitors are toxic and cause cells to undergo apoptosis by inhibiting survival signals from Akt [Blair et al., 1999; Vaillant et al., 1999]. In order to rule out a toxic effect of LY294002 as the basis for inhibited mitogenesis, additional experiments were therefore performed to determine the reversibility of the anti-mitogenic effect. Cultures maintained for three days with cpt-cAMP alone or in combination with LY294002 were rinsed, re-fed without the inhibitor and then labeled with BrdU for three days. The percentage of chromaffin cells labeled in cultures with cpt-cAMP during the three days after washout of LY294002 was 25.6 ± 8.2, vs. $27.7 \pm 4.7\%$ in cultures with cpt-cAMP never exposed to the inhibitor and $0.8 \pm 0.3\%$ in controls ($n = 3$ independent experiments).

Accumulation of PI 3-kinase lipid products leads to activation and phosphorylation of Akt, p70S6 kinase and other targets. Immunoblots demonstrated increased levels of phospho-Akt and phospho-p70S6 kinase in response to cpt-cAMP, consistent with increased PI 3-kinase activity (Figs. 1 and 2). Increased phosphorylation of Akt and p70S6 kinase was abolished by LY294002, as illustrated for p70S6 kinase in Figure 1.

Increased phosphorylation of ERKs 1 and 2 was also detected in response to cpt-cAMP (Fig. 2). In contrast to phosphorylation of Akt and p70S6 kinase, phosphorylation of ERKs showed little or no change in response to LY294002, suggesting little or no involvement of PI 3-kinase in the pathway leading to ERK phosphorylation.

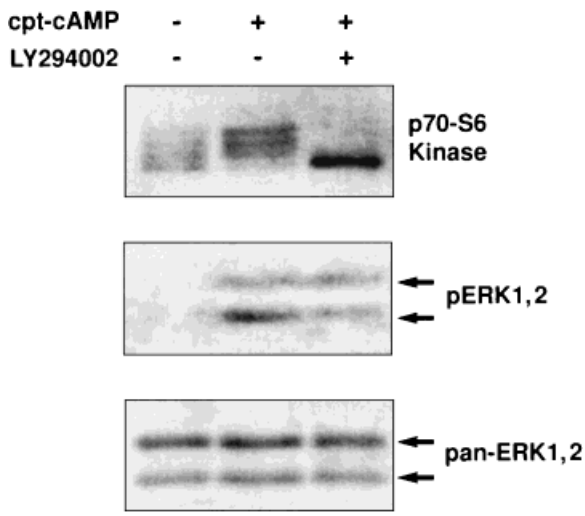

Fig. 1. Phosphorylation of p70S6 kinase, demonstrated by electrophoretic mobility shift [Chung et al., 1992], and phosphorylation of ERKs 1 and 2, demonstrated by phospho-specific antibodies [Johnson et al., 1997] in normal rat chromaffin cells stimulated by cpt-cAMP (300 μM) for 20 minutes. The figure represents a single blot cut into two sections, prepared from proteins resolved in a 7.5% gel. The section containing high molecular weight markers was stained for p70S6 kinase. The lower section was stained for phospho-ERKs, then stripped and re-probed for total ERKs as a loading control. Each lane was loaded with 6 μg of protein. The figure illustrates increased phosphorylation of p70S6 kinase in response to cpt-cAMP and abolition of the increase by LY294002. The apparent slight decrease in phosphorylation of the lower ERK band in the presence of LY294002 is due mostly or entirely to a slight difference in protein loading between lanes 2 and 3, as seen in the bottom panel. Selective abolition of cpt-cAMP-stimulated Akt phosphorylation by LY294002 was also observed both in normal chromaffin cells (not shown) and in PC12 cells (Fig. 3).

Effects of Cyclic Adenosine Monophosphate on Proliferation and Phosphorylation of Akt in PC12 Cells

PC12 cells differ from normal chromaffin cells in that they proliferate in the absence of exogenous mitogens and that cAMP is anti-mitogenic for these cells [Mark and Storm, 1997]. In spite of these biological differences from primary chromaffin cells, we found that cpt-cAMP increases levels of phosphorylated Akt in PC12 cells and this increase is blocked by LY294002 (Fig. 3).

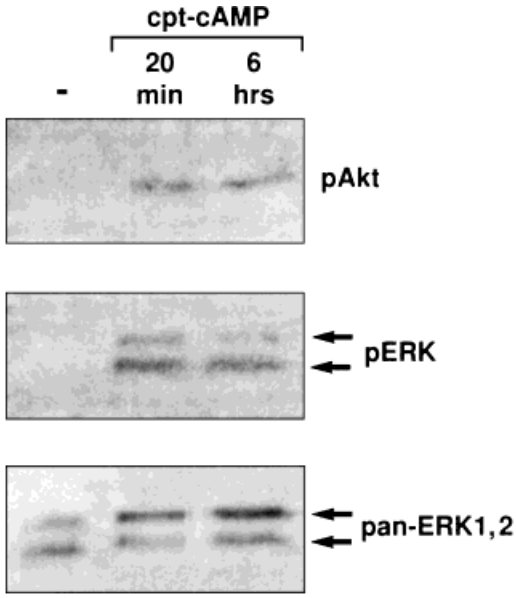

Fig. 2. Increased phosphorylation of Akt and of ERKs 1 and 2 demonstrated by phospho-specific antibodies in normal rat chromaffin cells stimulated by cpt-cAMP (300 μM) for 20 min or six hours. The figure represents a single blot cut into two sections as in Figure 2. Each lane was loaded with 8 μg of protein.

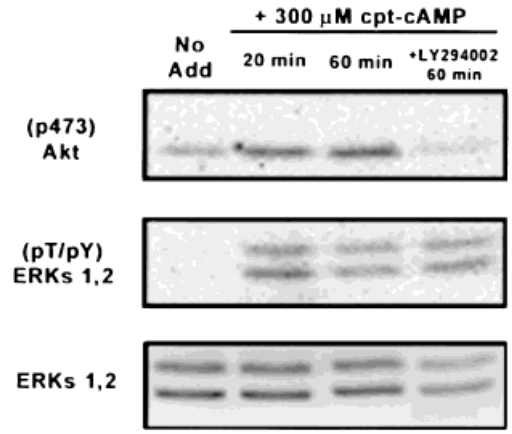

Fig. 3. Increased phosphorylation of Akt and of ERKs 1 and 2 demonstrated by phospho-specific antibodies in PC12 cells stimulated by cpt-cAMP (300 μM). The figure represents a single blot cut into two sections as in Figure 2. Each lane was loaded with 25 μg of protein.

Cyclic Adenosine Monophosphate Causes Phosphotyrosine-Independent Activation of Phosphatidylinositol 3-Kinase

Activation of Akt is initiated by interaction of its pleckstrin homology domain with lipid products of PI 3-kinase, phosphatidylinositol 3,4 bisphosphate (PI 3,4-P_2) and phosphatidylinositol 3,4,5 trisphosphate (PI 3,4,5-P_3), which are absent in quiescent cells and accumulate in response to activation of Type 1 PI 3-kinase [Franke et al., 1997]. Because we detected phosphorylation of Akt in cAMP-treated normal chromaffin and PC12 cells, we tested whether cAMP leads to activation of PI 3-kinase. Incubation with cpt-cAMP led to almost a ten-fold increase in total PI 3-kinase activity over 20 min, which persisted at 1 h and was sensitive to LY294002 (Fig. 4). Activity returned to basal levels by 6 h (not shown). A two-fold increase in PI 3-kinase activity also occurred in response to NGF, which is comparable to increases in total cellular activity associated with activation of PDGF receptor and non-receptor protein tyrosine kinases [Susa et al., 1992; Jain et al., 1996].

Type IA PI 3-kinase catalytic subunits p110α and p110β bind to p85 regulatory subunits and this complex is activated by interaction with specific motifs phosphorylated on tyrosine residues, including the NGF receptor [Franke et al., 1997]. As expected, NGF induced a substantial increase in anti-phosphotyrosine-immunoprecipitable PI 3-kinase activity (Fig. 5, top). In

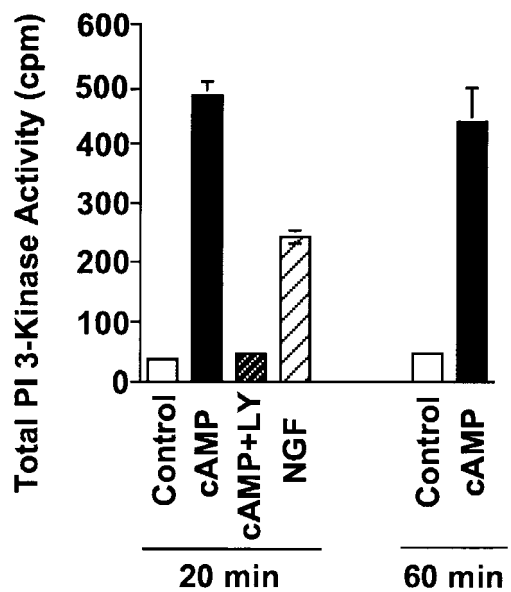

Fig. 4. Total PI 3-kinase activity in whole-cell lysates of PC12 cells. Activity was measured in duplicate samples using equal amounts of protein (250 ng) from PC12 cells treated with cpt-cAMP (300 μM), cpt-cAMP + LY294002 (10μM) or NGF (50 ng/ml) for the indicated times. Activity is shown as cpm of phosphate incorporated in to PI 3,4,5-P_3 over 20 minutes. Results are presented as the means ± standard error of duplicate samples. One representative result from three independent experiments is shown.

contrast, there was no increase in anti-phosphotyrosine-immunoprecipitable PI 3-kinase activity or p85 protein in response to cpt cAMP, indicating a phosphotyrosine-independent mechanism of activation. Pre-treatment with the tyrosine kinase inhibitor genistein did not inhibit increased PI 3-kinase activity caused by cpt cAMP (not shown), providing further evidence for phosphotyrosine-independent activation.

Phosphotyrosine-independent activation of p85-associated PI 3-kinase has been reported in response to activation of heterotrimeric G proteins [Kurosu et al., 1997; Murga et al., 1998]. We tested whether increase in intracellular cAMP augments p85-associated PI 3-kinase activity. Because p85 is an abundant protein and the antibodies to p85 subunit efficiently precipitate PI 3-kinase activity, we used a limited amount of whole cell lysate (100 μg) to assure quantitative recovery of p85 [Misra et al., 1999]. We found a significant increase in p85-associated lipid kinase activity that correlated with the time course for total increased activity in response to cpt-AMP (Fig. 5, center). Quantitative recovery of p85 was confirmed by lack of a p85 band in cell lysates after immunoprecipitation (Fig. 6).

We also detected a three-to four-fold increase in lipid kinase activity in anti-p110γ immune complexes in response to cpt-cAMP (Fig. 5, bottom). This increase was reproducibly evident after 1 h, in contrast to an increase in p85-associated activity within 20 min (Fig. 5), and was sensitive to LY294002. Therefore, cAMP leads to activation of both p110γ- and p85-associated Type I isoforms of PI 3-kinase in PC12 cells. Immunoblots of proteins immunoprecipitated with anti-p110γ showed faint bands when probed with anti-p85 which did not change in the presence of cpt-cAMP (not shown).

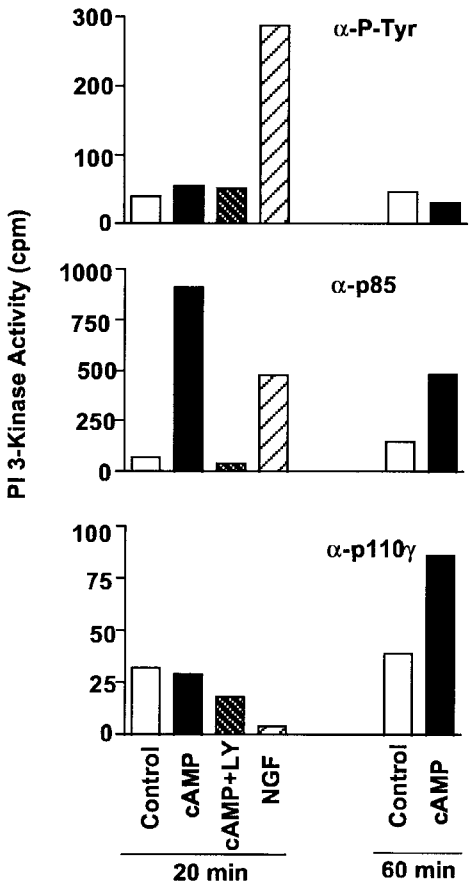

Fig. 5. PI 3-kinase activity in anti-P-Tyr, (top), anti-p85 (center) and anti-p110γ (bottom) immune precipitates from PC12 cells treated with cpt-cAMP (300 μM), cpt-cAMP + LY294002 (10μM) or NGF (50 ng/ml) for the indicated times. Cells were treated as described in Materials and Methods. PI 3-kinase activity measured in immune precipitates is presented as cpm of phosphate incorporated into PI 3,4,5-P_3. One representative result from three independent experiments is shown for each antibody. In additional experiments, immunoblots of the p85 immune precipitates were probed for p110 α, β, and γ isoforms and showed no changes in the amounts of these proteins, indicating that changes in PI 3-kinase activity caused by cyclic AMP represent increased specific activity.

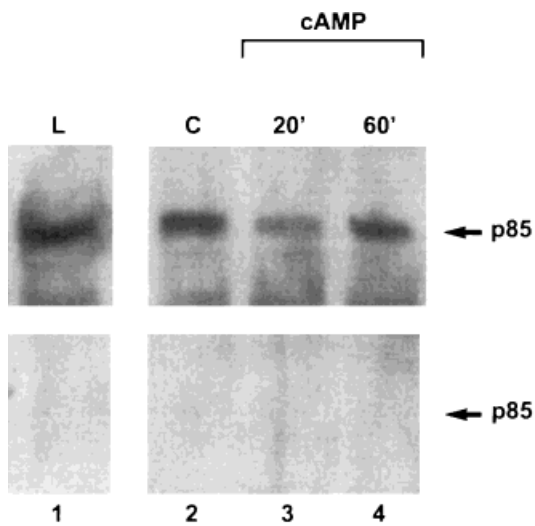

Fig. 6. Immunoblot showing depletion of p85 from PC12 cell lysates used for PI 3-kinase assays in Figure 5, center. The blot was prepared using 20 μg of protein from PC12 whole cell lysates before (upper panel) and after (lower panel) immunoprecipitation with anti-p85 antibodies. An equal amount of lysate from IL-3- dependent murine lymphocyte cell line, BaF3, was used as control (Lane 1). The arrow indicates migration position of p85.

DISCUSSION

We have found that the mitogenic effect of cAMP in normal chromaffin cells is sensitive to PI 3-kinase inhibition and correlates with activation of Akt and p70S6 kinase. In addition, using PC12 cells as a biochemical model, we have demonstrated that cAMP leads to activation of Type IA and IB PI 3-kinase by a novel mechanism. By inference, that mechanism may be involved in normal chromaffin cell proliferation.

Type I PI 3-kinases comprise a family of enzymes that includes four p110 catalytic subunits, designated α, β, γ, and δ. The p110 α, β, and δ isoforms, which are classified as Type IA, constitutively associate with p85, p55, or p50 regulatory subunits and are activated by subsequent binding to specific phosphotyrosine residues [Vanhaesebroeck and Waterfield, 1999]. Roles in DNA synthesis and cell transformation have recently been demonstrated for p110α and β [Vanhaesebroeck et al., 1999; Benistant et al., 2000]. The Type IB PI 3-kinase, p110γ, lacks a p85 binding domain and can be activated directly by interaction with heterotrimeric G proteins or p21Ras [Duronio et al., 1998; Vanhaesebroeck and Waterfield, 1999]. In PC12 cells, activation by this mechanism has been found to involve βγ dimers and $G\alpha_q$, $G\alpha_I$ but not $G\alpha_s$ [Murga et al., 1998]. Synergistic activation of p85/p110β by βγ dimers derived from $G\alpha_I$ and phosphotyrosyl peptides has been described in rat hepatocytes [Kurosu et al., 1997] and THP-1 human monocytes [Okada et al., 1996].

We were unable to immunoprecipitate PI 3-kinase activity with anti phosphotyrosine antibodies, effectively ruling out tyrosine phosphorylation in response to accumulation of intracellular cAMP. In contrast, increased PI 3-kinase activity in response to cAMP was associated with p85 and p110γ, suggesting a coordinate effect on activation of Type IA and IB PI 3-kinases. Activation of PI 3-kinase by cAMP in PC12 cells and, by inference, in normal chromaffin cells, therefore appears to differ markedly from activation by NGF. The latter occurs principally via association of p85 with phosphorylated tyrosine 490 of the intracellular domain of the NGF receptor, *trk* [Hallberg et al., 1998].

The signaling pathways linking cAMP to PI 3-kinase activation are not known. A plausible pathway involves G-protein regulation by protein kinase A (PKA) via the neural protein phosducin, which inhibits G-protein signaling and is itself inhibited by PKA [Bauer et al., 1992, 1998]. We have previously reported that cAMP-stimulated chromaffin cell proliferation is abolished by high concentrations of PKA inhibitor H-89 [Tischler et al., 1995]. However, lower, more specific concentrations of H-89 (10–30 μM) do not block cAMP-stimulated phosphorylation of Akt (J. F. Powers and A.S Tischler, unpublished data), suggesting that PKA is not involved in activation of PI 3-kinase by cAMP, although it may mediate other mitogenic cAMP effects.

PI 3-kinase activation by cAMP might occur independently of PKA by small G-proteins of the Ras superfamily, which can bind to Ras effectors. Activation of the Ras pathway by cAMP in normal chromaffin cells and PC12 cells was suggested in our experiments by increased phosphorylation of ERK1 and 2. Other investigators have also reported ERK phosphorylation in response to cAMP in PC12 cells [Vossler et al., 1997]. Although ERK phosphorylation in many circumstances serves as a downstream readout for activation of the Ras-mediated MAP kinase cascade consisting of Ras, Raf, and MEK, PKA inhibits Ras-mediated activation of the most extensively studied Raf isoform, Raf-1 [Vossler et al., 1997, MacNicol and MacNicol, 1999]. The small G-protein, Rap1, can be directly activated by cAMP-binding guanine nucleotide exchange proteins [Kawasaki et al., 1998] and in turn activate B-raf, providing an alternate route to ERK activation by cAMP [Vossler et al., 1997]. Rap1 has been proposed to mediate mitogenic and oncogenic effects of cAMP in other cell types [Altschuler and Ribeiro-Neto, 1998]. Our data suggest that if cAMP activates Rap1 in chromaffin cells, it does so upstream of PI 3-kinase or in a parallel pathway because, in contrast to Akt, ERK activation was not blocked by LY294002.

Because it is not possible to propagate a sufficient number of normal chromaffin cells to directly measure PI 3-kinase activity, it must be asked whether PI 3-kinase lipid products are, in fact, the critical determinant of mitogenic signaling by cAMP in these cells. The question is particularly compelling because cAMP has been reported to activate Akt in a PI 3-kinase-independent manner in a different cell type [Sable et al., 1997, Filippa et al., 1999]. In our experiments, cAMP increased phosphorylation

of two downstream effectors of PI 3-kinase activity, Akt and p70S6kinase, in chromaffin and PC12 cells and these changes correlated with direct measurements of increased PI 3-kinase activity in PC12 cells. The cAMP-induced mitogenic response of chromaffin cells, activation of PI 3-kinase and phosphorylation of Akt and p70S6kinase were sensitive to LY294002.

It must also be noted that mitogenesis by cAMP has been reported in some cells to be dependent on MAPK activation [Suzuki et al., 1999]. We have shown in the present investigation that inhibition of PI 3-kinase completely blocks the mitogenic effect of cAMP on chromaffin cells independently of effect on ERKs. However, in ongoing studies, we have found that the MAPK kinase (MEK) inhibitor U0126 [Favata et al., 1998] abolishes the proliferative responses of chromaffin cells to all mitogens tested, including cpt-cAMP and NGF (J.F. Powers and A.S. Tischler, unpublished). Our results are consistent with recent findings by Cass et al. [1999] that PI 3-kinase is required for cAMP-stimulated mitogenesis and suggest that mitogenesis requires mutually permissive interactions [Suzuki et al., 1999] between independently regulated signaling pathways involving ERKs and PI 3-kinase.

CyclicAMP can either stimulate or inhibit proliferation of different types of cells, and both responses have been correlated with activation of particular signaling intermediates such as p70S6 kinase [Monfar and Blenis, 1996] or ERKs1 and 2 [Sable et al., 1997]. The confusing association of the same effectors with opposite effects may indicate that specificity is determined by convergence of multiple signals. The rat chromaffin and PC12 cell models offer a unique opportunity to compare mitogenic signaling in normal and neoplastic cells of the same lineage. Normal chromaffin cells proliferate almost exclusively in response to exogenous mitogens, while proliferation of PC12 cells is autonomous and inhibited by at least two agents, NGF and cAMP, that are chromaffin cell mitogens. LY294002 inhibits cAMP-stimulated proliferation of normal chromaffin cells, but not constitutive proliferation of PC12 cells. An additional difference noted during the present studies was that forskolin, in contrast to cpt-cAMP, causes a decrease, rather than an increase, in phosphorylated p70S6 kinase in PC12 cells (J.F. Powers, unpublished), suggesting an inhibitory event downstream of PI 3-kinase specific to PC12 cells. Inhibition of p70S6 kinase accompanies anti-proliferative effects of cAMP in some cell types [Monfar and Blenis, 1996] and may account for the fact that, in our experience, forskolin exerts a greater antiproliferative effect on PC12 cells than cpt-cAMP (J.F. Powers and A.S. Tischler, unpublished). These and other differences between normal chromaffin cells and their neoplastic counterparts might provide clues to signaling aberrations responsible for chromaffin cell neoplasia.

REFERENCES

Akiyama T, Ogawara H. 1991. Use and specificity of genistein as inhibitor of protein-tyrosine kinases. Methods Enzymol 201:362–370.

Altschuler DL, Ribeiro-Neto F. 1998. Mitogenic and oncogenic properties of the small G protein Rap1b. Proc Natl Acad Sci USA 95:7475–7479.

Bauer PH, Bluml K, Schroder S, Hegler J, Dees C, Lohse MJ. 1998. Interactions of phosducin with the subunits of G-proteins. Binding to the alpha as well as the beta-gamma subunits. J Biol Chem 273:9465–9471.

Bauer PH, Muller S, Puzicha M, Pippig S, Obermaier B, Helmreich EJ, Lohse MJ. 1992. Phosducin is a protein kinase A-regulated G-protein regulator. Nature 358:73–76.

Benistant C, Chapuis H, Roche S. 2000. A specific function for phosphatidylinositol 3-kinase alpha (p85alpha–p110alpha) in cell survival and for phosphatidylinositol 3-kinase beta (p85alpha–p110beta) in de novo DNA synthesis of human colon carcinoma cells [In Process Citation]. Oncogene 19:5083–5090.

Blair LA, Bence-Hanulec KK, Mehta S, Franke T, Kaplan D, Marshall J. 1999. Akt-dependent potentiation of L channels by insulin-like growth factor- 1 is required for neuronal survival. J Neurosci 19:1940–1951.

Cass LA, Summers SA, Prendergast GV, Backer JM, Birnbaum MJ, Meinkoth JL. 1999. Protein kinase A-dependent and -independent signaling pathways contribute to cyclic AMP-stimulated proliferation. Mol Cell Biol 19:5882–5891.

Chung J, Kuo CJ, Crabtree GR, Blenis J. 1992. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell 69:1227–1236.

Duronio V, Scheid MP, Ettinger S. 1998. Downstream signalling events regulated by phosphatidylinositol 3-kinase activity. Cell Signal 10:233–239.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273: 18623–18632.

Filippa N, Sable CL, Filloux C, Hemmings B, Van Obberghen E. 1999. Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase. Mol Cell Biol 19:4989–5000.

Franke TF, Kaplan DR, Cantley LC. 1997. PI3K: downstream AKTion blocks apoptosis. Cell 88:435–437.

Gratzner HG. 1982. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science 218:474–475.

Greene LA, Tischler AS. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 73:2424–2428.

Hallberg B, Ashcroft M, Loeb DM, Kaplan DR, Downward J. 1998. Nerve growth factor induced stimulation of Ras requires Trk interaction with Shc but does not involve phosphoinositide 3-OH kinase. Oncogene 17:691–697.

Holgert H, Holmberg K, Hannibal J, Fahrenkrug J, Brimijoin S, Hartman BK, Hokfelt T. 1996. PACAP in the adrenal gland-relationship with choline acetyltransferase, enkephalin and chromaffin cells and effects of immunological sympathectomy. Neuroreport 8:297–301.

Jain SK, Susa M, Keeler ML, Carlesso N, Druker B, Varticovski L. 1996. PI 3-kinase activation in BCR/abl-transformed hematopoietic cells does not require interaction of p85 SH2 domains with p210 BCR/abl. Blood 88:1542–1550.

Johnson CM, Hill CS, Chawla S, Treisman R, Bading H. 1997. Calcium controls gene expression via three distinct pathways that can function independently of the Ras/mitogen-activated protein kinases (ERKs) signaling cascade. J Neurosci 17:6189–6202.

Kaplan DR, Stephens RM. 1994. Neurotrophin signal transduction by the Trk receptor. J Neurobiol 25:1404–1417.

Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. 1998. A family of cAMP-binding proteins that directly activate Rap1. Science 282:2275–2279.

Kurosu H, Maehama T, Okada T, Yamamoto T, Hoshino S, Fukui Y, Ui M, Hazeki O, Katada T. 1997. Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110beta is synergistically activated by the betagamma subunits of G proteins and phosphotyrosyl peptide. J Biol Chem 272:24252–24256.

Lillien LE, Claude P. 1985. Nerve growth factor is a mitogen for cultured chromaffin cells. Nature 317:632–634.

MacNicol MC, MacNicol AM. 1999. Nerve growth factor-stimulated B-Raf catalytic activity is refractory to inhibition by cAMP-dependent protein kinase. J Biol Chem 274:13193–13197.

Mark MD, Storm DR. 1997. Coupling of epidermal growth factor (EGF) with the antiproliferative activity of cAMP induces neuronal differentiation. J Biol Chem 272: 17238–17244.

Misra S, Ujhazy P, Varticovski L, Arias IM. 1999. Phosphoinositide 3-kinase lipid products regulate ATP-dependent transport by sister of P-glycoprotein and multidrug resistance associated protein 2 in bile canalicular membrane vesicles. Proc Natl Acad Sci USA 96:5814–5819.

Monfar M, Blenis J. 1996. Inhibition of p70/p85 S6 kinase activities in T cells by dexamethasone. Mol Endocrinol 10:1107–1115.

Murga C, Laguinge L, Wetzker R, Cuadrado A, Gutkind JS. 1998. Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinasegamma. J Biol Chem 273:19080–19085.

Okada T, Hazeki O, Ui M, Katada T. 1996. Synergistic activation of PtdIns 3-kinase by tyrosine-phosphorylated peptide and beta gamma-subunits of GTP-binding proteins. Biochem J 317:475–480.

Powers JF, Shahsavari M, Tsokas P, Tischler AS. 1999a. Nerve growth factor receptor signaling in proliferation of normal adult rat chromaffin cells. Cell Tiss Res 295:21–32.

Powers JF, Tischler AS, Cherington V. 1999b. Discordant effects of rapamycin on proliferation and p70S6 kinase phosphorylation in normal and neoplastic rat chromaffin cells. Neurosci Lett 259:137–140.

Sable CL, Filippa N, Hemmings B, Van Obberghen E. 1997. cAMP stimulates protein kinase B in a Wortmannin-insensitive manner. FEBS Lett 409:253–257.

Susa M, Keeler M, Varticovski L. 1992. Platelet-derived growth factor activates membrane-associated phosphatidylinositol 3-kinase and mediates its translocation from the cytosol. Detection of enzyme activity in detergent-solubilized cell extracts. J Biol Chem 267:22951–22956.

Suzuki S, Yamamoto I, Arita J. 1999. Mitogen-activated protein kinase-dependent stimulation of proliferation of rat lactotrophs in culture by 3′,5′-cyclic adenosine monophosphate. Endocrinology 140:2850–2858.

Tischler AS, McClain RM, Childers H, Downing J. 1991. Neurogenic signals regulate chromaffin cell proliferation and mediate the mitogenic effect of reserpine in the adult rat adrenal medulla. Lab Invest 65:374–376.

Tischler AS, Riseberg JC, Cherington V. 1994. Multiple mitogenic signalling pathways in chromaffin cells: a model for cell cycle regulation in the nervous system. Neurosci Lett 168:181–184.

Tischler AS, Riseberg JC, Gray R. 1995. Mitogenic and antimitogenic effects of pituitary adenylate cyclase-activating polypeptide (PACAP) in adult rat chromaffin cell cultures. Neurosci Lett 189:135–138.

Tischler AS, Riseberg JC, Hardenbrook MA, Cherington V. 1993. Nerve growth factor is a potent inducer of proliferation and neuronal differentiation for adult rat chromaffin cells in vitro. J Neurosci 13:1533–1542.

Tischler AS, Ruzicka LA, Donahue SR, DeLellis RA. 1989. Chromaffin cell proliferation in the adult rat adrenal medulla. Int J Dev Neurosci 7:439–448.

Tischler AS, Ruzicka LA, Riseberg JC. 1992. Immunocytochemical analysis of chromaffin cell proliferation in vitro. J Histochem Cytochem 40:1043–1045.

Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, Miller FD. 1999. Depolarization and neurotrophins converge on the phosphatidylinositol 3- kinase-Akt pathway to synergistically regulate neuronal survival. J Cell Biol 146:955–966.

Vanhaesebroeck B, Jones GE, Allen WE, Zicha D, Hooshmand-Rad R, Sawyer C, Wells C, Waterfield MD, Ridley AJ. 1999. Distinct PI(3)Ks mediate mitogenic signalling and cell migration in macrophages. Nat Cell Biol 1:69–71.

Vanhaesebroeck B, Waterfield MD. 1999. Signaling by distinct classes of phosphoinositide 3-kinases. Exp Cell Res 253:239–254.

Varticovski L, Daley GQ, Jackson P, Baltimore D, Cantley LC. 1991. Activation of phosphatidylinositol 3-kinase in cells expressing abl oncogene variants. Mol Cell Biol 11:1107–1113.

Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ. 1997. cAMP activates Map kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell 89:73–82.

Yao R, Cooper GM. 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003–2006.