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Carbachol Inhibits Insulin-Stimulated Phosphatidylinositol 3-Kinase Activity in SH-SY5Y Neuroblastoma Cells

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Abstract: SH-SY5Y human neuroblastoma cells express muscarinic M_3 receptors as well as insulin receptors, thus offering the opportunity to investigate possible cross-talk following activation of two distinct intracellular signal transduction pathways that convert the precursor phosphatidylinositol (PI) to its 3′ phosphate or its 4′ phosphate, respectively. In this study, the effect of carbachol on insulin-stimulated PI 3-kinase (PI3K) activity was examined in SH-SY5Y cells. Insulin addition to the cell medium induced a 10–26-fold increase in anti-phosphotyrosine-immunoprecipitable PI3K activity. Preincubation with 1 m*M* carbachol inhibited the insulin-stimulated PI3K activity in a time-dependent manner, with half-maximal and maximal inhibition times of 4 and 15 min, respectively. Atropine blocked the inhibitory effect of carbachol. Although carbachol did not change the amount of 85-kDa subunit protein regulatory unit associated with tyrosine-phosphorylated proteins, either in control or in insulin-stimulated cells, it appears to decrease the amount of associated 110-kDa catalytic subunit protein in the latter instance. Because PI3K activity from SH-SY5Y cells has been shown to be inhibited in vitro in the presence of cytidine diphosphodiacylglycerol (CDP-DAG) or phosphatidate (PA), we examined the presence of these lipids in SH-SY5Y cells that had been treated with carbachol. Formation of both lipids was increased in a time-dependent manner following carbachol addition, and their increased levels are proposed to account for the observed in vivo inhibition of PI3K. Addition of the cell-permeable homologue didecanoyl-CDP-DAG to intact cells inhibited insulin-stimulated PI3K activity up to 75%, with an IC_{50} of 0.5 μM, a result that further supports a proposed lipid-mediated inhibition of PI3K. Exogenously added didecanoyl-PA, however, did not affect PI3K activity. The possibility that stimulation of the PI 4-kinase-mediated signal transduction pathway leads to down-regulation of the PI3K-mediated signal transduction pathway in vivo, via inhibition of PI3K by CDP-DAG or by other consequences of phosphoinositidase C-linked receptor activation, is discussed. **Key Words:** Carbachol—Insulin—Phosphatidylinositol 3-kinase—Cytidine diphosphodiacylglycerol—Phosphatidate—SH-SY5Y neuroblastoma cells.
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Phosphatidylinositol (PI) 3-kinase (PI3K) mediates a central downstream signal transduction route for various growth factors, including cytokines, epithelial growth factor, platelet-derived growth factor, insulin, insulin-like growth factors (IGFs), and nerve growth factor (NGF) (Parker and Waterfield, 1992), and is permissive for several cellular responses, including mitogenesis (Rodriguez-Viciana et al., 1994), translocation of glucose transporters (Clarke et al., 1994), and differentiation (Carter and Downes, 1993). The enzyme is able to phosphorylate PI, PI 4-phosphate (PI4P), and PI 4,5-bisphosphate [PI(4,5)P_2] on the 3′ position of the inositol ring in vitro, yielding in each instance the 3-phosphate of the corresponding lipid (Panayotou and Waterfield, 1992; Parker and Waterfield, 1992). In vivo, PI(4,5)P_2 is the favored substrate (Hawkins et al., 1992). The mechanism by which insulin activates PI3K involves induction of insulin receptor tyrosine kinase activity followed by tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1). Tyrosine-phosphorylated residues within IRS-1 then associate with specific *src* homology domains of various target proteins, including PI3K (Myers et al., 1994). The recruitment of PI3K holoenzyme [containing both the 85-kDa (p85) regulatory subunit and the 110-kDa (p110) catalytic subunit] results in the enzyme's activation. p85 is thought to act as an adapter protein that couples the p110 catalytic subunit

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Abbreviations used: CDP-DAG, cytidine diphosphodiacylglycerol; DAG, diacylglycerol; IGF, insulin-like growth factor; IRS-1, insulin receptor substrate-1; NGF, nerve growth factor; p85, 85-kDa regulatory subunit of phosphatidylinositol 3-kinase; p110, 110-kDa catalytic subunit of phosphatidylinositol 3-kinase; PA, phosphatidate; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; PI4K, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

to tyrosine phosphoproteins via an SH2 domain (Hu and Schlessinger, 1994).

In the present study we used SH-SY5Y human neuroblastoma cells, which express insulin receptors (Mattsson et al., 1990) as well as muscarinic M_3 receptors (Offermanns et al., 1993), to examine PI3K activity following exposure of the cells to both insulin and carbachol. This investigation was prompted by a prior study in which we demonstrated that phosphatidate (PA) and cytidine diphosphodiacylglycerol (CDP-DAG) each block PI3K in vitro (Lavie and Agranoff, 1996). These two lipids are sequential biosynthetic precursors of PI, the common lipid substrate for both PI3K and PI 4-kinase (PI4K) (Fisher et al., 1992). It was furthermore known that labeled CDP-DAG levels are elevated during carbachol-stimulated phosphoinositide turnover cycle [which uses the PI4K pathway in these cells (Heacock et al., 1993)]. The question was thus raised of whether or not ligand-stimulated breakdown of PI(4,5)P_2 and its associated resynthesis via PA and CDP-DAG would affect stimulation of the PI3K-mediated pathway in vivo. Because insulin induces a mitogenic response and enhances neurite formation in SH-SY5Y cells (Recio-Pinto and Ishii, 1988; Mattsson et al., 1990), it was chosen for investigation of the possible effects of carbachol-stimulated activation of the PI4K pathway on the PI3K pathway in these cells.

The present results indicate that elevated intracellular CDP-DAG content resulting from stimulated PI(4,5)P_2 breakdown may contribute to regulation of the PI3K-directed signal transduction pathway in vivo. During the course of these studies, it was found that carbachol addition to insulin-stimulated cells leads to selective loss of p110 from the phosphotyrosine immunocomplex, indicating a possible additional mechanism for interaction of the two signal transduction pathways.

EXPERIMENTAL PROCEDURES

Materials

ATP, bovine serum albumin, carbachol, insulin from bovine pancreas, leupeptin, aprotinin, and PI from bovine liver were purchased from Sigma (St. Louis, MO, U.S.A.). Didecanoyl-PA was the product of Serdary Research Labs (London, Ontario, Canada). Didecanoyl cytidine diphosphodiacylglycerol (C_{10}-CDP-DAG) was the gift of A. K. Hajra. [γ-^{32}P]ATP (3,000 Ci/mmol) and [^{3}H]cytidine (30 Ci/mmol) were from DuPont NEN (Wilmington, DE, U.S.A.). Carrier-free $^{32}P_i$ was purchased from ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.). Silica gel 60 TLC plates were from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium, fetal bovine serum, and protein A-agarose were purchased from GibcoBRL (Gaithersburg, MD, U.S.A.). Anti-rat PI3K (anti-p85) and anti-phosphotyrosine (monoclonal IgG2b$_k$) antibodies were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-p110 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

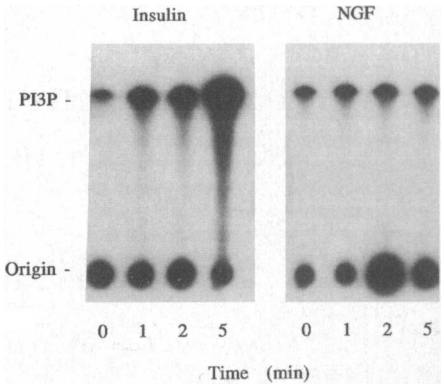

FIG. 1. Insulin and NGF stimulate PI3K activity in anti-phosphotyrosine immunoprecipitates of SH-SY5Y cells. Cells were grown as described in Experimental Procedures. Insulin (100 n*M*) or NGF (100 n*M*) was added to the medium and incubated for the indicated times at 37°C. Lysates were immunoprecipitated with anti-phosphotyrosine antibodies, and PI3K activity was measured as described in Experimental Procedures. Autoradiographs of the TLC of PIP are from one of three experiments that gave similar results.

Cell culture

SH-SY5Y human neuroblastoma cells (passages 60–80) were grown in Dulbecco's modified Eagle's supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 10% CO_2. For all the experiments using effectors (insulin, carbachol, etc.), cells were preincubated for 24 h in Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin.

Immunoprecipitation of PI3K activity from stimulated cells using anti-phosphotyrosine antibodies

Cells were grown in 150-mm-diameter dishes. Effectors were directly added to the medium, and the cells were incubated for the indicated times at 37°C. After hormonal treatment, the medium was aspirated, and the cells were washed twice with 15 ml of ice-cold phosphate-buffered saline (PBS; pH 7.4) before addition of 1 ml of buffer B [137 m*M* NaCl, 20 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 10 μg/ml of leupeptin, 2 μg/ml of aprotinin, 1 m*M* phenylmethylsulfonyl fluoride, and 1 m*M* sodium orthovanadate]. The cells were scraped free from the dishes and centrifuged at 6,000 *g* for 10 min in a 15-ml conical polystyrene centrifuge tube (Sardstet). The resulting supernatant was incubated overnight at 4°C with 5 μg per tube of anti-phosphotyrosine antibodies, followed by addition of protein A-agarose beads for 2 h. Immune complexes bound to the beads were washed three times with cold PBS (pH 7.4) containing 1% Nonidet P-40, twice with PBS containing 0.5 *M* LiCl and 0.1 *M* Tris-HCl (pH 7.5), and twice with 10 m*M* Tris-HCl (pH 7.5) containing 0.1 *M* NaCl and 1 m*M* EDTA.

PI3K assay

The reaction mixture for measuring PI3K activity contained 100 μl of the enzyme and 50 μl of PI micelles that were prepared by sonicating 800 μg of PI in 1 ml of 20 m*M* HEPES buffer (pH 7.6) containing 1 m*M* EDTA for 45 s (Kontes sonicator with a microtip probe). The mixture was preincubated for 15 min at room temperature, and the reaction was

started by addition of 50 μl of 40 m*M* $MgCl_2$ containing 200 μ*M* [γ-^{32}P]ATP (20 μCi per assay). The reaction was terminated after 15 min by addition of 750 μl of ice-cold $CHCl_3$/methanol/2 *M* HCl (20:40:0.5 by volume) followed by 250 μl of chloroform and 250 μl of 2 *M* HCl. The resulting organic phase and the interface were extracted with an equal volume of methanol/0.1 *M* EDTA (1:0.9 vol/vol). The organic phase was dried, resuspended in 25 μl of chloroform/methanol (1:1 vol/vol), and spotted on a TLC plate that was precoated with 1% potassium oxalate. The plate was developed in $CHCl_3$/methanol/water/7.7 *M* NH_4OH (60:47:11.3:2 by volume) (Serunian et al., 1991). ^{32}P-Labeled PI 3-phosphate (PI3P) was visualized by autoradiography and compared with iodine-stained PI phosphate marker, for which PI4P was used. Radioactivity in this spot was quantified as described in Experimental Procedures. Although PI3P and PI4P are not separated by this procedure, the possibility that PI4K activity was present was excluded on the basis of susceptibility to wortmannin and to Nonidet P-40 detergent.

Immunoblotting of the p85 subunit of PI3K

Cells, treated with various effectors, were lysed, followed by immunoprecipitation with anti-phosphotyrosine antibodies as described above. After washing, immune complexes were resolved on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to nitrocellulose paper and analyzed for the presence of the p85 by protein immunoblot, using anti-p85 antiserum. Bound antibodies were visualized by an enhanced chemiluminescence (ECL) detection system, using horseradish peroxidase conjugated to anti-rabbit IgG as the secondary antibody (Amersham). In a separate series of experiments, gels were reprobed with anti-p110 antibody.

Radiolabeling studies

Cells were labeled with either $^{32}P_i$ or [^{3}H]cytidine in oxygenated buffer A (142 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 3.6 m*M* $NaHCO_3$, 1 m*M* $MgCl_2$, 5.6 m*M* glucose, and 30 m*M* HEPES, pH 7.4). To label PA, cells grown in 60-mm-diameter culture dishes were washed twice with buffer A and preincubated in 5 ml of the same buffer containing 20 μCi of $^{32}P_i$ per dish for 10 min at 37°C. To label CDP-DAG, cells were washed as above and incubated in 5 ml of buffer A containing 10 μCi of [^{3}H]cytidine per dish for 1 h at 37°C. Following addition of the effectors for various time points, reactions were terminated on ice by aspirating the medium and adding 1 ml of ice-cold methanol/1 *M* HCl. The cells were scraped free from the dishes and transferred to glass test tubes. Lipids were extracted from the cell pellet by adding 1 ml each of water and chloroform, followed by vortex-mixing. The lower chloroform phase was dried with a Speed Vac and resuspended in 25 μl of chloroform/methanol (1:1 vol/vol). Lipids extracts were spotted on a 20- × 20-cm TLC plate and were developed in a $CHCl_3$/CH_3OH/acetic acid/acetone/water (40:13:12:15:8 by volume) solvent system. The plate was subjected to autoradiography, and the radioactive bands were identified by cochromatography with authentic standards. TLC spots of the radioactive lipids were scraped off into counting vials, and radioactivity was determined by liquid scintillation counting.

RESULTS

Stimulation of PI3K activity associated with anti-phosphotyrosine immunoprecipitates in SH-SY5Y cells is induced by insulin and NGF

Activation of PI3K is generally correlated with an increase in the amount of PI3K activity that can be immunoprecipitated with anti-phosphotyrosine antibodies. We found that in SH-SY5Y cells, insulin and NGF are each able to induce such an enzyme activation. As shown in Fig. 1, insulin induced a rapid increase in anti-phosphotyrosine-precipitable PI3K activity, which was clearly visible by 1 min and was maximal by 5 min. The response was transient and decreased thereafter (data not shown). Although the kinetics of stimulation by NGF were very similar, NGF's potency was much lower (threefold stimulation, compared with 20-fold).

Carbachol inhibits insulin-stimulated PI3K activity

We examined the effect of the muscarinic ligand carbachol on the activation of PI3K by insulin. Pretreatment of SH-SY5Y cells with 1 m*M* carbachol for 30 min markedly reduced the elevation in PI3P content produced by PI3K from insulin-stimulated cells (Fig. 2). PI3P content was also lower compared with control basal levels after treatment with carbachol alone. The inhibitory effect elicited by carbachol was blocked by preincubation of the cells with the muscarinic antagonist atropine, demonstrating that the effect of carbachol was receptor mediated. Figure 3 shows that the inhibition of insulin-induced PI3K activity by carbachol is time dependent. As preincubation time increased, enzyme-stimulated activity decreased, with half-maximal and maximal times of 4 and 15 min, respectively.

Investigation of possible mechanisms by which carbachol inhibits insulin-stimulated PI3K activity

Because PI3K is activated in insulin-stimulated cells by the binding of its p85 to tyrosine-phosphorylated

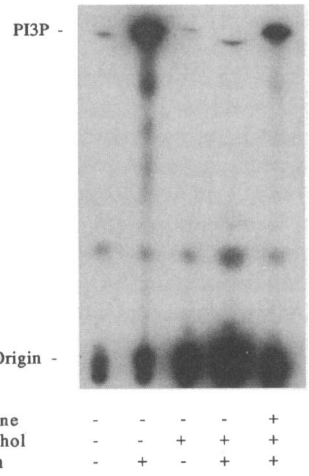

FIG. 2. Carbachol inhibits insulin-stimulated PI3K activity. Cells were preincubated for 10 min without or with atropine (10 μ*M*) and then for an additional 30 min in the absence or presence of 1 m*M* carbachol, followed by addition of 100 n*M* insulin for 5 min. Cellular extracts were then reacted with anti-phosphotyrosine antibodies and assayed for PI3K activity as described in Experimental Procedures. Carbachol produced an 87% decrease in this experiment, somewhat higher than the average inhibition seen in three experiments (56%).

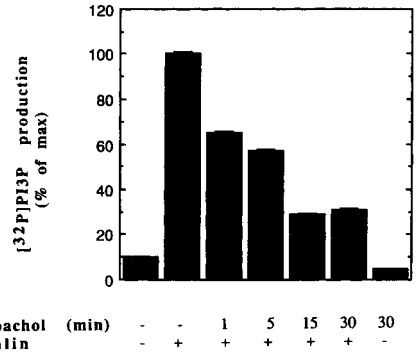

FIG. 3. Time dependence of carbachol inhibition of insulin-stimulated PI3K activation. Cells were treated with or without (1 m*M*) carbachol for the indicated times and then for an additional 5 min with 100 n*M* insulin, as indicated. Anti-phosphotyrosine immunoprecipitates were assayed for PI3K activity as described in Experimental Procedures. The phosphorylated lipid products were separated by TLC, and the corresponding PI phosphate band was scraped into vials and counted for radioactivity. PI3K activity is calculated as a percentage of insulin-stimulated, uninhibited kinase activity (100% = 7,091 ± 430 cpm). Data are mean ± SD (bars; half the range) values of duplicate determinations and are typical of three experiments.

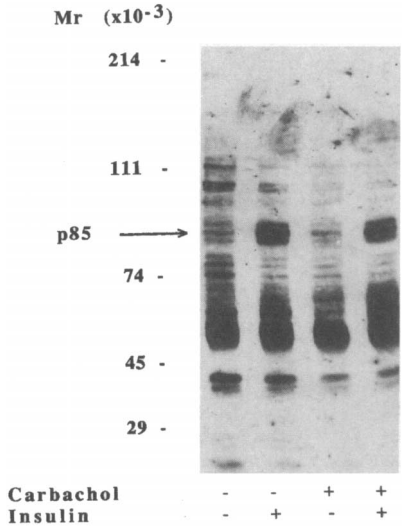

FIG. 4. Detection of PI3K in anti-phosphotyrosine immunoprecipitates by western blotting. Cells were treated for 30 min with or without 1 m*M* carbachol and then for an additional 5 min with or without 100 n*M* insulin, as indicated. The cellular extracts were immunoprecipitated with anti-phosphotyrosine antibodies, subjected to 7.5% SDS-PAGE, and then immunoblotted, using anti-p85 antibodies, as described in Experimental Procedures. The prominent spots above the 45-kDa mark represent IgGs.

proteins, we examined the possibility that carbachol interferes directly with this interaction. Cells were treated with carbachol and/or insulin for 30 and 5 min, respectively. Whole cell lysates were then subjected to immunoprecipitation with anti-phosphotyrosine antibodies, and the immunoprecipitates were analyzed by western blotting with anti-p85 antibodies. As shown in Fig. 4, p85 association with tyrosine-phosphorylated proteins is greatly enhanced in cells treated with insulin. Carbachol affected neither the control level of p85 nor the insulin-stimulated association of p85 with tyrosine-phosphorylated proteins (lane 4). The tentative conclusion that the observed inhibitory effect of carbachol on insulin-induced PI3K activation does not involve changes in binding of the heterodimer to tyrosine-phosphorylated protein was, however, challenged by further studies in which such gels were reprobed with anti-p110 antibodies. In contrast to the lack of effect seen on p85 binding, a significant decrease (54%) in amount of anti-p110-immunoreactive material in insulin-stimulated cells was seen in the presence of carbachol, as is further addressed in Discussion.

Because CDP-DAG and PA inhibit SH-SY5Y cell-derived PI3K activity in vitro (Lavie and Agranoff, 1996), it was of interest to examine the possibility that a carbachol-induced increase in CDP-DAG content could contribute to our in vivo findings. We first characterized the time course by which carbachol stimulates the formation of CDP-DAG and PA in SH-SY5Y cells. To assess changes in PA level, cells were incubated briefly with $^{32}P_i$ before addition of carbachol. The time course of labeled PA accumulation was then followed for 30 min. As shown in Fig. 5, labeled PA content is dramatically elevated as early as 1 min after addition of 1 m*M* carbachol and is maximal within 5 min, presumably reflecting phosphorylation of diacylglycerol (DAG) by DAG kinase. Similarly, addition of carbachol to [^{3}H]cytidine-prelabeled cells results in a drastic increase (up to fivefold) in amount of [^{3}H]-CDP-DAG compared with the control level. Carbachol elicits a half-maximal increase in [^{3}H]CDP-DAG level

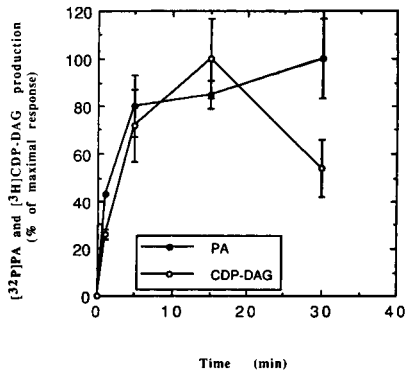

FIG. 5. Time-dependent accumulation of [^{3}H]CDP-DAG and [^{32}P]PA in carbachol-stimulated cells. SH-SY5Y cells were prelabeled with either $^{32}P_i$ or [^{3}H]cytidine, treated with 1 m*M* carbachol for the times indicated, and analyzed for [^{3}H]CDP-DAG and [^{32}P]PA as described in Experimental Procedures. Data are mean ± SEM (bars) values from two separate experiments, expressed as percentages of the maximal response for each lipid (100% = 5,870 ± 1,003 and 215,800 ± 38,220 cpm for [^{3}H]-CDP-DAG and [^{32}P]PA, respectively). The zero-time radioactivity level for [^{3}H]CDP-DAG was 1,100 ± 150 cpm, and that for [^{32}P]PA was 10,060 ± 703 cpm.

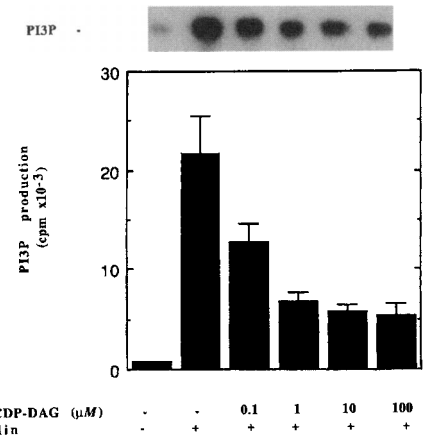

FIG. 6. C_{10}-CDP-DAG inhibits insulin-stimulated PI3K activity in SH-SY5Y cells. Cells were preincubated for 60 min with the indicated concentrations of C_{10}-CDP-DAG and then for an additional 5 min with 100 n*M* insulin. Anti-phosphotyrosine immunoprecipitates were assayed for PI3K activity as described in Experimental Procedures. The phosphorylated lipid products were separated by TLC, and the corresponding PI phosphate bands were scraped off and quantified by liquid scintillation spectrometry. **Lower panel:** Data are average ± SEM of duplicate determinations and are typical of three separate experiments. **Upper panel:** Autoradiographs of the region of the thin-layer chromatogram that contained PI3P.

within 3 min of incubation and is maximal within 15 min. The decrease in [^{3}H]CDP-DAG level evident by 30 min of incubation with carbachol is most likely due to further metabolism of CDP-DAG, primarily to PI. The elevation in [^{32}P]PA content precedes that of [^{3}H]CDP-DAG, reflecting the biosynthetic sequence in PI resynthesis after carbachol-stimulated breakdown of PI(4,5)P_2. To test directly the possibility that CDP-DAG is inhibiting PI3K activity in vivo, we used C_{10}-CDP-DAG (Benjamins and Agranoff, 1969). Cells were preincubated for 1 h without or with given concentrations of C_{10}-CDP-DAG and then stimulated with insulin for 5 min. As shown in Fig. 6, a 26-fold stimulation of PI3P formation by insulin was half-maximally reduced by 0.5 μM C_{10}-CDP-DAG and was maximally blocked at 10 μM. These values are very close to the IC_{50} value (6 μM) required for inhibition of PI3K by CDP-DAG in an in vitro assay system (Lavie and Agranoff, 1996). Addition of C_{10}-PA (0.01–100 μM), however, did not affect PI3K activity (data not shown).

DISCUSSION

PI3K participates in various signal-regulated cell processes induced by mitogenic growth factors and neurotrophic factors (Panayotou and Waterfield, 1992; Parker and Waterfield, 1992; Carter and Downes, 1993; Myers et al., 1994). Stimulation of PI3K activity by insulin in SH-SY5Y neuroblastoma cells resembles that observed with insulin in several other cell preparations, including Chinese hamster ovary cells (Ruderman et al., 1990), adipocytes (Shepherd et al., 1995), and rat liver and skeletal muscle (Folli et al., 1992), and is associated with an increase in anti-phosphotyrosine-immunoprecipitable activity. It is generally accepted that insulin induces an association of the enzyme with tyrosyl-phosphorylated Tyr-Met-X-Met or Tyr-X-X-Met motifs in IRS-1, which increases on insulin stimulation, without an apparent increase in amount of tyrosyl-phosphorylated PI3K (Sung and Goldfine, 1992; Myers et al., 1994). Although there are alternative possible mechanisms for insulin-induced increases in PI3K activity (Carter and Downes, 1993; Myers et al., 1994), the present results indicate that association with IRS-1 also occurs in SH-SY5Y cells. Western blots of insulin-treated cells with anti-PI3K antibodies revealed a dramatic increase in the amount of PI3K associated with an anti-phosphotyrosine-immunoprecipitable complex, indicating that the enzyme is associated with tyrosine-phosphorylated proteins in vivo. In accordance with the finding, PI3K activity measured in anti-phosphotyrosine immunoprecipitates was elevated 10–26-fold in insulin-stimulated cells. In contrast, SH-SY5Y cell PI3K protein and activity immunoprecipitated with anti-PI3K antibodies were not measurably affected by insulin treatment (data not shown), in agreement with what has been found in rat HTC cells (Sung and Goldfine, 1992). These results also indicate that the insulin-regulated PI3K activity present in anti-phosphotyrosine immunoprecipitates accounts for only a minor portion of cellular PI3K.

Insulin's principal physiological role in liver and muscle is readily understood, but this is less clear for the nervous system. Receptors for insulin and IGFs are present in various brain regions, and agonist binding leads to increased glucose, uridine, and thymidine uptake, as well as increased ornithine decarboxylase activity (De Pablo and De la Rosa, 1995). Insulin receptors are present also in the SH-SY5Y human neuroblastoma cell line and are known to induce mitogenesis and neurite formation (Recio-Pinto and Ishii, 1988; Mattsson et al., 1990). Although the data indicate that PI3K activation can mediate insulin signal transduction in a brain-derived cell line, it remains to be demonstrated that this insulin-stimulated activity in SH-SY5Y cells is a component of a mechanism whereby insulin exerts its neurotrophic effects on the CNS (De Pablo and De la Rosa, 1995). It should also be noted that some of the insulin-induced PI3K activity measured in the present study may be mediated by IGF receptors, as IGF receptors are present in SH-SY5Y cells (Mattsson et al., 1990) are known to cross-react with insulin receptors (Recio-Pinto and Ishii, 1988) and to activate PI3K (De Pablo and De la Rosa, 1995).

SH-SY5Y cells have been investigated extensively in regard to their muscarinic (M_3) acetylcholine receptors, which on activation stimulate phosphoinositide turnover (including release of DAG and inositol 1,4,5-

trisphosphate, followed by PA, and CDP-DAG increases, protein kinase C activation, and elevation in intracellular [Ca^{2+}]. In addition to possible roles in prostanoid production and catecholamine release in neurons (Abdel-Latif, 1986), muscarinic receptors have been linked, depending on the cell type examined, to increases (Gutkind et al., 1991) and decreases (Conklin et al., 1988) in mitogenesis. Conklin et al. (1988) have found that carbachol caused a marked inhibition of thymidine incorporation into A9 L cells transfected with M_1 and M_3 muscarinic receptors, which could be blocked by atropine. Our finding that PI3K activation following insulin addition to intact cells is inhibited by carbachol raised the possibility that the inhibition of mitogenesis by carbachol reflects its inhibition of insulin-induced PI3K activity.

These observations led us to study the nature of carbachol's action on the insulin signal to activate PI3K. Recent studies implicate tyrosine phosphorylation as both an initiator and a possible consequence of muscarinic receptor-mediated PI hydrolysis. Offermanns et al. (1993) have found that stimulation of muscarinic receptors increased tyrosine phosphorylation in SH-SY5Y cells. Thus, we investigated whether or not the tyrosine phosphorylation mechanism of insulin-induced activation of PI3K is altered on carbachol addition. We found that carbachol, either added alone or in the presence of insulin, did not change the amount of PI3K p85 associated with tyrosine-phosphorylated proteins. The availability of anti-p110 antibody during the course of these experiments prompted us to investigate the possibility that carbachol decreases the insulin-stimulated increase in the amount of p110 that is associated with the phosphotyrosine immunocomplex. Unlike the result obtained with anti-p85 shown in Fig. 4, we found a marked decrease in amount of bound p110. Because p110 is believed to bind to SH2 domains of p85 (Hu and Schlessinger, 1994), it may be that carbachol selectively dissociates the former from the immunocomplex. It is unclear at present whether this represents an independent consequence of activation of the muscarinic receptor or whether the inferred dissociation of the PI3K heterodimer is a consequence of CDP-DAG binding in vivo.

We investigated the effects of exogenously added CDP-DAG and PA, as we recently found that these phospholipid intermediates of the ligand-stimulated phosphoinositide cycle are capable of inhibiting PI3K activity in vitro (Lavie and Agranoff, 1996). We show here that the level of these lipids in intact SH-SY5Y cells is increased on carbachol addition. In accordance with previous studies (Thompson and Macdonald, 1976; Lee et al., 1991), the calculated intracellular concentrations of CDP-DAG and PA after carbachol stimulation (Lavie and Agranoff, 1996) would seem to suffice for potential in vivo regulation of PI3K activity. Addition of the membrane-permeable analogue C_{10}-CDP-DAG to cells bypasses carbachol-mediated receptor activation and results in a similar extent of inhibition as does carbachol. The lack of an analogous effect of added PA may reflect its lesser in vitro inhibitory action relative to CDP-DAG, failure to enter the cells, or its rapid breakdown. CDP-DAG and its analogues appear to enter cells intact (Turcotte et al., 1980). It should be noted that interpretation of these in vivo experiments on the basis of in vitro effects of CDP-DAG do not take into account issues of intracellular compartmentation. Because the levels of CDP-DAG in SH-SY5Y cells may vary with the inositol concentration in the culture medium and may be further modified in the presence of Li^+ (Stubbs and Agranoff, 1993), it will be of interest to examine further PI3K activity under these various conditions. Preliminary results (authors' unpublished data) indicate that constitutive levels of PI3K are decreased under conditions of low medium levels of inositol in the presence of Li^+, but no effect is seen on insulin stimulation.

In a prior study (Lavie and Agranoff, 1996), we demonstrated that CDP-DAG does not compete with PI3K's cosubstrates, PI or ATP. Possible effects of higher inositides of the PI 3- and PI 4-series under conditions of hormonal stimulation have not been examined. Of relevance is that PI 3,4,5-trisphosphate has been demonstrated to block PI3K in HIR cells (Rameh et al., 1995). The precise mechanism whereby CDP-DAG (or possibly PA) inhibits PI3K is unknown, nor is it presently known whether the apparent dissociation of the PI3K heterodimer seen in insulin-stimulated cells preincubated with carbachol is mediated via increased levels of CDP-DAG or by an independent mechanism. Nevertheless, the finding that such inhibition occurs in vivo gives further credence to the hypothesis that stimulation of the PI4K-mediated pathway, whereby the intracellular messengers inositol 1,4,5-trisphosphate and DAG are generated, may inhibit the PI3K-mediated signal transduction pathway, somewhat paradoxically, via increases in levels of metabolic precursors of PI, the common substrate of both PI3K and PI4K.

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