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Polyphosphoinositides Are the Major Source of Inositol Phosphates in Carbamoylcholine-Stimulated SK-N-SH Neuroblastoma Cells

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SUMMARY

The contribution of polyphosphoinositides to muscarinic receptor-stimulated phosphoinositide turnover has been evaluated for intact and digitonin-permeabilized human SK-N-SH neuroblastoma cells. Addition of carbamoylcholine to [^{3}H]inositol-prelabeled intact cells resulted in a rapid (5–10 sec) loss of phosphatidylinositol-4,5-bisphosphate and the concomitant appearance of radiolabeled inositol-1,4,5-trisphosphate, inositol-1,3,4-trisphosphate, and inositol tetrakisphosphate. In the presence of the agonist, production of these inositol polyphosphates remained enhanced for up to 45 min. Inositol mono- and bisphosphates steadily accumulated in response to receptor activation and in the presence of Li^+ comprised >95% of agonist-stimulated inositol phosphate formation at incubation times >5 min. The major inositol bisphosphate isomer was the 1,4-species. Of the two inositol monophosphates produced, radioactivity recovered in inositol-4-monophosphate increased continuously, whereas that in the inositol-1-monophosphate/inositol-3-monophosphate fraction was delayed in appearance but thereafter progressively accumulated. Omission of Ca^{2+} reduced carbamoylcholine-stimulated inositol phosphate release by >50% but did not significantly influence the ratio of inositol monophosphates formed. Upon addition of atropine to agonist-pretreated cells, radioactivity was lost from inositol phosphates in the following order: inositol-1,4,5-trisphosphate > inositol-1,3,4-trisphosphate > inositol-1,4-bisphosphate = inositol-4-monophosphate > inositol-1-monophosphate/inositol-3-monophosphate. Although carbamoylcholine addition to digitonin-permeabilized cells also resulted in a sustained release of inositol monophosphates, relatively more inositol-4-monophosphate was produced in these preparations. Omission of ATP from permeabilized cell incubations inhibited carbamoylcholine-stimulated inositol phosphate formation by >70%. Whole homogenates of SK-N-SH cells metabolized added inositol-1,4,5-trisphosphate and inositol-1,4-bisphosphate exclusively to inositol-4-monophosphate, whereas inositol-1,3,4,5-tetrakisphosphate was degraded to inositol-1- or 3-monophosphate. Measurement of inositol trisphosphate 3′-kinase and 5′-phosphatase activities revealed that, following permeabilization, 3′-kinase activity was diminished, whereas that of 5′-phosphatase was enhanced. The results indicate that occupancy of muscarinic cholinergic receptors in SK-N-SH cells elicits a continuous Ca^{2+}-dependent breakdown of the polyphosphoinositides rather than of phosphatidylinositol. In intact cells, inositol-1,4,5-trisphosphate produced upon receptor activation is metabolized both to inositol bisphosphate and inositol tetrakisphosphate via the 5′-phosphatase and 3′-kinase pathways, respectively. The latter pathway may account for much of the radioactivity present in the inositol-1-monophosphate/inositol-3-monophosphate fraction recovered from intact cells stimulated with carbamoylcholine. In contrast, the 5′-phosphatase route of degradation predominates in the permeabilized cell.

Agonist occupancy of a diverse group of cell-surface receptors is known to elicit the phosphodiesteratic breakdown of PIP_2, with the concomitant formation of two intracellular second messengers, I(1,4,5)P_3 and diacylglycerol (for reviews, see Refs. 1 and 2). Less certain, however, is whether the receptor-mediated hydrolysis of other inositol lipids, in particular that of PI, also occurs. It has been suggested that in some tissues a transient receptor-mediated breakdown of PIP_2 may precede a more sustained hydrolysis of PI, which, like that of PIP_2, is also mediated by activation of phospholipase C (3–5). An elevated intracellular [Ca^{2+}] that results from receptor activation could serve to trigger this hydrolysis of PI (6), because higher

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ABBREVIATIONS: PIP_2, phosphatidylinositol 4,5-bisphosphate; mAChR, muscarinic acetylcholine receptor; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PPI, phosphoinositide (PI, PIP, and PIP_2); IP_1, 1-D-*myo*-inositol monophosphate (isomeric positioning of phosphate groups are indicated when appropriate); IP_2, 1-D-*myo*-inositol bisphosphate; IP_3, 1-D-*myo*-inositol trisphosphate; IP_4, 1-D-*myo*-inositol tetrakisphosphate; IP_5, 1-D-*myo*-inositol pentakisphosphate; IP_6, 1-D-*myo*-inositol hexakisphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); TCA, trichloroacetic acid; HPLC, high pressure liquid chromatography; TLC, thin layer chromatography; CCh, carbamoylcholine; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; 2,3-DPG, 2,3-diphosphoglyceric acid.

concentrations of Ca^{2+} are required for the phospholipase C-mediated hydrolysis of PI than for that of PIP_2 (7). In contrast, from studies of the kinetics of inositol phosphate isomer metabolism in intact cells, it has been concluded that the continuous breakdown of PIP_2 alone is sufficient to account for inositol phosphate formation and that the observed IP_1 and IP_2 reflect the sequential dephosphorylation of IP_3 (and IP_4), rather than the hydrolysis of PI and PIP (8–10). In this context, it is noteworthy that in relatively few studies have the isomers of IP_1 (the major inositol phosphate formed) been routinely separated. Because $I(4)P_1$ can result only from PIP or PIP_2 breakdown and, furthermore, because $I(1)P_1$ can be derived from PI, this information is key to the identification of those lipids that undergo hydrolysis following receptor activation and can, thereby, provide insight into the relative contributions made by the IP_3 and diacylglycerol limbs of this signal transduction pathway. Furthermore, analysis of the onset and duration of the breakdown of individual inositol lipids following receptor activation may be germane to assessment of the rate at which stimulated PPI turnover desensitizes.

Human SK-N-SH neuroblastoma cells possess an abundance of mAChRs that are linked to PPI hydrolysis and Ca^{2+} mobilization (11, 12). Agonist occupancy of these receptors results in a rapid appearance of IP_1, IP_2, and IP_3 within 5–10 sec of agonist addition (12). In more prolonged incubations, the predominant inositol phosphate recovered is IP_1, which, in the presence of Li^+, accumulates continuously for at least 60 min with no indication of receptor desensitization (13). Whereas the increase in IP_3 is presumed to occur via the hydrolysis of PIP_2, the route of IP_2 and IP_1 formation has not been established for this cell line. In the present study, we have analyzed the inositol phosphate isomers produced following prolonged mAChR activation of intact and digitonin-permeabilized SK-N-SH cells to identify the lipid source of IP_1 and IP_2. The results obtained indicate that, under conditions in which PPI hydrolysis is sustained, the majority of inositol phosphates produced upon receptor activation are derived from the continuous breakdown of PIP_2 and/or PIP rather than PI.

Materials and Methods

myo-[2-^{3}H]Inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). [2-^{3}H](1)P_1 (5 Ci/mmol), [2-^{3}H]I(4)P_1 (4.5 Ci/mmol), [2-^{3}H]I(1,4)P_2 (4.5 Ci/mmol), [1-^{3}H]I(1,4,5)P_3 (20 Ci/mol), [1-^{3}H]I(1,3,4)P_3 (17 Ci/mmol), [2-^{3}H]I(1,3,4,5)P_4 (5 Ci/mmol), and [4,5-^{32}P]I(1,4,5)P_3 were obtained from New England Nuclear (Boston, MA). Unlabeled I(1,4,5)P_3 and I(1,3,4,5)P_4 were purchased from Calbiochem (San Diego, CA). GTPγS was obtained from Boehringer-Mannheim (Indianapolis, IN). CCh, I(1,4)P_2, digitonin, 2,3-DPG, and ATP were obtained from Sigma Chemical Co. (St. Louis, MO). Phytic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI) and a partial hydrolysate was prepared as previously described (14). Tissue culture supplies were obtained from Corning Glass Works (Corning, NY). Powdered Dulbecco's modified Eagle's medium and fetal calf serum were purchased from GIBCO (Grand Island, NY). Dowex-1 (100–200 mesh, ×8 in the formate form) was obtained from Bio-Rad (Rockville Center, NY). Silica gel G TLC plates (0.25 mm) were purchased from E. Merck (Darmstadt, West Germany). Supelclean LC-NH_2 columns were obtained from Supelco (Bellafonte, PA). The source of SK-N-SH cells was as previously described (11).

Culture and Characteristics of SK-N-SH Cells

Human SK-N-SH neuroblastoma cells were cultured under conditions that have been previously described (11). In the majority of experiments, cells that were 10–14 days post-passage were utilized. Cells were removed from the tissue culture dishes by the addition of Puck's D_1 solution (15), collected by centrifugation (300 × *g* for 1 min), and resuspended in buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM D-glucose, and 30 mM Na^+ HEPES buffer, pH 7.4). Although the SK-N-SH cell line has been reported to exhibit both neuroblast and epithelial phenotypes (16), under the culture conditions employed in this study, i.e., relatively prolonged culture at low initial subculture densities, only the neuroblast phenotype is found (17). We (18, 19) and others (20) have previously determined that the pharmacological and biochemical characteristics of the mAChR on SK-N-SH cells are identical to those of the receptor on the neuroblast subclone (SH-SY-5Y). Furthermore, the epithelial phenotype does not express mAChRs (17, 20).

Measurement of Inositol Phosphate Isomer Formation

Intact cells. SK-N-SH cells were allowed to prelabel for 3 days in Dulbecco's modified Eagle's medium/10% fetal calf serum containing 5 mCi/ml [^{3}H]inositol, in an atmosphere of 90% air/10% CO_2. Cells were detached, washed twice in buffer A, resuspended in buffer A, and incubated for the times indicated, in the presence or absence of 10 mM CCh. This concentration of agonist is sufficient to occupy >95% of the available surface mAChRs on these cells (11, 21). For rapid time-course experiments, reactions were terminated by the addition of an equal volume (0.5 ml) of 20% ice-cold TCA, and the TCA extract was washed with five 2-ml aliquots of H_2O-saturated diethyl ether and neutralized by the dropwise addition of 1 M $NaHCO_3$. Removal of TCA was accomplished within 1–2 hr of termination of the reactions, to minimize the possibility of phosphate migration (22). For experiments in which the separations of $I(1)P_1$/$I(3)P_1$ (an enantiomeric mixture) and $I(4)P_1$ were required, an alternative procedure was employed to terminate the reactions and, thereby, minimize the interference of salts in the subsequent HPLC step. For these experiments, reactions were terminated by the addition of 2 ml of ice-cold 0.9% NaCl (23), the cells were centrifuged for 3 min at 500 × *g*, and the supernatants were removed by aspiration. To cell pellets was added 0.5 ml of 5% TCA, and tubes were left on ice for 20 min to allow precipitation of protein. After centrifugation, the TCA extracts were processed as described previously. The neutralized extracts were filtered through a 0.45-μm filter (Micro Prep-Disc, Bio-Rad) and applied to a Whatman Partisil 10 SAX column fitted with a guard column (IC-PAK; Waters). Inositol phosphates were separated by elution with ammonium phosphate (pH 3.8) at a flow rate of 1 ml/min (23). For experiments in which optimum separation of the higher inositol phosphates was required, the following gradient was employed: 0.01 to 0.04 M for 30 min, then 0.54 to 0.57 M for 30 min, and a final elution with 1.0 M ammonium phosphate for 30 min. Under these conditions, I(1,4,5)P_3 eluted at least 4–5 min after I(1,3,4)P_3 and 10–15 min before IP_4 (isomers not identified). In experiments in which the IP_3 isomers and IP_4 were examined, 100 μg of phytate hydrolysate were included to optimize their recovery during the HPLC step, as previously recommended (24). When separation of the IP_1 isomers was the primary objective, an abbreviated protocol was employed in which the intermediate gradient step was omitted. $I(1)P_1$/$I(3)P_1$ eluted 3 min before $I(4)P_1$ (or its enantiomer, $I(6)P_1$). $I(2)P_1$, which was not routinely detected in our studies, elutes between these two major IP_1 fractions. In some experiments, IP_2 isomers were separated by means of the following gradient: 0.01 to 0.07 M ammonium phosphate for 25 min, then 0.26 to 0.61 M for 40 min, and a final elution with 1.0 M ammonium phosphate for 25 min. Under these conditions, the IP_2 fraction could be resolved into three isomeric species. The major species coeluted with authentic I(1,4)P_2. One minor peak of radioactivity eluted 2 min before the I(1,4)P_2 peak, and one 2–3 min after the major peak. These IP_2 isomers were tentatively identified as I(1,3)P_2 and I(3,4)P_2, respectively, based upon known HPLC elution profiles (25). Radioactivity was routinely monitored on-line by means of a Beckman System Gold 171 flow counter, with an efficiency of 12–16% when ACS scintillation fluid was employed or 20–30% with Beckman

Ready Flow III scintillation fluid (ratio of scintillant to eluate in each case, 4:1). Alternatively, fractions were collected and radioactivity was determined directly by liquid scintillation counting. Inositol phosphates were identified from the elution times of labeled inositol phosphate standards, i.e., I(1)P_1, I(4)P_1, I(1,4)P_2, I(1,3,4)P_3, I(1,4,5)P_3, and I(1,3,4,5)P_4. It should be cautioned, however, that the cochromatography of radiolabeled inositol phosphates isolated from cell extracts with authentic standards does not provide an unequivocal assignment of their structures, without further analysis (e.g., periodate oxidation). Recoveries of added inositol phosphates were typically >80%. When the metabolism of IP_5 and IP_6 was studied, these inositol phosphates were separated by high voltage electrophoresis (26).

Permeabilized cells. SK-N-SH cells were allowed to prelabel with [^{3}H]inositol for 3 days, detached from culture dishes, and permeabilized in KGEH buffer (139 mM K^+ glutamate, 2 mM ATP, 4 mM $MgCl_2$, 10 mM LiCl, 10 mM EGTA, and 30 mM Na^+ HEPES buffer, pH 7.4) containing 20 μM digitonin, as previously described (12). Permeabilized cells were centrifuged (300 × g, 1 min), washed in an equal volume of KGEH buffer (without digitonin), and then resuspended in the same buffer at a protein concentration of approximately 10 mg/ml. Reactions were allowed to continue for the times indicated at 37° in the presence or absence of 10 mM CCh plus 50 μM GTPγS, conditions previously determined to be optimal (12). The free [Ca^{2+}] was routinely maintained at 60 nM by the addition of $CaCl_2$. Reactions were terminated by rapid cooling to 0° followed by centrifugation at 500 × g for 5 min. Supernatants were collected and an equal volume of 10% TCA was added. Labeled inositol phosphates were isolated from the TCA supernatants as previously described for intact cells and extracts were neutralized with $NaHCO_3$. Before HPLC analysis, the water-soluble inositol phosphate fraction was applied to Supelclean LC-NH_2 columns for desalting (27), free [^{3}H]inositol was removed by washing of the columns with 5 × 1 ml of H_2O, and inositol phosphates were eluted with 1 ml of 1 M NH_4OH. After vacuum centrifugation, samples were resuspended in 1 ml of H_2O and analyzed by HPLC. In some experiments, inositol phosphates were fractionated by anion exchange chromatography, (12, 28).

Metabolism of [^{3}H]Inositol Phosphates in Homogenates of SK-N-SH Cells

Cells were homogenized in KGEH buffer (approximately 10 mg of protein/ml) with [Ca^{2+}] maintained at 60 nM. Aliquots of tissue homogenates (~500 μg of protein) were then incubated in the same buffer for the times indicated in the presence of either 10 μM [^{3}H]I(1,4,5)P_3, 10 μM [^{3}H]I(1,3,4,5)P_4, or [^{3}H]I(1,4)P_2 at concentrations of 10 or 100 μM. Reactions were terminated by the addition of an equal volume of 20% TCA, 100 μg of phytate hydrolysate were added, and samples were processed as described for permeabilized cells. Labeled inositol phosphates were then separated by HPLC.

Measurement of I(1,4,5)P_3 3′-Kinase and I(1,4,5)P_3 5′-Phosphatase Activities

Intact or permeabilized SK-N-SH cells were first homogenized in ice-cold 20 mM HEPES buffer (pH 7.0) containing 1 mM EGTA, 0.5 mM $CaCl_2$, and 10 mM LiCl. Enzyme assays were performed as previously described (14), using whole homogenates in a total volume of 200 μl with 10 μM [^{32}P]I(1,4,5)P_3 (50,000 cpm/assay) as substrate. For the 5′-phosphatase assays, the reaction mixture contained 4 mM $MgCl_2$, whereas that for the 3′-kinase contained 10 mM $MgCl_2$, 5 mM ATP, and 5 mM 2,3-DPG [an inhibitor of 5′-phosphatase activity (29)]. Reactions were initiated by the addition of substrate to prewarmed tissue (200–500 μg of protein) and allowed to proceed for 2 or 5 min. Reactions were terminated by the addition of chloroform/methanol, and inositol phosphates were extracted, desalted, and separated by high voltage electrophoresis (14). Electrophoretograms were exposed to X-ray film overnight, spots corresponding to IP_3, IP_4, and P_i were cut out, and radioactivity was determined by liquid scintillation counting. IP_3 5′-phosphatase activity was calculated as the loss of [^{32}P]I(1,4,5)P_3 radiolabel, whereas IP_3 3′-kinase activity was calculated from the amount of [^{32}P]IP_4 formed.

Analysis of [^{3}H]PPI

[^{3}H]PPI present in TCA precipitates were extracted following the addition of 1.5 ml of chloroform/methanol (1:2, by volume) and 0.5 ml of H_2O (30). Inositol lipids were separated on oxalate-impregnated TLC plates using the solvent system chloroform/methanol/acetone/acetic acid/water (40:15:15:12:8, by volume) (31). This system permits the one-dimensional separation of PI, PIP, and PIP_2. TLC plates were sprayed with Enhance and lipids were located by means of autoradiography at −70° for 2–3 weeks. Areas of the TLC plates corresponding to the individual lipids were scraped into a vial, 2 ml of 2% Triton X-100 were added, and vials were sonicated. Radioactivity was determined after the addition of 10 ml of Universol scintillation fluid. Protein was determined by the method of Geiger and Bessman (32).

Data Analysis

Values quoted are means ± standard errors for the numbers of separate experiments performed. Student's two-tailed t tests were used to evaluate the statistical differences of the means of unpaired sets of data.

Results

CCh-stimulated PPI breakdown in intact cells. In preliminary experiments, it was determined that the maximum specific activity of the inositol lipid fraction in SK-N-SH cells was attained after 2 days of prelabeling with [^{3}H]inositol and, thereafter, remained constant. A 3-day prelabeling period was routinely employed and, under these conditions, the relative proportions of ^{3}H label recovered in PIP_2, PIP, and PI were 5.3 ± 0.4, 14.2 ± 4.2, and 80.5 ± 4.5% of total, respectively (nine experiments). The addition of CCh to prelabeled cells resulted in a rapid reduction of PIP_2 radiolabel such that 24 ± 5% of that present initially was lost within 5 sec of agonist addition and 43 ± 6% after 60 sec (three experiments) (Fig. 1). Little further reduction of [^{3}H]PIP_2 labeling occurred thereafter. Whereas the addition of CCh also resulted in a 15–20% reduction in [^{3}H]PIP label and a 10% loss of [^{3}H]PI, these reductions occurred more slowly than those observed for [^{3}H]PIP_2. Reductions in chemical masses of PIP_2, PIP, and PI also were observed following CCh addition, similar in extent to changes in radiolabeling.[1] In the absence of CCh, label was lost from PIP_2 in prolonged incubations (15–45 min), whereas little or no loss of radiolabel from either PIP or PI was observed (Fig. 1).

Kinetics of inositol phosphate formation in intact SK-N-SH cells following CCh addition. Because it has been reported that Li^+ can influence the stimulated production of I(1,4,5)P_3 and IP_4 in rat brain (33), studies of the rapid release of inositol phosphate isomers following the activation of mAChRs on SK-N-SH cells were conducted in its absence. Upon the addition of CCh to prelabeled cells, an increase in [^{3}H]I(1,4,5)P_3 was detected within 5–10 sec of agonist addition and its enhanced release was sustained throughout the first 5 min of incubation (Fig. 2). A similar sustained production of [^{3}H]I(1,3,4)P_3 was also apparent following CCh addition, although I(1,3,4)P_3 was formed more slowly than I(1,4,5)P_3. Relatively small increases in the labeling of IP_4, the precursor of I(1,3,4)P_3, were observed upon CCh addition, indicating that IP_4 is rapidly metabolized in SK-N-SH cells. Agonist-induced

[1] C-H. Lee, S. K. Fisher, B. W. Agranoff, and A. K. Hajra, unpublished results.

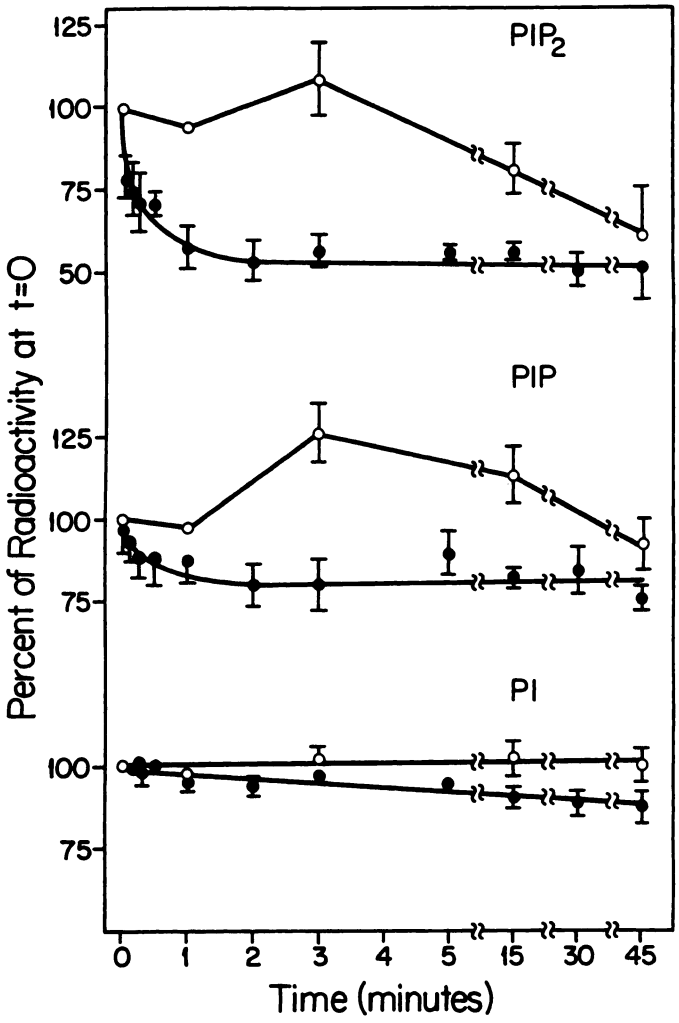

Fig. 1. Time-course of CCh-stimulated inositol lipid breakdown. Prelabeled SK-N-SH cells (approximately 2–3 mg of protein) were incubated for the times indicated at 37° in the absence (○) or presence (●) of CCh. Reactions were terminated by the addition of an equal volume of 20% TCA, and phospholipids were extracted and then separated by TLC. Lipids were quantitated following their localization by autoradiography. Results are expressed as percentage of radioactivity present at zero time. Values shown are the mean ± standard errors for three to five separate experiments. Where *error bars* are not shown, the standard error fell within the *symbol*. Incorporation of [^{3}H]inositol into lipid was approximately 300,000 dpm/mg of protein.

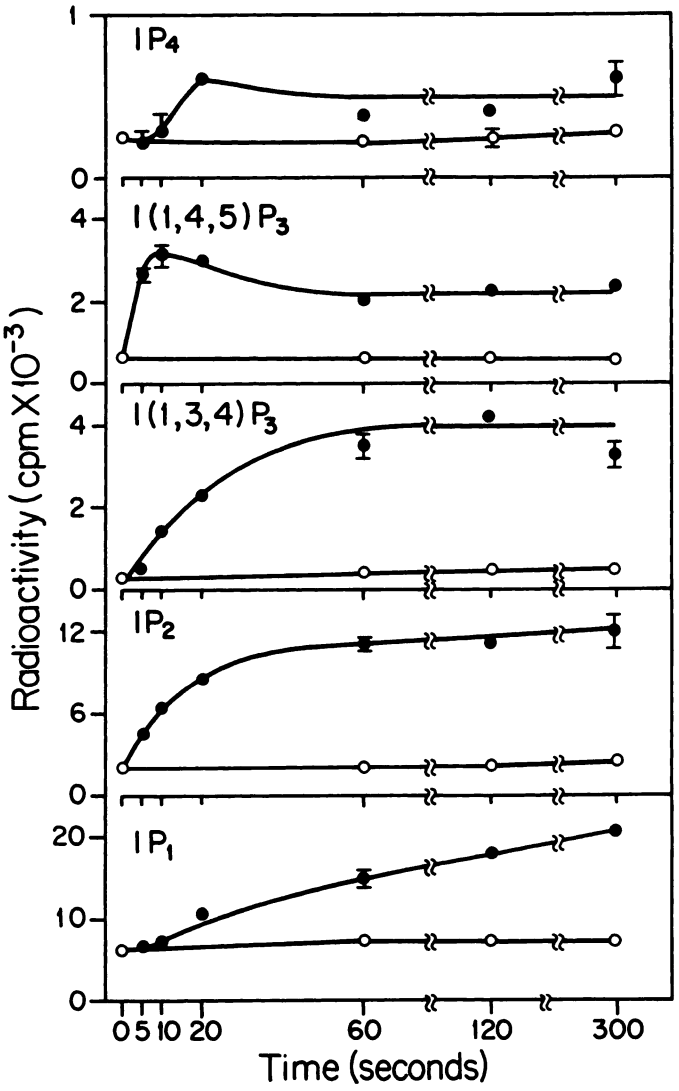

Fig. 2. Time-course of inositol phosphate formation in CCh-stimulated intact SK-N-SH neuroblastoma. Prelabeled cells (6 mg of protein) were incubated for the times indicated in the absence (○) or presence (●) of CCh, in the absence of Li^+. Reactions were terminated by the addition of an equal volume of 20% TCA and labeled inositol phosphates present in neutralized TCA extracts were separated and analyzed by HPLC on a Partisil 10 SAX column. Results shown are means ± standard errors for triplicate replicates obtained in a single experiment. Where *error bars* are not shown, the standard error fell within the *symbol*. Similar results were obtained in two further experiments.

increases in [^{3}H]IP_2 were consistently detected even at the shortest assay intervals (5 sec), at which time label in IP_2 typically exceeded that of $I(1,4,5)P_3$ by 2–4-fold. In the absence of Li^+, steady state levels of [^{3}H]IP_2 formation were achieved within 60 sec of CCh addition. IP_1 also accumulated in CCh-stimulated cells and steadily increased between 10 and 300 sec, even in the absence of Li^+ (Fig. 2). In some experiments, we examined the possibility that receptor-mediated changes in the metabolism of IP_5 and IP_6 also occurred in SK-N-SH cells. Although not extensively studied, no consistent change in the labeling of these inositol phosphates was observed upon the addition of CCh.

In more prolonged incubations, CCh (in the presence of 30 mM Li^+) elicited a large and sustained increase in all of the inositol phosphates. Even though the inositol polyphosphates constituted less than 3% of the total formed, substantial increases in the labeling of $I(1,3,4)P_3$, $I(1,4,5)P_3$, and $I(1,3,4,5)P_4$ were observed in the presence of CCh in incubations up to 45 min (Table 1). [^{3}H]IP_2 accumulated linearly for at least 30 min and thereafter at a slower rate, indicating that IP_2 degradation in SK-N-SH cells is inhibited by Li^+ (Fig. 3). Between 45 and 90 min of incubation, [^{3}H]IP_2 remained relatively constant and did not decline (data not shown). The major isomer formed (>85% of total) was $I(1,4)P_2$, and relatively little accumulation of either $I(1,3)P_2$ or $I(3,4)P_2$ was observed at any time period examined. The production of $I(4)P_1$, a marker of polyphosphoinositide hydrolysis (34), was linear over the entire 45-min incubation period and further increases occurred for up to 90 min of incubation (data not shown). The time course for appearance of $I(1)P_1/I(3)P_1$ differed from that of $I(4)P_1$ in that little increase was detected within the first 1–3 min, but thereafter the rate of accumulation progressively increased, such

TABLE 1

CCh stimulation of IP_3 and IP_4 upon prolonged incubation of intact cells

[^{3}H]Inositol-prelabeled cells (3–6 mg of protein) were incubated for the times indicated in the absence or presence of CCh. Li^+ was present in the assays at a concentration of 30 mM. Reactions were terminated by the addition of 20% TCA and inositol phosphate isomers present in neutralized TCA extracts were separated by HPLC. Results are expressed as the release of the inositol phosphate relative to control incubations and are derived from the means of triplicate replicates for CCh-stimulated cells and duplicate replicates for control incubations. Values shown are the means ± standard errors for three separate experiments.

Time	I(1,3,4)P_3	I(1,4,5)P_3	IP_4
min		*% of control*	
5	1063 ± 328	289 ± 104	200 ± 32
15	902 ± 252	284 ± 71	264 ± 34
45	680 ± 163	297 ± 48	292 ± 18

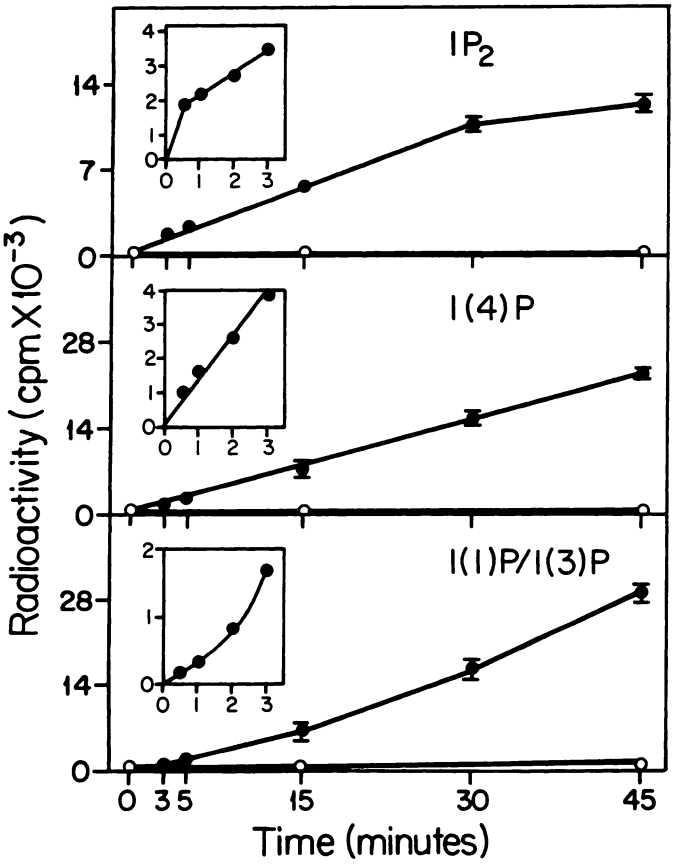

Fig. 3. CCh-stimulated formation of IP_2 and IP_1 isomers in prolonged incubations of intact cells. Prelabeled cells (2 mg of protein) were incubated for the times indicated in the absence (○) or presence (●) of CCh. Li^+ was present in the assays at a concentration of 30 mM. Reactions were terminated by the addition of ice-cold 0.9% NaCl, cells were centrifuged, and supernatants were removed by aspiration. Inositol phosphates were extracted from the cell pellets following the addition of 5% TCA and isomers present in the neutralized TCA extracts were separated by HPLC. Results shown are means ± standard errors for triplicate replicates. Where *error bars* are not shown, the standard error fell within the *symbol*. Similar results were obtained in three further experiments. *Insets*, CCh-stimulated release of IP_2, I(4)P_1, and I(1)P_1/I(3)P_1 in the first 3 min of incubation.

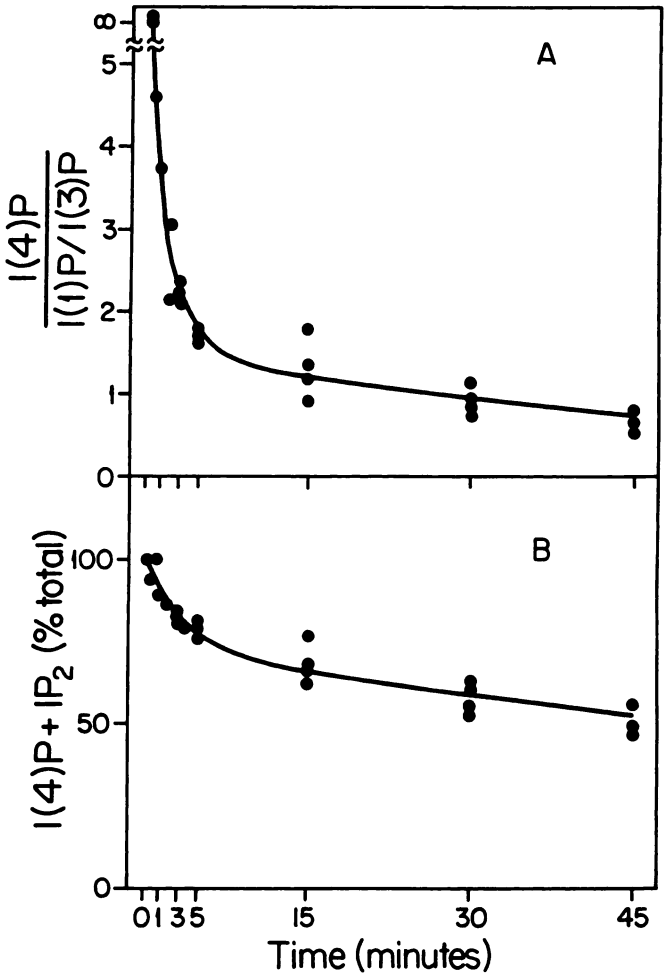

Fig. 4. The proportions of IP_1 isomers formed upon CCh addition differ as a function of assay time. A, Ratio of CCh-stimulated I(4)P_1 to I(1)P_1/I(3)P_1 formation as a function of time. B, The minimum proportion of radiolabeled inositol phosphates derived from polyphosphoinositide breakdown, i.e., I(4)P_1 plus IP_2/total inositol phosphates, as a function of time. Each point represents the result obtained in a separate experiment.

that after 45 min of incubation label recovered in the I(1)P_1/I(3)P_1 fraction was comparable to that in I(4)P_1. The ratio of I(4)P_1 to I(1)P_1/I(3)P_1 formed upon CCh addition as a function of time is shown in Fig. 4A. At short time intervals (<5 min) relatively more I(4)P_1 is produced, whereas after 45 min of incubation the ratio of I(4)P_1 to I(1)P_1/I(3)P_1 produced is close to unity. The minimum contribution of polyphosphoinositides to total inositol phosphate production can be calculated from the sum of radioactivities associated with I(4)P_1 and IP_2 [ignoring a quantitatively minor contribution from inositol polyphosphates, i.e., I(1,4,5)P_3, I(1,3,4)P_3, and IP_4]. Such calculations indicate that the hydrolysis of polyphosphoinositides accounts for at least 81 ± 1, 68 ± 4, 60 ± 2, and 54 ± 4% of the inositol phosphates released following 3, 15, 30, and 45 min of CCh stimulation, respectively (three or four experiments) (Fig. 4B).

Calcium dependence of IP_1 isomer formation. In SK-N-SH cells, mAChR activation results in a continuous influx of Ca^{2+}, which is prevented by inclusion of EGTA (12). Because it has been proposed that PI hydrolysis may be secondary to a sustained increase in the concentration of cytoplasmic Ca^{2+} (6), we determined whether the availability of extracellular Ca^{2+} influences the ratio of IP_1 isomers produced upon receptor activation. A Ca^{2+}-dependent breakdown of PI would be reflected by a larger production of [^{3}H]I(1)P_1. Although reduction of extracellular Ca^{2+} to 0.2–0.75 μM substantially reduced the extent of stimulated inositol phosphate production, as previ-

ously demonstrated (12), the ratio of IP_1 isomers produced upon CCh stimulation was not appreciably altered (Table 2).

Kinetics of inositol phosphate isomer degradation. To determine the rates at which the inositol phosphate isomers undergo degradation, prelabeled cells were first incubated in the presence of CCh for 15 min (to attain a steady state stimulation) and then the loss of radiolabel from each inositol phosphate fraction was monitored following the addition of 50 μM atropine to terminate agonist action. Radiolabel was rapidly lost from I(1,4,5)P_3 ($t_{½}$ ~ 9 sec), whereas a 12-fold slower decline in radioactivity present in the I(1)P_1/I(3)P_1 fraction was observed ($t_{½}$ ~ 119 sec) (Fig. 5). The other inositol phosphates, i.e., I(1,3,4)P_3, IP_2, and I(4)P_1, were degraded with $t_{½}$ values of approximately 23, 46, and 40 sec, respectively.

CCh-stimulated PPI turnover in digitonin-permeabilized cells. We have previously shown that muscarinic receptor-effector coupling, as determined from the stimulated release of IP_1, IP_2, and IP_3, is fully retained in permeabilized cells (12). Following permeabilization of [^{3}H]inositol-labeled SK-N-SH cells, the proportion of label associated with PIP_2, PIP, and PI was 2.8 ± 0.2, 9.3 ± 0.5, and 87.9 ± 0.6% of total recovered, respectively (10 experiments). The amount of radiolabel in PIP_2 (but not that in either PIP or PI) was significantly lower in permeabilized cells than in intact cells ($p < 0.05$), which may reflect the rapid turnover of this lipid even in quiescent cells. Addition of CCh resulted in a significant breakdown of prelabeled PIP_2 and PIP within 2–5 min, whereas in more prolonged incubations a decrease in labeled PI was also observed (Table 3). The time-course of inositol phosphate isomer formation in permeabilized cells differed from that in intact cells in two respects. First, whereas CCh addition resulted in an increase in [^{3}H]IP_2 formation, this was maximal between 5 and 15 min and declined thereafter, even in the presence of Li^+ (Fig. 6). Second, of the two IP_1 isomers formed, label in I(4)P_1 exceeded that in I(1)P_1/I(3)P_1 at all assay times, and (unlike that in intact cells) the ratio of I(4)P_1 to I(1)P_1/I(3)P_1 formed upon mAChR activation remained relatively constant during extended assay periods (4.8 ± 1.1, 3.4 ± 0.7, 4.9 ± 2.0, and 5.2 ± 1.4 at 5, 15, 30, and 45 min, respectively; three to five experiments). At these assay times, the minimum contribution of polyphosphoinositides to inositol phosphate formation was calculated to be 92 ± 3, 84 ± 3, 83 ± 5, and 83 ± 4% of total recovered, respectively (three to five experiments). Raising the [Ca^{2+}] from 60 to 300 nM (the peak concentration of Ca^{2+} observed following mAChR activation in the SK-N-SH cell; see Ref. 12) did not influence the ratio of IP_1 isomers formed (data not shown). Further evidence for the importance of polyphosphoinositides to mAChR-stimulated inositol phosphate release was obtained from experiments in which ATP was omitted (Fig. 7). Under these conditions, the CCh-stimulated release of [^{3}H]inositol phosphates was significantly im-

TABLE 2

Effect of calcium deprivation on CCh-stimulated IP_1 isomer formation in intact SK-N-SH cells

[^{3}H]Inositol-prelabeled cells (1.5 and 1.7 mg of protein in Experiments 1 and 2, respectively) were incubated for 30 min in buffer A containing either (a) 2.2 mM $CaCl_2$/4 mM EGTA, (b) no added $CaCl_2$, or (c) 2.2 mM $CaCl_2$, in the presence or absence of CCh. Reactions were terminated by the addition of ice-cold 0.9% NaCl, the cells were centrifuged, and supernatants were removed by aspiration. Inositol phosphates were extracted from the cell pellets following the addition of 5% TCA and isomers present in neutralized TCA extracts were separated by HPLC. Results (obtained from triplicate replicates) are expressed either as the ratio of the CCh-induced increases in the radiolabeled I(4)P_1 and I(1)P_1/I(3)P_1 fractions or as percentage of total inositol phosphate release obtained in the presence of 2.2 mM $CaCl_2$. The free extracellular [Ca^{2+}] present under conditions a and b were 0.2 and 0.75 μM as determined directly by fura-2 (free acid) fluorescence (12).

	(a) Ca^{2+}/EGTA		(b) No added Ca^{2+}		(c) 2.2 mM Ca^{2+}	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
I(4)P_1:I(1)P_1/I(3)P_1	1.08	1.04	1.06	1.00	0.75	0.72
Total inositol phosphates (%)	21	35	46	42	100	100

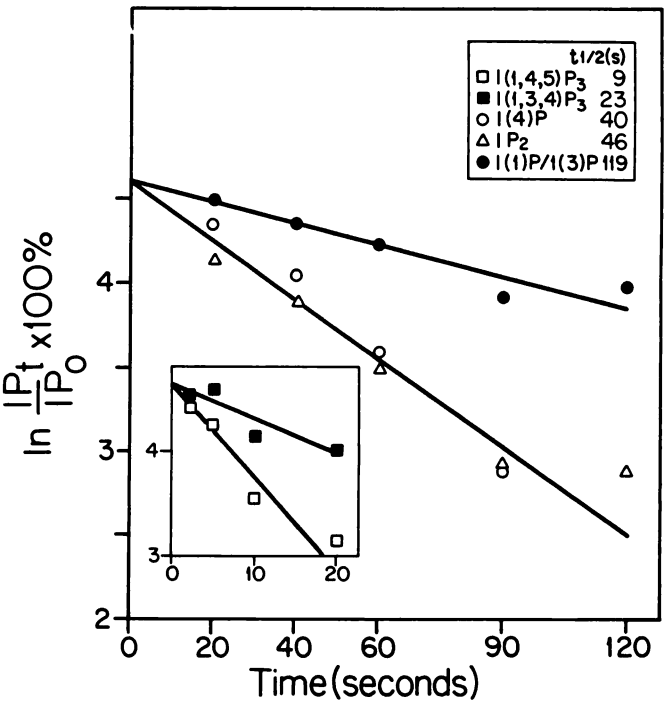

Fig. 5. Time-course of inositol phosphate isomer degradation following muscarinic receptor blockade. Prelabeled SK-N-SH cells (6 mg of protein) were incubated with either buffer A or CCh for 15 min. Atropine was then added and reactions were terminated at the times indicated either by the addition of TCA [for analysis of I(1,4,5)P_3 and I(1,3,4)P_3] or by the addition of ice-cold 0.9% NaCl [for analysis of IP_2, I(4)P_1, and I(1)P_1/I(3)P_1]. In the experiment shown, radioactivities associated with I(1,4,5)P_3, I(1,3,4)P_3, IP_2, I(4)P_1, and I(1)P_1/I(3)P_1 at time zero were 2,100, 1,800, 17,000, 6,300, and 16,000 cpm, respectively. The decay in the radioactivity associated with the inositol phosphates is plotted as ln $IP_t/IP_0 \times 100\%$ versus time, where IP_t and IP_0 are radioactivities associated with each isomer at times t or zero, respectively. The $t_{½}$ values are calculated as 0.693/k, where k = rate constant (slope), as determined by linear regression analysis. For the sake of clarity, a single line is drawn for the I(4)P_1 and IP_2 data.

TABLE 3

Time dependence of CCh-stimulated inositol lipid breakdown in permeabilized SK-N-SH cells

Prelabeled cells were incubated for the times indicated at 37° in the absence or presence of CCh. Reactions were terminated by rapid cooling to 0°, cells were centrifuged, supernatants were aspirated, and 0.5 ml of 20% TCA was added to the cell pellets. Lipids were extracted, separated, and quantitated as described in Materials and Methods. Results are expressed as percentage of radioactivity present in each lipid at zero time. Values shown are the means ± standard errors for three to nine separate experiments.

Time	PIP_2		PIP		PI	
	Control	CCh	Control	CCh	Control	CCh
min	*% of radioactivity at t = 0*					
2	ND[a]	84 ± 1	ND	76 ± 8	ND	98 ± 2
5	109 ± 5	82 ± 5	93 ± 2	70 ± 3	106 ± 2	96 ± 2
15	102 ± 12	75 ± 10	88 ± 4	71 ± 5	101 ± 3	90 ± 3
30	ND	64 ± 12	ND	61 ± 7	ND	85 ± 3
45	78 ± 13	50 ± 9	77 ± 5	63 ± 4	105 ± 6	86 ± 4

[a] ND, not determined.

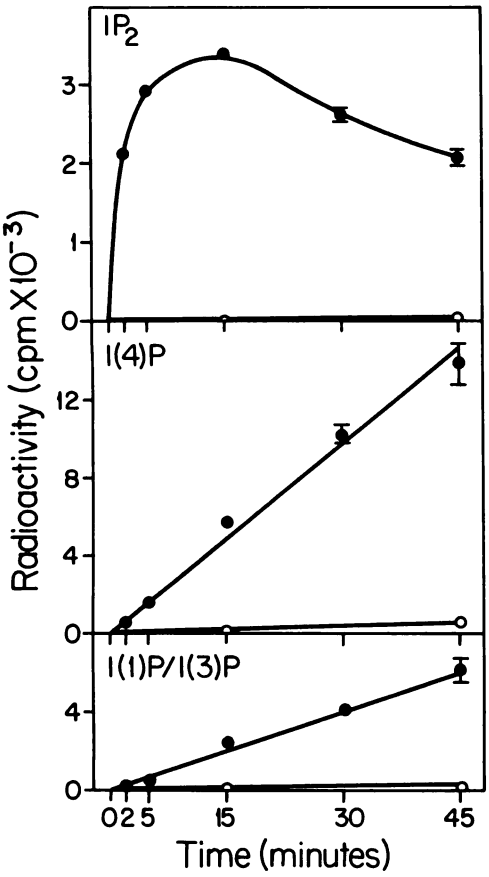

Fig. 6. Time-course of CCh-stimulated inositol phosphate formation in permeabilized SK-N-SH cells. Prelabeled cells (1 mg of protein) were incubated in KGEH for the times indicated in either the absence (○) or presence (●) of 10 mM CCh and 50 μM GTPγS. Reactions were terminated by rapid cooling to 0° followed by centrifugation. An equal volume of 10% TCA was added to the supernatants and inositol phosphates present in the neutralized TCA extracts were separated by HPLC. Results shown are means ± standard errors for triplicate replicates. Where *error bars* are not shown, the standard error fell within the *symbol*. Similar results were obtained in two additional experiments. Incorporation of [^{3}H]inositol into lipid was approximately 200,000 dpm/mg of protein.

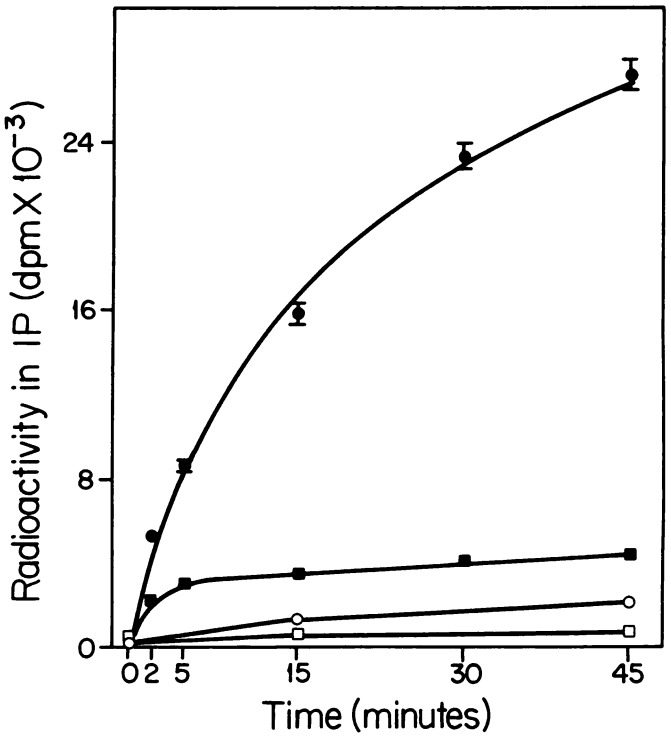

Fig. 7. CCh-stimulated inositol phosphate accumulation in permeabilized SK-N-SH cells requires the presence of ATP. Prelabeled cells were incubated for the times indicated in the absence (*open symbols*) or presence (*closed symbols*) of CCh and GTPγS with 2 mM ATP present (*circles*) or ATP omitted (*squares*). Results shown are means ± standard errors for triplicate replicates. Where *error bars* are not shown, the standard error fell within the *symbol*.

paired. In three separate experiments, omission of ATP inhibited CCh-stimulated [^{3}H]inositol phosphate formation by 76 ± 12 and 79 ± 3% at 5 and 30 min of incubation. In the absence of ATP, label in PIP and PIP_2 declined by 75 and 50% within 30 min, whereas label in PI was unaffected.

Because $I(4)P_1$ and IP_2 could hypothetically be derived from PIP_2 and/or PIP degradation, experiments were conducted in which CCh- plus GTPγS-stimulated inositol phosphate accumulation was monitored in the presence of increasing concentrations of unlabeled $I(1,4,5)P_3$ (Fig. 8). Preliminary experiments indicated that the addition of 100 μM $I(1,4,5)P_3$ completely blocked the metabolism of [^{32}P]$I(1,4,5)P_3$ when added to permeabilized cells. Although relatively little radioactivity was recovered in $I(1,4,5)P_3$ in permeabilized cells (thereby precluding its further study), inclusion of unlabeled $I(1,4,5)P_3$ enhanced the stimulated release of [^{3}H]IP_3 in a dose-dependent manner, with a maximum effect observed at a concentration of 1 mM (Fig. 8). In three separate experiments, inclusion of 1 mM $I(1,4,5)P_3$ increased IP_3 label to 320 ± 21% of control. Comparable reductions in radiolabel associated with the combined IP_1 and IP_2 fraction were observed. However, the latter amounted to <20% of the total stimulated [^{3}H]IP_1 and [^{3}H]IP_2 release. When permeabilized cells were first allowed to preincubate with 1 mM $I(1,4,5)P_3$ for 3 min at 37° before the addition of CCh and GTPγS, no additional increase in radiolabeled IP_3 was observed.

Metabolism of inositol phosphates by homogenates of SK-N-SH cells. When SK-N-SH cells were homogenized in KGEH and incubated with 10 μM [^{3}H]$I(1,3,4,5)P_4$, this compound was metabolized successively to $I(1,3,4)P_3$, IP_2, and $I(1)P_1/I(3)P_1$. No $I(4)P_1$ formation was detected (Fig. 9). In contrast, when homogenates were incubated with 10 or 100 μM [^{3}H]$I(1,4)P_2$ or, alternatively, 10 μM [^{3}H]$I(1,4,5)P_3$, the only IP_1 formed was $I(4)P_1$. Thus, radiolabel present in the $I(1)P_1/I(3)P_1$ fraction may be derived from breakdown of IP_4 formed previously from 3′-kinase action on $I(1,4,5)P_3$, whereas $I(4)P_1$ is formed solely from phosphatase breakdown of $I(1,4,5)P_3$ and/or $I(1,4)P_2$.

Measurement of IP_3 3′-kinase and IP_3 5′-phosphatase activities in intact and permeabilized cells. Because the 5′-phosphatase route of metabolism of $I(1,4,5)P_3$ appeared to predominate in permeabilized cells (see Fig. 6), the 3′-kinase (measured in the presence of 2,3-DPG) and 5′-phosphatase activities were determined in intact and permeabilized cells. Permeabilization resulted in a loss of IP_3 3′-kinase activity (63 ± 16% of control; four experiments), whereas an increase in the activity of IP_3 5′-phosphatase was detected (177 ± 39% of control; three experiments). Thus, following permeabilization, the relative enzymatic potential for the phosphatase route of $I(1,4,5)P_3$ degradation, already substantial, is further enhanced.

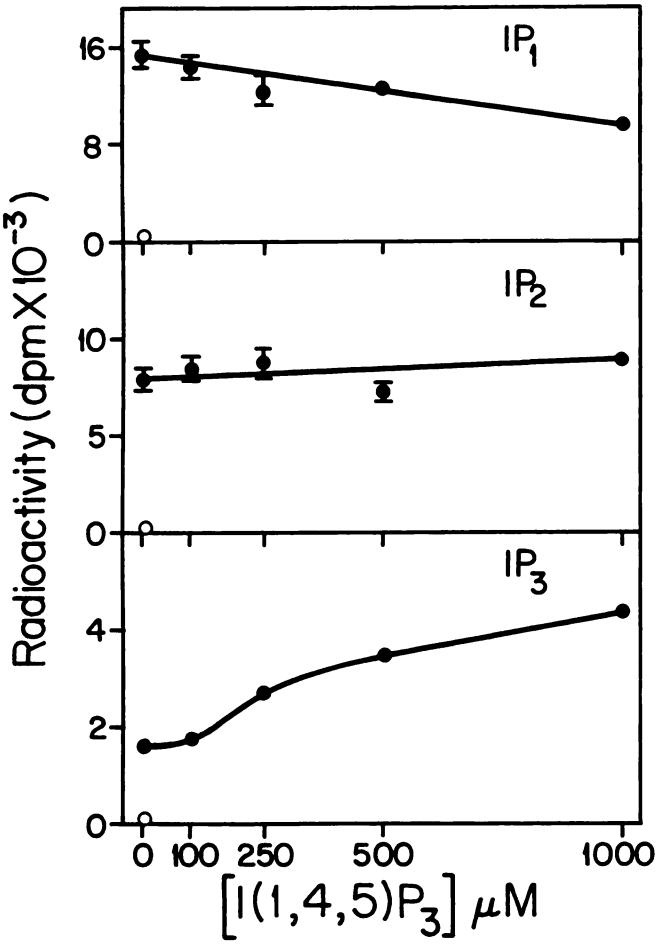

Fig. 8. Addition of exogenous I(1,4,5)P_3 to permeabilized cells increases the radiolabel recovered in the IP_3 fraction following mAChR activation. Prelabeled cells were incubated with CCh and GTPγS (●) for 5 min in the presence of I(1,4,5)P_3 at the concentrations indicated. Inositol phosphates were then fractionated by anion exchange chromatography. Results shown are means ± standard errors for triplicate replicates. Where *error bars* are not shown, the standard error fell within the *symbol*. Addition of I(1,4,5)P_3 to control incubations (○) was without effect.

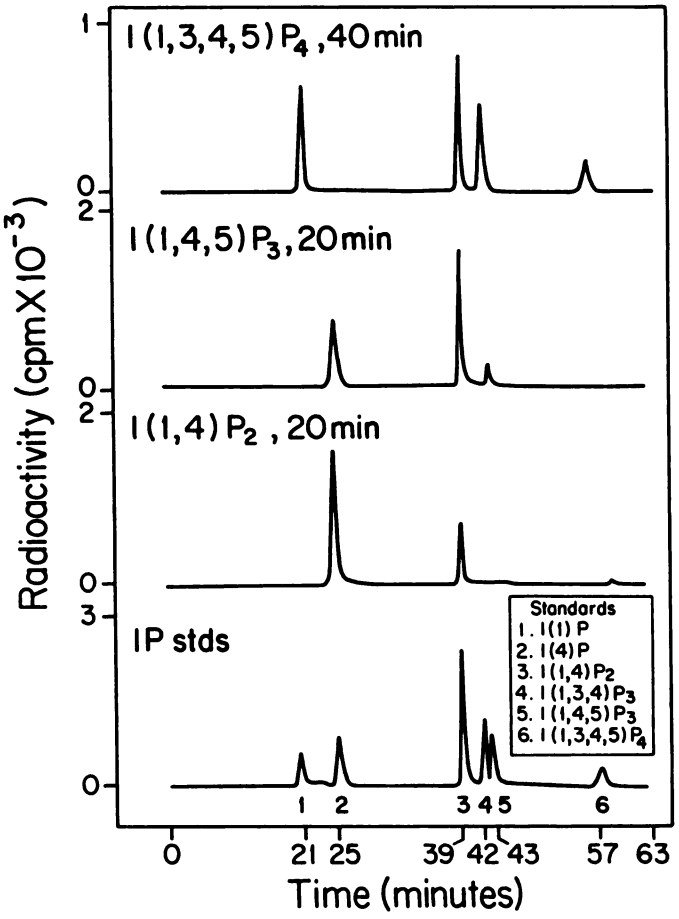

Fig. 9. Metabolism of labeled inositol phosphates by homogenates of SK-N-SH cells. Cell-free homogenates were prepared in KGEH buffer and approximately 500 μg of protein were incubated with the inositol phosphates, each at a concentration of 10 μM, for the times indicated.

Discussion

mAChR-stimulated PPI turnover in SK-N-SH cells, as monitored by the release of a total inositol phosphate fraction, is characterized by a relatively slow rate of desensitization (13). The results obtained in the present study indicate that a continuous breakdown of polyphosphoinositides, rather than of the quantitatively more abundant lipid PI, accompanies this prolonged receptor activation. Evidence in favor of a sustained hydrolysis of polyphosphoinositides can be summarized as follows. First, the addition of CCh to intact cells promotes a rapid and persistent breakdown of PIP_2 with the concomitant formation of radiolabeled IP_3 and IP_4, which is maintained for at least 45 min. Moreover, CCh addition to SK-N-SH cells results in a rapid increase in the mass of I(1,4,5)P_3 (from 20 pmol/mg of protein to 105 pmol/mg of protein within 5–10 sec, as measured by a radioreceptor assay), much of which is maintained in the continuous presence of the agonist (35). Second, in permeabilized cells omission of ATP (which prevents the phosphorylation of PI to PIP and PIP_2) results in a pronounced inhibition of CCh-stimulated [^{3}H]IP_1 production. Third, in both intact and permeabilized cells, a major metabolite, I(4)P_1, which can only be formed from PIP_2 or PIP breakdown, accumulates linearly in SK-N-SH cells, reflecting a continuous breakdown of the polyphosphoinositides. It is, nonetheless, true that in intact SK-N-SH cells, as has been previously observed for neutrophils (25) and for adrenal glomerulosa cells (36), an increasing proportion of radioactivity present in the IP_1 fraction at long incubation times is in I(1)P_1 and/or I(3)P_1. Although an explanation for the curvilinear nature of this accumulation is not yet apparent, it is probable that much of this radiolabel originates from the sequential dephosphorylation of I(1,3,4,5)P_4 formed from 3′-kinase action on I(1,4,5)P_3, because a 6-fold stimulation of I(1,3,4)P_3 formation was observed even after 45 min of agonist stimulation (Table 1). Because I(1,3,4)P_3 is known to undergo dephosphorylation to I(1,3)P_2 and I(3,4)P_2 and thereafter to I(3)P_1 and I(1)P_1 (37), a steady accumulation of I(1)P_1/I(3)P_1 radioactivity can be anticipated if the 3′-kinase pathway of I(1,4,5)P_3 metabolism is operating continuously and if the subsequent breakdown of IP_1 is prevented by inclusion of Li^+. In support of this possibility, SK-N-SH cell homogenates metabolized added [^{3}H]I(1,3,4,5)P_4 to [^{3}H]I(1)P_1/[^{3}H]I(3)P_1 but not to [^{3}H]I(4)P_1 (Fig. 9). Fourth, the possibility that a sustained influx of Ca^{2+} serves to initiate PI hydrolysis in SK-N-SH cells can be discounted, because the ratios of IP_1 isomers formed in the absence or presence of Ca^{2+} were similar (Table 2).

In a recent study, it was calculated that, following mAChR

activation of parotid gland cells, the metabolism of $I(1,4,5)P_3$ thus formed was sufficient to account for $I(1,4)P_2$ and $I(1,3,4)P_3$ formation and, moreover, for the accumulation of radiolabel in both $I(1)P_1/I(3)P_1$ and $I(4)P_1$ fractions (9). From these observations it was concluded that the breakdown of PIP_2 alone was sufficient to account for inositol phosphate formation in these cells. Comparable studies with SK-N-SH cells do not permit such an unequivocal conclusion because (a) the rates of degradation of all inositol phosphate isomers are relatively rapid in these cells, thereby precluding a ready assessment of flux rates because $t_{1/2}$ values for precursor and product inositol phosphates are not sufficiently dissimilar; see Fig. 5) and (b) quantitation of the rates at which the IP_1 isomers accumulate in the presence of Li^+ is complicated by the inhibitory influence of Li^+ on both $I(1,4)P_2$ and $I(1,3,4)P_3$ metabolism in SK-N-SH cells. Although quantitation of flux rates was not feasible in the present study, our results nonetheless suggest that, in SK-N-SH cells, the polyphosphoinositides undergo a continuous Ca^{2+}-dependent hydrolysis following mAChR activation and the rapid metabolism of the inositol polyphosphates thus formed constitutes the major (if not sole) source of inositol phosphates.

Although polyphosphoinositide breakdown is the primary event in both intact and digitonin-permeabilized cells, it is evident that the routes of metabolism of $I(1,4,5)P_3$ differ in the two preparations. In the intact cell, both the 5′-phosphatase and 3′-kinase pathways operate, whereas in permeabilized cells only the phosphatase route appears to be significant. In this context, a number of factors may account for these differential routes of metabolism. For example, approximately 30–40% of the 3′-kinase, a largely cytosolic enzyme (37), is lost upon permeabilization. Furthermore, the specific activity of the 5′-phosphatase, a membrane-bound enzyme (37), is enhanced upon permeabilization to an extent greater than that attributable to loss of cytosolic protein. This may reflect detergent activation of the 5′-phosphatase, as reported by Erneux *et al.* (38). In cell-free homogenates (as in permeabilized cells), the 3′-kinase activity is not readily detected and exogenous $I(1,4,5)P_3$ is metabolized exclusively to $I(1,4)P_2$ and $I(4)P_1$. However, in the presence of 2,3-DPG, an inhibitor of the 5′-phosphatase, 3′-kinase activity is readily detected. This raises the possibility that 2,3-DPG (or other unknown intracellular molecules whose effective concentrations are lowered upon either homogenization or cell permeabilization) may regulate the 5′-phosphatase activity *in vivo* and thereby redirect the metabolism of $I(1,4,5)P_3$. Although the use of permeabilized cell preparations provides an opportunity to manipulate the intracellular milieu and, thereby, probe underlying mechanisms of receptor-effector coupling, it is evident that in the SK-N-SH cell the routes of inositol phosphate metabolism are not identical to those found in intact cells. Notwithstanding this consideration, results from both intact and permeabilized cell preparations point to the underlying importance of polyphosphoinositide hydrolysis in mAChR-stimulated inositol phosphate formation.

There remains the possibility that both PIP_2 and PIP breakdown occur following mAChR activation, particularly for permeabilized SK-N-SH cells (and parotid gland cells; see Ref. 9) where the ratio of PIP_2 to PIP is significantly lower than in intact cells. In studies in which exogenous $I(1,4,5)P_3$ was added to permeabilized SK-N-SH cells to reduce the degradation of endogenously generated radiolabeled IP_3, a 2-fold increase in radioactivity was observed. However, this increase in IP_3 radioactivity corresponded to a loss of only 20% of the label found in the combined IP_1 and IP_2 fraction. It appears that exogenously added $I(1,4,5)P_3$ may not readily equilibrate with the endogenously generated pool, because relatively high concentrations of $I(1,4,5)P_3$ were required for a half-maximal effect (250 μM). In contrast, the addition of 100 μM $I(1,4,5)P_3$ fully blocked the degradation of exogenously added $[^{32}P]I(1,4,5)P_3$. The local concentrations of $I(1,4,5)P_3$ achieved following CCh addition may, therefore, be higher than anticipated. Exogenously added IP_3 also fails to completely block IP_2 and IP_1 generation in permeabilized RBL-2H3 leukemic cells (39), turkey erythrocytes (40), and parotid cells (9). These various results raise the possibility that the dephosphorylation of $I(1,4,5)P_3$ (and IP_4) may occur very rapidly and in a metabolic compartment to which exogenous IP_3 has only a limited access.

In summary, the present results indicate that in both intact and permeabilized SK-N-SH cells the polyphosphoinositides, rather than PI, are the preferred substrates for phospholipase C, even following prolonged mAChR activation. As predicted for other neural preparations in which mAChR-stimulated PPI turnover is also slow to desensitize (see Ref. 13 and references therein), a consequence of this sustained polyphosphoinositide breakdown in SK-N-SH cells is the continuous production of both intracellular second messengers, $I(1,4,5)P_3$ and diacylglycerol.[1] In contrast, events more distal to mAChR activation in neural tissues, such as the mobilization of intracellular Ca^{2+} (12, 41) and neuronal cell firing (42), rapidly desensitize upon the prolonged administration of muscarinic agonists.

Acknowledgments

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