Differential Regulation of Muscarinic Acetylcholine Receptor-sensitive Polyphosphoinositide Pools and Consequences for Signaling in Human Neuroblastoma Cells*

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In this study we have quantitatively assessed the basal turnover of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P) and M₃-muscarinic receptor-mediated changes in phosphoinositides in the human neuroblastoma cell line, SH-SYSY. We demonstrate that the polyphosphoinositides represent a minor fraction of the total cellular phosphoinositide pool and that in addition to rapid, sustained increases in [³H]inositol phosphates dependent upon extracellular receptor activation by carbachol, there are equally rapid and sustained reductions in the levels of polyphosphoinositides. Compared with phosphatidylinositol 4-phosphate (PtdIns(4)P), PtdIns(4,5)P₂ was reduced with less potency by carbachol and recovered faster following agonist removal suggesting protection of PtdIns(4,5)P₂ at the expense of PtdIns(4)P and indicating specific regulatory mechanisms(s). This does not involve a pertussis toxin-sensitive PtdIns(4)P and indicating specific regulatory mechanisms. The use of wortmannin also indicated that PtdIns(4)P is not a substrate for receptor-activated phospholipase C and that 15% of the basal level of PtdIns(4,5)P₂ is in an agonist-insensitive pool. We estimate that the agonist-sensitive pool of PtdIns(4,5)P₂ turns over every 5 s (0.23 fmol/cell/min) during sustained receptor activation by a maximally effective concentration of carbachol. Immediately following agonist addition, PtdIns(4,5)P₂ is consumed >3 times faster (0.76 fmol/cell/min) than during sustained receptor activation which represents, therefore, utilization by a partially desensitized receptor. These data indicate that resynthesis of PtdIns(4,5)P₂ is required to allow full early and sustained phases of receptor signaling. Despite the critical dependence of phosphoinositide and Ca²⁺ signaling on PtdIns(4,5)P₂ resynthesis, we find no evidence that this rate resynthesis is limiting for agonist-mediated responses.

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) is a minor membrane-associated phospholipid that is a substrate for enzymes involved in important cellular signal transduction pathways (1). Thus, PtdIns(4)P is a substrate for both phospholipase C (PLC) and phosphoinositide 3-kinase (PI3-K) activities. The importance of the signaling pathway initiated by PI3-K and resulting in the generation of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃) is rapidly emerging (2, 3). However, all estimates so far suggest that the PI3-K pathway utilizes only a small fraction of the PtdIns(4,5)P₂ pool compared with that hydrolyzed by PLC in a signaling cascade (4, 5). The latter enzyme liberates both inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and 1,2-diacylglycerol, which mobilize Ca²⁺ from intracellular stores and activate several isoforms of protein kinase C, respectively (6). Both Ins(1,4,5)P₃ and diacylglycerol are recycled to provide the substrates (myo-inositol and CMP-phosphatidate) necessary for phosphatidylinositol (PtdIns) resynthesis. PtdIns(4,5)P₂ can then be regenerated by the sequential phosphorylation of PtdIns and phosphatidylinositol 4-phosphate (PtdIns(4)P) by phosphatidylinositol 4-kinase (PtdIns 4-kinase) and phosphatidylinositol 4-phosphate 5-kinase (PtdIns(4)P 5-kinase), respectively (6). This resynthesis is vital in maintaining an agonist-sensitive PtdIns(4,5)P₂ pool, as the cellular content of PtdIns(4,5)P₂ is small in comparison to the rate at which it may be consumed during receptor-mediated activation of PLC (7). Although it is possible that changes in substrate/product concentrations, particularly the dramatic changes that might occur in the immediate vicinity of activated PLC, may play a role in regulating PtdIns(4,5)P₂ supply, the pathway is likely to possess more sophisticated regulatory features that enable supply to be matched to demand under agonist-stimulated conditions. In this context a number of potential regulatory mechanisms for PtdIns 4-kinase and/or PtdIns(4)P 5-kinase activities have been proposed (8–15), although a true understanding of the mechanisms and roles of such regulation remains elusive (2). Furthermore, although the resupply of PtdIns(4,5)P₂ must occur to allow sustained or repetitive phosphoinositide signaling, there is little information to indicate the extent to which its resynthesis contributes to regulatory aspects of signaling mediated by PLC-coupled receptors. It is unclear, for example, whether resynthesis of PtdIns(4,5)P₂ is required during acute agonist-mediated responses and indeed whether depletion of this substrate can...
contribute to the rapid receptor desensitization that is an almost universal feature of receptors activating this signal transduction pathway (16, 17).

Cells of the human SH-SY5Y neuroblastoma cell line have many features characteristic of fetal sympathetic ganglion cells and have been used extensively in studies from signal transduction to neurotransmitter release (18–22). Our laboratory has used this neuroblastoma extensively to examine mechanistic and regulatory aspects of muscarinic receptor-mediated phosphoinositide and Ca2+ signaling (19–22), and these have proved to be representative of other PLC-linked receptor types in differing cellular backgrounds (16, 17). In the current study we have, therefore, used SH-SY5Y cells to examine quantitatively the regulation of PtdIns(4,5)P2 hydrolysis and resynthesis during stimulation of their muscarinic acetylcholine receptors which are predominantly of the M3 subtype (23). In addition we have sought to assess the impact of reduced PtdIns(4,5)P2 supply on transmembrane signaling via PLC and to determine whether agonist-mediated depletion of PtdIns(4,5)P2 contributes to the rapid desensitization of muscarinic receptor-mediated phosphoinositide signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—Experiments were performed on SH-SY5Y cells (originally a gift from Dr. J. Biedler, Sloan-Kettering Institute, New York) between passages 70 and 90. Cells were maintained in minimum essential medium supplemented with 50 IU ml−1 penicillin, 50 μg ml−1 streptomycin, 2.5 μg ml−1 amphotericin B, 2 mM t-glutamine, and 10% fetal calf serum. Cultures were maintained at 37 °C in 5% CO2/humidified air and passaged weekly. For experiments, cells were harvested with 10 mM HEPES, 154 mM NaCl, 0.54 mM EDTA (pH 7.4) and placed on ice for 15 min and then samples added to 0.5–ml bed volume Dowex-50 (H+ form) columns. Columns were washed with 2 ml 2 × 2 ml of water. The pooled eluate was brought to pH 7 by addition of NaHCO3 and applied to a Dowex (AG1-X8) formate anion exchange column. The [3H]GroPls, [3H]GroPls4/4P, and [3H]GroPls4(4,5)P2 were then eluted as described elsewhere (24) and quantified by liquid scintillation spectrometry.

Measurement of PtdIns(4,5)P2 Mass—PtdIns(4,5)P2 mass was determined by assay of Ins(1,4,5)P3 released by alkaline hydrolysis following a previously described protocol (25). Briefly, dried lipid extracts, prepared as described above from cells not labeled with [3H]Insositol, were dissolved in 0.25 ml of 1 M KOH and heated to 100 °C for 15 min during which time they were vortex mixed at regular intervals. Tubes were then placed on ice for 15 min and then samples added to 0.5–ml bed volume Dowex-50 (H+ form) columns. Columns were washed (3 × 0.25 ml) with water. NaHCO3 (100 μl, 0 μl, 600 μl) and EDTA (100 μl, 30 μl) were then added to the pooled column eluates that were stored at 4 °C. The Ins(1,4,5)P3, which had been released from the PtdIns(4,5)P2, was assayed as described below within 48 h. Recoveries from each processing step were assessed (25) to allow levels of PtdIns(4,5)P2 to be extrapolated to the amount of PtdIns(4,5)P2.

Generation and Measurement of Ins(1,4,5)P3—Cell monolayers were preincubated in Krebs/HEPES, challenged, and the reaction terminated as described above. Experiments examining recovery following termination of carbachol action by addition of atropine were performed as described above. A series of experiments were also designed to examine the potential desensitization and resensitization of the peak (10 s) Ins(1,4,5)P3, response. Cells were treated as described above for experiments examining the recovery of inositol phosphates and phosphoinositides. However, following aspiration of the initial carbachol challenge and washing of the monolayer (2 × with 1 ml Krebs/HEPES), incubation was continued for the required recovery time in 1 ml of buffer before aspiration and rechallenge with carbachol (200 μl) for 10 s. Reactions were again stopped by the addition of an equal volume of 1 ml trichloroacetic acid. A 160-μl aliquot of the acidified aqueous phase was removed, processed, and assayed for Ins(1,4,5)P3 by a radioreceptor assay (26).

Measurement of Intracellular [Ca2+]i—The intracellular [Ca2+]i ([Ca2+]i) was determined in suspensions of fura-2-loaded cells. Briefly, confluent cells were harvested, washed with Krebs/HEPES, and resuspended in 2.5 ml of the same buffer. A 0.5-ml aliquot was removed and utilized as below but with the exclusion of the acetylcholine methyl ester of fura-2 (fura-2-AM) thereby allowing the determination of cellular autofluorescence. Fura-2-AM was added to the remaining cells at 5 μM and the cells left with gentle stirring for 40–60 min at room temperature. Intracellular content containing extracellular fura-2-AM was removed following gentle centrifugation of 0.5-ml aliquots. Cells were resuspended in 1 ml of buffer and incubated at 37 °C for 10 min prior to further centrifugation and resuspension in 3 ml of buffer at 37 °C. With emission at 509 nm, the 340:380 nm ratio was recorded every 3.8 s as an index of [Ca2+]i. Cells were challenged by the addition of 50 μl of carbachol to give a final concentration of 1 μM. The 340:380 ratio was recorded prior to challenge (Ca2+0) and 10 s after challenge (Ca2+1) with 10 mM caffeine. The 1 min of water. The pooled eluate was brought to pH 7 by addition of NaHCO3 and then placed on ice for 15 min and then samples added to 0.5–ml bed volume Dowex-50 (H+ form) columns. Columns were washed (3 × 0.25 ml) with water. NaHCO3 (100 μl, 600 μl) and EDTA (100 μl, 30 μl) were then added to the pooled column eluates that were stored at 4 °C. The Ins(1,4,5)P3, which had been released from the PtdIns(4,5)P2, was assayed as described below within 48 h. Recoveries from each processing step were assessed (25) to allow levels of PtdIns(4,5)P2 to be extrapolated to the amount of PtdIns(4,5)P2.

Effects of Li+ on Carbachol-mediated Phosphoinositide Signaling—The effects of Li+ on muscarinic receptor-mediated phosphoinositide signaling was determined as described above with the exception that the preincubation and incubation buffers were isositol-free and contained 10 mM Li+.

Effects of Wortmannin on Carbachol-mediated Phosphoinositide Signaling—Although wortmannin is better known for its ability to inhibit PI3-K (28), this fungal metabolite has recently been demonstrated to inhibit some isoforms of PtdIns 4-kinase (29, 30). We have, therefore, used wortmannin in an alternative strategy to Li+ to examine the immediate consequences of blocking the provision of PtdIns4P and PtdIns(4,5)P2 under basal and agonist-stimulated conditions. For experiments in which cells were pretreated with wortmannin, this was added during the final 10 min of incubation and incorporated with agonist additions. In experiments investigating the time course of the effects of wortmannin on inositol phosphates and phosphoinositides, wortmannin was added in a 10–μl volume.

Measurement of Inositol 1-P—Reagents of analytical grade were obtained from suppliers listed previously (20, 21, 24). [methyl-3H]Insositol was from Amersham Corp. (Little Chalfont, Buckinghamshire, UK) [3H]Insositol (1,4,5)P3 was from NEN Life Science Products (Stevenage, UK). Wortmannin was obtained from Sigma (Poole, UK). 2-(4-Morpholinyl)-8-phenyl-4-1-benzopyran-4-one (LY-294002) was obtained from Affiniti Research Products Ltd. (Exeter, UK).
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RESULTS

Agonist-stimulated Changes in Polyphosphoinositides

Incubation of SH-SYSY human neuroblastoma cells with [3H]inositol resulted in marked changes in the absolute and relative amounts of radioactivity incorporated into the phosphoinositides over the first 20 h (data not shown). However, there were little or no differences between cells labeled for either 44 or 48 h under basal or agonist-stimulated (1 mM carbachol, 5 min) conditions (data not shown), and the phosphoinositide pools were judged to be in equilibrium. In all subsequent experiments, cells were therefore labeled for 48 h and under these conditions [3H]PtdIns comprised the major fraction (94.3 ± 0.3% (n = 4)) of the inositol phospholipid pool, whereas [3H]PtdIns(4)P and [3H]PtdIns(4,5)P$_2$ represented only minor fractions (2.5 ± 0.1% (n = 4) and 3.2 ± 0.2% (n = 4) respectively). Upon addition of a concentration of carbachol that is maximal for the muscarinic receptor-mediated phosphoinositide-linked responses in these cells (1 mM), there was a rapid and marked accumulation of [3H]InosP$_4$ (Fig. 1a). We emphasize that this accumulation of [3H]InosP$_4$ is in the absence of a Li$^{+}$ block of inositol monophosphatase activity. Accumulation therefore represents the net result of both generation and metabolism to free inositol. Over the first 60 s of stimulation with carbachol there was an increase in [3H]InosP$_4$ to 291 ± 23% (n = 4) of basal levels that was sustained throughout the remaining period of agonist stimulation. In the same cell monolayers, there were rapid and marked decreases in the levels of [3H]PtdIns(4)P and [3H]PtdIns(4,5)P$_2$ upon agonist challenge to 32.4 ± 2.9% (n = 4) and 24.7 ± 2.8% (n = 4) of basal levels, respectively, by 60 s (Fig. 1b). These reductions were again sustained throughout the period of agonist challenge. In contrast, the level of [3H]PtdIns decreased to only 85.8 ± 4.2% (n = 4) of basal levels over the experimental period (900 s) (Fig. 1b). Given this relatively small reduction of [3H]PtdIns, it is unlikely that the specific activities of the [3H]-polyphosphoinositides change greatly during this period of stimulation, and therefore the changes in radioactivity are likely to accurately reflect the changes in mass.

The ability of carbachol to mediate the accumulation of [3H]InsP$_x$ and depletion of the phosphoinositides was concentration-related (Fig. 2). EC$_{50}$ values (log$_{10}$ M, determined at 60 s following agonist addition) were −5.43 ± 0.17 (n = 5) (3.7 μM) for [3H]InsP$_x$ accumulation, −5.82 ± 0.15 (n = 5) (1.5 μM) for the depletion of [3H]PtdIns(4)P, and −5.25 ± 0.11 (n = 5) (5.6 μM) for the depletion of [3H]PtdIns(4,5)P$_2$. The 3.7-fold difference between EC$_{50}$ values for agonist-stimulated changes in polyphosphoinositide levels was statistically significant (p < 0.02), indicating that carbachol was more potent at depleting [3H]PtdIns(4)P than [3H]PtdIns(4,5)P$_2$. The relatively minor reductions in [3H]PtdIns, particularly at lower concentrations of carbachol, precluded an accurate assessment of agonist potency for this response.

Comparative Rates of [3H]PtdIns(4)P and [3H]PtdIns(4,5)P$_2$ Resynthesis following Agonist Challenge

The kinetics of recovery from carbachol-induced alterations of [3H]InsP$_x$, [3H]PtdIns(4)P, and [3H]PtdIns(4,5)P$_2$ were de-


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Table I

Reversal by atropine of carbachol-mediated changes in PtdIns(4,5)P_2 and Ins(1,4,5)P_3

| Treatment | PtdIns(4,5)P_2 | Ins(1,4,5)P_3 |
|-----------|---------------|---------------|
| Basal     | 87.7 ± 5.0    | 4.4 ± 0.7     |
| 10 s, 1 mM carbachol | 15.8 ± 4.1  | 68.1 ± 6.1    |
| 60 s, 1 mM carbachol | 15.2 ± 2.6  | 38.0 ± 5.5    |
| 100 s following addition of 10 µM atropine | 88.8 ± 6.5 | 9.9 ± 1.5 |

Changes in [³H]inositol-labeled phospholipids reflect changes in mass levels. Further indicated by the measurement of PtdIns(4,5)P_2 mass during carbachol stimulation and following addition of atropine. Thus, challenge with carbachol for 60 s resulted in an approx. 80% reduction in the mass of PtdIns(4,5)P_2 which was approximately restored within 100 s of atropine addition (Table I, see also Fig. 7).

Extending the period of exposure to carbachol from 1 min (as above) to 5 min before washing had no effect on the rate or extent of recovery of either the [³H]inositol-labeled inositol phosphates or phosphoinositides (data not shown and Fig. 8).

Effect of Pertussis Toxin on the Extent and Rate of Recovery of Inositol Phosphates and Phosphoinositides

 Pretreatment of cells for 24 h with 100 ng/ml pertussis toxin had no effect on either the extent of depletion or the rate and extent of recovery of the [³H]inositol-labeled inositol phosphates or phosphoinositides following 5 min exposure to 1 mM carbachol (Fig. 4 and data not shown).

Effects of Li⁺ on Agonist-stimulated Inositol Phosphate and Phosphoinositide Levels

Incubation of cells with carbachol in the presence of 10 mM Li⁺ resulted in an accumulation of [³H]InsP_2, that was rapid over the 1st min of stimulation (increase of 90% of the basal value which was 13,877 ± 1495 (n = 4) dpm/well). Following this, there was a slower but sustained accumulation over a further 19 min of carbachol stimulation (increase of 50% of basal/min). The sustained linear increase between 1 and 20 min of stimulation demonstrate an effective block of inositol monophosphatase activity that will prevent the recycling of inositol back into the phospholipids. Despite this the carbachol-mediated depletion of the phospholipid was identical in the presence and absence of Li⁺ (data not shown). We were, therefore, unable to use Li⁺ to manipulate cellular levels of the phosphoinositides, and an alternative strategy using inhibitors of PtdIns 4-kinase (wortmannin and LY-294002) was employed.

Effects of Wortmannin and LY-294002 on Basal and Agonist-stimulated Inositol Phosphate and Polyphosphoinositide Levels

Basal Levels—Addition of 10 µM wortmannin to unstimulated cells produced a decrease in [³H]InsP_2 over a 10-min time course to 81.6 ± 20.8% (n = 3) of initial values (Fig. 5a). Over this time frame there was no effect of wortmannin on [³H]PtdIns levels (data not shown), but [³H]PtdIns(4,5)P_2 fell to 84.9 ± 7.3% (n = 3) of basal levels (Fig. 5c). In contrast there was a more dramatic decrease in [³H]PtdIns(4)P to 25.4 ± 1.1% (n = 3) of basal levels (Fig. 5b). The ability of wortmannin to

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induce these changes over a 10-min period was concentration-related (Fig. 6, a and c), but wortmannin was significantly ($p = 0.001$) more potent at reducing basal levels of $[^3H]$PtdIns(4)P than $[^3H]$PtdIns(4,5)P$_2$ with IC$_{50}$ values (log$_{10}$ M) of $-6.23 \pm 0.04$ ($n = 3$) (0.59 $\mu$M) and $-5.82 \pm 0.03$ ($n = 3$) (1.52 $\mu$M), respectively.

**Agonist-stimulated Levels**—When wortmannin was added 60 s after 1 mM carbachol there was an immediate reduction in the agonist-mediated accumulation of $[^3H]$InsP$_{2}$, which approached unstimulated levels by 10 min (Fig. 5a), whereas the $[^3H]$Ins(4,5)P$_2$ levels were further decreased below levels seen in unstimulated levels. This suggests that its ability to deplete cellular levels of PtdIns(4,5)P$_2$ is dependent upon the influx of extracellular Ca$^{2+}$ (29). This was supported by experiments in $\beta$-escin permeabilized SH-SY5Y cells in which we demonstrated that 10 $\mu$M wortmannin reduced the incorporation of $^{32}$P from $[32P]$ATP into PtdIns(4)P but not PtdIns(4,5)P$_2$ over a 10-min period under basal conditions (data not shown).

Addition of 10 $\mu$M wortmannin 10 min prior to challenge with 1 mM carbachol markedly attenuated the transient peak of Ins(1,4,5)P$_3$ accumulation and abolished the sustained component of the response (Fig. 7a). Challenge of cells with 1 mM carbachol also resulted in a biphasic elevation of [Ca$^{2+}$]$_i$, consisting of a rapid transient peak (901 nM) followed by a lower but sustained elevation (367 nM) (Fig. 7b). Addition of 10 $\mu$M wortmannin, 10 min prior to carbachol challenge, markedly attenuated the transient peak of [Ca$^{2+}$]$_i$ elevation (500 $\pm$ 12 nM) (Fig. 7b). This sustained phase of [Ca$^{2+}$]$_i$ elevation is dependent upon the influx of extracellular Ca$^{2+}$ via an as yet undefined mechanism but which most likely involves capacita-
Addition of 1 mM thapsigargin to these cells also resulted in capacitative Ca\(^{2+}\) entry and a sustained elevation of \([\text{Ca}^{2+}]_i\) of similar magnitude to that mediated by 1 mM carbachol, but this was unaffected by 10 \(\mu\)M wortmannin (data not shown). Thus, a direct block of \(\text{Ca}^{2+}\) entry does not underlie the ability of wortmannin to inhibit \(\text{Ca}^{2+}\) signaling (and potentially also phosphoinositide signaling through a reduction in the \(\text{Ca}^{2+}\) feed-forward activation/facilitation of PLC).

Simultaneous addition of carbachol and either 10 \(\mu\)M wortmannin or 100 \(\mu\)M LY-294002 had no effect on the peak accumulation of Ins(1,4,5)P\(_3\) at 10 s but significantly reduced the accumulation determined at 60 s following agonist addition (Fig. 7c).

By using a similar experimental protocol to that outlined above in which cells underwent a potentially desensitizing challenge with 1 mM carbachol for 5 min, we sought to examine recovery of the carbachol-mediated peak (10 s) Ins(1,4,5)P\(_3\) responses. This peak response showed desensitization with washout and recovery periods of less than 2 min which approximately followed the time course of recovery of PtdIns(4,5)P\(_2\) levels. By 2 min the response was fully restored (Fig. 8). Desensitization is often reflected in a reduction in agonist potency rather than a reduction in the maximal response, and we therefore conducted an identical series of experiments in which cells were rechallenged with a concentration of carbachol (25 \(\mu\)M) equivalent to the EC\(_{50}\) for the peak Ins(1,4,5)P\(_3\) response in these cells (20). Compared with rechallenge with 1 mM carbachol...
Regulation of Polyphosphoinositide Pools

Quantitative Estimates of Basal PtdIns(4,5)P₂ Utilization and Relative Increases during Receptor Activation

PtdIns(4,5)P₂ is hydrolyzed in the absence of added agonist, and we have estimated this basal turnover using wortmannin. Addition of wortmannin under basal conditions caused an immediate reduction in [³H]PtdIns(4,5)P₂ (Fig. 5b) but no reduction in [³H]PtdIns(4,5)P₂ for at least 2 min (Fig. 5c). Thus, during this 2-min period, although synthesis of [³H]PtdIns(4,5)P₂ was blocked due to inhibition of a PtdIns 4-kinase by wortmannin, there was sufficient [³H]PtdIns(4,5)P₂ to maintain the levels of [³H]PtdIns(4,5)P₂. The initial rate of decrease of [³H]PtdIns(4,5)P₂ may, therefore, provide an index of the basal consumption of PtdIns(4,5)P₂. This rate of 6839 dpm/min/well infers that an amount of PtdIns(4,5)P₂ equivalent to the total cellular pool (34,510 dpm/well) turns over every 5 min under basal (unstimulated) conditions. The rate of basal PtdIns(4,5)P₂ utilization is 6.4-fold less than the estimated rate of consumption during sustained receptor activation and at least 21.4-fold less than that occurring immediately upon agonist addition (see above). These determinations assume that recycling of PtdIns(4,5)P₂ to PtdIns (by a PtdIns(4,5)P 4-phosphatase) and hydrolysis of PtdIns(4,5)P by PLC are negligible following addition of wortmannin. Any contribution would lead to an overestimation of basal activity and an underestimation, therefore, of the relative increase in the consumption of PtdIns(4,5)P₂ following agonist addition. As a consequence of the potential overestimation of basal hydrolysis, our values of 21- and 6-fold over basal stimulation of PtdIns(4,5)P₂ hydrolysis during the immediate and sustained components of maximal muscarinic-receptor activation, respectively, are likely to be underestimates. Indeed, these values contrast with 150- and 60-fold basal stimulations reported previously (32) in these cells in which basal PtdIns(4,5)P₂ hydrolysis was assessed by the determination of [³H]InsP₃ accumulation in Li⁺-blocked cells. However, due to the uncompetitive nature of Li⁺ action, complete block may not occur under conditions of low flux through the enzyme (33) resulting in an underestimation of basal activity. It is likely that the true extent of stimulation lies between these values.
The measured basal level of PtdIns(4,5)P$_2$ was 87.7 pmol/well, whereas cells labeled to equilibrium with $[^{3}H]$inositol had $[^{3}H]$PtdIns(4,5)P$_2$ present at 34,510 dpm/well. This relationship has enabled us to derive estimates of PtdIns(4,5)P$_2$ levels and utilization in mass units. Furthermore, our measurement of cell number and protein content of a typical well (4.9 $\times$ 10$^4$ cells, 250 $\mu$g of protein) has allowed conversion of these estimates to a per cell or per unit cellular protein basis. Thus, the basal level of PtdIns(4,5)P$_2$ is 0.18 fmol/cell (1.1 $\times$ 10$^8$ molecules/cell) of which 0.15 fmol/cell exists in an agonist-sensitive pool. During sustained receptor activation with a maximally effective concentration of carbachol, PtdIns(4,5)P$_2$ levels fall to 0.05 fmol/cell of which 0.02 fmol/cell exists in an agonist-sensitive pool. During such sustained receptor activation, PtdIns(4,5)P$_2$ turns over at a rate of 0.23 fmol/cell/min (1.4 $\times$ 10$^8$ molecules/cell/min), while during the acute phase of receptor activation this rate is at least 0.76 fmol/cell/min (4.6 $\times$ 10$^8$ molecules/cell/min). From previous estimates of the catalytic activity of PLC-β (200 pmol/min/mg protein (34)), we can estimate that the equivalent of approximately 15,000 molecules of PLC would require to be maximally activated to achieve this hydrolytic rate.

DISCUSSION

PtdIns(4,5)P$_2$ is considered to be the physiological substrate for both PLC and PI3-K activities. Despite the dependence upon PtdIns(4,5)P$_2$ as the substrate for Ins(1,4,5)P$_3$ formation, this polyphosphoinositide constitutes a minor fraction (~5%) of the cellular inositol phospholipid content under basal conditions. The increasing realization that PtdIns(4,5)P$_2$, and perhaps PtdIns(4,5)P$_3$, are rate-limiting substrates for both PLC and PI3-K activities. Despite the dependence upon PtdIns(4,5)P$_2$ as the substrate for Ins(1,4,5)P$_3$ formation, this polyphosphoinositide constitutes a minor fraction (~5%) of the cellular inositol phospholipid content under basal conditions. The increasing realization that PtdIns(4,5)P$_2$, and perhaps PtdIns(4,5)P$_3$, are rate-limiting substrates for both PLC and PI3-K activities. Despite the dependence upon PtdIns(4,5)P$_2$ as the substrate for Ins(1,4,5)P$_3$ formation, this polyphosphoinositide constitutes a minor fraction (~5%) of the cellular inositol phospholipid content under basal conditions. The increasing realization that PtdIns(4,5)P$_2$, and perhaps PtdIns(4,5)P$_3$, are rate-limiting substrates for both PLC and PI3-K activities.

The sustained depletions of the polyphosphoinositides are related to the extent of agonist stimulation although carbachol was more potent at depleting PtdIns(4)P than PtdIns(4,5)P$_2$. This is inferred from the observation that PtdIns(4,5)P$_2$ is protected from dephosphorylation by carbachol, suggesting that substrate availability and product inhibition are unlikely to be the only factors governing the supply of PtdIns(4,5)P$_2$. Thus, mechanisms exist that coordinate polyphosphoinositide synthesis in accordance with demand, and the current data indicate that these mechanisms are of relevance in intact cells.

Regulation of polyphosphoinositide supply could potentially occur via an activation of the kinases responsible for their generation and/or a reduction in the activities of the phosphatases that appear to be involved in substrate cycling (13). However, it seems unlikely that the massive increase in demand for PtdIns(4,5)P$_2$ could be met by changes in polyphosphoinositide-phosphatase activities alone as this would require a persistently high level of substrate or “futile” cycling (13). While coordinated changes in phosphatase and kinase activities may occur, there is little information on the regulation of phosphatase activities. There is, however, a variety of experimental evidence to implicate regulation of both PtdIns-4-kinase and PtdIns(4,5)P$_2$-5-kinase (8–15). Of particular relevance to the current study is the suggestion that a G$_i$-mediated activation of rho is responsible for the regulation of PtdIns(4)P$_2$-5-kinase (15, 40). Thus, in HEK cells expressing recombinant muscarinic M$_4$ receptors, PtdIns(4,5)P$_2$ recovers to above basal levels in a pertussis toxin-sensitive manner following removal of agonist, and it has been argued that this allows an enhanced phosphoinositide response upon rechallenge (40). Such results are in contrast to those of the current study in which we observed a desensitization of the Ins(1,4,5)P$_3$ response and a complete lack of effect of pertussis toxin on the rate or extent of recovery of PtdIns(4,5)P$_2$ following muscarinic receptor activation. Whether the difference in these studies reflects cell specificity or a promiscuous coupling of M$_4$ receptors to G$_i$ at high expression levels in HEK cells is unclear. However, the current study demonstrates that at physiologically relevant levels of muscarinic receptor expression (300 fmol/mg protein (32)), pertussis toxin-sensitive G-proteins play no significant role in regulating resynthesis of PtdIns(4,5)P$_2$ or setting the absolute level of this substrate in SH-SY5Y cells indicating that this is not a universal phenomenon.

By the use of wortmannin we have demonstrated that approximately 15% (of basal levels) of PtdIns(4,5)P$_2$ is unavailable to agonist-stimulated PLC. Similar values have been obtained using Li$^+$ block in Chinese hamster ovary cells expressing recombinant muscarinic M$_4$ receptors (24). The reason for the inaccessibility of this fraction is unclear but may well relate to localization within the cell (41, 42), and this has the potential to impede regulatory control on the signaling pathway. Taking the agonist-insensitive pool into account, we calculate that the agonist-sensitive pool of PtdIns(4,5)P$_2$ turns over at a rate of approximately 12 times per min under sustained stimulation with a maximally effective concentration of carbachol. In the absence of resynthesis, the existing substrate pool would be consumed in less than 2 s of agonist addition. This contrasts somewhat with a previous estimate for PtdIns(4,5)P$_2$ turnover in these cells of 13–19 s (32) and emphasizes the critical dependence of peak (at 10 s) Ins(1,4,5)P$_3$ responses on PtdIns(4,5)P$_2$ resynthesis. Small changes in the absolute basal level of PtdIns(4,5)P$_2$ will, therefore, have little impact on phosphoinositide responses.

Wortmannin treatment of SH-SY5Y cells results in the loss of muscarinic receptor-mediated phosphoinositide and [Ca$^{2+}$], responses demonstrating the critical dependence of this signaling pathway on a wortmannin- (and LY-294002-) sensitive PtdIns 4-kinase as reported in bovine adrenal glomerulosa cells (29, 43). A recent report has indicated that phosphorylation of PtdIns(5)P at the D-4 position by the enzyme, previously identified as type II PtdInsP-5-OH kinase, provides another potential pathway for the generation of PtdIns(4,5)P$_2$. If this route of synthesis also relies upon a wortmannin-sensitive enzyme, the current data suggest that this pathway contrib-
utes little or not at all to the provision of PtdIns(4,5)P$_2$ in these cells under basal or agonist-stimulated conditions. The ability of wortmannin to further deplete PtdIns(4,5)P$_2$ during sustained receptor activation is also paralleled by a loss of $[^{3}H]$InsP$_3$ generation. This suggests that despite the ability of most PLC isoforms to hydrolyze PtdIns, PtdIns(4)P, and PtdIns(4,5)P$_2$ (45), the large cellular pool of PtdIns does not provide a substrate for PLC during muscarinic-receptor activation in SH-SY5Y cells. The issue of the substrate specificity of receptor-activated PLC is difficult to address in situ, and the ability of agents such as wortmannin to manipulate the phosphoinositide pools represents a novel approach to this problem. Furthermore, the ability of wortmannin to abolish the sustained [Ca$^{2+}$]$_i$ elevation during muscarinic receptor stimulation suggests that there is no Ca$^{2+}$ entry via a receptor-operated Ca$^{2+}$ channel (rather than a capacitative mechanism) which has been inferred in other systems (46, 47).

Although both wortmannin and LY-294002 are better known for their ability to block PI3-K, inhibition of this enzyme cannot account for the results of the present study. Thus, the effect of wortmannin on both basal and carbachol-stimulated (poly)phosphoinositide levels is minimal at concentrations that have been reported to abolish totally PI3-K-dependent responses (30–100 nM (29, 43)). Wortmannin (and LY-294002) appears to inhibit one isozyme of the multiple PtdIns 4-kinase activities that are present in many cell types (3), possibly the recently cloned type III PtdIns 4-kinase (48, 49). Although we acknowledge that wortmannin is not a specific inhibitor of PI-4 kinase (28, 29, 43), the data in the present study are totally consistent with an inhibition of this enzyme and indeed are in agreement with studies in which Li$^+$ has been used to manipulate cellular levels of the polyphosphoinositides in Chinese hamster ovary cells during stimulation of muscarinic M$_1$ receptors (24). The current study demonstrates that while Li$^+$ may be used to manipulate phosphoinositide pools in an agonist-dependent manner in some cell lines, this is not the case in SH-SY5Y cells. This is probably as a consequence of a large intracellular pool of free inositol, and a chronic inositol depletion strategy is required to render the cells sensitive to Li$^+$ (50). Given the similar temporal profiles and susceptibility to desensitization of PLC signaling mediated by muscarinic M$_1$ receptors expressed in Chinese hamster ovary cells (Li$^+$-sensitive) and SH-SY5Y cells (Li$^+$-insensitive) (16, 20, 22, 32), it is unlikely that the size of the inositol pool has a major impact on these aspects of phosphoinositide metabolism.

The present data emphasize the dynamic nature of the agonist-sensitive PtdIns(4,5)P$_2$ pool, and the use of wortmannin demonstrates that the immediate consequence of blocking the synthesis of PtdIns(4)P (and also therefore PtdIns(4,5)P$_2$) is the failure of agonist-induced Ins(1,4,5)P$_3$ generation and Ca$^{2+}$ mobilization. These data clearly indicate that the rate of supply of PtdIns(4,5)P$_2$ must be tightly and adequately matched to demand to prevent a full or partial failure of agonist-mediated phosphoinositide signaling. Indeed, a reduction in the availability of PtdIns(4,5)P$_2$ is a possible mechanism underlying the rapid, although often partial, desensitization of PLC activity that occurs within seconds of agonist occupation of many types of PLC-linked receptors including muscarinic receptors of SH-SY5Y cells (20, 22, 32). Whether depletion of PtdIns(4,5)P$_2$ contributes to or underlies such desensitization has proved difficult to resolve. However, we demonstrate here that the sustained reduction in the level of PtdIns(4,5)P$_2$ is related to the concentration of carbachol. This suggests that at submaximal agonist concentrations, there is PtdIns(4,5)P$_2$ available for hydrolysis and implies that limited substrate supply is not responsible for the desensitization phenomenon unless each molecule of PLC has access to a limited amount of PtdIns(4,5)P$_2$ and that this is replaced at a rate related to the extent of receptor activation.

The Ins(1,4,5)P$_3$ response to activation of muscarinic M$_3$ receptors in SH-SY5Y cells and indeed other cells consists of a rapid transient peak followed by a lower but sustained plateau phase (19–22). We have argued previously that these two phases of Ins(1,4,5)P$_3$ accumulation represent desensitization-sensitive and desensitization-resistant phases, respectively (16). In the current study we also demonstrate desensitization of the peak Ins(1,4,5)P$_3$ response using a rechallenge protocol. These studies indicate that pretreatment with a maximal concentration of carbachol results in desensitization of the Ins(1,4,5)P$_3$ peak response upon rechallenge with either a maximal or submaximal concentration of carbachol. Although recovery of the response to a maximal concentration of carbachol paralleled the recovery of PtdIns(4,5)P$_2$, recovery of the response to a submaximal concentration was slower. Thus, there are instances when there is sufficient PtdIns(4,5)P$_2$ to support a maximal Ins(1,4,5)P$_3$ response, and yet responses to a submaximal agonist concentration remain partially desensitized. This indicates that there are events unrelated to the recovery of PtdIns(4,5)P$_2$ (and other constituents of the signal transduction pathway) that are involved in the desensitization phenomenon. We have argued previously that this may be related to phosphorylation of the muscarinic M$_3$ receptor in a manner analogous to the phosphorylation and desensitization of the $\beta_2$-adrenoreceptor (16).

The current data indicate a critical dependence upon a wortmannin- and LY-294002-sensitive PtdIns 4-kinase for the synthesis of PtdIns(4,5)P$_2$ and indicate that the immediate consequence of blocking the synthesis of PtdIns(4,5)P$_2$ is the failure of receptor-mediated phosphoinositide and Ca$^{2+}$ signaling. Mechanisms clearly exist that enable the supply of PtdIns(4,5)P$_2$ to be matched to demand, and these mechanisms are effective even under conditions of maximal muscarinic receptor activation in SH-SY5Y cells. Thus, despite the critical dependence upon PtdIns(4,5)P$_2$ synthesis, our data indicate that agonist-mediated depletion of this lipid substrate is unlikely to account for acute receptor desensitization in SH-SY5Y cells. The pattern of PLC activation and extent of PtdIns(4,5)P$_2$ depletion directed by muscarinic receptor activation in this cell line is consistent with that mediated by many other receptor types in a variety of cellular systems (5, 16, 17, 51) suggesting that the current findings are likely to have general applicability to PLC signaling systems.

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