Accumulation and metabolism of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in muscarinic-receptor-stimulated SH-SY5Y neuroblastoma cells

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Stimulation of M_3 muscarinic receptors expressed by SH-SY5Y cells induced a dose- and time-related increase in the mass of Ins(1,4,5)P_3 (basal 38.3 ± 5.8 pmol/mg of protein) and Ins(1,3,4,5)P_4 (basal 6.1 ± 1.2 pmol/mg of protein). Comparison of radioreceptor mass assays with [³H]inositol labelling showed higher-fold stimulations with the former protocol. The later accumulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 mass was dependent upon extracellular Ca^{2+}.

INTRODUCTION

Agonist occupancy of several diverse cell-surface receptors including various muscarinic subtypes leads to a guanine-nucleotide-sensitive hydrolysis of phosphoinositides and the generation of diacylglycerol and Ins(1,4,5)P_3. Both of these products are believed to act as second messengers, with the latter interacting with a specific receptor and releasing Ca^{2+} from non-mitochondrial intracellular sites [1–3]. There is also increasing evidence that a phosphorylation product of Ins(1,4,5)P_3, i.e. Ins(1,3,4,5)P_4, may also control Ca^{2+} homeostasis by controlling Ca^{2+} entry across the plasma membrane or Ca^{2+} movement between different intracellular stores [4].

In recent studies we have used the human neuroblastoma SK-N-SH and particularly its neuronal clone SH-SY5Y as model systems to examine these potential actions of inositol poly-phosphates in Ca^{2+} homeostasis. These cells stably express a single (M_3) subclass of muscarinic receptor [5,6] that is linked to phosphoinositide metabolism and changes in intracellular Ca^{2+} [7,8]. In particular, we have recently detailed the characteristics of muscarinic-receptor-induced Ca^{2+} mobilization and entry across the plasma membrane in intact SH-SY5Y cells and have associated a dramatic early accumulation of Ins(1,4,5)P_3 mass with the release of the intracellular Ca^{2+} stores [9]. In the present studies we have extended these observations by assessing the mass accumulation of both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 using highly specific receptor assays. Furthermore, by labelling these cells with [³H]inositol and separating various isomeric species of [³H]inositol polyphosphates, we have been able to compare labelled and mass data and make estimates of the proportion of metabolism that occurs by phosphorylation or dephosphoryl-ation. Our observations are discussed in relation to accompanying changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) when cells are incubated in the presence or absence of extracellular Ca^{2+}.

MATERIALS AND METHODS

Cell culture

SH-SY5Y cells (passages 60–100) were cultured in minimum essential medium supplemented with 2 mM-L-glutamine, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 2.5 μg of fungi-zone/ml and 10% (v/v) foetal calf serum as described previously [5,9].

Measurement of [³H]inositol phosphate production

All experiments were performed with SH-SY5Y cells suspended in Krebs buffer of the following composition (mM): Na^+ (143.3), K^+ (5.9), Mg^{2+} (1.2), Ca^{2+} (1.3), Cl^- (128.3), $H_2PO_4^{2-}$ (2.2), HCO_3^- (24.9), SO_4^{2-} (1.2) and glucose (10), pH 7.4. SH-SY5Y cells were prelabelled for 24–48 h (48 h for h.p.l.c.) with 4–10 μCi of myo-[2-³H]inositol/ml (10 μCi/ml for h.p.l.c.). Suspensions of labelled cells were then stimulated (300 μl final volumes) with carbachol. All reactions were terminated with an equal volume of trichloroacetic acid (1 M). [³H]Inositol phosphates were extracted with Freon/octylamine and neutralized with bicarbonate as described previously [9]. [³H]Inositol phosphates were separated either by conventional Dowex anion-exchange chromatography [10,11] or by h.p.l.c. [12]. The latter separation was achieved with a Partisil (10 μm) SAX analytical column, and separation of isomers has been carefully validated by co-elution with authentic ³H-labelled inositol (poly)phosphate standards in separate runs and/or by co-elution with internal ¹⁴C- or ³²P-labelled inositol phosphate standards [12].

Measurement of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 mass

Assays were performed using radioreceptor methods previously described and evaluated in this laboratory for stereo- and positional specificity [13,14]. Briefly, intact SH-SY5Y cells (approx. 1 mg of protein), harvested with EDTA (this reduces membrane disturbance and increases reproducibility compared with scrape-harvesting; see [9]), were preincubated for 5 min at 37 °C. Carbachol was then added for the indicated time periods. The reaction was terminated, and Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were extracted and neutralized as described above. Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were quantified using bovine adrenal cortical and rat cerebellar binding proteins respectively at 4 °C [13,14]. Authentic Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (0.036–36 pmol) were prepared in buffer, taken through an identical extraction and used as standards. Appropriate treatment of standards is essential to obtain authentic tissue estimates. [³H]Ins(1,4,5)P_3 and [³²P]Ins(1,3,4,5)P_4 were used as radioligands {in some assays [³H]Ins(1,3,4,5)P_4 was used}. Non-specific binding was defined in the presence of excess D/L-Ins(1,4,5)P_3 (10 μM) for Ins(1,4,5)P_3 determinations and D/L-Ins(1,3,4,5)P_4 (10 μM) for Ins(1,3,4,5)P_4 determinations. Bound and free ligand were separated by rapid filtration. [³H]Ins(1,4,5)P_3 (17–20 Ci/mmol), [³H]Ins(1,3,4,5)P_4

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concn.; EC_{50}, concn. causing half-maximum stimulation.
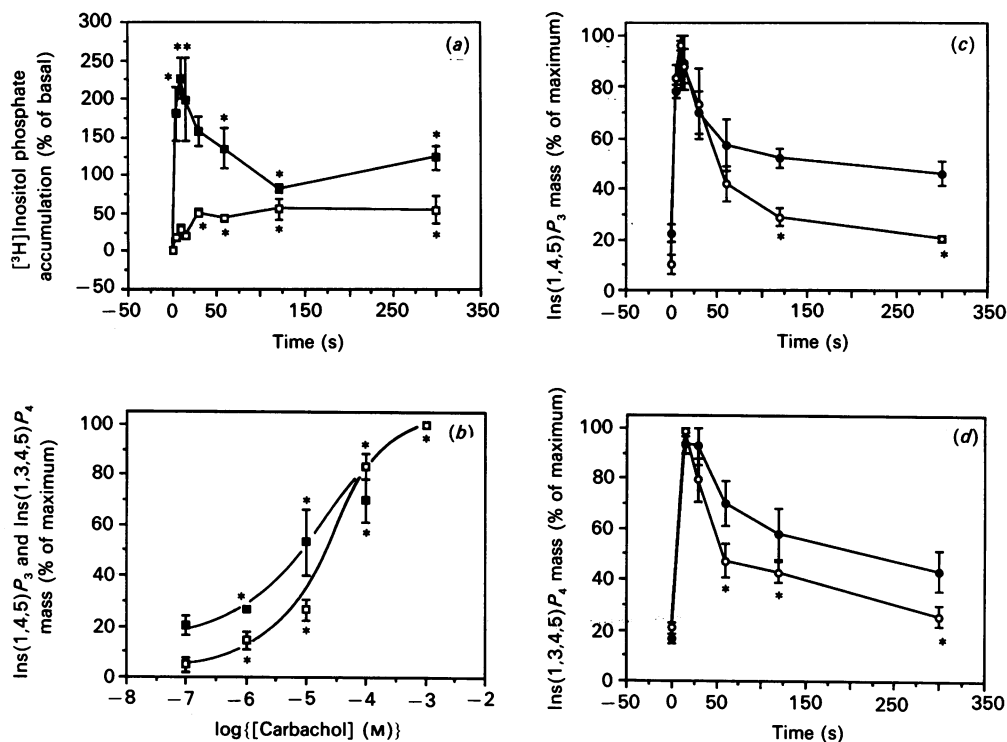* To whom correspondence should be addressed.

Fig. 1. Time- and dose-related accumulation of carbachol-stimulated inositol tris- and tetrakis-phosphate in SH-SY5Y human neuroblastoma cells

(a) Time-related accumulation of [^{3}H]InsP_3 (■) and [^{3}H]InsP_4 (□) in response to carbachol (1 mM). Cells were prelabelled with 4 μCi of myo-[^{3}H]inositol/ml for 24 h. Inositol phosphates were separated by Dowex anion-exchange chromatography (see the text). Data are means ± s.e.m. (n = 3). Basal radioactivity was as follows: [^{3}H]InsP_3, 204 ± 27 d.p.m.; [^{3}H]InsP_4, 270 ± 27 d.p.m. Basal radioactivity was unchanged at 300 s. * P < 0.05 compared with basal. (b) Dose-related accumulation of Ins(1,4,5)P_3 (■) and Ins(1,3,4,5)P_4 (□) mass. Basal Ins(1,4,5)P_3 mass was 24 ± 3 pmol/mg of protein, and Ins(1,3,4,5)P_4 mass was 4.5 ± 0.4 pmol/mg of protein. * P < 0.05 compared with basal. (c) and (d) Effect of removal of external Ca^{2+} (+0.1 mM-EGTA) on Ins(1,4,5)P_3 (c) and Ins(1,3,4,5)P_4 (d) mass accumulation in carbachol (1 mM)-stimulated SH-SY5Y human neuroblastoma cells. Removal of external Ca^{2+} (+0.1 mM-EGTA) did not alter the peak production of either polyphosphate. * P < 0.05 compared with Ca^{2+}-replete. ■, +Ca^{2+}; □, −Ca^{2+}. All data are means ± s.e.m. (n = 3–8).

(17–20 Ci/mmol) and [^{32}P]Ins(1,3,4,5)P_4 (100 Ci/mmol) used above were generously provided by NEN (Du Pont).

Data analysis

Data are expressed as means ± s.e.m. of at least three determinations. EC$_{50}$ (half-maximum stimulation) values were obtained by computer-assisted curve fitting using ALLFIT [15]. Where appropriate, statistical significance was assessed by Student's t test and was considered to be significant when P < 0.05.

RESULTS AND DISCUSSION

Previous studies in both the parent SK-N-SH cell and the neuronal clone SH-SY5Y from this [5,9,16] and other laboratories [6,8] have shown that muscarinic M$_3$ receptor occupation by appropriate agonists leads to increased phosphoinositide metabolism, release of intracellular stored Ca^{2+} and Ca^{2+} entry across the plasma membrane. The present studies, however, provide the first detailed analysis of inositol polyphosphate isomers using both labelling and mass assays and have allowed assessment of their potential role in Ca^{2+} signalling in response to muscarinic receptor stimulation in these cells.

In initial experiments, [^{3}H]inositol phosphates were separated by Dowex anion-exchange chromatography to describe the time course of accumulation. Addition of carbachol (1 mM) to prelabelled cells caused a time-related accumulation of InsP_3, reaching a peak of 226 % above basal some 5–10 s after agonist

addition. The pattern of InsP_3 production was biphasic, with a new apparent steady state being reached (approx. 100 % above basal values) by 2 min and maintained for at least a further 3 min. [^{3}H]InsP_4 reached a peak accumulation (50 %) some 30–60 s after muscarinic receptor stimulation and remained relatively constant for at least 5 min (Fig. 1a).

The time-related increases in [^{3}H]inositol polyphosphates described above were confirmed and extended by examining two time points (10 and 300 s) using h.p.l.c. (Table 1). Significant increases (162 %) in [^{3}H]Ins(1,4,5)P_3 were seen at 10 s, whereas at 5 min this had decreased to a value insignificantly different from basal values. Slower increases in [^{3}H]Ins(1,3,4)P_3 (229 %) and [^{3}H]InsP_4 (165 %) were also noted by analysis at 5 min. At this later time point, radioactivity eluting with characteristics of Ins1P_1 or Ins3P_1, Ins4P_1 and Ins(1,4)P_2/Ins(1,3)P_2 were all significantly increased by muscarinic receptor stimulation. Although the radioactivity eluting as InsP_4 was not definitively identified as Ins(1,3,4,5)P_4 [Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4 also co-elute under these conditions], the identification of [^{3}H]-Ins(1,3,4)P_3 and both [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,3)P_2 strongly suggests that metabolism of Ins(1,4,5)P_3 proceeds in stimulated cells via dephosphorylation or phosphorylation followed by dephosphorylation of Ins(1,3,4,5)P_4 [1].

The recent availability of methods to specifically assay the mass of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 using radioreceptor techniques has been particularly useful here. The problems of changing specific radioactivity during agonist stimulation, and particularly the problem of separating isomeric species of InsP_3

Table 1. H.p.l.c. analysis of inositol phosphate isomers in carbachol (1 mM)-stimulated SH-SY5Y cells

Cells were prelabelled with 10 μCi of myo-[^{3}H]inositol/ml for 48 h. Inositol phosphate isomers were separated by h.p.l.c. (see the text) Background radioactivity was subtracted from each fraction (Ins$P_{1/2}$ = 21, InsP_3 = 46, InsP_4 = 70 d.p.m.). Data are means ± S.E.M., n = 3–4 (% increases are given in parentheses); * $P < 0.05$ compared with basal.

Inositol phosphate	Basal	Radioactivity (d.p.m.) (%)	
		10 s	5 min
Ins1P_1	1125±75	1853±197 (64)*	2748±369 (144)*
Ins4P_1	545±141	787±135 (44)	1798±307 (230)*
Ins(1,3)P_2	83±23	125±40 (50)	273±79 (228)*
Ins(1,4)P_2	1162±29	1584±143 (36)	1996±338 (71)*
Ins(1,3,4)P_3	84±37	146±98 (73)	276±70 (229)*
Ins(1,4,5)P_3	207±31	542±12 (162)*	334±74 (61)
InsP_4	188±12	277±86 (47)	499±51 (165)*

and InsP_4, even with h.p.l.c., are overcome with assays that are stereo- and positionally specific. Indeed, they provide information on the potential errors of labelling experiments. When incubations were terminated at 10 s, carbachol induced a dose-related accumulation of Ins(1,4,5)P_3 mass. Maximum and half-maximum stimulation occurred at 1 mM and 42±18 μM respectively (Fig. 1b). As reported previously [9], carbachol (1 mM) caused a biphasic increase in Ins(1,4,5)P_3 mass, reaching a maximal level some 10 s after agonist addition and then declining to a new steady state (above basal) by 1 min, and at 5 min was still significantly ($P < 0.05$) increased above basal. Omission of Ca^{2+} (+0.1 mM-EGTA) from the incubation buffer significantly reduced the plateau phase without affecting the peak Ins(1,4,5)P_3 mass. Analysis of peak-to-plateau ratios show that in control cells the plateau was 49±4 % of the peak value, whereas in cells devoid of extracellular Ca^{2+} this value was only 21±1 % ($P < 0.05$) (Fig. 1c).

Resting concentrations of Ins(1,3,4,5)P_4 in SH-SY5Y cells (approx. 6.1±1.2 pmol/mg of protein) are less that those of Ins(1,4,5)P_3 in these cells, but are very similar to those we have reported in cerebral cortical slices [14], and to those reported using a metal-dye technique in HL60 cells [17] and in NG108 cells using a similar receptor assay [18]. Stimulation by carbachol increased the mass of Ins(1,3,4,5)P_4 from 6.1 pmol/mg of protein to 47.0 pmol/mg of protein by 30 s, and this accumulation remained unchanged for a further 90 s before declining to 23.3 pmol/mg of protein at 5 min, a level still significantly above basal (see Fig. 1d). The increase observed at 60 s was dose-dependent, with maximum and half-maximum stimulation occurring at 1 mM and 31±4 μM respectively (Fig. 1b). The EC$_{50}$ values for the mass of Ins(1,4,5)P_3 and the mass of Ins(1,3,4,5)P_4 did not differ significantly. In experiments in which extracellular Ca^{2+} was omitted (+0.1 mM-EGTA), the pattern of Ins(1,3,4,5)P_4 accumulation after carbachol was changed significantly. Under Ca^{2+}-free conditions, although this polyphosphate accumulated initially, a significant reduction in Ins(1,3,4,5)P_4 was observed at 1 min, and by 5 min after agonist addition Ins(1,3,4,5)P_4 had returned to the basal level. Omission of Ca^{2+} did not alter the basal levels of this polyphosphate (Fig. 1d).

It should be noted that the mass measurements of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 always gave much larger fold stimulations than comparable radioactive Dowex/h.p.l.c. peaks associated with labelled SH-SY5Y cells. Although these data would be consistent with a fall in specific radioactivity following receptor stimulation, we feel it is more likely that these discrepancies relate to other labelled (isomeric) species eluting in the [^{3}H]InsP_3 and [^{3}H]InsP_4 fractions and are proportionately more significant

in basal samples. These observations highlight the advantages of the mass assays.

The initial accumulation of Ins(1,4,5)P_3 {and perhaps of Ins(1,3,4,5)P_4 [19,20]} are almost certainly responsible for the release of Ca^{2+} from intracellular stores [7,21]. However, the significance of the sustained accumulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in SH-SY5Y cells is unclear. The time course of these responses parallels a phase of Ca^{2+} entry which has been carefully evaluated in previous studies in these cells [9]. This agonist-sensitive entry of Ca^{2+} is not mediated by voltage-sensitive Ca^{2+} channels [22], and we have discussed the possibility of a direct receptor-operated channel (although not involving a pertussis-toxin-sensitive G-protein [23]) or second-messenger-operated Ca^{2+} entry [24]. It should be further emphasized that Ca^{2+} entry in SH-SY5Y cells occurs at lower carbachol concentrations than are required for intracellular Ca^{2+} mobilization. Furthermore, the EC$_{50}$ value of carbachol stimulation of Ca^{2+} entry [9] is more than one order of magnitude lower than the EC$_{50}$ for its effects on Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 accumulation. This latter point argues against inositol phosphates mediating Ca^{2+} entry.

Two recent studies in which [^{3}H]Ins(1,3,4,5)P_4 has been separated from other inositol tetrakisphosphate isomers have reported a good temporal relationship between [^{3}H]Ins(1,3,4,5)P_4 [but not Ins(1,4,5)P_3] accumulation and Ca^{2+} influx in bradykinin-stimulated PC12 cells [25] and HL60 cells activated with fMetLeuPhe [26]. Results in the present study using mass assays indicate a close correlation of both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 with Ca^{2+} entry in SH-SY5Y cells. However, stimulating the cells with carbachol in the absence of extracellular Ca^{2+} markedly suppressed the later sustained accumulation of inositol polyphosphates. These data may be consistent with a model in which the later accumulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 is dependent upon Ca^{2+} entry rather than vice versa. However, since we know that Ca^{2+} entry is a very early event (probably occurring in concert with the discharge of the internal stores) in these cells [9], it still remains possible that Ins(1,4,5)P_3 and/or Ins(1,3,4,5)P_4 may open the Ca^{2+} channel and that the subsequent Ca^{2+}-dependent sustained accumulation of these second messengers may maintain the Ca^{2+} channel in the open state in a positive feedback system.

Thus, in conclusion, we have established using both labelling and mass assays the generation and accumulation of the second messengers Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 after muscarinic M$_3$ receptor stimulation in SH-SY5Y cells. The later sustained accumulation of these inositol polyphosphates appears to be dependent upon extracellular Ca^{2+}. A Ca^{2+}-stimulated hydrolysis

of phosphoinositides has been observed in these cells [16], and in detailed studies on the parent SK-N-SH cell, Fisher *et al.* [8] have demonstrated synergism for inositol phosphate generation between Ca^{2+} in the micromolar range and guanine nucleotides. Our data appear consistent with a direct receptor-operated Ca^{2+} entry that is separate from intracellular release, as recently established in bovine adrenal chromaffin cells [27].

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