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Muscarinic receptors coupled to phosphoinositide hydrolysis and elevated cytosolic calcium in a human neuroblastoma cell line SK-N-SH

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1 The effects of the muscarinic agonist carbachol on phosphoinositide metabolism and its relationship to alteration of intracellular calcium were examined in SK-N-SH human neuroblastoma cells. Muscarinic receptors on these cells are coupled to phospholipase C and the myo [2-^{3}H]-inositol phosphates resulting from receptor activation of cells labelled with [^{3}H]-inositol accumulate rapidly. The breakdown of both inositol monophosphate ($InsP_1$) and inositol bisphosphate ($InsP_2$) is sensitive to lithium with inhibition of the latter only observed at higher concentrations of this ion.

2 Use of the calcium indicator dye Fura 2 revealed that carbachol stimulates a biphasic increase in intracellular calcium.

3 Carbachol was able to stimulate both [^{3}H]-inositol phosphate production and intracellular calcium levels with respective EC_{50} values of $15.9 \pm 1.0\,\mu M$ and $10.7 \pm 3.2\,\mu M$, indicating that no amplification occurs between these steps in the signal transduction pathway.

4 Inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_3$) released $^{45}Ca^{2+}$ in a stereospecific and dose-related manner from intracellular stores of permeabilised cells.

5 These results suggest that this cell line may represent a useful model system to investigate receptor-mediated phosphoinositide metabolism and calcium homeostasis.

Introduction

Although it is now well established that several neurotransmitters stimulate phosphoinositide metabolism in the central nervous system, the recently discovered complexities of this metabolism (Nahorski, 1988; Irvine *et al.*, 1988) and calcium homeostasis cannot be easily delineated in cerebral preparations due to the cellular heterogeneity of the tissue. Indeed, there is still little evidence that inositol (1,4,5)-trisphosphate ($Ins(1,4,5)P_3$) can induce release of intracellular calcium in CNS neurones. To simplify interpretation of neurochemical data in cerebral tissue, several groups have recently used clonal neuroblastoma cell lines as a model homogeneous population of cells, as they express many characteristics of neuronal cells. The more commonly used cell lines are N1E-115 mouse neuroblastoma, which express a relatively low density of muscarinic (probably M_1) receptors linked to phosphoinositide metabolism (Fisher & Snider, 1987), and NG108-15 neurobastoma × glioma, which possess muscarinic receptors that inhibit adenylate cyclase (Harden *et al.*, 1988). However, preliminary investigations revealed that these cells displayed little or no production of inositol phosphates in response to muscarinic agonists (Baird & Nahorski, unpublished observations). In contrast the human cell line SK-N-SH, which was originally derived from bone marrow metasteses of a four year old girl (Biedler *et al.*, 1973), expresses a relatively high density of muscarinic receptors linked to phosphoinositide metabolism (Fisher & Snider, 1987; Fisher & Heacock, 1988). We have recently confirmed the relatively high density of M_3-muscarinic receptors on SK-N-SH cells by use of the radioligand [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) (Lambert *et al.*, 1989) and here examine the linkage to phosphoinositide metabolism and calcium metabolism in this cell line. In previous measurements of phosphoinositide metabolism with these cells (e.g. Yu & Sadée, 1986; Fisher & Snider, 1987) only long periods of incubation with muscarinic agonists have been investigated and lithium has always been

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present. The use of lithium to inhibit dephosphorylation of inositol phosphates, and hence act as a means of amplification, has been widely used in many cells but may perturb the relative rates of phosphorylation/dephosphorylation of Ins(1,4,5)P_3 (Batty & Nahorski, 1987). A more accurate estimation of the initial rates of accumulation of the separate [^{3}H]-inositol phosphates is best attempted in its absence. In the present study, we provide evidence that these cells accumulate separate [^{3}H]-inositol phosphates in response to the muscarinic agonist carbachol. Carbachol was also able to elicit rapid increases in intracellular calcium, with similar EC_{50} values to its ability to stimulate [^{3}H]-inositol phosphate production, and Ins(1,4,5)P_3 could be demonstrated to release calcium from intracellular stores in permeabilised SK-N-SH cells.

Methods

[^3H]-inositol phosphate determination

SK-N-SH cells (obtained from the American Tissue Culture Collection, U.S.A.) were grown in Minimal Essential Medium supplemented with 10% foetal calf serum, 100 iu ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, 2 mM glutamine and 2.5 $\mu g\,ml^{-1}$ Fungizone. Cultures were grown in 175 cm^2 tissue culture flasks (Gibco, U.K.) containing 50 ml of supplemented medium and maintained at 37°C in 5% CO_2/humidified air. Stock cultures were passaged every seven days (split ratio 1:3) and fed twice weekly. Experimental work was performed with cells of passages 50–70. Cells were seeded into 6-well plates at a density of 0.5 million cells per well. After 18–24 h myo-[2-^{3}H]-inositol (14.1 Ci $mmol^{-1}$; New England Nuclear), which had been cleaned by passing it through a small column of Dowex resin in the chloride form, was added to the monolayers of cells in Earles balanced salt solution (1.25 ml) containing amino acids. [^{3}H]-inositol was present at a final concentration of 1 μCi ml^{-1}. After at least a 24 h labelling period, the cells were washed with Krebs-Ringer Bicarbonate solution (KRB). Carbachol (1 mM) was added for the desired incubation period in KRB (1 ml). After termination of the reaction with cold perchloric acid (10% v/v), the monolayers of cells were removed with a rubber policeman. Following centrifugation the [^{3}H]-inositol phosphates were neutralised with Freon/octylamine (Downes *et al.*, 1986) and separated by Dowex AG-X resin in the Formate form (200–400 mesh) as described previously (Baird & Nahorski, 1986) and counted for radioactivity. When levels of $InsP_5$/$InsP_6$ were estimated these were eluted with 2 M ammonium formate/0.1 M formic acid, whilst inositol tetrakisphosphate ($InsP_4$) was eluted with 1 M ammonium formate/0.1 M formic acid. Where total [^{3}H]-inositol phosphates were measured the incubations were terminated by use of cold methanol and the chloroform was added to the suspension of cells after removal from the well, as described previously (Baird *et al.*, 1983). When present, atropine was added (at 37°C) 15 min before the addition of carbachol.

Determination of intracellular calcium concentration

Cells from near confluent flasks were scraped off and resuspended in KRB containing the calcium indicator dye Fura 2 AM (5 μM) for 45 min at 37°C. After loading, the cells were washed and resuspended in fresh KRB at room temperature. This regime reduced dye leakage. Intracellular calcium was measured in a 3 ml suspension of Fura 2-loaded SK-N-SH cells at 37°C in polypropylene cuvettes containing a magnetic stirrer bar. Fluorescence was measured in a Perkin-Elmer LS5B spectrofluorimeter. The excitation wavelengths were 340 and 380 nm with emission at 509 nm. The time taken to drive between 340 and 380 nm excitation intensities was 7.5 s. Intracellular calcium concentration was calculated from the fluorescence ratio at 340/380 nm according to Grynkiewicz *et al.* (1985). Maximum and minimum fluorescence values were determined by use of Triton X-100 (0.1%) and EGTA (3.0–4.5 mM) respectively.

Ins(1,4,5)P_3 induced calcium release

The method used was as described previously (Strupish *et al.*, 1988). Briefly, cells were permeabilised with saponin (100 $\mu g\,ml^{-1}$) for one minute and then suspended in a cytosol-like buffer containing oligomycin (2 $\mu g\,ml^{-1}$) and 2 μCi $^{45}Ca^{2+}$ ml^{-1} (free calcium concentration 200–300 nM) for 20 min, to load actively non-mitochondrial stores with $^{45}Ca^{2+}$. Aliquots (100 μl) of cell suspension were added to 100 μl of buffer containing varying concentrations of synthetic D/L-Ins(1,4,5)P_3 (kindly provided by Dr B.V.L. Potter, Department of Chemistry, Leicester University). After a one minute incubation the cells were centrifuged through silicone oil mixture (Dow-Corning 556/550,3:2, v/v). Following removal of buffer and oil the pellet was dissolved in Lumasolve (100 μl) (May & Baker, Dagenham, Essex) and then assayed for radioactivity.

Data analysis

The EC_{50} (concentration of drug producing 50% of maximal stimulation) and IC_{50} (concentration of

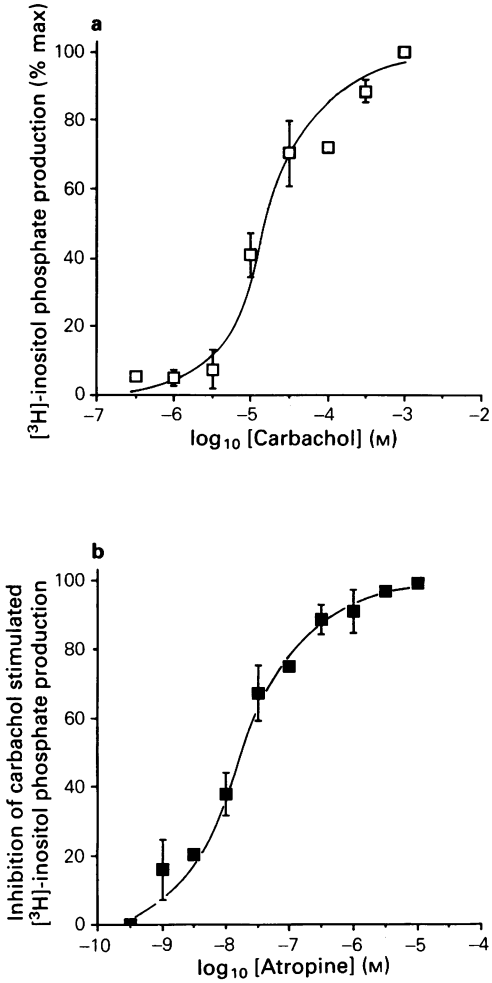

Figure 1 (a) The effects of a range of carbachol concentrations on production of [^{3}H]-inositol phosphates expressed as % of the response obtained with 1 mM carbachol (1160 ± 312 d.p.m. basal subtracted). (b) The inhibitory effect of atropine on carbachol (1 mM) stimulated [^{3}H]-inositol phosphate production (which amounted to 1539 ± 67 d.p.m., basal subtracted). In both cases the cells were stimulated for 30 min in the presence of lithium (10 mM; Na^+ concentration adjusted accordingly) and basal values of 760 ± 106 d.p.m. (a) and 859 ± 67 d.p.m. (b) were subtracted. Data are mean with vertical lines indicating s.e.mean (n = 3 in a) or range (n = 2 in b).

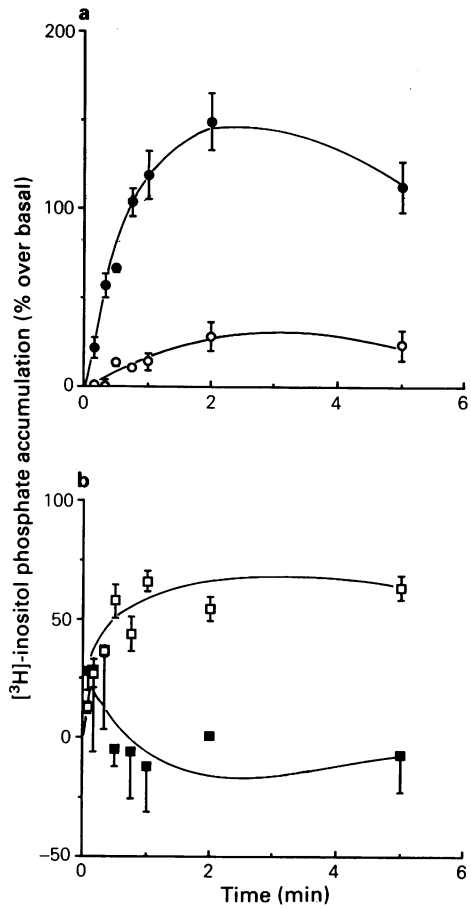

Figure 2 Time course of accumulating [^{3}H]-inositol phosphates in SK-N-SH cells in response to carbachol (1 mM). Cells were pre-incubated with myo-[2-^{3}H]-inositol and the [^{3}H]-inositol phosphates assayed as described. Data shown are the mean of at least three separate determinations performed using triplicate samples; vertical bars show s.e.mean. (Basal values were as follows: $InsP_1$ (○) 675 ± 54, $InsP_2$ (●) 174 ± 11, $InsP_3$ (□) 122 ± 8, $InsP_4$ (■) 142 ± 14 d.p.m.)

drug producing 50% inhibition) values were obtained by computer-assisted curve fitting by use of ALLFIT (De Lean *et al.*, 1978). K_i values for atropine were calculated according to Cheng & Prussoff (1973). Statistical significance was assessed by Student's t tests and differences were considered significant when $P < 0.05$.

Results

The effects of muscarinic receptor activation on [^{3}H]-inositol phosphate production

Carbachol stimulated the production of total [^{3}H]-inositol phosphates in a concentration-dependent

manner, maximum stimulation occurred in response to 1 mM carbachol (Figure 1a). The EC_{50} value for carbachol-stimulated [^{3}H]-inositol phosphate production was $15.9 \pm 1.0\,\mu$M (mean ± s.e.mean, $n = 3$). The muscarinic receptor antagonist atropine caused a dose-dependent inhibition of 1 mM carbachol stimulated total [^{3}H]-inositol phosphate production (Figure 1b), with a K_i of 0.27 ± 0.06 nM (mean ± range, $n = 2$). In other experiments the production of the separate inositol phosphates was examined. The data in Figure 2 show that following the addition of carbachol, [^{3}H]-inositol trisphosphate ([^{3}H]-$InsP_3$) increased to 57% after 30 s ($P < 0.05$) and remained stable for the following 5 min ($P < 0.05$). [^{3}H]-inositol bisphosphate ([^{3}H]-$InsP_2$) was maximal after 1 min increasing 149% ($P < 0.05$) and then decreasing slowly. [^{3}H]-inositol monophosphate ([^{3}H]-$InsP_1$) increased slowly reaching 42% over basal after 5 min. Levels of [^{3}H]-$InsP_4$ and higher inositol phosphates did not change significantly.

In order to compare the ability of lithium to influence the accumulation of the separate [^{3}H]-inositol phosphates, KRB containing 10 mM Li^+ (Na^+ concentration adjusted accordingly) was used. The results in Figure 3 show that carbachol increased [^{3}H]-$InsP_2$ and [^{3}H]-$InsP_1$ to 1140% and 406% after 30 min respectively ($P < 0.05$). Dose-response profiles to lithium show that the concentration of lithium needed to produce 50% of the maximal accumulation of [^{3}H]-$InsP_1$ was 0.60 ± 0.12 mM whilst that of [^{3}H]-$InsP_2$ was not complete even at 10 mM (mean value from two experiments ± range; data not shown). [^{3}H]-$InsP_3$ values were similar to those in the absence of lithium, although a small increase in [^{3}H]-$InsP_4$ levels to 23% above basal after 10 min was observed. No significant increase in higher [^{3}H]-inositol phosphates (likely to comprise of $InsP_5$ and $InsP_6$ eluted with 2.0 M ammonium formate/0.1 M formic acid) were observed, although the basal radioactivity in this fraction increased markedly after 24 h pre-incubation with myo-[2-^{3}H]-inositol in comparison with a 2 h preincubation (data not shown).

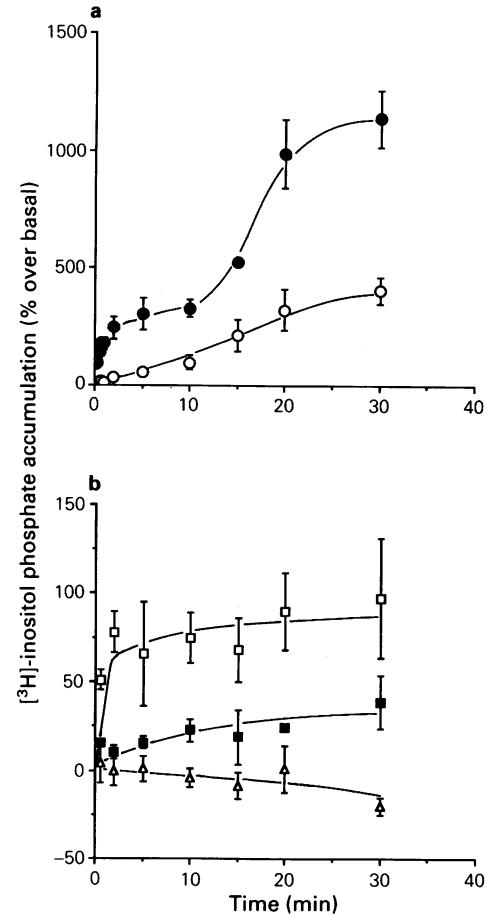

Figure 3 Time course of accumulating [^{3}H]-inositol phosphates in SK-N-SH cells in response to carbachol (1 mM) in a solution of KRB containing lithium at a concentration of 10 mM, with the Na^+ concentration adjusted accordingly. Cells were pre-incubated with myo-[2-^{3}H]-inositol and the [^{3}H]-inositol phosphates assayed as described. Data shown are the mean of at least three separate determinations performed using triplicate samples; vertical bars show s.e.mean. (Basal values were as follows: $InsP_1$ (○) 1026 ± 256, $InsP_2$(●) 208 ± 52, $InsP_3$ (□) 138 ± 17, $InsP_4$ (■) 106 ± 16, $InsP_5$ (△) 587 ± 164 d.p.m.)

Effect of carbachol on intracellular calcium

Addition of carbachol (1 mM) to suspensions of Fura 2-loaded cells caused a rapid initial spike in intracellular calcium, maximal after 15 s. This peak declined slowly to reach a new plateau phase above basal values (Figure 4a). Carbachol caused a dose-related increase in peak intracellular calcium, maximum stimulation occurring at 1 mM carbachol (Figure 4b). The EC_{50} value for carbachol-stimulated increase in intracellular calcium was $10.7 \pm 3.2\,\mu$M ($n = 4$). The actions of carbachol were shown to be atropine-sensitive with maximal inhibition occurring at 1 μM (data not shown).

Ins(1,4,5)P_3-induced calcium release from permeabilised cells

To assess in a more direct way whether Ins(1,4,5)P_3 could release calcium in these cells, the effects of D/L-Ins(1,4,5)P_3 in saponin permeabilised SK-N-SH cells loaded with $^{45}Ca^{2+}$ were also examined. D/L-Ins(1,4,

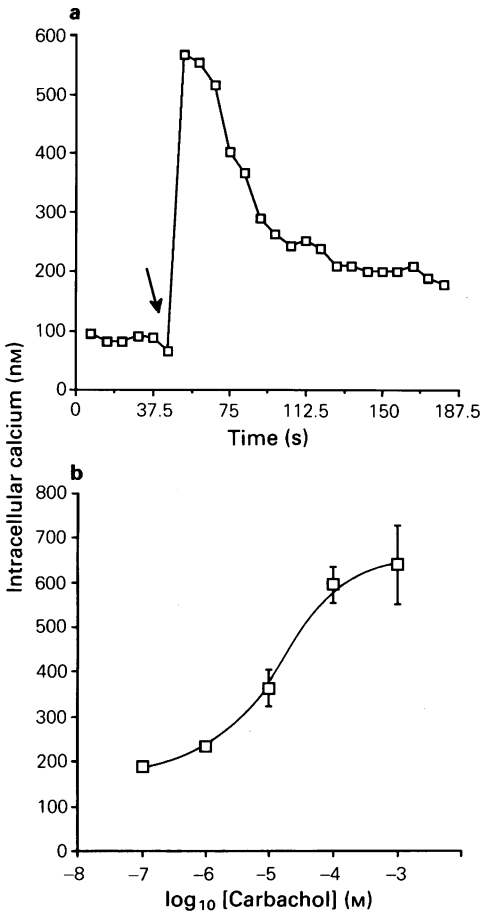

Figure 4 Upper panel (a) shows the effect of carbachol, 1 mM (indicated by the arrow) on intracellular calcium levels in Fura 2-loaded SK-N-SH cells. Fluorescence levels were measured at both 340 and 380 nm and the ratio was used to determine the concentration of intracellular calcium as described. The graph shown is typical of at least three others. Panel (b) shows the dose-response relationship of peak intracellular calcium levels to carbachol concentration. Data are the mean from 4 separate experiments; vertical bars show s.e.mean.

5)P_3 was able to release rapidly 50–60% of the $^{45}Ca^{2+}$ accumulated, with maximum and half-maximum release occurring at 10 and 1 μM, respectively. The synthetic unnatural L-isomer was ineffective at all concentrations used (Figure 5).

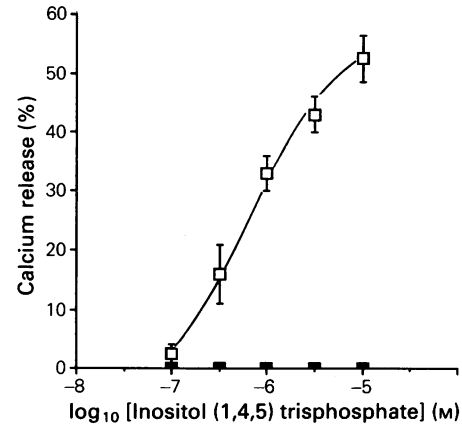

Figure 5 The effects of synthetic D/L-Ins(1,4,5)P_3 (□) and L-Ins(1,4,5)P_3 (■) added to permeabilised SK-N-SH cells loaded with $^{45}Ca^{2+}$. Data are the mean of three to six separate determinations; vertical bars show s.e.mean.

Discussion

Stimulation of muscarinic receptors on SK-N-SH neuroblastoma cells resulted in the rapid accumulation of the various [^{3}H]-inositol phosphates, which confirms and extends the previous studies of Yu & Sadée (1986), Fisher & Snider (1987) and Fisher & Heacock (1988). These cells clearly display a coupling of muscarinic receptors to phospholipase C, although in contrast to results obtained in cerebral cortical tissue (Batty & Nahorski, 1985; 1987) little $InsP_4$ appears to accumulate. It is difficult to interpret this latter data without further analysis of the proportions of the Ins(1,4,5)P_3 and inositol 1,3,4 trisphosphate (Ins(1,3,4)P_3) isomers in these experiments. For example, it remains possible that there is substantial flux through the tris-tetrakisphosphate pathway with little accumulation of [^{3}H]-InsP_4. Analysis of the isomeric composition of [^{3}H]-InsP_3 has not been performed here because of the relatively low extent of labelling of [^{3}H]-InsP_3 in these experiments. However, from our present understanding of receptor-mediated phosphoinositide metabolism it would seem certain that from the present results, at least initially, stimulated production of [^{3}H]-Ins(1,4,5)P_3 occurred. Whether this proceeds via dephosphorylation to inositol 1,4 bisphosphate (Ins(1,4)P_2) and/or phosphorylation to inositol 1,3,4,5 tetrakisphosphate (Ins(1,3,4,5)P_4) remains to be established. It is interesting to note that by use of the phenotypically stable SH-SY5Y cells derived from the parent SK-N-SH cells, mass measurements of Ins(1,4,5)P_3 have been performed and show that carbachol stimulates peak production (16 fold) after 10 s and then falls rapidly to a second steady state maintained above basal (Lambert & Nahorski, unpublished observations).

Many studies investigating phosphoinositide

metabolism have utilised the ability of lithium to inhibit the inositol phosphatases (see Berridge, 1984). The data in Figure 3 clearly show the ability of lithium to inhibit the hydrolysis of $InsP_1$ and $InsP_2$. The greater increase seen with 10 mM lithium on $InsP_2$ accumulation, over that seen with $InsP_1$, could relate to a major inhibition of $InsP_2$ breakdown and thus limit the accumulation of $InsP_1$. These data may also suggest that the major source of $InsP_1$ is from a lithium sensitive dephosphorylation of $InsP_2$. Since the dephosphorylation of Ins(1,4)P_2 to Ins(4)P_1 is the only known bis-phosphatase that is lithium-sensitive (see Nahorski, 1988), this again emphasizes the major route of Ins(1,4,5)P_3 metabolism in these cells is dephosphorylation to Ins(1,4)P_2 and probably Ins(4)P_1. The shape of the time courses for [^{3}H]-$InsP_1$ and [^{3}H]-$InsP_2$ in the presence of lithium are also of interest, in that the effects appear to be biphasic. This could relate to the uncompetitive inhibition of the phosphatases by lithium, with the degree of inhibition relating to the amount of substrate (Gee *et al.*, 1988). The lack of lithium-induced accumulation of [^{3}H]-$InsP_3$ may indicate that little Ins(1,3,4)P_3 is being formed, since at least one degradation route of this metabolite has been shown to be sensitive to lithium (Gee *et al.*, 1988). Furthermore, as Ins(1,3,4)P_3 is formed as a product of the action of the 5-phosphatase on $InsP_4$ (Batty & Nahorski, 1985), this would again emphasise that little $InsP_4$ was being produced from Ins(1,4,5)P_3 under these conditions. In view of the known calcium mobilising properties of Ins(1,4,5)P_3 and the accumulation of $InsP_3$ observed in this study, the effects of carbachol on intracellular calcium in SK-N-SH cells loaded with the calcium indicator dye Fura 2 were also evaluated. Carbachol was able to produce rapid and marked increases in levels of intracellular calcium (Figure 4), presumably due to release from an Ins(1,4,5)P_3-sensitive store. These studies have been repeated with the SH-SY5Y clone in which the time taken to change between wavelengths has been reduced to 3.8 s. Under these conditions we see at least one point on the upstroke of the calcium spike, peaking at 8.7 s (Lambert & Nahorski, 1989) and, by use of a single wavelength (340 nm), we have confirmed a similar time to reach a maximum level (unpublished observations). It is interesting to note that the production of total inositol phosphates (Figure 1a) and the elevation of intracellular calcium (Figure 4b) displayed similar EC_{50} values for carbachol, suggesting that receptor-mediated activation of phosphoinositide hydrolysis is tightly coupled to the mobilization of intracellular calcium in these cells.

Although analysis of [^{3}H]-Ins(1,4,5)P_3 was not undertaken in this study, it would seem probable in view of the results in permeabilised cells (Figure 5) that Ins(1,4,5)P_3 produced, as a consequence of muscarinic receptor activation, releases calcium from intracellular stores. These results are in good agreement with previous work from this laboratory with GH_3 anterior pituitary cells and 3T3 fibroblasts, where we have shown (Strupish *et al.*, 1988) that the mobilization of calcium from intracellular pools is stereospecific, with the D-isomer of Ins(1,4,5)-P_3 being active whilst the L-isomer and Ins(1,3,4)P_3 were inactive. Indeed D-Ins(1,4,5)P_3 had 2000 fold greater affinity than L-$InsP_3$ to receptor sites in cerebellum (Willcocks *et al.*, 1987). Thus, SK-N-SH cells display ATP-dependent high affinity uptake of $^{45}Ca^{2+}$ into non-mitochondrial stores which can be released by D-Ins(1,4,5)P_3.

In conclusion, we have established that the human neuroblastoma SK-N-SH cell line possesses muscarinic receptors coupled to phosphoinositide metabolism. The resulting inositol phosphates are associated with an increase in intracellular calcium. Addition of Ins(1,4,5)P_3 to permeabilised cells can release stored calcium, strongly suggesting that this is a physiological effect of the muscarinic receptor-stimulated formation of inositol phosphates and specifically Ins(1,4,5)P_3. Thus, SK-N-SH cells appear to provide a useful model neuronal system with which to define the regulation of muscarinic cholinergic post receptor events. However, this cell line has been shown to be phenotypically unstable, the population being plastic between both neuronal and epithelial cells types (Ross *et al.*, 1983). Recent work from this laboratory shows exclusive expression of muscarinic receptors on the more phenotypically stable neuronal subclone SH-SY5Y (Lambert *et al.*, 1989) and work currently in progress with this subclone may allow a further evaluation of the relationship between receptor occupancy, phosphoinositide metabolism and calcium homeostasis.

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