Quantitative comparisons of muscarinic and bradykinin receptor-mediated Ins (1,4,5)P₃ accumulation and Ca²⁺ signalling in human neuroblastoma cells

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1 Muscarinic and bradykinin receptor-mediated Ins(1,4,5)P₃ accumulation, Ca²⁺ mobilization and Ca²⁺ entry have been examined in human SH-SY5Y neuroblastoma cells. This has allowed both direct comparison of signalling events by two receptor types potentially linked to the same transduction pathway and an investigation of the interactions between the components of this pathway.

2 Stimulation of muscarinic receptors with carbachol produced biphasic accumulations of Ins(1,4,5)P₃ consisting of a rapid peak followed by a lower sustained phase. Both phases were dose-dependent but the potency of elevation at peak was significantly less than that of the sustained phase. Bradykinin also dose-dependently stimulated Ins(1,4,5)P₃ accumulation but responses were smaller and not sustained.

3 Lowering of [Ca²⁺]_i reduced basal Ins(1,4,5)P₃ levels. Peak Ins(1,4,5)P₃ elevation in response to carbachol and bradykinin were lowered by an amount approximating this reduction over the entire dose-response curves. Sustained Ins(1,4,5)P₃ elevation in response to carbachol showed a more marked absolute reduction. Agonist potencies were unaffected by lowering [Ca²⁺]_i. Thus, a consistent but small amount of PLC activity during rapid activation appears to be sensitive to lowered [Ca²⁺]_i, whilst activity during sustained stimulation is greatly facilitated by external Ca²⁺, probably through Ca²⁺ entry.

4 The temporal- and dose-dependency of carbachol-mediated Ins(1,4,5)P₃ accumulations were unaffected by loading cells with fura-2, thus allowing direct comparison of Ins(1,4,5)P₃ and [Ca²⁺]_i changes monitored by fura-2.

5 Changes in [Ca²⁺]_i by both agonists revealed temporal patterns that were similar to Ins(1,4,5)P₃ accumulations. Only carbachol stimulated a marked sustained [Ca²⁺]_i signal and this was fully dependent on external Ca²⁺.

6 All agonist-mediated [Ca²⁺]_i elevations occurred with significantly greater potency than that of the respective Ins(1,4,5)P₃ accumulations. Further examination of peak elevations in response to carbachol indicated that this was independent of Ca²⁺ entry. Thus, a major site for amplification of the potency of rapid agonist-mediated responses lies at the level of the Ins(1,4,5)P₃ receptor.

7 The transient nature of Ins(1,4,5)P₃ and [Ca²⁺]_i peaks followed by either lower but sustained levels with carbachol or a return to basal levels with bradykinin suggests rapid but partial desensitization of the muscarinic receptor and complete desensitization of the bradykinin receptor. This indicates receptor-specific desensitization. Further analysis of this was provided by detecting accumulations of [³H]-inositol phosphates ([³H]-InsPs) in Li⁺-blocked, *myo*-[³H]-inositol labelled cells. Carbachol produced a rapid accumulation over the first minute, followed by a slower linear accumulation for at least 29 min. At this point accumulations were dose-related with a potency similar to that of sustained Ins(1,4,5)P₃ accumulation. However, bradykinin produced a minor accumulation of [³H]-InsPs, maximal by 1 min. Thus, analysis of PLC activation by measurement of [³H]-InsPs over relatively long time frames will indicate the ability of agonists for predominantly sustained PLC activation, potentially driven by a partially desensitized receptor, as opposed to rapid activation by a fully sensitized receptor.

8 These data provide quantitative comparisons between and within components of the receptor-mediated phosphoinositide and Ca²⁺ signalling pathway, provide mechanistic insights into regulation of these components and characterize a model system in which heterologous interaction between two receptors linked to the same transduction pathway may be examined.

Keywords: Inositol 1,4,5-trisphosphate; intracellular calcium; desensitization; muscarinic receptor; bradykinin receptor; human neuroblastoma cells

Introduction

Upon agonist binding, a variety of cell surface receptors increase the activity of specific isoforms of phospholipase C (PLC), via GTP-binding proteins, to increase the hydrolysis of phosphatidylinositol 4,5-bisphosphate. This liberates both diacylglycerol and inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) which activate various isoforms of protein kinase C (PKC) and mobilize Ca²⁺ from intracellular stores respectively (Beridge, 1993; Putney & Bird, 1993) providing the means for

regulation of cellular events. Although the sequence of events following receptor activation is known, much remains to be uncovered regarding the nature and generality of regulatory features within the pathway. It is clear, for example, that the dose-response curves for many cellular and tissue responses in PLC-linked systems lie to the left of the PLC activation curve, which in turn may lie to the left of the apparent receptor occupation curve (Kenakin, 1987). However, mechanistic aspects of such amplification are unclear and, given the possible unsuitability of the commonly used measurement of total [³H]-inositol phosphates ([³H]-InsPs) in

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lithium-blocked cells for the determination of rapid PLC activation (see below), these fundamental relationships may require reassessment.

In a series of recent studies we have examined phosphoinositide signalling in the human neuroblastoma cell line SH-SY5Y that displays many of the characteristics of human foetal sympathetic ganglion cells (Ross *et al.*, 1983). These cells possess a predominant (Lambert *et al.*, 1989), although probably not exclusive (Wall *et al.*, 1991) M₃ receptor population and muscarinic receptor agonists stimulate phosphatidylinositol 4,5-bisphosphate hydrolysis via a pertussis-toxin insensitive G-protein (Lambert & Nahorski, 1990a) probably involving a member of the G_{q/11} family (Rhee & Choi, 1992). Activated SH-SY5Y cells display a rapid peak of Ins(1,4,5)P₃ followed by a lower sustained phase which is dependent upon extracellular Ca²⁺ (Lambert & Nahorski, 1990b; Lambert *et al.*, 1991). Consistent with agonist-induced increases in [Ca²⁺]_i in many cell types (Putney & Bird, 1993), the pattern of muscarinic-induced [Ca²⁺]_i elevation is also biphasic in SH-SY5Y cells and parallels the changes in Ins(1,4,5)P₃ levels. The initial peak arises predominantly from Ins(1,4,5)P₃-mediated liberation of Ca²⁺ from intracellular stores and the sustained phase is dependent upon Ca²⁺ entry (Lambert & Nahorski, 1990b; Murphy *et al.*, 1991).

Using muscarinic receptor stimulation in the SH-SY5Y cell line as a model phosphoinositide-linked receptor system, the present study sought to compare the determination of PLC activity by measurement of accumulation of [³H]-InsPs and Ins(1,4,5)P₃ mass and allow direct quantitative comparison with components of the Ca²⁺ signalling pathway. In addition, this work revealed a bradykinin receptor linked to activation of PLC in these cells, and this has, therefore, allowed the examination of these relationships for two PLC-linked receptors in the same cell type to assess the receptor specificity of such phenomena. Given both the reported Ca²⁺-sensitivity of some PLC isoforms (Rhee & Choi, 1992) and the Ca²⁺ influx across the plasma membrane caused by many agonists (Putney & Bird, 1993) we have also examined the influence of reductions in the extracellular [Ca²⁺]_e ([Ca²⁺]_e) on both [Ca²⁺]_i elevation and Ins(1,4,5)P₃ generation to assess further potential regulatory elements of the signalling pathway.

We provide evidence that measurement of accumulation of [³H]-InsPs reflects reduced but sustained PLC activity which may be indicative of a desensitized agonist-mediated process. Additionally we demonstrate a clear dissociation between the dose-response relationships for agonist-mediated Ins(1,4,5)P₃ generation and components of [Ca²⁺]_i elevation and highlight the complex interactions between Ins(1,4,5)P₃-induced Ca²⁺ mobilization, Ca²⁺ entry across the plasma membrane and the role of the latter in facilitating Ins(1,4,5)P₃ generation.

Methods

Cell culture

Cultures of the human neuroblastoma cell line, SH-SY5Y (originally given by Dr J. Biedler, Sloan-Kettering Institute, New York, U.S.A.) 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 L-glutamine and 10% (v/v) newborn-calf serum. Cultures were maintained at 37°C in 5% CO₂/humidified air. Stock cultures were passaged weekly at a 1:4–6 split following harvesting with 10 mM HEPES, 154 mM NaCl, 0.54 mM EDTA pH 7.4 (harvesting buffer) and fed or refed alternately 2–3 times per week.

Population [Ca²⁺]_i measurement

Confluent cultures of SH-SY5Y cells (P70-90) were harvested and reseeded in 3 ml of media at an equivalent density on 11

× 22 mm coverslips in 8 well multidishes. After leaving overnight to allow cell attachment, coverslips were placed in 3 ml Krebs/HEPES buffer (composition (mM): HEPES 10, NaHCO₃ 4.2, glucose 10, MgSO₄ 1.2, KH₂PO₄ 1.2, KCl 4.7, NaCl 118 and CaCl₂ 2) within acrylic cuvettes constructed to hold them diagonally across the cuvette. Cuvettes were placed in a Perkin-Elmer LS-5B spectrofluorimeter with a water jacket to maintain cuvette temperature at 37°C and aligned to minimize reflectance. Buffer was stirred continually with a magnetic follower. Excitation wavelengths were 340 and 380 nm, with a 3.8 s changeover time, and emission was recorded at 509 nm. Following determination of autofluorescence, 10 µl of fura-2-AM in DMSO was added (final fura-2-AM concentration 3.3 µM). After 40 min at room temperature with continual stirring, buffer was removed, the cuvette washed 3 times with 3 ml buffer at 37°C and left in 3 ml of buffer for 5 min at 37°C. When [Ca²⁺]_i was to be examined at reduced [Ca²⁺]_e, cuvettes were then washed 3 times with 3 ml of nominally Ca²⁺-free (low [Ca²⁺]_e) buffer and left for a further 5 min at 37°C in 3 ml of buffer. The [Ca²⁺]_e of low [Ca²⁺]_e buffer was approximately 2 µM as determined with fura-2 (Gryniewicz *et al.*, 1985). The [Ca²⁺]_e of standard buffer was 2 mM and this was defined as normal [Ca²⁺]_e. Agonists were added in 50 µl aliquots. With emission determined at 509 nm, the 340 nm/380 nm ratio was recorded as an index of [Ca²⁺]_i. However, the relationship between the 340 nm/380 nm ratio and [Ca²⁺]_i is non-linear and in particular will underestimate the magnitude of changes in [Ca²⁺]_i as the ratio approaches R_{max} (Gryniewicz *et al.*, 1985) thus invalidating EC₅₀ determination or direct comparison of changes at different ratio values. An *in situ* calibration was therefore performed in separate experiments, deriving R_{max} with either ionomycin or Br-A23187 (6–33 µM) and elevated [Ca²⁺]_e of up to 10 mM followed by EGTA to determine R_{min}. These values were used to convert all population 340/380 ratios to [Ca²⁺]_i (Gryniewicz *et al.*, 1985).

Measurement of D-Ins(1,4,5)P₃ mass

This was done by modification of a radioreceptor method previously assessed for stereo- and positional specificity (Challiss *et al.*, 1990). Confluent cultures (P70-90) were harvested and reseeded at an equivalent density in 24 well multidishes. After incubating overnight to allow cell attachment, wells were washed with 1 ml of Krebs/HEPES at 37°C and left for 30 min at 37°C with 1 ml of buffer per well. When Ins(1,4,5)P₃ generation was to be examined at low [Ca²⁺]_e, standard buffer was replaced by low [Ca²⁺]_e buffer for the last 5 min. Multidishes were maintained at 37°C, wells aspirated individually and 100 µl of buffer with the appropriate agonist concentration added immediately. Reactions were stopped with 100 µl of ice-cold 1 M TCA. For zero time, TCA was added prior to agonist. Multidishes were left at 4°C for at least 15 min and 40 µl of 10 mM EDTA added to 160 µl aliquots, followed by 200 µl of a 1:1 (v:v) mixture of tri-n-octyl-amine and 1,1,2-trichloro-trifluoro-ethane. After vortexing, samples were left at room temperature for 15 min then microfuged (10,000 g, 2 min) and 100 µl of the upper aqueous phase was taken to which 50 µl of 25 mM NaHCO₃ was added. Samples were stored at 4°C until assay of Ins(1,4,5)P₃ (<3 days).

D-Ins(1,4,5)P₃ was quantified in single 30 µl aliquots of duplicate generations in a final volume of 120 µl (25 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C using bovine adrenal cortical binding protein (~0.6 mg protein per tube) and D-myio-[³H]-inositol 1,4,5-trisphosphate (specific activity 44 Ci mmol⁻¹, 3.6 nCi per tube) as radioligand. Authentic D-Ins(1,4,5)P₃ (0.1 nM–3 µM) was used as a standard with non specific binding (<10% of total) defined by 10 µM Ins(1,4,5)P₃. Following a 30 min incubation, protein was collected with 2 ml of ice-cold buffer (25 mM Tris-HCl, 1 mM EDTA, 5 mM NaHCO₃, pH 8.0) and rapid filtration (Brandel Cell Harvester) onto Whatman GF/B glass fibre filters. After two

rapid 5 ml washes, filters were counted by liquid-scintillation spectrometry in 4 ml Emulsifier-Safe scintillation cocktail.

Protein content of wells (typically 100–200 µg) was determined in 0.1 M NaOH digests of at least two wells per multidish by the Bradford method using an acidic Coomassie Brilliant Blue G-250 solution and bovine albumin fraction V as standard. When *n* is given, this represents the number of duplicate generations of Ins(1,4,5)P₃.

Measurement of inositol phosphate accumulation

Cells were prelabelled with 3 µCi ml⁻¹ of *myo*-[³H]-inositol (86 Ci mmol⁻¹) for 48 h in 24 well multidishes. Following a 10 min preincubation in 250 µl Krebs/HEPES with 5 mM Li⁺, a further 250 µl of buffer containing agonist was added. Following incubation at 37°C, reactions were stopped with 500 µl 1 M TCA. An 800 µl aliquot was removed and 200 µl of 10 mM EDTA added. After vortexing with 1 ml of a 1:1 (v:v) mixture of tri-*n*-octyl-amine and 1,1,2-trichloro-trifluoro-ethane, 50 µl of 250 mM NaHCO₃ was added to 800 µl of the aqueous phase. This was applied to a Dowex AG1X8, 200–400 mesh column which was then washed with 10 ml of 25 mM ammonium formate. Inositol phosphates were washed off with 10 ml of 1 M ammonium formate/0.1 M formic acid (Baird *et al.*, 1989). A 2 ml aliquot of this was counted by liquid scintillation spectrometry following addition of 8 ml Floscint.

Materials

Reagents of analytical grade and highly purified Elgastat water were used throughout. Minimum Essential Medium, penicillin/streptomycin, amphotericin B, glutamine, newborn calf serum and tissue culture flasks were from Gibco. Multidishes were from Nunc. Fura-2-AM (pentaacetoxymethyl ester), ionomycin free acid and 4-Br-A23187 were from Calbiochem. Anhydrous dimethyl sulphoxide (DMSO) was from Aldrich. Tri-*n*-octylamine, 1,2,3-trichloro-trifluoro-ethane, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid (HEPES), Tris-HCl, atropine, carbachol, ethylene glycol-bis (β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), bradykinin, N-adamantaneacetyl-D-Arg, [Hyp³, Thi^{5,8}, D-Phe⁷]-bradykinin and bovine albumin fraction V were from Sigma. Triton X-100 was from Boehringer. Coomassie reagent was from Pierce. D-Ins(1,4,5)P₃ (K⁺ Salt) was from University of Rhode Island Foundation, U.S.A. D-*myo*-[³H]-inositol 1,4,5-trisphosphate and *myo*-[³H]-inositol were from Amersham. Emulsifier-Safe Scintillation Cocktail and Flo Scint IV were from Packard. Trichloroacetic acid (TCA) and all other reagents were from Fisons.

Statistical analysis

Dose-response curves were fitted by Graph-PAD (Academic Press Inc. 1986) using a standard four-parameter logistic equation. EC₅₀ mean values and standard errors of the Ins(1,4,5)P₃ data were generated from the mean ± standard error of the mean (s.e.mean) of log₁₀ EC₅₀ values generated from separate curves. However, for the [Ca²⁺]_i data, where individual data points were from different coverslips, EC₅₀ values were from curves fitted with mean data for each concentration and the s.e.mean quoted therefore reflects the standard error determined by the curve fitting routine. The degrees of freedom associated with comparisons of these data were taken as the mean number of values contributing to each data point minus 2. The lines representing the dose-response curves in the figures represent the best fit curves with uniform weighting to all points. Comparison of full dose-response curves was by two-way analysis of variance with log₁₀ transformations to account for heteroscedastic data where required. Other statistical comparisons were by Student's two-tailed unpaired *t* test. Acceptance of

significance for all tests was at *P* < 0.05. EC₅₀ values are presented as log₁₀ M mean ± s.e.mean (*n*). All other data are presented as mean ± s.e.mean (*n*).

Results

Carbachol- and bradykinin-mediated Ins(1,4,5)P₃ accumulation

Carbachol and bradykinin both increased Ins(1,4,5)P₃ levels. Following 1 mM carbachol there was a biphasic elevation consisting of a peak at 10 s followed by a reduction over the next 50 s to a lower, but sustained phase (Figure 1a). Following 10 µM bradykinin there was also a peak of Ins(1,4,5)P₃ at 10 s, which decreased rapidly over the next 50 s and then more slowly over the next 2–3 min to approximately basal levels (Figure 1a). Peak Ins(1,4,5)P₃ responses to carbachol and bradykinin were dose-dependent with EC₅₀ values of -4.63 ± 0.07 (11) (24 µM) and -6.58 ± 0.13 (9) (264 nM) respectively (Figure 2a and 2b). The sustained phase of Ins(1,4,5)P₃, determined at 300 s following carbachol addition, was also dose-dependent (not shown) with an EC₅₀ of -5.37 ± 0.23 (6) (4.3 µM), this being significantly (*P* < 0.02) lower than that of the peak EC₅₀. A maximal (10 µM)

Figure 1 Time course of changes in inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) mass following addition of 1 mM carbachol (■), 10 µM bradykinin (□) or buffer alone (x) to adherent SH-SY5Y cells at normal (a) or low (b) [Ca²⁺]_i. Data are mean ± s.e.mean, *n* = 3–5.

bradykinin challenge produced only a small (2.32 ± 0.27 (10) fold) increase in Ins(1,4,5)P₃ levels at 10 s compared to that in response to carbachol (8.75 ± 0.78 (11) fold) (Figures 1 and 2). The sustained phase of Ins(1,4,5)P₃ elevation in response to carbachol was 5.75 ± 0.78 fold (68% of peak response) compared to only 0.47 ± 0.25 fold (20% of peak response) for bradykinin.

Sensitivity of carbachol- and bradykinin-mediated Ins(1,4,5)P₃ accumulation to reductions in [Ca²⁺]_i

A 5 min preincubation of cells in low [Ca²⁺]_i buffer prior to agonist challenge significantly reduced the resting level of Ins(1,4,5)P₃ from 36.2 ± 4.8 (18) pmol mg⁻¹ protein to 9.0 ± 2.2 (6) pmol mg⁻¹ protein. This preincubation did not

Figure 2 Dose-response relationship for carbachol (a) and bradykinin (b) mediated peak changes in inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) mass in adherent SH-SY5Y cells at normal (●) or low (○) [Ca²⁺]_i. Data points are mean $\pm$ s.e.mean, $n = 7-11$ for carbachol, $n = 6-9$ for bradykinin at normal [Ca²⁺]_i and $n = 3$ for bradykinin at low [Ca²⁺]_i. EC₅₀ (log₁₀ M, mean $\pm$ s.e.mean (n)) were clearly not different whether determined in the presence of normal or low [Ca²⁺]_i for either carbachol (-4.63 ± 0.07 (11) (24 μ M) and -4.59 ± 0.23 (3) (26 μ M) respectively) or bradykinin (-6.58 ± 0.13 (9) (264 nM) and -6.17 ± 0.16 (3) (673 nM) respectively).

affect the time course of the responses to carbachol or bradykinin (Figure 1a and b). The reduction in the peak response to carbachol was of a similar absolute magnitude over the entire dose-response. However, as a proportion of the response in the presence of extracellular Ca²⁺ the reduction varied over the carbachol dose-response curve, decreasing from approximately 80% at 1 nM to approximately 20% at 1 mM. The EC₅₀ at low [Ca²⁺]_i was not significantly different from that in the presence of a normal [Ca²⁺]_i (-4.59 ± 0.23 (3) (26 μ M) and -4.63 ± 0.07 (11) (24 μ M) respectively) (Figure 2a). The elevation of Ins(1,4,5)P₃ during the sustained phase at low [Ca²⁺]_i was also dose-dependent with an EC₅₀ of -5.38 ± 0.11 (3) (4 μ M) (data not shown). This was not significantly different from the EC₅₀ determined in the presence of normal [Ca²⁺]_i (-5.37 ± 0.23 (6) (4.3 μ M)) but was significantly ($P < 0.05$) less than that of peak Ins(1,4,5)P₃ elevation at low [Ca²⁺]_i (-4.59 ± 0.23 (3) (26 μ M)). The accumulation of Ins(1,4,5)P₃ at 10 s in response to bradykinin was markedly reduced at low [Ca²⁺]_i (Figures 1b and 2b). Compared to the direct addition of TCA, the addition of buffer alone provoked an increase of 14.8 ± 0.4 (26) pmol mg⁻¹ protein at 10 s which remained elevated for the duration of stimulation (Figure 1a and b). This increase was virtually abolished in low [Ca²⁺]_i buffer (0.81 ± 0.84 (3) pmol mg⁻¹ protein) (Figure 1a and b).

Pertussis toxin sensitivity of carbachol- and bradykinin-mediated Ins(1,4,5)P₃ accumulation

Pretreatment of cells for 24 h with 100 ng ml⁻¹ pertussis toxin (added at the time of seeding of cells into multiwells) had no effect on the levels of Ins(1,4,5)P₃ during the sustained phase in response to carbachol (data not shown). However, there was a small but significant effect of pertussis toxin on the Ins(1,4,5)P₃ response to carbachol at 10 s, ($P < 0.01$ by two-way analysis of variance of dose-response curves). Thus, 1 mM carbachol elevated Ins(1,4,5)P₃ levels from 29.2 ± 5.1 (5) pmol mg⁻¹ protein to 290.3 ± 49.2 (5) pmol mg⁻¹ protein in untreated compared to 25.8 ± 2.1 (5) pmol mg⁻¹ protein to 210.9 ± 41.4 (5) pmol mg⁻¹ protein in pertussis toxin-treated cells. The EC₅₀ was, however, unaffected (data not shown). There was no significant effect of pertussis toxin on the Ins(1,4,5)P₃ response to bradykinin at 10 s (data not shown).

Ins(1,4,5)P₃ accumulation in fura-2 loaded cells

To allow direct comparison of dose-response curves for elevation of Ins(1,4,5)P₃ and [Ca²⁺]_i, carbachol stimulation of Ins(1,4,5)P₃ accumulation was determined in fura-2 loaded cells. Cells preloaded with fura-2, by incubation in 1 ml Krebs/HEPES containing 3.3 μ M fura-2-AM for 40 min at room temperature prior to carbachol stimulation, displayed time-course and dose-response relationships for Ins(1,4,5)P₃ generation that were identical to those in unloaded cells (data not shown). Thus any potential influence of cellular fura-2 on Ins(1,4,5)P₃ generation via Ca²⁺ buffering was not apparent over the full range of Ins(1,4,5)P₃ levels.

Carbachol- and bradykinin-mediated accumulation of [³H]-InsPs

In response to 1 mM carbachol, [³H]-InsPs showed a biphasic accumulation, being more rapid over the initial minute compared to a slower but still linear accumulation between 1 min and 30 min. By 1 min, accumulation was $133 \pm 7\%$ (3) over basal compared with $1501 \pm 139\%$ (3) at 30 min (Figure 3a). The response at 30 min was dose-related (Figure 3b) with an EC₅₀ of -5.54 ± 0.16 (3) (2.9 μ M) which was not significantly different from the sustained phase of Ins(1,4,5)P₃ accumulation (-5.37 ± 0.23 (6) (4.3 μ M)). In contrast, in response to 10 μ M bradykinin, [³H]-InsPs increased by $47 \pm 14\%$ (3) over basal by 1 min and showed no further increase over the subsequent 29 min (Figure 3a).

Carbachol and bradykinin mediated elevations of [Ca²⁺]_i

At normal [Ca²⁺]_e, carbachol at 100 nM or greater produced a characteristic rapid peak of [Ca²⁺]_i followed by a sustained phase (Figure 4). At 10 nM carbachol and below, although there was no characteristic [Ca²⁺]_i peak, there was evidence for the generation of a sustained component but this represented changes of less than 10 nM [Ca²⁺]_i. Changes in [Ca²⁺]_i were dose-dependent and the EC₅₀ values were not significantly different between peak (-6.26 ± 0.16 (0.55 μM) and sustained phases (-6.64 ± 0.22 (0.23 μM)) (Figure 5). Responses to maximal (1 mM) carbachol stimulation were prevented by 10 μM atropine while addition of atropine during the plateau phase returned [Ca²⁺]_i levels to basal (data not shown).

Challenge with bradykinin also resulted in an immediate, concentration dependent, increase in [Ca²⁺]_i with an EC₅₀ of

Figure 3 Time course of accumulation of [3H]-inositol phosphates ([3H]-InsPs) in adherent SH-SY5Y cells in response to 1 mM carbachol (○) and 10 μM bradykinin (●) (a) and the dose-response relationship for carbachol-mediated accumulation of [3H]InsPs after 30 min of agonist exposure (b): EC₅₀ value (mean ± s.e.mean (n) log₁₀ M) of -5.54 ± 0.16 (3) (2.9 μM). Data points are mean ± s.e.mean *n* = 3.

-7.80 ± 0.11 (16 nM) (Figures 6 and 7). There was little evidence for a sustained phase of [Ca²⁺]_i elevation at maximal (1 μM) bradykinin concentrations, basal levels being attained after approximately 1 min (Figure 6). At 130 s following bradykinin addition, the [Ca²⁺]_i was $10.4 \pm 23.3\%$ (16) of the peak response compared to $33.1 \pm 4.1\%$ (7) and $12.6 \pm 1.5\%$ (8) for the sustained carbachol response at normal and low [Ca²⁺]_e respectively. However, at sub-maximal concentrations, particularly 10 nM, the [Ca²⁺]_i response to bradykinin showed a rapid elevation followed by a slow decline such that at 130 s following stimulation (the point for sustained response determination) [Ca²⁺]_i was still elevated above basal but declining (Figure 6 inset). Those populations of cells which did respond to bradykinin at a concentration of 1 nM or below showed very small (5–10 nM) sustained

Figure 4 Representative traces of the time course of [Ca²⁺]_i elevation in populations of adherent SH-SY5Y cells in response to 1 mM carbachol, added at 50 s, at normal (upper peak) or low [Ca²⁺]_e (lower peak). At 180 s Ca²⁺ was added back to cells stimulated at low [Ca²⁺]_e. The bottom trace demonstrates the effect of Ca²⁺ readition to cells incubated, but not stimulated, at low [Ca²⁺]_e.

Figure 5 Dose-response relationships for carbachol mediated elevation of [Ca²⁺]_i in populations of adherent SH-SY5Y cells at peak (●) and sustained phase (■) in the presence of normal [Ca²⁺]_e and peak (○) at low [Ca²⁺]_e with Ca²⁺ readition for determination of sustained phase (□). EC₅₀ values (log₁₀ M, mean ± s.e.mean) for peak and sustained phases were not significantly different whether determined in the presence of normal [Ca²⁺]_e (-6.26 ± 0.16 (0.55 μM) and -6.64 ± 0.22 (0.23 μM) respectively) or at low [Ca²⁺]_e with Ca²⁺ readition (-5.68 ± 0.10 (2.1 μM) and -5.91 ± 0.18 (1.2 μM respectively). All data are mean ± s.e.mean, *n* = 3.

elevations of $[Ca^{2+}]_i$ (data not shown). The response to 10 nM bradykinin was fully inhibited by the bradykinin B_2 receptor antagonist, N-adamantaneacetyl-D-Arg[Hyp³, Thi^{5,8}, D-Phe⁷]-bradykinin at 10 μ M with recovery of response after removal of antagonist by washing (data not shown).

Sensitivity of carbachol- and bradykinin-mediated elevation of $[Ca^{2+}]_i$ to reductions in $[Ca^{2+}]_e$

Basal $[Ca^{2+}]_i$ was 59 ± 10 nM (8) at normal $[Ca^{2+}]_e$. Preincubation of cells in low $[Ca^{2+}]_e$ buffer at 37°C for 5 min prior to agonist challenge had a small but not statistically significant effect on basal $[Ca^{2+}]_i$ (47 ± 7 nM (8)). Subsequently, 1 mM carbachol increased $[Ca^{2+}]_i$ by 221 ± 35 nM (8) representing a numerical but not significant reduction of 38% compared to the response at normal $[Ca^{2+}]_e$ (358 ± 93 nM (7)). The response during the sustained phase was, however, more severely (73%) and significantly ($P < 0.01$) reduced from an elevation of 73 ± 12 nM (8) at normal to 20 ± 5 nM (7) at low $[Ca^{2+}]_e$ (see Figure 4 for example traces). Thus, the

Figure 6 Representative traces of the time course of $[Ca^{2+}]_i$ elevation in populations of adherent SH-SY5Y cells in response to 1 μ M bradykinin in the presence or absence of extracellular Ca^{2+} . At 180 s Ca^{2+} was added back to cells stimulated in its absence. The lower trace demonstrates the effect of Ca^{2+} readdition to cells incubated, but not stimulated, in the absence of extracellular Ca^{2+} . Inset is shown the time course of the response to 10 nM bradykinin in the presence of extracellular Ca^{2+} .

Figure 7 Dose-response relationship for bradykinin-mediated peak elevations of $[Ca^{2+}]_i$ in populations of adherent SH-SY5Y cells. Data are mean $\pm$ s.e.mean, $n = 3-18$. EC_{50} ($\log_{10}M$, mean $\pm$ s.e.mean) -7.80 ± 0.11 (16 nM).

sustained phase of $[Ca^{2+}]_i$ elevation was very dependent upon Ca^{2+} entry. To ensure determination of agonist potency for intracellular Ca^{2+} mobilization, independent of Ca^{2+} entry, the dose-dependence of carbachol elevation of $[Ca^{2+}]_i$ at peak was determined at low $[Ca^{2+}]_e$. In addition, the potency of carbachol for sustained elevation of $[Ca^{2+}]_i$ was evaluated by Ca^{2+} readdition to these cells (Figures 4 and 5). Determined by these methods, the EC_{50} values for peak (-5.68 ± 0.10 (2.1 μ M)) and sustained (-5.91 ± 0.18 (1.2 μ M)) phases were not significantly different from each other or their respective values determined at normal $[Ca^{2+}]_e$ (-6.26 ± 0.16 (0.55 μ M) and -6.64 ± 0.22 (0.23 μ M)) (Figures 4 and 5).

Under conditions of normal $[Ca^{2+}]_e$ a maximally effective concentration of bradykinin (1 μ M) evoked a change in $[Ca^{2+}]_i$ of 137 ± 37 nM (5). At low $[Ca^{2+}]_e$ this was reduced to 86 ± 21 nM (5) representing a numerical but not significant reduction of 37% (see Figure 6 for example traces).

In the continued presence of a maximal carbachol challenge, the readdition of extracellular Ca^{2+} (2 mM) to cells initially challenged at low $[Ca^{2+}]_e$ provoked a sustained increase in $[Ca^{2+}]_i$ (Figure 4) of 168 ± 10 nM (3). This compared to an increase of only 18 ± 1 nM (3) (Figures 4 and 6) following the readdition of extracellular Ca^{2+} to unstimulated cells and 57 ± 7 nM (6) upon readdition to cells challenged with bradykinin (Figure 6).

Comparison of agonist potencies for elevation of Ins(1,4,5) P_3 and $[Ca^{2+}]_i$

The potency of carbachol, as judged from EC_{50} values, was significantly greater for elevation of $[Ca^{2+}]_i$ than Ins(1,4,5) P_3 at both peak (-6.26 ± 0.16 (0.55 μ M) versus -4.63 ± 0.07 (11) (24 μ M) respectively, $P < 0.01$) and sustained (-6.64 ± 0.22 (0.23 μ M) versus -5.37 ± 0.23 (6) (4.3 μ M) respectively, $P < 0.05$) phases (Figure 8). Similarly bradykinin elevated $[Ca^{2+}]_i$ at peak more potently than Ins(1,4,5) P_3 (EC_{50} values of -7.80 ± 0.11 (16 nM) versus -6.58 ± 0.13 (9) (264 nM) respectively, $P < 0.01$) (Figure 8). This potency difference was maintained when peak $[Ca^{2+}]_i$ and Ins(1,4,5) P_3 responses

Figure 8 Comparison of normalized dose-response curves for bradykinin-mediated peak elevation of $[Ca^{2+}]_i$ (●) and inositol 1,4,5-trisphosphate (Ins(1,4,5) P_3) (○) and carbachol-mediated peak elevation of $[Ca^{2+}]_i$ (■) and Ins(1,4,5) P_3 (□). This figure was constructed with data collected from cells stimulated in the presence of extracellular Ca^{2+} (see Figures 2, 5 and 7). The EC_{50} value ($\log_{10}M$, mean $\pm$ s.e.mean) for elevation of $[Ca^{2+}]_i$ was significantly ($P < 0.01$) less than that for elevation of Ins(1,4,5) P_3 for both bradykinin (-7.80 ± 0.11 (16 nM) and -6.58 ± 0.13 (9) (264 nM) respectively) and carbachol (-6.26 ± 0.16 (0.55 μ M) and -4.63 ± 0.07 (11) (24 μ M) respectively).

Figure 9 Comparison of normalized dose-response curves for carbachol-mediated elevation of $[Ca^{2+}]_i$ (●) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) (○) in the absence of extracellular Ca²⁺. Data were taken from Figures 2 and 5. The EC₅₀ value (log₁₀ M, mean $\pm$ s.e.mean) for elevation of $[Ca^{2+}]_i$ (-5.68 ± 0.10 (2.1 μ M)) was significantly ($P < 0.01$) less than that for elevation of Ins(1,4,5)P₃ (-4.59 ± 0.23 (3) (26 μ M)).

were determined for carbachol at low $[Ca^{2+}]_e$ (EC₅₀ values of -5.68 ± 0.10 (2.1 μ M) versus -4.59 ± 0.23 (3) (26 μ M) respectively, $P < 0.05$) (Figure 9).

Discussion

The current work provides new information on the quantitative relationships between Ins(1,4,5)P₃ and Ca²⁺ signalling in a human neuroblastoma possessing muscarinic and bradykinin receptors. It is now accepted that these relationships are complex, with important regulatory features displayed by the signalling pathway and with Ca²⁺ playing a potentially crucial role at several levels (Wojcikiewicz *et al.*, 1993).

Carbachol- and bradykinin-mediated elevation of Ins(1,4,5)P₃ and $[Ca^{2+}]_i$

Stimulation of muscarinic receptors with carbachol confirmed that elevations of Ins(1,4,5)P₃ in SH-SY5Y cells are biphasic, consisting of a rapid peak followed by a lower but sustained phase (Lambert & Nahorski, 1990b; Lambert *et al.*, 1991). These changes are matched both in time and pattern by atropine-blockable elevations of $[Ca^{2+}]_i$, as previously reported in these cells (Akerman, 1989; Lambert & Nahorski, 1990b; Murphy *et al.*, 1991), the parental SK-N-SH cell line (Fisher *et al.*, 1989) and indeed consistent with the pattern of response in many other receptor systems (Putney & Bird, 1993). There is now substantial evidence that peak $[Ca^{2+}]_i$ responses are driven predominantly by the actions of Ins(1,4,5)P₃ on ligand-gated Ca²⁺ channels located on specialised components of the endoplasmic reticulum (Beridge, 1993) while sustained phases result from Ca²⁺ influx, by mechanisms still incompletely understood but almost certainly involving a capacitative entry following store depletion (Putney & Bird, 1993).

The current study also revealed a bradykinin-mediated PLC activation in these cells, in agreement with a preliminary report of $[Ca^{2+}]_i$ elevation by this peptide (Vaughan *et al.*, 1993). Measurement of $[Ca^{2+}]_i$ in fura-2 loaded single cells demonstrated that in many instances both bradykinin and

carbachol were able to elevate $[Ca^{2+}]_i$ in the same cell, indicating co-expression of bradykinin and muscarinic receptors (data not shown). In contrast to the maximal elevations of Ins(1,4,5)P₃ and $[Ca^{2+}]_i$ induced by carbachol, bradykinin produced relatively small peak elevations with little evidence of sustained phases. With the possible exception of NIH3T3 cells (Fu *et al.*, 1988), there is little evidence elsewhere of sustained PLC activation by bradykinin in neuronal or neuronal-like cells, although the decline of some responses may be slower (Fu *et al.*, 1988; Imaizumi *et al.*, 1989; Burgess *et al.*, 1989; Donie & Reiser, 1991; Iredale *et al.*, 1992). It is unknown whether this may be related, for example, to differences in the level of receptor expression. Although the significance and mechanism of the slight pertussis toxin sensitivity of peak Ins(1,4,5)P₃ responses to carbachol are unclear, the pertussis toxin insensitivity of the Ins(1,4,5)P₃ response to bradykinin in the present study is consistent with that in NG108-15 cells (Fu *et al.*, 1988). This suggests a possible shared pool of G_{q/11} between muscarinic M₃ and bradykinin receptors in SH-SY5Y cells which may have consequences for their simultaneous stimulation. The B₂ bradykinin receptor is likely to mediate PLC activation in SH-SY5Y cells given that neuronal bradykinin receptors are predominantly B₂ (see Wolsing & Rosenbaum, 1991), its established linkage to PLC activation (Derian & Moskowitz, 1986; Eggerickx *et al.*, 1992) and the inhibition of $[Ca^{2+}]_i$ responses by B₂ antagonists in this and a previous study (Vaughan *et al.*, 1993).

Sensitivity of carbachol- and bradykinin-mediated elevation of Ins(1,4,5)P₃ and $[Ca^{2+}]_i$ to reduction of $[Ca^{2+}]_e$

Accumulating evidence strongly suggests that the activity of many PLC isoforms may be regulated by the prevailing $[Ca^{2+}]_e$ (Rhee & Choi, 1992). In the current study, while both phases of Ins(1,4,5)P₃ accumulation displayed some sensitivity to reductions in $[Ca^{2+}]_e$, that of the sustained phase was clearly greater than that of the peak as previously demonstrated for maximal muscarinic receptor stimulation of these cells (Lambert & Nahorski, 1990b). We also demonstrate here a reduction in basal levels of Ins(1,4,5)P₃ at low $[Ca^{2+}]_e$ and a reduction in peak elevations of similar absolute size, approximately equivalent to the basal reduction, over the entire carbachol dose-response curve. Despite the clear dependence of sustained Ins(1,4,5)P₃ generation on extracellular Ca²⁺, this was not absolute as demonstrated by the dose-related elevation at low $[Ca^{2+}]_e$. This sustained elevation to maximal carbachol stimulation was not reduced further when $[Ca^{2+}]_e$ was buffered to approximately 100 nM with EGTA (data not shown) and is consistent with continued accumulation of [³H]-InsPs during a 5 min maximal carbachol stimulation of SH-SY5Y cells at 100 nM $[Ca^{2+}]_e$ (Wojcikiewicz *et al.*, 1994). Peak bradykinin-mediated Ins(1,4,5)P₃ accumulation was proportionately more sensitive to low $[Ca^{2+}]_e$ than that of carbachol. This marked sensitivity is similar to that reported for bradykinin stimulation in NIH3T3 and NG108-5 cells (Fu *et al.*, 1988) although that effect required EGTA buffering of Ca²⁺. In the current study, the absolute reductions in Ins(1,4,5)P₃ accumulations were, however, similar for both agonists, suggesting a consistent amount of PLC activity sensitive to reductions in $[Ca^{2+}]_e$ independent of the extent of agonist stimulation.

These data argue very strongly for a role of changes in $[Ca^{2+}]_i$ regulating both basal and agonist stimulated PLC activity. The greater sensitivity of the sustained compared to the peak response to carbachol to low $[Ca^{2+}]_e$ suggests either that Ca²⁺ regulation of PLC β becomes greater during prolonged activation or that a different PLC isoform is involved. Alternatively the replenishment of substrate required to maintain the response may be Ca²⁺-sensitive or the rapid mobilization of Ca²⁺ from intracellular stores may, through a feed-forward activation of PLC (Wojcikiewicz *et al.*, 1994) be

sufficient to mask any requirement for extracellular Ca²⁺ during the peak of Ins(1,4,5)P₃ generation. Such differences may account for the greater potency of the sustained phase compared to peak carbachol-mediated Ins(1,4,5)P₃ generation demonstrated here and reported recently for these cells and CHO cells expressing the cloned human muscarinic m3 receptor (Wojcikiewicz *et al.*, 1994).

Interestingly, in the present study, the addition of buffer alone containing 2 mM Ca²⁺ provoked a small increase in Ins(1,4,5)P₃ which appeared to be totally dependent upon extracellular Ca²⁺. Whether this truly reflects Ca²⁺ activation of PLC and whether it is a consequence of, for example, shear stress or damage to the cells is unclear.

Peak and sustained [Ca²⁺]_i elevations following muscarinic receptor activation, while displaying similar agonist potencies, also displayed a differential sensitivity to reductions in [Ca²⁺]_e with the sustained phase being more greatly affected. Although peak elevations of [Ca²⁺]_i to both carbachol and bradykinin were not significantly affected by low [Ca²⁺]_e, it should be noted that the estimates of difference are based upon peak heights only and differences in areas under the [Ca²⁺]_i versus time plots are more pronounced. This might suggest that either a proportion of intracellular Ca²⁺ stores display marked sensitivity to reduced [Ca²⁺]_e or alternatively that Ca²⁺ entry, initiated either directly or in response to Ca²⁺-store depletion (Putney & Bird, 1993) may contribute to the initial elevation of [Ca²⁺]_i by both agonists. Indeed early Ca²⁺ entry in response to carbachol in SH-SY5Y cells is consistent with an immediate quench of intracellular fura-2 fluorescence by Mn²⁺ when used to replace external Ca²⁺ (Lambert & Nahorski, 1990b).

Receptor desensitization and utility of measurement of [³H]-InsPs accumulation for determination of agonist-mediated PLC activation

The transient nature of [Ca²⁺]_i and particularly Ins(1,4,5)P₃ peak elevations following both carbachol and bradykinin imply desensitization of receptor/G-protein/PLC coupling. Given the biphasic nature of responses to carbachol compared with monophasic responses to bradykinin, desensitization of the muscarinic receptor must be partial while that of the bradykinin receptor may be complete or near complete. Interestingly the readdition of extracellular Ca²⁺ to cells challenged with bradykinin produced a sustained elevation of [Ca²⁺]_i apparently greater than that when added to cells incubated at low [Ca²⁺]_e but not challenged with agonist. Given the rapid return of [Ca²⁺]_i to approximately basal levels after bradykinin addition in the presence of extracellular Ca²⁺, the reason for this is unclear. Whether it is a consequence of bradykinin stimulation under non-physiological conditions which may, for example, reset the Ca²⁺ homeostatic mechanism or whether this truly reflects a persistent bradykinin-mediated Ca²⁺ entry via the same receptor that mediates PLC activation requires further study.

Mechanisms underlying rapid desensitization phenomena are not established but have the potential to include phosphorylation of the receptor (Wojcikiewicz *et al.*, 1993). This is analogous to the β -adrenoceptor kinase (β -ARK) mediating phosphorylation and desensitization of the β -adrenoceptor (Hausdorff *et al.*, 1990). Indeed, phosphorylation of a number of PLC-linked receptors has been reported (Klupepelberg *et al.*, 1992; Kwatra *et al.*, 1993) including that of the m3 receptor by PKC and an additional kinase distinct from either protein kinase A (PKA), Ca²⁺-calmodulin-dependent kinase or β -ARK (Tobin & Nahorski, 1993; Tobin *et al.*, 1993).

That desensitization of the bradykinin response in SH-SY5Y was not due to enhanced metabolism of Ins(1,4,5)P₃ was demonstrated by the lack of continued accumulation of [³H]-InsPs following the initial rapid but small increase in cells where inositol monophosphatase was uncompetitively blocked by lithium. Thus, when there is no sustained

Ins(1,4,5)P₃ generation and particularly when the peak response is small, measurement of accumulation of [³H]-InsPs may lack the required sensitivity for demonstration of PLC activation. Indeed this may account for previous reports of no bradykinin mediated PLC activation in either the parental SK-N-SH (Yu & Sadee, 1986) or SH-SY5Y cell lines (Ogino & Costa, 1993). The biphasic nature of the [³H]-InsPs responses in the present study are consistent with the pattern in AR4-2J pancreatoma cells stimulated with either bombesin or cholecystokinin (Menniti *et al.*, 1991) and our previous study of SH-SY5Y cells stimulated with carbachol (Wojcikiewicz *et al.*, 1994) where there were falls in the rates of accumulation following 20–30 s of exposure. Many studies have used the measurement of accumulation of [³H]-InsPs in lithium-blocked cells generally employing relatively long periods of agonist exposure and assumed linearity of accumulation over tens of minutes to reflect a lack of desensitization (Wojcikiewicz *et al.*, 1993). However, more detailed analysis may reveal biphasic accumulations where the change in rate may reflect the biphasic nature of the Ins(1,4,5)P₃ response. We have argued (Wojcikiewicz *et al.*, 1993) that this reflects a rapid desensitization of receptor-G_q-PLC coupling. Thus, although measurement of accumulation of [³H]-InsPs at extremely short time points (<30 s) may reflect initial levels of PLC activity, most studies will measure predominantly PLC activity during the sustained phase of Ins(1,4,5)P₃ generation. This is emphasized by the similarity of the EC₅₀ values for sustained carbachol-mediated Ins(1,4,5)P₃ generation and accumulation of [³H]-InsPs and their greater potency relative to peak Ins(1,4,5)P₃ generation shown here. Thus, early desensitization events may be missed and either different PLC isoforms may be active or there could be a differing inositol lipid substrate profile during prolonged activation. In addition to these pitfalls, measurement of [³H]-InsPs will also reflect the Ca²⁺ sensitivity of the sustained but not the peak phase of Ins(1,4,5)P₃ generation, which may differ. Thus, although several studies have determined muscarinic receptor activation of PLC by this method in SK-N-SH (Yu & Sadee, 1986; Fisher & Heacock, 1988; Fisher *et al.*, 1989; Baird *et al.*, 1989) and SH-SY5Y cells (Lambert & Nahorski, 1990b; Lambert *et al.*, 1991) the substantial sensitivity to reductions in [Ca²⁺]_e reported in SK-N-SH cells (Fisher *et al.*, 1989) is likely to reflect the sensitivity of a partially desensitized receptor.

Comparison of agonist potencies for elevation of Ins(1,4,5)P₃ and [Ca²⁺]_i

The present study has also revealed that the potencies of both carbachol and bradykinin for the peak elevation of [Ca²⁺]_i were substantially greater than those for peak Ins(1,4,5)P₃ elevation. This is unlikely to result from an enhanced PLC activation due to Ca²⁺ entry as the difference existed when responses to carbachol were determined at low [Ca²⁺]_e when little or no Ca²⁺ influx would occur. One potential difficulty in the interpretation of these data is that the magnitude of both Ins(1,4,5)P₃ and [Ca²⁺]_i elevations are a consequence of mechanisms designed to both elevate and decrease these variables. Thus, Ins(1,4,5)P₃ elevation will be limited by metabolism to both higher and lower inositol phosphates, while [Ca²⁺]_i elevation may be limited by buffering, uptake and extrusion systems. However, such differences in agonist potency are in accord with the amplification within PLC-linked systems such that dose-response curves for agonist-mediated PLC activation, as assessed by accumulation of [³H]-InsPs in lithium-blocked cells, generally lie to the left of receptor occupation curves and to the right of downstream events such as smooth muscle contraction (Kenakin, 1987). The present study and a previous demonstration of a 33 fold greater potency for fMet-Leu-Phe stimulation of Ca²⁺ mobilization compared to Ins(1,4,5)P₃ accumulation in neutrophils at low [Ca²⁺]_e (Thompson *et al.*, 1991) suggests that a potency difference

does exist during acute stimulation of fully sensitized receptors. This is likely, therefore, to reflect a true amplification step in the phosphoinositide pathway. Thus, the observed difference in agonist potency for Ins(1,4,5)P₃ and [Ca²⁺]_i elevation is an expected phenomenon, defining a site of amplification and allowing consideration of the mechanisms involved.

Mechanism for amplification within the phosphoinositide signal transduction pathway

There is evidence from single cells that agonist-mediated elevation of [Ca²⁺]_i is an 'all-or-none' response (Bootman *et al.*, 1992; Shao & McCarthy, 1993). The amplitude of agonist-mediated [Ca²⁺]_i elevation within a single cell may not, therefore, be proportionately related to the concentration of agonist. Thus [Ca²⁺]_i elevation will or will not occur at a given agonist concentration and this concentration may differ between cells of the same population. A consequence of this is that concentration-effect curves for agonist-mediated elevation of [Ca²⁺]_i in cell populations would represent cell recruitment profiles with more cells responding at higher agonist concentrations. Generation of Ins(1,4,5)P₃ above that required to trigger the [Ca²⁺]_i response of a single cell may therefore play no part in Ca²⁺ signalling and represent a spare capacity within the system. This is supported by the observation of the full Ca²⁺ mobilization at sub-maximal Ins(1,4,5)P₃ levels in populations of cells in the current study. The precise mechanisms underlying potential all-or-none [Ca²⁺]_i responses in single cells are unclear. Such responses may be a simple reflection of a threshold level of Ins(1,4,5)P₃ being attained with subsequent emptying of all of the Ca²⁺ from the rapidly releasable pool. If, however, heterogeneity of Ins(1,4,5)P₃ receptor sensitivity exists within a cell (see Oldershaw *et al.*, 1991; Bootman *et al.*, 1992), this would suggest cooperativity of the Ca²⁺ release process, such that the release of a small amount of Ca²⁺ causes the release of all available Ca²⁺. This may be via Ca²⁺-induced Ca²⁺

release whereby the release of a small amount of Ca²⁺ initiates the release of further Ca²⁺ from ryanodine-sensitive stores (see Putney & Bird, 1993). Alternatively the release of a small amount of Ca²⁺ may sensitize Ins(1,4,5)P₃ receptors such that release escalates at the same Ins(1,4,5)P₃ concentration until the elevated [Ca²⁺]_i inhibits further Ins(1,4,5)P₃ receptor activation (Bezprozvanny *et al.*, 1991).

Thus, the greater agonist potency for elevation of [Ca²⁺]_i relative to that of Ins(1,4,5)P₃ could occur as a consequence of all-or-none [Ca²⁺]_i responses in single cells mediated either by threshold levels of Ins(1,4,5)P₃ or the feed-forward interactions described. These feed-forward interactions could also account for the observed potency difference if agonist-mediated [Ca²⁺]_i elevation were graded as opposed to an all-or-none phenomenon. Whatever the mechanism, the potency difference defines a step which features as an important amplification stage within the signal transduction pathway.

Summary

In summary, this study has provided a detailed evaluation of the time- and concentration-dependent aspects of polyphosphoinositide and Ca²⁺ signalling by muscarinic and bradykinin receptor stimulation of the human SH-SY5Y neuroblastoma cell line. Clear differences in the ability of agonists to activate PLC and mobilize intracellular Ca²⁺ are revealed and the complexities of Ca²⁺ entry in these responses are highlighted. Finally, rapid regulatory features of this signalling pathway indicate that the experimental approaches for determining agonist-stimulated PLC activity require careful evaluation.

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