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Complex relationship between Ins(1,4,5)P_3 accumulation and Ca^{2+}-signalling in a human neuroblastoma revealed by cellular differentiation

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1 Differentiation of SH-SY5Y neuroblastoma cells induces morphological and biochemical changes consistent with a more neuronal phenotype. These cells may therefore provide a model for studying phenomena such as signal transduction in a neuronal context whilst retaining the advantages of a homogenous cell population expressing a well characterized array of G-protein coupled receptors.

2 This study examined the effects of differentiating SH-SY5Y cells on muscarinic- and bradykinin-receptor-mediated phosphoinositide and Ca^{2+} signalling. Retinoic acid (10 μM, 6 days) along with a lowered serum concentration produced phenotypic changes consistent with differentiation including reduced proliferation and increased neurite outgrowth.

3 Differentiation increased the magnitude and potency of rapid Ins(1,4,5)P_3 responses to a full muscarinic receptor agonist. Bradykinin receptor-mediated Ins(1,4,5)P_3 signalling was also potentiated following differentiation. Determination of agonist-evoked accumulation of [^{3}H]-inositol phosphates under lithium-block demonstrated these changes reflected enhanced phospholipase C activity which is consistent with observed increases in the expression of muscarinic and bradykinin receptors.

4 Despite the marked alterations in Ins(1,4,5)P_3 signalling following differentiation, elevations of intracellular [Ca^{2+}] were totally unaltered. Thus, in SH-SY5Y cells, the relationship between the elevations of Ins(1,4,5)P_3 and intracellular [Ca^{2+}] is agonist dependent and affected by the state of differentiation. This demonstrates that mechanisms other than the measured increase in Ins(1,4,5)P_3 regulate the elevation of intracellular [Ca^{2+}].

Keywords: SH-SY5Y; G-protein coupled receptor; Ins(1,4,5)P_3; Ca^{2+} signalling

Abbreviations: $[Ca^{2+}]_i$, intracellular [Ca^{2+}]; [^{3}H]-InsPs, total [^{3}H] inositol phosphates; [^{3}H]-NMS, 1-[N-methyl-^{3}H]scopolamine methyl chloride; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; PLC, phosphoinositide-specific phospholipase C; SH-SY5Y_{RA6} cells, SH-SY5Y cells treated for 6 days with 10 μM retinoic acid

Introduction

A large number of agonists, upon binding to their cell surface receptors, stimulate the activity of phosphoinositide-specific phospholipase C (PLC), with the resultant formation of the second messenger molecules inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (Berridge, 1993). Whilst Ins(1,4,5)P_3 releases Ca^{2+} from intracellular Ca^{2+} stores, diacylglycerol activates various isoforms of protein kinase C (PKC) thereby linking receptor activation to a wide range of cellular responses. More than 100 receptor types have so far been demonstrated to be linked to activation of the phosphoinositide signalling pathway (Kenakin, 1996) which therefore represents a key regulatory influence on cellular function.

We have been interested in both the activation and regulation of this phosphoinositide signalling pathway, particularly in a neuronal context and have examined this extensively in a human neuroblastoma cell line, SH-SY5Y. This cell line displays many of the characteristics of human foetal sympathetic ganglion cells (Ross *et al*., 1983) and would seem, therefore, a suitable model system for such studies. Activation of endogenously expressed muscarinic M_3 or bradykinin B_2 receptors of these cells results in a robust elevation of the intracellular [Ca^{2+}] ($[Ca^{2+}]_i$) (Fisher *et al*., 1989; Lambert & Nahorski, 1990; Lambert *et al*., 1990; Wojcikiewicz *et al*., 1994; Willars & Nahorski, 1995a,b; Willars *et al*., 1996; McDonald *et al*., 1994) and, at least for muscarinic receptor stimulation, also results in the activation of PKC (Willars *et al*., 1996). The temporal pattern of inositol phosphate and Ca^{2+} signalling in these cells is consistent with that mediated by activation of M_3 muscarinic receptors expressed as recombinant proteins in other cell types including fibroblasts (Wojcikiewicz *et al*., 1993). There is, however, evidence to suggest that the activation and regulation of phosphoinositide and Ca^{2+} signalling may differ in 'true' neuronal cells. Thus, in primary cultures of rat superior cervical ganglion cells, activation of PLC-coupled muscarinic (M_1) receptors does not provoke an elevation of $[Ca^{2+}]_i$ (Marsh *et al*., 1995). Indeed the biological effects of muscarinic receptor-mediated PLC activation appear to be *via* PKC whilst alterations in $[Ca^{2+}]_i$ are primarily a consequence of cellular depolarization following activation of ionotropic receptors. It is conceivable, therefore, that the balance between the Ca^{2+} and PKC limbs of the signalling pathway and indeed their regulation are different in true neuronal cells compared to that seen in many model cell lines.

SH-SY5Y cells (and other neuroblastomas) can be differentiated morphologically and biochemically to a more neuronal-like cell when cultured in the presence of low concentrations of retinoic acid (Pahlman *et al*., 1984; Adem

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et al., 1987; Kaplan *et al*., 1993). This differentiation, marked phenotypically by neurite extension, is also accompanied by an increased electrical excitability of cell membranes (Perez-Polo *et al*., 1979), an increase in noradrenaline content (Pahlman *et al*., 1984) and alterations in the expression of distinct G-protein subunits (Ammer & Schulze, 1994). Given the more neuronal phenotype displayed by differentiated SH-SY5Y cells, the current study was undertaken to examine the influence of retinoic acid-induced differentiation on phosphoinositide and Ca^{2+} signalling following activation of muscarinic M_3 and bradykinin B_2 receptors. Aspects of this work have previously been published as an abstract (Martin *et al*., 1997).

Methods

Cell culture

SH-SY5Y cells, passages 15–25, were maintained in Minimum Essential Medium supplemented with 5% newborn calf serum, 5% foetal calf serum, 2 mM glutamine, 100 iu ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} fungizone in 5% CO_2/humidified air at 37°C and fed or refed on alternate days. Cultures were passaged weekly, at which point they were also either seeded onto 24-well multidishes for subsequent determination of Ins(1,4,5)P_3 generation and the accumulation of total [^{3}H] inositol phosphates ([^{3}H]-InsPs) or seeded onto 22×11 mm glass coverslips for determination of $[Ca^{2+}]_i$. All experimental manipulations with viable cells were performed at 37°C.

Differentiation

Following seeding as described above and an overnight incubation to allow for cell attachment, differentiation was initiated by replacing the media with one containing 10 μM retinoic acid and 1% heat-inactivated foetal calf serum rather than the 10% serum described above. Cells were cultured for a further 6 days before use (SH-SY5Y_{RA6} cells) with media change every 2 days. Control (undifferentiated) cells were grown in media containing the vehicle for retinoic acid (0.1% ethanol v v^{-1}) for 24 h before use (SH-SY5Y cells). Prolonged exposure to ethanol was avoided as this has previously been reported to induce differentiation of these cells (Larsson *et al*., 1996).

Ins(1,4,5)P_3 mass measurement

Determination of Ins(1,4,5)P_3 mass was by a modification of a radioreceptor assay previously characterized for stereo- and positional-specificity (Challiss *et al*., 1990) and described in detail elsewhere (Willars & Nahorski, 1995a,b). Ins(1,4,5)P_3 was referenced to total cellular protein (Lowry *et al*., 1951) in 0.1 M NaOH digests of cell monolayers.

Measurement of [^{3}H]-inositol phosphate formation

Cells on 24-well multidishes were prelabelled for 48 h with 3 μCi ml^{-1} of [^{3}H]-inositol (73 Ci $mmol^{-1}$). Cells were then washed twice with 1 ml of Krebs-HEPES buffer (pH 7.4, composition (mM): HEPES 10, $NaHCO_3$ 4.2, glucose 11.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, KCl 4.7, NaCl 118 and $CaCl_2$ 1.3). Following a 15 min incubation in 400 μl Krebs-HEPES buffer containing 12.5 mM LiCl, a further 100 μl of buffer containing agonist was added (final LiCl concentration 10 mM). The reaction was terminated by the addition of 500 μl 1 M trichloroacetic acid. After 15 min on ice, 800 μl aliquots were taken and 200 μl of 10 mM EDTA added followed by 1 ml of a 1:1 (v v^{-1}) mixture of tri-n-octylamine and 1,1,2-trichloro-trifluoro-ethane. After vortexing, 800 μl of the upper aqueous phase was taken and neutralized with 50 μl of 250 mM $NaHCO_3$. Samples were stored at 4°C for <2 weeks. Samples were applied to a Dowex AG1X8, 200–400 mesh column which was washed with 10 ml of 25 mM ammonium formate. Inositol phosphates were eluted with 10 ml of 1 M ammonium formate/0.1 M formic acid (Baird *et al*., 1989). A 5 ml aliquot of this was counted by liquid scintillation spectrometry.

Measurement of [^{3}H]-NMS binding

[^{3}H]-NMS binding was measured as previously described (Lambert *et al*., 1989). Briefly, equilibrium binding assays were performed in a total volume of 200 μl at 37°C for 60 min. Cell membranes (approximately 100 μg of protein per tube) were incubated with increasing concentrations of [^{3}H]-NMS (0.07–6 nM) in assay buffer (MgCl 1 mM, HEPES 10 mM, pH 7.5). Non-specific binding was determined in the presence of 1 μM atropine. The reaction was terminated by separating bound and free ligand by rapid vacuum filtration onto Whatman GF/B filters with 2×5 ml washes of ice-cold assay buffer. Radioactivity on filters was detected by liquid scintillation spectrometry.

Measurement of [^{3}H]-bradykinin binding

[^{3}H]-bradykinnin binding was measured by modification of a previously described method (Liebmann *et al*., 1994). Equilibrium binding assays were performed in a total volume of 200 μl at 4°C for 60 min. Washed membranes (approximately 70 μg of protein per incubation) were incubated with increasing concentrations of [^{3}H]-bradykinin (0.02–3.0 nM) in buffer (TES 25 mM, 1,10-phenathroline 1 mM, bacitracin 140 μg ml^{-1}, captopril 10 μM, dithiothreitol 1 mM, bovine serum albumin 0.1%, GTP 100 μM, pH 6.8). The reaction was terminated by separating bound and free ligand by rapid filtration onto Whatman GF/B filters with 2×5 ml washes of ice cold 10 mM TES buffer, pH 6.8. Radioactivity on filters was detected by liquid scintillation spectrometry.

$[Ca^{2+}]_i$ measurement

The majority of experiments measuring $[Ca^{2+}]_i$ were performed using a ratiometric method in fura-2 loaded adherent populations of cells. Experiments examining the possible role of ryanodine receptors in muscarinic receptor-mediated responses were carried out on single cells. These methods are described in detail elsewhere (Willars & Nahorski, 1995a,b).

Materials

Tissue culture media and culture flasks were from GIBCO, multidishes were from Nunc. Fura-2-AM and ionomycin free acid were from Calibiochem (Nottingham, U.K.). D-*myo*-Ins(1,4,5)P_3 was obtained from University of Rhode Island Foundation, U.S.A. *myo*-[^{3}H]-Inositol and [^{3}H]-NMS, were from Amersham International (Little Chalfont, Bucks., U.K.). D-*myo*-[^{3}H]-Ins(1,4,5)P_3 and [^{3}H]-bradykinin were from DuPont NEN (Stevenage, U.K.). Tri-n-octylamine, 1,1,2-trichloro-trifluoro-ethane, retinoic acid, HEPES, Tris-HCl, Tween-20, atropine, methacholine, bradykinin, EGTA and EDTA were from Sigma (Poole, U.K.). Emulsifier Safe Scintillation Cocktail was from Packard (Groningen, Netherlands). All other reagents were supplied by Fisons (U.K.).

Data analysis

Dose-response curves were fitted by Graph-PAD PRISM (Graph Pad Software, Inc, San Diego, CA, U.S.A.) using a four parameter logistic equation with equal weighting to each point. EC_{50} mean values and associated s.e.means were generated from the $\log_{10}$ EC_{50} values generated from separate curves of at least three individual experiments. All EC_{50} values are given as $\log_{10}$ M. Statistical comparisons were by Student's two-tailed *t*-test or two-way analysis of variance where appropriate. Acceptance of significance for all tests was at $P<0.05$. Data are presented as means$\pm$s.e.mean with the number of separate experiments given in parentheses.

Results

Incubation of SH-SY5Y cells for 6 days in retinoic acid (10 μM) produced changes characteristic of cellular differentiation including cessation of cell division as judged by failure of the cells to increase coverage and by the expression of a more neuronal phenotype including a marked increase in neurite extension and the appearance of growth cones (data not shown). For the purposes of this paper, SH-SY5Y_{RA6} cells will also be referred to as differentiated cells.

Methacholine- and bradykinin-mediated Ins(1,4,5)P_3 accumulation

Maximal concentrations of methacholine (1 mM) evoked a biphasic elevation of Ins(1,4,5)P_3 in SH-SY5Y cells consisting of a rapid (10 s) peak followed by a lower but sustained phase (Figure 1). In SH-SY5Y_{RA6} cells the peak was significantly ($P<0.01$) greater (approximately 1.5 fold) compared to SH-SY5Y cells (Figure 1). The addition of buffer alone (i.e. without agonist) to SH-SY5Y cells provoked a minor elevation of Ins(1,4,5)P_3 (13 ± 6 (3) pmol mg protein^{-1}) which was maximal by 5 s and declined to basal levels by approximately 30 s. The addition of bradykinin (10 μM) evoked a small Ins(1,4,5)P_3 response in SH-SY5Y cells (Figure 1) which was numerically but not significantly greater than that evoked by addition of buffer alone. This response also peaked at 5 s and then declined to basal levels by approximately 30 s. Treatment of cells with retinoic acid significantly ($P<0.01$) increased the peak response to bradykinin (Figure 1) but had no effect on the response to buffer alone (data not shown). The peak Ins(1,4,5)P_3 accumulation in response to methacholine in SH-SY5Y cells was concentration-dependent with an EC_{50} value of -4.96 ± 0.03 (3) (11 μM) in SH-SY5Y cells. Retinoic acid treatment significantly ($P<0.01$) decreased the EC_{50} value to -5.36 ± 0.07 (3) (4 μM) (Figure 2).

Treatment of cells with nerve growth factor (5 days, 10 nM with 1% heat-inactivated foetal calf serum (as above)) also produced phenotypic changes consistent with differentiation. This protocol also significantly ($P<0.05$) increased both the potency (EC_{50} -5.11 ± 0.04 (3) versus -4.96 ± 0.03 (3)) and magnitude of peak Ins(1,4,5)P_3 responses to methacholine (responses to a maximal (1 mM) concentration of methacholine: 180.3 ± 4.7 (7) versus 107.6 ± 3.2 (5)).

It was not possible to determine an EC_{50} for bradykinin-mediated Ins(1,4,5)P_3 accumulation in SH-SY5Y cells due to the small magnitude of the response. However, peak Ins(1,4,5)P_3 accumulations in response to bradykinin in SH-SY5Y_{RA6} were concentration-dependent with an EC_{50} of -6.54 ± 0.05 (3) (290 nM).

Methacholine- and bradykinin-mediated accumulation of [^{3}H]-InsPs

To investigate whether the increase in methacholine- and bradykinin-mediated peak Ins(1,4,5)P_3 accumulation following retinoic acid treatment was the result of either enhanced PLC activity or a consequence of reduced metabolism of Ins(1,4,5)P_3 (Wojcikiewicz *et al*., 1993) we measured the accumulation of [^{3}H]-InsPs in cells in which inositol monophosphatase was blocked by lithium. A maximal concentration of methacholine (1 mM) caused a biphasic accumulation of [^{3}H]-InsPs, being more rapid over the initial 60 s compared with the linear accumulation between 1 min and 25 min. At 25 min the accumulation of [^{3}H]-InsPs in SH-SY5Y_{RA6} cells was significantly ($P<0.05$) greater than that in SH-SY5Y cells (Figure 3a). In both SH-SY5Y and SH-SY5Y_{RA6} cells bradykinin (10 μM) increased [^{3}H]-InsPs accumulation over the first minute but then showed no further increase over the subsequent 24 min. The incorporation of 1 mM captopril to inhibit or reduce the potential metabolism of bradykinin by angiotensin converting enzyme had no effect on the magnitude or profile of these accumulations in SH-SY5Y cells (data not

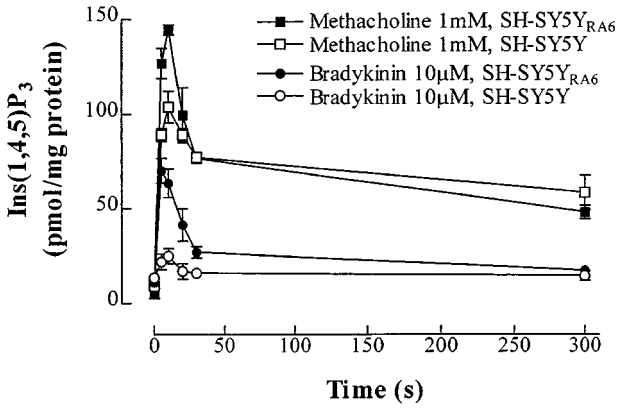

Figure 1 Time courses of agonist-mediated Ins(1,4,5)P_3 accumulation. Adherent populations of SH-SY5Y or SH-SY5Y_{RA6} cells were challenged with 1 mM methacholine or 10 μM bradykinin for the indicated times. Data are means$\pm$s.e.mean, $n=3$.

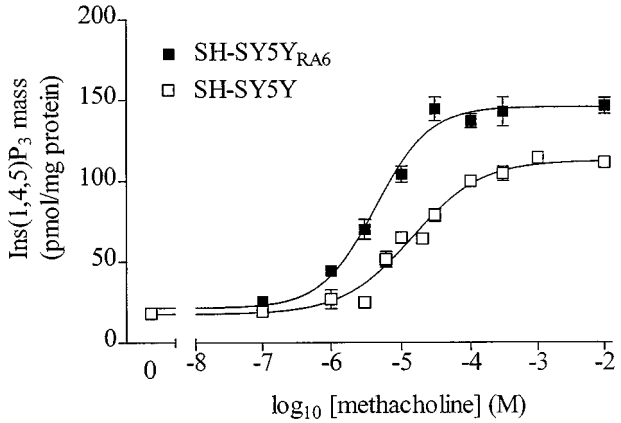

Figure 2 Concentration-response relationships of agonist-mediated Ins(1,4,5)P_3 accumulation. Methacholine-mediated peak changes in Ins(1,4,5)P_3 mass in adherent SH-SY5Y or SH-SY5Y_{RA6} cells. Data are mean$\pm$s.e.mean, $n=3$. EC_{50} values ($\log_{10}$ M, mean$\pm$s.e.mean) for methacholine were -4.96 ± 0.03 (11 μM) and -5.36 ± 0.07 (4.4 μM) in SH-SY5Y and SH-SY5Y_{RA6} cells respectively ($P<0.01$).

shown). The accumulation of [^{3}H]-InsPs in response to bradykinin was greater in SH-SY5Y$_{RA6}$ cells compared with SH-SY5Y cells ($P=0.019$, two-way analysis of variance) (Figure 3b). The addition of buffer alone to either SH-SY5Y or SH-SY5Y$_{RA6}$ cells did not stimulate [^{3}H]-InsPs accumulation (Figure 3b). Taken together, these data indicate that the enhanced Ins(1,4,5)P_3 responses following agonist occupation of either muscarinic- or bradykinin-receptors truly reflected enhanced PLC activation and was not simply a consequence of alterations in the metabolism of Ins(1,4,5)P_3.

Muscarinic- and bradykinin-receptor expression

Specific binding of the muscarinic antagonist [^{3}H]-NMS to membranes from SH-SY5Y and SH-SY5Y$_{RA6}$ cells was saturable. Muscarinic receptor number was significantly ($P<0.01$) increased following retinoic acid treatment as assessed by the B_{max} values (272±11 (4) versus 529±22 (4) fmol mg protein^{-1}) although the K_D values were unaffected (−9.23±0.03 M (4) (0.59 nM) versus −9.31±0.28 M (4) (0.49 nM)). Western blot analysis using an anti-M_3 polyclonal antibody (Tobin & Nahorski, 1993) also indicated that retinoic acid treatment resulted in an approximate doubling of muscarinic M_3 receptor protein (data not shown). Blots using a specific muscarinic M_1 protein antibody (Waugh *et al.*, 1995) failed to detect any M_1 receptor immunoreactivity in either undifferentiated or differentiated cells. Using a CHO cell line expressing recombinant human muscarinic M_1 receptors we have estimated the lower limit of detection using this antibody to be approximately 10 fmol mg protein^{-1} (data not shown).

Specific binding of [^{3}H]-bradykinin in the presence of GTP to membranes from SH-SY5Y and SH-SY5Y$_{RA6}$ cells was also saturable. The number of bradykinin receptors were significantly ($P<0.05$) increased following retinoic acid treatment as assessed by the B_{max} values (300±21 (4) versus 519±44 (4) fmol mg protein^{-1}) although the K_D values were unaffected (−9.74±0.03 M (4) (0.18 nM) versus −9.70±0.08 M (4) (0.2 nM). Although the K_D for binding of [^{3}H]-bradykinin was less than the EC_{50} for the measured functional responses, binding was determined under different conditions to the functional responses and may not therefore represent the true binding affinity in intact cells.

Methacholine- and bradykinin-mediated elevations of $[Ca^{2+}]_i$

SH-SY5Y cells has a basal $[Ca^{2+}]_i$ level of 98±4 nM (7). Stimulation with 1 mM methacholine produced a rapid peak of $[Ca^{2+}]_i$ (727±30 nM (7)) followed by a sustained phase (198±9 nM (7)) (Figure 4). Challenge of SH-SY5Y cells with 10 μM bradykinin also resulted in a peak $[Ca^{2+}]_i$ elevation (535±41 nM)) although basal levels were regained after approximately 1 min (Figure 4) indicating a lack of a sustained $[Ca^{2+}]_i$ elevation. Treatment of cells with retinoic acid had no effect on the magnitude or time course of peak $[Ca^{2+}]_i$ elevations in response to maximal concentrations of either methacholine or bradykinin.

Peak $[Ca^{2+}]_i$ elevations in response to methacholine or bradykinin in SH-SY5Y cells were concentration-dependent with EC_{50} values of −5.59±0.19 (7) (2.6 μM) and −7.65±0.2 (3) (22 nM) respectively. Treatment of cells with retinoic acid had no effect on agonist potency (Figure 5a,b).

Preincubation of SH-SY5Y cells in nominally Ca^{2+}-free buffer (Ca^{2+} excluded from the buffer) for 5 min prior to agonist challenge had no significant effect on maximal methacholine or bradykinin mediated peak $[Ca^{2+}]_i$ elevations although the sustained response to methacholine was abolished (data not shown).

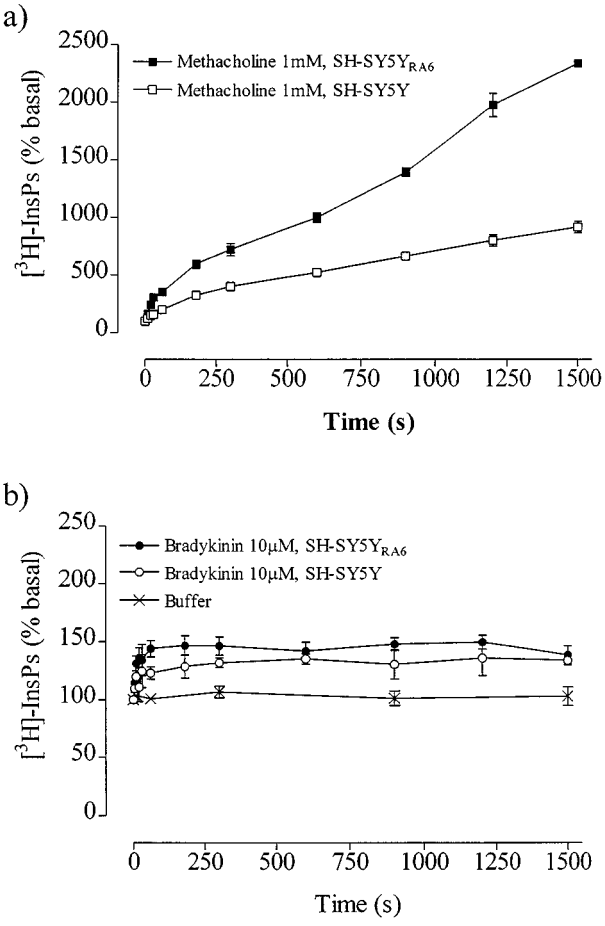

Figure 3 Time courses of agonist-mediated accumulation of [^{3}H]-InsPs in cells in which inositol monophosphatase activity was blocked with 10 mM Li^+. (a) SH-SY5Y or SH-SY5Y$_{RA6}$ cells were challenged with 1 mM methacholine for the indicated times. (b) SH-SY5Y or SH-SY5Y$_{RA6}$ cells were challenged with 10 μM bradykinin for the indicated times. The lack of effect of buffer addition alone is also shown. All data are means±s.e.mean, $n=3$.

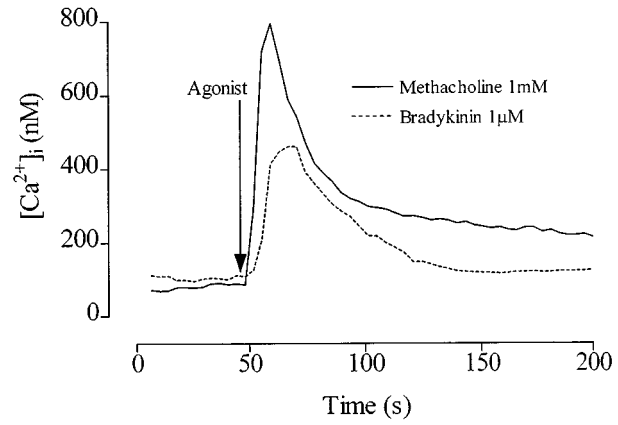

Figure 4 Time courses of agonist-mediated elevations of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined in adherent populations of fura-2 loaded SH-SY5Y cells during challenge with either 1 mM methacholine or 1 μM bradykinin added at 48 s and not removed. Data are representative of $n=3-6$ for each agonist.

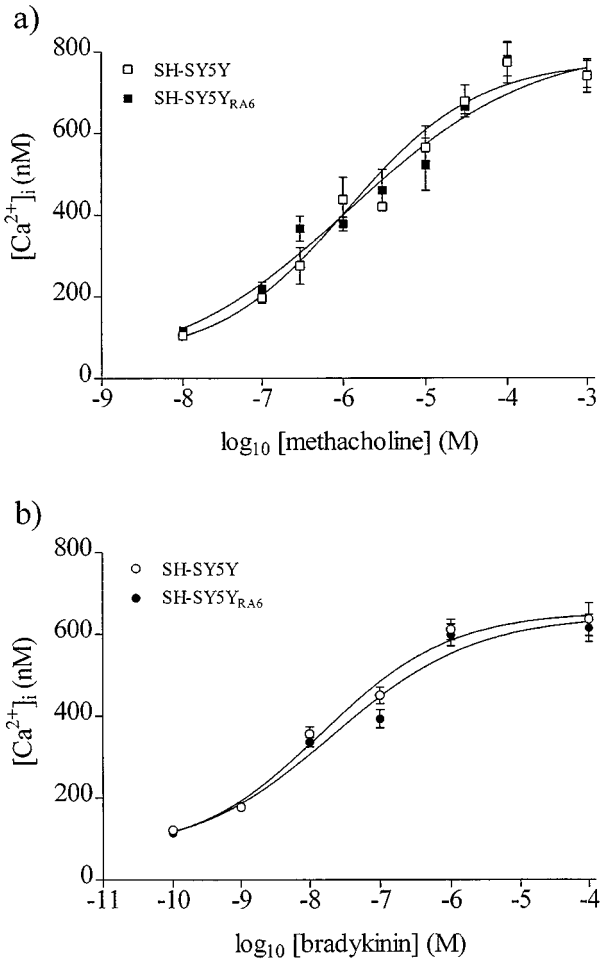

Figure 5 Concentration-response relationships for agonist-mediated peak elevations of $[Ca^{2+}]_i$. (a) Peak $[Ca^{2+}]_i$ responses to methacholine in populations of SH-SY5Y or SH-SY5Y$_{RA6}$ cells. (b) Peak $[Ca^{2+}]_i$ responses to bradykinin in SH-SY5Y or SH-SY5Y$_{RA6}$ cells. Data are mean ± s.e.mean, $n = 3-7$ for methacholine-mediated responses and $n = 3$ for bradykinin-mediated responses. EC_{50} values ($\log_{10}$ M, mean ± s.e.mean) were unaffected by retinoic acid treatment (-5.59 ± 0.19 (2.6 μM) and -5.94 ± 0.3 (1.1 μM) for methacholine and -7.65 ± 0.2 (22 nM) and -7.81 ± 0.2 (15 nM) for bradykinin in control and retinoic acid treated cells respectively.

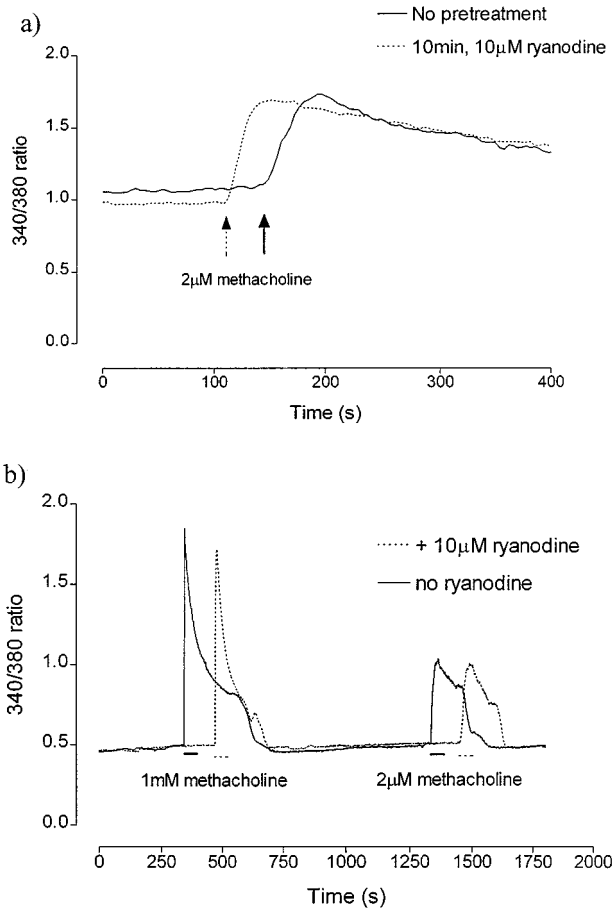

Figure 6 Lack of effect of ryanodine on methacholine-mediated elevation of $[Ca^{2+}]_i$. (a) Pre-incubation of SH-SY5Y cells with 10 μM ryanodine had no effect on the subsequent response to a sub-maximal concentration (2 μM) of methacholine. (b) Challenge of cells with a maximal concentration of methacholine (1 mM) in the presence of ryanodine (10 μM) had no effect on the subsequent response to a sub-maximal concentration of methacholine. All data are representative of a total of three experiments on single cells showing similar results.

Comparison of the relationships between peak Ins(1,4,5)P_3 accumulations and peak $[Ca^{2+}]_i$ elevations in response to methacholine and bradykinin

The current study confirmed that in SH-SY5Y cells the potency of methacholine for the peak elevation of $[Ca^{2+}]_i$ was greater than that for the peak elevation of Ins(1,4,5)P_3 (Willars & Nahorski, 1995a). The mechanisms underlying such amplification are unclear but one possibility is that this resides in the ability of Ca^{2+} itself to mediate Ca^{2+}-release at either ryanodine- or Ins(1,4,5)P_3 receptors. In this context we were unable to provoke an elevation of $[Ca^{2+}]_i$ by caffeine (10 or 100 mM) in these cells (data not shown) suggesting a lack of functional ryanodine receptors. That ryanodine receptors played no role in methacholine-mediated $[Ca^{2+}]_i$ responses in these SH-SY5Y cells was supported by data from two further experimental protocols using single-cell epifluorescence microscopy. Firstly, pretreatment of cells for 10 min with 10 μM ryanodine did not influence the $[Ca^{2+}]_i$ response to a concentration of methacholine approximating to the EC_{50} value for this response (2 μM) (Figure 6a). Secondly, the continuous presence of ryanodine (10 μM) had no effect on $[Ca^{2+}]_i$ responses to 2 μM methacholine when the cells had been challenged 15 min previously with a maximal concentration of methacholine (1 mM for 1 min) (Figure 6b). This second experimental protocol sought to overcome the potential use-dependent block of its receptors by ryanodine. Submaximal agonist concentrations were employed in case the experimental interventions resulted in a shift of agonist potency rather than a reduction in the maximal response.

Following differentiation, the increase in the potency of methacholine for peak Ins(1,4,5)P_3 responses but not peak $[Ca^{2+}]_i$ responses meant that the potency difference was no longer apparent. However, bradykinin was significantly ($P < 0.001$) more potent at mediating the peak elevation of $[Ca^{2+}]_i$ compared to that of Ins(1,4,5)P_3 in SH-SY5Y$_{RA6}$ cells.

One consequence of the potency difference described above is that in SH-SY5Y cells the agonists were able to evoke an increase in $[Ca^{2+}]_i$ without a measurable increase in Ins(1,4,5)P_3. Thus, low concentrations of methacholine elevated $[Ca^{2+}]_i$ but not Ins(1,4,5)P_3 whilst a concentration of bradykinin maximal for elevation of $[Ca^{2+}]_i$ did not produce a measurable increase of Ins(1,4,5)P_3. Indeed the same level of $[Ca^{2+}]_i$ elevation in response to methacholine rather than bradykinin was associated with an approximate 4 fold increase

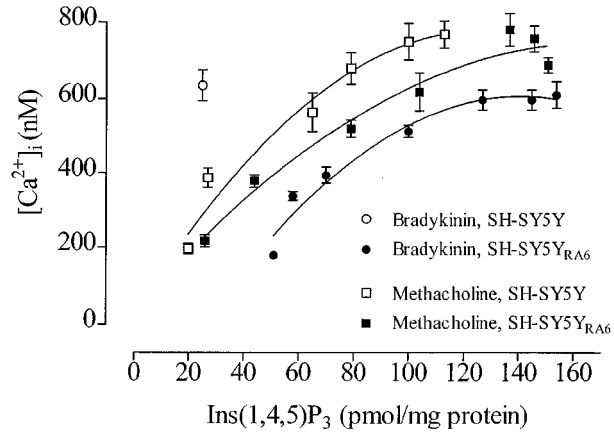

Figure 7 Relationship between peak Ins(1,4,5)P_3 accumulation and maximal $[Ca^{2+}]_i$ elevation. Data represent the peak Ins(1,4,5)P_3 level and the associated elevation of $[Ca^{2+}]_i$ following challenge with a range of either methacholine or bradykinin concentrations in adherent SH-SY5Y and SH-SY5Y$_{RA6}$ cells. Data points represent mean values ($n=3$) for peak Ins(1,4,5)P_3 mass accumulations and means$\pm$s.e.mean, $n=3-9$ for peak $[Ca^{2+}]_i$ elevations. Curves were generated using a second order polynomial equation.

of Ins(1,4,5)P_3 from 20–80 pmol mg protein^{-1}. These data suggest an agonist-dependent relationship between Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ elevations in these cells. To examine this aspect further we plotted the peak Ins(1,4,5)P_3 accumulation against the peak $[Ca^{2+}]_i$ elevation for both bradykinin and methacholine in SH-SY5Y and SH-SY5Y$_{RA6}$ cells (Figure 7). Curves to these data points (for a single agonist in either SH-SY5Y or SH-SY5Y$_{RA6}$ cells) could be best fit by a second order polynomial equation (GraphPAD Prism, GraphPAD Inc.). These curves clearly demonstrate that the relationship between the peak Ins(1,4,5)P_3 accumulation and the peak $[Ca^{2+}]_i$ elevation is dependent not only upon the agonist but also upon the state of differentiation of the cell. Compared to undifferentiated cells, differentiation resulted in a greater Ins(1,4,5)P_3 accumulation being associated with a particular elevation of $[Ca^{2+}]_i$ (Figure 7).

Discussion

Treatment of SH-SY5Y cells for 6 days with retinoic acid in the presence of reduced media serum content resulted in phenotypic changes consistent with cellular differentiation. These included the arrest of cell growth and appearance of neuritic extensions and growth cones. Such changes are typical of neuroblastomas differentiated by retinoic acid and indeed either activators or inhibitors of PKC (Heikkila *et al*., 1989; Shea & Beerman, 1991; Jalava *et al*., 1993). In the present study we found that this differentiation was accompanied by enhanced phosphoinositide signalling. This effect was not receptor-specific as it was apparent following activation of either muscarinic or bradykinin receptors. This enhanced signalling was reflected by an increased accumulation of Ins(1,4,5)P_3 following receptor activation and that this represented a true increase in PLC activity was demonstrated by an enhanced accumulation of [^{3}H]-InsPs against a Li^+-block of inositol monophosphatase activity. The pattern of change in the concentration-response curves for stimulation of Ins(1,4,5)P_3 accumulation by the full muscarinic receptor agonist methacholine demonstrate that differentiation results in the appearance of a functional receptor reserve for this response. Our data indicate that this is most likely accounted for by an increase in the number of muscarinic receptors although alterations in other signalling components also have the potential to contribute to the observed changes. Differentiation was also accompanied by an increase in the number of bradykinin receptors suggesting that this also underlies the enhanced bradykinin-mediated phosphoinositide signalling.

Increased receptor number following differentiation of SH-SY5Y cells with retinoic acid is consistent with previous reports of increased expression of muscarinic, opioid, PGE_1, and dopamine binding sites (Adem *et al*., 1987; Yu *et al*., 1988; 1990; Farooqui, 1994) and we demonstrate here that altered receptor number is reflected functionally at the level of phosphoinositide signalling. Increased receptor expression following differentiation is, however, in direct contrast with other studies in SH-SY5Y cells which were induced to differentiate with phorbol ester (Cioffi & Fisher, 1990). This particular differentiation protocol resulted in a reduction of muscarinic receptor number concomitant with a reduction in muscarinic receptor-mediated phosphoinositide hydrolysis. These data, along with the present study, indicate that the opposite effects of retinoic acid and phorbol ester on a muscarinic receptor number are paralleled by changes in phosphoinositide signalling. Thus, although retinoic acid has been reported to influence the abundance (Ammer & Schulz, 1994) and biochemical properties (Singh & Cerione, 1996) of G-protein α-subunits, differentiation-induced changes in receptor number may well be causally related to altered signalling. These contrasting effects of retinoic acid and phorbol ester on muscarinic receptor number and signalling also support the notion (Pahlman *et al*., 1984) that these agents produce differentiation *via* different routes.

The temporal profile of muscarinic- or bradykinin-receptor-mediated signalling observed in undifferentiated SH-SY5Y cells were in accord with those seen previously (Lambert & Nahorski, 1990; Wojcikiewicz *et al*., 1993; Willars & Nahorski, 1995a,b). Thus, stimulation of the cells with methacholine resulted in biphasic elevations of Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ consisting of a rapid transient peak followed by a lower but sustained phase. That the biphasic accumulation of Ins(1,4,5)P_3 is indicative of a rapid partial desensitization of the muscarinic receptor-mediated response was confirmed by the demonstration that the accumulation of [^{3}H]-InsPs against a Li^+-block of inositol monophosphatase activity (an index of total PLC activity (Wojcikiewicz *et al*., 1993)) was also biphasic. In contrast, challenge of cells with bradykinin resulted in transient accumulations of both Ins(1,4,5)P_3 and [^{3}H]-InsPs confirming the rapid and full desensitization of bradykinin-mediated phosphoinositide signalling in SH-SY5Y cells (Willars & Nahorski, 1995a). Despite the marked potentiation of phosphoinositide responses to both methacholine and bradykinin following differentiation the temporal profiles were essentially unchanged. Thus, differentiation did not alter the ability of either muscarinic- or bradykinin-receptor-mediated responses to undergo acute desensitization which may well result from receptor phosphorylation (Tobin & Nahorski, 1993; Blaukat *et al*., 1996).

Agonist potency for muscarinic receptor-mediated $[Ca^{2+}]_i$ elevation was greater than that for Ins(1,4,5)P_3 elevation as previously reported in these cells (Willars & Nahorski, 1995a) and for other agonists in other cell types (Thompson *et al*., 1991; McArdle *et al*., 1996; Yu & Hinkle, 1997). This potency difference was not, however, maintained following differentiation although in these cells the potency difference was present for bradykinin receptor-mediated responses. Thus, although signal amplification may occur, this is neither obligatory nor of

fixed magnitude. The process underlying amplification is unclear. One possibility is the ability of Ca^{2+} itself to mediate Ca^{2+}-release. This is, however, unlikely to be *via* an action on ryanodine receptors (see Results) although we cannot exclude a sensitization of the Ins(1,4,5)P_3 receptor by Ca^{2+} (Bezprozvanny *et al*., 1991).

The difference in agonist potency between Ins(1,4,5)P_3 generation and $[Ca^{2+}]_i$ elevation also implies that very little of the measured Ins(1,4,5)P_3 is actually required to mediated an elevation of $[Ca^{2+}]_i$. It is possible, therefore, that only a limited proportion of the total measured Ins(1,4,5)P_3 has access to the Ins(1,4,5)P_3 receptor. This is also supported by a comparison between the basal Ins(1,4,5)P_3 level and the ability of Ins(1,4,5)P_3 to release Ca^{2+} from the intracellular stores of these cells. Thus we estimate the basal level of Ins(1,4,5)P_3 to be in the low μM range (cell volume approximately 1000 μm^3, 2×10^6 cells mg protein^{-1} (calculated) and a basal Ins(1,4,5)P_3 level of 10 pmol mg protein^{-1} (see Results)). However, exogenously added Ins(1,4,5)P_3 is able to release $^{45}Ca^{2+}$ from internal stores of permeabilized SH-SY5Y cells with an EC_{50} of only approximately 100 nM (Wilcox *et al*., 1997). Thus, unless Ins(1,4,5)P_3 has limited access to its receptor through physical compartmentalization or buffering there would be enough to maximally release all Ca^{2+} from intracellular stores even in unstimulated cells.

The current data demonstrating the ability of retinoic acid treatment to enhance Ins(1,4,5)P_3- but not Ca^{2+}-signalling also highlight a lack of consistency in the relationship between Ins(1,4,5)P_3 accumulation and $[Ca^{2+}]_i$ elevation. Furthermore this relationship was agonist-specific as recently reported in HEK293 cells expressing recombinant and endogenous receptors coupled to the activation of PLC *via* G-proteins (Yu & Hinkle, 1997). These data suggest that factors other than the measured increase in Ins(1,4,5)P_3 affect the ability of an agonist to elevate $[Ca^{2+}]_i$. It must be remembered that the elevation of $[Ca^{2+}]_i$ is the net result of the movement of Ca^{2+} both into and out of the cytoplasm whilst the accumulation of Ins(1,4,5)P_3 is the result of both its generation and metabolism. It is possible that other mechanisms responsible for regulating $[Ca^{2+}]_i$ (e.g. the plasma membrane Ca^{2+}-ATPase) and/or the metabolism of Ins(1,4,5)P_3 are affected in an agonist dependent fashion and that these contribute significantly to the relative positions of the concentration-response curves. Differential regulation of these processes by bradykinin receptors and muscarininc receptors and their dependence upon the differentiated state of SH-SY5Y cells could well account for the variety in the relationship between elevations of $[Ca^{2+}]_i$ and Ins(1,4,5)P_3. Alternatively other factors which exert their effects in a manner dependent upon the nature of the agonist and the differentiated state of the cell may regulate the sensitivity of Ins(1,4,5)P_3 receptors. Indeed it has been previously suggested that the mobilization of intracellularly stored Ca^{2+} may be modulated by agonsits *via* a route distinct from the activation of PLC (Xu *et al*., 1996; Zeng *et al*., 1996). Alternatively, mechanisms of Ca^{2+} release which are independent of Ins(1,4,5)P_3 may either cause or contribute to the elevation of $[Ca^{2+}]_i$ and these may occur to different extents depending upon the nature of the receptor stimulated and the cellular context. For example, recent evidence has highlighted a role for sphingosine-1-phosphate in the $[Ca^{2+}]_i$ elevations in response to the stimulation of muscarinic M_3 receptors stably expressed in HEK-293 cells (Meyer zu Heringdorf *et al*., 1998).

In summary, the current study demonstrates that following differentiation of SH-SY5Y cells with retinoic acid, there are marked enhancements in phosphoinositide signalling in response to muscarinic- or bradykinin-receptor activation. The potentiation of phosphoinositide signalling but not Ca^{2+}-signalling following cellular differentiation highlight a major discrepancy between the elevation of Ins(1,4,5)P_3 and $[Ca^{2+}]_i$. This suggests that mechanism other than the measured increase in Ins(1,4,5)P_3 regulate $[Ca^{2+}]_i$ elevation and these aspects are currently under investigation.

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