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Receptor coupled events in bradykinin action: rapid production of inositol phosphates and regulation of cytosolic free Ca^{2+} in a neural cell line

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The addition of bradykinin to NG115-401L cells grown on coverslips results in the generation of rapid transient increases in intracellular $[Ca^{2+}]$ and inositol phosphates. Changes in intracellular Ca^{2+}, measured using the fluorescent indicator dye Fura-2, show two components; an initial rapid peak in $[Ca^{2+}]_i$ which is essentially independent of extracellular Ca^{2+}, and a sustained plateau dependent on the presence of extracellular Ca^{2+}. Analysis of bradykinin stimulated production of [^{3}H]inositol phosphates, by h.p.l.c., shows a rapid biphasic production of inositol 1,4,5-trisphosphate, inositol tetrakisphosphate and inositol bisphosphates, followed by a sustained rise in inositol 1,3,4-trisphosphate production. Quantitative measurements have indicated the presence of other, more polar, [^{3}H]inositol-labelled metabolites which do not show major changes on bradykinin stimulation. The initial phase of inositol phosphate production parallels the rapid transient increase in intracellular $[Ca^{2+}]$, however, the second phase of inositol phosphate production occurs when intracellular $[Ca^{2+}]$ is declining and implies a complex series of regulatory events following receptor stimulation. Similar time courses of inositol 1,4,5-trisphosphate and Ca^{2+} signals provides supporting evidence that inositol 1,4,5-trisphosphate is the second messenger coupling bradykinin receptor stimulation to release of Ca^{2+} from intracellular stores.

Key words: bradykinin/Ca^{2+}/inositol phosphates/neural cell line

Introduction

Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is a locally acting hormone, whose best characterized neural action is the stimulation of peripheral sensory neurones, which may contribute to its hyperalgesic and pro-inflammatory effects (Luttinger *et al.*, 1984). Functional bradykinin receptors have been identified on several neural cell lines, including the N1E-115 mouse neuroblastoma (Snider and Richelson, 1984), the C6 rat glioma (Sapirstein and Benos, 1984), the NG108-15 neuroblastoma × glioma hybrid (Yano *et al.*, 1984, 1985), and the NCB-20 Chinese hamster brain × neuroblastoma hybrid (Francel and Dawson, 1986); the activation of which stimulates breakdown of inositol lipids (Yano *et al.*, 1984) and accumulation of inositol phosphates (Yano *et al.*, 1985; Higashida *et al.*, 1985; Francel and Dawson, 1986). Recently we have shown that the NG115-401L neuroblastoma × glioma hybrid cell line (Ogura and Amano, 1983) also responds to bradykinin by a rapid breakdown of phosphoinositides and a concomitant production of inositol phosphates (A.Legget, S.I.Patterson, M.J.O.Wakelam and M.R.Hanley submitted).

Two wings of the inositol lipid pathway are now recognized. One is the regulation of protein kinase C by diglyceride (Nishizuka, 1984), and the other is the regulation of the discharge of Ca^{2+} from an intracellular store by the production of inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) (Berridge and Irvine, 1984). In neuronal cells these pathways may act independently to regulate plasma membrane ionic events, as suggested by the recent work of Higashida and Brown (1986). Herein we have focused on one pathway. In particular we have measured the magnitude and temporal relationship between inositol phosphates and calcium signals under identical conditions. To do this, a number of technical refinements in the methodology of inositol phosphate analysis, and the use of fluorescent Ca^{2+} indicators were necessary. Analysis of inositol phosphates has been complicated by the discovery of a number of inositol phosphate isomers in stimulated cells (Downes, 1986). Therefore one refinement was the use of an h.p.l.c. technique (adapted from that of Batty *et al.*, 1985) to identify and quantitate [^{3}H]inositol labelled metabolites at all times after stimulation with bradykinin. Another refinement was the use of Fura-2, a highly fluorescent indicator which permits the measurement of $[Ca^{2+}]$ in monolayers of cells without significantly increasing the Ca^{2+} buffering capacity of the cytoplasm. Its sensitivity to changes in $[Ca^{2+}]$ is greatly enhanced over other fluorescent indicators, such as Quin-2, by (i) its greater quantum yield and extinction coefficient giving an overall 30-fold greater fluorescence, and by (ii) monitoring fluorescence at two excitation wavelengths, which show opposing sensitivities to $[Ca^{2+}]$ and which can then be ratioed (Grynkiewicz *et al.*, 1985; Tsien *et al.*, 1985) (see Materials and methods). Responses were analysed in cells grown on coverslips under conditions which minimize the physical perturbation of cells during experimental manipulations.

Results

Inositol phosphate generation in response to bradykinin

Addition of bradykinin to suspensions of NG115-401L cells has been shown to result in the production of inositol phosphates accompanied by rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP) (A.Leggett, S.I.Patterson, M.J.O.Wakelam and M.R.Hanley submitted).

The characteristics of inositol phosphate production in cells grown and stimulated as monolayers were determined to establish any differences from cells in suspension. The time course of inositol phosphate generation was determined using Dowex AG1X8 ion exchange columns. At 5 s the levels of inositol tris- and bisphosphates (Ins P_3 and Ins P_2, respectively) are already elevated and reach peak values (of 3.5 and 4 times basal, respectively) at 15 s. They then decline to a minimum level, though still elevated over basal, within 1 min and then appear to rise again at 5 min. Inositol monophosphate is not elevated at 5 s; it is first seen to rise at 10 s and peaks at around 25 s after stimulation and this would indicate that receptor stimulation of phospholipase C may produce first a hydrolysis of polyphosphoinositides but not of PI itself. There is no indication of any change in higher [^{3}H]inositol labelled fractions though there may be a rise in the

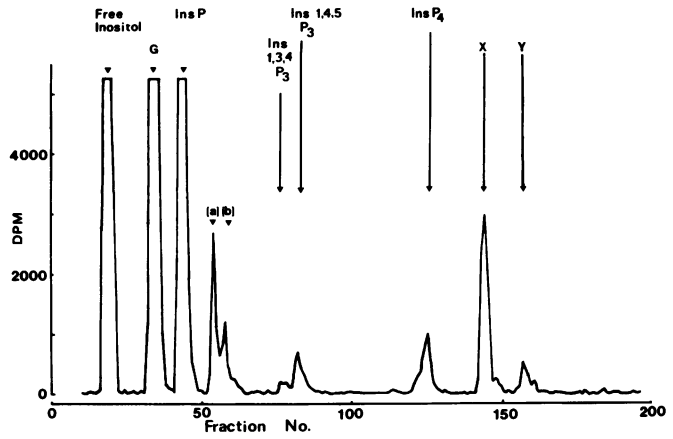

Fig. 1. Elution of [^{3}H]inositol metabolites from a Partisil SAX 10 μ h.p.l.c. column. NG115-401L cells were incubated with 10 mM Li^+ for 30 min without receptor stimulation. Column elution was a modified gradient from that of Batty *et al.* (1985): H_2O (2 min); a linear gradient 0–0.75 M ammonium formate (pH 3.7 10 min); linear gradient 0.75–1.0 M ammonium formate (6 min); isocratic at 1.0 M (5 min); linear gradient 1.0–1.7 M ammonium formate (10 min); linear gradient 1.7–3.5 M ammonium formate (10 min); and isocratic at 3.5 M (2 min). Fractions of 0.33 ml were collected using a flow rate of 1.2 ml/min. For counting, samples were diluted by the addition of 1 ml of water:methanol (1:1, v/v). Named peaks were identified by co-elution with radiolabelled standards. Other peaks are presumed to contain: G:GPI, (a) and (b) isomers of Ins P_2, X:Ins P_5, Y:Ins P_6. Routinely recoveries were >80%.

glycero-phosphoinositol (GPI) containing fraction (B) at 15 s and longer. These changes are purely intracellular as medium removed from either control or stimulated cells contains no detectable [^{3}H]inositol phosphates. This also indicates that there is no secretion of [^{3}H]inositol-labelled phosphates at up to 5 min after receptor stimulation.

There are two isomeric forms of IP_3 known to be produced on receptor stimulation, inositol 1,4,5- and 1,3,4-trisphosphates (Ins 1,4,5-P_3 and Ins 1,3,4-P_3, respectively) (Irvine *et al.*, 1984) which cannot be separated on Dowex columns. Information on the isomeric forms of inositol phosphates requires the use of a high resolution ion exchange system such as h.p.l.c. or f.p.l.c. These systems also allow full separation of the higher phosphates. Using a modification of the h.p.l.c. procedure reported by Batty *et al.*, 1985; the inositol phosphates produced on bradykinin stimulation of NG115-401L cells were analysed in greater detail. Figure 1 shows an elution profile with peaks identified by co-migration with radiochemical standards.

Figure 2 shows the time course of Ins 1,4,5-P_3, Ins 1,3,4-P_3 and Ins P_2 isomer production on bradykinin stimulation. At the earliest time point measured, 2 s, both Ins 1,4,5-P_3 and Ins P_2 are elevated to at least four times basal; however, their levels are declining by 5–7 s. At 10 s the levels of Ins 1,4,5-P_3 and Ins P_2 are again increasing, reaching a peak at 15 s, at a value in excess of nine times basal, these then decline but even at 1 min remain elevated over basal. Ins 1,3,4-P_3 is not detectable until at least 10 s after stimulation, after which it shows a steady rise

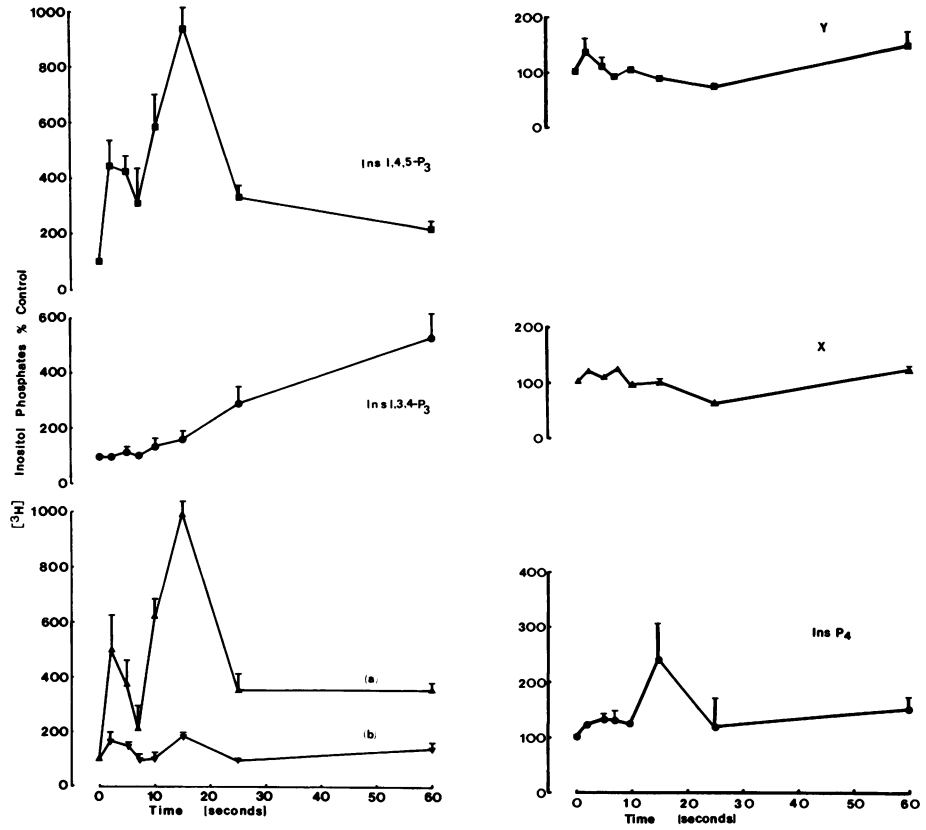

Fig. 2. The time course for bradykinin (40 μM)-induced production of [^{3}H]inositol phosphates NG115-401L cells on coverslips. Inositol phosphates separated by h.p.l.c. on Partisil SAX 10 μ ion exchange column. Each point is representative of three experiments performed in duplicate ± SEM. Control levels were as follows: Ins P_2 (a) 466 d.p.m., Ins P_2 (b) 740 d.p.m., Ins 1,3,4-P_3 158 d.p.m., Ins 1,4,5-P_3 655 d.p.m., Ins P_4 550 d.p.m., X 6374 d.p.m., Y 583 d.p.m.

with a maximal level being measured at 1 min. Ins P_4 is elevated at most time points with a maximal level at 15 s. Small increases in the higher phosphate-(Ins P_5 and Ins P_6) containing fractions, peaks X and Y, are found at the earliest time points, they decline, and then significantly increase by 1 min.

It is also interesting to note that there appear to be two forms of Ins P_2 [termed (a) and (b) as their structures are unknown, though one is very likely to be Ins 1,4 P_2]. Both appear to rise in line with Ins 1,4,5-P_3, Ins P_2 (a) shows a larger increase on stimulation.

Inclusion of 200 μM aspirin (acetyl salicylic acid) during pre-incubation and bradykinin stimulation had no apparent effect on the time course of production of inositol phosphates induced by bradykinn (Table I), suggesting that there is no involvement of prostaglandins or other cyclo-oxygenase products in the measured responses.

It should be noted that there is an apparent discrepancy between the maximal levels of stimulation seen in the analyses by Dowex column and by h.p.l.c. These appear to arise through the fact that the Dowex fractions give counts which are an average of two isomers, only one of which is increasing at any time, whilst h.p.l.c. gives the counts for each isomer individually.

Table I. Effect of aspirin on inositol phosphate production in response to bradykinin in NG115-401L cells (c.p.m. ± SD)

	Ins 1,3,4-P_3	Ins 1,4,5-P_3
Control	70 ± 15	177 ± 23
Control + aspirin	72 ± 12	158 ± 93
Stimulation (2 s)	62 ± 4	1361 ± 700
Stimulation (2 s) + aspirin	64 ± 5	1316 ± 156
Stimulation (15 s)	230 ± 15	3939 ± 948
Stimulation (15 s) + aspirin	238 ± 2	3837 ± 65

The cells were treated as described in Materials and methods, inositol phosphates were separated by h.p.l.c. on Partisil SAX 10 μ column. Results are from two experiments performed in duplicate.

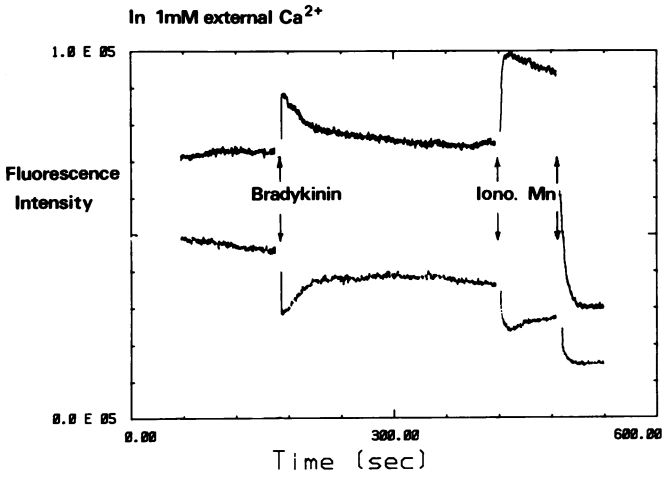

Fig. 3. Fluorescence collected at 500 nm for 340 nm (upper trace) and 380 nm (lower trace) excitation wavelengths from Fura-2-loaded NG115-401L cells attached to a coverslip and stimulated by 1.0 μM bradykinin (giving maximal release of $Ca^{2+}{}_i$ (see Figure 5); also indicated are the addition of 3 μM ionomycin (Iono) and 3 mM $MnCl_2$ (Mn), see Materials and methods for details. Breaks in the traces indicate periods of additions.

Bradykinin elevates $[Ca^{2+}]_i$

Figure 3 shows the raw fluorescence data collected from a coverslip containing NG115-401L cells in the presence of 1 mM $CaCl_2$ and the effect of adding bradykinin. Bradykinin stimulated an increase in fluorescence at 340 nm with a corresponding decrease in fluorescence at 380 nm, both of which then returned towards basal levels consistent with a transient elevation in $[Ca^{2+}]_i$. The calculated data from the same experiment is shown in Figure 4A. In the presence of extracellular Ca^{2+} (1 mM $CaCl_2$) bradykinin causes a rapid increase in $[Ca^{2+}]_i$ from basal levels of 105 ± 21 nM; n = 6, to a peak of 743 ± 173 nM; n = 6, after 10−15 s. Within 90 s $[Ca^{2+}]_i$ had declined back to a steady state level of 259 ± 42 nM; n = 5. This level of $[Ca^{2+}]_i$ was maintained for more than 5 min after stimulation. In the absence of extracellular Ca^{2+} (without $CaCl_2$ and with 1 mM EGTA) 1 μM bradykinin stimulates an equally rapid transient increase in $[Ca^{2+}]_i$ from the resting level of 114 ± 37 nM; n = 6, to a peak of 624 ± 127 nM; n = 6 (Figure 4B). $[Ca^{2+}]_i$ returns to basal levels, 123 ± 40 nM; n = 5, within 90 s after addition of bradykinin. The initial transient elevation in $[Ca^{2+}]_i$ is essentially independent of extracellular Ca^{2+}, whereas the sustained plateau depends on the presence of extracellular Ca^{2+}. The data show that bradykinin can cause the discharge of Ca^{2+} from an intracellular store and that the later phase of the response may reflect influx of Ca^{2+} ions across the plasma membrane.

With decreased data acquisition periods (100 ms) it is possible to observe the initial bradykinin stimulated $[Ca^{2+}]_i$ changes

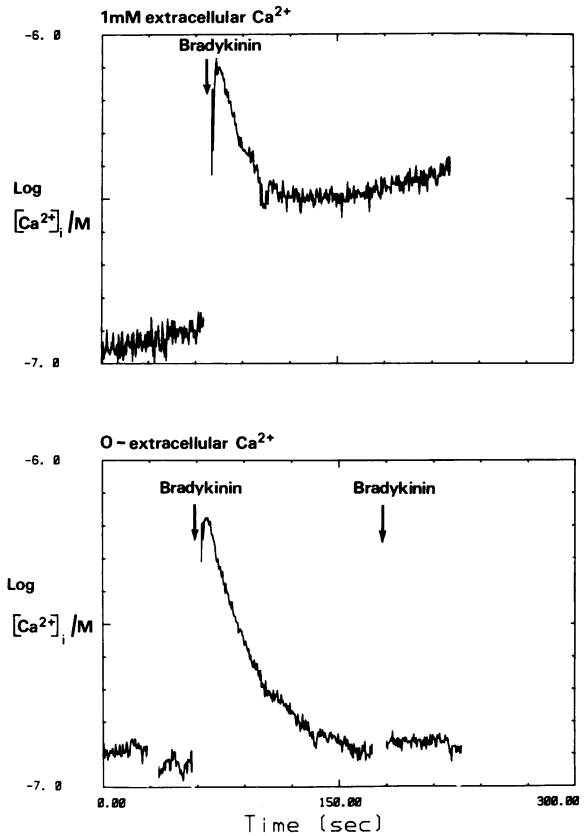

Fig. 4. Intracellular Ca^{2+} transients in Fura-2-loaded NG115-401L cells on coverslips, in response to 1 μM bradykinin, in the presence of 1 mM Ca^{2+} (upper) or 1 mM EGTA (lower). Each trace is representative of six separate determinations. See Materials and methods for details of $[Ca^{2+}]$ determination. Further additions of bradykinin do not elicit a response (lower trace) indicating that desensitization has occurred.

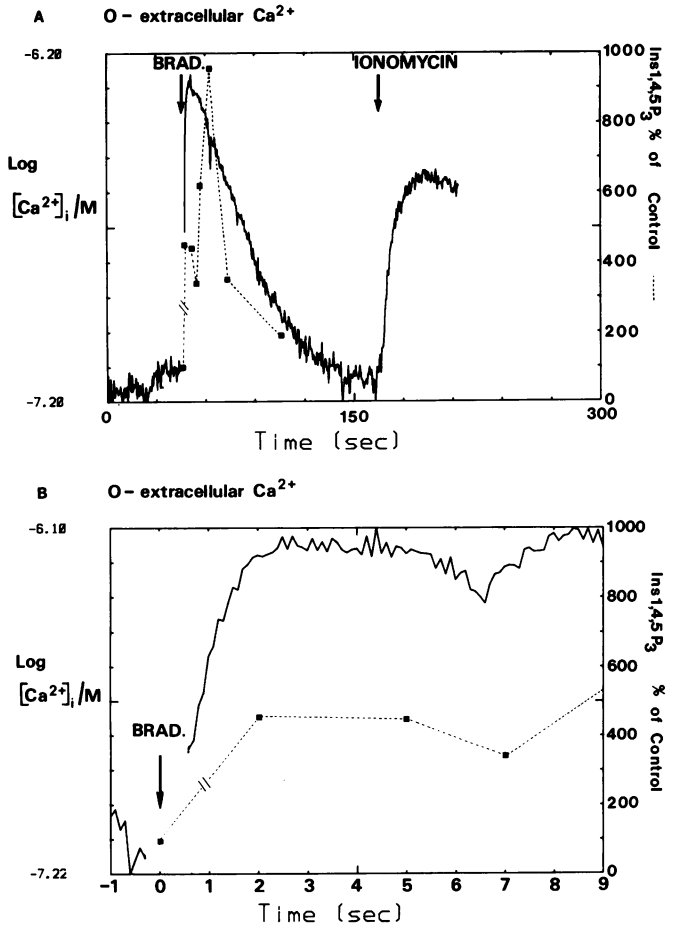

Fig. 5. Comparison of $[Ca^{2+}]_i$ and Ins 1,4,5-P_3 transients in NG115-401L cells on coverslips, in response to supramaximal bradykinin (A, 100 μM; B, 1 μM; each giving a maximal $[Ca^{2+}]_i$ value of ~790 nM) in the absence of extracellular Ca^{2+} (1 mM EGTA present). Fluorescence data acquisition times were 0.5 (A) and 0.1 (B). $[Ca^{2+}]_i$ ——; Ins 1,4,5-P_3 - - - -. Each trace is representative of at least three separate determinations. Fura-2 loading of these cells has no effect on inositol phosphate production (unpublished data).

in more detail as shown in Figure 5B. In the absence of external Ca^{2+}, on addition of bradykinin, there is already an increased level of $[Ca^{2+}]_i$ at the earliest data point (~0.7 s) and it reaches a peak value of around 740 nM within 2 s. In the presence of extracellular Ca^{2+} a similarly rapid rise in $[Ca^{2+}]_i$ is observed.

A comparison of the time course of Ins 1,4,5-P_3 generation and $[Ca^{2+}]_i$ signals for cells grown on coverslips and stimulated in Ca^{2+}-free medium (Figure 5A and B) shows that only the initial Ins 1,4,5-P_3, Ins P_4 and Ins P_2 transients are in the same time domain as the elevation in $[Ca^{2+}]_i$. There is no indication that any other inositol phosphates are elevated to any degree until at least 10 s after receptor stimulation at which point the $[Ca^{2+}]_i$ level is beginning to decline.

Discussion

The formation of inositol phosphates, when examined by h.p.l.c., can be shown to consist of three phases: a rapid production of Ins 1,4,5-P_3 and Ins P_2 which peaks within 2 s of stimulation; a second peak of Ins 1,4,5-P_3 and Ins P_2 occurs at 15 s and begins to decline by 25 s; and finally a steady increase in Ins 1,3,4-P_3 is seen beginning at 10–15 s and continues until at least 1 min after stimulation. This picture is essentially identical to that seen in NG115-401L cells in suspension. There is no evidence of Ins P production during this early phase as has been observed in other cell types (Downes and Wusteman, 1983) indicating that there is no direct breakdown of PI on receptor activation. This agrees with data on cells in suspension. Formation of Ins P_4 shows essentially similar characteristics to Ins 1,4,5-P_3 but the magnitude of the changes is much smaller. The apparently complex data are consistent with recent studies on other cells which suggest that the initial response to receptor activation is the breakdown of PIP_2 to give Ins 1,4,5-P_3. Ins 1,4,5-P_3 can be metabolized in two ways: by specific removal of its 5-phosphate to give Ins 1,4-P_2 (Downes *et al.*, 1982) or by kinase action to give Ins 1,3,4,5-P_4 (Irvine *et al.*, 1984; Batty *et al.*, 1985; Hawkins *et al.*, 1986). Ins 1,3,4,5-P_4 is rapidly degraded to Ins 1,3,4-P_3 (Hawkins *et al.*, 1986; Batty *et al.*, 1985) which is, in turn, metabolized to an unidentified inositol phosphate (Hawkins *et al.*, 1986). Ins 1,4,5-P_3 has been shown to release Ca^{2+} from intracellular stores in many different cells (Berridge and Irvine, 1984) including the closely related N1E-115 cell line (Ueda *et al.*, 1986), but thus far possible functions of any of the other inositol phosphates formed in stimulated cells have not been defined.

In the absence of external Ca^{2+}, activation of bradykinin receptors generates a rapid increase in $[Ca^{2+}]_i$ reaching a peak at 2 s and after 10 s begins to decline back to basal levels. In the NG115-401L the only inositol phosphates produced that correlate with the time course of release of intracellular Ca^{2+} are Ins 1,4,5-P_3, Ins P_2 and Ins P_4 transients. Of these only Ins 1,4,5-P_3 has been shown to be effective in $[Ca^{2+}]_i$ release (Berridge and Irvine, 1984). Further seconds after bradykinin stimulation, the $[Ca^{2+}]_i$ is declining, yet there is a second peak of Ins 1,4,5-P_3. This indicates that the fall in $[Ca^{2+}]_i$ is unlikely to be secondary to a reduction in the Ins 1,4,5-P_3 concentration. This may perhaps be due to exhaustion of the Ins 1,4,5-P_3-releasable Ca^{2+} pool; as implied in Figure 4B by the inability of a second pulse of bradykinin to cause a cytoplasmic Ca^{2+} rise. Both the significance and mechanism of this second phase of Ins 1,4,5-P_3 production are uncertain. However, it must be noted that the second phase is not dependent on extracellular Ca^{2+} (Figure 5A) or production of a prostanoid derived from the cyclo-oxygenase pathway (Table I). A similar kinetic comparison of the production of Ins 1,4,5-P_3 and the cytoplasmic Ca^{2+} rise stimulated by carbachol, in RIN m5F cells, has shown only a single phase of Ins 1,4,5-P_3 peaking at 5 s (Wollheim and Biden, 1986). Few other studies of inositol phosphate generation have reported on time points before 5 s. Indeed a peak at 10–15 s following receptor activation is consistently observed (Drummond *et al.*, 1984; Batty *et al.*, 1985; Rittenhouse and Sasson, 1985; Francel and Dawson, 1986) corresponding to the second peak in NG115-401L. However, in blowfly salivary glands it is clear that the first Ins P_3 response to serotonin occurs within 5 s of stimulation (Berridge *et al.*, 1984).

As noted above, evidence from other tissues suggests that Ins 1,3,4-P_3 is formed by a two-step process from Ins 1,4,5-P_3. Its slow and sustained formation in bradykinin stimulated NG115-401L cells is consistent with this notion, and with the relatively slow rate of breakdown of Ins 1,3,4-P_3 compared with Ins 1,4,5-P_3 first noted in carbachol-stimulated parotid glands (Irvine *et al.*, 1984). The h.p.l.c. trace shown in Figure 1 also shows two labelled compounds, more polar than Ins P_4, that have similar chromatographic properties to the Ins P_5 and Ins P_6 described by Heslop *et al.*, 1985. As yet we have insufficient

data to say whether these peaks are significantly influenced by bradykinin stimulation.

There is a second component in the $[Ca^{2+}]_i$ response in the form of a Ca^{2+} influx presumably through the opening of some form of membrane channel. It is unlikely that this is via activation of a voltage gated Ca^{2+} channel as the undifferentiated NG115-401L is non-excitable, and lacks voltage activated Na^+ and Ca^{2+} channels (unpublished data). It is possible that an intracellular messenger generated by the bradykinin stimulation may be capable of increasing plasma membrane permeability to Ca^{2+}. It has been proposed that Ca^{2+} channels sensitive to the $[Ca^{2+}]$ in the intracellular Ca^{2+} store exist, which on sensing a decrease in $[Ca^{2+}]$ could trigger a Ca^{2+} influx (Putney, 1986). Plasma membrane−endoplasmic reticulum contacts have been reported in muscle and in neurons (Henkart *et al.*, 1976) which could provide the site for such a mechanism to operate. A similar pattern of $[Ca^{2+}]_i$ transients has been reported in Fura-2-loaded monolayers of A10 and DDT_1 smooth muscle cells, on stimulation with arginine vasopressin and noradrenalin, respectively, in which a sustained influx of Ca^{2+} was attributed to receptor activated Ca^{2+} channels (Reynolds and Dubyak, 1986), and has also been seen in PC12 cells (Pozzan *et al.*, 1986) and platelets (Hallam *et al.*, 1984). Thus the two phases of cytoplasmic Ca^{2+} elevation may be a feature common to several, if not all, Ca^{2+} mobilising receptors.

In conclusion we have used improved radio-labelling and analytical procedures to measure rapid inositol phosphate and $[Ca^{2+}]_i$ signals in cells maintained under identical conditions. The resulting methodology should be generally applicable to a complete and quantitative analysis of the water-soluble products of inositol lipid hydrolysis. An important result emerging from these data is that Ins 1,4,5-P_3 parallels the $[Ca^{2+}]_i$ signal stimulated by bradykinin, thus providing strong evidence in favour of Ins 1,4,5-P_3 as the authentic second messenger coupling surface receptor occupancy to intracellular Ca^{2+} release.

Material and methods

Cell culture

Neuroblastoma × glioma hybrid cell line NG115 401L was cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37°C in 8% CO_2/92% air. Cells were passaged and plated out onto sterile 11 × 22 mm glass coverslips at 1 × 10^5/coverslip and allowed to grow for 3 days (around 90% confluence) prior to use.

Measurement of inositol phosphate production

Cells were incubated for 16−18 h with 5 μCi/ml [^{3}H]myo-inositol in DMEM (purchased inositol free from Gibco) containing 10 μM myo-inositol and 5% dialysed fetal calf serum. Preliminary experiments indicate this gives maximal labelling without cytotoxicity due to inositol deprivation. Cells were then washed twice with Hepes-buffered Hanks' basic salts [composition 1.0 mM $CaCl_2$, 5.4 mM KCl, 0.5 mM $MgCl_2.6H_2O$, 10.4 mM $MgSO_4$, 136.8 mM NaCl, 4.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 44 mM glucose, 0.05% bovine serum albumin (BSA), 20 mM Hepes, pH 7.4]. Cells were allowed to equilibrate in this for 15 min prior to stimulation. Immediately prior to use cells for Ca^{2+}-free experiments were transferred into the above medium from which $CaCl_2$ and BSA had been excluded and which contained 1.0 mM EGTA. Bradykinin was added in Ca^{2+}-free Hanks, 20 mM Hepes medium and the incubation stopped by addition of ice-cold 10% perchloric acid. The supernatant was removed and inositol phosphates were extracted by addition of a 1:1 mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane (Freon). After thorough mixing and centrifugation to separate phases the inositol-phosphate-containing upper phase was removed. After addition of 1 mM EDTA the inositol phosphate fractions were analysed by ion exchange chromatography on a 0.5 ml Dowex (AG1X8 Formate form column by sequential addition of (i) 2 × 5 ml H_2O (Fraction A); (ii) 2 × 6 ml 60 mM ammonium formate/5 mM disodium tetraborate (Fraction B); (iii) 2 × 5 ml 0.15 M ammonium formate/0.1 M formic acid (Fraction C); (iv) 2 × 5 ml 0.4 M ammonium formate/0.1 M formic acid (Fraction D); (v) 2 × 5 ml 0.8 M ammonium formate/0.1 M formic acid (Fraction E); and (vi) 1.2 M ammonium formate/0.1 M formic acid (Fraction F). Authentic standards applied to the columns elute as follows: free inositol Fraction A: inositol monophosphate Fraction C; inositol trisphosphate Fraction E; inositol tetrakisphosphate Fraction F. The Dowex columns, however, do not separate isomers of inositol phosphates that differ only in the distribution of phosphate groups around the ring. Inositol phosphates were analysed in greater detail using h.p.l.c., by ion exchange on a Partisil SAX 10 μ column with elution by a complex gradient going from 0 to 100% 3.5 M ammonium formate buffered to pH 3.7 with orthophosphoric acid, developed from that of Batty *et al.* (1985). Inositol phosphates were identified by comparison of their elution positions with those of known standards (these are indicated in Figure 1) and in a number of determinations a [^{32}P]-Ins 1,4,5-P_3 spike was included to provide an internal standard. Two [^{3}H]inositol-labelled compounds eluted from the h.p.l.c. later than standard Ins P_4. These have similar chromatographic characteristics to Ins P_5 and Ins P_6 identified in GH_4 pituitary cells (Heslop *et al.*, 1985) and are referred to as Peak X and Peak Y. Radioactivity in the eluates was determined by liquid scintillation counting.

Measurement of $[Ca^{2+}]_i$

Coverslips containing ~90% confluent monolayers of NG115-401L cells were placed in DMEM containing 2 μM Fura-2/AM (the penta-acetoxymethyl ester of the dye) and incubated for 45 min at 37°C. The coverslips, containing cells now loaded with ~25 μm Fura-2, were removed from the loading medium, washed twice with a Hepes-buffered saline (consisting of 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 44 mM glucose, 10 mM Hepes, pH 7.4) at 37°C and then kept in the same buffer, containing 0.5 mM $CaCl_2$ with 0.05% BSA, at room temperature until ready for use. Maintaining cells at room temperature prevented excessive dye leakage; under these conditions dye leakage was <10%/h.

Before addition of agonist, coverslips were placed across the diagonal of a quartz cuvette containing 1.5 ml of the same Hepes-buffered saline, with no added BSA, and containing either 1 mM $CaCl_2$ or 1 mM Na_2H_2 EGTA, and allowed to equilibrate to 37°C for 1−2 min in the 37°C thermostatted cuvette holder in a Spex dual-wavelength excitation fluorescence spectrophotometer. The cuvette and coverslip were arranged such that the coverslip was at an angle of 60° to the incident light and such that the incident light fell directly on the monolayer of cells without passing through the glass coverslip.

Excitation wavelengths alternated between 340 nm and 380 nm, with a bandpass on each of ~1 nm, at ~30 Hz using dual Xenon lamps and a chopping mirror assembly (Glen Creston Instruments Ltd, Stanmore, Middlesex, UK). The characteristics of the dye are such that at excitation wavelength 340 nm with 500 nm emission there is an increase in emitted fluorescence intensity with increasing $[Ca^{2+}]$, whilst at excitation wavelength 380 nm with 500 nm emission there is a corresponding decrease in emitted fluorescence intensity with increasing $[Ca^{2+}]$. Hence, ratioing the two simultaneously recorded signals enhances the overall sensitivity of the dye for changing $[Ca^{2+}]$ (see Tsien *et al.*, 1985; Poenie *et al.*, 1985; Grynkiewicz *et al.*, 1985). Emitted light was collected for 0.5-s periods, or 0.1 s where indicated, at 500 nm with a 2 nm bandpass simultaneously for 340 nm and 380 nm excitation. At the end of each experiment the divalent cation ionophore, ionomycin (3 μM), was added followed by 3 mM $MnCl_2$. Ionomycin effectively translocates Mn^{2+} ions across the plasma membrane and into the cytoplasm. The affinity of Fura-2 for Mn^{2+} is much greater than that for Ca^{2+}, so Mn^{2+} preferentially binds the dye resulting in an effective quenching of the dye's fluorescence. Residual fluorescence is entirely due to the glass coverslip and cells (Hallam *et al.*, 1984). The values for autofluorescence at 340 nm and at 380 nm are subtracted from the respective data traces and the ratio of collected data at 340 nm divided by data at 380 nm is calculated. Ratio data ($R_{340/380}$) could then be interpreted in terms of the equation

$$[Ca^{2+}] = Kd \frac{R_{340/380} - R_{MIN}}{R_{MAX} - R_{340/380}} \frac{S_{F2}}{S_{b2}}$$

see Grynkiewicz *et al.* (1985) for a more complete description of the calibration procedure. The Kd for Fura-2 at 37°C was taken to be 224 nmol. Using our Spex Laboratories instrument R_{MAX} was measured to be 18.9; R_{MIN} was 0.94; S_{f2}/S_{b2} was 10.3.

Materials

Inositol-free DMEM was obtained from Gibco, Paisley. Myo-[2-^{3}H(N)]inositol was obtained from New England Nuclear, Boston, MA; Fura-2/AM was from Molecular Probes, Junction City, Oregon; bradykinin was from Cambridge Research Biochemicals, Cambridge.

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