Differences between muscarinic-receptor- and Ca^{2+} -induced inositol polyphosphate isomer accumulation in rat cerebral-cortex slices

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Muscarinic-receptor stimulation or depolarization by elevated K^+ leads to increased accumulation of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$, $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ and several degradation products of these polyphosphates separated by h.p.l.c. On the other hand, agents such as ionomycin and maitotoxin, which increase intracellular Ca^{2+} directly, produce a small accumulation of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ and markedly increase $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$, but $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$, $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ and $[^3\text{H}]\text{Ins}(1,3)\text{P}_2$ are virtually unaffected. Ca^{2+} -dependent $[^3\text{H}]\text{inositol}$ polyphosphate metabolism may involve different pools of lipids and/or phosphoinositidases.

INTRODUCTION

It is now generally accepted that several cell-surface receptors are linked to phosphoinositidase C and that products of polyphosphoinositide hydrolysis are important intracellular messengers (for review, see Berridge & Irvine, 1989). In addition to the well-documented studies of receptor-mediated phosphoinositide breakdown, it is apparent that depolarization induced by elevated extracellular K^+ or veratrine can also stimulate phosphoinositide hydrolysis in several tissues, particularly those of a neuronal nature (Kendall & Nahorski, 1984, 1985, 1987; Bone & Michell, 1985; Baird & Nahorski, 1986, 1989, 1990; Court *et al.*, 1986; Eva & Costa, 1986; Hollingsworth *et al.*, 1986; Gusovsky *et al.*, 1986; Habermann & Laux, 1986; Gurwitz & Sokolovsky, 1987; Audigier *et al.*, 1988; Sasaguri & Watson, 1988; McDonough *et al.*, 1988). In several of these studies, indirect effects of depolarization can be eliminated and responses can be shown to be secondary to Ca^{2+} entry via voltage-sensitive channels. Of interest in this respect are reports that ionophores and toxins enhanced phosphoinositide metabolism in cerebral slices (Kendall & Nahorski, 1984; Baird & Nahorski, 1990) and other cell types [see Gusovsky *et al.* (1989a,b) and Baird & Nahorski (1990) for further references].

Thus, although the concept of Ca^{2+} -stimulated phosphoinositide hydrolysis appears to be widely reported, little attention has been paid to the individual inositol phosphates which constitute the metabolites produced by polyphosphoinositide hydrolysis under these circumstances. Indeed, most investigators have used the relatively crude approach of studying long incubation periods in the presence of Li^+ , which allows an accumulation of mainly InsP .

In previous work from this laboratory (Baird & Nahorski, 1990), we have shown that depolarization induced by K^+ or by using agents such as the ionophore ionomycin or the marine toxin maitotoxin, which act to increase intracellular Ca^{2+} directly, can produce different patterns of the $[^3\text{H}]\text{inositol}$ phosphates that could suggest different mechanisms of phosphoinositide metabolism. In the present report we have extended these observations by using a recently established h.p.l.c. separation (Batty *et al.*, 1989) to include a detailed analysis of separate $[^3\text{H}]\text{inositol}$ polyphosphate isomers produced under these conditions. We demonstrate that muscarinic-receptor stimulation differs from most conditions that simply elevate intracellular Ca^{2+} by virtue

of the isomeric composition of the $[^3\text{H}]\text{inositol}$ (poly)phosphates. Depolarization of slices by elevated K^+ resembles more the responses seen to receptor agonist and is consistent with a significant indirect component in its response.

MATERIALS AND METHODS

Materials

Carbachol and ionomycin were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) and Calbiochem (Cambridge, U.K.) respectively. Maitotoxin was generously provided by Dr. T. Yasumoto (Tohoku University, Sendai, 980, Japan). *myo*-[2- ^3H]Inositol (12–17 Ci/mmol) was purchased from New England Nuclear (Du Pont Biotechnology, Stevenage, Herts., U.K.). All other chemicals were from Fisons (Loughborough, Leics., U.K.).

Methods

These are as described previously (Baird & Nahorski, 1986). Briefly, cross-chopped rat cerebral-cortex slices ($350\ \mu\text{m} \times 350\ \mu\text{m}$), after 60 min preincubation in Krebs–Ringer bicarbonate buffer (119 mM-NaCl, 4.7 mM-KCl, 1.2 mM- MgSO_4 , 7H $_2\text{O}$, 1.2 mM- KH_2PO_4 , 25 mM- NaHCO_3 , 11.7 mM-glucose and 1.2 mM- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), were transferred to flat-bottomed vials containing *myo*-[2- ^3H]inositol (2.5 μCi) in 300 μl for a further 60 min. After this period, agents were added, for 5 min. Reactions were terminated by addition of HClO_4 (10%, w/v; 300 μl) and the $[^3\text{H}]\text{inositol}$ phosphates were extracted and neutralized as described originally by Downes *et al.* (1986).

H.p.l.c. separations of the $[^3\text{H}]\text{inositol}$ phosphates were performed by a modification of the method of Dean & Moyer (1988) as described previously (Batty *et al.*, 1989). Briefly, separation was achieved with a Partisil (10 μm) SAX analytical column (Technicol) equipped with a pre-column packed with Whatman Pellicular anion-exchange resin and eluted with gradients comprising water and $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ adjusted to pH 3.7 with H_3PO_4 . After sample injection (2 ml), free $[^3\text{H}]\text{inositol}$ was eluted by washing through with water for 10 min. $[^3\text{H}]\text{inositol}$ phosphates were eluted by applying three consecutive gradients at a flow rate of 1 ml/min. $\text{InsP}_1(\text{s})$ were resolved by applying a linear gradient to 0.6 mM- $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ over 30 min. $\text{InsP}_2(\text{s})$ were then separated by isocratic elution at 190 mM- $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ for 15 min, followed

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Fig. 1. H.p.l.c. analysis showing $[^3\text{H}]$ inositol polyphosphate isomer accumulation in rat cerebral-cortical slices

$[^3\text{H}]$ Inositol-labelled rat cortical slices were incubated with: (2) the muscarinic-receptor agonist carbachol (1 mM); (3) K^+ (18 mM); (4) carbachol (1 mM) in the presence of K^+ (18 mM); (5) ionomycin (10 μM); or (6) maitotoxin (0.1 $\mu\text{g/ml}$) for 5 min; (1) shows basal values. Data are means $\pm$ S.E.M. of 3-4 separate experiments.

by a linear increase in eluent concentration to 300 mM over a further 15 min. InsP₃(s) were separated by isocratic elution for 35 min at 500 mM-(NH₄)H₂PO₄. Ins(1,3,4,5)P₄ was then displaced from the column by a 15 min wash at 1.4 M-(NH₄)H₂PO₄. Samples were routinely spiked with 50–100 nmol each of AMP, ADP, ATP, GMP, GDP and GTP before injection. Nucleotides were detected by continuous u.v. monitoring of the column eluate at 254 nm. Authentic ³H-labelled standards and/or co-elution with internal ¹⁴C- or ³²P-labelled inositol standards were used to identify sample [³H]inositol phosphates (Batty *et al.*, 1989).

RESULTS AND DISCUSSION

The present experiments confirm several previous studies in establishing that a variety of stimuli other than those that are clearly receptor-mediated can stimulate phosphoinositide metabolism in rat cerebral-cortical slices. Thus, although it is well established that agonists acting through cell-surface receptors can stimulate phosphoinositidase C via a guanine nucleotide exchange protein, stimuli that elevate intracellular Ca²⁺ also result in inositol phosphate accumulation that is clearly secondary to increased concentrations of this ion [for references see Eberhard & Holz (1988) and Baird & Nahorski (1990)]. In the present experiments we have attempted to separate these responses by detecting changes in different [³H]inositol phosphate isomers separated by h.p.l.c.

It is now well established that the metabolism of Ins(1,4,5)P₃ can proceed by a dephosphorylation route via Ins(1,4)P₂ to Ins(4)P₁, or by phosphorylation to Ins(1,3,4,5)P₄ before dephosphorylation via Ins(1,3,4)P₃ to Ins(1,3)P₂ and Ins(3,4)P₂ and finally Ins(3)P₁ and Ins(1)P₁. Identification of these routes of metabolism in several tissues, including brain, has been revealed predominantly from broken-cell preparations (Inhorn *et al.*, 1987; Bansal *et al.*, 1987; Shears, 1989; Batty *et al.*, 1989). Furthermore, identification of individual isomeric species in whole cells has generally confirmed such metabolic routes after receptor stimulation. Of significance to the present studies, muscarinic-receptor stimulation of rat cerebral-cortex slices has been shown to lead to the accumulation of all of these metabolites, suggesting both phosphorylation and dephosphorylation of Ins(1,4,5)P₃ in stimulated cells (Batty *et al.*, 1989).

In the present experiments we have used this carefully validated h.p.l.c. separation to investigate receptor- and Ca²⁺-dependent responses. For such a detailed analysis we have chosen a single time point (5 min) in which steady-state accumulation of phosphates has been shown to be achieved (Batty & Nahorski, 1985, 1987; Batty *et al.*, 1989; Baird & Nahorski, 1986). Furthermore, studies with the Ca²⁺ ionophore ionomycin (10 μM) show that the [³H]inositol polyphosphates reach maximal values between 3 and 5 min (J. G. Baird & S. R. Nahorski, unpublished work). Detailed studies using maitotoxin have not been performed, although, in view of the similarity in magnitude of the response we obtained previously (Baird & Nahorski, 1990), together with its known effects on increasing [³H]inositol phosphates in other cell types [see Gusovsky *et al.* (1989b) and references therein], we would be confident that the response to this toxin is sustained.

In confirmation of previous results, muscarinic-receptor stimulation of rat cerebral-cortex slices resulted in accumulation of all isomeric species of [³H]InsPs (Fig. 1). Of interest, a similar pattern of metabolites was observed after slices had been depolarized with elevated extracellular K⁺. However, the latter response is complex, with a clear indirect component, since depolarization will release transmitters from nerve terminals which can act through receptor-G-protein activation of phosphoinositidase C. We have established, for example, that K⁺-evoked

accumulation of [³H]Ins(1,3,4,5)P₄ is, at least in part, suppressed by atropine (Baird & Nahorski, 1989). Although it is likely that there is an additional direct effect of Ca²⁺, entering via voltage-sensitive channels, on phosphoinositidase C, the action of K⁺ is complicated by the ability of Ca²⁺ also to enhance phosphorylation of Ins(1,4,5)P₃ by activation of its 3-kinase (Ryu *et al.*, 1987; Biden *et al.*, 1987; Takazawa *et al.*, 1988). This is clearly seen with enhanced, and in some cases synergistic, accumulation of several isomers when carbachol and K⁺ are used together (Fig. 1).

Dramatically different responses were obtained when cortical slices were exposed to the Ca²⁺ ionophore ionomycin or the pore-forming maitotoxin. Both these agents produced similar results that are highly suggestive that they are secondary to elevated intracellular Ca²⁺. Unlike the receptor agonist carbachol, these agents resulted in a dramatic accumulation of [³H]Ins(1,4)P₂ without any significant detection of Ins(1,3,4,5)P₄, Ins(1,3,4)P₃ or Ins(1,3)P₂. These data strongly suggest that the modest accumulation of Ins(1,4,5)P₃ induced by ionomycin and maitotoxin undergoes selective dephosphorylation to Ins(1,4)P₂. Indeed, the dramatic accumulation of the latter bisphosphate may indicate activation of the 5-phosphatase or breakdown (secondary to elevated Ca²⁺) of PtdInsP. This has been previously suggested both by us (Baird & Nahorski, 1986, 1990) and by others (Kolesnick & Gershengorn, 1984; Brammer & Weaver, 1989a; Brammer *et al.*, 1988), without clear separation of bisphosphate isomers.

Analysis of [³H]InsP isomers can only be partially resolved, since the enantiomers Ins(1)P₁ and Ins(3)P₁ cannot be separated on h.p.l.c. However, chiral separation and mass assays suggest that under carbachol stimulation > 90% is Ins(1)P₁ (W. R. Sherman, I. H. Batty & S. R. Nahorski, unpublished work). This would be consistent with major metabolism of Ins(1,3,4)P₃ proceeding via Ins(1,3)P₂ in cerebral cortex (Batty *et al.*, 1989). In the present experiments it was surprising to detect a relatively consistent ratio of Ins(1)P₁/Ins(4)P₁ with all stimuli. This result is not easy to interpret, since, in the absence of Li⁺ to block the monophosphatase, this ratio could be influenced by differences in the rates of hydrolysis of the isomers. However, if it is accepted that Ins(1,4)P₂ is selectively hydrolysed to Ins(4)P₁ (Inhorn *et al.*, 1987; Batty *et al.*, 1989), then accumulation of Ins(1)P₁ after treatment with ionomycin and maitotoxin in the absence of any flux through the Ins(1,3,4,5)P₄ pathway could indicate direct hydrolysis of PtdIns under conditions of elevated intracellular Ca²⁺. Moreover, using a synaptosomal preparation, Brammer & Weaver (1989b) have demonstrated an increase in Ins(1)P with no formation of Ins(1,3,4,5)P₄ in response to the ionophore A23187. They suggest that this would be consistent with hydrolysis of PtdIns directly stimulated by Ca²⁺.

These results have provided the first evidence for clear differences in the patterns of inositol phosphate isomer accumulation after receptor- and Ca²⁺-activated phosphoinositide metabolism. Of interest, however, the report by Gusovsky *et al.* (1989b) suggests that maitotoxin can stimulate InsP₃ and InsP₄ accumulation in a Ca²⁺-dependent manner in PC12 and NCB-20 cells. In contrast, using the clonal human neuroblastoma SH-SY6Y cells, we could find little significant accumulation of InsP₃ or InsP₄ after treatment with ionomycin (Baird *et al.*, 1989) or maitotoxin (J. G. Baird & S. R. Nahorski, unpublished work), even though InsP₂ and InsP₁ were markedly accumulated. These data suggest that certain cells may respond differently to elevated intracellular Ca²⁺. They highlight the care that is required in interpretation of results that do not evaluate the isomeric composition of inositol phosphates.

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