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DIFFERENTIAL EFFECTS OF ELEVATED CALCIUM ION CONCENTRATIONS ON INOSITOL PHOSPHOLIPID RESPONSES IN MOUSE AND RAT CEREBRAL CORTICAL SLICES

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Abstract—Inositol phospholipid turnover in cerebral cortical slices from mouse and rat was assessed using a [^{3}H]inositol pre-labelling technique followed by anion exchange chromatography to isolate [^{3}H]inositol phosphates ([^{3}H]InsP$_x$). In both mouse and rat cerebral cortical slices, elevating the $CaCl_2$ concentration of the Krebs medium from 1.3 to 4 mM did not significantly enhance the accumulation of [^{3}H]InsP$_x$ in the absence of any stimulus, or in the presence of glutamate (3 mM), depolarizing concentrations of KCl (25 mM), 5-hydroxytryptamine (0.3 mM), the calcium ionophore A23187 (33 μM) or carbachol (1 mM). However, the accumulations of [^{3}H]InsP$_x$ induced by histamine (1 mM) or noradrenaline (0.1 mM) were significantly increased by between 95 and 178% in cerebral cortical slices from both species by the elevation of extracellular calcium. Analysis of the individual inositol phosphates revealed that elevated ambient calcium enhanced the histamine-generated accumulations of [^{3}H]InsP$_2$, [^{3}H]InsP$_3$ and [^{3}H]InsP$_4$ by up to two-fold, while only the [^{3}H]InsP$_3$ response to carbachol was significantly increased. Under the same conditions, histamine, but not carbachol, selectively increased the accumulation of [^{3}H]PtdInsP$_2$ by up to 50%. The [^{3}H]InsP$_x$ responses to histamine and noradrenaline in combination with the calcium ionophore A23187 were greater-than-additive, inferring an enhancement of the receptor response by raised intracellular calcium. However, the combination of A23187 with glutamate or KCl resulted in significantly less-than-additive [^{3}H]InsP$_x$ responses. The [^{3}H]InsP$_x$ response to carbachol or 5-hydroxytryptamine was not significantly altered in the presence of A23187. Taken together, these results indicate heterogeneity between the mechanisms of inositol phospholipid turnover induced by these various stimuli in mammalian cerebral cortical slices.

The linkage of specific receptors to inositol phospholipid (PtdInsP$_x$) turnover giving rise to inositol 1,4,5-trisphosphate and diacylglycerol as a second messenger system is now well established [1]. Calcium ions appear to play a pivotal role in this system, either as an essential requirement for the enzyme concerned with the initial propagation of the response in this system, phosphoinositidase C [2], or as a mechanism by which the activation of phosphoinositidase C may be either instigated [3] or prolonged [4]. Thus it has been hypothesized that an agent may initiate PtdInsP$_x$ hydrolysis producing inositol 1,4,5-trisphosphate, leading to a release of intracellular Ca^{2+} [5], at the same time causing the entry of extracellular calcium, which may prolong the initial PtdInsP$_x$ turnover response through a direct stimulatory action on phosphoinositidase C [4].

There is evidence that calcium-stimulated phosphoinositidase C may be different to the (presumably receptor- and G-protein-linked) GTP-analogue-stimulated activity since studies in broken cells have identified differing pH optima and sensitivity to inhibitors for these activities [6]. The production of differing inositol polyphosphate metabolic profiles dependent on the receptor stimulated (muscarinic or histamine H_1 [7, 8]) has also led to the suggestion of heterogeneity in phosphoinositidase C activity. Certainly, there is good evidence from molecular biological approaches for a variety of isoforms of phosphoinositidase C [9], suggesting a diversity of function for this group of isoenzymes.

In the present study we have investigated the effect of elevated extra- and intracellular levels of calcium ions on the inositol phospholipid responses induced by a range of stimuli in slices of mouse and rat cerebral cortex. A preliminary account of this work has been presented to the British Pharmacological Society [10].

MATERIALS AND METHODS

Inositol phosphates accumulation. Due to alterations in calcium ion concentration which may alter the synthesis of PtdInsP$_x$ [11–14], we have adopted a protocol in which incorporation of [^{3}H]inositol is initially uniform prior to agonist-stimulated PtdInsP$_x$ turnover in a range of added calcium ion concentrations.

Inositol phosphates accumulation was carried out in slices from mouse or rat cerebral cortex using the following protocol. Cortices were dissected, chopped (350 μm × 350 μm), and allowed to equilibrate at 37° for 60 min with several changes of pre-gassed (O_2:CO_2 95:5) Krebs–Henseleit medium [composition: NaCl (118 mM); KCl (4.7); $MgSO_4 \cdot 7H_2O$ (1.2); KH_2PO_4 (1.2); glucose (11.7); $NaHCO_3$ (25); $CaCl_2$ (1.3)]. Following the 60 min equilibration

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period, slices were washed three times with nominally calcium-free medium. Aliquots (50 μL) of gravity-packed slices were then distributed into flat-bottomed mini-scintillation vials for [^{3}H]inositol incorporation in the presence of 5 mM LiCl and 1 unit/mL adenosine deaminase. Sufficient Krebs–Henseleit medium lacking $CaCl_2$ was added to give a final volume of 300 μL. In some experiments (Table 2), 25-μL aliquots of tissue were used, to which normal Krebs–Henseleit medium was added in the place of medium from which $CaCl_2$ had been omitted. Incorporation of radioactivity was allowed to proceed for 40 min. Agonist(s) were then added simultaneously with $CaCl_2$ to the appropriate concentration and the incubation proceeded for a further 45 min, before termination with 10% (v/v) perchloric acid. After 15 min at 4° the suspension was neutralized with 0.15 M KOH prior to centrifugation at 3000 *g* for 15 min. The supernatant layer was then removed and used for the determination of either total [^{3}H]inositol phosphates [15], or individual inositol polyphosphate fractions [16].

[^{3}H]*Inositol incorporation.* Incorporation of [^{3}H]inositol into phospholipids was assessed in some experiments by extracting the neutralized slices for at least 20 min in 200 μL 0.1 M HCl and 900 μL chloroform:methanol (1:2 v/v). The phases were separated by the addition of 300 μL chloroform and 300 μL distilled water and centrifugation at 3000 *g* for 10 min. Aliquots (200 μL) of the lower organic phase were removed, allowed to evaporate to dryness, then dissolved in liquid scintillation fluid for determination of radioactivity.

Assessment of the radiolabelling of individual inositol phospholipids was carried out according to Ref. 17. Briefly, the acidified chloroform extract containing the mixture of inositol phospholipids was deacylated at alkaline pH and the resulting [^{3}H]glycerophosphoinositols ([^{3}H]GroInsP$_x$) were separated by Dowex AG 1X8 anion exchange chromatography.

Chemicals. *myo*-2-[^{3}H]Inositol (540 GBq/mmol) was from NEN Du Pont (Boston, MA, U.S.A.). Dowex AG 1X8 resin was obtained from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were from Fisons or the Sigma Chemical Co. (Poole, U.K.).

Statistics. Data were compared for statistical significance using a two-way analysis of variance using the computer programme Statmode (Coleman Ltd). The first factor was the different experimental conditions and the second was the separate experiments. Unless otherwise indicated, analysis was performed with three replicate determinations per cell. Statistical analysis of the effect of individual experimental conditions was performed using the Newman–Keuls *post hoc* test.

RESULTS

The effect of ambient calcium ion concentration on the incorporation of [^{3}H]*inositol*

The effect of ambient calcium ion concentration on the distribution of [^{3}H]inositol in inositol phospholipids was assessed using the adapted protocol (see Materials and Methods). Acidified

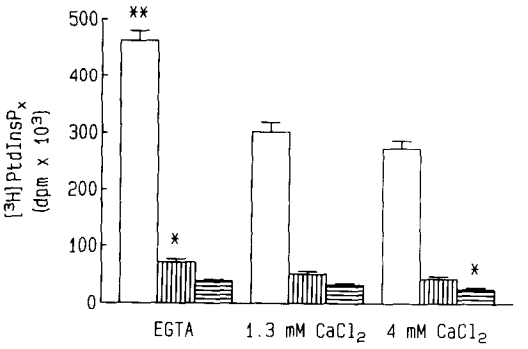

Fig. 1. The effect of ambient calcium ion concentration on the distribution of radiolabel in the inositol phospholipids. Mouse cerebral cortical slices were incubated in nominally calcium-free medium containing [^{3}H]inositol (35 kBq/tube) prior to incubation with EGTA or $CaCl_2$ in the absence of agonist. An acidified chloroform-soluble fraction was obtained from the neutralized slices and the individual glycerophosphoinositols were prepared and separated as described in Materials and Methods ([^{3}H]GroPIns (open bars), [^{3}H]GroPInsP (vertical shading) and [^{3}H]GroPInsP$_2$ (horizontal shading). Data are means ± SE of combined triplicates from three to four experiments. * $P < 0.05$, ** $P < 0.01$ vs 1.3 mM $CaCl_2$.

chloroform-soluble radioactivity (equating to total inositol phospholipids [^{3}H]PtdInsP$_x$ − PtdIns + PtdInsP + PtdInsP$_2$) was enhanced relative to 1.3 mM $CaCl_2$ in calcium-free medium containing 200 μM EGTA, and also in nominally calcium-free medium (data not shown). However, using this present protocol, at higher calcium ion concentrations there was no apparent alteration in the basal accumulation of [^{3}H]PtdInsP$_x$ compared to 1.3 mM $CaCl_2$. The presence of 1 mM histamine or carbachol had apparently only minor affects on incorporation of [^{3}H]inositol into the total inositol phospholipid fraction (data not shown). When the radiolabelled phospholipids from unstimulated slices were deacylated and separated on Dowex AG 1X8 formate form resin, the ratios of [^{3}H]GroPIns (PtdIns), [^{3}H]GroPInsP (PtdInsP) and [^{3}H]GroPInsP$_2$ (PtdInsP$_2$) were found to be largely unchanged with the variation in ambient calcium ion concentration (Fig. 1) PtdIns:PtdInsP:PtdInsP$_2$—EGTA 11.8:1.8:1; 1.3 mM $CaCl_2$ 9.9:1.6:1; 4 mM $CaCl_2$ 11.9:1.8:1). However, the presence of EGTA significantly enhanced the incorporation of [^{3}H]inositol into PtdIns and PtdInsP relative to 1.3 mM $CaCl_2$, while 4 mM $CaCl_2$ slightly but significantly reduced the accumulation of [^{3}H]PtdInsP$_2$.

Elevating ambient calcium

Using the protocol of a [^{3}H]inositol incorporation period in nominally calcium-free medium, the [^{3}H]InsP$_x$ response to 1 mM histamine increased in a linear fashion with increasing added calcium ions (Fig. 2A). In contrast, the response to carbachol was maximal at 1.3 mM added $CaCl_2$, and was not further increased by raising the ambient calcium ion concentration (Fig. 2B). As a result, the [^{3}H]InsP$_x$ response to histamine at 4 mM $CaCl_2$ proved to be approximately twice the carbachol response,

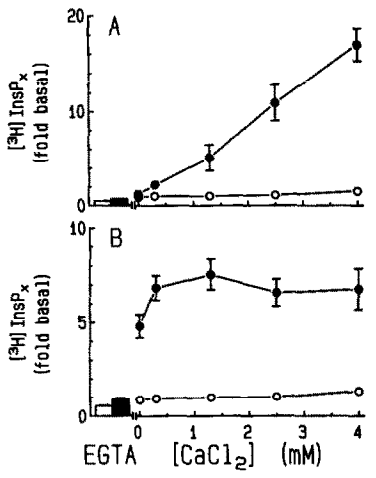

Fig. 2. The effect of raised extracellular calcium ion concentration on agonist-stimulated [^{3}H]InsP$_x$ accumulation. Mouse cerebral cortical slices were incubated in nominally calcium-free medium containing [^{3}H]inositol (35 kBq/tube) prior to incubation with EGTA (bars) or calcium (graph), and stimulated in the absence (open symbols) or presence of agonist (filled symbols). Data are means ± SE expressed as fold stimulation compared to basal accumulation at 1.3 mM $CaCl_2$. (A) [^{3}H]InsP$_x$ accumulation in the absence or presence of 1 mM histamine (basal accumulation 1230 ± 280 dpm, four experiments conducted in triplicate). (B) [^{3}H]InsP$_x$ accumulations in the absence or presence of 1 mM carbachol (basal accumulation 1273 ± 55 dpm, three experiments conducted in triplicate). Accumulations in the presence of either agonist were statistically significant at all concentrations of $CaCl_2$ and in the presence of EGTA ($P < 0.05$ vs basal).

although at 1.3 mM $CaCl_2$ the responses to the two agonists were not greatly different (Fig. 2A and B). The presence of EGTA (0.2 mM) in the absence of added calcium led to reduced but nevertheless significant responses to both agonists. Basal accumulations of [^{3}H]InsP$_x$ were only significantly affected in the presence of EGTA compared to accumulation at 1.3 mM $CaCl_2$ Fig. 2A and B).

When the [^{3}H]InsP$_x$ responses to various stimuli in mouse cerebral cortical slices were investigated at "normal" (1.3 mM) and "high" (4 mM) ambient calcium ion concentrations, it was confirmed that the response to histamine was enhanced while the carbachol response remained unaffected (Table 1). The [^{3}H]InsP$_x$ accumulations generated by glutamate, 5-hydroxytryptamine (5HT), depolarizing concentrations of KCl, and A23187 were similarly unaffected by raising the ambient calcium ion concentration. The [^{3}H]InsP$_x$ accumulation induced by noradrenaline was, however, more than doubled in the presence of elevated ambient calcium ion concentration.

Accumulations of [^{3}H]PtdInsP$_x$ were assessed in the same experiments. Basal accumulation was 126,968 ± 11,096 dpm and was not significantly affected by the elevation in ambient calcium, or the addition of the stimulatory agents (data not shown).

In rat cerebral cortical slices, the responses to histamine and noradrenaline were also selectively enhanced by elevating the ambient calcium ion concentration, without significantly affecting the responses to the other agents (Table 1). In the same experiments, neither elevation of ambient calcium ion concentration nor inclusion of the stimulatory agents had any significant effect on the accumulation of acidified chloroform-soluble radioactivity (basal accumulation = 105,214 ± 7984, data not shown).

Distribution of radiolabel in the inositol phospholipids and polyphosphates

We were prompted to investigate whether the change in ambient calcium ion concentration induced a change in the distribution of radiolabel in the inositol phospholipids and polyphosphates. Using the same protocol as described above, the inositol polyphosphates under basal, histamine- or carbachol-stimulated conditions at varying extracellular calcium concentration were separated into mono-, bis-, tris- and tetrakisphosphate fractions by sequential elution from Dowex AG 1X8 formate form resin [16]. Similarly, the acidified chloroform extracts from the same experiments were collected, deacylated, and the resultant [^{3}H]GroPInsP$_x$ were separated into [^{3}H]GroPIns, [^{3}H]GroPInsP, and [^{3}H]GroPInsP$_2$ fractions eluted sequentially from the same resin [17].

Basal accumulation of [^{3}H]InsP$_1$, [^{3}H]InsP$_2$, [^{3}H]InsP$_3$ and [^{3}H]InsP$_4$ were significantly increased to 254 ± 23, 209 ± 18, 136 ± 18 and 124 ± 15% control, respectively ($N = 7$, $P < 0.05$, two-way ANOVA), by raising the ambient calcium concentration from 1.3 to 4 mM. Both histamine and carbachol significantly stimulated the accumulation of all four species of inositol phosphate at both calcium ion concentrations (Fig. 3A–D). Although the accumulations of [^{3}H]InsP$_1$ elicited by histamine and carbachol at 1.3 mM $CaCl_2$ were comparable (Fig. 3A), histamine evoked a greater accumulation of the inositol polyphosphates than carbachol (Fig. 3B–D). The elevated ambient calcium ion concentration enhanced the histamine response in the [^{3}H]InsP$_1$ fraction non-significantly to 122% control (21,815 ± 11,976 dpm) but significantly raised accumulations of the polyphosphate fractions by approximately two-fold ([^{3}H]InsP$_2$ 20539 ± 2564 dpm, 197%; [^{3}H]InsP$_3$ 6721 ± 1909 dpm, 179%; [^{3}H]InsP$_4$ 711 ± 197 dpm, 205%; Fig. 3B–D).

In the presence of carbachol, on the other hand, the raised extracellular calcium led to slight, non-significant reductions in the accumulations of [^{3}H]InsP$_1$, [^{3}H]InsP$_2$, and [^{3}H]InsP$_4$, with a smaller (compared to the histamine-evoked [^{3}H]InsP$_3$ response) but significant enhancement of the [^{3}H]InsP$_3$ response (1154 ± 325 dpm, 230% control, Fig. 3).

In the same experiments the amount of radiolabel recovered in the individual inositol phospholipids was assessed. No significant changes were observed in the labelling of [^{3}H]GroPIns with agonist or the change in extracellular calcium ion concentration (data not shown). Carbachol slightly, but significantly, increased the labelling of the [^{3}H]GroPInsP fraction to 112 ± 6% control in 1.3 but not 4 mM $CaCl_2$ (data not shown). The raised ambient calcium concentration elicited a significant reduction in the

Table 1. The effect of ambient calcium concentration on [^{3}H]InsP$_x$ accumulation induced by various agents in mouse and rat cerebral cortical slices

Species	Mouse		Rat	
[$CaCl_2$] (mM)	1.3	4.0	1.3	4.0
Glutamate	1534 ± 292	1458 ± 581 (95)*	1445 ± 306	1254 ± 403 (87)
5HT	1982 ± 576	1546 ± 567 (78)	1393 ± 403	1085 ± 55 (78)
High KCl	3961 ± 1108	3037 ± 1089 (77)	2573 ± 398	2270 ± 572 (88)
A23187	3994 ± 826	4055 ± 969 (102)	2929 ± 677	2948 ± 901 (101)
Histamine	6176 ± 860	17,191 ± 379 (278)†	3074 ± 778	8008 ± 1579 (260)†
Noradrenaline	7510 ± 1250	16,175 ± 2509 (215)†	6189 ± 679	12,090 ± 1969 (195)†
Carbachol	9693 ± 2971	9398 ± 2261 (97)	5630 ± 1241	5617 ± 1199 (100)

Data are means ± SE of [^{3}H]InsP$_x$ accumulations expressed as dpm-basal from three to four separate experiments conducted in triplicate as delineated in Materials and Methods using 26 (mouse) or 30 kBq (rat) of [^{3}H]inositol/tube. The following concentrations were employed: glutamate 3 mM; 5HT 1 mM; high KCl 31 mM; A23187 33 μM; histamine 1 mM; noradrenaline 0.1 mM; and carbachol 1 mM. Basal accumulations were 1298 ± 231 and 2247 ± 442; 1052 ± 122 and 1758 ± 263 at normal (1.3 mM) and high (4 mM) $CaCl_2$, for mouse and rat, respectively.

* Per cent response in 1.3 mM $CaCl_2$.

† $P < 0.01$ vs response in 1.3 mM $CaCl_2$.

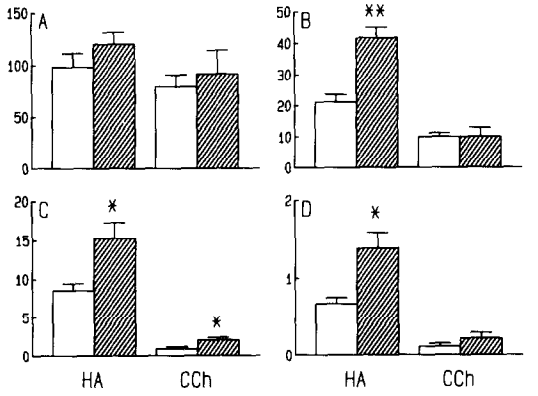

Fig. 3. The distribution of radiolabel in the inositol polyphosphates from mouse cerebral cortical slices. Data are means ± SE of combined triplicates from 5 to 7 experiments expressed as dpm × 10^3. Agonist (histamine, HA or carbachol, CCh) incubation was carried out in the presence of 1.3 (open bars) or 4 mM $CaCl_2$ (shaded bars) for 45 min, as stated in Materials and Methods. Dowex AG 1X8 formate form columns were used to separate (A) [^{3}H]InsP$_1$, (B) [^{3}H]InsP$_2$, (C) [^{3}H]InsP$_3$ and (D) [^{3}H]InsP$_4$ according to Brown *et al.* [16]. All accumulations were statistically significant compared to basal ($P < 0.05$ vs basal). * $P < 0.05$, ** $P < 0.01$ 4 vs 1.3 mM $CaCl_2$.

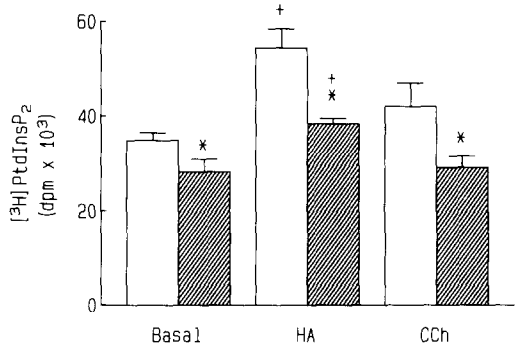

Fig. 4. The influence of ambient calcium on the labelling of PtdInsP$_2$ in mouse cerebral cortical slices. Agonist (histamine, HA or carbachol, CCh) incubation was carried out in the presence of 1.3 (open bars) or 4 mM $CaCl_2$ (shaded bars) for 45 min. Acidified chloroform extracts of the neutralized slices were deacylated and separated on Dowex AG 1X8 as described previously [17]. Data are means ± SE of combined triplicates from 4 to 6 experiments. * $P < 0.05$ vs 1.3 mM $CaCl_2$, + $P < 0.05$ agonist vs basal.

labelling of [^{3}H]GroPInsP$_2$ under either basal or agonist-stimulated conditions (Fig. 4). The presence of histamine, but not carbachol, however, resulted in an enhanced labelling of this fraction at both 1.3 and 4 mM $CaCl_2$ (156 ± 11% and 136 ± 4% control, respectively).

The calcium ionophore A23187

Since an increase in the extracellular calcium ion concentration revealed heterogeneity in the [^{3}H]InsP$_x$ responses to various agents, we investigated whether an increase in intracellular calcium ion concentration using the ionophore A23187 would also result in heterogeneity. Mouse cerebral cortical slices were incubated throughout in medium containing 1.3 mM $CaCl_2$. The ionophore, at a concentration previously shown to be maximally effective at enhancing the [^{3}H]InsP$_x$ response to noradrenaline in rat cerebral cortical slices (33 μM, [3]) was added simultaneously with the primary agent. The accumulations of [^{3}H]InsP$_x$ under control conditions and in the presence of A23187 are shown in Table 2.

In the presence of A23187 the [^{3}H]InsP$_x$ responses to glutamate and a depolarizing concentration of KCl were reduced to less-than-additive levels, while the responses to histamine and noradrenaline were enhanced by up to 80%. The 5HT response was enhanced to a similar degree, but failed to reach

Table 2. A23187 and the [^{3}H]InsP$_x$ accumulations induced by various agents in mouse cerebral cortical slices

Agent	1.3 mM $CaCl_2$ InsP$_x$ (dpm)	(dpm-basal)	+A23187 (dpm)	(dpm-A23187)
Basal	993 ± 128		5853 ± 516*	
5HT	2358 ± 304*	1365 ± 269	8238 ± 468†	2384 ± 100 (175)‡
Glutamate	2567 ± 376*	1574 ± 374	5560 ± 354	−293 ± 297 (−19)
High KCl	3610 ± 611*	2617 ± 594	6458 ± 706	694 ± 531 (27)
Carbachol	8560 ± 1717*	7567 ± 1719	14,214 ± 2009†	8361 ± 1814 (110)
Histamine	8877 ± 1608*	7884 ± 1613	17,207 ± 1674†	11,354 ± 1464 (144)§
Noradrenaline	8991 ± 1264*	7998 ± 1238	20,220 ± 3410†	14,367 ± 2465 (180)§

Data are means ± SE from triplicate determinations in three to four separate experiments, with 25 μL of tissue slices incubated continuously in medium containing 1.3 mM $CaCl_2$ and 29 kBq of [^{3}H]inositol, as delineated in Materials and Methods. Agents were present at the same concentrations as in Table 1.

* $P < 0.01$ vs basal response in 1.3 mM $CaCl_2$.

† $P < 0.01$ vs A23187 basal response.

‡ Per cent response in 1.3 mM $CaCl_2$.

§ $P < 0.01$ vs response in 1.3 mM $CaCl_2$ (two-way ANOVA conducted with mean values from each experiment—one entry per cell).

significance at the 5% level. In contrast, the carbachol-elicited [^{3}H]InsP$_x$ accumulation was unchanged in the presence of calcium ionophore.

DISCUSSION

In the present study, in which the effects of raising both ambient and intracellular calcium ion concentrations were investigated, we have observed heterogeneity in the [^{3}H]InsP$_x$ responses to various stimuli. These responses are of three types: (i) glutamate, KCl and A23187 give [^{3}H]InsP$_x$ responses of similar magnitudes which are not enhanced in the presence of elevated ambient calcium (Table 1), and combining the former two with A23187 (i.e. in the presence of raised intracellular calcium) results in less-than-additive responses (Table 2); (ii) the [^{3}H]InsP$_x$ accumulations generated by carbachol and 5-HT are not affected by elevations in extra- or intracellular calcium levels (Fig. 2B; Tables 1 and 2); (iii) the [^{3}H]InsP$_x$ responses to noradrenaline and histamine are enhanced by elevations in ambient or intracellular calcium ion concentrations (Fig. 2A; Tables 1 and 2).

It is likely that the PtdInsP$_x$ breakdown induced by the first group of agents (glutamate, KCl and A23187) may be, in whole or in part, a consequence of facilitated calcium ion entry. In membranes from rat cerebral cortex, the ability of calcium ions to directly stimulate phosphoinositidase C activity has been observed [6], and this may prove to be the means by which this group of agents gives rise to accumulations of [^{3}H]InsP$_x$. This view is supported by our preliminary studies in which we observed that the response to A23187 was completely blocked in the presence of EGTA, increased over the range 0 to 0.3 mM $CaCl_2$ and was maximal at 1.3 mM (data not shown). Thus the [^{3}H]InsP$_x$ response to A23187 is sensitive to ambient calcium ion levels but the effect is maximal at 1.3 mM and is not further enhanced at higher calcium ion concentrations (Table 1). The effect of KCl has also been shown to be calcium-dependent [3], and is enhanced by dihydropyridine calcium channel activators, and reduced by dihydropyridine calcium channel blockers [18], indicating the involvement of calcium ion entry. Similarly, glutamate has been shown to cause an influx of calcium ions into cerebral cortical slices [19], indicating the potential for calcium ion stimulation of phosphoinositidase C activity with this agent.

If these agents cause PtdInsP$_x$ hydrolysis through facilitation of calcium ion entry, the combination of other agents from this group with A23187, in which the normal gradient of calcium ion entry across plasma membranes is much reduced, would be expected to lead to an accordingly much reduced [^{3}H]InsP$_x$ signal. Indeed, we observed less-than-additive responses to glutamate and KCl when combined with A23187 (Table 2). The [^{3}H]InsP$_x$ response to a combination of glutamate and KCl was also less-than-additive (data not shown).

The accumulations of [^{3}H]InsP$_x$ elicited by carbachol were not affected by elevations in either ambient or intracellular calcium (Fig. 2B; Tables 1 and 2), although we observed a slight (compared to the increase in the histamine response) but significant increase in the accumulation of [^{3}H]InsP$_3$ (Fig. 3C). The [^{3}H]InsP$_x$ response to carbachol in mouse cerebral cortical slices is maximal at 1.3 mM Ca^{2+}, and does not increase at higher Ca^{2+} concentrations (Fig. 2B; Table 1). This latter result is in apparent contradiction with the recent study of Baird *et al.* [14], who observed an inhibition of the carbachol response in rat cerebral cortex at higher calcium ion concentrations. This effect was apparently due to a reduced incorporation of [^{3}H]inositol into phospholipid by the elevated calcium ion concentration thereby resolving the apparent contradiction. The protocol we have adopted involves a [^{3}H]inositol incorporation period in the absence of added calcium ions in order that incubations in the presence of agonist (with varying added calcium) should better reflect calcium-influenced agonist-induced [^{3}H]InsP$_x$

accumulation rather than a combination of the effects of calcium and agonist on [^{3}H]inositol incorporation into phospholipid and its subsequent catabolism. However, we did observe a significant reduction in the labelling of [^{3}H]PtdInsP$_2$ in slices incubated for 45 min in 4 mM $CaCl_2$ compared to 1.3 mM $CaCl_2$. We also noted an enhanced labelling of [^{3}H]PtdIns and [^{3}H]PtdInsP in the presence of EGTA compared to 1.3 mM $CaCl_2$ in accord with a previous investigation of the effects of calcium-free medium on the distribution of [^{3}H]inositol in phospholipids [3].

It is likely that the muscarinic receptor of mouse and rat cerebral cortical slices is linked to a phosphoinositidase C isoenzyme that is Ca^{2+}-sensitive (since EGTA greatly reduces the response to carbachol, Fig. 2B). However, this isoenzyme is probably different from that stimulated by the first group of agents, since this former (muscarinic-linked) isoenzyme is not further stimulated by raising calcium ions above the normal (submicromolar [20, 21]) intracellular levels through the action of A23187.

Finally, histamine and noradrenaline [^{3}H]InsP$_x$ responses are raised by both elevated extra- and intracellular calcium. In contrast to the effects of carbachol (Fig. 2B), the [^{3}H]InsP$_x$ accumulation generated by histamine in mouse and rat cerebral cortical slices rises apparently linearly with increasing ambient calcium levels (Fig. 2A, data not shown). Furthermore, the bis-, tris- and terakisphosphate fractions generated by histamine were all enhanced by a similar factor by raising the ambient calcium ion concentration to 4 mM, while this elevation resulted in an enhancement of the carbachol-stimulated [^{3}H]InsP$_3$ response alone.

Analysis of the distribution of radiolabel in the inositol phospholipids also revealed differences in the effects of muscarinic and H_1 receptor activation. Stimulation of the former led to a slight increase in the labelling of PtdInsP at 1.3 mM, whereas stimulation of the latter produced major enhancements of the labelling of PtdInsP$_2$ at both 1.3 and 4 mM. The mechanism of this increase in labelling is not known but may result from G-protein-linked activation of PtdInsP kinase, a phenomenon which has recently been suggested to occur in brain membranes with GTP analogues [22]. Dissimilarities between muscarinic and histamine H_1 [^{3}H]InsP$_x$ responses in brain have also been observed from other studies on the effects of lithium [7, 8] and variations in temperature [23].

It is suggested that the receptors stimulated by this third group of agonists are linked to phosphoinositidase C isoenzyme(s) which are Ca^{2+}-requiring [EGTA markedly attenuates the effects of 1 mM (Fig. 2A), and completely blocks the response to 0.3 mM histamine (data not shown)]. However, in contrast to the muscarinic-linked phosphoinositidase C, elevated calcium ion concentrations enhance the production of InsP$_x$. Conceivably, either in parallel to, or as a consequence of PtdInsP$_x$ hydrolysis, both these latter two groups of agonists may stimulate calcium ion entry [20, 21], and so have the potential to augment the production of InsP$_x$.

In summary, therefore, we observe heterogeneity in the [^{3}H]InsP$_x$ accumulations generated by three groups of agents, based on the differential influence of elevated calcium ions. These disparities may well arise through differences in the phosphoinositidase C isoenzyme(s) stimulated, directly or indirectly, by these agents.

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