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Temporal changes in the calcium-dependence of the histamine H_1-receptor-stimulation of cyclic AMP accumulation in guinea-pig cerebral cortex

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1 2-Chloroadenosine (2CA) causes a maintained rise in adenosine 3':5'-cyclic monophosphate (cyclic AMP) content of guinea-pig cerebral cortical slices which is augmented by addition of histamine. We have investigated the temporal profile of the sensitivity of this response to calcium.

2 Rapid removal of extracellular calcium with EGTA (5 mM) at 2CA (30 μM)-induced steady state caused a slight increase in the cyclic AMP response to 2CA alone and completely abolished the augmentation produced by histamine (0.1 mM) added 20 min later. When EGTA was added only 2 min before histamine, the augmentation was reduced by 72%.

3 The calcium sensitivity of the histamine response was also indicated in studies in which EGTA was added 1 or 3 min after histamine at 2CA-induced steady state. Following addition of EGTA at either of these times, the augmentation was not maintained.

4 When calcium was rapidly removed with EGTA once a steady state level of cyclic AMP had been achieved with histamine, the augmentation response was maintained. This was despite the fact that EGTA had a similar effect on both extracellular free calcium and tissue calcium content when it was applied before or after histamine.

5 The 2CA response was augmented by phorbol esters (which mimic the actions of diacylglycerol) in a calcium-independent manner.

6 These results suggest that calcium is important for the initiation and early stages of the histamine-induced augmentation response. The apparent lack of calcium sensitivity of the response at later stages could mean that calcium is not involved in the maintenance of the response or that the intracellular machinery involved in the augmentation process becomes more sensitive to calcium as the response progresses, such that it becomes able to operate at a much lower level of intracellular calcium. A possible role for diacylglycerol in the maintenance of the response is discussed.

Introduction

Histamine can elevate adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in brain slices by two mechanisms. It can stimulate adenylate cyclase directly via histamine H_2-receptors. Alternatively, it can act through H_1-receptors to augment the cyclic AMP responses to H_2-, vasoactive intestinal peptide (VIP)- or adenosine A_2-receptor stimulation (Palacios *et al.*, 1978; Al-Gadi & Hill 1987; Hill *et al.*, 1981; Magistretti & Schorderet, 1985; Hollingsworth & Daly, 1985; Garbarg & Schwartz, 1988; Hill & Kendall, 1989). The augmentation of the adenosine response is rapid in onset and requires a maintained stimulation of H_1-receptors; addition of the H_1-receptor antagonist mepyramine causes a rapid fall in cyclic AMP levels to those obtained with adenosine alone (Donaldson *et al.*, 1988b). Kinetic studies have indicated that this augmentation is exerted via an action on adenylate cyclase (i.e. on the G_s regulatory protein or the catalytic unit) rather than by inhibition of the enzyme which breaks down cyclic AMP, phosphodiesterase (Donaldson *et al.*, 1988b).

Little is yet known about the intracellular mechanisms by which neurotransmitters indirectly potentiate cyclic AMP accumulation. However, the production of another second messenger seems to be

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involved since the effect is lost in membrane preparations (Hegstrand *et al.*, 1976; Green *et al.*, 1977; Coupet & Szuchs-Meyers, 1981; Green, 1983; Hill, 1987). It has been suggested that the products of inositol phospholipid breakdown, the inositol phosphates and diacylglycerol, promote increased activity of the cyclase via calcium mobilisation and protein kinase C activation, respectively (Schwabe *et al.*, 1978; Hollingsworth *et al.*, 1985; Danoff & Young, 1987). Histamine H_1-receptor stimulation is accompanied by an increased hydrolysis of inositol phospholipids in mammalian brain slices and hence can give rise to both an increase in intracellular calcium and the formation of diacylglycerol (Daum *et al.*, 1984; Donaldson & Hill, 1986; Hill, 1987). Phorbol esters, which mimic the action of diacylglycerol by activating protein kinase C, have been reported to mimic fully the effects of H_1-receptor stimulation in potentiating the cyclic AMP response to 2-chloroadenosine (2CA) in a synaptoneurosome preparation of guinea-pig cerebral cortex (Hollingsworth *et al.*, 1985). However, since the concentrations of phorbol ester used were much higher than those normally required to activate protein kinase C, the significance of this observation has been challenged (Danoff & Young, 1987).

An involvement of calcium in the augmentation response has been suggested, since a reduction in extracellular free calcium concentration prior to agonist addition has been shown to abolish the synergism between histamine and adenosine analogues in slices of guinea-pig cerebral cortex (Schwabe *et al.*, 1978). Furthermore, in rabbit cerebral cortex the indirect H_1-receptor-mediated cyclic AMP response has been found to be reduced in calcium-free Krebs or by addition of EGTA (Al-Gadi & Hill, 1987). In slices of guinea-pig hippocampus, the H_1-receptor-mediated augmentation of the direct H_2-receptor-induced cyclic AMP response appears to involve both calcium ions and diacylglycerol (Garbarg & Schwartz, 1988).

The rise in intracellular calcium in response to histamine H_1-receptor activation has been studied in some detail in both vascular and tracheal smooth muscle, with calcium-sensitive fluorescent dyes (Reynolds & Dubyak, 1986; Matsumoto *et al.*, 1986; Takuwa *et al.*, 1987; Kotlikoff *et al.*, 1987). Following H_1-receptor activation in these cells, there appears to be a large transient rise in intracellular calcium which peaks around 1–2 min then falls to a plateau somewhat above basal. The contractile response, however, remains constant despite the fall in intracellular calcium. This observation has led several authors to suggest that the initiation of the contractile response is dependent upon mobilization of calcium while contraction is maintained by the activation of protein kinase C (Park & Rasmussen, 1985; Takuwa *et al.*, 1987). If the rise in intracellular calcium following H_1-receptor activation in guinea-pig brain slices were also transient, then the calcium sensitivity of the response might be expected to change with time. In the present study we have therefore investigated in detail the temporal profile of the calcium sensitivity of the augmentation by histamine of the cyclic AMP response to 2CA in slices of guinea-pig cerebral cortex, by examining the effect of a sudden reduction in external free calcium (and associated change in intracellular calcium e.g. see Al-Gadi & Hill, 1987) with ethylene-glycol-*bis* (β-amino ethylether)N,N′-tetraacetic acid (EGTA) at various times before and after histamine addition at 2CA-induced steady state levels of cyclic AMP.

Methods

Measurement of [^{3}H]-cyclic AMP accumulation

Cyclic AMP accumulation was determined by the [^{3}H]-adenine prelabelling technique, essentially as described previously (Donaldson *et al.*, 1988b). Briefly, slices (300 × 300 μm) of guinea-pig cerebral cortex were labelled with [^{3}H]-adenine, washed three times with Krebs-Henseleit solution at 37°C and finally allowed to settle under gravity. Slices (1 ml packed volume) were transferred to a 20 ml glass vial containing 8 ml Krebs-Henseleit solution (calcium concentration 2.5 mM). Slices were stirred at a rate sufficient to prevent them settling. The H_2-receptor antagonist tiotidine (30 μM) was included in the incubation medium in order to eliminate the direct actions of histamine (acting via H_2-receptors). The direct cyclic AMP stimulus was provided by the stable adenosine analogue 2CA, and the effects of endogenous adenosine were eliminated by inclusion of adenosine deaminase (1.2 u ml^{-1}) in the incubation medium. At various times before and after EGTA addition, 300 μl aliquots of the slice suspension were taken for analysis of cyclic AMP. A concentration of 5 mM EGTA was used, since this concentration was calculated, using constants for Mg^{2+}, Ca^{2+}, and H^+ binding to EGTA given by Sillen & Martell (1964), to reduce extracellular free calcium to below 0.1 μM. Addition of 5 mM EGTA to Krebs solution causes a decrease in pH (from 7.5 to 6.7) because of H^+ release on calcium chelation. Consequently in all experiments it was added in combination with sufficient 1 M NaOH to maintain a pH of 7.5 in the incubation medium.

In experiments using calcium channel antagonists and some of the experiments using phorbol esters, incubations were performed in flat bottomed insert vials. In these experiments, 50 μl portions of gravity packed slices were added to 240 μl of Krebs medium

containing tiotidine (30 μM), calcium channel antagonists, phorbol esters or adenosine deaminase (1.2 u ml^{-1}) where appropriate. Tubes were gassed with 95% O_2:5% CO_2, capped and incubated for 10–20 min at 37°C in a shaking water bath. Agonist was added after this step in 10 μl of medium and the tubes gassed again and incubated for an additional 10 min.

Incubations or aliquots from them (300 μl) were stopped and cyclic AMP extracted by mixing with 200 μl ice cold 1 M HCl. The [^{3}H]-cyclic AMP was then isolated by sequential Dowex-alumina chromatography, as described previously (Donaldson *et al.*, 1988a). The recovery of [^{3}H]-cyclic AMP off the columns was corrected for by spiking the samples with 100 μl [^{14}C]-cyclic AMP (0.001 μCi, 0.24 μM).

Calcium content of the slices

The calcium content of guinea-pig cerebral cortical slices was monitored at various times before and after addition of EGTA (5 mM) by atomic absorption spectrophotometry (Pye-Unicam series 2). Aliquots (300 μl) of the slice suspension were added rapidly to 1 ml ice-cold calcium-free Krebs and the slices precipitated by spinning at 12,000 *g* in a Beckman Microfuge B for 5 s. The supernatant was discarded and the slices rapidly washed a second time in 1 ml ice-cold calcium-free Krebs. The tissue calcium was finally extracted in 250 μl of 5% trichloroacetic acid. The sample was diluted with 250 μl of a solution containing 200 mM NaCl, 200 mM KCl and 2% $LaCl_3$. Tissue debris was precipitated by brief centrifugation at 12,000 *g*. The calcium content of the supernatant was then analysed by atomic absorption spectrophotometry. Calcium standards were prepared in twice distilled water containing 100 mM KCl, 100 mM NaCl, 1% $LaCl_3$ and 2.5% trichloroacetic acid. The protein content of slices was determined by the method of Lowry *et al.* (1951).

In preliminary experiments, the effect on the tissue calcium content of including 1 mM EGTA in the ice-cold calcium-free wash buffer, was investigated. No significant difference was observed in the recorded tissue calcium content with the two different wash buffers. Consequently, EGTA was not included in the wash medium in all subsequent experiments.

Measurements of extracellular free calcium

Extracellular free calcium concentration was determined by use of a calcium electrode (Orion Research Inc., Cambridge, MA) with a silver/silver chloride reference electrode. Calcium standards were prepared over the range 2.5 mM to 10 μM in de-ionised twice-distilled water (further purified with an Elgastat UHQ) containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4. Zero calcium was determined in an aliquot of this buffer containing 5 mM EGTA. The standard curve (mV vs log [Ca]) was linear over this range. The response to a free calcium concentration below 0.1 μM was obtained with an aliquot of the same buffer containing 5 mM EGTA.

Data analysis

In time course studies, cyclic AMP was measured at single time points and the lines through the data points were drawn by inspection. Each experiment was repeated at least three times. The mean effect of each intervention is given in the text along with the s.e.mean. *n* in the text refers to the number of separate experiments. Concentration-response curves for phorbol esters were fitted by non-linear regression using the programme Graphpad. The equation fitted was:

$$\% \text{ of maximum response} = \frac{E_{max} \times D^n}{D^n + (EC_{50})^n}$$

where D is the concentration of phorbol ester, n is the Hill coefficient, EC_{50} is the concentration of phorbol giving 50% maximal response and E_{max} is the maximal stimulation.

Chemicals

Dowex 50W, H^+ form (200–400 mesh), 2-chloroadenosine, adenosine deaminase (type VI), neutral alumina (type WN-3), imidazole, mepyramine maleate, histamine dihydrochloride, ethyleneglycol-*bis*(β-amino ethylether)N,N′-tetraacetic acid (EGTA), 4β-phorbol 12-myristate 13-acetate, 4β-phorbol 12,13-dibutyrate, 4α-phorbol, nifedipine, nickel chloride, cadmium chloride, polymyxin B sulphate and H-7 (1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine dihyrochloride) were purchased from Sigma. [8-^{3}H]-adenine (specific activity, 26 Ci $mmol^{-1}$) was obtained from Amersham International, and [8 – ^{14}C]-cyclic AMP (specific activity 42.4 mCi mol^{-1}) from Dupont-N.E.N. Tiotidine was a gift from I.C.I. PN-200-110 (isopropyl 4-(2,1,3,benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methyoxy-carbonyl-pyridine-3-carboxylate) was obtained from Sandoz and staurosporin from Boehringer Mannheim.

Results

Effect of EGTA

Addition of 30 μM 2CA to guinea-pig cerebral cortical slices resulted in an increase in cyclic AMP levels,

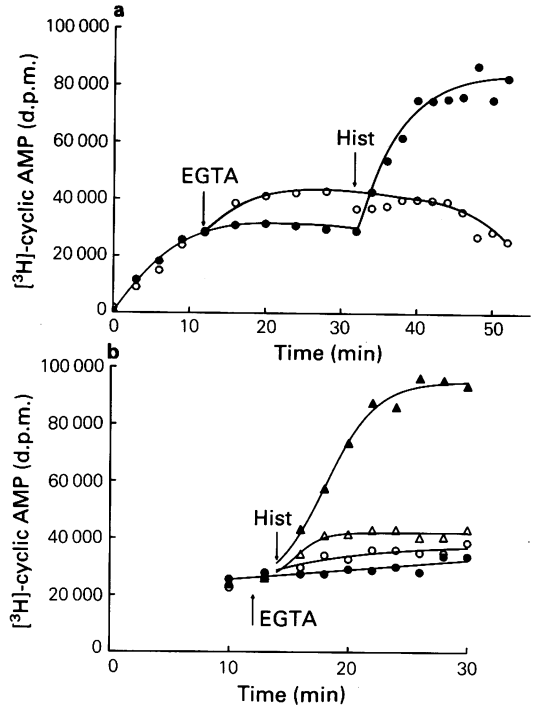

Figure 1 Effect of 5 mM EGTA on the cyclic AMP response to 2-chloroadenosine (2CA) (30 μM) and 0.1 mM histamine in guinea-pig cerebral cortical slices. (a) EGTA added 20 min prior to histamine at 2CA steady state: (●) control, (○) EGTA. 2CA was added at zero time. Histamine (Hist) was added 32 min after 2CA. (b) Effect of addition of EGTA 2 min prior to histamine at 2CA steady state. 2CA (30 μM) was added at zero time: (●) 2CA alone; (○) 5 mM EGTA added at 2CA steady state; (▲) 0.1 mM histamine added at 2CA steady state; (△) 5 mM EGTA added at 2CA steady state followed by 0.1 mM histamine 2 min later. Experiments (both a and b) were performed in the presence of adenosine deaminase (1.2 u ml^{-1}). The H_2-receptor antagonist tiotidine (30 μM) was also included in the incubation medium in order to prevent the direct actions of histamine via H_2-receptors (Donaldson *et al.*, 1988b). Data were obtained in single experiments. Similar results were obtained on (a) two or (b) five other occasions.

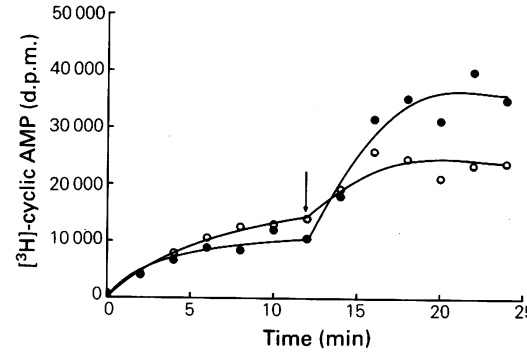

Figure 2 Comparison of the histamine-induced augmentation of the cyclic AMP response to 2-chloroadenosine (2CA) in guinea-pig cerebral cortical slices in calcium-containing (2.5 mM) and calcium-free Krebs: (●) calcium-containing; (○) calcium-free. 2CA (30 μM) was added at zero time and histamine (0.1 mM) 12 min later (at arrow). In both cases slices were prelabelled with [^{3}H]-adenine in calcium-containing Krebs as described under Methods. The H_2-receptor antagonist tiotidine (30 μM) and adenosine deaminase (1.2 u ml^{-1}) were included in the incubation medium. Data were obtained in a single experiment. Similar results were obtained on two other occasions.

which reached steady state within 10 min (46.1 ± 3.4 fold over basal, $n = 29$). EGTA (5 mM) had no significant effect on basal levels of cyclic AMP ($n = 6$) but elevated the cyclic AMP response to 2CA (30 μM), both when it was added 20 min or 2 min before 2CA (data not shown) and 12 min after (i.e. during 2CA-induced steady state) (Figure 1a). Addition of EGTA 2 min before or 12 min after 2CA (Figure 1a), resulted in an increase in the steady state level of cyclic AMP in the presence of 2CA of 1.61 ± 0.61 fold ($n = 3$) and 1.34 ± 0.06 fold ($P < 0.05$ paired t test, $n = 3$) over the response to 2CA in the absence of EGTA, respectively. When EGTA was added 20 min before 2CA, it produced a maximum elevation of the 2CA response of 1.35 ± 0.03 ($n = 3$, $P < 0.01$ paired t test). However, in this case a steady state level of cyclic AMP was not maintained in the presence of EGTA (data not shown).

Addition of 0.1 mM histamine to guinea-pig cerebral cortical slices at 2CA steady state, caused cyclic AMP levels to rise and achieve a new steady state, again within 10 min (3.70 ± 0.17 fold over the steady state response to 2CA, $n = 28$) (Figure 1). When 5 mM EGTA was added 2 min before histamine (0.1 mM) at 2CA steady state, histamine-induced augmentation was markedly reduced (by 71.7 ± 8.4% of the response in the absence of EGTA, $n = 6$, Figure 1b). If EGTA was added 20 min before the histamine ($n = 3$, Figure 1a), the histamine-induced augmentation was totally abolished.

These results suggested that the histamine response was in some way dependent on calcium. However, it is possible that these effects of EGTA on cyclic AMP accumulation may not be due to the chelation of calcium, but to another property of the EGTA. In order to investigate this possibility, we compared the histamine-induced cyclic AMP

response in normal Krebs (2.5 mM calcium) and in nominally calcium-free Krebs (50 μM calcium in the presence of slices, 5 μM calcium in the absence). In these experiments, slices were preincubated and labelled with [^{3}H]-adenine in normal Krebs buffer. The slices were then divided into two batches. One batch was washed and incubated in normal Krebs; the other was washed with calcium-free Krebs and the experiment carried out in this latter buffer. In calcium-free conditions the histamine-induced augmentation was reduced by $43.2 \pm 1.5\%$ ($n = 3$, Figure 2) whilst the response to 2CA alone was unaffected (Figure 2). These experiments therefore provided further evidence for a role for calcium in the augmentation response.

The next series of experiments investigated the effect of a sudden reduction in external calcium after the histamine had been added. In contrast to the results of experiments in which EGTA was added before histamine, addition of EGTA once a steady state level of [^{3}H]-cyclic AMP had been achieved with histamine (in the presence of 2CA) was without significant effect on the augmentation response (Figure 3a). Following EGTA addition, cyclic AMP levels fell by only $3.3 \pm 2.3\%$ ($n = 5$) over 10 min, even though a large significant fall ($P < 0.05$, $44.1 \pm 4.5\%$ over 10 min, $n = 3$) was obtained following addition of the H_1-receptor antagonist mepyramine (1 μM) (data not shown but see Donaldson *et al.*, 1988b).

In order to investigate at what point EGTA became ineffective in reducing the cyclic AMP response to histamine, we examined the effect of adding EGTA 1 min or 3 min after histamine (at 2CA steady state). At these times a new steady state level of cyclic AMP had not yet been achieved (see Figure 1). Following EGTA addition at either of these times, the augmentation was substantially reduced (Figure 3b) and although cyclic AMP levels continued to rise for 2–4 min after EGTA addition they then gradually fell towards basal ($n = 3$ in each case).

These results therefore suggested that calcium was important for the initiation and early stages of the augmentation response to histamine, but was less important for its maintenance, once a steady state had been attained. However, this apparent change in the calcium sensitivity of the response could be explained if EGTA was having different effects on calcium levels at the different time points. The effects of EGTA on both extracellular free calcium and tissue calcium content during cyclic AMP steady states in the presence of 2CA or 2CA plus histamine were therefore measured (Figure 4). The effect of EGTA on extracellular free calcium was apparently the same at the two steady states. In the presence of 2CA or 2CA plus histamine, EGTA addition caused

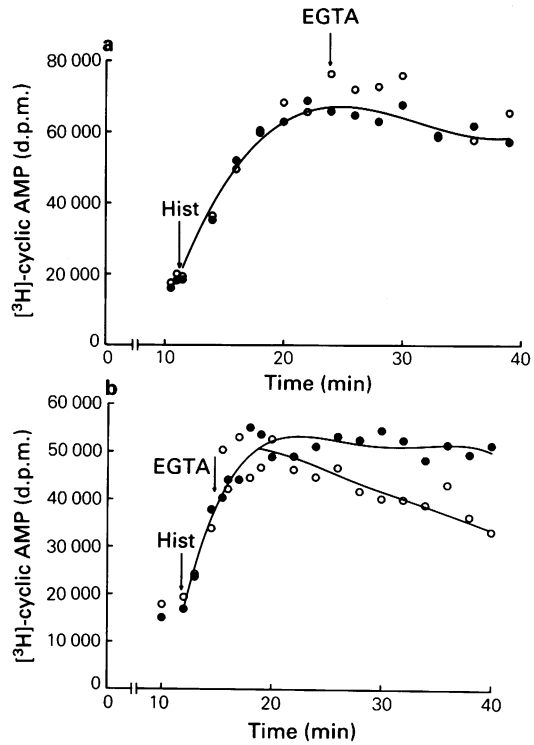

Figure 3 Effect of addition of EGTA (a) at histamine steady state or (b) 3 min after histamine (Hist) on the histamine-induced augmentation of the 2-chloroadenosine (2CA) cyclic AMP response in guinea-pig cerebral cortex: (●) control; (○) EGTA. 2CA (30 μM) was added at zero time. Histamine (0.1 mM) was added 12 min after 2CA. EGTA (5 mM) was added (a) 12 or (b) 3 min after histamine. The experiment was performed in the presence of adenosine deaminase (1.2 u ml^{-1}) and tiotidine (30 μM). Data were obtained in single experiments. Similar results were obtained on (a) four or (b) two other occasions.

extracellular free calcium to fall rapidly to below 10 μM ($n = 3$) (Figure 4a and b). EGTA also produced a rapid and large reduction in the tissue calcium content (Figure 4c and d). Addition of EGTA at either 2CA- or histamine-induced steady states caused tissue calcium content to fall by 50–70% ($n = 3$ in each case) within 2 min. A more detailed analysis of the fall in tissue calcium content over the first minute following EGTA addition revealed that calcium levels were unaffected for the first 20 s, but then fell rapidly over the next 20 s ($33.3 \pm 2.4\%$ in 20 s, $n = 3$, data not shown). However, since the fall in tissue calcium content was similar at the two cyclic AMP steady states, it could not account for

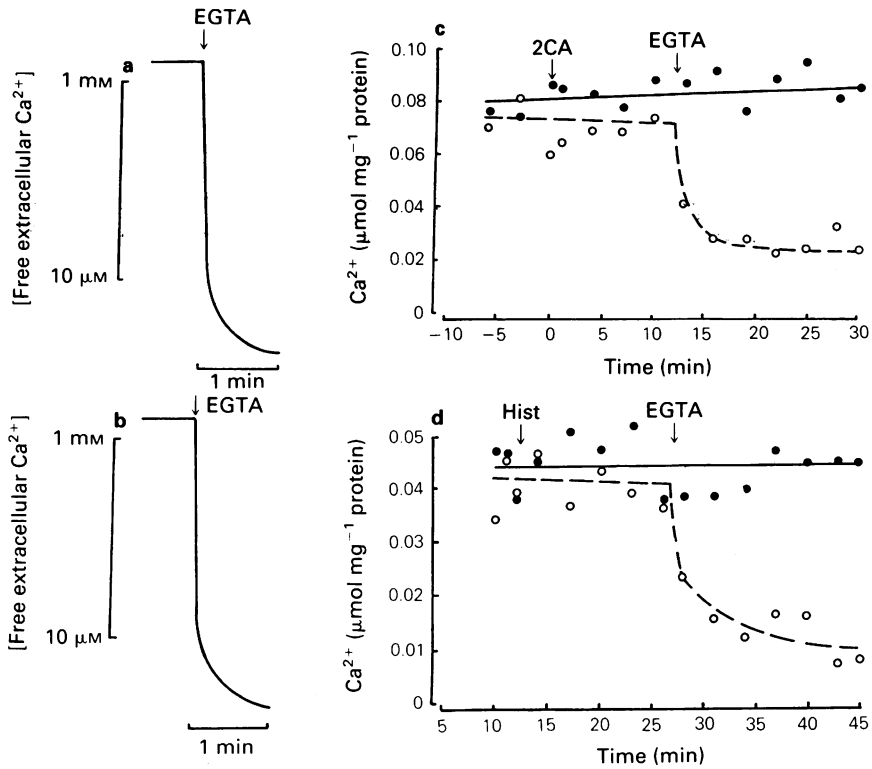

Figure 4 Effect of 5 mM EGTA on extracellular free calcium concentration (a and b) and tissue calcium content (c and d) in a suspension of guinea-pig cerebral cortical slices incubated with 2-chloroadenosine (2CA) or 2CA + histamine. In (a) and (c) EGTA was added at 2CA (30 μM) steady state (i.e. 12 min after 2CA). In (b) and (d) histamine (Hist, 0.1 mM) was added 12 min after 2CA and EGTA 12 (b) or 14 (d) min later (i.e. at histamine steady state): control (●); EGTA (○). Extracellular free calcium concentration was determined with a calcium electrode and tissue calcium content measured as described under methods. Data were obtained in single experiments but are representative of those obtained on two other occasions.

the apparent differences in calcium sensitivity at the different time points.

Calcium channel antagonists

In addition to chelating extracellular calcium, EGTA also had a rapid profound effect on tissue calcium content. It was therefore difficult to draw any conclusions as to the origin of the calcium involved in the histamine augmentation response from these experiments. We therefore performed a number of experiments using calcium channel antagonists to assess whether an influx of extracellular calcium was involved in the effect of histamine. An example of two of these experiments is shown in Table 1. The dihydropyridines, PN-200-110 and nifedipine were without significant effect on the cyclic AMP responses to 2CA (30 μM) and 2CA plus histamine (0.1 mM) ($n = 3$). However, the divalent cation nickel (1 mM) whilst having no significant effect on the cyclic AMP response to 2CA (30 μM) significantly reduced the histamine-induced augmentation (by 40.8 ± 2.6% of that in the absence of nickel, $n = 3$, $P < 0.01$, paired t test). Another divalent cation, cadmium (20 μM) also reduced the histamine response (by 29.6 ± 7.6%, $n = 3$). However, this concentration of cadmium also reduced the cyclic AMP response to 2CA to a similar extent (22.3 ± 3.5%, $n = 3$, $P < 0.05$, paired t test).

Effect of phorbol esters

The phorbol ester 4β-phorbol 12,13-dibutyrate (4βPDB) strongly augmented the maximal stimu-

Table 1 Effect of various calcium channel antagonists on the accumulation of cyclic AMP elicited by 2-chloroadenosine (2CA) (30 μM) or 2CA (30 μM) plus histamine (0.1 mM) in guinea-pig cerebral cortical slices

	Accumulation of [^{3}H]-cyclic AMP (d.p.m.) in the presence of	
	2CA	*2CA + histamine*
Experiment 1		
Control	23440 ± 901	78859 ± 11461
Nifedipine (1 μM)	23173 ± 906	87435 ± 2554
Nifedipine (10 μM)	25050 ± 515	91222 ± 3001
PN 200–110 (1 μM)	22484 ± 825	85563 ± 3658
PN 200–110 (10 μM)	24090 ± 702	84756 ± 3859
Experiment 2		
Control	36767 ± 675	140972 ± 6985
Cadmium (20 μM)	31125 ± 1673	131567 ± 6340
Nickel (1 mM)	34940 ± 1301	89789 ± 1322*
Cadmium (20 μM) + nickel (1 mM)	36751 ± 1664	89641 ± 3867*

Slices were incubated with antagonists for 10 min before the addition of agonist for a further 10 min as described under Methods. Control indicates the response to either 2CA or 2CA plus histamine in the absence of any calcium antagonist. Data represent the mean ± s.e.mean of three incubations in single experiments. *$P < 0.001$ compared to 2CA + histamine control (one-way analysis of variance). Similar results were obtained on two other occasions and the statistical data for the three paired experiments is given in the text.

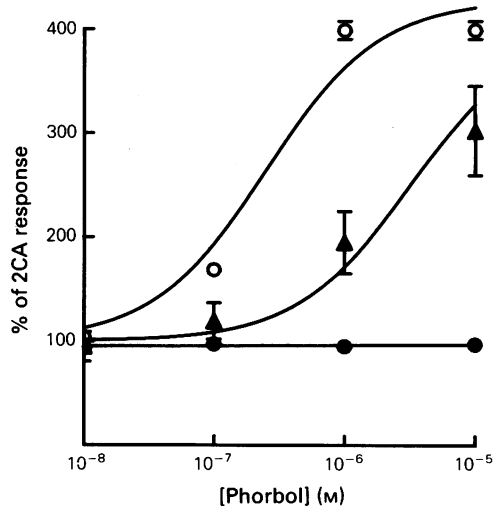

Figure 5 Effect of various phorbol esters on the stimulation of cyclic AMP accumulation elicited by 2-chloroadenosine (2CA) 30 μM in slices of guinea-pig cerebral cortex: (●) 4α-phorbol; (▲) 4β-phorbol 12-myristate 13-acetate (4β-PMA), (○)4β-phorbol 12,13-dibutyrate (4β-PDB). Slices were preincubated with phorbols for 20 min prior to addition of 2CA for a further 10 min. Data are expressed as % stimulation over the response to 2CA alone. Each point represents the mean of three incubations with s.e.mean shown by vertical bars. Where error bars are not shown, they are within the size of the symbol. Adenosine deaminase (1.2 u ml^{-1}) was included in the incubation medium.

lation of cyclic AMP accumulation elicited by 30 μM 2CA in a concentration-dependent manner (EC_{50} 2.3×10^{-7} M, Figure 5). Another phorbol ester, 4β-phorbol 12-myristate 13-acetate (4βPMA), also augmented the response to 2CA (30 μM) (Figure 5). A maximum response was not achieved with the highest concentration used (10 μM) so an EC_{50} value could not be calculated. This latter phorbol ester, however, appeared to be approximately 10 fold less potent than 4β-PDB (Figure 5). In contrast, the inactive phorbol ester 4α-phorbol had no stimulatory effect at concentrations up to 10 μM (Figure 5). All three phorbol esters were without effect on basal levels of cyclic AMP (data not shown).

The time course of the accumulation of cyclic AMP following addition of a low dose of 1 μM 4β-PMA at 2CA steady state is shown in Figure 6. This low dose was selected, since the need to avoid high doses and thus non-specific effects has been stressed (Nishizuka, 1984). Following addition of 4β-PMA, cyclic AMP levels rose to a new steady state (1.94 ± 0.12 fold over the response to 2CA

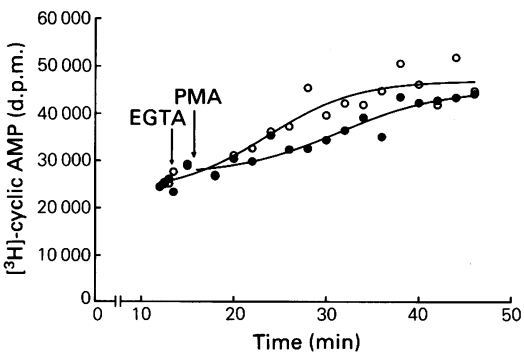

Figure 6 Effect of addition of 5 mM EGTA 2 min before 4β-phorbol 12-myristate 13-acetate (β-PMA) (1 μM) at 2-chloroadenosine (2CA) cyclic AMP steady state in slices of guinea-pig cerebral cortex. (●) 4β-PMA, (○) 4β-PMA + EGTA. 2CA (30 μM) was added at zero time. EGTA was added 12 min after 2CA and 4β-PMA 2 min after EGTA. The experiment was performed in the presence of adenosine deaminase (1.2 u ml^{-1}). Data were obtained in a single experiment. Similar results were obtained in 5 other experiments.

alone, $n = 8$) within 25 min. Similar results were obtained with 4β-PDB. Following addition of this latter phorbol (at a concentration of 0.1 μM) in the presence of 2CA, cyclic AMP levels rose to 1.94 ± 0.10 ($n = 3$) over the response to 2CA alone, again within 25 min. The time course of the augmentation produced by the phorbols was notably slower than that produced by histamine (Figure 1). In addition, the phorbol ester response was insensitive to removal of extracellular calcium with EGTA. Following addition of 5 mM EGTA 2 min before 4β-PMA at 2CA steady state, the augmentation produced by the phorbol ester was slightly increased (Figure 6, $n = 6$). This increase was however consistent with the elevation of the 2CA response by EGTA.

The possibility of using protein kinase C inhibitors to investigate the role of protein kinase C in the histamine-induced augmentation response was investigated. However, all the inhibitors used were unsatisfactory. Polymyxin B and H-7 showed no inhibition of the response to 1 μM 4β-PMA at concentrations up to 0.1 mM (data not shown). Staurosporin produced a very weak inhibition of the 4β-PMA (1 μM) response at concentrations of 0.1 μM and above. However, these concentrations of staurosporin also augmented the 2CA cyclic AMP response on their own (by 1.67 ± 0.17 fold, $n = 5$).

Discussion

Stimulation of H_1-receptors in guinea-pig cerebral cortical slices augmented the cyclic AMP response to 2CA, thus confirming the results of several previous studies (Daly, 1977; Hill *et al.*, 1981; Schwartz *et al.*, 1982; Hough & Green, 1984; Hollingsworth & Daly, 1985; Shonk & Rall, 1987; Donaldson *et al.*, 1988a,b; Hill & Kendall, 1989). The mechanism by which histamine produces this effect is uncertain. However, the products of the inositol phospholipid breakdown which occurs in response to H_1-receptor activation (Daum *et al.*, 1984; Carswell *et al.*, 1985; Claro *et al.*, 1986; Donaldson & Hill, 1986), have been implicated as mediators of this response (Schwabe *et al.*, 1978; Hollingsworth & Daly, 1985; Al-Gadi & Hill, 1987; Garbarg & Schwartz, 1988). The two products of inositol phospholipid hydrolysis, the inositol phosphates and diacylglycerol, are thought to mediate their effects by elevating intracellular calcium and activating protein kinase C respectively. The present study concentrates on the role of calcium in the augmentation response. In previous studies, histamine and adenosine have been added simultaneously under calcium-free conditions and the effects on the cyclic AMP accumulating after 10 min examined (Schwabe *et al.*, 1978; Al-Gadi & Hill, 1987). We have now investigated the time course of the effect of a sudden reduction in the external calcium concentration with EGTA, at various times before and after the addition of histamine at 2CA-induced steady state.

Addition of EGTA to guinea-pig cerebral cortical slices was found to produce a slight increase in the cyclic AMP response to 2CA, whether it was added 2 or 20 min before 2CA or at 2CA steady state. A similar elevation of the cyclic AMP response to adenosine in guinea-pig cerebral cortical slices by EGTA has been reported previously (Schwabe *et al.*, 1978). The reason for this is not known. One possible explanation of the effect of EGTA on the adenosine response could be that in the absence of extracellular calcium, the adenosine receptor undergoes a conformational change to a more active form.

The histamine-induced cyclic AMP response in cerebral cortical slices was much more dependent on calcium than the response to 2CA. When extracellular calcium was removed 20 min before histamine was added at 2CA steady state, the histamine-induced augmentation was completely abolished, while if EGTA was added just 2 min before histamine, a substantial reduction in the augmentation (72%) was still observed. These results therefore suggest that calcium has a role in the histamine-induced cyclic AMP response. However, it was difficult to determine from these experiments whether the calcium involved in the augmentation process was predominantly of an intra- or extracellular origin. Although EGTA was used to chelate extracellular calcium, it also had a profound effect on tissue calcium content. Reductions in the tissue calcium content were detectable after only 20 s and were greater than 30% within 1 min. The determination of tissue calcium is only a very crude method of assessing intracellular calcium levels and will partly reflect calcium binding to the extracellular membranes and entrapment of free calcium in the extracellular matrix within the slices. However, the fact that the inclusion of 1 mM EGTA in the wash solution did not reduce the measurements of tissue calcium content, would suggest that the calcium may be predominantly intracellular. Furthermore, the 20 s delay observed following addition of 5 mM EGTA before a measurable fall in slice calcium content was seen, also suggests that the calcium measured is predominantly intracellular.

In order to address the question of the origin of the calcium involved in the histamine response we performed a number of experiments using calcium channel antagonists to prevent any influx of extracellular calcium. Three different tyes of voltage-sensitive calcium channels have been defined by

electrophysiological and pharmacological techniques, the T, N and L channels (Hofmann *et al.*, 1987). T channels are blocked by 0.1 mM nickel (Hofmann *et al.*, 1987), L channels by the dihydropyridines (Triggle & Janis, 1987; Miller, 1987), whilst both N and L channels are blocked by 20 μM cadmium (Hofmann *et al.*, 1987). Furthermore millimolar concentrations of nickel have been reported to block a voltage-insensitive calcium channel responsible for refilling intracellular calcium stores, without affecting release of intracellular calcium (Hallam *et al.*, 1989; Carter *et al.*, 1988). Of the calcium channel antagonists tested, only Ni^{2+} (1 mM) produced a marked and selective attenuation of the H_1-receptor-mediated augmentation of cyclic AMP accumulation, although some 60% of the response to histamine remained in the presence of this antagonist. These results therefore suggest that the histamine-induced augmentation of the cyclic AMP response to 2CA in guinea-pig cerebral cortex is mediated predominantly by a release of stored intracellular calcium and is dependent to a lesser extent on influx of extracellular calcium. The small influx of calcium may enter via the voltage-independent channels responsible for refilling intracellular pools (Hallam *et al.*, 1989) or via the voltage-dependent T channels. It was not possible to eliminate the T channels as a specific antagonist for these channels was not available. These results are not inconsistent with data obtained by other workers in vascular and tracheal smooth muscle (Reynolds & Dubyak, 1986; Takuwa *et al.*, 1987). They discovered, using calcium-sensitive fluorescent dyes, that agonist-induced contraction is mediated by an initial mobilisation of intracellular calcium, followed by an influx of extracellular calcium.

Interestingly, when we studied the effects of EGTA addition after the histamine response was established, we discovered apparent differences in the calcium sensitivity of the response. When EGTA was added 1 or 3 min after histamine, the augmentation was not maintained. However, if EGTA was added once steady state had been achieved with histamine, it was apparently without effect. This was in marked contrast to the effect observed following addition of the H_1-receptor antagonist mepyramine at the histamine steady state (Donaldson *et al.*, 1988b and confirmed in the present study when the effects of EGTA and mepyramine additions were compared in the same experiment). Mepyramine addition caused cyclic AMP levels to fall to a level equivalent to that obtained with 2CA alone. Thus the apparent change in calcium sensitivity cannot be explained by a change at the receptor, since the H_1-receptor stimulus was still required to maintain the augmentation once steady state had been reached. The difference in calcium sensitivity could also be explained if EGTA were having different effects at the different time points. However, this seems unlikely since EGTA had similar effects on both extracellular free calcium and tissue calcium content, when it was added either at 2CA- or histamine-induced steady states.

There are thus two possible interpretations of this data. Firstly, it could be that calcium is important for the initiation and early stages of the augmentation, but is not involved in the maintenance of the response. Alternatively, it could be that the intracellular machinery involved in the augmentation response becomes more sensitive to calcium as the response progresses, so that it becomes able to operate at a much lower intracellular calcium concentration. In this respect it is interesting that in both vascular and tracheal smooth muscle, there is a large transient rise in intracellular calcium following receptor activation (Reynolds & Dubyak, 1986; Takuwa *et al.*, 1987) which peaks at around 1–2 min and then falls to a plateau somewhat above basal, whilst the contractile response remains unchanged. The question remains as to how the response is maintained if it does not involve calcium, or how changes are brought about to enable the response to operate at a lower level of calcium. One possibility is that diacylglycerol, another product of inositol phospholipid hydrolysis, is involved. This hypothesis is supported by the observed augmentation of the cyclic AMP response to 2CA produced by phorbol esters (which mimic the actions of diacylglycerol) in the present study. As mentioned previously phorbol esters have been shown to augment the cyclic AMP response to 2CA in a synaptoneurosome preparation of guinea-pig cerebral cortex (Hollingsworth *et al.*, 1985). It should however be noted that the physiological significance of this phorbol ester response has recently been challenged (Danoff & Young, 1987) since higher concentrations are required in slice preparations to produce the same effect as in a purified preparation. However, in view of the additional diffusional barriers that the slice preparation possesses these findings are not surprising. Garbarg & Schwartz (1988) have recently shown that the concentrations of 4β-PMA and 4β-PDB as used in the present study were effective in augmenting the cyclic AMP response to H_2-receptor activation in guinea-pig hippocampus. The fact that the response to 4β-PMA was insensitive to EGTA provides further support for a role of diacylglycerol in the augmentation response at the later time points.

The hypothesis that the intracellular machinery becomes more sensitive to calcium as the response progresses, is attractive in view of data obtained in other tissues. Bruschi *et al.* (1988) have presented some evidence for the existence of factors in vascular smooth muscle which sensitize the contractile machinery to calcium. These authors also proposed

that this 'sensitization' may be brought about by a phosphorylation of the myosin light chain. Protein kinase C is a phosphorylating enzyme which, while being calcium-dependent, has been reported to lower the calcium requirements for cellular activation (Nishizuka, 1986). One could therefore envisage that in guinea-pig brain, the diacylglycerol produced upon H_1-receptor activation could activate protein kinase C, which would in turn phosphorylate adenylate cyclase and thus make it more sensitive to calcium. This theory is particularly appealing since it has been demonstrated *in vitro* that protein kinase C can phosphorylate the catalytic unit of adenylate cyclase purified from bovine brain (Yoshimasa *et al.*, 1987).

In conclusion, the calcium sensitivity of the histamine-induced cyclic AMP response changes with time. The earlier but not the later stages of the augmentation response are particularly sensitive to calcium removal. A role for diacylglycerol in maintaining the histamine response at later time points is suggested.

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