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The role of calcium in the cyclic AMP response to histamine in rabbit cerebral cortical slices

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1 The effect of calcium on the H_1- and H_2-receptor components of the cyclic AMP response to histamine in rabbit cerebral cortical slices has been investigated.

2 Removal of calcium ions from the incubation medium during the preparation, preincubation and final incubation of brain slices significantly reduced the cyclic AMP responses to adenosine, histamine and the H_2-selective agonist, impromidine.

3 Removal of calcium ions from the incubation medium during only the final incubation with agonists did not influence the responses to adenosine, histamine, impromidine and the H_1-selective agonist, 2-thiazolylethylamine.

4 Final incubation of rabbit cerebral cortical slices in calcium-free buffer containing EGTA (1 mM) however, selectively reduced the cyclic AMP responses to the H_1-agonists histamine and 2-thiazolylethylamine without affecting the response to impromidine or adenosine.

5 These latter incubation conditions significantly reduced the maximal extent of the augmentation of impromidine- or adenosine-stimulated cyclic AMP accumulation produced by H_1-receptor stimulation, without affecting the EC_{50} values of the H_1-agonists. Calcium-free/EGTA conditions did not, however, alter the dose-response parameters for the response to the H_2-agonist, impromidine.

6 These data provide further evidence that the two histamine receptor systems affect cyclic AMP accumulation in rabbit cerebral cortical slices by different mechanisms.

Introduction

Histamine produces a large accumulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in guinea-pig (Daly, 1977; Hill *et al.*, 1981; Daum *et al.*, 1982) and rabbit (Kakiuchi & Rall, 1968; Al-Gadi & Hill, 1985) brain slices. This response to histamine can be mediated by both H_1- and H_2-receptor subtypes (Daly, 1977; Palacios *et al.*, 1978; Hill *et al.*, 1981; Al-Gadi & Hill, 1985). However, in brain membrane preparations the effect of histamine on adenylate cyclase activity is mediated exclusively via histamine H_2-receptors (Hegstrand *et al.*, 1976; Green *et al.*, 1977; Green, 1983).

In slices of rabbit cerebral cortex, the H_1-component of the cyclic AMP response to histamine appears to be indirect and requires the simultaneous stimulation of histamine H_2-receptors or another receptor system (e.g. adenosine A_2) which is linked directly to the cyclic AMP synthesizing enzyme, adenylate cyclase (Al-Gadi & Hill, 1985). Thus, stimulation of H_1-receptors produces a large augmentation of the cyclic AMP response to the selective H_2-agonist impromidine, or of that produced by adenosine, but is without effect on the cyclic AMP generating system on its own (Al-Gadi & Hill, 1985). A similar H_1-mediated augmentation of the response to H_2-agonists, adenosine or vasoactive intestinal polypeptide (VIP) has also been reported in slices of mouse cerebral cortex (Magistretti & Schorderet, 1985), guinea-pig hippocampus (Palacios *et al.*, 1978) and guinea-pig cerebral cortex (Hill *et al.*, 1981; Daum *et al.*, 1982; Hollingsworth *et al.*, 1985). Since it is not possible to detect an H_1-effect in membrane fractions of guinea-pig hippocampus and cerebral cortex (Hegstrand *et al.*, 1976; Green *et al.*, 1977; Hough *et al.*, 1980; Kanof & Greengard, 1979), it seems likely that these indirect cyclic AMP responses to histamine are mediated by another second messenger such as calcium ions (Schwabe *et al.*, 1978) or diacylglycerol (Hollingsworth & Daly, 1985).

In guinea-pig brain slices a rapid reduction in the extracellular concentration of free calcium ions with

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the chelating agent EGTA, immediately prior to agonist administration, abolished the synergism between histamine and 2-chloroadenosine (Schwabe *et al.*, 1978). The present study was undertaken to determine whether a reduction in the extracellular calcium ion concentration had a differential effect on the H_1- and H_2-components of the cyclic AMP response to histamine in rabbit cerebral cortical slices.

Methods

Preparation and preincubation of cerebral cortical slices (stage 1)

Rabbits (New-Zealand White, 2.5 kg) of either sex were killed by cervical dislocation, the brain removed and the cerebral cortex quickly dissected out on ice. Slices (300 × 300 μm) were cross cut with a McIlwain tissue chopper and incubated at 37°C in calcium containing (2.5 mM $CaCl_2$) or nominally calcium-free Krebs-Henseleit medium (75 ml per g of tissue) constantly gassed with O_2:CO_2 (95:5) in a shaking water bath for 30 min. During this preliminary preincubation period the medium was changed at 10 min intervals.

Drug treatment of slices (stage 2)

At the end of this preliminary incubation (stage 1), slices were washed with a calcium-containing or a calcium-free Krebs medium (which in most experiments contained 1 mM EGTA) and then suspended in the appropriate Krebs medium at a concentration of 200 mg wet weight per ml. Aliquots (50 μl, 10 mg wet weight) of the cortical slice suspension were then added to 240 μl of Krebs medium and incubated for 20 min at 37°C under an atmosphere of O_2:CO_2 (95:5). Agonists were added after this step in 10 μl of medium and the incubation continued for a further 10 min. Tissue cyclic AMP was released by heating the sample at 100°C for 10 min and the tissue debris then removed by brief centrifugation (11,600 *g* for 2 min). Duplicate 20 or 50 μl samples of the supernatant were taken for cyclic AMP determination by a sensitive protein binding assay (Brown *et al.*, 1972). The tissue pellets were solubilized by heating in 1 M NaOH and the protein concentration determined by the method of Lowry *et al.* (1951).

Calcium content of media and slices

The calcium contamination of calcium-free Krebs buffer and the medium that had been in contact with brain slices was monitored by atomic absorption spectrophotometry (Pye-Unicam SP90 series 2). Measurements of calcium standards were made in deionized double distilled water treated with the chelating ion exchange resin Chelex 100. The calcium content of Krebs-Henseleit medium prepared without the addition of $CaCl_2$ was 9.2 ± 0.7 μM (n = 11).

In a number of experiments the calcium content of slices of rabbit cerebral cortex was measured in parallel incubations. At the end of the experiment the incubation medium was removed and assayed for calcium. The remaining slices were rapidly washed in calcium-free medium and then solubilized in 1 ml of 0.1 M NaOH at 90°C. The calcium content of the solubilized slices was then analysed by atomic absorption spectrophotometry and the protein content determined by the method of Lowry *et al.* (1951).

Calculation of free calcium-ion concentrations

By using atomic absorption spectrophotometry to determine the total calcium content of buffer solutions at appropriate stages of the experiment, the concentration of free calcium in the presence of EGTA at 37°C was calculated according to Sillen & Martell (1964). The logarithms of the association constants (Sillen & Martell, 1964) used in the computation were as follows: H^+ to $EGTA^{4-}$, 9.46; H^+ to $HEGTA^{3-}$, 8.85; H^+ to H_2EGTA^{2-}, 2.68: H^+ to H_3EGTA^-, 2.00; Ca^{2+} to $EGTA^{4-}$, 11.0; Ca^{2+} to $HEGTA^{3-}$, 5.32; Mg^{2+} to $EGTA^{4-}$, 5.21; Mg^{2+} to $HEGTA^{3-}$, 3.37.

Data analysis

Concentration-response curves, obtained in the presence and absence of 2.5 mM calcium, were fitted simultaneously to a Hill equation using the programme ALLFIT (De Lean *et al.*, 1978) as described previously (Donaldson & Hill, 1986a). The equation fitted was: % of maximum response = $E_{max} \times D^n/(D^n + [EC_{50}]^n)$ where D is the agonist concentration, n is the Hill coefficient, EC_{50} is the concentration of agonist giving half maximal stimulation and E_{max} is the maximal stimulation. ALLFIT was used to test for differences in the response parameters, obtained in the presence and absence of calcium, by inspecting the effect on the residual variance of forcing them to be equal (De Lean *et al.*, 1978). The appropriateness of a particular constraint was evaluated by analysis of the residual variances using the following statistical test (Munson & Rodbard, 1980; Donaldson & Hill, 1986a):

$$F = \frac{(R_2-R_1)/(df_2-df_1)}{R_1/df_1}$$

where R_1 and R_2 are the residual sums of squares and df_1 and df_2 are the degrees of freedom associated with the original fit and the analysis with shared parameters respectively.

Drugs

Adenosine and Chelex 100 were obtained from Sigma and histamine acid phosphate from BDH. Gifts of 2-thiazolylethylamine dihydrochloride (2-(2-aminoethyl)thiazole) and impromidine trihydrochloride (both from Smith Kline & French) are gratefully acknowledged).

Results

Removal of calcium during stage 1 and stage 2

The preparation, preincubation (stage 1) and incubation (stage 2) of slices of rabbit cerebral cortex in calcium-free Krebs medium produced a marked decrease in the size of the cyclic AMP response elicited by histamine (0.1 mM), impromidine (0.1 mM) and adenosine (0.1 mM) without a significant change in the basal accumulation of cyclic AMP (Figure 1). For both histamine and the H_2-selective agonist impromidine, this effect appeared to be due primarily to a decrease in the size of the maximum response (Figures 2 and 3). For example, analysis of the concentration-response curve for impromidine (Figure 3), by the method of De Lean *et al.* (1978), indicated that there was a significant decrease ($P < 0.005$) in the maximum response from 54 ± 3 to 17 ± 2% of the response to 1 mM histamine (measured in the presence of calcium) without any significant change in the EC_{50} value (0.026 ± 0.012 and 0.068 ± 0.037 μM in the presence and absence of calcium respectively). It is worth noting that the maximum response to the H_2-selective impromidine in calcium-containing medium was only 54% of that produced by histamine (1 mM). This is consistent with our previous finding that a large proportion of the response to histamine in this tissue is due to an indirect H_1-receptor action (Al-Gadi & Hill, 1985).

The calcium content of the calcium-free medium

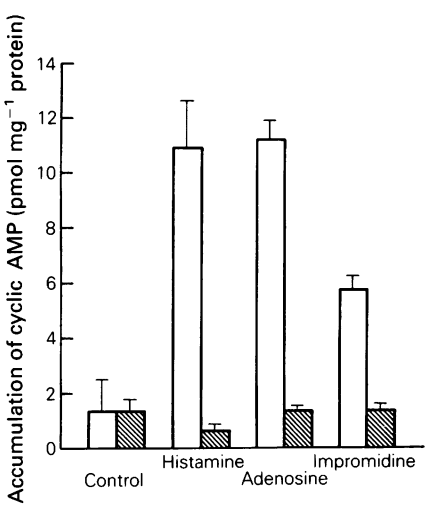

Figure 1 The effect of calcium removal, during the preparation, preincubation and incubation of slices of rabbit cerebral cortex, on the cyclic AMP accumulation produced in response to histamine (0.1 mM), adenosine (0.1 mM) or impromidine (0.1 mM). The open columns represent the responses measured in normal Krebs medium containing 2.5 mM Ca^{2+} while the hatched columns show the responses obtained in calcium-free medium. In the control slices 2.5 mM Ca^{2+} was present in the Krebs medium throughout the experiment (i.e. in both stage 1 and stage 2). Values represent the mean of six replicate determinations in a single experiment; vertical lines show s.e.means. Two other experiments gave essentially similar results.

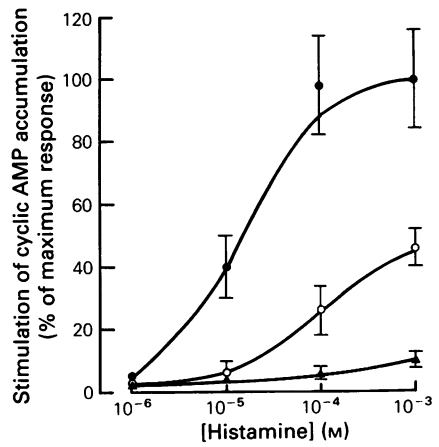

Figure 2 Concentration-response curves of the cyclic AMP accumulation induced by histamine in rabbit cerebral cortical slices obtained in the presence (●) and absence (○, ▲) of 2.5 mM calcium. For measurements made in the absence of calcium, slices were prepared, preincubated and incubated in calcium-free medium. During the preincubation the calcium-free medium in contact with the slices was normally changed at 10 min intervals (▲), however, in some experiments it was left in contact with the slices for the entire 30 min period (○). To normalize responses from different slice preparations, responses are expressed as a percentage of the response to 1 mM histamine which was measured in the presence of 2.5 mM calcium in each experiment. Each point represents the combined mean of 2–4 experiments; vertical lines show s.e.means. In each experiment, six replicate determinations were made at each concentration of histamine.

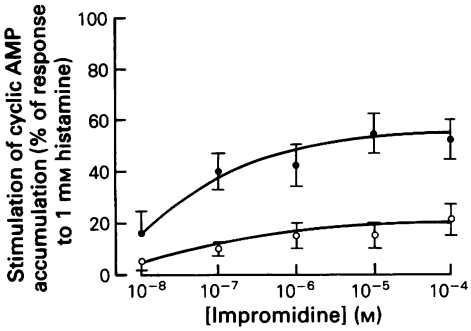

Figure 3 Concentration-response curves of the cyclic AMP accumulation induced by impromidine in rabbit cerebral cortical slices obtained in the presence (●) and absence (○, during stages 1 and 2, see Methods) of 2.5 mM calcium. To normalize responses from different slice preparations, responses are expressed as a percentage of the response to 1 mM histamine (in the presence of calcium) which was measured in each experiment. Each point represents the combined mean (± s.e.mean) of two experiments. In each experiment, six replicate determinations were made at each concentration of impromidine in the presence and absence of calcium.

remained low during these experiments, normally between 5 and 14 μM calcium. However, the extensive washing procedure and prolonged period in contact with calcium-free medium, during both preincubation and incubation phases of the experiment, resulted in a substantial reduction (by 82%) in the total calcium content of the incubated brain slices. In five experiments the calcium content of the slices was measured in duplicate parallel incubations at the end of the experiment. Under calcium-free conditions the mean calcium content of the slices was 21 ± 2 nmol mg^{-1} protein ($n = 5$) while that of slices incubated throughout in Krebs solution containing 2.5 mM calcium was 119 ± 12 nmol mg^{-1} protein ($n = 5$). A great deal of the calcium appeared to be lost from the slices during the preincubation stage of the experiment since the calcium concentration of the 'calcium-free' medium decanted from the slices during this stage was 21 ± 2 μM, 14 ± 2 μM and 11 ± 2 μM ($n = 7$) for the first, second and third 10 min intervals respectively. If the medium was not changed at 10 min intervals during the stage 1 preincubation, then the depletion of slice calcium was less severe (slice calcium = 40 ± 5 nmol mg^{-1} protein, $n = 5$) and the response to histamine more pronounced (Figure 2).

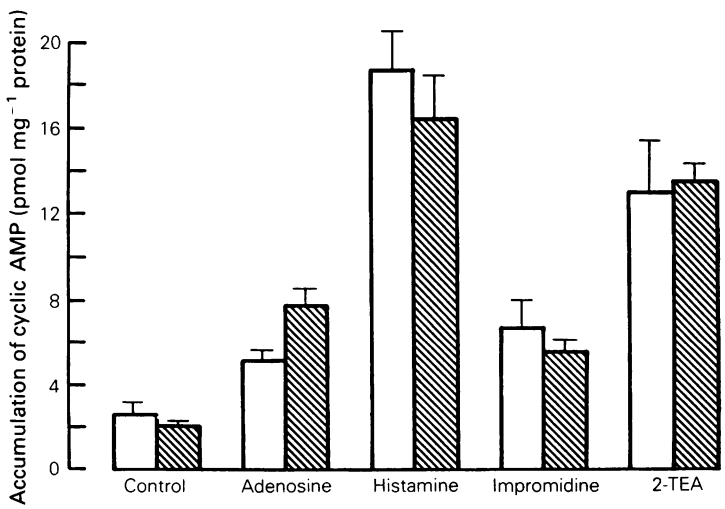

Figure 4 The effect of calcium removal during the final incubation (stage 2) on the cyclic AMP responses to adenosine (0.1 mM), histamine (0.1 mM), impromidine (0.1 mM) and 2-thiazolylethylamine (2-TEA, 1 mM) in rabbit cerebral cortical slices. The open columns represent the responses measured in normal Krebs buffer containing 2.5 mM calcium while the hatched columns show the responses obtained in calcium-free medium. Both sets of slices were prepared and preincubated (during stage 1) in normal Krebs medium before the incubation (stage 2) phase of the experiment. Values represent the mean of 6 replicate determinations in a single experiment; vertical lines show s.e.means. A second experiment gave very similar results.

Removal of calcium during stage 2

In two experiments, slices were preincubated in calcium-containing Krebs medium (during stage 1) prior to incubation in calcium-free buffer during stage 2. Under these conditions the calcium content of the slices was maintained (116 nmol mg^{-1} protein and 119 nmol mg^{-1} protein in the absence and presence of calcium respectively) and the response to histamine was not altered (Figure 4). The responses to adenosine, impromidine and 2-thiazolylethylamine (2-TEA) were also not attenuated by this intervention (Figure 4). It should be noted that a response to the H_1-agonist 2-TEA alone was obtained (Figure 4) because of its effect on H_2-receptors at high (i.e. 1 mM) concentrations (Al-Gadi & Hill, 1985). Under these conditions the cyclic AMP response to 2-TEA, like that to histamine, has two components: a direct H_2-effect and a marked H_1-effect which acts to amplify the H_2-component (Al-Gadi & Hill, 1985). Addition of 2.5 mM EGTA, to slices incubated in normal Krebs medium, 2 min before the addition of agonists according to Schwabe *et al.* (1978) similarly failed to reduce the responses to histamine, adenosine or impromidine (data not shown). The calcium content of the slices treated with EGTA (113 nmol mg^{-1} protein) was again similar to that of the untreated slices (114 nmol mg^{-1} protein). The concentration of extracellular free calcium in these experiments (in the presence of EGTA) was estimated to be approximately 10 μM, i.e. very similar to the calcium concentration in calcium-free Krebs.

A differential effect on the responses to agents with an indirect (H_1) cyclic AMP component (histamine and 2-TEA) was achieved when slices (previously prepared and incubated in calcium-containing medium) were deprived of calcium and treated with 1 mM EGTA during the incubation (stage 2) phase of the experiment (Figure 5). Under these conditions a significant inhibition ($P < 0.005$) of the response to histamine (0.1 mM) and 2-TEA (1 mM) was observed, while the responses to agents with only a direct H_2-(impromidine) or A_2- (adenosine) component were not significantly affected (Figure 5). The extracellular concentration of free calcium under these conditions was estimated to be 3.7 ± 0.2 nM ($n = 7$; Table 1). However, the presence of 1 mM EGTA in the calcium-free medium during the stage 2 incubation also produced a signficant reduction (by 43%) in the calcium content of the slices. In these experiments, the calcium content of slices incubated in normal Krebs medium was 93 ± 5 nmol mg^{-1} protein ($n = 11$) while that in slices incubated without calcium in the presence of EGTA was 53 ± 4 nmol mg^{-1} protein ($n = 11$).

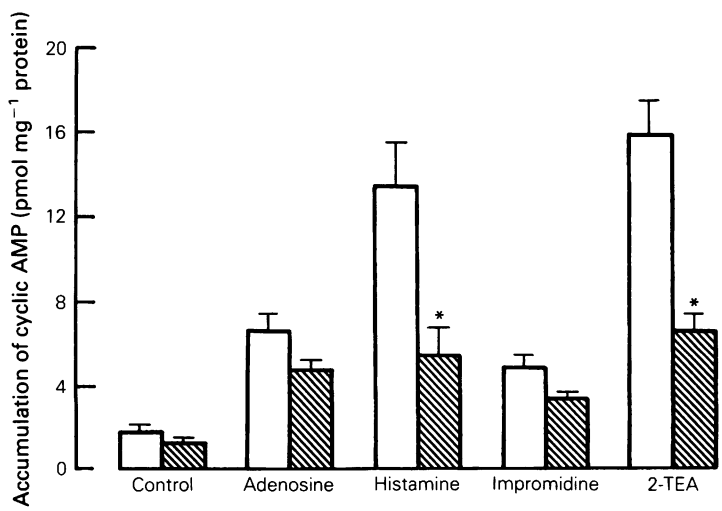

Figure 5 Effect of incubation of slices of rabbit cerebral cortex (during stage 2) in calcium-free medium containing 1 mM EGTA on the cyclic AMP responses to adenosine (0.1 mM), histamine (0.1 mM), impromidine (0.1 mM) and 2-thiazolylethylamine (2-TEA, 1 mM). The open columns represent the responses measured in normal calcium-containing Krebs medium while the hatched columns show the responses obtained in calcium-free medium. Both sets of slices were prepared and preincubated (during stage 1) in calcium-containing Krebs-Henseleit solution. Values represent the combined mean ± s.e.mean of 6 replicate determinations obtained in each of three separate experiments. *$P < 0.005$ compared to values obtained in calcium-containing medium (analysis of variance).

Table 1 Analysis of the calcium content of slices of rabbit cerebral cortex incubated in calcium-free medium containing 1 mM EGTA during stage 2

Experiment	*Ca^{2+} content* (nmol mg^{-1} protein) *of slices incubated in:* 2.5 mM Ca^{2+}	Ca^{2+}-free	*Final [Ca^{2+}] of Ca^{2+}-free medium [Total Ca^{2+}] (μM)†* measured	calculated*	*[Free Ca^{2+}]* (nM)#
1	92.2	58.3	84	86	3.4
2	123.3	77.1	110	117	4.5
3	98.2	60.4	95	96	3.6
4	100.8	62.9	100	96	4.1
5	84.9	44.5	90	103	3.5
6	73.8	39.7	90	87	3.5
7	94.3	63.0	78	79	3.1
Mean ± s.e.mean	95.4 ± 5.8	58.0 ± 4.7	92 ± 4	95 ± 5	3.7 ± 0.2

Values for the Ca^{2+}-content of slices incubated in the presence or absence of 2.5 mM Ca^{2+} were obtained by atomic absorption spectrophotometry as described under Methods. Both sets of slices were prepared and preincubated in calcium-containing medium. (†) The total calcium concentration in the calcium-free medium at the end of the experiment (free + chelated) was either measured directly by atomic absorption spectrophotometry or (*) calculated from the difference in calcium content of the slices incubated in the presence and absence of 2.5 mM calcium. (#) The free calcium concentration present in the calcium-free medium containing 1 mM EGTA was calculated from the measured [Total Ca^{2+}] as described under Methods.

Table 1 shows that the loss of calcium from the slices can be readily accounted for by the appearance of calcium (most of which is chelated with EGTA) in the extracellular medium. These incubation conditions were subsequently used to investigate the different calcium-sensitivities of the direct (H_2-) and indirect (H_1-) cyclic AMP response to histamine.

Differential effect on H_1- and H_2-receptor responses

Figure 6 shows the effect of calcium-free EGTA (1 mM) conditions on the dose-response curve to the selective H_2-agonist impromidine in rabbit cerebral cortical slices. This procedure produced no significant change in either the maximum response or the EC_{50} value suggesting that the direct H_2-effect on cyclic AMP accumulation is relatively resistant to these calcium-depleting conditions. In contrast, the H_1-mediated augmentation of impromidine- or adenosine-stimulated cyclic AMP accumulation in rabbit cerebral cortex was markedly reduced following calcium depletion (Figures 7 and 8). The maximum size of the augmentation of the cyclic AMP response to impromidine (1 μM) produced by histamine was significantly reduced ($P < 0.005$; analysis of variance according to De Lean *et al.*, 1978) from 105 ± 4 to 29.5 ± 1.9% of the response to 1 mM histamine (measured in the presence of calcium). The EC_{50} values obtained under these conditions, 13 ± 3 μM (+ calcium) and 27 ± 9 μM (− calcium) were not significan-

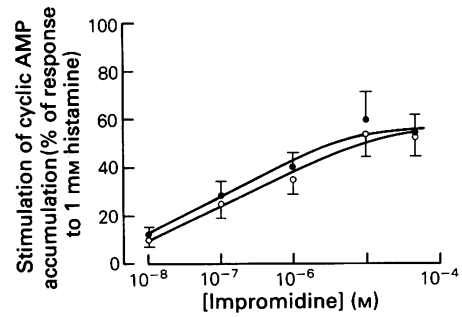

Figure 6 Concentration-response curves of the cyclic AMP response to impromidine in rabbit cerebral cortical slices obtained in the presence (●) and absence (○; calcium-free medium containing 1 mM EGTA) of 2.5 mM calcium during stage 2. Both sets of slices were prepared and preincubated (during stage 1) in calcium-containing Krebs medium. To normalize responses from different slice preparations, responses are expressed as a percentage of the response to 1 mM histamine measured in the presence of calcium. Each point represents the combined mean (± s.e.mean) of two experiments. In each experiment six replicate determinations were made at each concentration of impromidine in the presence and absence of calcium.

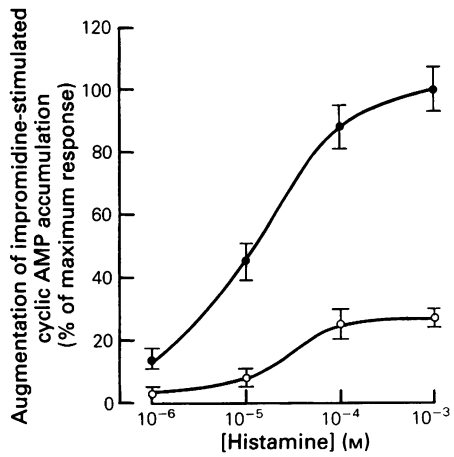

Figure 7 Concentration-response curves of the augmentation of impromidine-stimulated cyclic AMP accumulation induced by histamine in rabbit cerebral cortical slices obtained in the presence (●) and absence (○; calcium-free medium containing 1 mM EGTA) of 2.5 mM calcium during stage 2. Both sets of slices were prepared and preincubated in calcium-containing medium. Impromidine (1 μM) and histamine were added simultaneously. The augmentation produced by histamine was taken to be the difference between the accumulation of cyclic AMP elicited by impromidine in the presence and absence of different concentrations of histamine. Responses are expressed as a percentage of that produced by 1 mM histamine. Each point represents the combined mean for eight replicates in each of three experiments; vertical lines show s.e.mean.

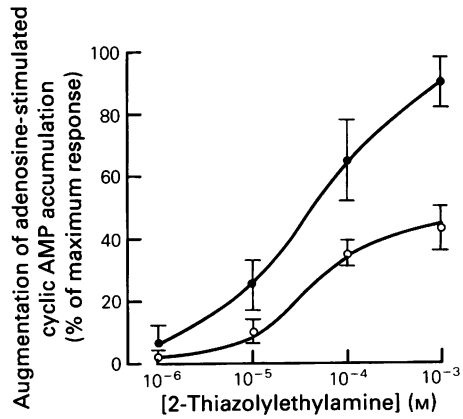

Figure 8 Concentration-response curves of the augmentation of adenosine-stimulated cyclic AMP accumulation elicited by 2-thiazolylethylamine (2-TEA) obtained in the presence (●) and absence (○; calcium-free medium containing 1 mM EGTA) of 2.5 mM calcium during stage 2. Adenosine (0.1 mM) was present in every incubation. Responses are expressed as a percentage of the augmentation produced by 1 mM histamine. Each point represents the combined mean (± s.e.mean) of two experiments. In each experiment, eight replicas were made at each agonist concentration. The mean control responses to adenosine (0.1 mM) alone were 5.6 ± 0.8 and 4.4 ± 0.7 pmol cyclic AMP mg^{-1} protein in the presence and absence of 2.5 mM calcium respectively.

tly different. Similarly, the augmentation of the response to adenosine (0.1 mM) produced by the H_1-agonist, 2-TEA, was also reduced (Figure 8). In these latter experiments the maximum extent of the augmentation response was significantly reduced ($P < 0.005$) from 94.6 ± 3.8 to 46.9 ± 2.7% of that produced by histamine which was measured in the presence of calcium in each experiment. There was, however, no significant change in the EC_{50} for 2-TEA (31.4 ± 7.1 μM and 37.6 ± 9.7 μM in the presence and absence of 2.5 mM calcium respectively).

Discussion

The cyclic AMP response to histamine in slices of rabbit cerebral cortex is composed of two components: a direct H_2-receptor-mediated effect and an indirect H_1-component which is dependent upon the simultaneous activation of histamine H_2- or adenosine A_2-receptors (Al-Gadi & Hill, 1985). This latter component acts to produce a large augmentation of the cyclic AMP response to H_2- or A_2-receptor stimulation (Al-Gadi & Hill, 1985). The present studies indicate that both of these responses to histamine are dependent to some extent on the presence of calcium in the extracellular medium.

When calcium ions were omitted from the incubation medium during the preparation, preincubation and incubation of rabbit cerebral cortical slices, the responses to histamine (mediated by H_1- and H_2-receptors), impromidine (H_2- only) and adenosine (A_2) were dramatically reduced. The effect on the direct responses to impromidine and adenosine is almost certainly due to the extensive depletion of the calcium stores in the brain slices, which are reduced by approximately 80%, since these responses are relatively resistant to less prolonged periods in calcium-free medium (see for example Figure 5). This reliance of the H_2- and adenosine-receptor effects on calcium is somewhat different from the situation normally observed in isolated membranes where agonist-induced increases in adenylate cyclase activity are routinely demonstrated in the presence of EGTA buffers (Hegstrand *et al.*, 1976; Green *et al.*, 1977;

Kanof & Greengard, 1979; Anand-Srivastava & Johnson, 1980). However, in a number of tissues, and in brain membranes in particular, there is growing evidence that calcium may exert a biphasic regulatory control over adenylate cyclase such that it stimulates enzyme activity at low calcium concentrations and inhibits cyclase activity at higher concentrations (Brostrum *et al.*, 1975; Cheung *et al.*, 1975; Piascik *et al.*, 1980; 1981). The fact that these two effects occur at free calcium concentrations thought to exist intracellularly (Piascik *et al.*, 1980) is therefore consistent with our finding that the responses to impromidine and adenosine in rabbit cerebral cortical slices are inhibited when the calcium content is depleted by 80%. Under these conditions the various non-ionic pools of calcium in the plasma membrane, endoplasmic reticulum and mitochondria (Rasmussen & Barret, 1984) are presumably depleted to such an extent that the free calcium concentration is no longer maintained at a level sufficient to have a stimulant effect on adenylate cyclase. Furthermore this sensitivity of the direct cyclic AMP responses (e.g. to impromidine and adenosine) to changes in slice calcium content would seem to be an essential prerequisite to any proposal that calcium is the intracellular messenger through which the indirect facilitatory H_1-effect on cyclic AMP accumulation is mediated.

The dependence of the H_1-receptor component of the cyclic AMP response to histamine on the presence of a functioning H_2-receptor system (Al-Gadi & Hill, 1985) means that the observed loss of responsiveness to histamine under severe calcium depleting conditions (e.g. Figures 1 and 2) cannot be taken as evidence that the H_1-response is also calcium-dependent. Evidence for this can only come from experiments which show a reduction in the H_1-response under conditions where the direct H_2- or adenosine responses are relatively well maintained. This was achieved in the present study when slices of rabbit cerebral cortex were prepared and incubated in calcium-containing medium before incubation with agonists in calcium-free medium containing 1 mM EGTA (see for example Figure 5). Under these less severe calcium-depleting conditions the responses to histamine and 2-TEA, which have a marked H_1-component (Al-Gadi & Hill, 1985), were substantially reduced while the responses to adenosine and the H_2-selective agonist impromidine were not significantly affected. A more direct evaluation of the effect of calcium on the H_1-receptor response was made by investigating the augmentation produced by H_1-agonists of the cyclic AMP response to a fixed concentration of impromidine or adenosine. With this approach the H_1-nature of the cyclic AMP response to these agonists is much clearer (Palacios *et al.*, 1978; Hill *et al.*, 1981; Daum *et al.*, 1982; Al-Gadi & Hill, 1985). For example, cyclic AMP responses to 2-TEA can be produced at much lower and more H_1-selective concentrations than are required if a directly acting agonist is not simultaneously added (Palacios *et al.*, 1978; Al-Gadi & Hill, 1985). These studies showed that calcium-free/EGTA conditions reduced the maximal H_1-responses to histamine and 2-TEA by 72% and 50% respectively without any significant effect on the EC_{50} values or the basal response to impromidine or adenosine alone.

The reason for the differential effect of these experimental conditions on indirect and direct cyclic AMP responses in rabbit cerebral cortical slices is not clear. The free calcium ion concentration in the extracellular medium in these experiments was estimated to be 3.7 nM while the calcium content of the slices was reduced by approximately 40%. It therefore seems likely that the H_1-receptor response is either (a) dependent on the influx of extracellular calcium or (b) dependent upon the mobilization of calcium ions from an intracellular pool which can be depleted by these less severe conditions. Studies performed in calcium-free medium without the addition of EGTA, in which slices are not depleted of calcium, indicate that the extracellular calcium concentration can be reduced to 10 μM without any deleterious effect on direct and indirect cyclic AMP responses. Similarly, a rapid reduction in free-calcium concentration to around 10 μM, with 2.5 mM EGTA immediately before agonist administration, was without effect on the responses to adenosine, 2-TEA, impromidine and histamine. These data suggest that if an influx of extracellular calcium is involved in the H_1-receptor response in this tissue, the system can tolerate a rather low concentration of extracellular calcium.

A more attractive proposition is that H_1-receptor stimulation mobilizes intracellular calcium to mediate its potentiating effect on cyclic AMP accumulation in this tissue. The method employed to monitor the calcium content of brain slices is obviously rather crude and will partly reflect calcium binding to the extracellular membranes and entrapment of free calcium in the extracellular matrix within the slices. Nevertheless, it does provide a simple method of measuring total slice calcium content and provides an indication that the intracellular stores may be depleted in calcium-free/EGTA buffers.

Our understanding of how stimulation of hormone or neurotransmitter receptors in the plasma membrane can elicit a mobilization of intracellular calcium has been greatly advanced in recent years by the discovery that agonists which mobilize cell calcium can stimulate the hydrolysis of inositol phospholipids (Berridge, 1984; Berridge & Irvine, 1984; Nahorski *et al.*, 1986). It is now believed that it is phosphatidylinositol bisphosphate which is the primary target for agonist action and that this polyphosphoinositide is cleaved to form two second messengers, diacylglycerol

and inositol-1,4,5-trisphosphate ($InsP_3$) (Downes & Wusteman, 1983; Berridge, 1984; Berridge & Irvine, 1984). $InsP_3$ is released into the cytoplasm and there is growing evidence, although mainly from peripheral tissues, that it is via the action of $InsP_3$ that calcium is released from intracellular stores (Streb *et al.*, 1983; Burgess *et al.*, 1984; Berridge & Irvine, 1984; Somlyo *et al.*, 1985). In this respect it is interesting that H_1-receptor stimulation has been shown to stimulate inositol phospholipid hydrolysis in mammalian brain slices (Brown *et al.*, 1984; Daum *et al.*, 1984; Donaldson & Hill, 1985; 1986a; Hollingsworth *et al.*, 1985). Moreover, studies in guinea-pig cerebellum have shown that H_1-receptor stimulation can lead to the production of inositol trisphosphate (Donaldson & Hill, 1986b), although it remains to be established whether this is the appropriate isomer for calcium mobilization (Irvine *et al.*, 1984; 1985).

The other product of inositol phospholipid hydrolysis is diacylglycerol which acts on the phospholipid-dependent protein kinase C to increase its sensitivity to activation by calcium ions (Nishizuka, 1984; Berridge & Irvine, 1984; Rasmussen & Barrett, 1984). Recently, phorbol esters which mimic the action of diacylglycerol have been shown to augment the cyclic AMP accumulation elicited by 2-chloroadenosine in a vesicle preparation of guinea-pig cerebral cortex (Hollingsworth *et al.*, 1985). Thus, both arms of the inositol phospholipid second messenger system may act conjointly to mediate the indirect effects of H_1-receptor stimulation on cyclic AMP accumulation.

In summary, the present studies have shown that the direct H_2- and the indirect H_1-components of the cyclic AMP response to histamine in rabbit cerebral cortical slices have different requirements for calcium. These data provide further evidence that the two histamine receptor systems affect cyclic AMP accumulation in brain slices by different mechanisms.

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