Inositol phospholipid hydrolysis in human brain; adenosine inhibition of the response to histamine

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1 Inositol phospholipid hydrolysis was examined in human cerebral cortex slices by a [³H]-inositol prelabelling assay.

2 Enhancement of [³H]-inositol phosphates accumulation was observed in the presence of carbachol, noradrenaline, histamine, 5-hydroxytryptamine (5-HT) and depolarizing concentrations of KCl.

3 Despite having no effect alone, adenosine (and its analogue 2-chloroadenosine) selectively inhibited the direct response to histamine.

4 The inhibition due to adenosine was antagonized by theophylline, but not by 8-cyclopropyltheophylline.

Introduction

It is becoming increasingly apparent that the effects of neurotransmitters and hormones on second messenger formation in the central nervous system can be influenced, positively and negatively, by the simultaneous activation of other neurotransmitter receptor systems (Hill & Kendall, 1989). These neuromodulations show a remarkable degree of species specificity; for instance, we have previously shown that histamine-stimulated inositol phospholipid hydrolysis is enhanced in the presence of adenosine and some adenosine analogues in guinea-pig cerebral cortex (Hill & Kendall, 1987), while the response is inhibited in mouse cerebral cortex (Kendall & Hill, 1988). It is thus impossible to make predictions concerning the occurrence of such interactions in human brain. We have therefore examined the effects of adenosine on histamine-stimulated inositol phospholipid hydrolysis in slices of human cerebral cortex taken during neurosurgical procedures.

A preliminary account of some of these data has been communicated to the British Pharmacological Society (Kendall & Firth, 1989).

Methods

Tissue preparation

Pieces of cerebral cortex were taken during neurosurgical operations which involved corticectomy. The indications for the procedures varied, including sub-cortical tumour removal or biopsy and cerebral decompression, and tissue from a variety of cortical regions was included in the study. In all cases the tissue would normally have been discarded. The mean age of the donors was 50.2 years and the range was 23–77 years. There were approximately equal numbers of men and women.

Immediately following removal, the tissue was placed in ice-cold Krebs-Hensleit buffer of the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 1.3, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, glucose 11.7, equilibrated with 95% O₂/CO₂ to pH 7.4. The tissue was taken to the laboratory (in less than 5 min) where it was cut into slices (350 µm × 350 µm × about 1 mm) with a McIlwain tissue chopper. The slices were dispersed in Krebs solution at 37°C in a glass conical flask gassed with 95% O₂/5% CO₂, stoppered and then incubated for 60 min with 3 intermediate changes of buffer. The tissue was treated as being potentially infectious and all transfer pro-

cedures, chopping and gassing were done in a microbiological safety cabinet in a designated human tissue laboratory.

Incorporation of [³H]-inositol and agonist stimulation of total inositol phosphates formation

Twenty five µl aliquots of gravity packed slices were dispensed into flat-bottomed plastic insert vials containing 25 kBeq [³H]-*myo*-inositol, 5 mM LiCl and Krebs buffer. The vials were gassed and shaken in a water bath at 37°C for 45 min. When appropriate, antagonists were added (10 µl), followed by agonists (10 µl) 10 min later. The final incubation volume was 300 µl. Agonist incubations were stopped, usually after 45 min, by the addition of 100 µl, 10% (w/v) perchloric acid. The samples were cooled on ice for 15 min and were then neutralized by the addition of an appropriate volume of 0.15 M KOH. After centrifugation (1000 g for 10 min at 4°C) 0.8 ml of the sample supernatant was taken and added to 2.25 ml of Tris buffer (50 mM, pH 7.0) before separation of total [³H]-inositol phosphates ([³H]-IP) by anion exchange chromatography on Dowex-1 resin in the Cl⁻ form. [³H]-inositol was eluted from the columns with 20 ml H₂O and [³H]-IP with 3 ml 1 M HCl. [³H]-IP were quantified by liquid scintillation counting after addition of 10 ml scintillation cocktail.

Separation of individual [³H]-inositol phosphates

In some experiments agonist incubations were stopped with 10% perchloric acid after 5 min incubations, before estimation of separate [³H]-inositol phosphates by the gradient h.p.l.c. procedure described by Whitworth & Kendall (1989).

Estimation of [³H]-inositol incorporation into phospholipids

After removal of the aqueous supernatant, slices were treated with 0.94 ml chloroform/methanol/conc. HCl (100/200/1) followed by 0.31 ml chloroform and 0.31 ml H₂O. Following centrifugation to separate the phases, an aliquot (200 µl) of the lower, chloroform phase was taken, evaporated overnight at room temperature and counted in scintillation fluid for estimation of [³H]-inositol incorporation into phospholipids.

Data analysis

The IC₅₀ for inhibition curves was determined from log probit analysis, and the inhibition constant (K_i) calculated from the

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relationship;

$$K_i = \frac{IC_{50}}{\left(1 + \frac{C}{EC_{50}}\right)}$$

where C is the concentration of histamine or adenosine and EC_{50} the concentration of histamine that produces 50% of its maximum response (for the experiment illustrated in Figure 4) or the dissociation constant for adenosine inhibition of the response to histamine (Figure 5).

Results

Total [3H]-inositol phosphate accumulation

After 45 min incubations there were significantly enhanced accumulations of [3H]-IP in the presence of the muscarinic agonist carbachol (10^{-3} M), noradrenaline (3×10^{-4} M), histamine (10^{-3} M), 5-hydroxytryptamine (5-HT, 3×10^{-4} M) and elevated KCl (31 mM) (Figure 1).

The incorporation of [3H]-inositol into the phospholipids was somewhat variable. In the absence of agonist there was an incorporation of 4249 ± 1198 d.p.m. ($n = 10$) per 200 μ l aliquot of the chloroform phase. This was increased after incubation with agonists; in the presence of histamine (10^{-3} M) by $143 \pm 10\%$ ($n = 8$) and in the presence of carbachol (10^{-3} M) by $173 \pm 16\%$ ($n = 7$) of basal levels. Basal and stimulated accumulations of [3H]-IP varied from sample to sample. Unstimulated levels ranged from 300 d.p.m. to over 3,500 d.p.m., and the stimulations due to 10^{-3} M histamine from 1.5 to 5.3 fold over basal.

The increases in [3H]-IP accumulation were concentration-related (Figure 2) with EC_{50} values of; carbachol 5×10^{-5} M; noradrenaline, 1×10^{-5} M and histamine 4×10^{-5} M (all $n = 2$). The responses were abolished in the presence of the Ca^{2+} chelator EGTA (2 mM) (data not shown).

Separate [3H]-inositol phosphates

It would appear that the majority of [3H]-IP accumulating upon stimulation is accounted for by [3H]-inositol monophosphate ([3H]-IP₁) (Figure 3). Although the basal levels of the higher phosphates were relatively large, following 5 min stimulation with 10^{-3} M carbachol, the increases in [3H]-inositol bisphosphate ([3H]-IP₂), [3H]-inositol trisphosphate ([3H]-IP₃) and [3H]-inositol 1,3,4,5-tetrakisphosphate ([3H]-IP₄) fractions were minor, in the absence of LiCl. When 5 mM LiCl was included in the incubations, the effect of carbachol was greatly enhanced with regard to [3H]-IP₁; there were small additional increases in [3H]-1,3,4-IP₃ and [3H]-1,3,4,5-IP₄, and the [3H]-1,4,5-IP₃ fraction was reduced somewhat.

Figure 1 [3H]-inositol phosphates ([3H]-IP) accumulation in slices of human cerebral cortex. Each column represents the mean of three separate experiments with tissue from three different donors, each performed in triplicate; bars show s.e.mean. The accumulations are expressed as percentages of those in the absence of stimulation. CCh = carbachol 10^{-3} M; NA = noradrenaline 3×10^{-4} M; Hist = histamine 10^{-3} M; 5-HT = 5-hydroxytryptamine 3×10^{-4} M; KCl, 31 mM.

Figure 2 Dose-response relationships for [3H]-inositol phosphates ([3H]-IP) accumulation in the presence of carbachol (CCh), noradrenaline (NA) and histamine (Hist). Each curve represents data from a separate tissue sample, and the experiments (performed in triplicate) were repeated on another occasion. EC_{50} values were estimated by inspection. Vertical lines show s.e.mean.

Effect of adenosine on [3H]-inositol phosphate accumulation

Despite having no significant effect alone, adenosine reduced, in a concentration-dependent manner, the response to histamine (Figure 4), with an IC_{50} of $4 \pm 0.7 \times 10^{-5}$ M ($n = 4$) in the presence of 10^{-3} M histamine, giving a K_i of 1.5×10^{-6} M.

This effect of adenosine was selective for the histamine response, of the three agonists examined, since the [3H]-IP accumulations due to carbachol and noradrenaline were unaffected by the presence of adenosine. The mean response to 3×10^{-4} M noradrenaline was $235 \pm 22\%$ of basal in the absence and $255 \pm 74\%$ in the presence of 3×10^{-4} M adenosine ($n = 3$). The response to 10^{-3} M carbachol was $350 \pm 25\%$ of basal in the absence and $370 \pm 15\%$ in the presence of 3×10^{-4} M adenosine ($n = 3$).

The adenosine analogue 2-chloroadenosine (10^{-4} M) was as effective as the parent compound (data not shown), although no concentration-response experiments were performed.

Adenosine antagonists

The inhibitory effect of 10^{-4} M adenosine on histamine-stimulated [3H]-IP accumulation was completely reversed by the non-selective adenosine receptor antagonist theophylline

Figure 3 Separation of [3H]-inositol phosphates from human brain slices in the absence of stimulation and LiCl (open columns) following 5 min stimulation with 10^{-3} M carbachol (diagonally-hatched columns) or 10^{-3} M carbachol plus 5×10^{-3} M LiCl (cross-hatched columns). The effects of LiCl alone were not determined. Twenty five μ l tissue aliquots were prelabelled for 60 min with 240 kBq [3H]-myo-inositol before agonist addition. [3H]-inositol phosphates were extracted by the freon/trioctylamine method and five samples were combined for separation by gradient h.p.l.c. Abbreviations: IP₁, inositol monophosphate; IP₂, inositol bisphosphate; (1,3,4)-IP₃, inositol 1,3,4-trisphosphate; (1,4,5)-IP₃, inositol 1,4,5-trisphosphate; IP₄, inositol 1,3,4,5-tetrakisphosphate. The experiment was repeated on two further occasions with qualitatively similar results, but variations in absolute levels of radioactivity precluded their combination.

Figure 4 Inhibition of histamine-stimulated [³H]-inositol phosphates ([³H]-IP) accumulation by adenosine in human cerebral cortex slices. The data represent the mean of triplicate incubations with tissue from a single donor. The experiment was repeated on three further occasions. (a) The open column represents control; the diagonally-hatched column, adenosine (3×10^{-4} M) and the horizontally-hatched column, histamine (10^{-3} M). (b) The data points represent the effect of increasing concentrations of adenosine in the presence of histamine. Vertical bars show s.e.mean in (a) and (b).

(Figure 5). In two separate experiments the K_i values calculated for theophylline were 7.4×10^{-7} M and 6.0×10^{-7} M. In contrast, 8-cyclopropyltheophylline, at concentrations up to 10^{-4} M, was, in three separate experiments, unable to reverse the inhibitory effects of 3×10^{-4} M adenosine significantly.

Discussion

Previous studies from our own and other laboratories (Hollingsworth *et al.*, 1986; Hill & Kendall, 1987; Kendall & Hill, 1988) have shown that the neuromodulator, adenosine (Snyder, 1985) can selectively influence inositol phospholipid hydrolysis due to histamine in both a positive and negative fashion in the brains of experimental animals. Considering the

Figure 5 Reversal of the adenosine inhibition of histamine-stimulated [³H]-inositol phosphates ([³H]-IP) accumulation by theophylline in human cerebral cortex slices. The data represent the mean of triplicate incubations with combined tissue from two donors. The experiment was repeated on another occasion, with essentially identical results. The inhibition constant for theophylline was calculated from the relationship given under Methods. (a) The open column represents control, the diagonally-hatched column, histamine (10^{-3} M) and the cross-hatched column, histamine plus adenosine (10^{-4} M). (b) The data points represent increasing concentrations of theophylline in the presence of histamine and adenosine. In (a) and (b), vertical bars show s.e.mean.

species diversity in the effects of adenosine (enhancement in the guinea-pig, inhibition in the mouse, no apparent effect in the rat), it was not possible to make predictions concerning the human response, so that it was necessary to assess directly the prospective interaction in human brain.

Given the paucity of studies on human central nervous system tissue, it was also thought to be a useful aim to examine some general features of agonist-stimulated phosphoinositide hydrolysis, which is now accepted to be a ubiquitous intracellular signalling system (Berridge, 1987), leading to Ca^{2+} mobilization and protein kinase C activation.

The accumulations of [³H]-IP in human cortical slices in the presence of LiCl (which prevents the dephosphorylation of inositol monophosphate, Berridge *et al.*, 1982) were generally comparable with those seen in rat brain (Brown *et al.*, 1984), apart from the muscarinic response which was somewhat smaller (Figure 1). The responses were apparently dependent upon extracellular Ca^{2+} , being abolished in the presence of the Ca^{2+} chelator EGTA (2 mM), as is the case in rat brain (Kendall & Nahorski, 1984).

The amount of [³H]-inositol incorporated into the phospholipids was less than that observed in rat, mouse and guinea-pig brain and there was a significant enhancement of incorporation due to histamine and carbachol stimulation. Whether this is a direct effect on the incorporation process or a reflection of a reduction in the phosphoinositide pool due to stimulated breakdown, can only be speculated upon given the available data. There were relatively small increases in the accumulations of the higher phosphates following 5 min stimulations with 10^{-3} M carbachol, but whether this reflects a low rate of polyphosphoinositide metabolism or a rapid flux of label through the higher inositol phosphates would need further investigation (see Batty & Nahorski, 1989). As expected, lithium enhanced the accumulation of [³H]-IP₁ and, probably, [³H]-IP₂ and [³H]-1,3,4-IP₃.

In rat and mouse brain slices lithium-induced decreases in [³H]-1,4,5-IP₃ and [³H]-1,3,4,5-IP₄ have been demonstrated (Batty & Nahorski, 1987; Whitworth & Kendall, 1988), but in the human slices there was no indication of a reduction in [³H]-IP₄ accumulation, although [³H]-1,4,5-IP₃ stimulation by carbachol may be reversed in the presence of 5 mM LiCl.

It is clear, however, that the response to histamine was inhibited by adenosine in human brain slices. The degree of inhibition was greater than that previously seen in mouse cerebral cortex, with complete reversal at the top of the adenosine concentration range. Also the effect was selective, in that the responses to carbachol and noradrenaline were unchanged.

There is some preliminary evidence that the adenosine receptor mediating the modulator response in human brain is different from that in the mouse. Adenosine was more potent in human cortex having a K_i of $1.5 \mu\text{M}$ compared with $16 \mu\text{M}$ in the mouse (Kendall & Hill, 1988), although this could be explained by more efficient receptor/effector coupling in the human cortex. On the other hand, the non-selective adenosine receptor antagonist theophylline (Bruns *et al.* 1986) was more potent in the human tissue, K_i versus adenosine about 700 nM compared with $12 \mu\text{M}$ in the mouse (Kendall & Hill, 1988). In addition the adenosine A₁-selective antagonist 8-cyclopropyltheophylline (H.H. Stein, personal communication) was without effect in human brain despite blocking the response to adenosine in mouse brain with a K_i of $0.9 \mu\text{M}$. Clearly a more detailed pharmacological investigation would be needed to clarify the identity of the adenosine receptor involved.

The complicating factors involved in the interpretation of data generated from experiments with human tissue should be taken into account before the involvement of different receptors is invoked. For instance, these studies used small segments of tissue from various areas of the human cortex compared with the whole cortices employed in animal experiments. Regional differences in the responses may therefore be important and could account, in part, for some of the observed variability in basal and stimulated [³H]-IP accumu-

lation. Also, the human donors were anaesthetized when their tissue was taken and, although the preincubation procedure would be expected to clear the tissue of anaesthetic, residual effects (see Miller, 1985) cannot be ignored. Further, the donors comprised a heterogeneous group in terms of sex, age, disease state and genetic make-up so that a greater degree of inter-experimental variance compared with in-bred laboratory animals would be expected.

In conclusion, agonist-stimulated inositol phospholipid hydrolysis can be demonstrated in human cerebral cortex

slices by a [^3H]-inositol prelabelling assay. The system has many features in common with those previously demonstrated in the brains of experimental animals, including modulation of the direct response to histamine by adenosine. The determination of the pharmacological profile of the receptors involved must remain the subject of future investigations.

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