Modulation of cyclic AMP formation by putative metabotropic receptor agonists

¹Jayne Cartmell, *John A. Kemp, Stephen P.H. Alexander, †Haruhiko Shinozaki & David A. Kendall

Department of Physiology and Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH; *Merck, Sharp and Dohme Research Labs, Neuroscience Research Centre, Terling's Park, Harlow, EM20 2QR and †The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

1 The effects of various metabotropic glutamate receptor agonists on [³H]-cyclic AMP accumulation and phosphoinositide hydrolysis were investigated in guinea-pig cerebral cortical slices prelabelled with [³H]-adenine or [³H]-inositol.

2 1-Aminocyclopentane-1S,3R-dicarboxylate (1S,3R-ACPD), L-2-amino-4-phosphonobutanoate (L-AP4) and (2S,3S,4S)- α -(carboxycyclopropyl)glycine (L-CCG-I), elicited concentration-dependent inhibitions of forskolin-stimulated [³H]-cyclic AMP accumulation, with IC₅₀ values of 2.1 ± 0.3 , 71 ± 17 and 0.2 ± 0.1 μ M respectively.

3 1S,3R-ACPD and L-CCG-I increased the cyclic AMP responses to histamine H₂ receptor stimulation with EC₅₀ values of 7 ± 2 μ M and 19 ± 2 μ M respectively. 1S,3R-ACPD (EC₅₀ value 17 ± 2 μ M) and L-CCG-I (EC₅₀ value 15 ± 3 μ M) potentiated the cyclic AMP responses to the adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA, 10 μ M). This potentiating effect of L-CCG-I was reduced in the presence of a protein kinase C inhibitor, and also in the absence of extracellular calcium. In contrast, L-AP4 inhibited the NECA response in a concentration-dependent manner, with an IC₅₀ value of 120 ± 20 μ M.

4 L-AP4 (at concentrations up to 1 mM) failed to stimulate phosphoinositide hydrolysis in guinea-pig cerebral cortical slices, but both 1S,3R-ACPD (EC₅₀ value 35 ± 6 μ M) and L-CCG-I (approximately 160 μ M) elicited concentration-dependent stimulations of phosphoinositide turnover.

5 These results confirm the existence of at least two distinct subtypes of metabotropic receptor in guinea-pig cortex. The data also substantiate previous studies indicating the importance of the agonist L-CCG-I as a pharmacological tool for distinguishing metabotropic receptor subtypes.

Keywords: Metabotropic receptors; excitatory amino acid receptors; ACPD; L-AP4; L-CCG-I

Introduction

Excitatory amino acid receptors are broadly classified into two main families. The first major group consists of receptors thought to contain integral cation-specific channels and these are termed 'ionotropic receptors'. The second group are responsible for slower signalling via second messenger transduction mechanisms and are known as 'metabotropic receptors' (mGluRs) (Monaghan *et al.*, 1989; Mayer & Miller, 1990). Glutamate, the most abundant excitatory amino acid in the vertebrate central nervous system, activates both groups of receptors, and in order to investigate distinct properties of metabotropic receptors, the use of agonists more selective than glutamate is necessary. The introduction of *trans*-1-aminocyclopentane-1,3-dicarboxylate (*t*-ACPD, a racemic mixture of the 1S,3R- and 1R,3S-isomers of ACPD), a rigid glutamate analogue selective for metabotropic receptors (Palmer *et al.*, 1989), enabled the metabotropic receptor-mediated effects on second messenger transduction systems to be elucidated. The subsequent purification of *trans*-ACPD and *cis*-ACPD lead to the isolation of the four isomers of ACPD, 1S,3R-, 1S,3S-, 1R,3S- and 1R,3R- (Curry *et al.*, 1988). The 1S,3R-isomer of ACPD is more selective for metabotropic receptors than the racemic mixture *t*-ACPD (Cartmell *et al.*, 1992), but 1S,3R-ACPD does not distinguish subtypes of the metabotropic receptor recently cloned by Nakanishi and co-workers (mGluR₁-mGluR₆; Nakanishi, 1992), although we have recently found that 1S,3R-ACPD is significantly more potent an agonist of metabotropic receptors negatively

coupled to adenylyl cyclase than phospholipase C-linked metabotropic receptors in guinea-pig cerebral cortex (Cartmell *et al.*, 1993). The metabotropic receptor subtypes are grouped according to distinct transduction mechanisms; mGluR₁ and mGluR₅ are coupled to phospholipase C, and mGluR₂, mGluR₃, mGluR₄ and mGluR₆ appear to be negatively-coupled to adenylyl cyclase (Masu *et al.*, 1991; Houamed *et al.*, 1991; Tanabe *et al.*, 1992; Abe *et al.*, 1992).

It has recently been shown that isomers of a second rigid glutamate analogue α -(carboxycyclopropyl) glycine (CCG) have agonist properties at metabotropic receptors. Nakagawa *et al.* (1990) reported that the (2S,3S,4S) isomer of α -(carboxycyclopropyl) glycine (L-CCG-I) elicited significant chloride currents (induced through phosphoinositide breakdown) in *Xenopus* oocytes injected with rat brain poly (A)⁺ RNA. L-CCG-I is also an effective stimulant of phosphoinositide metabolism in both hippocampal synaptoneurosome (Nakagawa *et al.*, 1990) and mGluR₁-expressing Chinese hamster ovary (CHO) cells (Hayashi *et al.*, 1992). Forskolin-stimulated cyclic AMP accumulation was inhibited by L-CCG-I in mGluR₂-expressing CHO cells with a potency about 100 fold greater than that at the mGluR₁ and mGluR₄ receptors. This evidence strongly suggests that L-CCG-I is a potent agonist at metabotropic receptors and may serve as an important tool in distinguishing putative receptor subtypes.

It has been reported that L-2-amino-4-phosphonobutyrate (L-AP4), an unconstrained glutamate analogue, inhibits neurotransmitter release via a G-protein-coupled glutamate receptor (Trombley & Westbrook, 1992) and, recently L-AP4 has been shown to have a potency in the high nanomolar range for inhibition of cyclic AMP formation in CHO cells trans-

¹ Author for correspondence.

fects with the mGluR₄ receptor, but is without effect on second messenger formation in any of the other mGluR-expressing cells (Tanabe *et al.*, 1993).

On the basis of their effects on transfected cells, L-CCG-I and L-AP4 would appear to be potentially useful agents for distinguishing between mGluR-mediated events in the central nervous system. Therefore, in this study we have compared the effects of L-CCG-I and L-AP4 with those of 1S,3R-ACPD on second messenger generation in slices of guinea-pig cerebral cortex.

Methods

The accumulation of [³H]-adenosine 3':5'-cyclic monophosphate ([³H]-cyclic AMP) was assessed in guinea-pig cerebral cortical slices prelabelled with [³H]-adenine as previously described (Donaldson *et al.*, 1990). Briefly, after washing the slices, antagonists were added for 15 min and excitatory amino acid (EAA) agonists for 5 min, before the addition of forskolin (30 μ M), NECA (10 μ M, in the presence of 1.2 u ml⁻¹ adenosine deaminase) or histamine (1 mM, in the presence of 3 μ M mepyramine and 10 μ M rolipram). The incubation was terminated after 10 min by the addition of 1 M HCl. [³H]-cyclic AMP was isolated by sequential Dowex 50/alumina column chromatography.

Agonist-stimulated phosphoinositide turnover was measured in slices labelled with [³H]-inositol, for 45 min in the presence of 5 mM LiCl (Alexander *et al.*, 1990). The incubation was terminated with 7.5% perchloric acid and [³H]-inositol phosphates were isolated by Dowex 1 (chloride form) column chromatography.

Curves were fitted to data using a logistic equation from the computer programme GraphPad (GraphPad, California, U.S.A.). Statistical significance was determined by Student's *t* test.

[³H]-adenine (888 GBq mmol⁻¹) and [¹⁴C]-cyclic AMP (1.6 GBq mmol⁻¹) were purchased from Amersham International, Bucks. [³H]-inositol (640 GBq mmol⁻¹) was obtained from NEN Du Pont, Herts. Forskolin, histamine, mepyramine, [ethylenebis (oxyethylenenitrilo)] tetraacetic acid, 2-amino-3-phosphonopropionate, L-2-amino-4-phosphonobutyrate and L-2-amino-5-phosphonopentanoate were all purchased from Sigma Chemical Company Ltd., Poole, Dorset. 6,7-Dinitroquinoxaline-2,3-dione and 1-aminocyclopentane-1S,3R-dicarboxylate were obtained from Tocris Neuramin, Bristol. 5-Methyl-10,11-dihydro-5H-dibenzo[*ad*]cyclohepten-5,10-imine maleate was manufactured at Merck, Sharp and Dohme Neuroscience Centre, Terling's Park, Harlow and 5'-N-ethylcarboxamidoadenosine was obtained from Research Biochemicals Incorporated, St. Albans, Herts. Adenosine deaminase was purchased from Boehringer Mannheim, Sussex. Rolipram and Ro 31-8220 (3-[(1-[3-(2-isothionreido)propyl]-indol-3-yl)-4-(1-methyl indol-3-yl)-3-pyrrolin-2,5-dione]) were gifts from Schering, Germany and Hoffmann La Roche, U.K., respectively.

Results

Inhibition of forskolin-stimulated cyclic AMP accumulation

L-CCG-I had no direct effects on basal levels of [³H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices, but inhibited forskolin-stimulated [³H]-cyclic AMP accumulation in a concentration-dependent manner with an IC₅₀ value of 0.24 $\pm$ 0.06 μ M (*n* = 5) (Figure 1b). L-CCG-I inhibited the forskolin response by 91 $\pm$ 4% (*n* = 5), a value similar to the inhibition of the forskolin cyclic AMP response by 1S,3R-ACPD (85 $\pm$ 2%, Cartmell *et al.*, 1993). The inhibition of forskolin-stimulated cyclic AMP accumulation by L-CCG-I was not reversed by the EAA receptor antagonists 6,7-

Figure 1 (a) The effect of L-CCG-I on phosphoinositide turnover in guinea-pig cerebral cortical slices in the presence of 5 mM LiCl. Slices were incubated with increasing concentrations of L-CCG-I for 45 min. Unlabelled column represents control values. Data are d.p.m. from a single experiment conducted in quadruplicate and are represented as mean $\pm$ s.e.mean values. (b) The inhibitory effects of L-CCG-I on forskolin-stimulated [³H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices, in the presence of adenosine deaminase (1.2 u ml⁻¹). Slices were incubated with 30 μ M forskolin in the absence or presence of increasing concentrations of L-CCG-I added 5 min previously. Unlabelled column represents control values, Forskolin indicates forskolin alone. Data are d.p.m. from a single experiment conducted in quadruplicate. The mean $\pm$ s.e.mean IC₅₀ value for five such experiments was 0.24 $\pm$ 0.06 μ M. Data are represented as mean $\pm$ s.e.mean values. (c) The effects of L-CCG-I on NECA-stimulated [³H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices, in the presence of adenosine deaminase (1.2 u ml⁻¹). Slices were incubated with 10 μ M NECA in the absence or presence of increasing concentrations of L-CCG-I added 5 min previously. Unlabelled column represents control values, NECA indicates NECA alone. Data are d.p.m. from a single experiment conducted in quadruplicate. The mean $\pm$ s.e.mean IC₅₀ value for three such experiments was 15 $\pm$ 3 μ M. Data are represented as mean $\pm$ s.e.mean values.

dinitroquinoxaline-2,3-dione (DNQX, 100 μ M), L-2-amino-5-phosphonopentanoate (L-AP5, 100 μ M) and 2-amino-3-phosphonopropionate (DL-AP3, 1 mM) (data not shown). However, the effects of the inotropic receptor antagonists are complicated by the finding that DNQX and L-AP5 alone also inhibited the forskolin response by 39 ± 3 and $30 \pm 3\%$ respectively. L-CCG-I appears to be approximately 10 fold more potent than 1S,3R-ACPD (IC_{50} 2.14 ± 0.29 μ M ($n = 4$); Cartmell *et al.*, 1993).

L-AP4 also inhibited forskolin-stimulated cyclic AMP accumulation in a concentration-dependent manner but with an IC_{50} value of 71 ± 17 μ M ($n = 5$) (Figure 2), indicating a lower potency of L-AP4 in comparison with 1S,3R-ACPD and L-CCG-I. The inhibition of the forskolin cyclic AMP response by L-AP4 was also not reversed by the inotropic receptor antagonists DNQX (100 μ M), L-AP5 (100 μ M) and DL-AP3 (1 mM).

Effects on receptor-mediated cyclic AMP formation

In order to investigate the effects of putative metabotropic receptor agonists on receptor-mediated [3 H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices, adenosine A_{2b} receptors were stimulated with 5'-N-ethylcarboxamido-adenosine (NECA, 10 μ M), in the presence of 1.2 μ M $^{-1}$ adenosine deaminase; and histamine H_2 receptors stimulated with 1 mM histamine, in the presence of 3 μ M mepyramine and 10 μ M rolipram. L-AP4 elicited inhibitory effects on both adenosine A_{2b} receptor- and histamine H_2 receptor-stimulated [3 H]-cyclic AMP accumulation (Figure 2). IC_{50} values obtained for these inhibitory effects were 120 ± 20 μ M ($n = 3$) and 71 ± 14 μ M ($n = 3$) respectively. At an L-AP4 concentration of 1 mM the NECA [3 H]-cyclic AMP response was inhibited by $86 \pm 4\%$ ($n = 3$) and the [3 H]-cyclic AMP response to histamine was inhibited by $80 \pm 2\%$ ($n = 3$).

In contrast to these inhibitory effects, L-CCG-I enhanced the [3 H]-cyclic AMP responses to both adenosine A_{2b} receptor- (Figure 1c) and histamine H_2 receptor- stimulation (Table 1). The response to NECA was enhanced by L-CCG-I with an EC_{50} value of 15 ± 3 μ M ($n = 3$); a maximal potentiation ($297 \pm 14\%$ of the NECA response) was achieved at 100 μ M. The EC_{50} value obtained for L-CCG-I-elicited potentiation is similar to that previously obtained for the enhance-

Figure 2 The inhibitory effects of L-AP4 upon forskolin (30 μ M, $\circ$), NECA (10 μ M, Δ), and histamine (1 mM, $\blacksquare$) stimulated [3 H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices. To normalize responses from different experiments data are expressed as a percentage of the response to the stimulus. Data are expressed as mean $\pm$ s.e.mean. For abbreviations, see text.

ment of the NECA response by 1S,3R-ACPD (17 ± 2 μ M ($n = 5$); Cartmell *et al.*, 1993). L-CCG-I also had a potentiatory effect on the [3 H]-cyclic AMP response to histamine with an EC_{50} value of 19 ± 2 μ M ($438 \pm 42\%$ increase in the histamine response at 100 μ M) compared to a value of 7 ± 2 μ M ($n = 3$) for the potentiation of the histamine [3 H]-cyclic AMP response by 1S,3R-ACPD.

Phosphoinositide hydrolysis

In addition to potentiating the [3 H]-cyclic AMP responses to NECA and histamine, L-CCG-I stimulated phosphoinositide hydrolysis in a concentration-dependent manner. Due to constraints imposed by the availability of the material, concentrations of L-CCG-I greater than 1 mM (at which a maximum effect had not been achieved) could not be examined. The EC_{50} value is therefore in excess of 160 μ M (the best fit estimate for the curve in Figure 1a). L-CCG-I is therefore a less potent agonist at the phospholipase C-coupled metabotropic receptor(s) than 1S,3R-ACPD (EC_{50} value 35 ± 6 μ M, $n = 8$; Table 1). L-CCG-I-stimulated phosphoinositide hydrolysis was not inhibited by the inotropic receptor antagonists 5-methyl-10, 11-dihydro-5H-dibenzo [*a,d*] cyclohepten-5, 10-imine maleate (MK-801, 100 μ M), L-AP5 (1 μ M) or DNQX (100 μ M). L-AP4 failed to stimulate significantly phosphoinositide hydrolysis at 1 mM (78 ± 26 d.p.m. over basal, $n = 3$).

The mechanism of L-CCG-I enhancement of cyclic AMP accumulation

In brain slice preparations, stimulation of many receptor systems linked to phosphoinositide hydrolysis e.g. histamine H_1 , α_1 -adrenoceptors, 5-HT $_2$ can indirectly potentiate cyclic AMP formation mediated by other receptor systems (Hill & Kendall, 1989). This augmentation is thought to be due to products of phosphoinositide turnover, increasing protein kinase C activity and intracellular Ca^{2+} concentration (Donaldson *et al.*, 1990). In order to investigate the possible role of protein kinase C in the augmentation of the NECA [3 H]-cyclic AMP response by L-CCG-I, the effects of the protein kinase C inhibitor Ro 31-8220 were studied. Slices were incubated for 5 min with L-CCG-I (100 μ M) prior to a 10 min stimulation with either NECA (10 μ M) or forskolin (30 μ M), in the presence or absence of 10 μ M Ro 31-8220 (Davis *et al.*, 1989). Ro 31-8220 had no effect on basal levels of [3 H]-cyclic AMP but reduced the potentiation of the NECA [3 H]-cyclic AMP response by both L-CCG-I (100 μ M) and β -phorbol-12,13-dibutyrate (PDBu, 1 μ M) (Figure 3a). Potentiation of the NECA response by L-CCG-I was $547 \pm 58\%$ ($n = 3$) of the [3 H]-cyclic AMP response to NECA in the absence and $356 \pm 50\%$ ($n = 3$) in the presence of the PKC inhibitor. Similarly, the PDBu-elicited increase in the NECA [3 H]-cyclic AMP response was reduced from $628 \pm 83\%$ in the absence to $172 \pm 23\%$ in the presence of Ro 31-8220 (10 μ M). These data suggest that protein kinase C plays a role in the potentiation of the [3 H]-cyclic AMP response to NECA by L-CCG-I.

The effects of removing Ca^{2+} from the extracellular medium were investigated by incubating slices in Ca^{2+} -free Krebs solution containing 250 μ M EGTA. The presence of EGTA had no effect on basal levels of cyclic AMP, nor on the inhibition of forskolin-stimulated cyclic AMP accumulation by 100 μ M L-CCG-I (although the forskolin response was increased in the absence of extracellular Ca^{2+} presumably due to the deactivation of Ca^{2+} -dependent phosphodiesterases, Figure 3b). Conversely, the L-CCG-I-elicited potentiation of the NECA [3 H]-cyclic AMP response was reduced by omission of Ca^{2+} from the extracellular medium. The L-CCG-I-elicited increase in the NECA [3 H]-cyclic AMP response was reduced from $634 \pm 32\%$ ($n = 3$) in the presence to $193 \pm 25\%$ ($n = 3$) in the absence of extracellular Ca^{2+} . Interestingly, the potentiating effects of PDBu on the NECA

Table 1 Values of agonist potencies (μM) of 1S,3R-ACPD, L-CCG-I and L-AP4

	Forskolin-stimulated cyclic AMP	NECA-stimulated cyclic AMP	Histamine-stimulated cyclic AMP	Phosphoinositide hydrolysis
1S,3R-ACPD	$2.14 \pm 0.29(7)\downarrow$	$17 \pm 2 (5)\uparrow$	$7 \pm 2 (3)\uparrow$	$35 \pm 6(8)\uparrow$
L-CCG-I	$0.24 \pm 0.06(5)\downarrow$	$15 \pm 3 (3)\uparrow$	$19 \pm 2 (3)\uparrow$	$\sim 160(3)\uparrow$
L-AP4	$71 \pm 17(5)\downarrow$	$120 \pm 20(3)\downarrow$	$71 \pm 14(3)\downarrow$	No effect

Values are mean $\pm$ s.e.mean of 3–8 experiments (n given in parentheses).

$\uparrow$ represents increased response

$\downarrow$ represents inhibition of response.

For abbreviations, see text.

Figure 3 (a) The effect of the protein kinase C inhibitor Ro-31-8220 (10 μM) on potentiation of the NECA-stimulated [^3H]-cyclic AMP response. NECA indicates incubation with 10 μM NECA only and PDBu indicates incubation with 1 μM PDBu only. Hatched columns represent data from slices incubated in the presence of Ro-31-8220 (10 μM). Data are d.p.m. from a single experiment conducted in quadruplicate and are represented as mean $\pm$ s.e.mean values. Two similar experiments gave essentially identical results. (b) The effects of EGTA (250 μM) on the inhibition of forskolin-stimulated [^3H]-cyclic AMP accumulation and potentiation of the NECA-stimulated [^3H]-cyclic AMP response by L-CCG-I (100 μM). Forskolin indicates incubation with 30 μM forskolin, and NECA represents incubation with 10 μM NECA. Unlabelled columns represent control values, hatched columns represent data from slices incubated in the presence of EGTA (250 μM). Data are d.p.m. from a single experiment conducted in quadruplicate and are represented as mean $\pm$ s.e. mean values. Two similar experiments gave essentially identical results.

response were also reduced ($\sim 400\%$ in the presence and $\sim 200\%$ in the absence of extracellular Ca^{2+}), confirming that the action of PKC is Ca^{2+} -dependent and suggesting that the concentration of EGTA used (250 μM) was sufficient to reduce cytoplasmic Ca^{2+} . These results implicate an important role for the phosphoinositide-linked metabotropic receptor in 1S,3R-ACPD- and L-CCG-I-elicited potentiations of adenosine A_{2b} - and histamine H_2 -receptor-mediated responses.

Discussion

The aim of this study was to determine the selectivity, in a brain-derived preparation, of two agonists, L-CCG-I and L-AP4 which have been shown to have different potencies for metabotropic receptor subtypes expressed in model cell systems.

L-CCG-I stimulated phosphoinositide hydrolysis in a concentration-dependent manner, but due to a limited availability of the compound the maximum concentration that could be achieved was 1 mM at which inositol phosphates accumulation was still increasing. The EC_{50} of L-CCG-I-stimulated inositol phosphates was therefore in excess of 160 μM , somewhat less potent than the EC_{50} of 5 μM reported for the compound in studies on rat hippocampal synaptoneuroosomes (Nakagawa *et al.*, 1990) or the 50 μM determined for mGluR $_1$ receptors expressed in CHO cells (Hayashi *et al.*, 1992). The differences could be due to the existence of different receptor reserves in the various preparations or to the mediation of the responses in the brain preparations by metabotropic receptors other than mGluR $_1$ (e.g. mGluR $_5$) for which the potency of L-CCG-I is not yet known. L-CCG-I is also a less potent agonist of the mGluR $_1$ receptor than quisqualate, glutamate, ibotenate or *trans*-ACPD (Tanabe *et al.*, 1993).

L-AP4 was without effect on basal phosphoinositide hydrolysis in guinea-pig cerebral cortex although it has previously been suggested that it is an antagonist in rat cerebral cortex (Schoepp *et al.*, 1990) and it is conceivable therefore that some of the effects of L-AP4 on neurotransmitter release could be due to blockade of Ca^{2+} -mobilizing metabotropic receptors, for instance those involved in the metabotropic receptor-mediated enhancement of glutamate release (Herrero *et al.*, 1992).

In contrast to their effects on forskolin-stimulated cyclic AMP formation, glutamate (Donaldson *et al.*, 1990) and 1S,3R-ACPD (Cartmell *et al.*, 1993, Table 1) enhanced adenosine A_{2b} receptor-mediated responses. In the present study, L-CCG-I was observed to potentiate both NECA- and histamine-stimulated cyclic AMP formation with EC_{50} of 15 μM and 19 μM respectively. PDBu also enhanced the cyclic AMP response to NECA, and this enhancement was completely reversed in the presence of the protein kinase C inhibitor, Ro 31-8220. The augmentation elicited by L-CCG-I was only partially reversed by Ro 31-8220 suggesting that protein kinase C (presumably activated by diacylglycerol generated by stimulation of phosphoinositide turnover) mediates only part of the potentiated response (Figure 3). Calcium influx also appears to be involved in the augmented response to L-CCG-I since a reduced extracellular Ca^{2+} concentration resulted in a reduction of the enhanced response. Similarly, the ACPD-evoked phosphoinositide response in guinea-pig cerebral cortex requires the presence of extracellular calcium (Alexander *et al.*, 1990). These findings suggest that L-CCG-I-evoked potentiations of receptor-stimulated cyclic AMP formation are mediated by products of phosphoinositide hydrolysis. The potency of 1S,3R-ACPD in potentiating receptor-stimulated cyclic AMP formation was

in accordance with that for stimulating phosphoinositide hydrolysis (Table 1) suggesting a similar mechanism, but L-CCG-I was at least 10 fold more potent in potentiating the cyclic AMP responses to histamine and NECA compared with its effect on phosphoinositide hydrolysis. It is therefore possible that the L-CCG-I-mediated potentiations involve a considerable amplification step although it is puzzling that a similar amplification does not appear to be involved in the enhanced cyclic AMP response to 1S,3R-ACPD—this mechanism clearly requires further investigation.

Since L-AP4 failed to stimulate phosphoinositide hydrolysis, it is not surprising that L-AP4 did not increase the [3 H]-cyclic AMP responses to either NECA or histamine. In fact L-AP4 inhibited these responses in a concentration-dependent manner (IC_{50} values $120 \pm 20 \mu M$ and $71 \pm 14 \mu M$ respectively), and these reductions might also be involved in L-AP4-mediated inhibition of synaptic neurotransmission. The inhibitory actions of L-AP4 are not due to non-specific toxic effects since in preliminary experiments on cultured rat astrocytes, L-AP4 (up to a concentration of 1 mM) had no effect on isoprenaline-stimulated cyclic AMP formation (Cartmell *et al.*, unpublished observations).

When adenylyl cyclase was stimulated by forskolin (30 μM), L-CCG-I elicited a concentration-dependent inhibition of [3 H]-cyclic AMP accumulation. L-CCG-I was approximately 10 fold more potent (IC_{50} 0.24 μM) in this regard than the selective metabotropic receptor agonist 1S,3R-ACPD (IC_{50} value $2.14 \pm 0.29 \mu M$, $n = 7$; Cartmell *et al.*, 1993, Table 1). Somewhat surprisingly, the extent of this inhibition at 1 μM was $91 \pm 4\%$ ($n = 5$) of the forskolin response (1S,3R-ACPD also caused an $85 \pm 2\%$ inhibition of the forskolin [3 H]-cyclic AMP response at 10 μM , Cartmell *et al.*, 1993). This contrasts with the much smaller inhibitions of the forskolin response mediated by some other receptors negatively coupled to adenylyl cyclase (e.g. α_2 -adrenoceptor, Duman & Enna, 1986) in brain slices. The simplest explanation for this difference is that most of the cells responding to forskolin contain metabotropic but not α_2 -adrenoceptors. The EC_{50} for L-CCG-I mediated inhibition of the forskolin response (240 nM) is very close to the 300 nM quoted by Hayashi *et al.* (1992) for L-CCG-I at the mGluR₂ but much

more potent than at the mGluR₄ receptor (IC_{50} 50 μM) expressed in CHO cells. Whether the steepness of the inhibition curve (Figure 2b) indicates the presence of multiple co-operating receptors or an additional, non-receptor-mediated effect of L-CCG-I remains to be determined.

L-AP4 also inhibited forskolin-stimulated [3 H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices (IC_{50} value $71 \pm 17 \mu M$, $n = 5$), but was much less potent than either 1S,3R-ACPD or L-CCG-I. The shallow concentration-response curve may be due to L-AP4 acting at a number of receptor subtypes with different potencies. Indeed, on the basis of the data from transfected receptor studies (Nakanishi, 1992; Thomsen *et al.*, 1992; Tanabe *et al.*, 1993), L-AP4 would be expected to have a potency at the mGluR₄ receptor in the high nanomolar range and it is likely that the guinea-pig cortex contains only a minor proportion of mGluR₄s with the majority of the inhibition of the forskolin response due to L-AP4 being mediated by other cyclase-linked mGluRs.

We may therefore conclude that there are at least two different subtypes of metabotropic glutamate receptors in guinea-pig cerebral cortex. The first group of receptors are coupled to phosphoinositide turnover via phospholipase C, and the second group are negatively linked to adenylyl cyclase. Furthermore, interaction between phospholipase C-linked and adenylyl cyclase-coupled receptors appears to cause an indirect enhancement of cyclic AMP accumulation. It is apparent from these results (Table 1) that 1S,3R-ACPD, L-AP4 and L-CCG-I are all more potent inhibitors of cyclic AMP accumulation than stimulators of phosphoinositide hydrolysis, but it is evident that L-CCG-I is by far the most selective for metabotropic receptors negatively-coupled to adenylyl cyclase in this tissue preparation. These data confirm previous studies indicating that L-CCG-I is an important pharmacological tool for the differentiation of metabotropic glutamate receptor subtypes.

We are grateful for support from the Wellcome Trust and Medical Research Council.

References

- ABE, T., SUGIHARA, H., NAWA, H., SHIGEMOTO, R., MIZUNO, N. & NAKANISHI, S. (1992). Molecular characterisation of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/ Ca^{2+} signal transduction. *J. Biol. Chem.*, **267**, 13361–13368.
- ALEXANDER, S.P.H., HILL, S.J. & KENDALL, D.A. (1990). Excitatory amino acid – induced formation of inositol phosphates in guinea-pig cerebral cortical slices: Involvement of inotropic or metabotropic receptors? *J. Neurochem.*, **55**, 1439–1441.
- CARTMELL, J., CURTIS, A.R., KEMP, J.A., KENDALL, D.A. & ALEXANDER, S.P.H. (1993). Subtypes of ACPD receptor distinguished by stereoisomers of the rigid glutamate analogue, 1-aminocyclopentane-1,3-dicarboxylate. *Neurosci. Lett.*, **153**, 107–110.
- CARTMELL, J., KEMP, J.A., ALEXANDER, S.P.H., HILL, S.J. & KENDALL, D.A. (1992). Inhibition of forskolin-stimulated cyclic AMP formation by *t*-ACPD in guinea-pig cerebral cortical slices. *J. Neurochem.*, **58**, 1964–1966.
- CURRY, K., PEET, M.J., MAGNUSON, D.S. & MCLENNAN, H. (1988). Synthesis, resolution, and absolute configuration of the isomers of the neuronal excitant 1-amino-1,3-cyclopentanedicarboxylic acid. *J. Med. Chem.*, **31**, 864–867.
- DAVIS, P.D., HILL, C.H., KEECH, E., LAWTON, G., NIXON, J.S., SEDGWICK, A.D., WADSWORTH, J., WESTACOLT, D. & WILKINSON, S.E. (1989). Potent selective inhibitors of protein kinase C. *FEBS Lett.*, **259**, 61–63.
- DONALDSON, J., KENDALL, D.A. & HILL, S.J. (1990). Discriminatory effects of forskolin and EGTA on the indirect cyclic AMP responses to histamine, noradrenaline, 5-hydroxytryptamine, and glutamate in guinea-pig cerebral cortical slices. *J. Neurochem.*, **54**, 1484–1491.
- DUMAN, R.S. & ENNA, S.J. (1986). A procedure for measuring α_2 -adrenergic receptor-mediated inhibition of cyclic AMP accumulation in rat brain slices. *Brain Res.*, **384**, 391–394.
- HAYASHI, Y., TANABE, Y., ARAMORI, I., MASU, M., SHIMAMOTO, K., OHFUNE, Y. & NAKANISHI, S. (1992). Agonist analysis of 2-(carboxycyclopropyl)glycine isomers for cloned metabotropic receptor subtypes expressed in Chinese hamster ovary cells. *Br. J. Pharmacol.*, **107**, 539–543.
- HERRERO, I., MIRAS-PORTUGAL, M.T. & SANCHEZ-PRIETO, J. (1992). Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation. *Nature*, **360**, 163–166.
- HILL, S.J. & KENDALL, D.A. (1989). Cross-talk between different receptor-effector systems in the mammalian CNS. *Cell. Signal.*, **1**, 135–141.
- HOUAMED, K.M., KUIJPER, J.L., GILBERT, T.L., HALDEMAN, B.A., O'HARA, P.J., MULVIHILL, E.R., ALMERS, W. & HAGEN, F.S. (1991). Cloning, expression, and gene structure of a G-protein-coupled glutamate receptor from rat brain. *Science*, **252**, 1318–1321.
- MASU, M., TANABE, Y., TSUCHIDA, K., SHIGEMOTO, R. & NAKANISHI, S. (1991). Sequence and expression of a metabotropic glutamate receptor. *Nature*, **349**, 760–765.
- MAYER, M.L. & MILLER, R.J. (1990). Excitatory amino acid receptors second messengers and regulation of intracellular Ca^{2+} in mammalian neurons. *Trends Pharmacol. Sci.*, **11**, 254–260.
- MONAGHAN, D.T., BRIDGES, R.J. & COTMAN, C.W. (1989). The excitatory amino acid receptors; their classes, pharmacology and distinct properties in the function of the central nervous system. *Annu. Rev. Pharmacol. Toxicol.*, **29**, 365–402.

- NAKAGAWA, Y., SAITOH, K., ISHIHARA, T., ISHIDA, M. & SHINOZAKI, H. (1990). (2S,3S,4S) α -(carboxycyclopropyl)glycine is a novel agonist of metabotropic glutamate receptors. *Eur. J. Pharmacol.*, **184**, 205–206.
- NAKANISHI, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. *Science*, **258**, 597–603.
- PALMER, E., MONAGHAN, D.T. & COTMAN, C.W. (1989). Trans-ACPD, a selective agonist of the phosphoinositide-coupled excitatory amino acid receptor. *Eur. J. Pharmacol.*, **160**, 585–587.
- SCHOEPP, D., BOCKAERT, J. & SLADECZEK, F. (1990). Pharmacological and functional characteristics of metabotropic glutamate receptors. *Trends Pharmacol. Sci.*, **11**, 508–515.
- TANABE, Y., MASU, M., ISHII, T., SHIGEMOTO, R. & NAKANISHI, S. (1992). A family of metabotropic receptors. *Neuron*, **8**, 169–179.
- TANABE, Y., NOMURA, A., MASU, M., SHIGEMOTO, R., MIZUNO, N. & NAKANISHI, S. (1993). Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. *J. Neurosci.*, **13**, 1372–1378.
- THOMSEN, C., KRISTENSEN, P., MULVIHILL, E., HALDEMAN, B. & SUZDAK, P.D. (1992). L-2-Amino-4-phosphonobutyrate (L-AP4) is an agonist at the type IV metabotropic glutamate receptor which is negatively coupled to adenylate cyclase. *Eur. J. Pharmacol. (Mol. Pharmacol.)*, **227**, 361–362.
- TROMBLEY, P.Q. & WESTBROOK, G.L. (1992). L-AP4 inhibits calcium currents and synaptic transmission via a G-protein coupled glutamate receptor. *J. Neurosci.*, **12**, 2043–2050.

(Received June 29, 1993)

Revised October 1, 1993

Accepted October 6, 1993)