GABA_B receptor-mediated stimulation of adenylyl cyclase activity in membranes of rat olfactory bulb

¹Maria C. Olinas & ^{*,1}Pierluigi Onali

¹Section on Biochemical Pharmacology, Department of Neurosciences, University of Cagliari, via Porcell 4, 09124 Cagliari, Italy

1 Previous studies have shown that GABA_B receptors facilitate cyclic AMP formation in brain slices likely through an indirect mechanism involving intracellular second messengers. In the present study, we have investigated whether a positive coupling of GABA_B receptors to adenylyl cyclase could be detected in a cell-free preparation of rat olfactory bulb, a brain region where other G_i/G_o-coupled neurotransmitter receptors have been found to stimulate the cyclase activity.

2 The GABA_B receptor agonist (–)-baclofen significantly increased basal adenylyl cyclase activity in membranes of the granule cell and external plexiform layers, but not in the olfactory nerve-glomerular layer. The adenylyl cyclase stimulation was therefore examined in granule cell layer membranes.

3 The (–)-baclofen stimulation (pD₂=4.53) was mimicked by 3-aminopropylphosphonic acid (pD₂=4.60) and GABA (pD₂=3.56), but not by (+)-baclofen, 3-aminopropylphosphonic acid, muscimol and isoguvacine. The stimulatory effect was counteracted by the GABA_B receptor antagonists CGP 35348 (pA₂=4.31), CGP 55845 A (pA₂=7.0) and 2-hydroxysaclofen (pK_i=4.22). Phaclofen (1 mM) was inactive.

4 The (–)-baclofen stimulation was not affected by quinacrine, indomethacin, nordihydroguaiaretic acid and staurosporine, but was completely prevented by pertussis toxin and significantly reduced by the α subunit of transducin, a $\beta\gamma$ scavenger. The $\beta\gamma$ subunits of transducin stimulated the cyclase activity and this effect was not additive with that produced by (–)-baclofen.

5 In the external plexiform and granule cell layers, but not in the olfactory nerve-glomerular layer, (–)-baclofen enhanced the adenylyl cyclase stimulation elicited by the neuropeptide pituitary adenylyl cyclase activating polypeptide (PACAP) 38.

6 Conversely, the adenylyl cyclase activity stimulated by either forskolin or Ca²⁺/calmodulin (Ca²⁺/CaM) was inhibited by (–)-baclofen in all the olfactory bulb layers examined.

7 These data demonstrate that in specific layers of rat olfactory bulb activation of GABA_B receptors enhances basal and neurotransmitter-stimulated adenylyl cyclase activities by a mechanism involving $\beta\gamma$ subunits of G_i/G_o. This positive coupling is associated with a widespread inhibitory effect on forskolin- and Ca²⁺/CaM-stimulated cyclic AMP formation.

Keywords: GABA_B receptors; adenylyl cyclase; G protein $\beta\gamma$ subunits; rat olfactory bulb

Abbreviations: 3-APA, 3-aminopropylphosphonic acid; 3-APPA, 3-aminopropylphosphonic acid; α_{iGDP} , the α subunit of transducin in the GDP-bound form; $\beta\gamma_i$, the $\beta\gamma$ subunits of transducin; BSA, bovine serum albumin; CaM, calmodulin; DTT, dithiothreitol; PACAP, pituitary adenylyl cyclase activating polypeptide; PMA, phorbol 12-myristate, 13-acetate

Introduction

The metabotropic GABA_B receptors constitute a distinct subclass of receptors for the inhibitory amino acid GABA (Bowery, 1993). These receptors are widely distributed in the central nervous system, where they are located at both pre- and post-synaptic sites. Drugs acting on GABA_B receptors have been found to be useful in the treatment of spasticity and may have other potential clinical applications, such as absence epilepsy, cognitive impairment and depression (Malcangio & Bowery, 1995; Wojcik & Holopainen, 1992). Recently, the GABA_B receptor has been cloned from rat brain and found to show sequence similarity to the metabotropic receptors for the excitatory neurotransmitter glutamate (Kaupmann *et al.*, 1997). These studies have also led to the identification of two molecular forms of the GABA_B receptor which display a different N-terminal sequence but similar pharmacological properties (Kaupmann *et al.*, 1997). GABA_B receptors have been shown to interact with pertussis toxin-sensitive G proteins of the G_i/G_o family (Morishita *et al.*, 1990) and to activate

different signalling mechanisms, including stimulation of K⁺ conductance, inhibition of Ca²⁺ channel activities and modulation of cyclic AMP formation (Bowery, 1993; Wojcik & Holopainen, 1992). With regard to the latter mechanism, both inhibitory and stimulatory effects have been reported. Thus, GABA_B receptors have been found to inhibit adenylyl cyclase activity in homogenates of different brain areas (Wojcik & Neff, 1984) and this effect has been shown to be prevented by pertussis toxin, indicating the involvement of G_i/G_o (Xu & Wojcik, 1986). A negative coupling to adenylyl cyclase has also been observed for the cloned receptor expressed in host cells (Kaupmann *et al.*, 1997). On the other hand, in brain slice preparations the GABA_B receptor agonist baclofen, besides inhibiting forskolin-stimulated cyclic AMP accumulation, has been found to potentiate the adenylyl cyclase stimulation elicited by different neurotransmitters, such as noradrenaline, vasoactive intestinal peptide, adenosine and prostaglandins (Enna & Karbon, 1984; Hill & Dolphin, 1984; Karbon & Enna, 1985; Watling & Bristow, 1986; Schaad *et al.*, 1989). Evidence has been provided that the facilitation of cyclic AMP formation

*Author for correspondence.

occurs indirectly as a consequence of phospholipase A₂ stimulation by GABA_B receptors (Duman *et al.*, 1986; Enna & Karbon, 1987). Moreover, baclofen has been found to enhance agonist binding to β -adrenergic receptors, thus suggesting that the GABA_B receptor potentiation of isoproterenol-stimulated cyclic AMP formation may be partially due to an enhanced coupling of β -adrenergic receptor to the adenylyl cyclase stimulatory G protein G_s (Scherer *et al.*, 1989).

The recent identification of adenylyl cyclases II and IV, two enzyme isoforms which can be stimulated by G protein $\beta\gamma$ subunits synergistically with activated G_s (Tang & Gilman, 1992), has raised the possibility of a novel mechanism by which GABA_B receptors may directly couple to cyclic AMP stimulation. In fact, by interacting with G_i/G_o, GABA_B receptors may promote the release of a sufficiently high amount of $\beta\gamma$ subunits to stimulate type II/IV adenylyl cyclase activities (Tang & Gilman, 1992). We have recently shown that in rat olfactory bulb, a brain area expressing a high level of type II adenylyl cyclase (Feinstein *et al.*, 1991), other G_i/G_o-coupled receptors, such as the acetylcholine muscarinic and opioid receptors, stimulate basal adenylyl cyclase activity and potentiate the responses of G_s-linked neurotransmitter receptors (Olanas & Onali, 1993). In the present study, we report that activation of GABA_B receptors stimulates adenylyl cyclase activity in discrete layers of the rat main olfactory bulb and provide evidence that this positive coupling involves the action of $\beta\gamma$ subunits of G_i/G_o. Part of this work has previously been presented in an abstract form (Olanas & Onali, 1996a).

Methods

Microdissection of olfactory bulb and membrane preparation

Male Sprague-Dawley rats (200–350 g) were killed by decapitation and the olfactory bulbs were rapidly removed and kept on an ice-cold phosphate buffered saline containing 2.5 mM CaCl₂. With the use of a tissue slicer, the bulbs were cut into 300 μ m thick coronal sections, which were kept in the same saline solution. With the aid of a stereoscopic microscope equipped with a diascope illuminator base, each slice was free-hand dissected into three portions of the main olfactory bulb: (1) the olfactory nerve-glomerular layer; (2) the external plexiform layer; (3) the granule cell layer plus the white matter. The tissue layers from individual slices were pooled and homogenized in an ice-cold buffer containing (mM): HEPES-NaOH 10, EGTA 1, dithiothreitol (DTT) 1 and MgCl₂ 1 (pH 7.4) using a teflon-glass tissue grinder. The homogenate was centrifuged at 27,000 $\times$ g for 20 min. The pellet was resuspended in the same buffer at a protein concentration of 0.7–1.0 mg ml⁻¹ and used immediately for the adenylyl cyclase assay.

Adenylyl cyclase assay

The enzyme activity was assayed by monitoring the conversion of [α -³²P]-ATP into [³²P]-cyclic AMP. Unless otherwise indicated, the reaction mixture (final volume = 100 μ l) contained (mM): HEPES-NaOH buffer (pH 7.4) 50, MgCl₂ 2.3, [α -³²P]-ATP (70–90 c.p.m. pmol⁻¹) 0.2, [³H]-cyclic AMP (80 c.p.m. nmol⁻¹) 0.5, GTP 0.1, EGTA 0.3, DTT 1.3, 3-isobutyl-1-methylxanthine 1, phosphocreatine 5, 50 u ml⁻¹ of creatine kinase, 50 μ g of bovine serum albumin (BSA), 10 μ g of bacitracin and 10 kallikrein inhibitor units of aprotinin. The incubation was started by adding the tissue preparation (50–60 μ g of protein) and carried out at 30°C for 10 min. [³²P]-cyclic

AMP was isolated according to Salomon *et al.* (1974). When the effects of the transducin subunits were examined, 10 μ l of the membrane preparation (20–25 μ g of protein) were preincubated with an equal volume of a solution containing either the $\beta\gamma$ subunits, the α subunit or the appropriate vehicle for 60–90 min at ice-bath temperature. Thereafter (–)-baclofen was added (10 μ l) immediately followed by the addition of the reaction mixture (20 μ l). The concentration of transducin subunits reported in the figures refer to the final concentrations in the adenylyl cyclase assay. Assays were performed in duplicate.

Intracerebral injection of pertussis toxin

Pertussis toxin was dissolved in a solution containing 50 mM sodium phosphate buffer and 250 mM NaCl (pH 7.4) and injected into the olfactory bulbs (3.0 μ g for each bulb) as previously described (Olanas & Onali, 1993). Control animals received an equal volume of vehicle containing 3.0 μ g of BSA. At 72 h after the injections, the animals were killed and the membranes were prepared from vehicle- and toxin-treated bulbs. Three tissue preparations were tested.

Protein content was determined by the method of Bradford (1976) using BSA as a standard.

Statistical analysis

Results are given as mean $\pm$ s.e.mean. Concentration-response curves were analysed by a least squares curve-fitting computer program (Graph Pad Prism, San Diego, CA, U.S.A.). Agonist concentrations producing half maximal effects (EC₅₀ values) were converted to the negative logarithmic form (pD₂), as these values are log-normally distributed. Antagonist pA₂ values were calculated from Arunlakshana-Schild regressions (Arunlakshana & Schild, 1959) in which the log of dose ratios (DR)-1 is plotted as a function of the antagonist concentration. The pA₂ values were calculated by using the PHARM/PCS programme of Tallarida & Murray (1987). In experiments examining the effects of a single concentration of antagonist, the inhibition constant (K_i) was calculated according to the equation:

$$EC_{50b} = EC_{50a}(1 + I/K_i)$$

where EC_{50a} and EC_{50b} are the concentrations of the agonist producing half maximal effect in the absence and in the presence of the antagonist, respectively, and I is the concentration of the antagonist. For comparison with pA₂ values, K_i values were converted to the negative logarithmic form (pK_i). Statistically significant differences between concentration-response curves were determined by two-way analysis of variance. Statistical significance of the difference between means was determined by Student's *t*-test.

Materials

[α -³²P]-ATP (30–40 Ci mmol⁻¹), [2,8-³H]-cyclic AMP (25 Ci mmol⁻¹) were obtained from New England Nuclear-Du Pont (Bad Homburg, Germany). (–)-Baclofen, (+)-baclofen, CGP 55845 A (3-N[1-(S)-(3,4-dichlorophenyl)ethyl]amino-2-(S)-hydroxy-propyl-P-benzyl-phosphinic acid) and CGP 35348 (3-aminopropyl)(diethoxymethyl) phosphinic acid) were obtained from Ciba-Geigy, Basel, Switzerland. Phaclofen, 2-hydroxybaclofen, 3-aminopropylphosphinic acid and 3-aminopropylphosphonic acid were purchased from Tocris Cookson Ltd (Langford, Bristol, U.K.). Muscimol, isoguvacine, GABA, nordihydroguaiaretic acid, indomethacin, phorbol 12-myristate, 13-acetate (PMA), quinaquine and

calmodulin (bovine brain) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pituitary adenylate cyclase-activating polypeptide (PACAP) 38 was from Peninsula Laboratories Inc. (Merseyside, U.K.). Forskolin and pertussis toxin were from Calbiochem (La Jolla, CA, U.S.A.) and Research Biochemical Inc. (Natick, MA, U.S.A.), respectively. Staurosporine was from Kyowa Hakko Kogyo Co (Tokyo, Japan). Transducin $\beta\gamma$ subunits ($\beta\gamma_t$) and α subunit in the GDP-bound form (α_{iGDP}), each purified from bovine retina, were kindly provided by Dr Heidi E. Hamm, Northwestern University, Chicago, IL, U.S.A.. Stock solutions of $\beta\gamma_t$ and α_{iGDP} were made in a storing buffer containing 50% glycerol, 75 mM Tris-HCl, 3.75 mM DTT, 75 μ M phenylmethanesulfonyl fluoride (pH 7.3). Dilutions were made in 10 fold diluted storing buffer containing 0.1% BSA immediately before the experiments.

Results

Effects of (–)-baclofen on adenylyl cyclase activity of different layers of main olfactory bulb

As shown in Table 1, the addition of 1 mM (–)-baclofen failed to affect adenylyl cyclase activity of olfactory nerve-glomerular layer. Conversely, in the external plexiform layer and in the granule cell layer, (–)-baclofen stimulated basal adenylyl cyclase activity by 27.7 and 42.6%, respectively. As the stimulatory effect was higher in the granule cell layer, the characterization of the stimulatory effect was performed in tissue samples microdissected from this layer.

Effects of GABA receptor agonists on basal adenylyl cyclase activity

As illustrated in Figure 1A, the stimulatory effect of (–)-baclofen was saturable and concentration-dependent with a pD_2 value of 4.53 ± 0.03 . The GABA_B receptor agonist 3-aminopropylphosphonic acid (3-APA) (Hills *et al.*, 1989) mimicked the stimulatory effect of (–)-baclofen with a pD_2 of 4.60 ± 0.10 . Its maximal stimulation corresponded to approximately 80% of that of (–)-baclofen. Conversely, the less active enantiomer (+)-baclofen (Bowery, 1993) and 3-aminopropylphosphonic acid (3-APPA) were without effects at concentrations up to 1 mM. The stimulation of adenylyl cyclase activity was also observed with GABA ($pD_2 = 3.56 \pm 0.08$), but not with the selective GABA_A receptor agonists muscimol and isoguvacine (Figure 1B).

Effects of GABA_B receptor antagonists

As shown in Figure 2, the adenylyl cyclase stimulation elicited by (–)-baclofen was progressively shifted to the right by increasing concentrations of the GABA_B receptor antagonists CGP 35348 and CGP 55845 A (Bittiger *et al.*, 1993). Schild plot analysis of the antagonism yielded a pA_2 value of 4.31 ± 0.06 and a slope of 0.96 ± 0.03 for CGP 35348 and a pA_2 of 7.0 ± 0.2

and a slope of 0.92 ± 0.4 for CGP 55845 A. 2-Hydroxysaclofen, tested at the concentration of 300 μ M, counteracted the (–)-baclofen stimulation with a pK_i of 4.22 ± 0.10 , whereas phaclofen was inactive at the concentration of 1 mM (results not shown). *Per se*, none of the antagonists tested had effects on basal adenylyl cyclase activity.

Effects of agents affecting phospholipid metabolism

Neither quinacrine (200 μ M), a non selective inhibitor of phospholipase A₂, nor indomethacin (10 μ M) and nordihy-

Figure 1 Effects of GABA receptor agonists on adenylyl cyclase activity in membranes of rat olfactory bulb granule cell layer. Data are the mean, and vertical lines show s.e.mean, of 3–5 experiments. 3-APA, 3-aminopropylphosphonic acid; 3-APPA, 3-aminopropylphosphonic acid.

Table 1 Effects of (–)-baclofen on adenylyl cyclase activity of different layers of rat olfactory bulb

Membrane source	n	Adenylyl cyclase (pmol min ⁻¹ mg protein ⁻¹)	
		Control	(–)-baclofen (1 mM)
Olfactory nerve-glomerular layer	3	50.1 ± 1.8	51.0 ± 1.5
External plexiform layer	5	82.5 ± 2.3	105.4 ± 2.9*
Granule cell layer	5	120.5 ± 3.8	171.9 ± 4.1*

N = number of determinations. Each experiment was performed on a separate tissue preparation. * $P < 0.001$.

Figure 2 Antagonism of (–)-baclofen-induced stimulation of adenylyl cyclase by CGP 35348 and CGP 55845. **A**, The enzyme activity stimulated by (–)-baclofen was assayed in the presence of either vehicle (control) or antagonist at the indicated concentrations. Data are the mean, and vertical lines show s.e.mean, of three experiments for each antagonist.

droguaiaric acid (10 μM), two inhibitors of arachidonic acid metabolism *via* cyclooxygenase and lipoxygenase pathways, respectively, affected the (–)-baclofen stimulation of adenylyl cyclase activity (results not shown). Incubation of membranes with the phorbol ester PMA (1 μM) increased basal adenylyl cyclase activity by $55.5 \pm 3.5\%$ and this effect was completely prevented by the coaddition of the protein kinase inhibitor staurosporine (0.5 μM). However, at the same concentration staurosporine did not affect the stimulatory effect of 1 mM (–)-baclofen (results not shown).

Effects of the intracerebral injection of pertussis toxin

As shown in Figure 3, the intrabulbar injection of pertussis toxin completely prevented the adenylyl cyclase stimulation elicited by 1 mM (–)-baclofen. The toxin treatment, however, failed to affect the enzyme stimulation elicited by the β-adrenergic agonist isoproterenol (10 μM).

Figure 3 Effects of treatment of rat olfactory bulb with pertussis toxin (pt) on the stimulation of adenylyl cyclase activity by either 1 mM (–)-baclofen (bacl) or 10 μM isoproterenol (iso). Data are the mean, and vertical lines show s.e.mean, of three experiments performed on three separate tissue preparations. * $P < 0.001$ vs basal activity; a not significantly different from basal.

Effects of α_{iGDP} and $\beta\gamma_t$

Preincubation of tissue membranes with increasing concentrations of α_{iGDP} , a scavenger of G protein $\beta\gamma$ subunits (Federman *et al.*, 1992), resulted in a progressive reduction of the adenylyl cyclase stimulation by 1 mM (–)-baclofen (Figure 4). At the concentration of 1.5 μM, α_{iGDP} reduced the enzyme stimulation by $75.4 \pm 5.0\%$. Basal adenylyl cyclase activity was slightly reduced by the exposure to α_{iGDP} (~15% reduction at 1.5 μM). On the other hand, incubation of tissue membranes with $\beta\gamma_t$ (0.4 μM) caused a 2 fold stimulation of adenylyl cyclase activity (Figure 5). In membranes pretreated with $\beta\gamma_t$ the addition of 1 mM (–)-baclofen failed to produce a further increase in adenylyl cyclase activity.

Effects of (–)-baclofen on PACAP stimulation of adenylyl cyclase activity

The neuropeptide PACAP 38 (10 nM) increased the adenylyl cyclase activity of olfactory nerve-glomerular layer, external plexiform layer and granule cell layer by 220 ± 7 , 150 ± 5 and $80 \pm 3\%$, respectively ($n = 3$). The addition of (–)-baclofen (1 mM) failed to affect the PACAP-stimulated enzyme activity in the olfactory nerve-glomerular layer, but potentiated it by 22.5 ± 2.0 and $26.0 \pm 3.0\%$ ($P < 0.01$, $n = 3$) in the external plexiform and granule cell layers, respectively. As shown in Figure 6A, in membranes of granule cell layer (–)-baclofen enhanced the maximal stimulation of the enzyme activity by PACAP 38 without significantly changing the potency of the neuropeptide (pD_2 values were: control 9.74 ± 0.09 , (–)-baclofen 9.79 ± 0.10). Moreover, the concurrent stimulation of adenylyl cyclase by PACAP 38 (0.1 nM) resulted in a marked amplification of the stimulatory effect elicited by (–)-baclofen (Figure 6B).

Effects of (–)-baclofen on forskolin- and Ca^{2+} /CaM-stimulated adenylyl cyclase activities

The diterpene forskolin (10 μM) stimulated adenylyl cyclase activity of olfactory nerve-glomerular layer, external plexiform layer and granule cell layer by 17, 7.5 and 3.3 fold, respectively.

Figure 4 Effect of $\alpha_t\text{GDP}$ on $(-)$ -baclofen stimulation of adenylyl cyclase activity in membranes of rat olfactory bulb. Tissue membranes were preincubated with the indicated final concentrations of $\alpha_t\text{GDP}$ for 60 min at ice-bath temperature. Thereafter, adenylyl cyclase activity was assayed in the absence and in the presence of 1 mM $(-)$ -baclofen. Data are the mean, and vertical lines show s.e.mean, of three experiments.

Figure 5 Lack of additivity of the stimulatory effects of $\beta\gamma_t$ and $(-)$ -baclofen on adenylyl cyclase activity of olfactory bulb membranes. Tissue membranes were preincubated for 60 min at ice-bath temperature with either vehicle or $\beta\gamma_t$ at the final concentration of $0.4 \mu\text{M}$. Thereafter, adenylyl cyclase activity was assayed in the absence and in the presence of 1 mM $(-)$ -baclofen. Data are the mean, and vertical lines show the s.e.mean, of three experiments. * $P < 0.001$ vs basal; ^anot significantly different from $\beta\gamma_t$ alone.

$(-)$ -Baclofen (1 mM) inhibited the forskolin-stimulated enzyme activity by $18 \pm 2\%$ ($P < 0.01$), $10.5 \pm 1.2\%$ ($P < 0.05$) and $16.5 \pm 1.5\%$ ($P < 0.01$) in olfactory nerve-glomerular layer, external plexiform layer and granule cell layer, respectively. The addition of $\text{Ca}^{2+}/\text{CaM}$ ($2 \mu\text{M}$ free Ca^{2+} and $2.5 \mu\text{M}$ CaM) enhanced the adenylyl cyclase activity by 290, 250 and 90% in olfactory nerve-glomerular layer, external plexiform layer and granule cell layer, respectively. $(-)$ -Baclofen (1 mM) inhibited the $\text{Ca}^{2+}/\text{CaM}$ -stimulated enzyme activity by $45 \pm 5\%$ in the granule cell layer and by $30 \pm 4\%$ in the other layers

($P < 0.001$). As shown in Figure 7, the $(-)$ -baclofen inhibition of $\text{Ca}^{2+}/\text{CaM}$ -stimulated enzyme activity of the granule cell layer was concentration dependent with a pD_2 of 4.3 ± 0.1 . $(+)$ -Baclofen was completely inactive.

Discussion

The present study shows that baclofen causes stimulation of adenylyl cyclase activity in membranes of specific layers of the main olfactory bulb. The pharmacological profile of this response is consistent with the involvement of GABA_B receptors. Thus, the enzyme stimulation displays a marked stereoselectivity to baclofen, with the $(-)$ -enantiomer being the active and the $(+)$ -enantiomer the inactive form (Bowery, 1993). The GABA_A receptor agonists isoguvacine and muscimol fail to stimulate the enzyme activity, further demonstrating the specificity of the $(-)$ -baclofen effect. Analysis of various functional responses has led to the identification of GABA_B receptors with high- and low-affinity for baclofen (Wojcik & Holopainen, 1992). The potency of $(-)$ -baclofen in stimulating adenylyl cyclase activity is in the micromolar range, suggesting the involvement of low-affinity GABA_B receptors. The different pharmacological activity of two GABA analogues 3-aminopropylphosphonic acid and 3-aminopropylphosphonic acid, the first one stimulating adenylyl cyclase with an efficiency slightly lower than that of $(-)$ -baclofen and the second one being completely inactive, agrees with that observed at GABA_B receptors potentiating the β -adrenoceptors-induced stimulation of cyclic AMP accumulation in rat cortical slices (Scherer *et al.*, 1988; Pratt *et al.*, 1989).

The $(-)$ -baclofen stimulation of adenylyl cyclase activity is antagonized in a concentration-dependent and surmountable manner by the selective GABA_B receptor antagonists CGP 35348 and CGP 55485 A. Both compounds yield Schild plots with slope factors close to unity, indicating a competitive type of inhibition. In terms of absolute values, the antagonist potencies are close to those previously reported to block GABA_B receptors coupled to cyclic AMP regulation in the brain (Knight & Bowery, 1996; Cunningham & Enna, 1996). Also the potency of 2-hydroxybaclofen in antagonizing the $(-)$ -baclofen stimulatory effect ($\text{pK}_i = 4.2$) agrees with the reported affinity for GABA_B receptors (Bowery, 1993). The lack of blockade by a high concentration of phaclofen (1 mM) indicates that the receptors are insensitive to this drug. Phaclofen-insensitive GABA_B receptors coupled to cyclic AMP have previously been described in rat cortical slices (Scherer *et al.*, 1988; Robinson *et al.*, 1989).

The $(-)$ -baclofen stimulation of adenylyl cyclase is not affected by quinacrine, indomethacin and nordihydroguaiaretic acid, indicating that in membranes this effect occurs independently of phospholipase A₂ activation and arachidonic acid metabolism. The protein kinase C stimulator PMA causes a significant stimulation of adenylyl cyclase activity and this effect is completely blocked by the protein kinase inhibitor staurosporine, indicating that a protein kinase C-catalyzed phosphorylation can affect cyclic AMP formation. However, staurosporine has no effect on the $(-)$ -baclofen stimulatory effect, ruling out the involvement of protein kinase C.

In vivo treatment with pertussis toxin completely prevents the $(-)$ -baclofen stimulation of adenylyl cyclase activity. This finding correlates with the previous observation that pertussis toxin prevents not only the inhibitory (Xu & Wojcik, 1986) but also the facilitatory effects of GABA_B receptors on cyclic AMP formation (Wojcik *et al.*, 1989). The pertussis toxin sensitivity of the GABA_B receptor-mediated stimulation suggests the

Figure 6 (A) Concentration-dependent stimulation of adenylyl cyclase activity of granule cell layer by PACAP 38 in the absence (control) and in the presence of 1 mM (–)-baclofen. Data are the mean, and vertical lines show the s.e.mean, of three experiments and represent the net stimulation of enzyme activity (reported as pmol cyclic AMP min⁻¹ mg⁻¹ protein) elicited by PACAP above control values. Control values were: basal 115 ± 3 , (–)-baclofen (1 mM) 160 ± 4 pmol cyclic AMP min⁻¹ mg⁻¹ protein. $P < 0.01$ for the difference between the curves by analysis of variance (B): Concentration-dependent stimulation of adenylyl cyclase activity by (–)-baclofen in the absence (control) and in the presence of 0.1 nM PACAP. Data are the mean, and vertical lines show the s.e.mean, of three experiments and represent the net stimulation of enzyme activity (reported as pmol cyclic AMP min⁻¹ mg⁻¹ protein) elicited by (–)-baclofen above control values. Control values were: basal 112 ± 2 , 0.1 nM PACAP 175 ± 4 pmol cyclic AMP min⁻¹ mg⁻¹ protein. $P < 0.01$ for the difference between the curves by analysis of variance.

possibility that this response occurs through the release of $\beta\gamma$ subunits from G_i/G_o and the activation of type II/IV adenylyl cyclase isoforms (Tang & Gilman, 1992). We found that the (–)-baclofen stimulation of adenylyl cyclase is markedly reduced by the addition of α_{iGDP} , a $\beta\gamma$ scavenger (Federman

Figure 7 Concentration-dependent inhibition of Ca²⁺/CaM-stimulated adenylyl cyclase activity of granule cell layer by (–)-baclofen and lack of effect by (+)-baclofen. Data are the mean, and vertical lines show the s.e.mean, of three experiments and represent the net stimulation of enzyme activity (reported as pmol cyclic AMP min⁻¹ mg⁻¹ protein) elicited by the addition of 2.0 μ M free Ca²⁺ and 2.5 μ M CaM. The concentration of free Ca²⁺ was obtained with an EGTA/Ca²⁺ buffer and was calculated with a computer program (EQCAL, Biosoft, U.K.).

et al., 1992). Moreover, exogenously added $\beta\gamma$ subunits of transducin increase the enzyme activity and this effect is not additive with that produced by (–)-baclofen. Because the $\beta\gamma$ -induced stimulation of type II/IV adenylyl cyclases is amplified when the enzymes are concomitantly activated by G_s (Tang & Gilman, 1992), we investigated whether GABA_B receptors could enhance the cyclic AMP formation elicited by G_s -coupled neurotransmitter receptors. We found that (–)-baclofen significantly potentiates the stimulation of adenylyl cyclase elicited by PACAP 38, a neurotransmitter that acts through G_s -linked receptors (Olanas & Onali, 1996b). The concurrent activation of PACAP receptors also results in a marked amplification of the (–)-baclofen stimulatory effect, as expected in the case of a synergistic interaction between $\beta\gamma$ subunits and G_s . Taken together, these observations indicate that the positive coupling of GABA_B receptors to cyclic AMP is mediated through $\beta\gamma$ subunits which then activate type II/IV adenylyl cyclases, two enzyme isoforms expressed in the olfactory bulb (Feinstein *et al.*, 1991; Olanas *et al.*, 1998).

As GABA_B receptors have been found to inhibit forskolin-stimulated cyclic AMP formation in the brain (Hill & Dolphin, 1984; Karbon & Enna, 1985), it was important to see whether (–)-baclofen could elicit the same response in the olfactory bulb. It was found that (–)-baclofen inhibits the forskolin-stimulated adenylyl cyclase activity in each layer examined. However, the extent of the maximal inhibition is modest (10–18%), possibly because forskolin stimulates all isoforms of adenylyl cyclase (Tang & Gilman, 1992), including types II and IV which may be stimulated by (–)-baclofen. We therefore examined the effect of (–)-baclofen on the enzyme activity stimulated by Ca²⁺/CaM, which activates cyclase isoforms I, III and VIII but is inactive on types II and IV (Sunahara *et al.*, 1996). Previous studies have shown that types I and III are expressed in rat olfactory bulb (Xia *et al.*, 1991; Glatt & Snyder, 1993). The Ca²⁺/CaM-stimulated enzyme activity is inhibited by (–)-baclofen to a greater extent than the forskolin-stimulated activity in each microdissected tissue layer examined. The inhibition occurs at the same agonist

concentrations required to cause the stimulatory effect and is stereospecific. Thus, as previously observed in brain slices (Hill & Dolphin, 1984; Karbon & Enna, 1985), GABA_B receptors can either stimulate or inhibit adenylyl cyclase. This dual control may provide a mechanism by which GABA_B receptors can enhance the signalling of G_s-linked neurotransmitter receptors regulating Ca²⁺-insensitive adenylyl cyclases and inhibit the Ca²⁺/CaM-dependent cyclic AMP formation induced by Ca²⁺-mobilizing neurotransmitters. Interestingly, whereas the inhibitory effect displays a wide distribution in the olfactory bulb, the enhancement of basal and G_s-stimulated adenylyl cyclase activities is only observed in the external plexiform and granule cell layers. Previous studies have demonstrated that in rat olfactory bulb GABA_B receptors are more concentrated in the glomerular layer than in external plexiform and granule cell layers (Bowery *et al.*, 1987; Chu *et al.*, 1990). Thus, the distribution of the positive coupling to cyclase is not a function of the receptor density but likely

depends on the colocalization at specific synaptic sites of GABA_B receptors, G_i/G_o, G_s-linked receptors and type II/IV adenylyl cyclases. While this study was in progress, Uezono *et al.* (1997) reported that in *Xenopus* oocytes expressing rat brain cortex poly (A)⁺ RNA and cRNA for type II adenylyl cyclase baclofen induced a $\beta\gamma$ -mediated stimulation of cyclic AMP production. To our knowledge, the present study provides the first evidence that in brain membranes, where receptors, G proteins and effector molecules are expressed in native conditions, activation of GABA_B receptors stimulate adenylyl cyclase activity through $\beta\gamma$ subunits.

We are grateful to Dr H.E. Hamm, Northwestern University, Chicago, U.S.A. for the gift of transducin subunits and to Dr W. Froestl, CIBA-GEIGY, Basel, Switzerland, for providing us with CGP 35348 and CGP 55845 A.

References

- ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–57.
- BITTIGER, H., FROESTL, W., MICKEL, S.J. & OLPE, H.-R. (1993). GABA_B receptor antagonists: from synthesis to therapeutic application. *Trends Pharmacol. Sci.*, **14**, 391–393.
- BOWERY, N.G. (1993). GABA_B receptor pharmacology. *Annu. Rev. Pharmacol. Toxicol.*, **33**, 109–147.
- BOWERY, N.G., HUDSON, A.L. & PRICE, G.W. (1987). GABA_A and GABA_B receptor site distribution in the rat central nervous system. *Neuroscience*, **20**, 365–383.
- BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
- CHU, D.C.M., ALBIN, R.L., YOUNG, A.B. & PENNEY, J.B. (1990). Distribution and kinetics of GABA_B binding sites in rat central nervous system: a quantitative autoradiographic study. *Neuroscience*, **34**, 341–357.
- CUNNINGHAM, M.D. & ENNA, S.J. (1996). Evidence for pharmacologically distinct GABA_B receptors associated with cAMP production in rat brain. *Brain Res.*, **720**, 220–224.
- DUMAN, R.S., KARBON, E.W., HARRINGTON, C. & ENNA, S.J. (1986). An examination of the involvement of phospholipases A₂ and C in the α -adrenergic and γ -aminobutyric acid receptor modulation of cyclic AMP accumulation in rat brain slices. *J. Neurochem.*, **47**, 800–810.
- ENNA, S.J. & KARBON, E.W. (1984). GABA_B receptors and transmitter-stimulated cAMP accumulation in rat brain. *Neuropharmacology*, **23**, 821–822.
- ENNA, S.J. & KARBON, E.W. (1987). Receptor regulation: evidence for a relationship between phospholipid metabolism and neurotransmitter receptor-mediated cAMP formation in brain. *Trends Pharmacol. Sci.*, **8**, 21–24.
- FEDERMAN, A.D., CONKLIN, B.R., SCHRADER, K.A., RANDALL, R.R. & BOURNE, H.R. (1992). Hormonal stimulation of adenylyl cyclase through G_i protein $\beta\gamma$ subunits. *Nature*, **356**, 159–161.
- FEINSTEIN, P.G., SCHRADER, K.A., BAKALAYAR, H.A., TANG, W.-J., KRUPINSKI, J., GILMAN, A.G. & REED, R.R. (1991). Molecular cloning and characterization of a Ca²⁺/calmodulin-insensitive adenylyl cyclase from rat brain. *Proc. Natl. Acad. Sci. USA*, **88**, 10173–10177.
- GLATT, C.E. & SNYDER, S.H. (1993). Cloning and expression of an adenylyl cyclase localized to the corpus striatum. *Nature*, **361**, 536–538.
- HILL, D.R. & DOLPHIN, A.C. (1984). Modulation of adenylate cyclase activity by GABA_B receptors. *Neuropharmacology*, **23**, 829–830.
- HILLS, J.M., DINGS DALE, R.A., PARSONS, M.E., DOLLE, R.E. & HOWSON, W. (1989). 3-Aminopropylphosphinic acid—a potent, selective GABA_B receptor agonist in the guinea-pig ileum and rat anococcygeus muscle. *Br. J. Pharmacol.*, **97**, 1292–1296.
- KARBON, E.W. & ENNA, S.J. (1985). Characterization of the relationship between γ -aminobutyric acid B agonists and transmitter-coupled cyclic nucleotide-generating systems in rat brain. *Mol. Pharmacol.*, **27**, 53–59.
- KAUPMANN, K., HUGGEL, K., HEID, J., FLOR, P.J., BISCHOFF, S., MICKEL, S.J., MCMASTER, G., ANGST, C., BITTIGER, H., FROESTL, W. & BETTLER, B. (1997). Expression cloning of GABA_B receptors uncovers similarity to metabotropic glutamate receptors. *Nature*, **386**, 239–246.
- KNIGHT, A.R. & BOWERY, N.G. (1996). The pharmacology of adenylyl cyclase modulation by GABA_B receptors in rat brain slices. *Neuropharmacology*, **35**, 703–712.
- MALCANGIO, M. & BOWERY, N.G. (1995). Possible therapeutic application of GABA_B receptor agonists and antagonists. *Clin. Neuropharm.*, **18**, 285–305.
- MORISHITA, R., KATO, K. & ASANO, T. (1990). GABA_B receptors couple to G proteins G₀, G₀* and G₁₁ but not to G₁₂. *FEBS Lett.*, **271**, 231–235.
- OLIANAS, M.C. & ONALI, P. (1993). Synergistic interaction of muscarinic and opioid receptors with G_s-linked neurotransmitter receptors to stimulate adenylyl cyclase activity of rat olfactory bulb. *J. Neurochem.*, **61**, 2183–2190.
- OLIANAS, M.C. & ONALI, P. (1996a). Positive coupling of GABA_B receptors to adenylyl cyclase in rat olfactory bulb. *Proc. Soc. Neurosci.*, **22**, 1293.
- OLIANAS, M.C. & ONALI, P. (1996b). Characterization of the G protein involved in the muscarinic stimulation of adenylyl cyclase of rat olfactory bulb. *Mol. Pharmacol.*, **49**, 22–29.
- OLIANAS, M.C., INGIANNI, A. & ONALI, P. (1998). Role of G protein $\beta\gamma$ subunits in muscarinic receptor-induced stimulation and inhibition of adenylyl cyclase activity in rat olfactory bulb. *J. Neurochem.*, **70**, 2620–2627.
- PRATT, G.D., KNOTT, C., DAVEY, R. & BOWERY, N.G. (1989). Characterization of 3-aminopropyl phosphinic acid (3-APPA) as a GABA_B agonist in rat brain tissue. *Br. J. Pharmacol.*, **96**, 141P.
- ROBINSON, T.N., CROSS, A.J., GREEN, A.R., TOCZEK, J.M. & BOAR, B.R. (1989). Effects of the putative antagonists phaclofen and δ -aminovaleric acid on GABA_B receptor biochemistry. *Br. J. Pharmacol.*, **98**, 833–840.
- SALOMON, Y., LONDOS, C. & RODBELL, M. (1974). A highly sensitive adenylate cyclase assay. *Anal. Biochem.*, **58**, 541–548.
- SCHAAD, N.C., SCHORDERET, M. & MAGISTRETTI, P.J. (1989). Accumulation of cyclic AMP elicited by vasoactive intestinal peptide is potentiated by noradrenaline, histamine, adenosine, baclofen, phorbol esters, and ouabain in mouse cerebral cortical slices: studies on the role of arachidonic acid metabolites and protein kinase C. *J. Neurochem.*, **53**, 1941–1951.
- SCHERER, R.W., FERKANY, J.W. & ENNA, S.J. (1988). Evidence for pharmacologically distinct subsets of GABA_B receptors. *Brain Res. Bull.*, **21**, 439–443.

- SCHERER, R.W., FERKANY, J.W., KARBON, E.W. & ENNA, S.J. (1989). γ -Aminobutyric acid_B receptor activation modifies agonist binding to β -adrenergic receptors in rat brain cerebral cortex. *J. Neurochem.*, **53**, 989–991.
- SUNAHARA, R.K., DESSAUER, C.W. & GILMAN, A.G. (1996). Complexity and diversity of mammalian adenylyl cyclases. *Annu. Rev. Pharmacol. Toxicol.*, **36**, 461–480.
- TALLARIDA, R.J. & MURRAY, R.B. (1987). *Manual of Pharmacologic Calculations with Computer Programs*. Springer-Verlag, New York.
- TANG, W.-J. & GILMAN, A.G. (1992). Adenylyl Cyclases. *Cell*, **70**, 869–872.
- UEZONO, Y., UEDA, Y., UENO, S., SHIBUYA, I., YANAGIHARA, N., TOYOHARA, Y., YAMASHITA, H. & IZUMI, F. (1997). Enhancement by baclofen of the G_s-coupled receptor-mediated cAMP production in *Xenopus* oocytes expressing rat brain cortex poly(A)⁺ RNA: a role of G-protein $\beta\gamma$ subunits. *Biochem. Biophys. Res. Comm.*, **241**, 476–480.
- WATLING, K.J. & BRISTOW, D.R. (1986). GABA_B receptor-mediated enhancement of vasoactive intestinal peptide-stimulated cyclic AMP production in slices of rat cerebral cortex. *J. Neurochem.*, **46**, 1756–1762.
- WOJCIK, W.J. & HOLOPAINEN, I. (1992). Role of central GABA_B receptors in physiology and pathology. *Neuropsychopharmacology*, **6**, 201–214.
- WOJCIK, W.J. & NEFF, N.H. (1984). γ -Aminobutyric acid B receptors are negatively coupled to adenylate cyclase in brain, and in the cerebellum these receptors may be associated with granule cells. *Mol. Pharmacol.*, **25**, 24–28.
- WOJCIK, W.J., ULIVI, M., PAEZ, X. & COSTA, E. (1989). Islet-activating protein inhibits the β -adrenergic receptor facilitation elicited by γ -aminobutyric acid_B receptors. *J. Neurochem.*, **53**, 753–758.
- XIA, Z., REFSDAL, C.D., MERCHANT, K.M., DORSA, D.M. & STORM, D.R. (1991). Distribution of mRNA for the calmodulin-sensitive adenylyl cyclase in rat brain: expression in areas associated with learning and memory. *Neuron*, **6**, 431–443.
- XU, J. & WOJCIK, W.J. (1986). γ -Aminobutyric acid_B receptor-mediated inhibition of adenylate cyclase in cultured cerebellar granule cells: blockade by islet activating protein. *J. Pharmacol. Exp. Ther.*, **239**, 568–573.

(Received June 22, 1998

Revised October 30, 1998

Accepted November 9, 1998)