British Journal of Pharmacology (1996) 119, 223-228 

# PK 11195 blockade of benzodiazepine-induced inhibition of forskolin-stimulated adenylate cyclase activity in the striatum

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**1** The effects of benzodiazepine receptor antagonists on the inhibition of forskolin-stimulated adenylate cyclase (AC) activity by various benzodiazepine (BZ) and indoleamine agonists in the rat striatum were investigated.

**2** A biphasic inhibition of forskolin-stimulated AC activity by the peripheral-type agonist, Ro5-4864, and a multiphasic inhibition by the non-selective BZ, diazepam, was observed. One phase of AC inhibition is consistent with a $G_i$-coupled receptor-mediated action, whereas the other phases appear to involve a direct effect on the enzyme itself.

**3** While the central-type antagonist, flumazenil, had no effect on the ability of Ro5-4864 to inhibit AC activity, the peripheral-type receptor ligand, PK 11195, abolished the first phase of inhibition.

**4** PK 11195 and pertussis toxin were found to block the inhibitory effect of various BZs and the indoleamines, melatonin and 2-iodomelatonin, on induced AC activity.

**5** Saturation binding studies, conducted at 30°C with [$^3$H]-diazepam revealed a single binding site in the rat striatum ($K_D = 19.3 \pm 0.80$ nM) which significantly decreased in affinity in the presence of GTP ($K_D = 30.5 \pm 2.6$ nM; $P < 0.05$). No significant change in $B_{max}$ was observed.

**6** These findings indicate the presence of $G_i$-coupled BZ receptors in the rat striatum. Thus, suppression of cyclic AMP production may contribute to the diverse neuropharmacological effects of BZs, melatonin and related drugs.

**Keywords:** Ro5-4864; diazepam; melatonin; 2-iodomelatonin; PK 11195; benzodiazepine; adenylate cyclase; cyclic AMP; $G_i$ protein; pertussis toxin

## Introduction

Benzodiazepines are effective anticonvulsant, anxiolytic and hypnotic drugs. It has been suggested that their therapeutic effects are mediated by specific central benzodiazepine (BZ) receptors which are coupled to $\gamma$-aminobutyric acid$_A$ ($GABA_A$) receptors (Tallman *et al.*, 1980; Olsen & Tobin, 1990). In addition to these central-type BZ receptors, high densities of BZ binding sites have been found in a variety of peripheral tissues (Braestrup & Nielsen, 1983; Parola *et al.*, 1993). These peripheral binding sites differ from the central-type BZ receptor in that they are not coupled to $GABA_A$ receptors and have different pharmacological and molecular characteristics. For example, clonazepam has a high affinity for central-type receptors but is very weak at peripheral sites. By contrast, Ro5-4864 has a high affinity for peripheral-type binding sites but very low affinity for central-type receptors. The peripheral-type BZ receptor (PBR) is present in not only peripheral tissues, such as the kidney, but also the central nervous system (Braestrup & Squires, 1977; Marangos *et al.*, 1982). Recent cloning of a PBR component responsible for the receptor binding properties, showed no apparent sequence homology with any of the cloned $GABA_A$ receptor subunits (Parola *et al.*, 1993). Thus, the peripheral binding sites are distinctly different from the central-type BZ receptors.

Recent studies have suggested the presence of another BZ receptor which is coupled to an inhibitory guanine nucleotide binding protein ($G_i$) in the central nervous system (CNS) (Dan'ura, 1988; Niles & Hashemi, 1990). Dan'ura *et al.* (1988) found that micromolar concentrations of diazepam could inhibit adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] (AC) activity in rat whole brain and this effect could be inhibited by pertussis toxin. Furthermore, Ro15-1788 (flumazenil), a central-type BZ receptor antagonist, had no effect on AC activity or on its inhibition by diazepam (Dan'ura *et al.*, 1988). This finding was later confirmed by Niles & Hashemi (1990) who also found that flumazenil failed to block the inhibition of forskolin-stimulated AC activity by Ro5-4864 and pharmacological concentrations of the indoleamine hormone, melatonin, in the rat striatum. Moreover, it was suggested that micromolar concentrations of melatonin and related indoleamines could inhibit AC activity via $G_i$ - coupled BZ receptors, which are quite distinct from the physiological high-affinity receptors for melatonin (Niles & Hashemi, 1990). Since Ro5-4864, an agonist at peripheral-type BZ receptors, was found to inhibit forskolin-stimulated AC activity in rat brain (Niles & Hashemi, 1990), we investigated the effects of PK 11195, a potential antagonist at these receptors, on this inhibitory effect of various BZs and indoleamines.

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## Methods

### *Membrane preparation*

Male Sprague-Dawley rats weighing 200-275 g were housed 2 per cage with free access to food and water. Animals were maintained under a 12 h light: 12 h dark lighting regimen for at least one week before use. For adenylate cyclase assays, animals were killed by decapitation approximately 3 h after lights on. Striatal tissue was dissected from rat brains on ice and hand homogenized in cold 50 mM HEPES/NaOH buffer containing 5 mM $MgCl_2$, 1 mM dithiothreitol and 0.2 mM EGTA (pH 7.4 at 30°C). The homogenate was centrifuged at 39,000 *g* for 10 min and membranes were subsequently washed twice by resuspension and centrifugation in the above buffer. Finally, membranes were resuspended in 50 mM HEPES/NaOH (pH 7.4 at 30°C) buffer for assays.

For binding assays, the striatum was rapidly dissected out on ice. Fresh striatal tissue was homogenized in 50 volumes of 50 mM Tris/HCl (pH 7.4 at 4°C) for 5 s by a polytron homogenizer. The homogenate was centrifuged at 39,000 *g* for

15 min at 4°C and the pellet was washed twice in the same buffer. Membranes were then resuspended in 50 mM Tris/HCl (pH 7.4 at 30°C) at a concentration of approximately 5 mg $ml^{-1}$ (wet weight/volume) for binding assays.

### *Adenylate cyclase assays*

Assays were performed as we have previously described (Niles & Hashemi, 1990) with slight modifications. Striatal membrane aliquots containing about 80–100 μg protein were preincubated with various drugs at 30°C for 30 min. Following preincubation, a reaction mixture containing 4 mM $MgCl_2$, 100 μM GTP, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM adenosine 3′:5′-cyclic monophosphate (cyclic AMP), 1 mg $ml^{-1}$ BSA, 1 mM rolipram, 20 mM creatine phosphate and 20 units $ml^{-1}$ of creatine phosphokinase was added to a final volume of 150 μl. Forskolin (50 μM) or dopamine (100 μM) was added to all tubes except basal and background samples and 0.5 mM ATP containing ~1 μCi [$\alpha^{32}$P]-ATP was then added to all tubes and the incubation carried out at 30°C for 10 min. The reaction was stopped by adding 4 mM cyclic AMP and boiling for 5 min. Samples were centrifuged at 10,000 *g* for 10 min in a refrigerated Mikro Rapid centrifuge. [$^3$H]-cyclic AMP was added to monitor recovery and the [$^{32}$P]-cyclic AMP formed was separated from the supernatant by sequential chromatography on Dowex and alumina columns as described by Salomon *et al.* (1974).

### *Pertussis toxin treatment*

Pertussis toxin experiments were carried out as we have previously described (Niles *et al.*, 1991) with some modifications. Fresh rat striatal tissue was placed into culture dishes containing 2 ml of α-minimum essential medium in 10 mM HEPES (pH 7.4 at 37°C) supplemented with 1% foetal bovine serum. Each striatum was manually cut into 6 sections and immersed in the medium which was saturated with 95% $O_2$/5% $CO_2$. Pertussis toxin was reconstituted in 1 ml of 1 mg $ml^{-1}$ of BSA in deionized water and then added at a concentration of 8.5 μg $ml^{-1}$ to one culture dish while an equivalent volume of the reconstitution buffer was added to another dish. The dishes were then incubated at 37°C for 15 ½ h under 95% $O_2$/5% $CO_2$. After incubation, the culture dishes containing tissue slices and medium were frozen at −20 °C until used for adenylate cyclase (AC) assays. For assays, culture dishes were thawed at room temperature and tissue and medium transferred to centrifuge tubes, hand homogenized in 50 mM HEPES/NaOH buffer containing 5 mM $MgCl_2$, 1 mM dithiothreitol and 0.2 mM EGTA (pH 7.4 at 30°C). The homogenate was centrifuged at 39,000 *g* for 10 min. Tissue pellets were washed twice by resuspension and centrifugation and then resuspended in 50 mM HEPES/NaOH (pH 7.4 at 30°C) buffer for AC assays as described above.

### *Receptor binding assays*

For saturation binding assays, tissue preparations were incubated at 30°C for 60 min with varying concentrations of [$^3$H]-diazepam (0.25–83 nM) with or without 1 mM GTP. Non-specific binding was determined in the presence of 10 μM diazepam. After incubation, samples were filtered and washed with 4 × 3 ml ice-cold 50 mM Tris/HCl (pH 7.4 at 4°C). Samples were then counted in a Beckman LS 6500 liquid scintillation counter (efficiency about 70%). Protein concentrations were determined by the method of Lowry *et al.* (1951).

### *Chemicals*

The following drugs were used: adenosine 5′[α-$^{32}$P]-triphosphate triethylammonium salt (3,000 Ci $mmol^{-1}$), and [$^3$H]-diazepam (85 Ci $mmol^{-1}$) (Amersham, Oakville, Canada); [2,8-$^3$H]-adenosine 3′,5′-cyclic monophosphate (ICN Radiochemicals, Montreal, Canada); 2-iodomelatonin and Ro5-4864 (7-chloro-5-(4-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one; Research Biochemicals Inc, Natick, U.S.A.); PK 11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide; a gift from Rhône Poulenc Santé, Vitry sur Seine, France); flumazenil (a gift from Hoffmann-La Roche Ltd Basle, Switzerland); rolipram (a gift from Schering AG, Berlin); melatonin and all other chemicals (Sigma, St. Louis, MO, U.S.A.).

### *Data analysis*

Drug $EC_{50}$ values in AC assays were calculated by non-linear regression analysis by use of the computer program CDATA (EMF software). The $EC_{50}$ values from at least three separate experiments are expressed as means with 95% confidence limits. Statistical analyses of enzyme data were performed by one-way analysis (ANOVA) and Scheffe's test. Saturation binding data were analysed by non-linear regression by use of BDATA program (EMF software) to determined the equilibration dissociation constant ($K_D$) and the maximum number of binding sites ($B_{max}$).

## Results

### *Effect of diazepam and Ro5-4864 on forskolin-stimulated AC activity*

Functional studies with Ro5-4864 and diazepam ($10^{-11}$–$10^{-3}$ M) revealed a concentration-dependent inhibition of forskolin-stimulated AC activity as shown in Figure 1. An F-test indicated a significantly better fit to two-site and three-site models for Ro5-4864 and diazepam, respectively ($P<0.05$). For the first phase of inhibition by Ro5-4864, this drug had an $EC_{50}$ of 1.36 nM (0.64–2.08). The second phase of inhibition by Ro5-4864 was steep with an $EC_{50}$ of 131 μM (87.9–174.0). Diazepam, which was more potent than Ro5-4864, inhibited forskolin-stimulated AC activity with an $EC_{50}$ of 0.43 nM (0.33–0.53) for the first phase and 1.0 μM (0.68–1.32) and 194.0 μM (157.0–231.1) for the other phases.

In other studies, diazepam was also found to inhibit dopamine-stimulated AC activity in the rat striatum. However, it was more potent against forskolin-stimulated AC, presumably due to the greater stimulation produced by forskolin (Table 1).

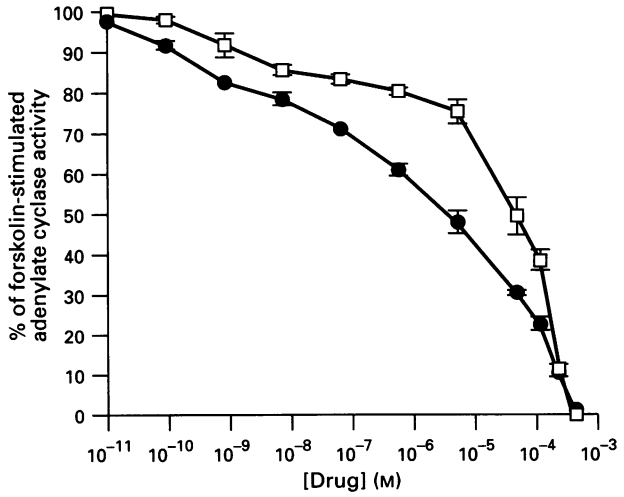


**Figure 1** The effects of diazepam and Ro5-8464 on forskolin-stimulated adenylate cyclase (AC) activity in rat striatum. Membranes were preincubated with the indicated drug concentrations at 30°C for 30 min; (●) diazepam; (□) Ro5-4864, AC assays were performed as described in Methods. Results are presented as a percentage of forskolin-stimulated AC activity. Data are the means of 3–4 experiments carried out in duplicate; vertical lines show s.e.mean.

**Table 1** Effect of diazepam on dopamine- or forskolin-stimulated adenylate cyclase activity in the rat striatum

| Drug | *Adenylate cyclase activity* (pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$) *Dopamine* | *% inhibition* | *forskolin* | *% inhibition* |
|---|---|---|---|---|
| none | 562±46.9 | | 1474±160 | |
| Diazepam (10 μM) | 392±36.1* | 30.2 | 746±79.2* | 49 |
| Diazepam (100 μM) | 264±21.1** | 53.0 | 467±66.4** | 68 |

Fresh striatal membranes were preincubated with diazepam at 30°C for 30 min. Dopamine (100 μM) or forskolin (50 μM) was added and following incubation at 30°C for 10 min, enzyme activity was measured. Basal enzyme activity was 282±18.9 pmol cyclic AMP $mg^{-1}$ $min^{-1}$. Mean±s.e.mean are presented for $n=3$. *$P<0.05$; **$P<0.005$.

### *Effect of flumazenil on Ro5-4864 inhibition of AC activity*

Previously, single point assays have shown that the central-type BZ receptor antagonist, flumazenil, failed to block the inhibitory effect of diazepam and Ro5-4864 on forskolin-stimulated AC activity (Niles & Hashemi, 1990). In the present study, a detailed examination of the effects of flumazenil, in dose-response studies, indicated that this drug, enhanced the effect of Ro5-4864 at lower doses ($10^{-11}$–$10^{-10}$ M, $P<0.001$ vs Ro5-4864 in the absence of flumazenil) (Figure 2). No difference was observed for basal AC activity in the absence or presence of flumazenil (376.3±14.2 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$ and 369.3±11.3 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$, respectively). There was also no difference in forskolin-stimulation of AC activity in the absence or presence of flumazenil (2213±67 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$ and 2193±94 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$, respectively). Thus, the enhanced effect of Ro5-4864 in the presence of flumazenil is not due to an additive effect between these compounds.

### *Effect of PK 11195 on BZ and indoleamine inhibition of AC activity*

There was no difference in basal AC activity in the absence or presence of PK 11195 (265.7±11.1 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$ and 270.3±7.2 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$, respectively). In addition, PK 11195 did not affect forskolin-stimulated AC activity (1965±253 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$ and 1966±227 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$, in the absence or presence of PK 11195, respectively). Preliminary experiments indicated that in the presence of 1 μM, 5 μM or 10 μM of PK 11195, the inhibitory effect of Ro5-4864 on AC activity was blocked. For example, in control incubates, Ro5-4864 (10 μM) suppressed stimulated AC activity by 26±2.2% as compared with 11±2.8%, 4±1.0% and 6±1.0%, in the presence of 1 μM, 5 μM and 10 μM of PK 11195, respectively. On the basis of these findings, 5 μM PK 11195 was used in subsequent dose-response experiments, which indicated that the first phase of Ro5-4864-induced inhibition of AC activity was blocked with no significant effect on the second phase of inhibition (Figure 2).

PK 11195 also blocked the inhibitory effect of diazepam and flunitrazepam as well as that of the indoleamines, melatonin and its iodinated analog, 2-iodomelatonin, on forskolin-stimulated AC activity in the striatum (Figure 3). Diazepam was found to be the most potent of the BZs examined, in inhibiting forskolin-stimulated AC activity, while 2-iodomelatonin was significantly more potent than melatonin (Figure 3).

### *Effect of pertussis toxin on BZ and indoleamine inhibition of AC activity*

Pretreatment of striatal tissues with pertussis toxin did not affect basal or forskolin-stimulated AC activity. Control values were: basal: 117.6±5.7 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$, forskolin: 458±121 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$.

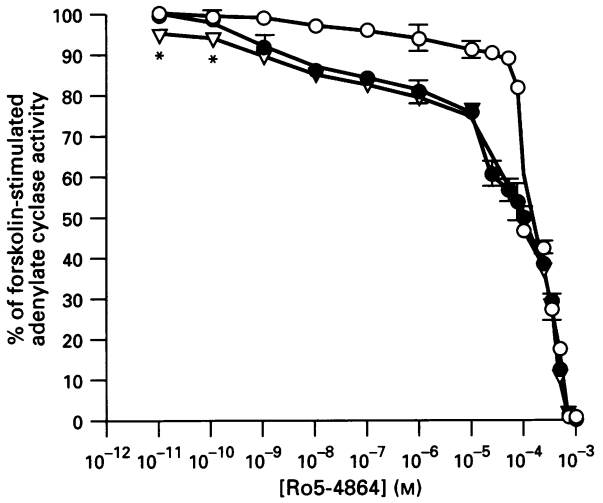


**Figure 2** The effects of benzodiazepine (BZ) antagonists on Ro5-4864-induced inhibition of adenylate cyclase (AC) activity. Striatal membranes were preincubated with the indicated concentrations of Ro5-4864, in the absence (●) or presence (○) of 5 μM of PK 11195 or 1 μM of Ro15-1788 (▽). AC assays were performed as described in Methods and results are presented as described in Figure 1. Data are means of 3–4 experiments carried out in duplicate; vertical lines show s.e.mean; *$P<0.001$ vs control.

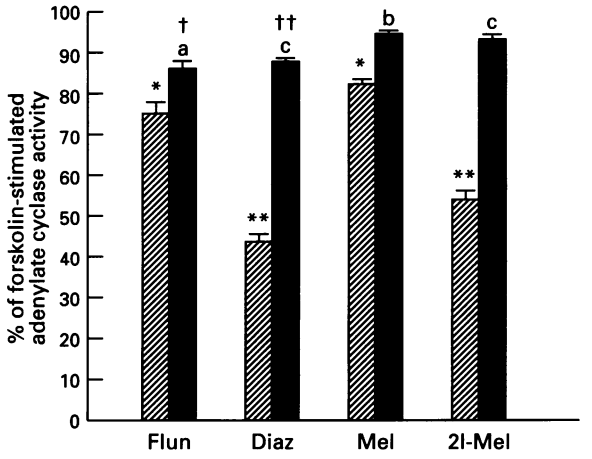


**Figure 3** Effect of PK 11195 on the inhibitory action of benzodiazepines (BZs) and indoleamines on stimulated adenylate cyclase (AC) activity. Striatal membranes were preincubated with the indicated drug concentrations, in the absence or presence of PK 11195 (5 μM). Flun, flunitrazepam (500 μM); Diaz, diazepam (10 μM); Mel, melatonin (500 μM); 2I-Mel, 2-iodomelatonin (500 μM). Hatched columns, drug alone; solid columns, +PK 11195. AC assays were performed as described in Methods. Data are means±s.e.mean (vertical lines) of 3–4 experiments carried out in triplicate. *$P<0.01$ and **$P<0.001$ vs control. $^{a}P<0.05$, $^{b}P<0.001$ and $^{c}P<0.0001$ vs drug in the absence of PK 11195, †$P<0.05$ and ††$P<0.01$ vs PK 11195 control.

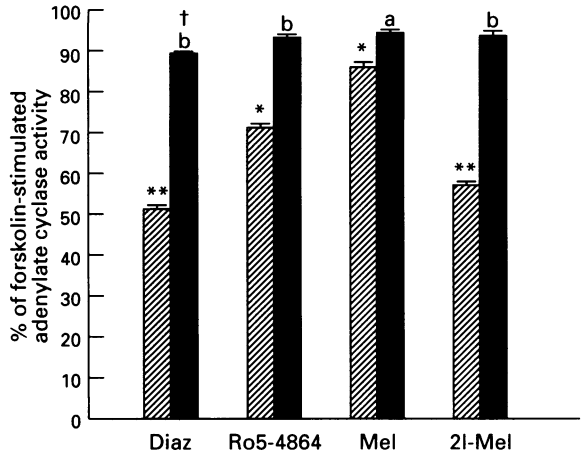


**Figure 4** Effect of pertussis toxin on the inhibitory action of benzodiazepines (BZs) and indoleamines on stimulated adenylate cyclase (AC) activity. Striatal slices were pretreated with pertussis toxin (8.5 $\mu g\,ml^{-1}$) for 15½ h as described in Methods. Subsequently, membranes were prepared and preincubated with Diaz, diazepam (10 $\mu M$); Ro5-4864 (50 $\mu M$); Mel, melatonin (500 $\mu M$) and 2I-Mel, 2-iodomelatonin (500 $\mu M$). Hatched columns, effect of drug alone; solid columns, + pertussis toxin. AC assays were performed as described in Methods. Data are means ± s.e.mean (vertical lines) of 3 experiments carried out in either duplicate or triplicate. $^{*}P<0.01$ and $^{**}P<0.001$ vs control. $^{a}P<0.005$ and $^{b}P<0.001$ vs drug in the absence of pertussis toxin treatment. $\dagger P<0.05$ vs pertussis toxin control.

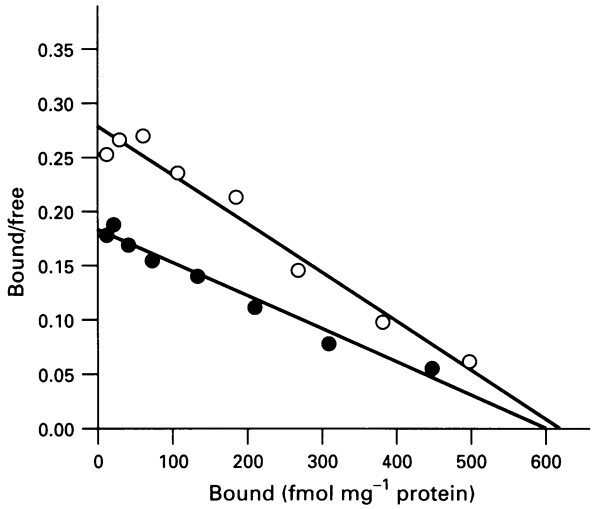


**Figure 5** Scatchard plot of [$^3$H]-diazepam binding in the rat striatum in the absence (○) and presence (●) of GTP (1 mM). Striatal membranes were incubated at 30°C for 60 min with [$^3$H]-diazepam (0.25–83 nM) in the presence or absence of diazepam (10 $\mu M$). Means of triplicate determination are presented for one of three separate experiments.

Following pertussis toxin treatment, AC activities were: basal 114 ± 9.2 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$; forskolin; 446 ± 131 pmol cyclic AMP $mg^{-1}$ protein $min^{-1}$. However, the toxin blocked the inhibitory effect of diazepam and Ro5-4864, as well as that of melatonin and 2-iodomelatonin, on forskolin-stimulated AC activity (Figure 4). While pertussis toxin did not affect basal or forskolin-stimulated AC activity, with prolonged incubation (15½ h), enzyme activity was decreased as compared with that in fresh tissues (see Table 1).

### *Effect of GTP on [$^3$H]-diazepam binding*

Saturation binding with [$^3$H]-diazepam in the rat striatum revealed a sensitivity to guanosine 5′-triphosphate (GTP) as shown in Figure 5. In the absence of GTP, binding parameters were: $K_D = 19.3 \pm 0.8$ nM; $B_{max} = 631.9 \pm 21.1$ fmol $mg^{-1}$ protein, $n = 3$. In the presence of GTP, a modest but significant ($P<0.05$) decrease in affinity was seen, but binding site density was not affected: $K_D = 30.5 \pm 2.6$ nM; $B_{max} = 688.4 \pm 76.6$ fmol $mg^{-1}$ protein, $n = 3$.

## Discussion

In the present study, the multiphasic dose-response curves observed for benzodiazepine inhibition of AC activity suggests that more than one site or mechanism is involved. The first phase of inhibition is very similar to that mediated by other inhibitory receptor types including striatal and hippocampal $G_i$ - coupled receptors such as 5-hydroxytryptamine $(5\text{-HT})_{1A}$ (De Vivo & Maayani, 1986) and dopamine $(D)_2$ (Olianas & Onali, 1987). In contrast, the extremely steep and nearly linear secondary phase of inhibition observed with micromolar amounts of BZs supports earlier evidence that high concentrations of these drugs can act directly on the AC enzyme (Dan'ura *et al.*, 1988).

Interestingly, while Ro5-4864 exhibited a biphasic inhibitory action against AC activity, inhibition by diazepam was triphasic. The high-affinity component ($EC_{50}$ = 0.43 ± 0.05 nM) of inhibition by diazepam, like that of Ro5-4864 ($EC_{50}$ = 1.36 ± 0.37 nM), appears to be receptor mediated as discussed below. As seen in Figure 2, the Ro5-4864 curve plateaus between concentrations of 1 $\mu M$ and 10 $\mu M$ in a manner typical of a receptor-mediated effect. However, at similar concentrations, the diazepam curve does not plateau, but continues a dose-dependent inhibition of AC activity. Thus, the secondary phase of the diazepam inhibitory curve ($EC_{50}$ = 1 ± 0.16 $\mu M$) may involve both the $G_i$-coupled BZ receptor and a direct effect on the AC enzyme. This is supported by the finding that pertussis toxin, which blocks $G_i$-protein mediated activity, was not as effective in reversing the inhibitory action of diazepam on AC activity as it was with Ro5-4864, as shown in Figure 4.

The fact that the central-type BZ antagonist, flumazenil, failed to block the inhibitory effects of Ro5-4864 on AC activity in this study, as well as that of diazepam in previous studies (Dan'ura *et al.*, 1988; Niles & Hashemi, 1990), indicates that it is inactive at the $G_i$-coupled BZ receptor. In contrast to flumazenil, the peripheral-type BZ receptor ligand, PK 11195, blocked the first phase of inhibition by Ro5-4864, suggesting involvement of a receptor with PBR-like pharmacological characteristics. Although PK 11195 may act as a partial agonist in some systems, it did not affect basal or stimulated AC activity, but blocked the inhibitory effect of various other BZs and pharmacological doses of indoleamines, which are thought to act at the same BZ receptor (Niles & Hashemi, 1990). This suggests that PK 11195 acts as a functional antagonist at the $G_i$-coupled BZ receptor. Furthermore, the inhibition of forskolin-stimulated AC activity in the rat striatum by BZs and indoleamines, was blocked following pretreatment with pertussis toxin. These results provide further evidence for the presence of a $G_i$-coupled BZ receptor, in the striatal plasma membrane, which mediates the first phase of AC inhibition by various BZs, melatonin and indoleamine analogues.

Melatonin has been shown to inhibit AC activity via a high-affinity receptor in vertebrate brain which is coupled to a pertussis toxin-sensitive G-protein (Niles *et al.*, 1991; Ying *et al.*, 1992). However, it should be emphasized that the inhibition of striatal AC activity, by melatonin and 2-iodomelatonin, does not involve the high-affinity $G_i$-coupled receptor for melatonin. This is indicated by evidence that: (1) micromolar concentrations of these indoleamines are required to inhibit AC activity in the striatum, as compared to the picomolar to nanomolar concentrations which are effective at the high-affinity receptor for melatonin; (2) autoradiographic and other studies in the rat brain have shown that the high-affinity $G_i$-

coupled receptor for melatonin is primarily localized in the suprachiasmatic nucleus of the hypothalamus, but not present in the striatum; (3) the ability of the PBR antagonist, PK 11195, to block the inhibitory effect of melatonin and 2-iodomelatonin in the striatum provides further evidence that a BZ receptor, and not a melatonin receptor, is involved.

Although diazepam is a non-selective BZ agonist, it was more potent than Ro5-4864 in inhibiting stimulated AC activity, therefore, [$^3$H]-diazepam was used in binding experiments which were conducted at 30°C, in order to minimize binding to the temperature-sensitive central site. Both the binding and functional data suggest that a high (nanomolar)-affinity $G_i$-coupled BZ receptor mediates inhibitory signalling via the AC pathway by BZ agonists. This is in contrast to the micromolar affinity site described by Dan'ura *et al.* (1988). However, this discrepancy could involve tissue differences as their studies were carried out in rat whole brain preparations while striatal tissues were utilized in this study.

In conclusion, the multiphasic nature of AC inhibition observed suggests the involvement of multiple sites or mechanisms of BZ action on this signalling pathway, with the high-affinity phase of inhibition being receptor mediated. Therefore, $G_i$-coupled BZ receptors in the brain may mediate suppression of the ubiquitous second messenger, cyclic AMP, contributing to the diverse psychotropic effects of the BZs, melatonin and related indoleamines. There is evidence that cyclic AMP/protein kinase A (PKA)-dependent mechanisms are involved in the regulation of excitatory synaptic transmission in the CNS (Colwell & Levine, 1995). In keeping with the above, the $\alpha_2$-adrenoceptor agonist, clonidine, which inhibits adenylate cyclase activity and cyclic AMP synthesis in the rat brain (Kitamura *et al.*, 1985), is a potent anticonvulsant (Papanicolaou *et al.*, 1982) and anxiolytic drug (Scheinin *et al.*, 1989). It is therefore possible that the anticonvulsant (Albertson *et al.*, 1981; Champney & Champney, 1992; Golombek *et al.*, 1992) anxiolytic (Guardiola-Lemaitre *et al.*, 1992; Pierrefiche *et al.*, 1993) and other central effects of benzodiazepines, melatonin and related drugs, may involve modulation of the cyclic AMP/protein kinase A pathway. An important question is whether behaviourally effective doses of BZs, such as diazepam, are present in the brain in concentrations sufficient to alter significantly cyclic AMP production, and thus involve this pathway in the neuropharmacological actions of these drugs. Diazepam has been shown to produce its anxiolytic and anticonvulsant effects in rats with $ED_{50}$s of 1.25 mg kg$^{-1}$, i.p. and 1.31 mg kg$^{-1}$, i.p., respectively (Caccia *et al.*, 1980). Fifteen minutes, post-injection of diazepam (1.25 mg kg$^{-1}$, i.p.), $0.04 \pm 0.01$ $\mu$g ml$^{-1}$ or about 140 nM of the drug was detectable in plasma. More importantly, over 5 times as much diazepam was present in the brain (Caccia *et al.*, 1980), which is enough to cause a significant suppression of central production of cyclic AMP, assuming that *in vivo* efficacy is reasonably comparable to that observed *in vitro* (see Figure 1). Thus, in addition to the ability of these drugs to enhance GABAergic activity (Haefely, 1989; Tenn & Niles, 1995), inhibition of cyclic AMP production in the CNS appears to be a second mechanism underlying the neuropharmacological effects of BZs, melatonin and analogues.

This work was supported by a NSERC (Canada) Strategic grant.

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*(Received January 15, 1996*
*Revised May 29, 1996*
*Accepted June 6, 1996)*