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Mitochondrial benzodiazepine receptors regulate steroid biosynthesis

(peripheral-type benzodiazepine receptor function/cholesterol transport/adrenocortical cells)

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ABSTRACT **Recent observations on the steroid synthetic capability within the brain open the possibility that benzodiazepines may influence steroid synthesis in nervous tissue through interactions with peripheral-type benzodiazepine recognition sites, which are highly expressed in steroidogenic cells and associated with the outer mitochondrial membrane. To examine this possibility nine molecules that exhibit a greater than 10,000-fold difference in their affinities for peripheral-type benzodiazepine binding sites were tested for their effects on a well-established steroidogenic model system, the Y-1 mouse adrenal tumor cell line. 4′-Chlorodiazepam, PK 11195, and PK 14067 stimulated steroid production by 2-fold in Y-1 cells, whereas diazepam, flunitrazepam, zolpidem, and PK 14068 displayed a lower (1.2- to 1.5-fold) maximal stimulation. In contrast, clonazepam and flumazenil did not stimulate steroid synthesis. The potencies of these compounds to inhibit ^{3}H-labeled PK 11195 binding to peripheral-type benzodiazepine recognition sites correlated (r = 0.985) with their potencies to stimulate steroid production. Similar findings were observed in bovine and rat adrenocortical cell preparations. These results suggest that ligands of the peripheral-type benzodiazepine recognition site acting on this mitochondrial receptor can enhance steroid production. This action may contribute specificity to the pharmacological profile of drugs preferentially acting on the benzodiazepine recognition site associated with the outer membrane of certain mitochondrial populations.**

Benzodiazepines elicit their primary therapeutic actions in the central nervous system through specific binding sites on γ-aminobutyric acid (GABA)-gated chloride channels (1). In addition to these sites another class of binding sites for benzodiazepines was identified in peripheral tissues (2) and has been referred to as peripheral-type benzodiazepine recognition sites (PBRs). PBRs are also abundant in neural tissue (3, 4) where they appear to be preferentially associated with astroglial cells (5). Since the physiological role of PBRs is not presently known, the effects resulting from benzodiazepine interactions with PBRs in peripheral tissues and in the brain are poorly characterized. Ligands that can bind to the PBR with high affinity have been reported to induce convulsions (6) and exhibit proconflict actions (7, 8). The mechanisms by which these behavioral effects are generated remain unclear.

The central nervous system can synthesize steroids (9, 10). In another set of investigations, steroids were demonstrated to be potent modulators of type A GABA ($GABA_A$) receptors (11, 12), implying a physiological role of steroids as regulators of GABAergic transmission. These findings are significant when one considers that PBRs are extremely abundant in steroidogenic cells (13, 14). Furthermore, metabolism of cholesterol to pregnenolone, the first intermediate in the steroid biosynthetic pathway, occurs in mitochondria where PBRs are associated with the outer mitochondrial membrane (15, 16). Consequently, several groups have demonstrated that benzodiazepines can influence steroid production in several steroidogenic systems (17–19).

To investigate whether PBRs play a central role in steroid production, we have used the well-characterized Y-1 adrenocortical cell line as a model system (20, 21). The data presented here strongly support a role for PBR in the regulation of steroidogenesis. One might infer that the complex central actions of various benzodiazepines could include effects on neurosteroid production mediated through centrally located mitochondrial PBRs.

METHODS

Cells. The Y-1 mouse adrenal tumor cell line used in these studies was obtained from American Type Culture Collection. Stock cultures were grown in modified Waymouth's MB752/1 medium containing 20 mM Hepes, $NaHCO_3$ (1.2 g/liter), 15% (vol/vol) horse serum, and 2.5% (vol/vol) fetal calf serum (pH 7.4). Before use, the cells were washed for three 30-min periods with serum-free medium to eliminate serum components that may interfere with the assays and then incubated as indicated in the presence of drugs under investigation. Bovine fasciculata-reticularis and adrenocortical cells from adult Sprague–Dawley rats (300 g) were prepared as described (22).

Radioligand Binding Assays. Y-1 cell cultures were scraped from the flasks (75 mm^2) in 5 ml of Kreb's buffer (8, 29), dispersed by repetitive pipetting, and centrifuged at 1200 × g for 5 min. The cell pellets were resuspended in Kreb's buffer and larger cellular aggregates were allowed to settle for 5 min. The finely dispersed cell suspensions were taken for further experimentation.

Binding assays were performed in a total volume of 250–400 μl of Kreb's buffer containing ^{3}H-labeled PK 11195 [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide] (0.07–20 nM) with 5 μg of cellular protein. Incubations were conducted at 37°C for 30 min and bound radioligand was collected by filtration through Whatman GF/C filters and rapidly washed four times with 5 ml of 25 mM Tris·HCl (pH 7.4) containing 1 μM PK 11195. Radioactivity was determined by liquid scintillation counting. Nonspecific binding was measured in the presence of 10 μM nonradioactive PK 11195.

Measurement of Steroid Production. These experiments were performed with the Y-1 cells plated in 12 × 22 mm wells

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Abbreviations: PBR, peripheral-type benzodiazepine recognition site; GABA, γ-aminobutyric acid; PK 11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; PK 14067 and PK 14068, (−)- and (+)-*N*,*N*-diethyl-2-methyl-3-[4-(2-phenyl)quinolinyl]propanamide, respectively.

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and incubated for the times shown in a final volume of 1 ml of serum-free medium at 37°C. At the end of the incubation period the cell medium was saved, centrifuged at 1500 × *g* for 10 min, and stored at −20°C until use. Steroid production was determined by measuring 20α-hydroxyprogesterone, the main steroid product of these cells (20, 21), using a radioimmunoassay with an antibody donated by G. Nieswender (Colorado State University, Fort Collins). Cortisol and corticosterone, indices for steroid production in bovine and rat adrenocortical preparations, respectively, were also measured by radioimmunoassay using commercial kits (Baxter Scientific Products, McGaw Park, IL, and Endocrine Sciences, Tarzana, CA, respectively). Analysis of the radioimmunoassay data was performed using the IBM-PC RIA DATA REDUCTION program (version 4.1) from Jaffe (Silver Spring, MD).

Protein Measurements. Protein was quantitated by the method of Bradford (23) using bovine serum albumin as a standard.

RESULTS

Binding of PBR Ligands in Y-1 Cells. Preliminary experiments to measure ^{3}H-labeled PK 11195 binding in Y-1 cells at 37°C demonstrated rapid association kinetics reaching equilibrium within 15 min, which was stable for at least 2 hr and fully reversible by adding excess PK 11195 (data not shown). Scatchard analysis of ^{3}H-labeled PK 11195 binding in Y-1 cells revealed a single class of recognition sites with a dissociation constant of 1.8 nM and a B_{max} of 54 pmol/mg of protein (Fig. 1). The high density of PBRs in Y-1 total cell membranes is consistent with the abundance reported (24) in rat adrenal mitochondrial preparations.

Nine ligands, exhibiting a range greater than four orders of magnitude in their affinities for PBRs, were examined for their potencies to inhibit ^{3}H-labeled PK 11195 binding to Y-1 cells at 37°C (Fig. 2*A*). These compounds demonstrated a rank order of ligand displacement potency (PK 11195 > PK 14067 > 4′-chlorodiazepam > zolpidem > PK 14608, diazepam, flunitrazepam > clonazepam, flumazenil), which is consistent with reports (16) on PBRs from rat tissues [PK 14067 and PK 14068, (−)- and (+)-*N*,*N*-diethyl-2-methyl-3-[4-(2-phenyl)quinolinyl]propanamide, respectively; these compounds are referred to in ref. 25 as (−)- and (+)-*N*,*N*-diethyl-α-methyl-2-phenyl-4-quinoline propanamide]. In particular it should be noted that PK 14067 was more than two orders of magnitude more potent than its stereoisomer PK

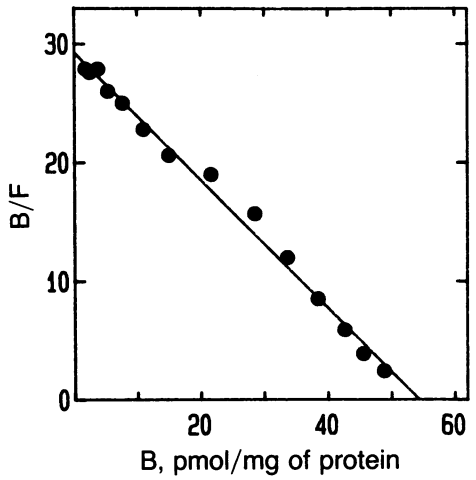

FIG. 1. Scatchard analysis of ^{3}H-labeled PK 11195 binding in Y-1 cells showing the specific binding of ^{3}H-labeled PK 11195. The value of B/F is expressed × 10^3. B, bound ligand; F, free ligand.

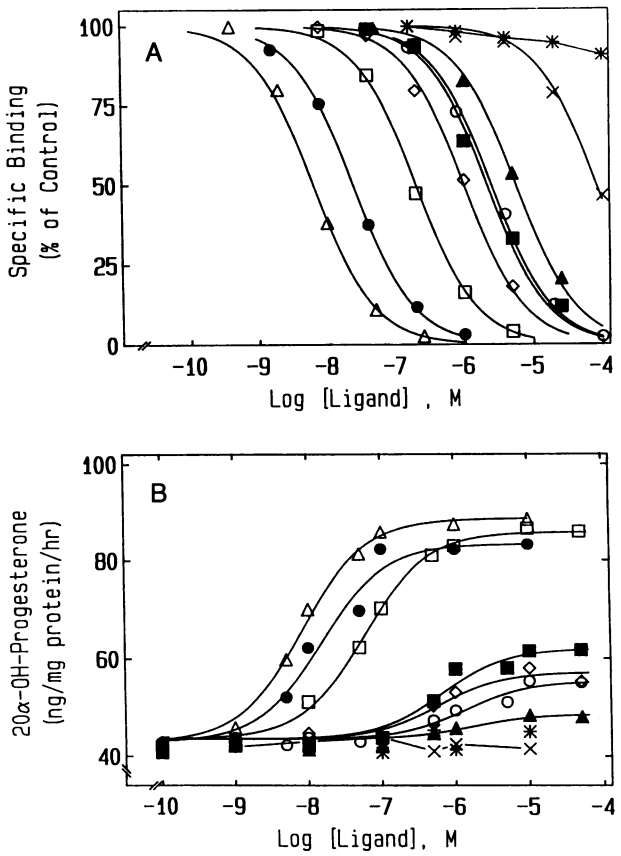

FIG. 2. Binding specificity of PBRs and stimulation of steroidogenesis in Y-1 cells. (*A*) Potencies of nine compounds to compete against ^{3}H-labeled PK 11195 (0.8 nM) for binding in Y-1 cells. (*B*) Secretion of 20α-hydroxyprogesterone (20α-OH-Progesterone) was measured in cultures of Y-1 cells treated for 4 hr with various concentrations of ligand as indicated. △, PK 11195; ●, PK 14067; □, 4′-chlorodiazepam; ◇, zolpidem; ○, PK 14068; ■, diazepam; ▲, flunitrazepam; ×, clonazepam; *, flumazenil. Curves were generated by nonlinear regression analysis of the data.

14068 (25). Also at 37°C, benzodiazepines, 4′-chlorodiazepam, diazepam, and flunitrazepam showed a relatively low potency compared to PK 11195, which is in agreement with a report (26) that demonstrated that the binding of benzodiazepines to PBRs is a temperature-sensitive enthalpy-driven process.

Stimulation of Steroidogenesis by PBR Ligands. Each ligand was tested for its effect in Y-1 cells on secretion of 20α-hydroxyprogesterone, the final product in the pathway of steroid synthesis in Y-1 cells. All three compounds that exhibited high potencies in displacing ^{3}H-labeled PK 11195 binding (K_i < 200 nM) stimulated steroid secretion in a concentration-dependent manner, each exhibiting a maximal stimulation of about 2-fold greater than the basal level of steroid secretion (Fig. 2*B*). In contrast, clonazepam and flumazenil failed to stimulate steroid production whereas the remaining four ligands stimulated steroid secretion; however, the maximal stimulation achieved was only 20–50% above the basal levels. When the inhibition constants of this series of ligands to compete with ^{3}H-labeled PK 11195 for binding were compared with their potencies to stimulate steroid secretion (Fig. 3), a highly significant correlation was found (r = 0.985), suggesting a direct relationship between ligand occupancy of the PBRs and steroidogenic activity.

Stimulation of Steroidogenesis in Normal Adrenocortical Cells. In case the effects of PBR ligands in the Y-1 cell line are not representative of the effects observed in cells from adrenal cortical tissue, we also tested several PBR ligands for

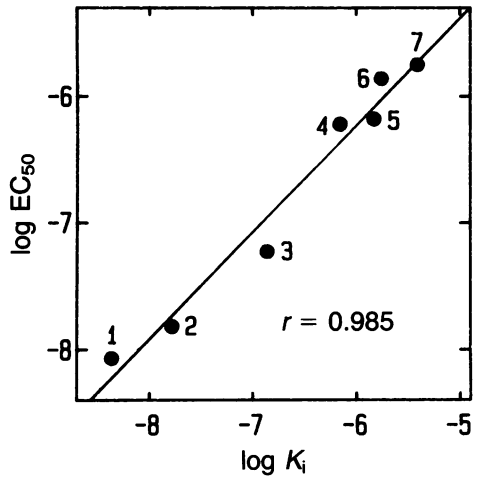

FIG. 3. Correlation of affinity for the PBR with steroidogenic stimulatory potency. The log K_i calculated for each ligand in Fig. 2*A* (except clonazepam and flumazenil) is plotted against the log of the concentration necessary to attain half-maximal stimulation of the corresponding ligand (log EC_{50}) as determined from the analysis of Fig. 2*B*. Data points: 1, PK 11195; 2, PK 14067; 3, 4′-chlorodiazepam; 4, zolpidem; 5, diazepam; 6, PK 14068; 7, flunitrazepam.

corticosterone and cortisol secretion in dissociated rat and bovine adrenal cortical tissue, respectively. These experiments demonstrate that the high-affinity PBR ligands stimulated steroid production in suspensions of adrenocortical cells as well (Fig. 4). Hence, the Y-1 cell line is a reliable and convenient model system for studying the effects of PBR ligands on steroidogenesis, and the effects observed in Y-1 cells are likely to be found *in vivo*.

It should also be noted in Fig. 4 that 4′-chlorodiazepam is much more potent at stimulating steroidogenesis in rat adrenocortical cells than in bovine preparations. This observation is consistent with the species-dependent differences described for the binding of 4′-chlorodiazepam to PBRs (27). Therefore, this experiment provides additional support that steroidogenesis is specifically mediated by the interaction of ligand and PBR rather than by another mechanism elicited by the ligand.

DISCUSSION

Although a number of laboratories have shown that certain benzodiazepines and other ligands that bind to PBRs enhance steroidogenesis (17–19), the results presented here unambiguously demonstrate that these effects are mediated through the PBR. We have used a series of nine compounds with apparent association constants for PBRs that cover four orders of magnitude. The potencies of these ligands to stimulate steroidogenesis in Y-1 cells correlated very closely with their potencies to compete against ^{3}H-labeled PK 11195 for binding to PBRs (r = 0.985). This provides substantial proof that high-affinity PBR ligands regulate steroid production when they bind to the mitochondrial benzodiazepine recognition site.

Similar effects were observed in primary cultures of rat and bovine adrenocortical cells, verifying the use of Y-1 cells as a model system for these studies. The finding that bovine adrenocortical cultures are relatively unresponsive to 4′-chlorodiazepam at high nanomolar concentrations is consistent with the low affinity of bovine PBRs reported for this ligand (27). This experiment further supports the proposal that specific association of the ligands with the PBR is required for the steroidogenic effects.

Currently, the role of the PBR in the steroidogenic pathway remains to be elucidated. Metabolism of cholesterol to pregnenolone, a common intermediate for all steroid hormone biosynthesis, requires transport of cholesterol from extramitochondrial stores to the inner mitochondrial membrane at the site where the C27-side-chain-cleavage cytochrome P-450 enzyme is located. The rate-determining step in this process appears to occur around the incorporation of cholesterol into the mitochondria and its subsequent transport to the inner mitochondrial membrane (28). Although it is fundamental to understanding the regulation of steroid synthesis, this mechanism is poorly characterized. Because of the outer mitochondrial membrane location of the PBR (15, 16), it is probable that PBRs physiologically participate in this process by binding an endogenous ligand, such as the diazepam-binding inhibitor (18, 29) or its cellular processing products (8). Thus, the mimicry of such physiological mechanisms by PBR synthetic ligands might account for their ability to enhance steroidogenesis. In fact, subsequent studies have shown that PBR ligands promote cholesterol delivery to the inner mitochondrial membrane by a mechanism that follows the transport of exogenous cholesterol to the mitochondria (K.E.K., A.G.M., and V.P., unpublished results).

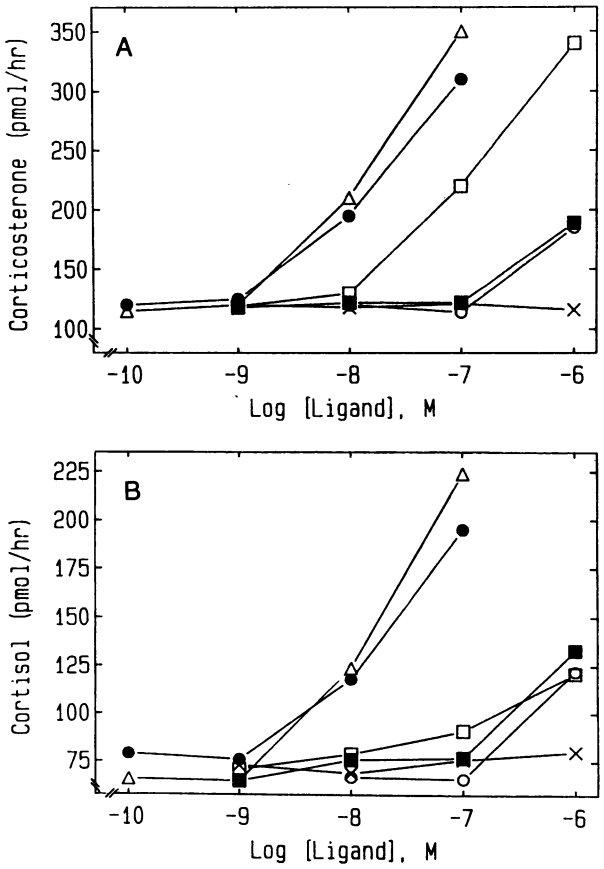

FIG. 4. Stimulation of steroidogenesis in bovine and rat adrenocortical cells. Steroid synthesis was measured in cultures of rat (*A*) or bovine (*B*) adrenocortical cells treated for 4 hr with various concentrations of ligands as indicated. Symbols are as indicated in Fig. 2.

Investigations by several groups studying other actions of drugs believed to be mediated by PBRs demonstrated that PK 11195 antagonizes the actions of 4′-chlorodiazepam in pro-conflict activity (7, 8), sensitivity to audiogenic seizures (30), and chemotaxis of human monocytes (31). The studies shown in this report demonstrate that PK 11195 and 4′-chlorodiazepam stimulate steroidogenesis. This finding might indicate heterogeneity in recognition sites for PBR ligands participating in various cellular processes. The possibility that PBRs may play multiple roles is supported by the presence of

significant levels of these recognition sites in tissues that do not synthesize steroids (14, 24).

The pharmacological profile of benzodiazepines appears to allow for the identification of two drug subtypes: those acting on GABA-gated Cl^- channels (clonazepam) and those acting on steroidogenesis (4′-chlorodiazepam). Clarification is needed as the process whereby this can be achieved is still incompletely understood. However, the brain has the capacity to synthesize steroids (9, 10), and the ability of certain steroids to facilitate or inhibit GABAergic transmission (11, 12) implies the physiological importance of brain steroids in the central nervous system. The findings reported here suggest that benzodiazepines may interact with the PBR not only in peripheral tissues but also in the central nervous system and that these sites of action might contribute to the identification of a class of benzodiazepines with a specific pharmacological profile. Studies of the molecular mechanisms mediating this action of benzodiazepines should be extended to astroglial primary cell cultures to examine whether effects similar to those obtained in the experiments reported here can be obtained with cells derived from the neural crest.

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1. Costa, E. & Guidotti, A. (1979) *Annu. Rev. Pharmacol. Toxicol.* **19,** 531–545.
2. Braestrup, C. & Squires, R. F. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3805–3809.
3. Shoemaker, H., Boles, R. G., Horst, W. D. & Yamamura, H. I. (1983) *J. Pharmacol. Exp. Ther.* **225,** 61–69.
4. Villiger, J. W. (1985) *Neuropharmacology* **24,** 95–98.
5. Gallager D. W., Mallorga, P., Oertel, W., Henneberry, R. & Tallman, J. (1981) *J. Neurosci.* **1,** 218–225.
6. Weissman, B. A., Cott, J., Paul, S. M. & Skolnick, P. (1983) *Eur. J. Pharmacol.* **90,** 149–150.
7. Mizoule, J., Gauthier, A., Uzan, A., Renault, C., Dubroeucq, M. C., Gueremy, C. & Le Fur, G. (1985) *Life Sci.* **36,** 1059–1068.
8. Slobodyansky, E., Guidotti, A., Wambebe, C., Berkovich, A. & Costa, E. (1989) *J. Neurochem.* **53,** 1276–1284.
9. Le Goascogne, C., Robel, P., Gouezou, M., Sananes, N., Baulieu, E. & Waterman, M. (1987) *Science* **237,** 1212–1215.
10. Yi Hu, Z., Bourreau, E., Jung-Testas, I., Robel, P. & Baulieu, E. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8215–8219.
11. Majewska, M. D., Harrison, N. L., Schwartz, R. D., Barker, J. L. & Paul, S. M. (1986) *Science* **232,** 1004–1007.
12. Mienville, J. M. & Vicini, S. (1989) *Brain Res.* **489,** 190–194.
13. De Souza, E. B., Anholt, R. R. H., Murphy, K. M. M., Snyder, S. H. & Kuhar, M. S. (1985) *Endocrinology* **116,** 567–573.
14. Anholt, R. R. H., De Souza, E. B., Oster-Granite, M. L. & Snyder, S. H. (1985) *J. Pharmacol. Exp. Ther.* **233,** 517–526.
15. Anholt, R. R. H., Pedersen, P. L., De Souza, E. B. & Snyder, S. H. (1986) *J. Biol. Chem.* **261,** 576–583.
16. Hirsch, J. D., Beyer, C. F., Malkowitz, L., Loullis, C. C. & Blume, A. J. (1989) *Mol. Pharmacol.* **35,** 164–172.
17. Ritta, M. N. & Calandra, R. S. (1989) *Neuroendocrinology* **49,** 262–266.
18. Besman, M. J., Yanagibashi, K., Lee, T. D., Kawamura, M., Hall, P. F. & Shively, J. E. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4897–4901.
19. Barnea, E. R., Fares, F. & Gavish, M. (1989) *Mol. Cell. Endocrinol.* **64,** 155–159.
20. Kowal, J. (1970) *Recent Prog. Horm. Res.* **26,** 623–687.
21. Schimmer, B. P. (1981) in *Functionally Differentiated Cell Lines*, ed. Sato, G. (Liss, New York), pp. 61–92.
22. Yanagibashi, K., Papadopoulos, V., Masaki, E., Iwaki, T., Kawamura, M. & Hall, P. F. (1989) *Endocrinology* **124,** 2383–2391.
23. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
24. Antkiewicz-Michaluk, L., Guidotti, A. & Krueger, K. E. (1988) *Mol. Pharmacol.* **34,** 272–278.
25. Dubroeucq, M. C., Benavides, J., Doble, A., Guilloux, F., Allam, D., Vaucher, N., Bertrand, P., Gueremy, C., Renault, C., Uzan, A. & Le Fur, G. (1986) *Eur. J. Pharmacol.* **128,** 269–272.
26. Le Fur, G., Vaucher, N., Perrier, M. L., Flamier, A., Benavides, J., Renault, C., Dubroeucq, M. C., Gueremy, C. & Uzan, A. (1983) *Life Sci.* **33,** 449–457.
27. Awad, M. & Gavish, M. (1987) *J. Neurochem.* **49,** 1407–1414.
28. Hall, P. F. (1984) *Int. Rev. Cytol.* **86,** 53–95.
29. Guidotti, A., Forchetti, C. M., Konkel, D., Bennett, C. D. & Costa, E. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3531–3535.
30. Benavides, J., Guilloux, F., Allam, D. E., Uzan, A., Mizoule, J., Renault, C., Dubroeucq, M. C., Gueremy, C. & Le Fur, G. (1984) *Life Sci.* **34,** 2613–2620.
31. Ruff, M. R., Pert, C. B., Weber, R. J., Wahl, L. M., Wahl, S. M. & Paul, S. M. (1985) *Science* **229,** 1281–1283.