REFERENCES AND NOTES

1. R. Shrimpton, R. Giugliano, N. M. Rodrigues, *Acta Amazonica* **9**, 117 (1978).
2. M. Petrere, Jr., *ibid.* **8** (suppl. 2), 1 (1978); *Cienc. Cult. (Maracaibo)* **37**, 1987 (1985).
3. N. J. H. Smith, *Man, Fishes and the Amazon* (Columbia Univ. Press, New York, 1981); M. Goulding, *Man and Fisheries in the Amazon Frontier* (Junk, The Hague, 1981).
4. R. Eckmann, *Anim. Res. Dev.* **21**, 59 (1985).
5. P. B. Bayley, thesis, Dalhousie University, Halifax (1982).
6. W. J. Junk and C. Howard-Williams, in *The Amazon*, H. Sioli, Ed. (Junk, The Hague, 1984), pp. 269–293; W. J. Junk, *ibid.*, pp. 215–243.
7. M. Goulding, *The Fishes and the Forest* (Univ. of California Press, Berkeley, 1980); M. C. L. Ribeiro, thesis, Instituto Nacional de Pesquisas da Amazônia/Fundação Universidade de Amazonas, Manaus (1983); M. Goulding and M. L. Carvalho. *Rev. Bras. Zool.* **1**, 107 (1982).
8. G. Marlier, *Cad. Amazonia* **2**, 5 (1968); H. A. Knoppel, *Amazoniana* **2**, 257 (1970); F. M. Carvalho, *ibid.* **8**, 525 (1984); C. A. R. M. Araujo-Lima and E. Hardy, *ibid.*, in press.
9. E. B. Haines, *Oikos* **29**, 254 (1977); B. Fry, A. Joern, P. L. Parker, *Ecology* **59**, 498 (1978); E. B. Haines and C. L. Montague, *ibid.* **60**, 48 (1979); B. Peterson, R. W. Howarth, F. Lipschultz, D. Ashendorf, *Oikos* **34**, 173 (1980); E. H. Hughes and E. B. Sherr, *J. Exp. Mar. Biol. Ecol.* **67**, 227 (1983); B. J. Peterson, R. W. Howarth, R. H. Garritt, *Science* **227**, 1361 (1985).
10. Nine fish and 37 plant species were sampled. The Characiformes fish species were *Semaprochilodus taeniurus, Prochilodus nigricans, Potamorhina latior, Potamorhina altamazonica*, and *Psectrogaster rutiloides*. The catfish species were *Pterygoplichthys multiradiatus, Loricariichthys platimetopon, Hoplosternum thoracatum*, and *Hemiancistrus scaphirhynchus*. Only adult fishes were sampled.
11. POC samples were collected exclusively in lakes. We collected POC samples to estimate phytoplankton $\delta^{13}C$ values. POC samples with more than 60% phytoplankton were classified as pure phytoplankton samples. POC samples were not collected in the river because phytoplankton biomass is typically very low [T. R. Fisher, *Comp. Biochem. Physiol.* **62A**, 1 (1979); R. C. Wissmar, J. E. Richey, R. F. Stallard, J. M. Edmond. *Ecology* **62**, 1622 (1981)]. The samples were concentrated by continuous centrifugation or by filtering whole-water samples through precombusted glass fiber filters (Gelman, GFF). Periphyton and macrophytes were collected in lakes and rivers. Tree parts were collected in lake drainage basins.
12. Plant samples were washed with tap water and a 1*N* HCl solution and then rinsed several times with deionized water. All samples were dried at 60°C. After drying, vascular plants were ground to a fine powder with a mortar and pestle. A skinless, boneless sample of muscle was taken from the upper body of each fish, dried, and ground as above.
13. Samples for isotopic analysis were prepared by combustion of ~10 mg of dry matter with CuO, in sealed, evacuated Pyrex tubes. The samples were burned overnight at 550°C and purified by passage through alcohol–dry ice traps. The purified sample was collected in a tube under liquid nitrogen, in a special vacuum line. A set of CENA's substandards [pure charcoal from C_3 and C_4 plants, calibrated to Pee Dee belemnite standard (PDB) limestone] was prepared with each sample batch; thus, no correction for oxygen was necessary, as its source was the same for the samples and the substandards. All carbon-13 values represent the mean of two separate analyses from the same sample (SEM = 0.25 $\delta^{13}C$ unit) and are reported relative to PDB limestone:

$$\delta^{13}C = \frac{(^{13}C/^{12}C_{sample} - {}^{13}C/^{12}C_{PDB})}{(^{13}C/^{12}C_{PDB})} \times 1000$$

Analyses were performed in a Micromass model 602-E mass spectrometer fitted with double inlet and double collector systems.
14. J. I. Hedges *et al.*, *Limnol. Oceanogr.*, in press. (Sixteen wood, 15 leaf, and 4 macrophyte values from their table 1 were included in the analysis.)
15. Since the organic matter encountered in stomach analyses is largely unrecognizable, it is not clear whether these fishes are obtaining their energy directly from phytoplankton by consuming their sedimented remains or indirectly by consuming the remains of organisms at higher trophic levels. The $\delta^{13}C$ results merely indicate that the carbon in these fishes is derived from a food chain of which the initial stage is phytoplankton.
16. One caveat should be mentioned here. The existence of a major unrecognized autotrophic energy source with a $\delta^{13}C$ value lower than phytoplankton would tend to invalidate this conclusion, making it impossible to determine the relative energy contribution of phytoplankton. The only autotrophs known to have values significantly lower than those that we report for phytoplankton are the chemolithotrophic bacteria [B. Fry and E. Sherr, *Contrib. Mar. Sci.* **27**, 15 (1984)]. The importance of autotrophic bacteria in Amazon waters is unknown. However, given their strict growth requirements, it is unlikely that they contribute significant quantities of organic carbon to the system.
17. The maximum carbon contribution from alternative plant sources was calculated as:

$$x = (\delta_3 - 1 - \delta_2)/(\delta_1 - \delta_2)$$

where x is the proportional contribution of the alternative carbon source, δ_2 and δ_3 are the upper limits of the 95% confidence intervals for the mean $\delta^{13}C$ values of phytoplankton and fish species, respectively, and δ_1 was the lower limit of the 95% confidence interval for the mean $\delta^{13}C$ value of the alternative carbon source. An increase of 1 delta unit per trophic level was assumed to be due to fractionation [M. De Niro and S. Epstein, *Geochim. Cosmochim. Acta* **42**, 495 (1978); G. H. Rau *et al.*, *Ecology* **64**, 1314 (1983)].

18. We thank L. Py-Daniel for identifying the siluriform species, C. C. Fernandes and T. Pimentel for help in collecting and preparing the samples, and N. A. Menezes for critically reviewing the manuscript. We also thank M. N. Goes, P. Vose, J. Richey, J. Hedges, P. Bayley, and P. Quay for valuable advice and support during the course of this study. This research was supported by NSF grant DEB 81-07522, Sudam-Polamazonia and International Atomic Energy Agency grant Amazonia I/BRA/0/010. Contribution 16 of the Carbon in the Amazon River Experiment Project and contribution 1 of the Amazonia I Project.

9 June 1986; accepted 15 September 1986

Expression of Bovine 17α-Hydroxylase Cytochrome P-450 cDNA in Nonsteroidogenic (COS 1) Cells

MAURICIO X. ZUBER,* EVAN R. SIMPSON, MICHAEL R. WATERMAN

Cortisol production requires the activity of only 17α-hydroxylase, whereas the formation of sex steroids requires both 17α-hydroxylase and 17,20-lyase activities. Studies in reconstituted enzyme systems have suggested that a single steroid hydroxylase, 17α-hydroxylase cytochrome P-450 (P-450$_{17\alpha}$), catalyzes both activities. By expression of bovine adrenocortical P-450$_{17\alpha}$ in COS 1 (transformed monkey kidney) cells, which normally contain no detectable P-450$_{17\alpha}$, it has now been established in situ that a single polypeptide chain does catalyze both the 17α-hydroxylase and the 17,20-lyase reactions. This heterologous system supports 17α-hydroxylation of pregnenolone and progesterone with equal efficiency, but catalyzes about five times as much 17,20-lyase activity when 17α-hydroxypregnenolone is the substrate than when 17α-hydroxyprogesterone is the substrate. For these activities to be observed in COS 1 cells, newly synthesized apocytochrome P-450$_{17\alpha}$ must bind heme and insert into the endoplasmic reticulum such that endogenous cytochrome P-450 reductase can support hydroxylation. Thus, COS 1 cells are a useful system for expression and study of various forms of cytochrome P-450.

STUDIES OF MICROSOMAL 17α-HYdroxylase cytochrome P-450 (P-450$_{17\alpha}$), as with other eukaryotic cytochromes P-450, have been complicated by problems associated with purification from their membrane (microsomal or inner mitochondrial) environments and subsequent reconstitution of their activities in vitro. The similarities in physical and biochemical characteristics of different cytochromes P-450 (*1*) together with potential artifacts generated during solubilization, purification, and reconstitution procedures (*2*) have made it difficult to unambiguously assign one or more activities to an individual form of cytochrome P-450. Preparations of purified adrenocortical and testicular P-450$_{17\alpha}$ possess 17α-hydroxylase activity necessary for the production of cortisol, as well as 17,20-lyase activity required for sex steroid formation (*3*). However, the ratio of these activities changes during purification procedures (*3*) and in vivo under differing physiological conditions (*4*). Also, in humans, deficiencies associated with these activities have been reported for either 17α-hydroxylase activity (*5*) or 17,20-lyase activity (*6*). Thus, after identifying and characterizing a complementary DNA (cDNA) clone specific for bovine P-450$_{17\alpha}$ (*7*), we sought to clarify the uncertainty surrounding the reported dual activities associated with the P-450$_{17\alpha}$ polypeptide chain.

Our strategy was to analyze the activities

M. X. Zuber and E. R. Simpson, Departments of Biochemistry and Obstetrics and Gynecology and the Cecil H. and Ida Green Center, University of Texas Health Science Center, Dallas, TX 75235.
M. R. Waterman, Department of Biochemistry, University of Texas Health Science Center, Dallas, TX 75235.

*Present address: Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138.

catalyzed by bovine P-450$_{17\alpha}$ in situ by expressing this enzyme in mammalian cells that do not normally express the P-450$_{17\alpha}$ gene. An SV40 expression vector containing a cDNA encoding the complete amino acid sequence of bovine P-450$_{17\alpha}$ (pcD-17α2) has been used. A successful system for expression from such pcD vectors (*8*) is provided by COS 1 cells from the kidney of the African Green monkey (*9*). These cells are potentially useful for expression of a mammalian cytochrome P-450 enzyme, such as P-450$_{17\alpha}$, since they originate from kidney that contains constitutive forms of cytochrome P-450 necessary for the metabolism of fatty acids (*10*), vitamin D (*11*), and xenobiotics (*12*). Thus kidney endoplasmic reticulum contains cytochrome P-450 reductase, the ubiquitous flavoprotein necessary for P-450$_{17\alpha}$ function. In addition, Northern blot analyses of bovine or human kidney RNA indicate that these tissues do not express the P-450$_{17\alpha}$ gene (*7*). Thus, COS 1 cells should be an ideal model system to support microsomal cytochrome P-450$_{17\alpha}$ activity. These cells have been used for the expression of another microsomal enzyme, uridine diphosphate-glucuronyltransferase (*13*), but the only eukaryotic expression system previously used for study of cytochrome P-450 is yeast, in which rat liver P-450$_c$–catalyzing aryl hydrocarbon hydroxylase activity has been expressed (*14*). Our results demonstrate that both 17α-hydroxylase and 17,20-lyase activities are catalyzed by bovine P-450$_{17\alpha}$ when it is expressed in COS 1 cells.

Transfection of COS 1 cells with pcD-17α2 yielded detectable P-450$_{17\alpha}$ by indirect immunofluorescence (Fig. 1). Furthermore, the subcellular distribution of P-450$_{17\alpha}$ as determined by immunofluorescence coincided with that of the kidney microsomal flavoprotein cytochrome P-450 reductase (Fig. 1). When Northern blot analyses were performed (Fig. 2A), only RNA from cells transfected with pcD-17α2, and not those transfected with a different pcD vector or mock-transfected cells, hybridized with a P-450$_{17\alpha}$–specific probe. As expected, the signal indicated longer transcripts than the one found in RNA from cultured bovine adrenocortical cells (BAC) since part of the SV40 sequence in the vector is also transcribed. Immunoblot analysis of cell protein from pcD-17α2 transfected cells revealed a protein that comigrated with the P-450$_{17\alpha}$ from cultured BAC (Fig. 2B). Taken together, these results indicate that bovine adrenal P-450$_{17\alpha}$ is synthesized in the pcD-17α2 transfected COS 1 cells and is probably inserted into the correct subcellular compartment, the endoplasmic reticulum.

Table 1. Rates of formation of selected steroid products in COS 1 cells transfected with pcD-17α2. Rates were determined by measuring the conversion of ^{14}C-labeled substrates after 1/2-hour incubations with progesterone and pregnenolone and 3-hour incubations with 17α-hydroxylated substrates as described in the legend to Fig. 3. After autoradiography, the spots corresponding to the indicated substrates and products were scraped and the radioactivity was determined by scintillation spectrometry (*18*). Control cells or cells transfected with pBAdx-6 showed conversion rates not detectable above background levels.

Substrate	Concentration (μ*M*)	Product	Amount per plate (nmol/hour)
Pregnenolone	10	17α-OH pregnenolone	3.80
Progesterone	10	17α-OH progesterone	3.92
17α-OH pregnenolone	10	Dehydroepiandrosterone	0.69
17α-OH progesterone	10	Androstenedione	0.17
17α-OH pregnenolone	1.0	Dehydroepiandrosterone	0.190
17α-OH progesterone	1.0	Androstenedione	0.021
17α-OH pregnenolone	0.1	Dehydroepiandrosterone	0.044
17α-OH progesterone	0.1	Androstenedione	0.003

For COS 1 cells to support steroid 17α-hydroxylase activity, certain criteria must be met. Protoheme must be bound as a prosthetic group by the apocytochrome P-450$_{17\alpha}$, and the bovine adrenocortical P-450$_{17\alpha}$ must be inserted into the endoplasmic reticulum such that the endogenous monkey kidney cytochrome P-450 reductase of the COS 1 cells can transfer electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to P-450$_{17\alpha}$. In addition, the COS 1 cells must provide an adequate supply of NADPH to support 17α-hydroxylation. As shown in Fig. 3, all these criteria are satisfied since pcD-17α2 transfected cells catalyzed the production of 17α-hydroxyprogesterone from progesterone. Furthermore, the 17α-hydroxylated

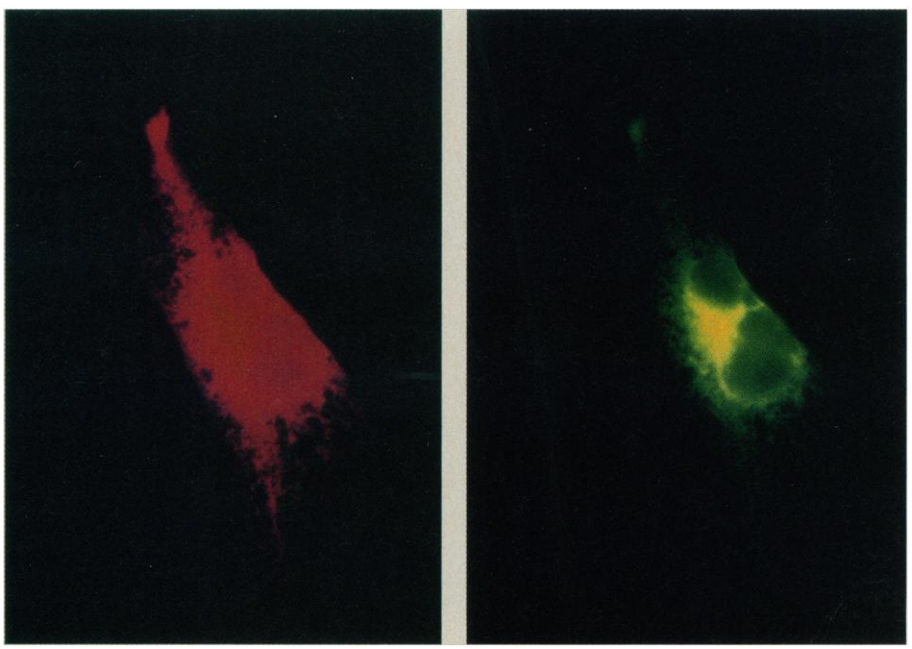

Fig. 1. Immunofluorescence of bovine P-450$_{17\alpha}$ (left) and endogenous monkey cytochrome P-450 reductase (right) in the same COS 1 cell. The night before transfection, COS 1 cells were passaged from a confluent 100-mm culture dish by trypsinization. They were placed in 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The suspension was diluted 1 to 100 with the same medium, and 0.5 ml was added to multiwell dishes containing cover slips. Transfection was accomplished by incubation of the cells for 1 hour with diethylaminoethyl-dextran (250 μg/ml; Pharmacia) and pcD-17α2 (10 μg/ml) in serum-free DMEM at 37°C (*15*). After 5 hours of incubation with chloroquine (52 μg/ml) (*16*) in DMEM containing fetal calf serum, the cells were washed and incubated for an additional 66 hours in the same medium without chloroquine. Immunofluorescence (Zeiss Photomicroscope III) of P-450$_{17\alpha}$ was determined (*17*) by using rabbit immunoglobulin G (IgG) specific for pig testis P-450$_{17\alpha}$ as primary antibody and rhodamine-conjugated goat antibody to rabbit IgG as secondary antibody. Immunofluorescence of cytochrome P-450 reductase was determined by using rabbit IgG specific for pig liver cytochrome P-450 reductase as primary antibody and fluorescein-conjugated goat antibody to rabbit IgG as the secondary antibody (Cooper Biomedical).

product was further converted to androstenedione by 17,20-lyase activity, thus demonstrating that both activities reside on the P-$450_{17\alpha}$ polypeptide chain. Similarly, when pregnenolone was used as substrate, 17α-hydroxypregnenolone and dehydroepiandrosterone (DHEA) were formed. In fact, at the end of 8 hours most of the pregnenolone had been converted to DHEA. This last result suggested a much higher lyase activity when 17α-hydroxypregnenolone was the substrate than when 17α-hydroxyprogesterone was the substrate. To investigate the relative rates for the different substrates for P-$450_{17\alpha}$, we measured the initial rates at substrate concentrations of 10 μ*M* (Table 1). The rates of 17α-hydroxylation were similar for pregnenolone and progesterone and approximately five times those for the conversion of 17α-hydroxypregnenolone into DHEA at the same substrate concentration (Table 1). The amount of androstenedione formed from 10 μ*M* 17α-hydroxyprogesterone was, in contrast, 1/20 of that of 17α-hydroxyprogesterone from progesterone (Table 1). When the 17,20-lyase activity was also measured at 1/10 and 1/100 times the substrate concentrations, the amount of androstenedione formed was again much lower than that of DHEA formation.

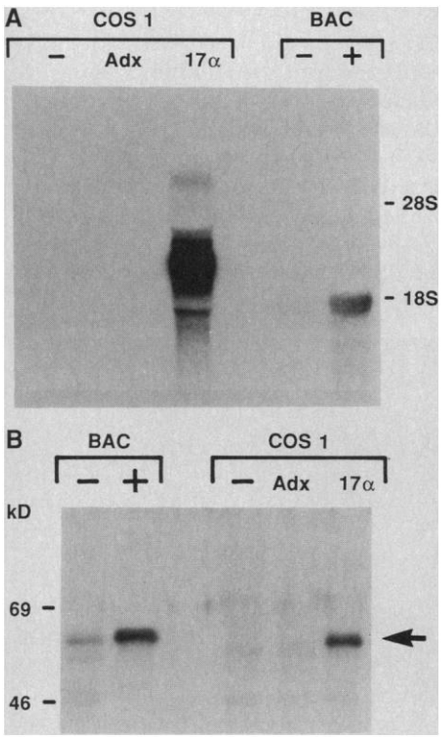

Fig. 2. (A) Northern blot analysis of P-$450_{17\alpha}$ messenger RNA from total RNA (10 μg) prepared (*18*) from BAC and COS 1 cells. The night before transfection COS 1 cells were passaged from a confluent 100-mm culture dish by trypsinization. They were placed in 5 ml of DMEM containing serum, and 1 ml of the suspension was added to each of five 100-mm culture dishes containing 10 ml of the same medium. Transfection was carried out as described in Fig. 1 by means of pcD-17α2 (10 μg/ml) or pBAdx-6 (10 μg/ml) (Adx, a control plasmid in the same pcD vector, but containing the complete coding sequence of bovine adrenodoxin). Also shown is an analysis of RNA from mock-transfected COS 1 cells (−) and from BAC cells maintained in the absence (−) or presence of 1 μ*M* ACTH (+) for 12 hours. (B) Immunoblot analysis (*18*) of P-$450_{17\alpha}$ from cell homogenates from COS 1 and BAC cells as described in (A). In all cases 50 μg of protein was used except for adrenocorticotropin-stimulated BAC cells (10 μg). The second antibody was ^{125}I-labeled goat antibody to rabbit IgG, and the arrow denotes the position of migration of purified pig adrenal P-$450_{17\alpha}$.

Conversion of 1 μ*M* 17α-hydroxypregnenolone to DHEA occurred at a rate of 0.2 nmol per hour per plate. When the same concentration of pregnenolone was used, more than 1 nmol of DHEA was formed by 2 hours, indicative of a rate higher than 0.5 nmol per hour per plate. Thus, it is possible that lyase activity is facilitated when the product of the 17α-hydroxylase reaction (which is the substrate for the lyase reaction) is already in contact with the enzyme. Further studies are necessary, however, to prove the concerted nature of this reaction.

Our studies demonstrate that bovine adrenal P-$450_{17\alpha}$ expressed in COS 1 cells catalyzes both 17α-hydroxylase and 17,20-lyase activities in situ, thus confirming the observations of Hall and colleagues (*3*) who used purified enzyme preparations. These results emphasize the fundamental role that this enzyme plays in the pathways of steroid hormone biosynthesis, located as it is at an important branch point in these pathways. How each of the activities is controlled to maintain a physiological balance between glucocorticoid production on the one hand and adrenal C19 steroid production on the other remains to be elucidated. Perhaps the previously observed changes in ratios of 17α-hydroxylase to 17,20-lyase activities upon purification (*3*) and in response to physiological conditions (*4*) are the result of the interaction of P-$450_{17\alpha}$ with the environment of the endoplasmic reticulum. Variations in these activities associated with human deficiency states (*5, 6*) presumably result from mutations that alter the interactions of substrates with the enzyme. Thus, P-$450_{17\alpha}$ is an example of a more general class of cytochromes P-450, namely, those that are involved in metabolism of endogenous substrates and that catalyze multistep reactions that use a single active site; other examples include cholesterol side-chain cleavage cytochrome P-450, 11β-hydroxylase cytochrome P-450, and aromatase cytochrome P-450. From studies based on site-directed mutagenesis, COS 1 cells transfected with pcD vectors should provide a useful system with which to study structure-function relations within this interesting group of enzymes.

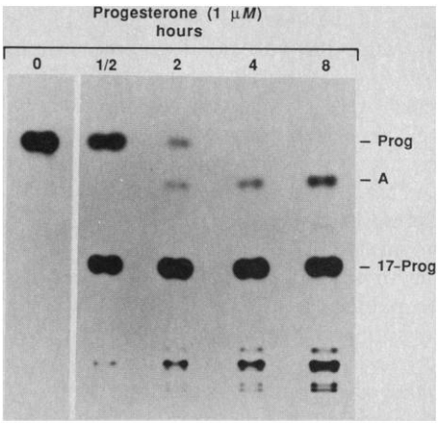

Fig. 3. Autoradiogram of thin-layer chromatography plate showing conversion of progesterone (Prog) to 17α-hydroxyprogesterone (17-Prog) and androstenedione (A) by COS 1 cells transfected with pcD-17α2. Transfected cells maintained in serum-containing DMEM were incubated at 37°C with 1 μ*M* progesterone containing 4 × 10^4 cpm of [^{14}C]progesterone (0.1 μ*M*). By the time of the assay, each 100-mm culture dish contained about 0.25 mg of total cell protein. At the indicated times, the culture medium was removed, and the steroids were extracted with dichloromethane and chromatographed on a coated silica gel plate with a mixture of chloroform and ethylacetate (75:25% by volume) prior to autoradiography (*18*). Identity of steroid products was determined by comigration with authentic standards and confirmed by radioimmunoassay and high-performance liquid chromatography. The polar metabolites below 17-Prog are unidentified.

REFERENCES AND NOTES

1. R. I. Murray, M. T. Fisher, P. G. Debrunner, S. G. Sligar, in *Metalloproteins*, P. Harrison, Ed. (Macmillan, New York, 1985), p. 157; G. T. Miwa and A. Y. H. Lu, in *Cytochrome P-450: Structure, Mechanism and Biochemistry*, P. R. Ortiz de Montellano, Ed. (Plenum, New York, 1986), p. 77; S. D. Black and M. J. Coon, *ibid.*, p. 161.
2. M. J Coon, *Methods Enzymol.* **52**, 200 (1958).
3. S. Nakajin and P. F. Hall, *J. Biol. Chem.* **256**, 3871 (1981); ——, M. Onoda, *ibid.*, p. 6134; S. Nakajin, M. Shinoda, M. Haniu, J. E. Shively, P. F. Hall, *ibid.* **259**, 3971 (1984); K. Suhara, Y. Fujimura, M. Shiroo, M. Katagiri, *ibid.*, p. 8729.
4. R. J. Schiebinger *et al.*, *J. Clin. Invest.* **67**, 1177 (1981); M. J. O'Hare and A. M. Neville, in *The Human Adrenal Cortex* (Springer-Verlag, New York, 1982), p. 91.
5. M. Zachmann, E. A. Werder, A. Prader, *J. Clin. Endocrinol. Metab.* **55**, 487 (1982).
6. F. Larrea *et al.*, *Acta Endocrinol.* **103**, 400 (1983); I. S. Salti, H. Hajj, S. Dhib-Jalbut, *J. Steroid Biochem.* **17**, 155 (1982).
7. M. X. Zuber, M. E. John, T. Okamura, E. R. Simpson, M. R. Waterman, *J. Biol. Chem.* **261**, 2475 (1986).
8. H. Okayama and P. Berg, *Mol. Cell. Biol.* **3**, 280 (1983).
9. Y. Gluzman, *Cell* **23**, 175 (1981).
10. K. Ichikara, E. Kusunose, M. Kusunose, *Biochim. Biophys. Acta* **239**, 178 (1971); A. Ellin, S. V. Jakobsson, J. B. Schenkman, S. Orrenius, *Arch. Biochem. Biophys.* **150**, 64 (1972); S. Jakobsson, H. Thor, S. Orrenius, *Biochem. Biophys. Res. Commun.* **39**, 1073 (1970).
11. J. G. Ghazarian, C. R. Jefcoate, J. C. Knutson, W. H. Orme-Johnson, H. F. DeLuca, *J. Biol. Chem.*

249, 3028 (1974); J. A. Kulkowski, T. Chan, J. Martinez, J. G. Ghazarian, *Biochem. Biophys. Res. Commun.* **90**, 50 (1979).
12. C. L. Litterist, E. G. Mimnaugh, R. L. Reagan, T. E. Gram, *Drug Metab. Dispos.* **3**, 259 (1975); J. H. Dees, B. S. S. Masters, U. Muller-Eberhard, E. F. Johnson, *Cancer Res.* **42**, 1423 (1982).
13. P. I. Mackenzie, *J. Biol. Chem.* **261**, 6119 (1986).
14. K. Oeda, T. Sakaki, H. Ohkawa, *DNA* **4**, 203 (1985); H. Murakami, K. Yabusaki, H. Ohkawa, *ibid.* **5**, 1 (1986).
15. L. M. Sompayrac and K. J. Danna, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7575 (1981).
16. H. Luthman and G. Magnusson, *Nucleic Acids Res.* **11**, 1295 (1983).
17. M. G. Roth, C. Doyle, J. Sambrook, M.-J. Gething, *J. Cell Biol.* **102**, 1271 (1986); J. F. Ash, D. Louvard, S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5584 (1977).
18. M. X. Zuber, E. R. Simpson, P. F. Hall, M. R. Waterman, *J. Biol. Chem.* **260**, 1842 (1985).
19. Supported by USPHS grants AM 28350 and HD 13234 and grant I-624 from the Robert A. Welch Foundation. M.X.Z. was supported in part by USPHS training grant 5-T 32HD07190. We thank L. Cortez for technical assistance, J. I. Mason for helpful conversations, Y. Gluzman for providing COS 1 cells, P. F. Hall for providing anti–pig testis P-450$_{17\alpha}$ IgG and pig adrenal P-450$_{17\alpha}$, and B. S. Masters for providing anti–pig liver cytochrome P-450 reductase IgG.

3 July 1986; accepted 16 September 1986

A G Protein Couples Serotonin and $GABA_B$ Receptors to the Same Channels in Hippocampus

R. ANDRADE, R. C. MALENKA, R. A. NICOLL*

Both serotonin and the selective γ-aminobutyric acid$_B$ ($GABA_B$) agonist, baclofen, increase potassium (K^+) conductance in hippocampal pyramidal cells. Although these agonists act on separate receptors, the potassium currents evoked by the agonists are not additive, indicating that the two receptors share the same potassium channels. Experiments with hydrolysis-resistant guanosine triphosphate (GTP) and guanosine diphosphate analogs and pertussis toxin indicate that the opening of the potassium channels by serotonin and $GABA_B$ receptors involves a pertussis toxin–sensitive GTP-binding (G) protein, which may directly couple the two receptors to the potassium channel.

MEMBRANE RECEPTORS OFTEN signal cellular responses via second messenger systems. One ubiquitous feature of such indirect coupling systems is their dependence on guanosine triphosphate (GTP)–binding proteins (G proteins) for the transmembrane signaling process. A number of distinct G proteins have been identified and some of the intracellular second messengers and effectors associated with their actions elucidated [for review, see (*1*)]. A great deal is now known about the role of these G proteins and assorted second messengers in controlling metabolic and secretory processes in a variety of cells. G proteins are present in extraordinarily high concentrations in the mammalian central nervous system (CNS) (*2*); for example, G_O accounts for 1% of membrane protein. Moreover, on the basis of the GTP sensitivity of the receptor binding of neurotransmitter agonists (*3, 4*) it is believed that G proteins are associated with many types of brain neurotransmitter receptors. However, little is known about the functional roles these G proteins play in controlling neuronal excitability and, hence neuronal communication in the CNS. Here we demonstrate that serotonin (5-HT) and γ-aminobutyric acid$_B$ ($GABA_B$) receptors modulate the same potassium channels through a mechanism that involves a pertussis toxin–sensitive G protein. We suggest, as has been proposed for muscarinic inhibition of the heart (*5*), that G proteins may directly couple receptors to the K^+ channels in mammalian brain.

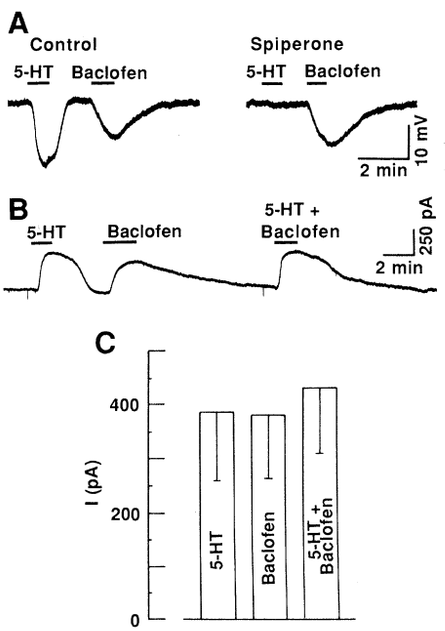

Fig. 1. 5-HT and baclofen hyperpolarize pyramidal cells by separate receptors but through a common conductance mechanism. (A) The hyperpolarization to 5-HT (10 μ*M*), but not to baclofen (10 μ*M*), is abolished by application of spiperone (5 μ*M*) for 20 minutes. The drugs were added to the superfusion medium and were applied by switching the inflow to the drug-containing medium. The resting membrane potential was −58 mV. (B) Continuous voltage clamp record of the outward currents evoked by 5-HT (30 μ*M*) and baclofen (30 μ*M*) applied in the bath alone or together. Note that the response evoked by the combined application of the two drugs is no larger than that evoked by 5-HT alone. (C) Bar graph of responses, such as those in (B), recorded from five cells. The bracket represents the standard deviation for the responses.

The methods we have used here are similar to those used in other studies from our laboratory (*6*). Rat hippocampal slices, 400-μm thick, were cut and placed in a holding chamber for at least 1 hour. A single slice was then transferred to the recording chamber and held between two nylon nets, submerged beneath a continuously superfusing medium that had been gassed with 95% O_2 and 5% CO_2. The composition of this medium was 119 m*M* NaCl, 2.5 m*M* KCl, 1.3 m*M* $MgSO_4$, 2.5 m*M* $CaCl_2$, 1.0 m*M* NaH_2PO_4, 26.2 m*M* $NaHCO_3$, and 11.0 m*M* glucose. The temperature of the medium was maintained between 29 and 31°C.

Application of either 5-HT (*7*) or baclofen (*8*), a $GABA_B$ agonist (*9*), to CA1 pyramidal cells, causes large hyperpolarizations, which are due to a selective increase in K^+ conductance; neither of the responses desensitizes with repeated applications. Although both compounds increase K^+ conductance, they do so via distinct receptors. There are no antagonists for $GABA_B$ receptors, but spiperone blocks responses to 5-HT, which are mediated by a 5-HT1a receptor (*10*). Concentrations of spiperone that abolish responses to 5-HT have no effect on responses to baclofen (Fig. 1A), indicating that these two agonists act through different receptors. If the increase in K^+ conductance evoked by these two receptors resulted from entirely separate mechanisms one would expect that the maximal currents generated by the two agonists would summate. This is not the case (Fig. 1, B and C). When near maximal concentrations of 5-HT and baclofen are applied together, the current is no larger than that produced by 5-HT alone. The results of five experiments are summarized in Fig. 1C, and indicate that the size of the maximal responses to the two agonists are similar and that there is no additivity between the currents activated by the two receptors. These findings indicate that 5-HT and baclofen act on different receptors, but control the same conductance.

5-HT1a agonists (*11*) and baclofen (*12*) inhibit adenylate cyclase. It is therefore pos-

Departments of Pharmacology and Physiology, University of California, San Francisco, CA 94143.

*To whom correspondence should be sent.