skin hyperpigmentation, and growth and development, and are thus generally consistent with an acquired (neuro)ectodermal dysplasia. The acne present at birth and persistent in some children is a specific effect of the class of polycyclic, polyhalogenated hydrocarbons, but may also be a part of the apparent effects on ectodermal structures. The increased frequency of bronchitis may be due to a specific pulmonary lesion, which has been seen in adults (*12*) and children (*11*) exposed to this class of agents, or due to a more generalized immune disorder (*13, 14*). The developmental effects are consistent with those seen in rhesus monkeys exposed transplacentally (*15*), and the behavioral problems may be secondary to the developmental delay or a form of direct toxicity (*16*).

These children have been exposed only by transplacental passage of the chemicals or by breast milk exposure. It is impossible to separate cleanly effects that persist because of structural changes during the fetal period from those that persist because of continued internal exposure. Transplacental passage of the chemicals has been documented in autopsy studies (*10*), and it is reasonable to suspect that the chemicals will persist in the children. There were metabolic changes in the placentae of some of these children (*17*) and a few have mild hepatic porphyria (*18*).

The kinds of toxicities seen are consistent with PCBs, but the exposures are relatively low. The children of workers exposed to PCBs uncontaminated by polychlorinated dibenzofurans (PCDFs) do not show nearly so much toxicity, but the mothers achieve blood PCB levels that are comparable to those seen in the outbreaks (*19*). The most likely reason is the presence of the very toxic PCDFs (*2*) in the cooking oil. Qualitatively, the PCBs and PCDFs are similar in toxicity, but the PCDFs are active at much lower doses. The oil in Taiwan had about 100 ppm PCBs, and about 0.1 ppm PCDFs (*20*). Although there has not been a human exposure to PCDFs in the absence of PCBs, it is reasonable to assume that much of the toxicity seen in both outbreaks is due at least in part to PCDF contamination.

REFERENCES AND NOTES

1. S-T Hsu *et al.*, *Environ. Health Perspect.* **59**, 5 (1985).
2. Y. Masuda, H. Kuroki, T. Yamaryo, K. Haraguchi, M. Kuratsune, S-T Hsu, *Chemosphere* **11**, 199 (1982).
3. P. C. Kahn *et al.*, *J. Am. Med. Assoc.* **259**, 1661 (1988).
4. M. Rutter, J. Tizard, K. Whitmore, in *Education, Health, and Behaviour: Psychological and Medical Study of Childhood Development* (Wiley, New York, 1970), pp. 412–21.
5. W. J. Rogan, *Teratology* **26**, 259 (1982).
6. I. Funatsu *et al.*, *Kurume Med. J.* **19**, 43 (1972).
7. I. Funatsu *et al.*, *Fukuoka Acta Med.* **62**, 139 (1971).
8. M. Harada, *Bull. Inst. Const. Med. Kumamoto Univ.* **25** (suppl.), 1 (1976).
9. K-C Wong and M-Y Hwang, *Clin. Med. (Taipei)* 7, 83 (1981).
10. S-J Lan, S-Y Tang, Y-C Ko, *Kaohsiung J. Med. Sci.* **3**, 64 (1987).
11. K-L Law, B-T Hwang, I-S Shaio, *Clin. Med. (Taipei)* 7, 88 (1981).
12. N. Shigematsu *et al.*, *Environ. Res.* **16**, 92 (1978).
13. K-J Chang, K-H Hsieh, T-P Lee, T-C Tung, *ibid.* **28**, 329 (1982).
14. K-J Chang, K-H Hsieh, S-Y Tang, T-C Tung, *J. Toxicol. Environ. Health* **9**, 217 (1982).
15. R. E. Bowman, M. P. Heironimus, J. R. Allen, *Pharmacol. Biochem. Behav.* **9**, 49 (1978).
16. R. E. Bowman and M. P. Heironimus, *Neurobehav. Toxicol. Teratol.* **3**, 15 (1981).
17. T-K Wong *et al.*, *Lancet* **i**, 721 (1985).
18. B. C. Gladen, W. J. Rogan, N. B. Ragan, F. W. Spierto, *Arch. Environ. Health* **43**, 54 (1988).
19. I. Hara, *Environ. Health Perspect.* **59**, 85 (1985).
20. P. H-S Chen, M-L Luo, C-K Wong, C-J Chen, *Am. J. Ind. Med.* **5**, 133 (1984).

21. We thank M-L Hwang, L. Huang, and J. Cwi, SRA, Baltimore; C-C Yeh, National Taiwan University Hospital; S-T Hsu, National Institute of Preventive Medicine, Taipei; M. Malison, Applied Epidemiology Training Program, Taipei; C-C Lin, Provincial Health Department, Taiwan; J-C Su, National Science Council, Taipei; (the late) J. D. Niswander and R. W. Miller of NIH. Supported by NIEHS contracts and by grant NSC74-0301-H002-29 from the National Science Council, Taiwan, ROC.

9 February 1988, accepted 24 May 1988

Molecular Cloning of Odorant-Binding Protein: Member of a Ligand Carrier Family

JONATHAN PEVSNER, RANDALL R. REED, PAUL G. FEINSTEIN, SOLOMON H. SNYDER*

Odorant-binding protein (OBP) is found in nasal epithelium, and it selectively binds odorants. Three complementary DNAs encoding rat odorant-binding protein have now been cloned and sequenced. One clone contains an open reading frame predicted to encode an 18,091-dalton protein. RNA blot analysis confirms the localization of OBP messenger RNA in the nasal epithelium. This OBP has 33 percent amino acid identity to α_2-microglobulin, a secreted plasma protein. Other members of an α_2-microglobulin superfamily bind and transport hydrophobic ligands. Thus, OBP probably binds and carries odorants within the nasal epithelium to putative olfactory receptors.

ANIMALS CAN DETECT SUBNANOMOLAR concentrations of odorants in ambient air despite a thousandfold lesser sensitivity of olfactory receptors to direct stimulation by odorants (*1*) and the requirement that the highly lipophilic odorants traverse a hydrophilic mucus to reach the receptors. A specific odorant-binding protein (OBP) may satisfy both these requirements (*2, 3*). A globular protein with a subunit molecular size of 20 kD, OBP is found in nasal glands and secreted into the nasal mucus where it has been detected by the binding of radiolabeled odorants. The OBP binds a variety of odorants including 2-isobutyl-3-methoxypyrazine, 3,7-dimethyloctan-1-ol, methyldihydrojasmonate, and amyl acetate (*4*). A pyrazine-binding protein, purified from bovine nasal epithelium (*3*), shares many physical properties with bovine OBP. We have sequenced 15 amino-terminal amino acids of bovine OBP. These match the partial sequence of the pyrazine-binding protein (*5*), confirming that the two proteins are the same. We now describe the cloning and sequence analysis of three cDNAs for the mRNA encoding rat OBP. We also show that OBP is part of a family of small homologous proteins, most of which appear to serve as carriers for small lipophilic molecules.

We utilized the binding of the odorants 2-isobutyl-3-[^{3}H]methoxypyrazine and 3,7-dimethyl-[^{3}H]octan-1-ol as an assay to purify rat OBP to homogeneity by DEAE-cellulose chromatography and reversed-phase high-performance liquid chromatography (HPLC) (*2, 4*). In reversed-phase HPLC only a single discrete peak of protein is apparent, and SDS–polyacrylamide gel electrophoresis reveals a single band of 20 kD (*2, 4*). Direct amino-terminal amino acid sequencing of the HPLC purified protein yields the sequence H_2N-Ala-His-His-Glu-Asn-Leu-Asp-Ile-Ser-Pro-Ser-Glu-Val-Asn-Gly-Asp. On the basis of the frequency of codon utilization (*6*), we constructed a mixed oligonucleotide probe (21-mer) containing 32 distinct sequences. We screened a rat olfactory cDNA library in λgt 10 (*7*) with the oligonucleotide and isolated 16 independent clones. Positive phages were subcloned into the plasmid vector Bluescript

J. Pevsner and S. H. Snyder, Departments of Neuroscience, Pharmacology and Molecular Sciences, Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205.
R. R. Reed and P. G. Feinstein, Laboratory of Genetics, Department of Molecular Biology and Genetics, Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

*To whom correspondence should be addressed.

(Stratagene) and sequenced by the Sanger random chain termination method (*8*). The complete sequence was determined for three of these clones.

We detected a long open reading frame that was predicted to encode a protein of 172 amino acids. The predicted ATG start codon at nucleotides 17 to 19 is surrounded by a consensus sequence typical of eukaryotic ribosomal start sites (*9*), CTAACATGG. This predicted initiator methionine is followed by a region of 14 amino acids, 13 of which are hydrophobic. The residues immediately following this presumed signal sequence correspond to the amino-terminal protein sequence indicated above. Eight amino acids of the amino-terminal sequence determined by amino acid sequence analysis were not encoded in the oligonucleotide probe. The fact that these eight amino acids in the cDNA correspond exactly to the sequence determined from amino acid sequence analysis confirms that the isolated cDNA represents OBP mRNA. Features of the determined sequence indicating that we isolated a cDNA clone containing a complete coding sequence include (i) a characteristic ribosome-binding sequence at the putative initiating methionine, (ii) a stop codon (TAA) at nucleotides 533 to 535, (iii) a polyadenylation signal (AATAAA) at nucleotides 737 to 742, and (iv) a polyadenylate sequence consisting of 43 consecutive adenine nucleotides at the 3′ end. Sequence analysis of a second clone reveals three single nucleotide substitutions compared to the initial clone sequence. All of these nucleotide changes occur in the 3′ untranslated region.

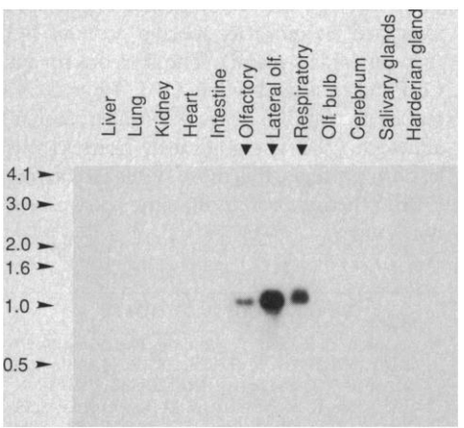

Fig. 1. RNA blot analysis. Total RNA was purified, treated with glyoxal, and separated by electrophoresis on a 1% agarose gel (*34*). RNA was transferred to Nytran (Schleicher & Schuell) and hybridized for 12 hours at 42°C with a ^{32}P-labeled OBP cDNA clone, washed twice for 20 min in 0.3*M* NaCl, 30 m*M* sodium phosphate, and 0.5% SDS [2× saline sodium citrate (SSC)] at 22°C, washed for 20 min in 0.5× SSC at 42°C, and analyzed by autoradiography. Molecular sizes (in kilobases) were determined from a glyoxal-treated 1-kb ladder (BRL) in adjacent lanes. Olfactory, olfactory epithelium; lateral olf., lateral olfactory epithelium including the lateral nasal glands; respiratory, nasal respiratory epithelium; olf. bulb, olfactory bulb. The blot was also hybridized to ribosomal RNA to confirm that each lane contained an equal amount of RNA.

OBP is selectively localized to the nose with no OBP detected in numerous other tissues by ^{3}H-labeled odorant binding (*2*) or by protein immunoblots with antibodies to OBP. To examine the tissue distribution of the mRNA for OBP, we conducted RNA blot analysis. Detectable signal is apparent only in nasal epithelium (Fig. 1). The largest amount of mRNA is detected in the lateral nasal gland (LNG), also known as Steno's gland (*10*). In situ hybridization also reveals a selective localization of OBP mRNA in the LNG (*11*). The OBP mRNA observed in olfactory and respiratory epithelium might reflect contaminating LNG tissue. The size of the OBP mRNA (about 1 kb) is 200 nucleotides larger than the cloned cDNA, possibly reflecting greater in vivo polyadenylation. The significance of the larger size of the mRNA in the respiratory epithelium compared to the LNG and the olfactory epithelium is not clear, but may also reflect different extents of polyadenylation.

Our observation that apparently homogeneous OBP can bind many odorants of widely varying chemical structure might suggest a microheterogeneity in OBP protein sequences with different forms of OBP binding different odorants. The two OBP cDNAs with different sequences imply that there are at least two genes in the OBP family. To estimate the size of the OBP gene family, we probed rat genomic DNA digests with full-length and 5′ fragments from an OBP cDNA (Fig. 2). The apparently identical bands seen with both probes support the notion that each hybridizing species represents a separate gene. This DNA blot analysis suggests a limited family size of about two to four members.

Computer analysis revealed a statistically significant similarity of OBP to a group of proteins with similar properties (Table 1 and Fig. 3) (*12, 13*). The *Z* score is a statistical measure of the relatedness of a protein sequence (OBP) to another protein relative to that other protein's sequence randomly shuffled (*14*). The *Z* scores above 3 may be significant, whereas scores above 10 are highly significant. The *Z* scores for OBP include α_2-microglobulin ($Z = 38.1$), major urinary protein ($Z = 26.0$), bovine OBP. ($Z = 22.4$), bovine β-lactoglobulin ($Z = 11.1$), and protein BG ($Z = 4.6$). Regions of similarity within the family of proteins related to OBP occur at several sites (Fig. 3) (*12, 13*). Two consensus sequences are apparent, beginning at amino acid residues 17 and 89 of mature OBP. The first sequence is Asn-hyd-acidic-hyd-X-basic-hyd-X-Gly-X-Trp-aro-X-hyd-hyd-hyd-hyd, where acidic is Asp or Glu, basic is Arg or Lys, hyd is a hydrophobic residue (Ile, Leu, Met, Phe, Tyr, or Val), aro is aromatic (Phe or Tyr), and X is any residue. The second consensus sequence is aro-X-hyd-hyd-X-Thr-Asp-Tyr-Asp-X-aro-hyd-hyd-hyd. While OBP is strongly related to members of the α_2-microglobulin superfamily overall, it lacks portions of both consensus sequences.

The structures of retinol-binding protein (*15*), β-lactoglobulin (*15*), and insecticyanin (*17*) have been determined by x-ray crystallography at high resolution. These three proteins are remarkably similar and contain an eight-stranded antiparallel β barrel motif that forms the core of the molecule. The first consensus sequence (containing Gly-X-Trp) occurs near the end of the first β barrel, while the second sequence (containing Thr-

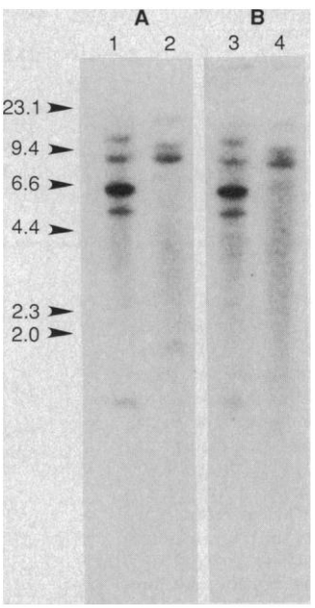

Fig. 2. DNA blot analysis. Genomic DNA was isolated from rat liver and digested for 10 hours at 37°C with Hinc II (lanes 2 and 4) or Bam HI (lanes 1 and 3) (New England Biolabs). DNA (15 μg per lane) was separated by electrophoresis on a 1% agarose gel and transferred to Nytran. The cDNA clone 800, which contains restriction sites for Bam HI and Hinc II at nucleotides 120 to 125 and 527 to 532, respectively, was digested with Hinc II to generate a 5′ fragment (nucleotides 41 to 570 of clone 8F3). Genomic DNA blots were hybridized for 12 hours at 65°C to ^{32}P-labeled clone 800 (**A**) or the 5′ cDNA fragment (**B**). Filters were washed twice for 15 min in 0.3*M* NaCl, 30 m*M* sodium phosphate, 0.1% SDS at room temperature, then twice for 10 min in 75 m*M* NaCl, 7.5 m*M* sodium phosphate, 0.1% SDS at 65°C, air-dried, and analyzed by autoradiography. Molecular sizes (in kilobases) were determined from λ/Hind III markers (BRL) in adjacent lanes.

Asp-Tyr) occurs near the sixth β sheet. None of the amino acids in the consensus sequences is in contact with the bound ligand.

All of the proteins in this family have a similar subunit molecular weight. Many of them bind and serve as carriers for small lipophilic molecules, while the function of some is undetermined. Insecticyanin binds the small lipophilic molecule biliverdin IX (*18*). Apolipoprotein D binds cholesterol and cholesteryl esters and has been proposed as a carrier for cholesterol (*19*). One of the best characterized of these proteins is retinol binding protein. It serves as a carrier for retinol, transferring retinol from plasma to the pigmented epithelium of the retina (*20*). Other related proteins then bind retinol intracellularly in the pigmented epithelium of the eye and transfer the retinol to the photoreceptors (*21*). Carrier proteins for retinol are necessary to transfer this extremely lipophilic molecule through various hydrophilic tissue spaces and to facilitate the incorporation of retinol into the opsin molecule (*21*).

We suggest that OBP functions in an analogous fashion to retinol binding protein, a notion consistent with the membership of OBP in a family of carrier proteins. By binding to OBP, lipophilic odorants could more readily traverse the hydrophilic environment of the nasal passages to reach the odorant receptors on the cilia of olfactory neurons. Our localizations of OBP mRNA by in situ hybridization (*11*) and OBP itself by immunohistochemistry (*4, 11, 22*) suggest a mechanism for these apparent functions of OBP. Rat OBP and its mRNA are localized to the lateral nasal glands on the lateral wall of the nasal passages, just anterior to the olfactory turbinates (*11*). These glands elaborate a mucus, secreted through ducts into the tip of the nose as an atomized spray permitting maximal contact with volatile odorants (*10*).

We first identified OBP by its binding of radiolabeled odorants (*2*). In a different approach to identifying olfactory-specific proteins, Lee and co-workers (*23*) screened a cDNA library from frog olfactory epithelium with radiolabeled polyadenylated RNA from olfactory or respiratory epithelium. They selected olfactory-specific clones and identified an olfactory-specific protein, BG (for Bowman's gland). The *Z* score for rat OBP relative to bovine OBP ($Z = 22.4$) and to protein BG ($Z = 4.6$) indicates that, although OBP is significantly homologous to both proteins, it is more similar to bovine OBP. Whether protein BG binds odorants is not known.

Table 1. Physical properties of the ligand-binding carrier family. MW, molecular weight; AA, number of amino acids; N.D., not determined.

Protein	MW	AA	Species, tissue	Ligand	Reference
OBP	18,091	172	Rat nasal epithelium	Odorants	(*2–4*)
α_2-Microglobulin	18,709	162	Rat liver	Unknown	(*24*)
Major urinary protein	18,730	162	Mouse liver	Unknown	(*25*)
Protein BG	20,300	182	Frog olfactory epithelium	Unknown	(*23*)
α_1-Acid glycoprotein	18,944	167	Human urine	Drugs, steroids	(*25*)
Retinol binding protein	22,868	199	Human liver	Retinol	(*13, 27*)
Apolipoprotein D	19,300	169	Human plasma	Cholesterol	(*19*)
Insecticyanin	21,382	189	*Manduca sexta* (tobacco hornworm) hemolymph	Biliverdin IX	(*18*)
Androgen-dependent secretory protein	18,500	184	Rat epididymis	Unknown	(*28*)
Protein HC; α_1-microglobulin	20,619	182	Human plasma, urine, spinal fluid	Unknown	(*29*)
β-Lactoglobulin	18,281	162	Bovine whey	Retinol, butane	(*30, 31*)
Purpurin	21,924	196	Chick retina	Retinol, heparin	(*32*)
Pregnancy-associated endometrial α_2-globulin	25,000	N.D.	Human placenta	Unknown	(*33*)

Fig. 3. Amino acid sequence alignments of rat OBP and 13 homologous proteins. The amino acid residues of OBP are numbered. Initial alignments were made with the DFASTP program of Lipman and Pearson (*14*) on a VAX/VMS computer; final alignment was manual. Abbreviations and sources of protein sequence data are A2M, α_2-microglobulin (*24*); MUP, mouse major urinary protein (*25*); OBP (b), bovine OBP (*5*); BBLG, bovine β-lactoglobulin (*30*); HC, protein of heterogeneous charge (*29*); BG, protein of Bowman's gland (*23*); PEG, pregnancy-associated endometrial α_2-globulin (*34*); ADSP, androgen-dependent secretory protein (*28*); PURP, purpurin (*32*); RBP, retinol binding protein (*13, 27*); APOD, apolipoprotein D (*19*); A1AG, α_1-acid glycoprotein (*26*); and THI, tobacco hornworm insecticyanin (*18*). Abbreviations of the amino acids are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The amino acid residues are shaded where seven or more residues in a column are either identical, acidic (Asp or Glu), basic (Arg or Lys), aromatic (Phe or Tyr), or hydrophobic (Ala, Ile, Leu, Met, Phe, Trp, Tyr, Val). Two consensus sequences are boxed.

REFERENCES AND NOTES

1. U. Pace, E. Hanski, Y. Salomon, D. Lancet, *Nature* **316**, 255 (1985); P. B. Sklar, R. R. H. Anholt, S. H. Snyder, *J. Biol. Chem.* **261**, 15538 (1986).
2. J. Pevsner, R. R. Trifiletti, S. M. Strittmatter, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3050 (1985).
3. E. Bignetti *et al.*, *Eur. J. Biochem.* **149**, 227 (1985).
4. J. Pevsner, P. B. Sklar, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4942 (1986).
5. A. Cavaggioni, R. T. Sorbi, J. N. Keen, D. J. C. Pappin, J. B. C. Findlay, *FEBS Lett.* **212**, 225 (1987).
6. R. Lathe, *J. Mol. Biol.* **183**, 1 (1985).
7. U. Gubler and B. H. Hoffman, *Gene* **25**, 263 (1983).
8. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977). The nucleotide sequences of the rat OBP clones have been submitted to the GenBank, Los Alamos National Laboratory, Los Alamos, NM 87545. The accession number for rat OBP is J03093.
9. M. Kozak, *Nucleic Acids Res.* **12**, 857 (1984).
10. F. Bojsen-Møller, *Anat. Rec.* **150**, 11 (1964).
11. J. Pevsner, P. Hwang, P. B. Sklar, J. C. Venable, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2383 (1988).

12. S. Pervaiz and K. Brew, *Science* **228**, 335 (1985); L. Sawyer, *Nature* **327**, 659 (1987); J. Godovac-Zimmermann, *Trends Biochem. Sci.* **13**, 64 (1988).
13. S. Pervaiz and K. Brew, *FASEB J.* **1**, 209 (1987).
14. D. J. Lipman and W. R. Pearson, *Science* **227**, 1435 (1985).
15. M. E. Newcomer *et al.*, *EMBO J.* **3**, 1451 (1984).
16. M. Z. Papiz *et al.*, *Nature* **324**, 383 (1986).
17. H. M. Holden, W. R. Rypniewski, J. H. Law, I. Rayment, *EMBO J.* **6**, 1565 (1987).
18. C. T. Riley *et al.*, *J. Biol. Chem.* **259**, 13159 (1984).
19. D. Drayna *et al.*, *ibid.* **261**, 16535 (1986).
20. L. Rask *et al.*, *Scand. J. Clin. Lab. Invest.* **40**, 45 (1980).
21. S. Ottonello, S. Petrucci, G. Maraini, *J. Biol. Chem.* **262**, 3975 (1987).
22. F. Avanzini *et al.*, *Cell Tissue Res.* **247**, 461 (1987).
23. K.-H. Lee, R. G. Wells, R. R. Reed, *Science* **235**, 1053 (1987).
24. R. D. Unterman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3478 (1981).
25. A. J. Clark, P. M. Clissold, R. A. Shawi, P. Beattie, J. Bishop, *EMBO J.* **3**, 1045 (1984).
26. G. A. Ricca and J. M. Taylor, *J. Biol. Chem.* **256**, 11199 (1981).
27. V. Colantuoni *et al.*, *Nucleic Acids Res.* **22**, 7769 (1983).
28. D. E. Brooks, A. R. Means, E. J. Wright, S. P. Singh, K. K. Tiver, *J. Biol. Chem.* **1**, 4956 (1986).
29. T. Takagi, K. Takagi, T. Kawai, *Biochem. Biophys. Res. Commun.* **98**, 997 (1981); C. Lopez, A. Grubb, F. Soriano, E. Mendez, *ibid.* **103**, 919 (1981).
30. G. Preaux, G. Braunitzer, B. Schrank, A. Stangl, *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1595 (1979).
31. C. Tanford, *The Hydrophobic Effect: Formation of Micelles and Biological Membranes* (Wiley, New York, 1980).
32. P. Berman *et al.*, *Cell* **51**, 135 (1987).
33. S. C. Bell and J. W. Keyte, *Nature* **329**, 296 (1987); S. C. Bell, J. W. Keyte, G. T. Waites, *J. Clin. Endocrinol. Metab.* **65**, 1067 (1987).
34. P. Thomas, *J. Clin. Endocrinol. Metab.* 77, 5201 (1980).
35. We thank C. Riley for assistance in purifying and sequencing the proteins; E. Barbosa for providing the cDNA library; M. Amzel, S. Bedarkar, P. Hwang, and P. Sklar for helpful discussions; and N. Bruce for manuscript preparation. Supported by a grant from International Flavors and Fragrances, Inc. J.P. and S.H.S. are respective recipients of NIH training grant GM-07626 and Research Scientist Award DA-00074. R.R. is supported in part by a grant from the National Cancer Institute (P01 CA16519-13).

29 December 1987; accepted 13 May 1988

$GABA_A$-Receptor Function in Hippocampal Cells Is Maintained by Phosphorylation Factors

ARMIN STELZER, ALAN R. KAY,* ROBERT K. S. WONG

Gamma aminobutyric acid (GABA) mediates fast synaptic inhibition in the central nervous system by activating the chloride-permeable $GABA_A$ channel. The $GABA_A$ conductance progressively diminishes with time when the intracellular contents of hippocampal neurons are perfused with a minimal intracellular medium. This "run down" of the GABA-activated conductance can be prevented by the inclusion of magnesium adenosine triphosphate and calcium buffer in the intracellular medium. The amount of chloride conductance that can be activated by GABA is determined by competition between a calcium-dependent process that reduces the conductance and a phosphorylation process that maintains the conductance.

THE REDUCTION OF GABA-MEDIATED synaptic inhibition by pharmacological agents (*1*) or tetanic stimulation of afferent fibers (*2, 3*) causes convulsive discharges in the hippocampus. Inhibition may be reduced in the short term by intracellular accumulation of Cl^-, desensitization of the GABA receptor (*4, 5*), or elevation of intracellular calcium ($[Ca^{2+}]_i$) (*6*). There is little information on the long-term regulation of the GABA conductance.

A number of voltage-gated (*7*) and ligand-gated channels (*8*) are regulated by protein phosphorylation. The operation of such a mechanism may be signaled by the lability of the channel when recording from cells in the whole-cell mode of patch clamping (*9*); on penetrating the cell, small diffusible molecules and ions are rapidly lost (within 1 to 2 min). When the intracellular medium is supplemented with nucleotides and buffers to control the divalent ion concentration, it is sometimes possible to maintain channel activity (*10*). We used this approach to attempt to define the intracellular conditions necessary for the stability of the $GABA_A$ conductance in hippocampal neurons.

Acutely dissociated hippocampal neurons were prepared from 650-μm slices of the CA1 subfield of guinea pig hippocampus by trypsin digestion (*11*). Neurons were dissociated and used 3 to 10 hours after removal of the brain. Whole-cell voltage clamp was used to monitor the current response to GABA (50 to 200 μ*M*) applied by pressure ejection. The intracellular recording pipette contained 130 m*M* tris methanesulfonate; 10 m*M* Hepes; 10 m*M* BAPTA {[1,2-bis(2-aminophenoxy)ethane - *N,N,N′,N′′* - tetraacetic acid]}; 0.1 m*M* leupeptin (a Ca^{2+}-activated neutral protease inhibitor); *p*H, 7.3. We used the Ca^{2+} chelator BAPTA because it is *p*H-insensitive and binds Ca^{2+} faster than EGTA (*12*). The external solution contained 120 m*M* NaCl; 5 m*M* CsCl; 2 m*M* $CaCl_2$; 1 m*M* $MgCl_2$; 15 m*M* tetraethylammonium chloride; 5 m*M* 4-aminopyridine; 10 m*M* Hepes; 25 m*M* D-glucose; *p*H, 7.4. This solution was applied at a rate of 5 ml/min, close to the cell to prevent agonist-induced desensitization. All experiments were performed at 18° to 22°C.

Current responses to GABA were elicited in CA1 pyramidal neurons by pressure ejection (pulse duration 20 to 80 ms) at a low frequency (0.016 Hz) to avoid cumulative desensitization. The current was completely blocked by 10^{-6} μ*M* picrotoxin and reversed at the Cl^- equilibrium potential, which suggests it resulted from the activation of the $GABA_A$ receptor (*13*). The peak amplitude of the GABA-activated outward current (at a holding potential of −10 mV) progressively declined to less than 10% within 10 min of penetrating the cell (Fig. 1A), in spite of the presence of 10 m*M* intracellular BAPTA to prevent accumulation of $[Ca^{2+}]_i$ (*6, 11*). Short voltage pulses (−10 mV, 10 ms) were applied before eliciting GABA responses to monitor the leak conductance and input capacitance. A short hyperpolarizing voltage pulse (−10 mV, 10 ms) was applied at a fixed point during the current response to assess the conductance increase and the GABA rever-

Table 1. Effect of intracellular contents on GABA response in acutely dissociated and cultured hippocampal cells. GABA currents were elicited approximately 1 min after entry into the whole-cell recording mode and every 1 min thereafter. Duration of GABA pulses was 20 to 80 ms and was constant for a given experiment. The intracellular solution had the composition specified in text with concentration of Mg^{2+}, ATP, or BAPTA as indicated in the table. The rate of run down was quantified by measuring the percentage of current remaining 10 min after penetration of the cell.

ATP (m*M*)	Mg^{2+} (m*M*)	BAPTA (m*M*)	Percent GABA current ± SEM remaining after 10 min (*n*)
Acutely dissociated neurons			
			9.8 ± 3.9 (8)
0	0	10	33.0 ± 7.4 (10)
2	4	10	130.9 ± 14.7 (12)
	4	10	6.1 ± 1.2 (12)
2	0	10	16.5 ± 2.8 (10)
2	4		21.8 ± 5.9 (6)
*1 mM Ca^{2+}**			
2	4	1†	23.6 ± 7.0 (7)
2 mM AMP-PCP			
0	4	10	22.5 ± 6.0 (10)
Cultured rat hippocampal cells			
0	4	10	54.1 ± 11.3 (9)
2	4	10	116.7 ± 9.3 (4)

*$[Ca^{2+}]_i$ = 5 μ*M*. †EGTA.

Department of Neurology and Department of Physiology and Cellular Biophysics, College of Physicians and Surgeons, Columbia University, New York, NY 10032.

*Present address: AT&T Bell Laboratories, 1C456, 600 Mountain Avenue, Murray Hill, NJ 07974.