Identification of Specific Transducin α Subunits in Retinal Rod and Cone Photoreceptors

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Transducin is a guanyl nucleotide-binding protein that couples rhodopsin photolysis to hydrolysis of guanosine 3′,5′-monophosphate in rod photoreceptor cells of vertebrate retinas. Several complementary DNA clones encoding transducin subunits have recently been characterized. One clone, isolated from a bovine retina complementary DNA library, encodes a previously unidentified polypeptide with an amino acid sequence 78% identical to the sequence of the α subunit of bovine rod outer segment transducin. Antibodies to a synthetic peptide with amino acid sequence derived specifically from this novel polypeptide recognize a 41-kilodalton polypeptide in homogenates of bovine retina. Localization of this polypeptide in bovine retina by indirect immunofluorescence demonstrates that it is expressed only in cone outer segments. Antibodies to specific sequences found only in the rod transducin α subunit recognize a polypeptide localized only in the rod outer segment. Therefore, bovine rod and cone cells each express structurally related yet significantly different forms of transducin.

THE MECHANISM OF PHOTOTRANSduction in vertebrate retinal rod photoreceptor cells has recently been clarified by several biochemical and physiological observations. Light photolyzes rhodopsin, stimulates guanosine 3′,5′-monophosphate (cyclic GMP) hydrolysis, and ultimately hyperpolarizes the rod outer segment plasma membrane (*1*). Rhodopsin photolysis is coupled to cyclic GMP metabolism through transducin, a guanosine 5′-triphosphate (GTP)–binding protein that activates a cyclic GMP phosphodiesterase (*2, 3*). The resulting changes in cyclic GMP metabolism hyperpolarize the photoreceptor cell through specific cyclic GMP–sensitive cation channels in the rod outer segment (ROS) plasma membrane (*4*).

The phototransduction mechanism of cone photoreceptors is less well understood. Cyclic GMP–regulated cation channels and cyclic GMP phosphodiesterases have been detected in cones (*5, 6*). Recent studies have suggested that transducin is present only in rods but that related forms of transducin may also be present in cones (*7*). Here we demonstrate that a recently isolated complementary DNA (cDNA) clone (*8*) encodes a form of transducin α subunit that is expressed in cones but not in rods of the bovine retina.

Transducin is a heterotrimeric protein with a 39-kD α subunit that binds GTP and activates cyclic GMP phosphodiesterase (*3*). The α subunit is also a substrate for adenosine 5′-diphosphate (ADP) ribosylation catalyzed by both cholera toxin and pertussis toxin (*9, 10*). The 36-kD β subunit and the 8-kD γ subunit form a complex that is required for transducin to interact with photolyzed rhodopsin (*11*).

Transducin is a member of a family of guanyl nucleotide-binding regulatory proteins referred to as G proteins (*12*). Other well-characterized members of this family include G_s and G_i, which regulate adenylate cyclase, and G_o, a G protein with unknown function. Each of these proteins has a heterotrimeric structure similar to transducin. The α subunits of G_s, G_i, and G_o are distinct, whereas the β subunits appear to be identical on the basis of peptide maps, amino acid composition, and immunoreactivity (*13, 14*). The γ subunits of G_i, G_s, and G_o appear to be identical but they are different from the γ subunit of transducin (*14*).

Homologies were recently reported between cDNA clones corresponding to the α subunits of bovine transducin, G_i, and G_s (*8, 15, 16*). The amino acid sequence of the transducin α subunit is 60% identical to the sequence of the G_i α subunit and 43% identical to the G_s α subunit. Four short sequences are well conserved in the primary structure of G protein α subunits and in other guanyl nucleotide-binding proteins, including *ras* and elongation factors (*17*). These sequences form the guanyl nucleotide-binding site in elongation factor EF-Tu, and also appear to play a similar role in *ras* proteins and in G proteins (*18*).

Complementary DNA clones thought to correspond to the transducin α subunit were independently isolated from bovine retinal cDNA libraries by four different laboratories. Three of these clones (*15*) are identical and encode a polypeptide with an amino acid sequence identical to sequences deter-

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Tα I   1" MGAGASAEEK----HSRELEKKLKEDAEKDARTVKLLLLGAGESGKSTIVKQMKIIHQDG
          ::.::::::.:    .:.:::::::.:::.:.:.:::::::::::::::::::::::::::
Tα II  1' MGSGASAEDKELAKRSKELEKKLQEDADKEAKTVKLLLLGAGESGKSTIVKQMKIIHQDG
                  region I

      57" YSLEECLEFIAIIYGNTLQSILAIVRAMTTLNIQYGDSARQDDARKLMHMADTIEEGTMP
          :: ::::::. :::::::.:::::::.::: :: :.:.. .  :..:.: ..:::.:::::::
      61' YSPEECLEYKAIIYGNVLQSILAIIRAMPTLGIDYAEVSCVDNGRQLNNLADSIEEGTMP
                                          region II

                                                              cholera toxin
     117" KEMSDIIQRLWKDSGIQACFDRASEYQLNDSAGYYLSDLERLVTPGYVPTEQDVLRSRVK
           :. ..:..:::::.:.::::::::.::::::::.:::..:.:...: :.: ::::::::::
     121' PELVEVIRKLWKDGGVQACFDRAAEYQLNDSASYYLNQLDRITAPDYLPNEQDVLRSRVK
           region III                                region IV

     177" TTGIIETQFSFKDLNFRMFDVGGQRSERKKWIHCFEGVTCIIFIAALSAYDMVLVEDDEV
          ::::::::.:: ::::::::::::::::::::::::::::::: ::::::::::::::::
     181' TTGIIETKFSVKDLNFRMFDVGGQRSERKKWIHCFEGVTCIIFCAALSAYDMVLVEDDEV

     237" NRMHESLHLFNSICNHRYFATTSIVLFLNKKDVFSEKIKKAHLSICFPDYNGPNTYEDAG
          ::::::::::::::::..::.::::::::::.: :::::.::::::.:.: :.:::::
     241' NRMHESLHLFNSICNHKFFAATSIVLFLNKKDLFEEKIKKVHLSICFPEYDGNNSYEDAG

                                                          pertussis toxin
     297" NYIKVQFLELNMRRDVKEIYSHMTCATDTQNVKFVFDAVTDIIIKENLKDCGLF
          :::: :::.:::::.::::::::::::::::::::::::::::::::::::::
     301' NYIKSQFLDLNMRKDVKEIYSHMTCATDTQNVKFVFDAVTDIIIKENLKDCGLF
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Fig. 1. Comparison of the amino acid sequences encoded by the $T_\alpha I$ (*15*) and $T_\alpha II$ (*8*) cDNA clones. Regions where the sequences are most divergent are designated I to IV. The cholera and pertussis toxin ADP-ribosylation sites are also shown. Identical amino acids (:) and functionally homologous amino acids (·) are indicated.

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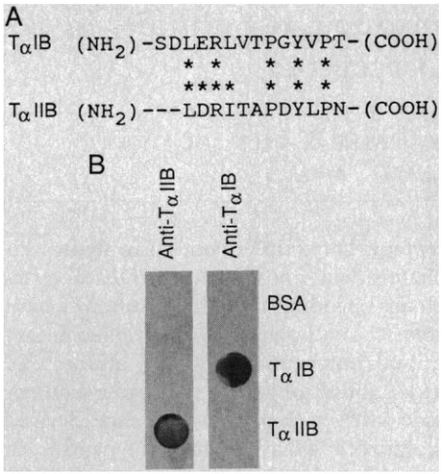

Fig. 2. (A) Synthetic peptides used to generate antibodies that specifically recognize either the T_αI or the T_αII polypeptides. The peptides were coupled to BSA with glutaraldehyde (*23*). An additional cysteine (not shown) was included at the amino terminal of peptide T_αIB to use if an alternative coupling method were required. (B) Specificity of polyclonal antibodies to peptides T_αIB and T_αIIB. BSA or BSA-peptide conjugate (10 μg) was dotted onto nitrocellulose as shown. The dot blots were treated with antibodies purified on the basis of affinity to their respective peptides and incubated with alkaline phosphatase–conjugated goat antibody to rabbit IgG (Cappel Laboratories). The blots were developed with nitro blue tetrazolium and 5-bromo-3-indoyl phosphate to detect bound antibodies.

mined from tryptic fragments of the purified ROS transducin α subunit (*19*). The sequence deduced for the ROS transducin α subunit is labeled T_αI in Fig. 1. A fourth cDNA clone, also isolated from a bovine retinal cDNA library (*8*), encodes a slightly different polypeptide, T_αII (Fig. 1). This polypeptide is only 78% homologous to T_αI, with most of the differences clustered in four separate regions labeled I to IV.

The polypeptide encoded by T_αII cDNA is more similar to the rod transducin α subunit (T_αI) than to other G proteins. The T_αII polypeptide is 78% identical to T_αI, 58% identical to $G_{i\alpha}$, and only 38% identical to $G_{s\alpha}$ (*16*). Furthermore, the amino acids immediately surrounding the cholera toxin ADP-ribosylation site in transducin (Arg^{174} in T_αI) (*20*) are nearly identical in T_αII. Sequences of T_αI and T_αII that surround the pertussis toxin ADP-ribosylation site (Cys^{347} in T_αI) (*21*) are also identical. Primary structures of other G proteins in these regions are significantly different (*16*).

T_αI and T_αII cDNA clones hybridize with messenger RNA transcripts from bovine retina on Northern blots and not with RNA from bovine brain, liver, kidney, spleen, or heart when high stringency conditions are used (*22*). T_αII cDNA recognizes 5.5- and 6-kb transcripts in retina, whereas T_αI cDNA recognizes a significantly more abundant 2.3-kb retinal transcript.

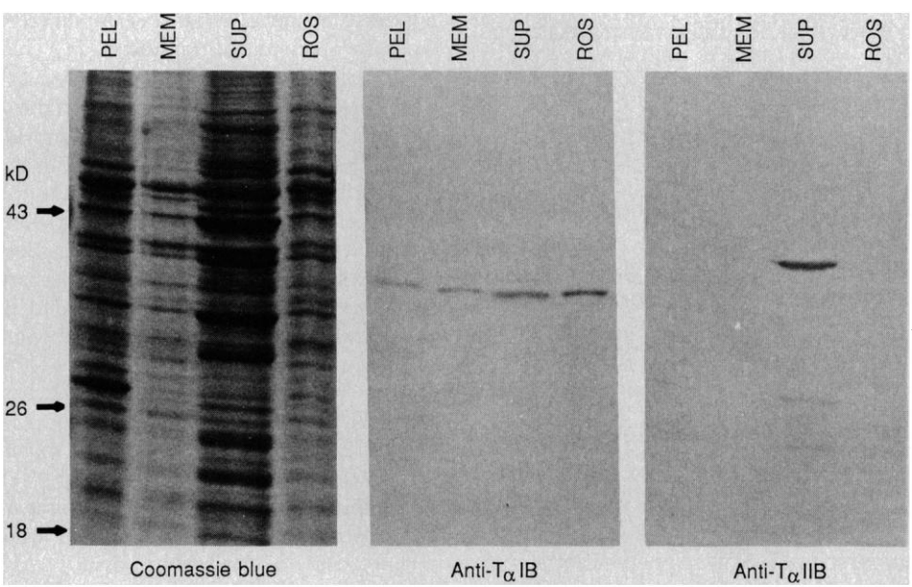

Fig. 3. Identification of the polypeptides encoded by the T_αI and T_αII cDNA clones. Fifty bovine retinas were stirred in sucrose solution–ROS buffer (*3*) (final density, 1.13) and the suspension was fractionated by centrifugation at 16,000*g* for 30 minutes in an HB-4 rotor (Dupont). ROS were collected as a paste on top of the sucrose solution and most of the retinal membranes were collected as a pellet (PEL) at the bottom of the tube. The sucrose solution containing soluble proteins (SUP) and lower density membranes (MEM) was further fractionated by 2× dilution in ROS buffer and centrifuged. Portions of each fraction corresponding to 0.4% of a retina were separated by electrophoresis on three 12% SDS polyacrylamide gels, and one gel was stained with Coomassie brilliant blue. The other two gels were electroblotted onto nitrocellulose. Each blot was treated with the designated antibody purified by peptide affinity and bound antibody was detected with affinity-purified alkaline phosphatase–conjugated goat antibody to rabbit IgG.

To localize the T_αI and T_αII polypeptides in retina, antibodies were raised that recognize short synthetic polypeptides with sequences derived from T_αI or T_αII (Fig. 2A). These polypeptides correspond to region IV near the cholera toxin ADP-ribosylation site on transducin. The polypeptides differ from each other at 7 of 12 overlapping positions.

The synthetic polypeptides were conjugated to bovine serum albumin (BSA) and injected into rabbits (*23*). The polyclonal antibodies produced were affinity-purified by the use of appropriate peptides coupled to cyanogen bromide–activated Sepharose. Each antibody recognizes the appropriate peptide without detectable cross-reactivity (Fig. 2B).

Affinity-purified antibodies to T_αIB and T_αIIB (anti-T_αIB and anti-T_αIIB) recognize specific polypeptides from bovine retinas. Bovine retinas were fractionated to separate ROS membranes from cytoplasmic proteins and other membranes. Equivalent amounts of each fraction were analyzed by SDS–polyacrylamide gel electrophoresis (Fig. 3) (*24*). Identical gels were either stained for protein with Coomassie brilliant blue or immunoblotted with affinity-purified antibodies to the peptides.

Anti-T_αIB detects a 39-kD polypeptide in ROS membranes and in other fractions (Fig. 3). This immunoreactive polypeptide copurifies with transducin α subunit upon further purification by GTP analog–specific elution from photolyzed ROS membranes (*25*). In contrast, the affinity-purified anti-T_αIIB detects a 41-kD polypeptide found primarily in the soluble protein fraction (Fig. 3). Weakly reactive lower molecular weight bands are also detected on the blots shown in Fig. 3. The intensities of these bands has varied in different preparations; therefore, these bands may represent proteolytic fragments of the 39- and 41-kD proteins. A weak band at 39 kD in ROS membranes is sometimes detected with antibody to T_αIIB. The relation between this protein and the major 39- and 41-kD immunoreactive polypeptides is uncertain. The affinity-purified antibodies do not cross-react with immunoblotted tryptic fragments of purified G_i or G_o.

Polypeptides immunoreactive to anti-T_αIB and to anti-T_αIIB were localized cytologically by indirect immunofluorescence on cryostat sections of bovine retina (*26*). Localization of the affinity-purified antibodies was determined with fluorescein-conjugated goat antibody to rabbit immunoglobulin G (IgG) as the secondary antibody. A control section of bovine retina treated with nonimmune serum from a rabbit that was later injected with peptide T_αIB (Fig. 4A) shows a bright yellow-gold autofluorescence of li-

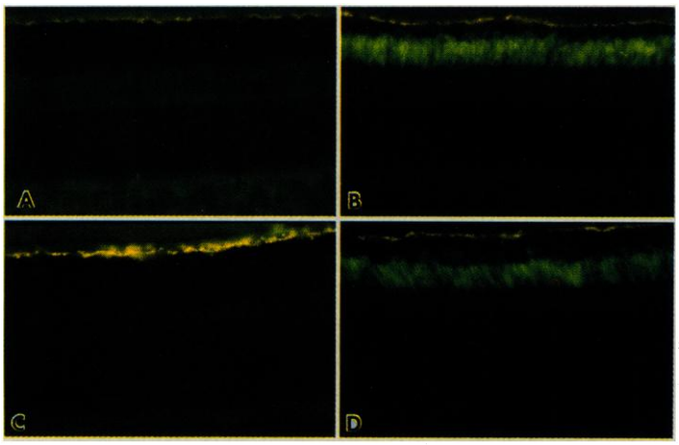

Fig. 4 (left). Localization of anti-T_αIB binding in bovine retina by indirect immunofluorescence. Magnification, ×150. (A) Section treated with nonimmune serum from a rabbit later injected with peptide T_αIB. The retinal pigment epithelium layer at the top of the figure shows yellow-gold autofluorescence due to lipofuscin granules. The retina and choroid show weak green background fluorescence. Nonspecific staining with the FITC-secondary antibody is highest in (A) because this preimmune serum was not affinity-purified with protein A prior to use on sections. (B) Affinity-purified anti-T_αIB binds specifically to the long, thin outer segments of the rod photoreceptors (ROS) but not to cone outer segments. (C) Prior incubation of the primary anti-T_αIB antibody with a tenfold molar excess of T_αIB peptide prevents specific staining of ROS. (D) Prior incubation of anti-T_αIB with a tenfold molar excess of T_αIIB peptide does not block anti-T_αIIB binding to ROS.

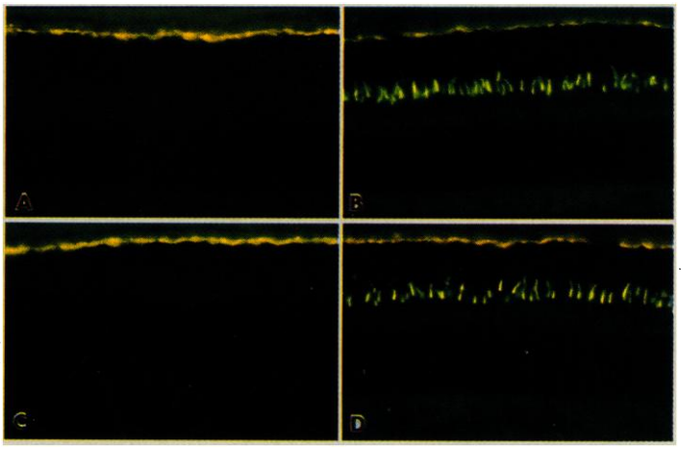

Fig. 5 (right). Localization of anti-T_αIIB binding in bovine retina by indirect immunofluorescence. Magnification, ×150. (A) Section treated with IgG fraction from preimmune serum from a rabbit later injected with peptide T_αIIB. The green background fluorescence is weaker than in Fig. 4A because the IgG fraction was used rather than whole serum. (B) Affinity-purified anti-T_αIIB binds specifically to the short, conical outer segments of cone photoreceptors but not to rod outer segments. (C) Prior incubation of the primary anti-T_αIIB with 100 times molar excess of T_αIIB blocks specific staining of cone outer segments. Twenty times molar excess of peptide was sufficient to block the anti-T_αIIB signal in most experiments. (D) Prior incubation of the primary antibody to T_αIIB with 100 times molar excess of T_αIB peptide does not interfere with anti-T_αIIB binding to cone outer segments.

pofuscin granules in the retinal pigment epithelium and weak nonspecific green background fluorescence in the retina and choroid. In other control experiments, no specific immunostaining was obtained with serum from another rabbit that was injected with a synthetic peptide–adjuvant mixture corresponding to a nonretinal protein.

Affinity-purified anti-T_αIB, binds specifically to the long, thin outer segments of rod photoreceptors (Fig. 4B). The anti-T_αIB immunoreactive 39-kD transducin α subunit is therefore localized to ROS, in agreement with previous reports (*7*). Prior incubation of the primary anti-T_αIB with excess T_αIB peptide prevents subsequent binding to ROS (Fig. 4C), whereas prior incubation with excess T_αIIB peptide does not prevent anti-T_αIB binding to ROS (Fig. 4D).

Similar indirect immunofluorescence experiments were performed with affinity-purified anti-T_αIIB. A section treated with nonimmune serum from a T_αIIB rabbit exhibits normal autofluorescence of the retinal pigment epithelium and background staining of the retina after incubation with the fluorescein-labeled secondary antibody (Fig. 5A). Affinity-purified anti-T_αIIB binds specifically to the short, conical outer segments of cones and not to ROS (Fig. 5B). The 41-kD polypeptide immunoreactive with anti-T_αIIB, therefore, appears to be a cone-specific form of transducin α subunit. Preliminary incubation with excess T_αIIB peptide prevents anti-T_αIIB from binding to cone outer segments (Fig. 5C), whereas preliminary incubation with excess T_αIB peptide does not interfere with specific anti-T_αIIB binding (Fig. 5D).

On the basis of these results, the TαI cDNA clone encodes a 39-kD transducin α subunit, $T_{r\alpha}$, that is found only in rods, whereas the T_αII cDNA clone encodes a cone-specific 41-kD transducin α subunit, $T_{c\alpha}$. Immunoblot analysis of purified preparations of transducin (*3*) detects almost entirely $T_{r\alpha}$, with little or no $T_{c\alpha}$. Therefore, most biochemical characterizations of bovine transducin have been performed with preparations containing predominantly T_r. Purification and biochemical characterization of T_c are now possible with the antibody reagents described here. Direct amino acid sequence analysis of the purified 41-kD protein is also necessary to confirm its identity as the polypeptide encoded by the T_αII cDNA clone.

Several lines of evidence suggest that rods and cones use similar but not identical biochemical pathways to respond to light. The photosensitive pigments of human rods and cones show significant homology in domains that are thought to interact with transducin (*27*). Cyclic GMP phosphodiesterase is present in both rod and cone outer segments, although several lines of evidence indicate that the two molecules have structural differences (*6*). In addition, a cyclic GMP–dependent cation conductance similar to the rod conductance has been identified in cone outer segments (*5*). However, clear morphological and physiological differences exist between rods and cones. Detailed biochemical comparisons of rod and cone transducins should help to elucidate mechanisms that underlie these differences.

REFERENCES AND NOTES

1. L. Stryer, *Annu. Rev. Neurosci.* **9**, 87 (1986); W. A. Hagins, R. D. Penn, S. Yoshikami, *Biophys. J.* **10**, 380 (1970); T. Tomita, *Q. Rev. Biophys.* **3**, 179 (1970).
2. M. W. Bitensky, R. E. Gorman, W. H. Miller, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 561 (1971); R. Yee and P. A. Liebman, *J. Biol. Chem.* **253**, 8902 (1978).
3. B. K.-K. Fung, J. B. Hurley, L. Stryer, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 152 (1981).
4. E. E. Fesenko, S. S. Kolesnikov, A. L. Lyubarsky, *Nature (London)* **313**, 310 (1985); K.-W. Yau and K. Nakatani, *ibid.* **317**, 252 (1985).
5. L. Haynes and K. W. Yau, *ibid.*, p. 61; W. H. Cobbs, A. E. Barkdoll, E. N. Pugh, *ibid.*, p. 64.
6. R. L. Hurwitz, A. H. Bunt-Milam, J. A. Beavo, *J. Biol. Chem.* **259**, 8612 (1984); R. L. Hurwitz, A. H. Bunt-Milam, M. Chang, J. A. Beavo, *ibid.* **260**, 568 (1985).
7. G. B. Grunwald, P. Gierschik, M. Nirenberg, A. Spiegel, *Science* **231**, 856 (1986); T. van Veen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 912 (1986); Y. Fukada and T. Akino, *Photobiochem. Photobiophys.*, in press.
8. M. L. Lochrie, J. B. Hurley, M. I. Simon, *Science* **228**, 96 (1985).
9. M. E. Abood, J. B. Hurley, M. C. Pappone, H. R. Bourne, L. Stryer, *J. Biol. Chem.* **257**, 10540 (1982).
10. C. Van Dop *et al.*, *ibid.* **259**, 23 (1984).
11. T. Shinozawa *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1408 (1980); B. K.-K. Fung, *J. Biol. Chem.* **258**, 10495 (1983).
12. A. G. Gilman, *Cell* **36**, 577 (1984).
13. D. R. Manning and A. G. Gilman, *J. Biol. Chem.* **258**, 7059 (1983).
14. P. Gierschik, J. Codina, C. Simons, L. Birnbaumer, A. M. Spiegel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 727 (1985).
15. T. Tanabe *et al.*, *Nature (London)* **315**, 242 (1985);

D. C. Medynski *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4311 (1985); K. Yatsunami and G. Khorana, *ibid.*, p. 4316.
16. T. Nukada *et al.*, *FEBS Lett.* **197**, 220 (1986); T. Nukada *et al.*, *ibid.*, p. 305.
17. K. R. Halliday, *J. Cyclic Nucleotide Prot. Phosphoryl. Res.* **9**, 435 (1984).
18. F. Jurnak, *Science* **230**, 32 (1985); F. M. McCormick *et al.*, *ibid.*, p. 78.
19. J. B. Hurley, M. I. Simon, D. B. Teplow, J. D. Robishaw, A. G. Gilman, *ibid.* **226**, 860 (1984).
20. C. Van Dop, M. Tsubokawa, H. R. Bourne, J. Ramachandran, *J. Biol. Chem.* **259**, 696 (1984).
21. D. R. Manning, B. A. Fraser, R. A. Kahn, A. G. Gilman, *ibid.*, p. 749; R. E. West *et al.*, *ibid.* **260**, 14428 (1985).
22. R. Miake-Li and C. Raport, unpublished results.
23. Peptides were synthesized with an Applied Biosystems automated peptide synthesizer and cleaved from the solid-phase resin with either hydrofluoric acid or trifluoromethane sulfonic acid. Amino acid analysis was carried out on each peptide to determine its purity. Approximately 1 μmol of each peptide was conjugated to 0.15 μmol of BSA by dropwise addition of 0.4 μmol of fresh glutaraldehyde, incubated for 1 hour at room temperature, and then dialyzed extensively against phosphate-buffered saline (PBS). An emulsion of 400 μg of conjugate with an equal volume of Freund's complete adjuvant was injected intradermally in the backs of two New Zealand White rabbits. Rabbits were boosted with 100 μg of peptide-BSA conjugates with incomplete Freund's adjuvant at 2 weeks, bled at 3 weeks, boosted again at 4 weeks, and then bled every 2 weeks. Immunization of each rabbit used for a given peptide caused production of antibodies with identical specificity. Peptide-Sepharose resins were prepared by the manufacturer's recommended method with 6.5 mg of pure peptide and 0.7 g of cyanogen bromide–activated Sepharose 4B (Pharmacia). Antisera were affinity-purified by incubation with peptide-Sepharose overnight. Bound antibodies were then eluted with 100 m*M* glycine (*p*H 3.0) into 100 m*M* tris (*p*H 7.5).
24. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
25. H. Kuhn, *ibid.* **283**, 587 (1980).
26. Bovine eyes were obtained immediately after death of the animal and fixed as eye cups for 6 hours at room temperature in 4% formaldehyde in 0.13*M* phosphate buffer (*p*H 7.4). The retinas were dissected and stored overnight in 30% sucrose in 0.13*M* phosphate buffer at 4°C. Cryostat sections were cut at 16 μm, mounted on glass slides, and dried at room temperature. Rubber rings (10-mm diameter) were mounted with fingernail polish on slides to form incubation wells over the sections. The sections were first treated for 30 minutes with 1% horse serum in tris-buffered saline (TBS) containing 0.3% Triton X-100 at room temperature, drained without rinsing, and incubated overnight at 4°C with the nonimmune (diluted 1:50), specific (20 μg/ml affinity-purified antibody), or adsorbed antiserum, each in TBS containing 0.3% Triton X-100 and 3% BSA. The sections were rinsed twice with phosphate buffer (15 minutes each), and incubated for 30 minutes at room temperature in dim red light with fluorescein isothiocyanate (FITC)–goat antibody to rabbit IgG (Cappel Laboratories) diluted 1:50 in PBS with 0.3% Triton X-100. The sections were rinsed twice with phosphate buffer and then covered with a drop of glycerol containing 2% DABCO (Sigma) and 10% phosphate buffer and then a glass cover slip. The sections were photographed immediately with Ektachrome color film (ASA 400) with a Zeiss microscope equipped for epifluorescence. For further details see (*6*).
27. J. Nathans, D. Thomas, D. S. Hogness, *Science* **232**, 193 (1986).
28. Supported by the Howard Hughes Medical Institute and NIH grants EYO 1311 (A.H.B.-M.) and CORE grant EYO 1730. A.H.B.-M. is a William and Mary Greve International Scholar of Research to Prevent Blindness, Inc. We are grateful to P. Chou and J. Scott for peptide synthesis; R. Wade for amino acid analysis; B. Clifton, P. Siedlak; and R. Jones for photographic help; F. Dahlan for technical assistance, and J. Beavo and J. Saari for helpful discussions.

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Stimulation of Neuronal Acetylcholine Receptors Induces Rapid Gene Transcription

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Cholinergic agonists rapidly and transiently induced transcription of the c-*fos* proto-oncogene and one or more actin genes in neuronally differentiated PC12 cells. Transcription was activated within minutes after stimulation of the nicotinic acetylcholine receptor and required an influx of extracellular Ca^{2+} ions through voltage-sensitive calcium channels. Nicotine activation proceeded by a different pathway from activation by nerve growth factor, whose stimulation of these genes is independent of extracellular Ca^{2+} ions. These findings suggest that neurotransmitters may rapidly activate specific gene transcription in nondividing neuronally differentiated cells. They also suggest a functional role for neurotransmitter induction of c-*fos* and actin expression in the nervous system.

ALTHOUGH THE BEST CHARACTERized function of neurotransmitters is the regulation of transsynaptic communication in the central and peripheral nervous systems, neurotransmitters also regulate longer term changes in the cellular properties of neurons and other postsynaptic cells. Well-studied examples of this include the transsynaptic regulation over a period of several days of levels of neuropeptide synthesis (*1*) and of synthetic enzymes such as tyrosine hydroxylase (*2*), the latter by a mechanism dependent on new RNA transcription (*3*). We report here that activation of cholinergic receptors also causes extremely rapid induction of specific gene expression. We show that two genes, the c-*fos* proto-oncogene and actin, which have previously been shown to be activated by growth factors (*4–8*), are induced within minutes after nicotinic agonists bind to their receptors on nondividing neuronally differentiated PC12 cells. Induction by nicotine and nerve growth factor (NGF) show distinctly different dependencies upon flux of extracellular Ca^{2+} into the cell, indicating different mechanisms for c-*fos* induction through the neurotransmitter and growth factor receptors. These results suggest that rapid gene activation in nondividing, neuronally differentiated cells may be closely coupled to neurotransmitter stimulation.

For these studies we used the pheochromocytoma cell line PC12 (*9–11*), which expresses both nicotinic and muscarinic acetylcholine receptors (*10, 12, 13*). PC12 cells differentiate to nondividing, sympathetic neuron–like cells when treated with NGF for several days (*9, 10*). In response to this treatment they become electrically excitable (*11*) and exhibit increased levels of both types of acetylcholine receptor (*12, 13*). Exposure of neuronally differentiated PC12 cells to 100 μ*M* nicotine, an agonist of nicotinic acetylcholine receptors, resulted in a tenfold increase in c-*fos* transcription within 5 minutes (Fig. 1, A and B) as measured by a nuclear run-off transcription assay (*4, 14*). Actin transcription was also rapidly induced but to a lesser extent. The activation of c-*fos* transcription by nicotine was transient; it was maximal within 5 to 15 minutes after drug addition and decreased to the level present in unstimulated cells within 30 to 60 minutes (Fig. 1, A and B). While the kinetics of induction and repression of c-*fos* and actin transcription in response to nicotine were very similar to those previously reported in response to growth factors such as epidermal growth factor and NGF in PC12 cells (*15–17*) or platelet-derived growth factor in 3T3 fibroblast cells (*4–8*), induction by nicotine is mediated through a distinct pathway. The stimulation of c-*fos* and actin by nicotine is specific inasmuch as this cholinergic agonist does not significantly affect the expression of several other genes including α-tubulin (Fig. 2) and c-*raf*, a proto-oncogene (*18*) that encodes a protein with sequence homology to tyrosine kinases (Fig. 1A). The level of transcription of another growth factor–inducible nuclear proto-oncogene, c-*myc* (*4, 16, 19, 20*), appears unaffected within the first hour after treatment with nicotine (Fig. 1A). However, a slight stimulation of c-*myc* transcription

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