ous symptoms in as yet unaffected family members.

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21. Control samples were from unrelated maternal lineages. These included 10 blacks (2 Africans, 1 HeLa cell line, and 7 American blacks), 10 Asians (9 Chinese and 1 Japanese), 14 Caucasians (Europeans), and 11 Finns (paternal lineage controls from Finnish pedigrees 1 to 4). Finnish pedigrees 1, 2, and 6 of this study are pedigrees A, D, and B, respectively, in (*3*). Patients from the five Finnish pedigrees are individual VII1 of pedigree 1; individuals V5, V7, V8, V12, V18, VI6, VI7, and V3 of pedigree 2 (Fig. 3); and the probands of pedigrees 3, 4, and 5. We tested individuals IV4 and IV17 from the discordant Finnish pedigree 6.
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26. Additional base substitutions (Cambridge to LHON) included two structural (G to A at nt1438, in the 12*S* rRNA, and G to A at nt2706, in the 16*S* rRNA) and 15 synonymous mutations: G to T, nt3423; A to G, nt4169; T to C, nt6221; C to T, nt6587; C to T, nt7028; T to C, nt9540; T to C, nt10370; A to G, nt10819; T to C, nt10873; T to C, nt11335; C to T, nt12705; A to T, nt13893; T to C, nt14152; T to C, nt14212; and A to C, nt15256. Peripheral lymphocytes from III5 (Fig. 1) were isolated by Ficoll-Hypaque gradients and transformed with Epstein-Barr virus (*8*). Mitochondria were isolated by differential centrifugation, and the mtDNA was purified by two sequential CsCl–ethidium bromide gradients [R. E. Giles, I. Stroynowski, D. C. Wallace, *Somatic Cell Genet.* **6**, 543 (1980)]. Purified mtDNA was digested with Taq I or various combinations of Bam HI, Eco RI, Hind III, Pst I, or Xba I, and the fragments were cloned into M13. MtDNA fragments were identified by hybridization with regional probes [(*17*); J. E. Garrison, E. Hardeman, R. Wade, L. Kedes, P. Gunning, *Gene* **38**, 177 (1985)] and partial sequencing. All sequencing was done by the dideoxy chain termination procedure [F. Sanger, A. R. Coulson, B. G. Barrell, A. J. H. Smith, B. A. Roe, *J. Mol. Biol.* **143**, 161 (1980)]. Sequences read and compiled [C. Queen and L. J. Korn, *Nucleic Acids Res.* **12**, 581 (1984)] were nt 1220–3899, 3945–5269, 5275–8005, 8284–13610, 13625–14260, and 14803–15260. Abbreviations for the amino acid residues 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.
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31. We thank R. P. Erickson (Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI), L. I. Grossman (Department of Molecular Biology and Human Genetics, Wayne State University School of Medicine, Detroit, MI), H. A. Gardner (Oshawa General Hospital, Oshawa, Ontario), H. H. Kazazian, Jr. (Department of Pediatrics, Johns Hopkins University Hospital, Baltimore, MD), E. J. Novotny, Jr. (Department of Neurology, Yale University Medical School, New Haven, CT), and R. Spector (Atlanta, GA) for contributing LHON pedigree samples to this study. We also acknowledge the technical assistance of T. Horton. Supported by a grant from the Sigrid Juselius Foundation (E.N.), and an NIH grant (NS21328) and an Emory University Pediatric Research Foundation grant (D.C.W.).

5 July 1988; accepted 22 September 1988

Cyclic AMP–Responsive DNA-Binding Protein: Structure Based on a Cloned Placental cDNA

JAMES P. HOEFFLER,* TERRY E. MEYER, YUNGDAE YUN, J. LARRY JAMESON,† JOEL F. HABENER

Cyclic AMP (cAMP) is an intracellular second messenger that activates transcription of many cellular genes. A palindromic consensus DNA sequence, TGACGTCA, functions as a cAMP-responsive transcriptional enhancer (CRE). The CRE binds a cellular protein of 38 kD in placental JEG-3 cells. A placental λgt11 library was screened for expression of specific CRE-binding proteins with the CRE sequence as a radioactive probe. A cDNA encoding a protein of 326 amino acids with the binding properties of a specific CRE-binding protein (CREB) was isolated. The protein contains a COOH-terminal basic region adjacent to a sequence similar to the "leucine zipper" sequence believed to be involved in DNA binding and in protein-protein contacts in several other DNA-associated transcriptional proteins including the products of the c-*myc*, c-*fos*, and c-*jun* oncogenes and GCN4. The CREB protein also contains an NH_2-terminal acidic region proposed to be a potential transcriptional activation domain. The putative DNA-binding domain of CREB is structurally similar to the corresponding domains in the phorbol ester–responsive c-*jun* protein and the yeast transcription factor GCN4.

ACTIVATION OF SECOND MESSENGER pathways by hormones results in the transcriptional stimulation of many cellular genes (*1*). Phorbol esters (for example, 12-O-tetradecanoylphorbol 14-acetate, TPA) and cAMP activate the protein kinase C- and A-signaling pathways, respectively. Similar consensus DNA regulatory elements are involved in the stimulation of gene transcription by these agents (*2, 3*); the octameric cAMP-response element (CRE, 5′-TGACGTCA-3′) differs from the heptameric TPA-response element (TRE, 5′-TGAGTCA-3′) by only a single base. Earlier studies suggested that transcriptional stimulation by both cAMP and TPA can be mediated through a common DNA sequence present in the 5′ regulatory region of the enkephalin gene, 5′-TGCGTCA-3′ (*3*). However, a DNA-binding protein of 47 kD (AP-1 or c-Jun) was isolated and shown to mediate TPA but not cAMP induction of SV40 gene transcription through a mechanism involving sequence-specific binding to the TRE motif (*4*). Similarly, a 43-kD protein (CREB) was identified that binds to a CRE sequence in the 5′ regulatory region of the rat somatostatin gene (*5*). Utilizing in vitro mutagenesis, we have recently demonstrated that the cAMP- and phorbol ester–activated transcriptional responsiveness of these related DNA elements can be dissociated by the addition or subtraction of a single base, and further, that different DNA-binding proteins interact with these related

Laboratory of Molecular Endocrinology, Massachusetts General Hospital, and Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02114.

*To whom correspondence should be addressed.
†Current address: Thyroid Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114.

CRE and TRE sequences (*6*).

Analysis of the cloned cDNA for the human proto-oncogene c-*jun* revealed a DNA-binding protein with structural and functional properties of AP-1, suggesting that AP-1 may be c-Jun (*7*). This finding has provided opportunities for more extensive studies of the relation between signal transduction mediated by protein kinase C and oncogenes. To further our understanding of CREB, we used a screening technique recently described by Singh and co-workers (*8*) to isolate a cDNA encoding an expressed protein that binds specifically to the CRE recognition site.

A primary screening of a human placental expression library with a synthetic CRE duplex probe yielded 23 positive recombinant phage plaques. After plaque purification through four successive screenings, only five positive clones remained. A recombinant phage that did not bind the radioactive probe was also plaque-purified as a negative control. To establish the specificity of the binding of the radioactive probes, we utilized an array of synthetic oligonucleotide duplexes for which transcriptional activities and protein-binding characteristics have been elucidated (*6, 9*). These duplex DNAs fall into four groups (*10*). The first group consists of CRE sequences that contain the 8-bp palindrome 5′-TGACGTCA-3′, flanked by several bases that are known to be permissive for both transcriptional activity and specific protein binding (*6, 9*). The second group consists of the identical oligonucleotide duplexes in which the core 8-bp element was mutated by the deletion of a single base to form the phorbol ester–responsive sequence 5′-TGAGTCA-3′. Although structurally very similar to CREs, these sequences exhibit functional and binding properties that allow exclusion of recombinant phage expressing TRE-binding proteins and other proteins that may recognize the CRE/TRE motifs nonspecifically. The third group corresponds to "inactive CREs." These oligonucleotide duplexes contain the CRE motif 5′-TGACGTCA-3′, but are flanked by the sequences that are not permissive for either cAMP-stimulated gene transcription or specific protein binding to the CRE. The final group contained general DNA-binding specificity controls, including two known cis-acting regulatory elements present in the 5′ flanking region of the α-gonadotropin gene (URE and CAAT), as well as a mixture of two noncomplementary synthetic oligonucleotides included as controls to screen for single-stranded DNA-binding proteins.

Two of the five recombinant phages initially identified fulfilled all of the binding criteria specific to the native CREB protein from JEG-3 human choriocarcinoma cells (Fig. 1); they bound specifically to all active CREs and did not bind to the other sequences. Analysis of the cDNA inserts from these two phages (4_1 and G_1) indicated that they contained identical 2.4-kb DNA inserts and probably represent duplicates of the same phage. Confirmation that the β-galactosidase fusion protein in the bacterial lysogen extract was responsible for the binding to the radioactive CRE probe was obtained by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analyses of proteins in the lysogen extracts to which the labeled CRE probe was cross-linked with ultraviolet light. The CRE probe tagged specifically a fusion

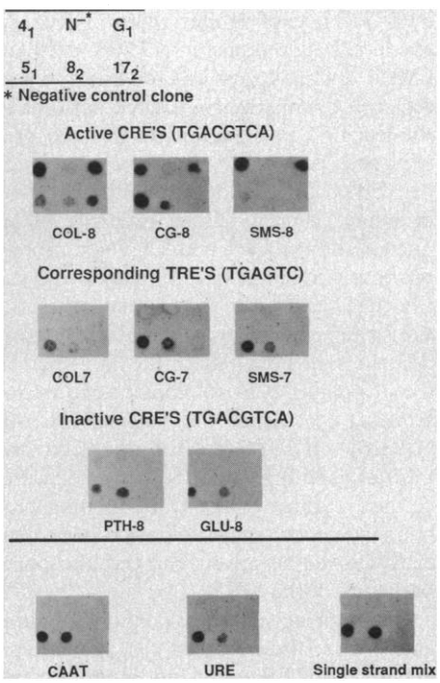

Fig. 1. Detection and specificity of CRE binding to a positive recombinant fusion protein in a λgt11 expression library containing human placental cDNA. Five microliters of six concentrated plaque-purified phage clones (4_1, N^-, G_1, 5_1, 8_2, and 17_2) were spotted onto freshly poured lawns of Y1090 host cells positioned as indicated in the legend at the top. After a 12-hour incubation at 37°C, large single plaques of each clone had formed, and isopropyl β-D-thiogalactopyronoside–induced proteins were bound to nitrocellulose filters. Separate filters from multiple identical plates were probed as described (*8*) with the following radioactive duplex oligonucleotides (*10*). Only the sense strand is listed here, and the C in parentheses represents the single base difference between the CREs and TREs (the C is present in the oligonucleotides with −8 suffixes and absent in the corresponding -7 oligonucleotides). Conserved bases are shown in capital letters: COL-7 and COL-8 [5′-gatccggcTGA(C)GTCAtcaagcta-3′]; CG-7 and CG-8 [5′-gatccaaatTGA(C)GTCAtggtaa-3′]; SMS-7 and SMS-8 [5′-gatccttggcTGA(C)GTCAgagagaga-3′]; PTH-8 [5′-gatccggagTGACGTCAtctgtaa-3′]; GLU-8 [gatccggctcattTGACGTCAaaattcattca-3′]; CAAT [5′-GATCCACCAAGTACCCTTCAATCATTGGATGGA-3′]; and URE [5′-GATCCAAACAAAAATGACCTAAGGGTTGAAACAAGATAA-3′]. Single strand mix was a mixture of two noncomplementary synthetic oligonucleotides from above. The plaque in the top center position denoted N^- corresponds to a nonbinding control clone carried through the cloning procedure. The cDNA library produced from human placental tissue (34 weeks) was obtained from Clontech Laboratories, Inc., Palo Alto, CA. The oligo dT-primed mRNA produced 1.0×10^6 independent clones in λgt11. The inserts ranged from 0.8 to 3.6 kb and averaged 1.8 kb in size. We screened 1×10^6 plaques from the amplified library.

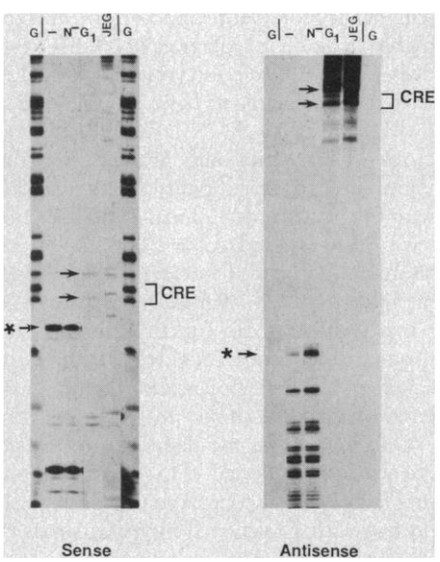

Fig. 2. Exonuclease III protection footprinting of the CRE by DNA-binding activity in lysogen extracts of phage G1. The radioactive probe used consisted of a CRE flanked by the native sequences found surrounding this element in the somatostatin gene linked to a 144-bp fragment of the α-gonadotropin gene promoter extending from −100 to +44 (*6*). Both sense and antisense DNA strands were 5′ end-labeled with ^{32}P and cut with a restriction endonuclease; the single end–labeled DNAs were isolated by electrophoresis on 4% polyacrylamide gels. Binding reactions with lysogen and JEG-3 whole-cell extracts were performed as described previously for gel-shift assays (*6, 9*). The radioactive probes in the presence of cell extracts were then exposed to 100 units of exonuclease III for 10 min at 37°C. The final radioactive products were analyzed by electrophoresis on 8% sequencing gels. Fragments cleaved at guanine (*6*) flank each set of lanes, and the other lanes are labeled at the top. (−) no extract, (N^-) negative recombinant phage lysogen extract, (G_1) G_1 recombinant phage lysogen extract, (JEG) JEG-3 whole cell extract. Seventy-five micrograms of each extract was used for binding reactions. The position of the CRE on each strand is denoted with a boxed area, the major sites of blockade to the progressive exonuclease III digestion are marked with arrows, and the natural half-stops corresponding to completed digestion of one of the two strands of the duplex DNA are indicated by starred arrows.

protein fragment of 140 kD that also stained with antiserum to β-galactosidase indicating that the CRE-binding domain of the expressed placental protein was contiguous with the β-galactosidase (*11*).

To demonstrate that the fusion protein bound specifically to the CRE element in the context of a cellular promoter, we performed a footprint analysis (*12*) with a DNA construct consisting of the CRE oligonucleotide duplex from somatostatin joined to the promoter sequence of the α-gonadotropin gene at position −100 (*6*). The bacterial lysogen extract and extracts of JEG-3 cells provided similar protection of the CRE (Fig. 2).

The amino acid sequence of 326 residues (equivalent to a molecular size of 35,044 daltons) deduced from the nucleotide sequence of the subcloned cDNA shows several structural features characteristic of DNA-binding transcription factors belonging to a new class recognized as "leucine zipper" proteins (*13*) (Fig. 3). This class of proteins includes Myc, Fos, C/EBP, GCN4, and c-Jun (*13*). A hypothetical leucine zipper sequence in which four leucines are spaced seven residues apart is located near the COOH-terminus of the protein. A computer search for sequence similarities between CREB and c-Jun revealed a single region of 61% identity of amino acids (19 of 31 residues) between positions 270 and 300 of CREB and 254 and 284 of c-Jun. These regions of similarity are located adjacent to the leucine zipper regions of the two proteins and constitute basic domains in which over 50% of the residues are either arginine or lysine. There is also a similarity of sequence in this region with GCN4, a protein previously noted to have similarity to c-Jun (*7*). It is tempting to speculate that the similarities of sequences limited to this basic domain somehow reflect the fact that all these proteins bind to similar palindromic sequences; either 5′-TGACGTCA-3′ (CREB) or 5′-TGAGTCA-3′ (c-Jun and GCN4). The high positive charge densities of these regions of the DNA-binding proteins would be compatible with close contact with the negatively charged phosphate backbone of the DNA.

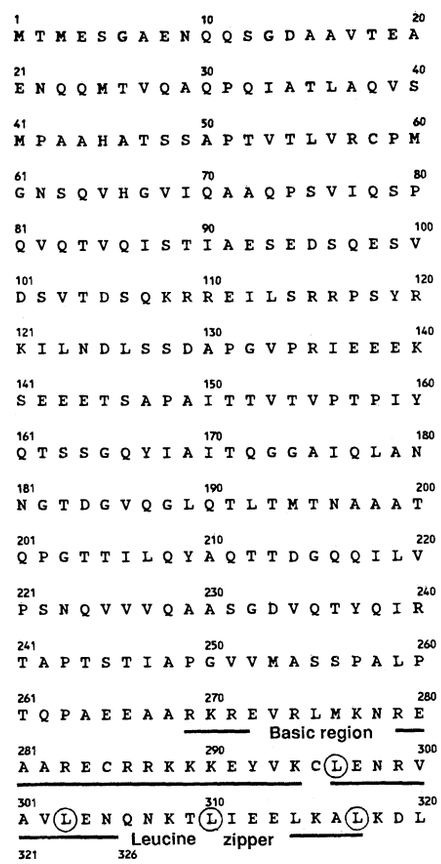

Fig. 3. Primary structure of CREB. The basic region and the putative leucine zipper sequence located at the COOH-terminus of the protein are underlined. The periodic array of leucine residues (circled) spaced seven residues apart would form the hypothetical α helix involved in protein-protein contacts (*13*). Preliminary evidence indicates that the methionine at position 1 is the translational start site in vivo. Amino acid sequence is in single letter code (*18*). The full DNA sequence is being deposited at GenBank; it is available from the authors on request.

Although no additional regions of similarity were discerned for the primary sequence of CREB and c-Jun, by the criteria of Garnier *et al.* and Kyte and Doolittle (*14*), comparison of the predicted secondary structures showed that the zipper regions at the COOH-terminus of the two proteins consist entirely of α helix (*13*). However, the remainder of the sequences located NH_2-terminal to the basic domains of both proteins are predominantly random coil and are highly acidic. The sequences of CREB (residues 1 to 268) and c-Jun (residues 1 to 225) have ratios of acidic to basic residues of 2.3 and 2.0, respectively (*15*). These acidic regions of transcriptional proteins may be important activator regions for interactions with the basic transcriptional machinery and have been referred to as "acid blobs" or "negative noodles" to describe the conformationally poorly defined structure of a polypeptide that can function almost irrespective of sequence, provided that there are a sufficient number of acidic residues clustered or scattered about (*16*).

On the basis of the deduced protein sequence of this cloned cDNA, we propose that the cDNA encodes a full-length CREB protein with a calculated molecular mass of 35,044 daltons. This conclusion is consistent with our finding by Southwestern blot analyses of a 38-kD CREB protein present in extracts of JEG-3 human choriocarcinoma cells, assuming that the cellular protein is post-translationally modified (*11*). The apparent discrepancy in size between this human placental CREB of 38 kD and the 43-kD CREB identified in rat adrenal cells (PC-12) by Montminy and Bilezikjian (*5*) could be due to species-specific differences in primary structure, posttranslational modifications, or the existence of multiple CREB proteins that are part of a larger family of CREB transcriptional activators. Recent reports have suggested that a 45-kD cellular transcription factor (ATF) that is regulated by the adenovirus EIA protein is similar or identical to CREB and that ATF/CREB can be regulated in vivo by both EIA and cAMP (*17*). Isolation of the cDNA-encoding CREB will facilitate studies aimed at addressing the basis for the molecular heterogeneity of CREB and CREB-like proteins and the interactions of CREB-like, Fos-related, and Jun-related proteins in the transcriptional activation of genes.

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10. The "active CREs" consisted of the CRE octamer element flanked by several bases as they occur in the cAMP-responsive, chorionic gonadotropin α-subunit (CG-8) and somatostatin (SMS-8) genes and the collagenase gene (COL-8) in which the TRE heptamer was converted to a transcriptionally active CRE octamer (*6*). These sequences could impart transcriptional stimulation in response to 8-bromo–cAMP, when linked to a minimal promoter element, and could successfully compete for binding to a labeled "active CRE" in gel-shift assays. The corresponding TREs (CG-7, SMS-7, and COL-7) produced band-shift patterns different from those of the CREs and could not compete for binding to a labeled "active CRE." Finally, the "inactive CREs"

consisted of the CRE octamer in the contexts of the surrounding bases of the cAMP-unresponsive parathyroid hormone (PTH-8) and glucagon (GLU-8) genes and gave no transcriptional response to 8-bromo-cAMP, nor could they produce specific gel-shift patterns or compete for binding to a labeled "active CRE."
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15. The sequence of CREB between residues 1 and 268 contains 25 glutamic acids and aspartic acids and 11 lysines and arginines. The corresponding sequence of c-Jun between residues 1 and 225 contains 22 glutamic acids and aspartic acids and 11 lysines and arginines.
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18. Abbreviations for the amino acid residues 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.
19. The authors thank G. Bruce for initial help with the λgt11 library, F. Traynor and M. LeSieur for expert experimental assistance, P. Sharp for information regarding the screening technique prior to publication, and J. Canniff for preparing the manuscript. Supported in part by NIH grants DK 25532 and DK 30457.

16 August 1988; accepted 7 November 1988

Newly Identified Brain Potassium Channels Gated by the Guanine Nucleotide Binding Protein G_o

A. M. J. VANDONGEN, J. CODINA, J. OLATE, R. MATTERA, R. JOHO, L. BIRNBAUMER, A. M. BROWN

Potassium channels in neurons are linked by guanine nucleotide binding (G) proteins to numerous neurotransmitter receptors. The ability of G_o, the predominant G protein in the brain, to stimulate potassium channels was tested in cell-free membrane patches of hippocampal pyramidal neurons. Four distinct types of potassium channels, which were otherwise quiescent, were activated by both isolated brain G_o and recombinant $G_o\alpha$. Hence brain G_o can couple diverse brain potassium channels to neurotransmitter receptors.

NEURONAL POTASSIUM CURRENTS are coupled by the G proteins G_i or G_o (*1–4*) to acetylcholine M2, adrenergic α_2, serotonin (5HT-1a), dopamine (D2), adenosine (A_1), γ-aminobutryic acid ($GABA_B$), opioid δ and μ, and somatostatin receptors (*1, 2*). Neuronal Ca^{2+} currents may be coupled similarly (*5*). Important evidence indicating this coupling is that the effects of the relevant neurotransmitter can be (i) mimicked by intracellular perfusion of guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS); (ii) inhibited by intracellular perfusion of guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS); or (iii) blocked by treatment of cells with pertussis toxin (PTX). Results with antibodies to G_o support this view (*1, 3–5*). The K^+ currents were in some cases identified as inwardly rectifying (*1, 2*) but the corresponding single channel K^+ currents have not been described; in fact there are only a few reports on neuronal single channel K^+ currents (*6–8*). We used single channel recordings to investigate the K^+ currents that are coupled to these receptors, and we tested whether G_o might, like G_k for atrial and clonal pituitary K^+ channels (*9*) or G_s for dihydropyridine-sensitive Ca^{2+} channels (*9*), gate or modulate neuronal K^+ channels directly.

In the first series of experiments (Fig. 1A), a highly purified preparation of preactivated G_o from bovine brain (*10, 11*) stimulated single channel K^+ currents in inside-out patches in a concentration-dependent manner. The major α subunit in this fraction was α_{39}; α_{41} and α_{40} were present at less than 5%, and α_z (*12*) may also have been present. We also tested another fraction that elutes before G_o and designated it "pre-G_o" (Fig. 2A, lane 1) (*11*). It contained mostly G_i with an α subunit of an apparent molecular mass of 41,000 daltons, another G protein with an α subunit of apparent molecular mass of 40,000 daltons, and a PTX-insensitive contaminant of about 55,000 daltons. The G proteins were activated with GTPγS (*11*), yielding activated G_o (G_o^*) and activated pre-G_o (pre-G_o^*); unbound nucleotide was removed by dialysis until the molar ratio of GTPγS to G protein was less than two. The activated G proteins were diluted at least by a factor of 100,000 in our bathing solutions. In some experiments, GTPγS at 100 μ*M* was tested to evaluate the effects of activating endogenous G proteins.

Our purified brain G_o could not be completely resolved into individual G proteins; therefore the reconstitution experiments infer, but do not prove, the identity of the putative activators. This difficulty was resolved in a second series of experiments by using a recombinant $G\alpha_o$ that was uncontaminated by any other Gα subunit. The recombinant was designated $r(+9)\alpha_o$ because it is a fusion polypeptide with nine amino acids added to its natural amino terminus (*13*). This recombinant and other Gα subunits fused in the same way were (i) expressed in *Escherichia coli*, with the use of techniques pioneered by Tabor and Richardson (*14, 15*), (ii) recovered from lysates of the bacteria, (iii) preactivated with GTPγS, and (iv) partially purified by column chromatography (Fig. 2) (*15*).

We tested G_o^* in 66 excised inside-out patches, and positive responses occurred in 34 of them. Of these 34 patches, 15 contain-

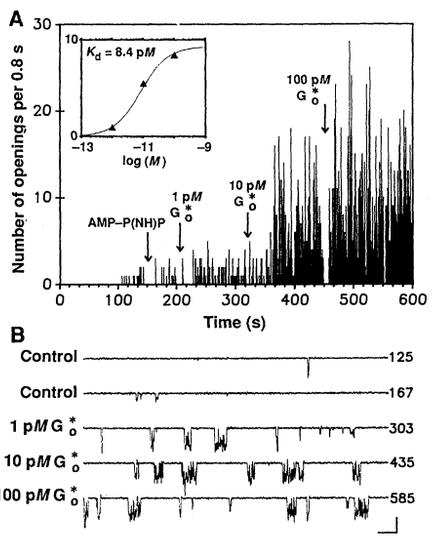

Fig. 1. Brain G_o^* gates single K^+ channel currents in hippocampal pyramidal cells of neonatal rats (*18*) in a concentration-dependent manner. Single K^+ channel currents were recorded from excised inside-out membrane patches (*19–22*). (**A**) The effect of increasing concentrations of G_o^* on a 55-pS K^+ channel (Table 1) is shown by plotting the number of openings per 0.8 s as a function of time for this continuous recording. The nonydrolyzable ATP analog 5′-adenylylimidodiphosphate [AMP-P(NH)P] was added first to inhibit single ATP-sensitive K^+ channel currents that are present in these cells. The inset shows the values averaged for 1 min at each concentration. The points were fitted with the Michaelis relation to give the curve shown. (**B**) Representative traces at different stages of activation. The elapsed time in seconds is indicated to the right of each trace. Holding potential was −80 mV and both bath and pipette solutions contained KMES. Calibrations are 50 ms (*x*) and 3.0 pA (*y*).

A. M. J. VanDongen, R. Joho, A. M. Brown, Department of Physiology and Molecular Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.
J. Codina, J. Olate, R. Mattera, Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.
L. Birnbaumer, Departments of Physiology and Molecular Biophysics and Cell Biology, Baylor College of Medicine, One Baylor Place, Houston, TX 77030.