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22. Isoelectric focusing gels contained 4% (v/v) of *p*H 5 to 7 and 1% (v/v) of *p*H 3 to 10 carrier ampholyte [Servalyt (Serva)], 9*M* urea, and 2% Triton X-100 (*16*). After incubation in 2% SDS, 10% glycerin, and 60 m*M* tris (*p*H 6.8) for 15 min, gels were run on a discontinuous SDS-PAGE system with a linear gradient of 5 to 20%. Proteins were electroblotted onto nitrocellulose, membranes were blocked for 1 hour (0.05*M* tris (*p*H 7.5), 0.5% casein, and 0.5% Tween 20), incubated for 12 hours at 4°C with MAb IA10, washed in blocking buffer, and incubated for 1 hour with alkaline phosphatase–conjugated goat antibody to mouse immunoglobulin G (Promega, Biotec Madison). Blots were developed with 98 μ*M* nitrobluetetrazolium, 92 μ*M* 5-bromo-4-chloro-3-indolyl phosphate in 1*M* diethanolamine, and 0.5 m*M* $MgCl_2$, *p*H 9.8.
23. The method used for the generation of tryptic peptides was as follows. The r-65-kD hsp of *M. bovis* was isolated from *E. coli* M1103 (*4*) containing the full-length gene of 65-kD hsp in the expression vector pPLc 236. The soluble constituents of bacteria were fractionated by ammonium sulfate precipitation. The precipitate that formed between 20% and 55% saturation of ammonium sulfate was bound on the anion exchanger Q-Sepharose Fast Flow (Pharmacia) in 50 m*M* tris, *p*H 8.0, and the 65-kD hsp was eluted with a linear gradient of 0 to 0.3*M* NaCl. For trypsin digestion, the 65-kD hsp was treated with 8*M* urea, and after dialyzing against 0.1*M* ammonium bicarbonate, up to 100 mg of protein was cleaved overnight at room temperature with 25 U of immobilized trypsin [*N*-tosyl-L-phenylalanine chloromethyl ketone–treated, attached to beaded agarose, (Sigma)]. Ovalbumin was denaturated and trypsinized as described (*18*).
24. This work was supported by the following grants to S.H.E.K.: United Nations Development Programme, World Bank, World Health Organization (WHO) Special Program for Research and Training in Tropical Diseases; WHO as part of its Program for Vaccine Development; Sonderforschungsbereich 322; and the A. Krupp Award for young professors. T.K. is supported by the A. v. Humboldt Foundation. We thank P. Conradt for his help with the fluorescence-activated cell sorter. The technical assistance of U. Väth, D. Preuss, and C. Kronfeld, as well as the secretarial help of R. Mahmoudi, is gratefully acknowledged. The r-IFN-γ was produced by Genentech and provided by Boehringer Ingelheim. We are grateful to J. D. A. v. Embden and J. Thole for clone M1103 expressing the mycobacterial 65 kD hsp, to W. J. Welch for MAb against the human 70-kD hsp, to R. Kubo for MAb against the αβ T cell receptor, to H. Waldmann for anti-CD4 and anti-CD8 MAb, and to R. A. Young for communicating the amino acid sequence of the human 65-kD hsp. We thank H. Wagner and U. Koszinowski for helpful discussions.

3 April 1989; accepted 23 June 1989

Primary Structure of the β Subunit of the DHP-Sensitive Calcium Channel from Skeletal Muscle

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Complementary DNAs for the β subunit of the dihydropyridine-sensitive calcium channel of rabbit skeletal muscle were isolated on the basis of peptide sequences derived from the purified protein. The deduced primary structure is without homology to other known protein sequences and is consistent with the β subunit being a peripheral membrane protein associated with the cytoplasmic aspect of the sarcolemma. The protein contains sites that might be expected to be preferentially phosphorylated by protein kinase C and guanosine 3′,5′-monophosphate–dependent protein kinase. A messenger RNA for this protein appears to be expressed in brain.

THE DIHYDROPYRIDINE (DHP)-SENSITIVE Ca^{2+} channel seems to play an important role in excitation-contraction coupling, and when purified from rabbit skeletal muscle, it contains three main subunits with molecular masses of 165,000 (α_1), 55,000 (β), and 32,000 daltons (γ) (*1–9*). Both the α_1 and the β subunits are substrates for protein kinases in vitro, and phosphorylation of these subunits may serve to regulate the in vivo function of the Ca^{2+} channel (*1–5*). A disulfide-linked dimer of 130,000- (α_2) and 28,000-dalton (δ) polypeptides has been observed in preparations of the channel either in variable (*6*) or constant (*7*) amounts with respect to the other subunits. The subunits have been reconstituted and form functional Ca^{2+} channels that are modulated by phosphorylation (*10*) and by monospecific antibodies for the α_1, β, and γ subunits (*11*). The α_1 polypeptide is the principal transmembrane subunit of the channel and forms the ion-conducting pore (*12*). This polypeptide binds DHPs, phenylalkylamines, and benzothiazepines (*1, 2, 6–9*) and is readily phosphorylated in vitro at Ser^{687} by adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (*4*). Microinjection of an expression plasmid carrying the α_1-subunit cDNA restores a DHP-sensitive Ca^{2+} current and excitation-contraction coupling in dysgenic muscle (*13*). It is not known whether these functions require the presence of the other channel subunits. The α_2 polypeptide has been cloned (*14*) and is present in dysgenic muscle cells (*15*), which may also contain the β and γ subunits.

We now report the primary structure of the β subunit from rabbit skeletal muscle as deduced from cloned cDNA and the tissue distribution of the corresponding mRNA. A total of eight peptides, six of which were unique, were isolated from two separate proteolytic digests of the isolated β subunit (*16*). In one case the purified channel containing the β subunit was phosphorylated by cAMP-dependent protein kinase before separation of the subunits. Peptide 1 contained 32% of the recovered radioactivity (recovered radioactivity was 74% of the radioactivity initially present in the β subunit). The remaining radioactivity was associated with several peptides. Four oligodeoxyribonucleotides, each containing 14 nucleotides (nt), which were complementary to all possible cDNA sequences encoding the amino acid sequence Asp-Met-Met-Gln-Lys (excluding the third nucleotide residue of the Lys codon) in peptide 2, were synthesized. These oligomers were used as specific primers for reverse transcription of the β-subunit mRNA. An equimolar mixture of 64 synthetic 14-nt oligodeoxyribonucleotides, which were complementary to all the possible cDNA sequences corresponding to the amino acid sequence Gly-Tyr-Glu-Val-Thr (excluding the third nucleotide residue of the Thr codon) in peptide 2, was used to probe 2×10^5 transformants. Two clones, pCaCHβ1-I and pCaCHβ10-I, which both contained the coding region for part of peptide 2, were isolated. In addition, clone pCaCHβ1-I contained the cDNA encoding peptides 1, 3, 4, and 7. This clone was used as a probe for cloning larger cDNA sequences (Fig. 1A).

The 1835-nt cDNA sequence obtained contains an open reading frame encoding a sequence of 524 amino acids (Fig. 1B). The calculated molecular mass of 57,868 daltons is similar to that estimated by SDS–polyacrylamide gel electrophoresis (55 kD). The cDNA contains all sequenced peptides. The NH_2-terminus of the isolated β subunit was blocked and could not be sequenced. We selected the first methionine (amino acid 1) rather than the second methionine (amino acid 7) as the translation initiation site be-

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cause it is the first in frame ATG triplet that appears downstream of the stop codon TGA (nucleotides −51 to −49) and because the nucleotide sequence surrounding this initiation codon agrees with the consensus sequence for eukaryotic initiation codons, except for the nucleotide at position −3 where a T substitutes for a purine. This substitution is frequently linked with a nonfunctional upstream ATG codon (*17*), and such a codon is found at nucleotides −57 to −55.

An analysis of the amino acid sequence of the β subunit for local hydropathicity (Fig. 1C) reveals the absence of a typical membrane-spanning region (segments of at least 19 residues with an average hydropathicity index of greater than 1.6) (*18*). Additionally, the NH_2-terminal sequence does not resemble a hydrophobic signal sequence. The protein appears to be hydrophilic, although it contains hydrophobic regions. These properties are consistent with biochemical data suggesting that the β subunit does not interact with the cell membrane but is tightly associated with an intracellular domain of the α_1 subunit (*2*). As judged by the probabilistic method of Garnier *et al.* (*19*), the β subunit contains four major α-helical domains, which have been designated I (amino acids 73 to 101), II (amino acids 212 to 237), III (amino acids 344 to 371), and IV (amino acids 414 to 455) (Fig. 1C). The arrangement of hydrophobic residues within domains II, III, and IV resembles a heptad repeat, in which most of the first and fourth residues of every seven are hydrophobic (for example, amino acids 418, 422, 425, 429, 432, 436, 439, 443, 446, 450, and 453 in domain IV). Such heptad repeats of hydrophobic residues are thought to participate in interactions between cytoskeletal proteins (*20*). By the use of the Dayhoff MDM 78 matrix (*21*), the β subunit shows weak relatedness to some isoforms of actin, keratin, glial fibrillary acidic protein, myosin, and tropomyosin with alignment scores of 1.6 to 1.9. Each α-helical domain contains a similar stretch of eight amino acids (boxed in Fig. 1B), which starts and ends with Leu or Ile and is interdispersed by negatively charged residues. This arrangement of acidic residues may function in the binding of divalent cations. The β subunit specifically binds Ca^{2+} with low affinity (apparent dissociation constant ≈1 m*M*) (*22*) as measured by the method of Hummel and Dreyer (*23*). This low affinity may be due to the isolation procedure, which includes a denaturation step. No sequence similarity was found between the β subunit and the EF-hand of high-affinity Ca^{2+}-binding proteins or with calsequestrin (*24*).

Several potential phosphorylation sites are present in the β subunit; this finding is consistent with in vitro phosphorylation studies (*1–5*). The serine (Ser^{182}) of peptide 1 was phosphorylated in vitro by cAMP-dependent protein kinase, although the amino acid sequence around this phosphorylation site does not contain the basic motif Arg-Arg-X-Ser (where X represents any amino acid) typical of substrates of this enzyme (*25*). Another potential phosphorylation site for cAMP-dependent protein kinase is Thr^{205}, which is preceded by the sequence Arg-Arg-Pro. The time course of phosphate incorporation into the β subunit catalyzed by cAMP-dependent protein kinase in vitro is slow (*3*), and so phosphorylation of these residues may not be physiologically relevant. The β subunit is also phosphorylated by both protein kinase C and guanosine 3′,5′-monophosphate (cGMP)–dependent protein kinase, and at least three different tryptic phosphopeptides have been identified (*3*). Two of these peptides were phosphorylated both by protein kinase C and by cAMP-dependent protein kinase (*2, 3*). There are 12 potential phosphorylation sites for protein kinase C [(Ser or Thr)-X-(Arg or Lys)] (*26*) in the β subunit (Fig. 1B). Analysis of the trypsin cleavage sites in the pri-

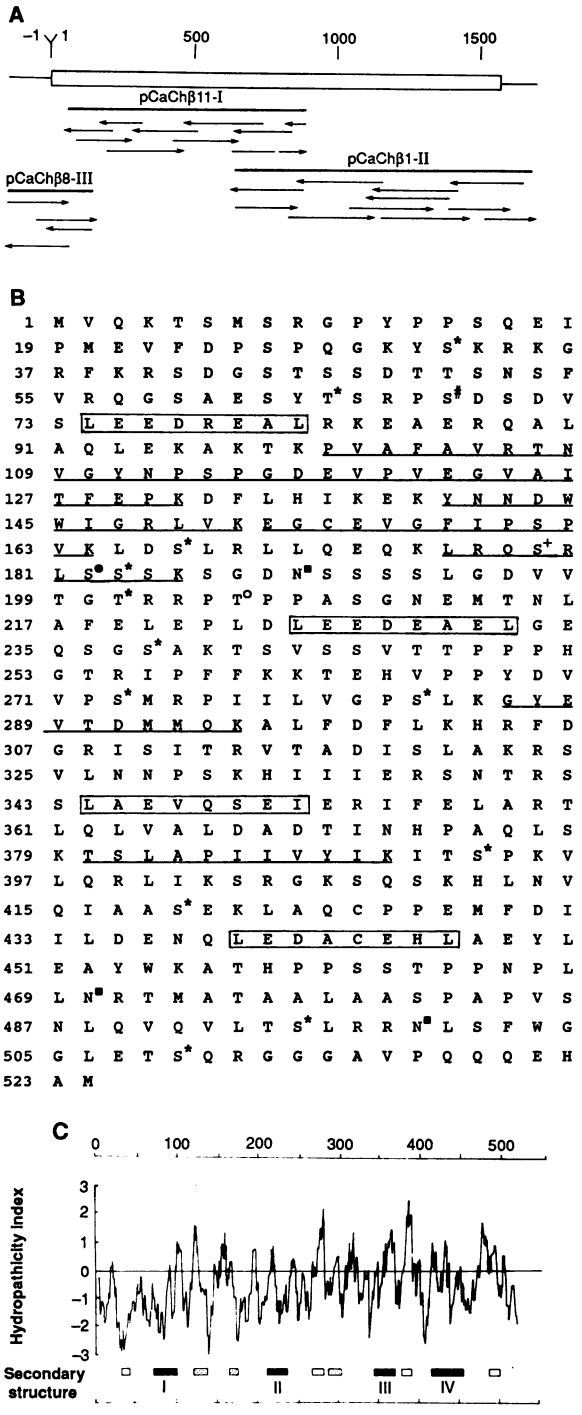

Fig. 1. (**A**) Cloning and sequencing strategy for the β subunit. The protein-coding region is indicated by a closed box. The extent of cDNA inserts of the individual clones (*32*) used for sequence analysis (*33*) (indicated by arrows) are shown by thick lines. (**B**) Deduced amino acid sequence of the β subunit. Cloning procedures, if not stated otherwise, were carried out as described (*34*). The six peptides determined by sequence analysis are underlined. Similar stretches of eight amino acids, contained in the long α helices, are boxed. Predicted phosphorylation sites for protein kinase C (*), cGMP-dependent protein kinase (+), casein kinase II (#), and cAMP-dependent protein kinase (○) are indicated. Site phosphorylated by cAMP-dependent protein kinase (●) is identified. Potential N-glycosylation sites (■) are at amino acid positions 189, 470, and 499. (**C**) Hydropathy profile and predicted secondary structure, computed according to Kyte and Doolittle (*18*); the window size is 19 residues, plotted at one-residue intervals. The positions of predicted secondary structures (stretches of ten or more amino acids) are shown: long α helices (I, II, III, and IV, solid boxes), short α helices (dotted boxes), and β sheets (open boxes).

mary structure identifies two potential tryptic peptides (amino acids 181 to 185 and 203 to 240) that contain phosphorylation sites for both kinases (Fig. 1B). Protein kinase C increases the number of Ca^{2+} channels in skeletal muscle and fibroblasts (*27*). There is also evidence that cGMP-dependent protein kinase alters Ca^{2+} channel function in specific neurons of *Helix aspersa* and cardiac muscle (*28*). The cGMP-dependent protein kinase phosphorylates at least one site in the β subunit that is not phosphorylated by protein kinase C (*3*). The β-subunit sequence Arg-X-Ser^{179}-Arg is a potential phosphorylation site for cGMP-dependent protein kinase (*25*). The presence of at least two regions that are rich in proline (P), glutamine (E), serine (S), and threonine (T) (amino acids 67 to 77 and 204 to 239) within the primary structure may explain the sensitivity of the β subunit to proteases (*1–3*). These PEST regions are common to rapidly degraded proteins (*29*) and are often targets of casein kinase II. Ser^{68}, being followed by Asp-Ser-Asp, represents a potential casein kinase II phosphorylation site (*25*). Casein kinase II phosphorylates the β subunit apparently at two sites in vitro (*3*), and it has been proposed that these might be constitutive phosphorylation sites of the channel complex.

The tissue distribution of the mRNA for the β subunit was analyzed by RNA blots of polyadenylated [poly(A)$^+$] RNA from several rabbit tissues (*30*) (Fig. 2). Various cDNA fragments derived from both the noncoding and the coding sequence of the β subunit were used as probes. The probes derived from the coding sequence hybridized to two poly(A)$^+$ RNA species of skeletal muscle with estimated sizes of 1900 and 1600 nt. The size of the large species is consistent with the size of the cloned cDNA (1835 nt). In addition, a cDNA fragment consisting of most of the 5′ noncoding sequence hybridized to the large species only. The less abundant and smaller mRNA may differ in the 5′ untranslated sequence as a result of alternative mRNA processing. In poly(A)$^+$ RNA from brain, a probe derived from the coding sequence hybridized to a species of 3000 nt. The intensity of this signal was similar to that of the 1900-nt species in skeletal muscle. No β subunit–specific hybridization was observed with poly(A)$^+$ RNA from smooth muscle or heart. Thus homologous, but not identical, β-subunit mRNAs appear to exist in different tissues. The lack of hybridization signals from heart and smooth muscle may be due either to the lower concentration of β-subunit mRNA in these tissues or to the presence of β subunits with considerably different nucleotide sequences. A similar tissue-specific expression was found with the mRNA encoding the α_1 subunit, whereas the α_2-subunit mRNA was detected in all tissues examined (*14*).

The primary structure of the β subunit lacks significant homology with any protein in the Swiss protein and the GenBank nucleotide sequence databases. Polypeptides of similar size as the β subunit appear to be associated with other voltage-activated ion channels, such as the Na^+ channel (*2*) and various K^+ channels (*31*). However, the primary structures of these proteins have not yet been determined.

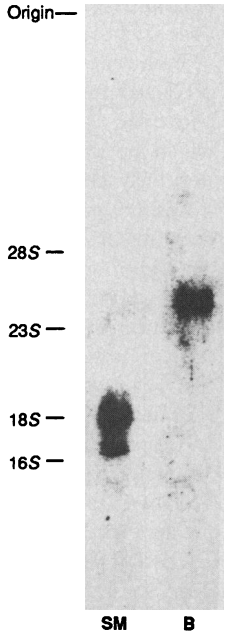

Fig. 2. RNA blot analysis of rabbit RNA with β-subunit cDNA probes. Samples of poly(A)$^+$ RNA (20 μg) from skeletal muscle (SM) and brain (B) were denatured with 1*M* glyoxal and 50% dimethyl sulfoxide, subjected to electrophoresis in a 1.5% agarose gel, and transferred to Biodyne membranes. The hybridization probe was the Sac I (nucleotide 695)–Pfl MI (nucleotide 1608) fragment labeled by random priming. Identical results were obtained when the Pst I (nucleotide 515)–Ava II (nucleotide 841) and Sty I (nucleotides 56 and 389) fragments were used as probes (*22*).

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16. The Ca^{2+} channel complex (0.86 mg), purified from rabbit skeletal muscle microsomes (*3*), was incubated for 2 hours at 37°C with 1 μ*M* of the catalytic subunit of cAMP-dependent protein kinase, 0.1 m*M* [γ-^{32}P]adenosine 5′-triphosphate (5 cpm/fmol), and 5 m*M* $MgCl_2$. The phosphorylated β subunit was separated from the other subunits by gel filtration (*6*). Fractions containing the β subunit were combined, dialyzed, and dissolved as described (*4*). Endoproteinase Lys-C was added at a protein to Lys-C ratio of 50:1, and the mixture was incubated at 25°C for 17 hours. The digest was loaded directly onto a LiChrosorb RP-18 column equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted with a gradient of 1% acetonitrile per minute at a flow rate of 1 ml/min. Absorbance at 216 nm and ^{32}P content of the eluted material were measured. From two different channel preparations a total of eight fractions were collected, which then were purified further on a second LiChrosorb RP-18 column with a gradient of 0.2% acetonitrile per minute at a flow rate of 1 ml/min, and sequenced (*4*). Peptides 1, 5, 6, and 8 and peptides 2, 3, 4, and 7 were from the first and second digests, respectively. The amino acid sequences were as follows: 1, LRQSRLS(^{32}P)SSK; 2, GYEVTDMMQK; 3, EGXEVGFIPSPVK; 4 and 6, PVAFAVRTNVGYNPSPGDEVPVEGVAITFEPK; 5, TSLAPIIVYIK; and 7 and 8, YNNDWWIGRLVK. The localization of the phosphoserine residue of peptide 1 was unambiguous because only the phenylthiohydantoin derivative of dithiothreitol-dehydroalanine, which is formed quantitatively from phosphoserine [H. E. Meyer, E. Hoffmann-Posorske, C. C. Kuhn, L. M. G. Heilmeyer, Jr., in *Modern Methods in Protein Chemistry*, H. Tschesche, Ed. (de Gruyter, Berlin, 1988), vol. 3, pp. 185–212], was detected in the seventh cycle. Abbreviations for the amino acid residues are as follows: 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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32. The cDNA resulting from primer extension of 4.3 nmol of 5′-TT(T/C)TGCATCAT(A/G)TC-3′ (corresponding to the sequence DMMQK of peptide 2) with 20 μg of rabbit skeletal muscle poly(A)$^+$ RNA was cloned in pBR322. Screening of approximately 2×10^5 transformants with an equimolar mixture of the 64 synthetic probes, 5′-GTNAC(T/C)TC(A/G)TANCC-3′, which were synthesized as four pools (corresponding to the sequence GYEVT of peptide 2), yielded two clones, pCaChβ1-I (nt 236 to 884) and pCaChβ10-I (nt 407 to 884) that contained part of the coding sequence for peptide 2. Screening of the same primer extension library (approximately 3×10^5 transformants) with the Alu I (nt 276)–Pst I (nt 515) fragment of pCaChβ1-I gave 15 positive clones including pCaChβ11-I (nt 53 to 884) and pCaChβ6-I (nt 105 to 884). The Pst I (nt 515)–Ava II (nt 841) fragment was used for screening approximately 5×10^5 transformants of an oligo(dT) library, and one positive clone, pCaChβ1-II (nt 635 to 1680), was obtained. Elongation of a third synthetic primer (1.8 nmol), complementary to nt 144 to 159 [with 30 μg of poly(A)$^+$ RNA], and screening of the resulting clones (approximately 1.5×10^5 transformants) with the Sty I (nt 56)–Dde I (nt 138) fragment yielded 12 positive clones, including pCaChβ8-III (nt −155 to 143). All clones had an identical length and restriction enzyme map except pCaChβ3-III (nt 34 to 417), which arose from priming at nt 419 to 434. Sequencing of the cDNA was performed on both strands (Fig. 1A). In addition, pCaChβ6-I, pCaChβ1-I, pCaChβ10-I, and pCaChβ3-III were sequenced. The nucleotide differences among the individual clones were as follows: at nt 252, G (pCaChβ11-I, pCaChβ1-I, and pCaChβ3-III) or A (pCaChβ6-I); at nt 438, C (pCaChβ11-I, pCaChβ6-I, and pCaChβ1-I) or T (pCaChβ10-I). The differences did not result in amino acid substitution.
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35. Supported by Deutsche Forschungsgemeinschaft, Thyssen, and Fond der Chemischen Industrie. The GenBank accession number of the nucleotide sequence of the β subunit of the DHP-sensitive calcium channel is M25817.

4 April 1989; accepted 17 July 1989

Molecular Characterization of the Human β_3-Adrenergic Receptor

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Since the classification of β-adrenergic receptors (β-ARs) into β_1 and β_2 subtypes, additional β-ARs have been implicated in the control of various metabolic processes by catecholamines. A human gene has been isolated that encodes a third β-AR, here referred to as the "β_3-adrenergic receptor." Exposure of eukaryotic cells transfected with this gene to adrenaline or noradrenaline promotes the accumulation of adenosine 3′,5′-monophosphate; only 2 of 11 classical β-AR blockers efficiently inhibited this effect, whereas two others behaved as β_3-AR agonists. The potency order of β-AR agonists for the β_3-AR correlates with their rank order for stimulating various metabolic processes in tissues where atypical adrenergic sites are thought to exist. In particular, novel β-AR agonists having high thermogenic, antiobesity, and antidiabetic activities in animal models are among the most potent stimulators of the β_3-AR.

ADRENERGIC RECEPTORS MEDIATE the physiological actions of the hormones adrenaline and noradrenaline. Four subtypes of these catecholamine receptors, the α_1-, α_2-, β_1-, and β_2-adrenergic receptors (α_1-, α_2-, β_1-, and β_2-AR) have been identified on the basis of their pharmacological properties and physiological effects. Chemical agents that selectively block or stimulate these receptors are used extensively in clinical medicine. Despite the efficacy of these compounds, they may produce side effects, in part because of interaction with other homologous receptors. Improvement of drug selectivity thus necessitates a complete characterization of each receptor that mediates the physiological actions of catecholamines.

The genes encoding the α_1-, α_2-, β_1-, and β_2-AR have been isolated (*1–4*). They belong to a family of homologous genes that encode integral membrane receptors (*5, 6*), which presumably have seven membrane-spanning domains and which are coupled to regulatory G proteins. Several of the genes in this family have been characterized, and probes derived from these genes have been used to identify additional receptor subtypes (*7, 8*).

Additional subtypes of β-AR have been suggested to mediate the sympathetic control of various metabolic processes in the digestive tract (*9, 10*), adipose tissue (*11–13*), and skeletal muscle (*14, 15*). Evidence for the existence of such atypical β-AR sites includes their low affinity for standard β-AR blockers. Recently, β-AR agonists have been synthesized (*16*) that are potent stimulators of metabolic rate, adipose tissue thermogenesis, ileum relaxation, and soleus muscle glycogen synthesis (*10, 11, 14–16*), but these agonists have minimal effects at β_1 and β_2 sites. However, the existence of a β-AR different from the currently defined β_1- and β_2-AR is controversial (*17*).

We screened a human genomic library with the entire coding regions of the genes for the turkey β_1- and the human β_2-AR (*18*). Among 43 positive clones, two carried the gene coding for the human β_1-AR and another two the gene for the β_2-AR. A family of 14 homologous clones (*19*) displayed sequences homologous to both probes in a 2.1-kb Bam HI–Bgl II fragment. From one clone, this fragment was entirely sequenced and shown to contain a gene coding for a polypeptide of 402 amino acids with a predicted size of 42,881 daltons.

The amino acid sequence of this protein (Fig. 1) is 50.7 and 45.5% identical to that of the human β_1- and β_2-AR, respectively (the β_1-AR and β_2-AR are 48.9% identical). The protein shares the structural characteristics of receptors of the G protein–linked family. It has seven clusters of 21 to 27 mostly hydrophobic amino acids, presumed to constitute α-helical membrane-spanning domains. These hydrophobic segments form the catecholamine binding site of the β-AR (*20*) and are highly conserved between the predicted protein and the two β-ARs. In particular, Asp^{79} and Asp^{113} of the β_2-AR, which possibly act as counterions for the positively charged amine of adrenergic ligands, are present at analogous positions in the three proteins. Similarly, other functionally important residues (*20*), such as Cys^{106}, Cys^{184}, Asn^{318}, and Pro^{323} of the β_2-AR sequence, are conserved. As with other G protein–linked receptors, the predicted protein has potential Asn-linked glycosylation sites in the NH_2-terminal region and has Ser and Thr residues in its third cytoplasmic loop and COOH-terminal region that could be substrates for protein kinases, which may mediate receptor desensitization.

To characterize the product of the cloned gene, we introduced it into Chinese hamster ovary (CHO) cells (*21*). The transfected cells expressed specific and saturable binding sites for [^{125}I]iodocyanopindolol ([^{125}I]-ICYP), a β-AR ligand (Fig. 2A); the calcu-

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