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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.

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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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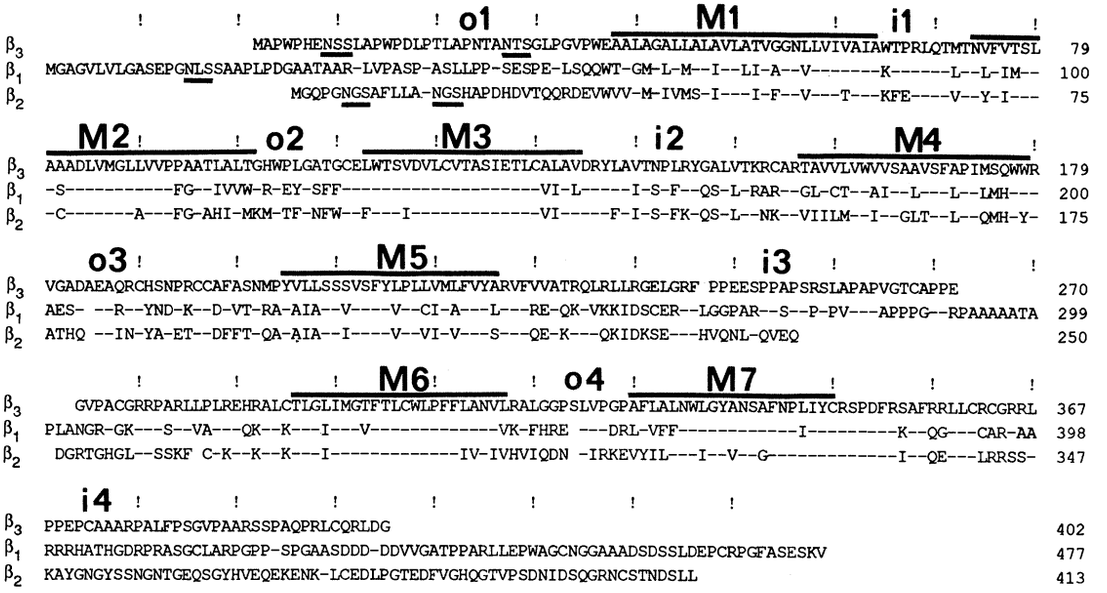

Fig. 1. Amino acid sequences (*26*) of the human β_1- (*3*) and β_2-AR (*4*) have been aligned with that of the human β_3-AR. In the β_1- and β_2-AR sequences, hyphens indicate identity with β_3-AR. To maximize homologies, deletions represented by empty spaces have been introduced in the three sequences. The seven presumed α-helical membrane-spanning domains (M1 to M7) are highlighted by solid bars and are separated by extracellular (o1 to o4) and intracellular (i1 to i4) loops. In the NH_2-terminal region, consensus sequences for asparagine-linked glycosylation sites (NXS/T) are underlined (where X represents any amino acid). (!) marks every tenth amino acid residue.

lated dissociation constant ($K_d \simeq 500$ p*M*) was almost ten times as large as that for the β_1- or β_2-AR. The expressed protein was detected on an SDS-polyacrylamide gel by prior affinity-labeling with [^{125}I]iodocyanopindololdiazirine ([^{125}I]ICYPD) and had an apparent molecular mass of 65 kD (Fig. 2B). The difference in size from that predicted from the amino acid sequence could reflect glycosylation at one or both of the sites found in the NH_2-terminal region of the molecule (Fig. 1). This protein will be referred to as the β_3-adrenergic receptor (β_3-AR).

The human β_1- and β_2-AR were separately introduced into CHO cells so as to compare the pharmacological properties of the three receptors in an identical environment. The three cell lines expressing each of the three β-ARs were designated CHO-β_1, CHO-β_2, and CHO-β_3, respectively. β-Adrenergic agonists increased the intracellular concentration of adenosine 3′,5′-monophosphate (cAMP) in CHO-β_3 cells (Fig. 3 and Table 1) but had no effect on nontransfected CHO cells. This effect was stereospecific as (−)-isoproterenol was almost 30 times as potent as (+)-isoproterenol. α-Adrenergic agents (such as phentolamine and clonidine), serotonin, and histamine had no effects on cAMP accumulation nor on ICYP binding (*22*). Large concentrations of dopamine resulted in partial stimulation of adenylate cyclase activity (Table 1). The rank order of potency of six β-AR agonists in CHO-β_3 cells (Fig. 3B) was different from that obtained for β_1- and β_2-AR–mediated responses but similar to that determined for adrenergic stimulation of both lipolysis in rat fat cells (*11, 16*) and relaxation of guinea pig ileum (*10*). In particular, the product BRL 37344, which is a potent agonist at the postulated atypical adrenergic sites of guinea pig ileum (*10*) and rat fat cells and soleus muscle (*11, 14–16*), is among the most effective β-AR agonists in CHO-β_3 cells (Fig. 3B), whereas it is less efficient in CHO-β_2 cells and almost ineffective in CHO-β_1 cells (*22*).

Most of the classical β-AR antagonists were ineffective in blocking isoproterenol-induced cAMP accumulation in CHO-β_3 cells (Fig. 4). Indeed, these compounds were also unable to block ICYP binding, and no specific binding was obtained with [^{3}H]dihydroalprenolol or ^{3}H-labeled CGP 12177. An effective blockade, either of cAMP accumulation or of ICYP binding, was obtained only with CGP 20712A and ICI 118551 (Fig. 4 and Table 1), two antagonists previously classified as β_1- and β_2-selective, respectively. A low affinity for standard β-blockers has been previously de-

Table 1. Stimulation and inhibition of cAMP accumulation and inhibition of [^{125}I]ICYP binding on intact CHO-β_3 cells. In two separate experiments performed in duplicate, CHO-β_3 cells from a single harvest were exposed (*24*) to 10^{-5}*M* of each agonist, and basal (less than 2 pmol per 10^6 cells) and agonist-induced (40 to 80 pmol per 10^6 cells) concentrations of cAMP were determined. Results represent the percentage (mean ± SD) stimulation achieved by each agonist relative to the effect of (−)-noradrenaline. For agonists, activation constants (K_{act} ± SEM) for cAMP accumulation were calculated from the curves shown in Fig. 3; for antagonists, inhibition constants (K_i ± SEM) were derived from dose-response curves (not shown) obtained with various concentrations of competitors (*24*). Inhibition constants (K_i ± SEM) of ICYP binding were obtained from receptor binding assays performed (*25*) in the presence of 200 p*M* of [^{125}I]ICYP and various doses of competitors.

	Accumulation of cAMP		Inhibition of ICYP binding
	Percentage of noradrenaline value	K_{act} or K_i (n*M*)	K_i (n*M*)
Agonists			
(−)-Isoproterenol	90.2 ± 2.1	3.9 ± 0.4	620 ± 220
BRL 37344	87.2 ± 15.4	5.9 ± 1.3	183 ± 10
(−)-Noradrenaline	100	6.3 ± 0.7	475 ± 75
Fenoterol	103.7 ± 8.5	25.6 ± 1.5	235 ± 35
(−)-Adrenaline	100.4 ± 3.8	49.2 ± 5.3	20,650 ± 2,810
(+)-Isoproterenol	97.3 ± 10.9	111 ± 1	18,950 ± 3,060
Partial agonists			
Oxprenolol	53.3 ± 6.8	76.7 ± 12.6	70 ± 10
Pindolol	55.0 ± 5.2	153 ± 12	11 ± 2
Salbutamol	76.9 ± 0.8	266 ± 23	53,000 ± 10,000
Prenalterol	64.9 ± 0.4	481 ± 22	810 ± 120
BRL 28410	52.6 ± 0.1	2,480 ± 320	14,800 ± 4,200
Dopamine	68.9 ± 8.6	10,700 ± 1,100	
Antagonists			
ICI 118551		770 ± 80	257 ± 34
CGP 20712A		6,700 ± 870	2,300 ± 450

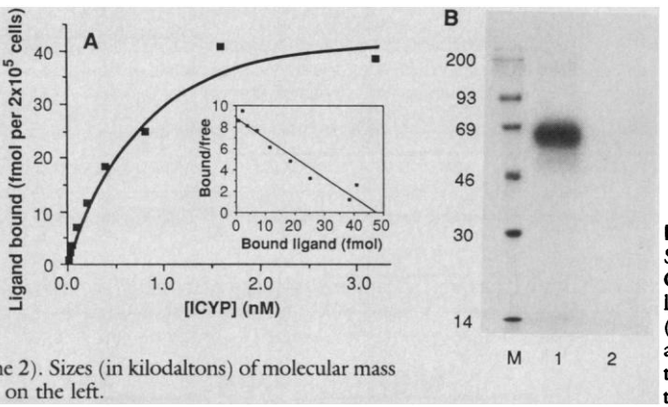

Fig. 2. (**A**) Isotherm and Scatchard analyses (inset) of ICYP binding to intact CHO-β_3 cells. Results represent the mean of two experiments done in duplicate (SEM = 0 to 9.4%). Under the conditions used (*25*), there were 46 ± 5 fmol of ICYP binding sites per 2 × 10^5 cells (K_d ± SEM = 490 ± 90 p*M*). (**B**) Photoaffinity labeling (*25*) of the β_3-AR with [^{125}I]ICYPD alone (lane 1) or in the presence of 10^{-4}*M* ICI 118551 (lane 2). Sizes (in kilodaltons) of molecular mass markers (lane M) are indicated on the left.

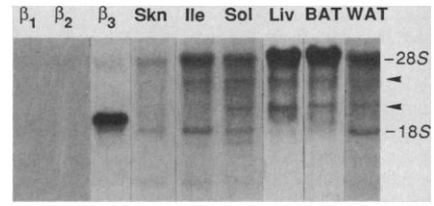

Fig. 5. Analyses of β_3-AR mRNA expression. Samples represent either total RNA (2 μg) from CHO-β_1, CHO-β_2, and CHO-β_3 cells or poly(A)$^+$ RNA (5 μg) from rat skin (Skn), ileum (Ile), soleus muscle (Sol), liver (Liv), brown adipose tissue (BAT), and mouse white adipose tissue (WAT). Nonspecific binding of the probe to ribosomal RNA (18*S* and 28*S*) was observed in spite of highly stringent experimental conditions (*27*). However, specific signals (arrowheads) were visible in RNA from adipocytes, liver, skeletal muscle, and ileum, which were not detected in RNA from rat brain, skin, heart, and lung (representative result shown for skin).

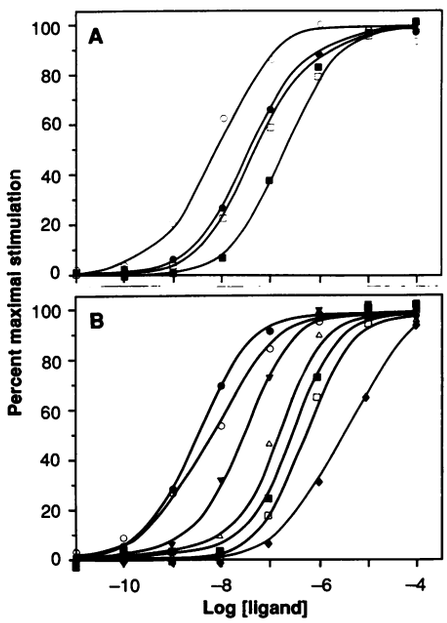

Fig. 3. The cAMP accumulation in CHO-β_3 cells exposed (*24*) to (**A**) (−)-noradrenaline (○), (−)-adrenaline (●), oxprenolol (□), and pindolol (■) and (**B**) (−)-isoproterenol (●), BRL 37344 (○), fenoterol (▼), (+)-isoproterenol (△), salbutamol (■), prenalterol (□), and BRL 28410 (◆). The basal concentration of cAMP varied from one preparation of cells to another (less than 2 to 40 pmol per 10^6 cells), as did the concentration at maximal stimulation (40 to 260 pmol per 10^6 cells). Some of the agonists, even at optimal doses, only partially stimulated adenylate cyclase (Table 1). To standardize the data with respect to these variations, results are expressed as the mean percentage (n = 4 to 6) of the maximal response induced by each ligand. The x-axis represents the logarithm of the concentration (in moles per liter) of ligands.

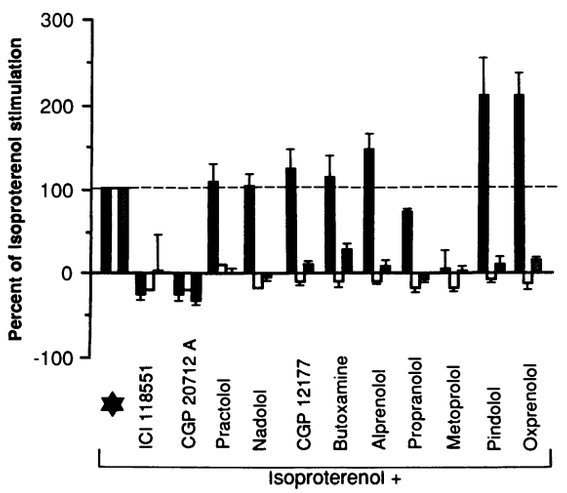

Fig. 4. Effect of β-AR antagonists on isoproterenol-induced cAMP accumulation in CHO-β_1 (hatched bars), CHO-β_2 (open bars), and CHO-β_3 (solid bars) cells. Cells were incubated (*24*) with 10^{-4}*M* of indicated inhibitors before the addition of isoproterenol. Results are expressed as the percentage [mean ± SD (n = 4 to 6)] of the reference cAMP concentration (dotted line) induced by isoproterenol alone (star). In CHO-β_1 and CHO-β_2 cells, cAMP concentrations were blocked at basal values (horizontal axis) in the presence of any of the inhibitors. In CHO-β_3 cells, only ICI 118551 and CGP 20712A were effective inhibitors (Table 1); metoprolol had no effect at concentrations less than 10^{-5}*M*. The stimulatory effects of oxprenolol and pindolol were additive to that of isoproterenol; when isoproterenol was used at saturating doses (10^{-6}*M* and above) no such additivity was observed.

scribed as suggesting the existence of atypical β-ARs (*9–16*).

Pindolol and oxprenolol are β-AR blockers used in humans therapeutically for the treatment of cardiovascular diseases, although they also display slight sympathomimetic activity in other tissues (*23*). These opposite activities correlate with their properties in transfected CHO cells where the compounds behaved as partial β_3-AR agonists (Fig. 3A and Table 1) but totally blocked β_1- and β_2-AR–mediated cAMP accumulation (Fig. 4).

To investigate the tissue distribution of the β_3-AR, we hybridized RNA from various tissues to a β_3-AR–specific probe (Fig. 5). In RNA from adipocytes, liver, soleus muscle, and ileum, transcripts of 2.8 kb and 4.1 kb were visible; these could either arise from the utilization of distinct promoters or polyadenylation sites or could represent the product of homologous atypical β-AR genes. In these tissues, a predominant role for classical β_1- and β_2-AR is well established. The higher sensitivity of the β_3-AR to noradrenaline than to adrenaline suggests that modulation of β_3-AR activity may arise through sympathetic innervation in response to situations such as stress, high energy intake, or cold acclimatization. Expression of β_3-ARs could thus be restricted to areas in the close vicinity of noradrenergic nerves, resulting in the low amounts of β_3-AR mRNA observed.

Our results (*19*) (Fig. 5) do not show whether the β_3-AR is the only atypical β-AR. Data indicating distinct atypical β-ARs in adipocytes, ileum, and soleus muscle (*10, 14*) are not definitive because they were obtained in different species and in systems heterogeneous in terms of receptor population. On the basis of the available data, we suggest, however, that the β_3-AR, which displays low affinity for standard β-AR blockers but marked responsiveness to the agonist BRL 37344, is the atypical receptor (or one of a class of atypical receptors) postulated to mediate catecholamine action on metabolic rate.

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18. The library was constructed in the Bam HI sites of the EMBL4 vector from size-selected (15 to 20 kb) fragments of human placental DNA partially digested with Sau 3A. For plaque purification, we used as probes the 1.3-kb Nco I–Aha III restriction fragment of the human β_2-AR (*4*) and the 1.8-kb Eco RI fragment of the turkey β_1-AR [Y. Yarden *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6795 (1986)].
19. The two clones containing the β_1-AR gene were overlapping, whereas those for the β_2-AR gene were identical. The 14 β_3-AR clones consisted of a group of six identical clones, a second group of three identical clones, a third group of two identical clones, and three independent clones. This situation could result from preferential growth, during library amplification, of the bacterial clones carrying the β_3-AR gene. Alternatively, it could reflect the existence of several highly homologous atypical β-AR genes.
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21. The coding region of the β_3-AR gene was inserted, under the control of the SV40 early promoter, in an expression vector [L. A. Larsky, D. Dowbenko, C. C. Simonsen, P. W. Berman, *Biotechnology* **2**, 527 (1984)] that contained the murine dihydrofolate reductase (DHFR) gene to allow for the selection of transfectant cells. Introduction of this construct into DHFR-deficient CHO cells [G. Urlaub and L. A. Chasin, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4216 (1980)] and screening for mRNA expression of the β_3-AR gene were as described (*4*).
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24. Preconfluent cells (10^6) were incubated at 37°C for 30 min in 1 ml of Hank's buffer containing 20 m*M* Hepes (*p*H 7.4), 1 m*M* ascorbic acid, 0.1 m*M* isobutylmethylxanthine, and various concentrations of agonists, and the amount of cAMP in cells was then determined (cAMP assay kit, Amersham). For studies on the inhibition of cAMP accumulation, cells were incubated at 37°C for 30 min with inhibitors before addition of 5×10^{-9} *M* (−)-isoproterenol and incubation for another 30-min period.
25. For receptor binding assays, 2×10^5 cells were incubated for 1 hour at 37°C in 1 ml of Hank's buffer containing 20 m*M* Hepes (*p*H 7.4), 0.1% bovine serum albumin, and 6.25 to 3200 p*M* ICYP. Nonspecific binding was determined as that occurring in the presence of 10^{-4} *M* isoproterenol. In competition assays, 4 μ*M* desipramine was added to the buffer to decrease nonspecific binding. Separation of bound from free ligand was achieved by filtration on glass filters soaked for 1 hour in 0.3% polyethyleneimine. Photoaffinity labeling was performed by incubating receptors (0.2 n*M*; 8×10^5 cells per milliliter) with 2 n*M* [^{125}I]ICYPD for 75 min at 37°C in a buffer containing 10 m*M* tris-HCl (*p*H 7.4), 150 m*M* NaCl, 5 m*M* $MgCl_2$, and 2 m*M* $CaCl_2$. Specificity of the labeling was demonstrated by addition of 10^{-4} *M* ICI 118551 to the reaction.
26. Abbreviations for 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.
27. RNA blots were incubated for 4 to 6 hours at 60°C in hybridization buffer [50% formamide, 5× SSC (standard saline citrate), 50 m*M* tris-HCl (*p*H 7.5), 0.1% sodium pyrophosphate, 1% SDS, 5 m*M* EDTA, denatured salmon sperm DNA (100 μg/ml), yeast transfer RNA (50 μg/ml), and 0.2% each of polyvinylpyrrolidone, Ficoll, and bovine serum albumin] before hybridization for 16 hours at 60°C. The probe was labeled with [^{32}P]UTP by transcription with T7 RNA polymerase from a template comprising the COOH-terminal region of the receptor (from Gly^{365}) and 180 bp of 3′ untranslated region. Filters were rinsed three times for 5 min each in 2× SSC, incubated for 15 min at room temperature with ribonuclease A (1 μg/ml in 2× SSC), and washed for 2 hours at 55°C in 0.1× SSC and 0.1% SDS.

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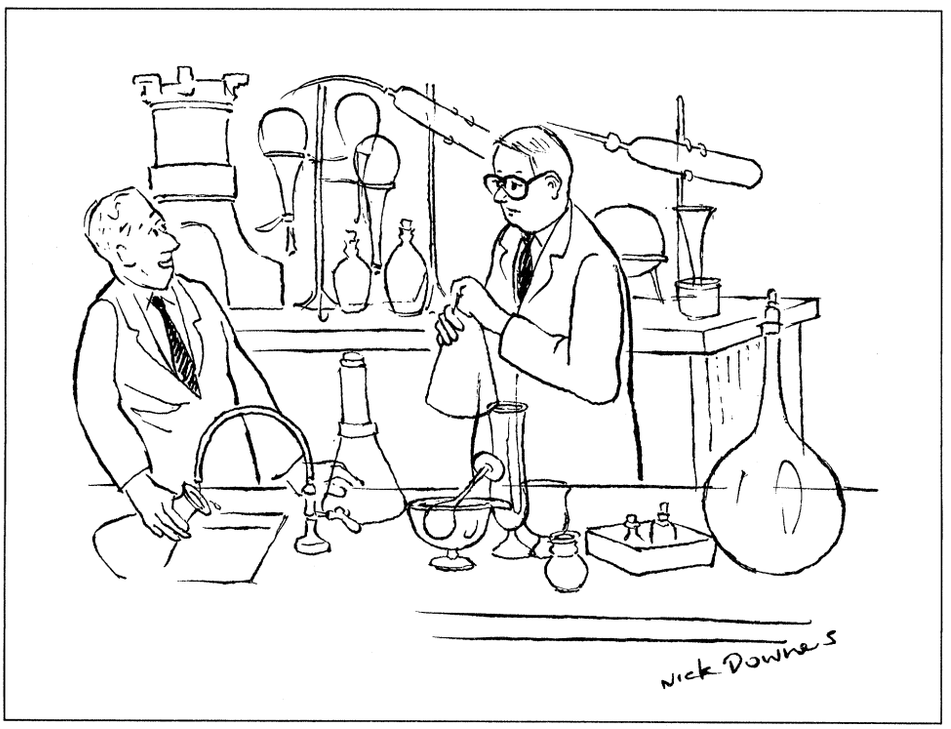

"Heard any good anecdotal evidence lately?"