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16. In G. Stent's interpretation of the Hebb postulate [*Proc. Natl. Acad. Sci. U.S.A.* **70**, 997 (1973)], postsynaptic depolarization is a necessary part of the cellular events that lead to synaptic elimination. In our study, heterosynaptic suppression was observed under voltage-clamp conditions, thus excluding the involvement of membrane depolarization. However, activation of ACh receptors could lead to postsynaptic changes, such as Ca^{2+} influx [E. R. Decker and J. A. Dani, *J. Neurosci.* **10**, 3413 (1990)], in the absence of membrane depolarization.
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Expression Cloning of an Adenylate Cyclase–Coupled Calcitonin Receptor

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A calcitonin receptor complementary DNA (cDNA) was cloned by expression of a cDNA library from a porcine kidney epithelial cell line in COS cells. The 482–amino acid receptor has high affinity for salmon calcitonin (dissociation constant $K_d \approx 6$ nM) and is functionally coupled to increases in intracellular cyclic adenosine monophosphate (cAMP). The receptor shows no sequence similarity to other reported G protein–coupled receptors but is homologous to the parathyroid hormone–parathyroid hormone–related peptide (PTH-PTHrP) receptor, indicating that the receptors for these hormones, which regulate calcium homeostasis, represent a new family of G protein–coupled receptors.

CALCITONIN IS A 32–AMINO ACID hormone that lowers serum calcium concentrations by increasing renal calcium excretion and inhibiting osteoclast-mediated bone resorption (*1*). A major pathway for intracellular signaling by calcitonin is through increases in cytosolic cAMP (*2*). The calcitonin receptor is thought to couple to $G_{s\alpha}$, the heterotrimeric guanosine triphosphate (GTP)–binding protein that is sensitive to cholera toxin (*3*). The receptor can also couple to an additional signaling pathway via a pertussis toxin–sensitive G protein in isolated osteoclasts (*4*) and in LLC-PK_1 cells (*5*).

We cloned the calcitonin receptor by expression in COS cells using a previously reported strategy (*6*). A size-fractionated cDNA library was constructed (7) from LLC-PK_1 cells (*8*), a porcine kidney epithelial cell line that expresses $\sim 3 \times 10^5$ calcitonin receptors per cell with an apparent dissociation constant (K_d) of ~3 nM (Fig. 1A). Pools of mini-prep cDNA (*9*) containing 10^4 recombinants were transfected into COS cells and screened for binding to radioiodinated salmon calcitonin (SCT) by emulsion autoradiography (*10*). After screening 30 pools representing 3×10^5 clones, we identified two positive pools from which two positive clones with cDNA inserts 2.2 and 3.9 kilobases in length were isolated. The 2.2-kilobase clone (3J8-14-F1) was a truncated version of the 3.9-kilobase clone (2B5-0-1) but encoded the same open reading frame. We studied and characterized the properties of the 2.2-kilobase clone.

Radioiodinated SCT binds to LLC-PK_1 cells and to COS cells transfected with the cloned calcitonin receptor (CTR) cDNA (Fig. 1). Transfected COS cells expressed $\sim 2 \times 10^6$ receptors per cell (assuming 10% of the transfected cells expressed the receptor) with an apparent K_d of ~6 nM, similar to that expressed by LLC-PK_1 cells. Bovine PTH(1-34) [see (*11*)] did not compete for binding of radioiodinated SCT to the CTR transfectants.

The cloned receptor is functionally coupled to increase in intracellular cAMP (Fig.

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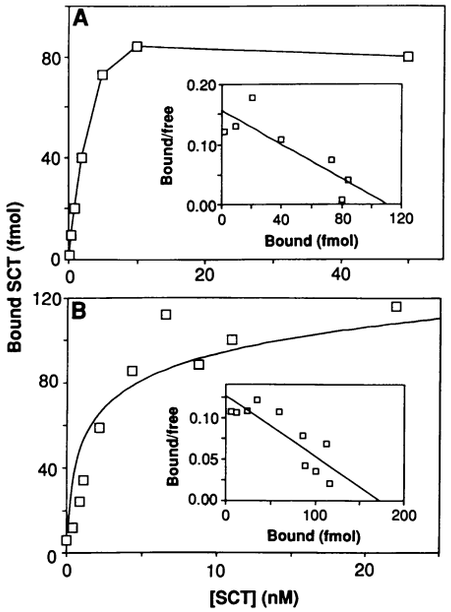

Fig. 1. (**A**) Calcitonin binding to LLC-PK$_1$ cells. (**B**) Calcitonin binding to COS cells transfected with clone 3J8-14-F1. Insets show Scatchard analysis of the binding data. Binding assays were performed as described (*16*).

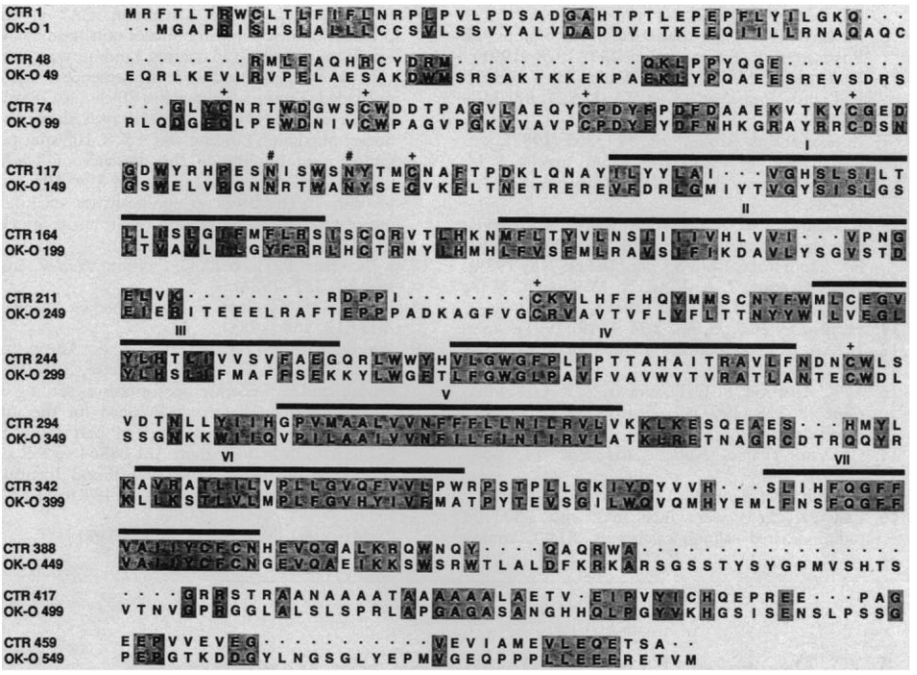

Fig. 3. An alignment of CTR versus the PTH-PTHrP receptor (OK-O) from opossum kidney cells (*11*) was generated with UWGCG program GAP (*11*). The following similarities were allowed: A:G, C:S.Y, D:E.G.N.Q, E:Q, F:I.L.W.Y, H:Q, I:I.V, K:R, L:M.V, R:W, and W:Y. Shaded boxes represent identity or similarity. The bars above the sequences represent the transmembrane domains indicated by the KKD method (*18*). The symbol "#" indicates N-linked glycosylation sites and "+" indicates conserved cysteines. The GenBank accession number is M74420 for the calcitonin receptor and M74445 for the PTH-PTHrP receptor.

2). A fourfold increase in the concentration of intracellular cAMP was observed after incubation of CTR-transfected COS cells with calcitonin, but there was no increase when cells mock-transfected with β-galactosidase were stimulated with calcitonin. Isoproterenol, an agonist of the β-adrenergic receptor, activated adenylate cyclase in both mock- and CTR-transfected cells.

Northern (RNA) blot analysis of poly A$^+$ mRNA from LLC-PK$_1$ cells and pig organs showed a single ~4.2-kilobase transcript. Expression of mRNA was most abundant in the brain and was present in other tissues (data not shown).

Analysis of the deduced amino acid sequence of the CTR (Fig. 3) revealed a molecule with an unusual structure. Searches of nucleic acid and protein sequence databases have not identified sequences similar to CTR. A Kyte-Doolittle hydropathy analysis (*12*) indicates seven or eight regions of hydrophobic amino acid sequences that could generate transmembrane domains. The CTR has no significant sequence identity (<12%) to any of the ~120 cloned receptors that are thought to span the membrane seven times and to interact with G proteins (*13*).

The NH_2-terminal hydrophobic domain, a putative hydrophobic signal sequence (*14*), precedes a long NH_2-terminal domain (147 amino acids with three potential N-linked glycosylation sites) that is presumed to be extracellular. There is a short cytosolic loop between helices V and VI that is not similar to corresponding regions of other adenylate cyclase–coupled receptors; this region is thought to couple to $G_{s\alpha}$. This unusual structural feature of the CTR could account for the observed coupling of the receptor to different G proteins in cultured osteoclasts (*4*) and the coupling during different phases of the cell cycle in LLC-PK$_1$ cells (*5*).

There is a striking degree of amino acid sequence similarity observed between the CTR and the PTH-PTHrP receptor, which is also different from other G protein–coupled receptors (Fig. 3) (*11*). Although the PTH-PTHrP receptor is more than 100 amino acids longer than the CTR, overall there is ~32% identity and ~56% similarity between the sequences of the two receptors. A stretch of 17 out of 18 amino acids around the putative transmembrane domain VII are identical. Also, two out of four N-linked glycosylation sites and the position of seven out of eight potentially extracellular cysteines are conserved (Fig. 3). Major differences between the two receptors appear to lie in their NH_2-terminal and COOH-terminal domains, where gaps exist in the CTR sequence relative to the PTH-PTHrP sequence. Both receptors also activate adenylate cyclase (Fig. 2) (*11*). The structural similarity of the CTR and the PTH-PTHrP receptor suggests that they represent members of a new class of seven transmembrane-spanning G protein–coupled receptors that activate adenylate cyclase.

Noted added in proof: After resubmission of this paper, we noted that the recently published rat secretin receptor (*15*) is similar in sequence to both the calcitonin and PTH/PTHrP receptors, with 30 and 42% identity, respectively. Thus, it is a member of this new receptor family.

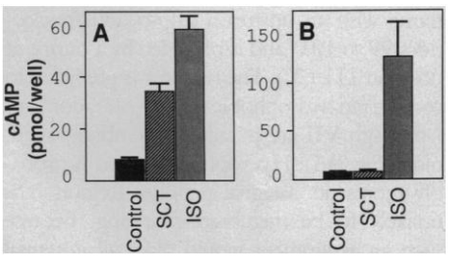

Fig. 2. The cAMP response in (**A**) calcitonin receptor–transfected COS cells and (**B**) COS cells mock-transfected with β-galactosidase. SCT = 1 μM salmon calcitonin and ISO = 4 μM isoproterenol. The cAMP assays were performed as described (*8*).

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A G Protein–Linked Receptor for Parathyroid Hormone and Parathyroid Hormone–Related Peptide

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The complementary DNA encoding a 585–amino acid parathyroid hormone–parathyroid hormone–related peptide (PTH-PTHrP) receptor with seven potential membrane-spanning domains was cloned by COS-7 expression using an opossum kidney cell complementary DNA (cDNA) library. The expressed receptor binds PTH and PTHrP with equal affinity, and both ligands equivalently stimulate adenylate cyclase. Striking homology with the calcitonin receptor and lack of homology with other G protein–linked receptors indicate that receptors for these calcium-regulating hormones are related and represent a new family.

PTH REGULATES CA AND P METABOLISM by binding to specific G protein–coupled receptors in bone and kidney (*1*), thereby activating adenylate cyclase and phospholipase C (*2, 3*). PTHrP, which shares 8 of 13 NH_2-terminal residues with PTH, causes the hypercalcemia of malignancy syndrome (*4, 5*). Both peptides appear to bind to the same ~80-kD receptor glycoprotein (*5–9*).

A random-primed, size-selected cDNA library (1.2×10^7 independent transformants) was constructed in pcDNAI (*10*) from opossum kidney (OK) cells (*3*). COS-7 cells were transfected with 26 plasmid pools, each representing ~10,000 independent colonies, and screened by [^{125}I]PTHrP(1-36) photoemulsion autoradiography (*11, 12*). Two independent clones, OK-O and OK-H, were isolated; each contained ~1.8-kb inserts.

Both receptors, transiently expressed in COS-7 cells, bound PTH(1-34) and PTHrP(1-36) equivalently [K_d (dissociation constant) ≈ 4 nM, compared to ~0.5 nM in native OK cells] when either peptide was used as radioligand (Fig. 1). PTH(3-34) and PTH(7-34) bound with progressively lower affinities with these expressed receptors (K_d ≈ 17 and 230 nM, respectively), showing affinities similar to their affinities in native OK cells (~5 and ~100 nM, respectively). Scatchard analysis revealed $>10^6$ receptor copies per transfected COS-7 cell (20% average transfection efficiency, range 10 to 40%). When OK-O and OK-H were stably expressed in LLC-PK$_1$ porcine renal cells at a density of 10,000 to 100,000 receptors per cell, both of the receptors' apparent K_d's for PTH(1-34) were ~0.3 nM (n = 10) (*13*). Therefore, the lower affinity of these receptors for PTH(1-34) and PTHrP(1-36) in COS-7 cells most likely reflects inefficient G protein coupling. Both peptides stimulated cAMP accumulation in COS-7 cells expressing either OK-O or OK-H equivalently [ED_{50}'s of ~0.4 nM (Fig. 1)] and had similar ED_{50}'s for both ligands in native OK cells. Northern blot analysis of total and poly(A)$^+$ RNA from OK cells revealed an apparently single, approximately 3.0-kb hybridizing species (*14*).

Nucleotide sequencing (*15*) of both strands of OK-O revealed an open reading frame encoding a 585–amino acid protein [figures 2B and 4 in (*16, 17*), respectively]. OK-H is identical to OK-O with the exception of one additional nucleotide at the 3′ end and a deletion of 16 nucleotides at the 5′ end. Further, one of six consecutive G's (nucleotides 1629 to 1634) is deleted and causes a shift in reading frame. Therefore, the resulting 515–amino acid protein encoded by OK-H is shorter than OK-O, and its last eight residues (508 to 515) differ. The region including these multiple G's was amplified from genomic OK cell DNA by polymerase chain reaction and cloned into pcDNAI. Because six individual clones all contained six G's, OK-H is likely to represent a cloning artifact.

Searches for similar sequences in nucleic acid and protein databases showed no related proteins: comparison of the deduced peptide sequence of OK-O with those of 120 reported G protein–linked receptors revealed less than 10% conservation of 35 "signatures," features that are at least 80% conserved among these other receptors (*18, 19*).

OK-O likely contains the entire coding region of the PTH-PTHrP receptor. The ATG codon (nucleotides 114 to 116) probably encodes the initator methionine because it is in frame with an upstream stop-codon (nucleotides 99 to 101) and is preceded by a purine at position 111 (*20*). The receptor is predicted to contain ten hydrophobic regions (*21*); domains I through VII are putatively membrane-spanning (Fig. 2A). Hydrophobic region A probably represents a signal peptide. Region B is unlikely to be membrane-spanning, because such an assignment would place all potential N-glycosylation sites inside the cell. Hydrophobic region C is predicted to reside within the unusually long, first putative extracellular loop. This assignment allows positive charges to line the intracellular borders of several of the

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