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Molecular cloning of two receptors from rat brain with high affinity for salmon calcitonin*

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Two receptors with high affinity for salmon calcitonin were cloned from the nucleus accumbens region of rat brain. The deduced 479 amino acid sequence of cDNA clone L2175-D20 (designated C1a receptor) is 78% and 66% identical with those reported for human and porcine calcitonin receptors, respectively. Clone U3237-A2 codes for a receptor (designated C1b) that is identical to C1a except for a 37 amino acid insert in the second extracellular domain. COS-7 cells transfected with either transcript bound [^{125}I]salmon calcitonin with high affinity (K_d = 8 pM for C1a; K_d = 48 pM for C1b) and responded to salmon calcitonin with increases in cAMP. Tissue distribution studies revealed C1a transcript in rat brain, skeletal muscle, kidney and lung, whereas C1b was predominantly found in brain.

Calcitonin; Receptor; Cloning; PCR; Expression; Rat brain

1. INTRODUCTION

Calcitonin receptor cDNAs have recently been cloned from porcine LLC-PK$_1$ cells [1] and human BIN-67 cells [2]. They are members of an emerging subfamily of G protein-coupled receptors (GPCRs) that include receptors for secretin [3], parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP) [4,5], glucagon [6], glucagon-like peptide 1 [7], vasoactive intestinal peptide [8] and growth hormone releasing hormone [9]. The members of this receptor subfamily share 26–51% identity with each other but ≤ 12% [1] with other GPCRs at the amino acid level.

Binding sites with high affinity for salmon calcitonin are concentrated in certain brain regions including hypothalamus, brainstem, striatum, nucleus accumbens and thalamus [10]. In this report, we describe two closely related receptors cloned from rat nucleus accumbens. The receptors were expressed in COS-7 cells for measurement of hormone binding and cAMP production. Tissue distribution was evaluated by both Northern blot and PCR methods. Salmon calcitonin-specific binding sites in brain have been termed C1 sites [11] and we refer to the two receptors described in this report as C1a and C1b receptors.

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*The nucleotide sequences described herein have been submitted to the GenBank and assigned accession numbers L14617 (clone L2175-D20) and L14618 (clone U3237-A2).

2. MATERIALS AND METHODS

2.1. *cDNA library screening*

An oligo(dT)-primed rat nucleus accumbens cDNA library was cloned in eukaryotic expression vector pcDNAI (Invitrogen; San Diego, CA) and transformed into *E. coli* MC1061/P3. Plasmid DNA was prepared from 72 sublibrary pools of ~5000 clones each and subjected to PCR amplification [12] utilizing a pair of degenerate oligonucleotides corresponding to conserved transmembrane (TM) regions of the porcine calcitonin, opossum PTH/PTHrP and rat secretin receptors. A312 is a mixture of sixteen 18-mers corresponding to the TM3 sense strand: 5′-GA(A/G)GG(G/C)(G/C)TCTA(C/T)CTTCAC-3′. A313 is a mixture of sixty-four 17-mers corresponding to the TM7 antisense strand: 5′-(T/C)(C/G)(A/G)TTG(C/A)(A/G)GAA(G/A)CAGTA-3′. Products were Southern blotted and probed [13] with a third degenerate oligonucleotide (A315) corresponding to a different TM7 region of the calcitonin and PTH/PTHrP receptors: 5′-GCAACGAA(G/A)AATCCCTGGAA-3′. Two positive pools were subdivided and rescreened until single bacterial transformants were isolated.

2.2. *5′ RACE*

Amplification and isolation of the upstream 5′ ends of the receptor cDNAs was accomplished using the technique of rapid amplification of cDNA ends [14,15] (5′ RACE system; Gibco BRL, Gaithersburg, MD) following the manufacturer's protocol. Briefly, 100 ng rat nucleus accumbens poly(A)$^+$ RNA was converted to first strand cDNA using A333 gene-specific primer (TM7; antisense strand): 5′-CCCTGGAAATGAATCAGAGAG-3′. Subsequent PCR amplification employed gene-specific primer A361 (E4 domain; antisense strand): 5′-(CAU)$_4$ATAATCATAGATCTTCCCAAGC-3′.

Specific amplification products were identified by Southern blotting and probing with a 656 bp TM2–7 probe from clone pcDNAI-237. They were subsequently subcloned and partially sequenced.

2.3. *Construction of full-length receptor cDNAs*

Corresponding 5′RACE and cDNA library clone fragments were ligated into *Pst*I–*Xba*I linearized eukaryotic expression plasmid pcDNAI and introduced into *E. coli* MC1061/P3. For the C1a receptor, pAMP1-L2 provides the ~1.2 kb sequence from a unique *Pst*I site

in the 5'-untranslated region (~60 bp downstream of the 5'-end of the PCR amplified fragment) to a unique *StuI* site in the I3 domain. pcDNAI-175 provides the corresponding ~2.2 kb sequence from the same *StuI* site through the poly(A) tail at the 3'-end of the cDNA followed by an *XbaI* site introduced by the cloning. Similarly for the C1b receptor, 5'RACE clone pAMP1-U3 provides the ~1.3 kb *PstI*-*StuI* fragment ligated to its corresponding ~2.2 kb *StuI*-*SpeI* fragment from cDNA library clone pcDNAI-237.

2.4. *DNA sequencing*

DNA was sequenced by the dideoxy chain-termination method [16] using a combination of nested deletions and synthetic oligonucleotide primers with modified phage T7 DNA polymerase (Sequenase; United States Biochemicals, Cleveland, OH).

2.5. *Tissue-specific distribution of transcripts*

A Northern blot containing 2 μg of poly(A)$^+$ RNA from eight rat tissues (Clonetech, Palo Alto, CA) was probed [13] with a 1244 bp *Hin*dIII–*Ear*I coding region fragment from clone L2175-D20. The blot was washed in 2 × SSC/0.1% SDS at 50°C and exposed to XAR film at −80°C with intensifying screens.

RT-PCR using 50 ng of poly(A)$^+$ RNA from a variety of rat tissues was accomplished using reagents from the GeneAmp RNA PCR kit (Perkin-Elmer-Cetus; Norwalk, CT) following the manufacturers instructions. First strand cDNA was amplified using upstream primer A356 (TM2; sense strand) 5'-GTTGAGGTTGTGCCCAATGGA-3' and downstream primer A333 for 30 cycles. Products were Southern blotted and probed with a 656 bp TM2–7 probe from clone pcDNAI-237. The blot was washed in 0.2 × SSC/0.1% SDS at 65°C and exposed to film.

2.6. *Transient transfection*

Monolayers of COS-7 cells in T-150 tissue culture flasks were transfected with 14 μg plasmid DNA and 120 μg Lipofectin reagent (Gibco BRL) in 10 ml Optimem media (Gibco BRL) containing 5.5 μM 2-mercaptoethanol. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 4 h after which the transfection medium was replaced with DMEM (low glucose, 1000 mg/l)/10% fetal bovine serum/2% L-glutamine/1% penicillin–streptomycin. 60 h post-transfection, cell homogenates were prepared in ice-cold 20 mM HEPES, aliquoted and stored at −80°C.

2.7. *[^{125}I]salmon calcitonin binding*

Membranes from transfected COS cells were incubated (in quadruplicate) for 1 h at 23°C with [^{125}I]iodotyrosyl-salmon calcitonin (2,000 Ci/mmol; Amersham, Arlington Heights, IL) in a buffer composed of 20 mM HEPES, pH 7.4/1 mg·ml^{-1} BSA/1 mg·ml^{-1} bacitracin/1 μg·ml^{-1} phosphoramidon/5 μg·ml^{-1} bestatin-HCl and were collected on glass fiber filters pretreated with 0.3% polyethyleneimine. Filters were washed with ice cold PBS, dried and assayed for radioactivity. Membrane concentrations were chosen in which specific binding was linear with protein concentration (1.3 μg·ml^{-1} for C1a; 13 μg·ml^{-1} for C1b). Binding of [^{125}I]salmon calcitonin was measured at concentrations varying from 1–150 pM to obtain total binding, and again in the presence of 100 nM unlabeled salmon calcitonin to obtain nonspecific binding. Competition curves were generated by measuring the binding of 8 pM or 20 pM [^{125}I]salmon calcitonin to C1a-expressing and C1b-expressing membranes, respectively, in the presence of 10^{-12} to 10^{-6} M competing peptide. Apparent inhibition constants (K_is) were derived from IC_{50} values using the Cheng–Prusoff equation (Inplot 4.03, GraphPAD, San Diego, CA). Proteins were measured according to the method of Bradford with BSA as control. Unlabeled peptides were from Bachem (Torrance, CA).

2.8. *Stimulation of cAMP production*

24 h after transfection cells were subcultured at 1 × 10^5 cells/0.2 ml medium/well in 96-well tissue culture plates. After an additional 24 h, cells were preincubated for 20 min with 100 μl Dulbecco's phosphate-buffered saline, pH 7.3 (DPBS, Sigma) containing 0.1 mg·ml^{-1} BSA and 0.05 mg·ml^{-1} isobutylmethyl xanthine, followed by 25 min incubation with added hormone. The response was halted by acidification with 25 μl 10% trichloroacetic acid followed by neutralization with 25 μl 0.8 M Tris (hydroxymethyl) aminomethane. Immunoreactive cAMP in supernatants was quantitated after acetylation using a scintillation proximity assay (Amersham, Arlington Heights, IL).

3. RESULTS AND DISCUSSION

3.1. *Molecular cloning*

We identified two distinct calcitonin receptor isoforms using mixtures of oligonucleotides corresponding to conserved regions in TM3 and TM7 of the calcitonin, PTH/PTHrP and secretin receptors as primers to amplify and clone potentially homologous sequences from a rat nucleus accumbens cDNA library. Partial DNA sequencing of two clones, pcDNAI-175 and pcDNAI-237, revealed that both were similar to the porcine calcitonin receptor [1] but that neither was full-length. The two cDNAs were identical over the region sequenced (TM2–7) except for a 111 bp insert in the E2 domain of pcDNAI-237. We employed 5' RACE to amplify the unknown sequence at the 5'-ends of the receptor transcripts. One product (pAMP1-L2) was identical to pcDNAI-175 from TM2 through E4 while a second product (pAMP1-U3) was identical to pcDNAI-237 over the same region, including the 111 bp insert in E2. Full-length sequences for each of the two receptors suitable for expression were constructed by ligating corresponding 5'RACE and cDNA library sequences into the eukaryotic expression vector pcDNAI.

The nucleotide sequences of both clones and their deduced C1a and C1b receptor sequences are presented in Fig. 1. The receptors differ only by a 37 amino acid segment present in the E2 domain of C1b. Except for the 111 bp insert encoding this segment, there are only two nucleotide differences (both of which are silent at the amino acid level) suggesting these receptors may be alternatively spliced transcripts of a single message. Two dopamine D_2 receptor isoforms that differ by a 29 amino acid insert in the I3 domain occur in human [19],

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Fig. 1. Nucleotide and deduced amino acid sequences of rat clone L2175-D20 coding for the C1a receptor. The numbering of the nucleotides and the amino acids is shown at the right and left of each line, respectively. The seven predicted membrane-spanning domains [17] are underlined. Four potential extracellular N-linked carbohydrate attachment sites are double underlined. The predicted signal sequence cleavage site [18] is indicated by a filled triangle (▲). Clone U3237-A2 coding for the C1b receptor was sequenced from nucleotide positions 1–1697 and is identical except for a T at position 1093, a C at position 1186, and the following 111 bp insert between positions 891 and 892: GCATATTTTTCATCATAACAC-ATATATGTGGACAATGCAGTGGGAACTGTCACCACCCTTACCCCTGAGTGCACACGAGGGAAAGATGGACCCTCATGACAGT-GAAGTGAT. This insert codes for an additional 37 residues between amino acid positions 216 and 217: MHIFHHNTYMWTMQWELSPPLPL-SAHEGKMDPHDSEV.

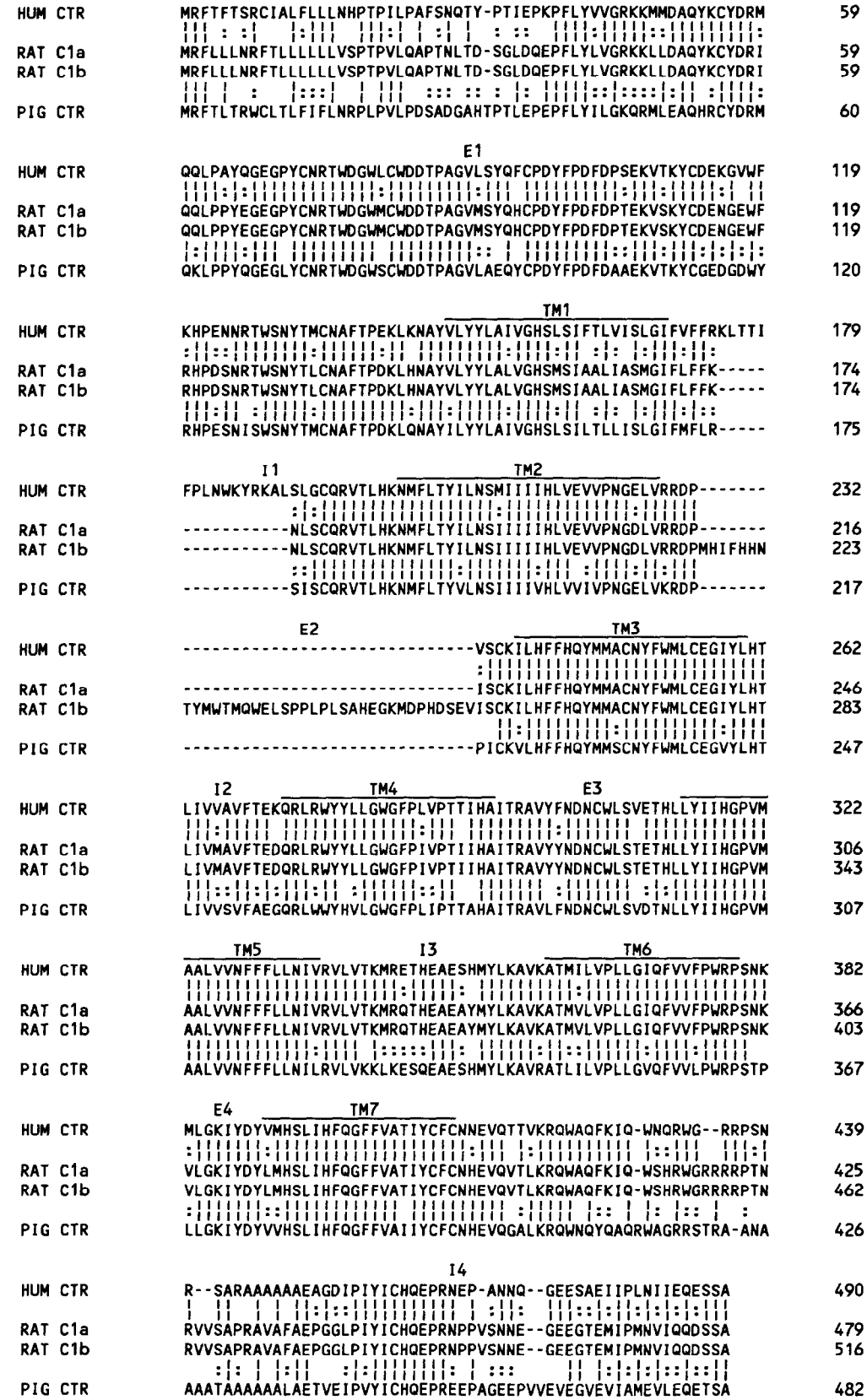

Fig 2. Alignment of the rat C1a and C1b receptors with the human [2] and porcine [1] calcitonin receptors. Amino acid identities are shown by a vertical line (¦) and conservative amino acid replacements by a colon (·). Gaps have been introduced to maximize the alignment. The seven potential transmembrane (TM1–7) domains [17] are indicated by horizontal lines. Extracellular (E1–4) and intracellular (I1–4) domains are marked. The numbering of the amino acids for the receptors is shown in the right margin.

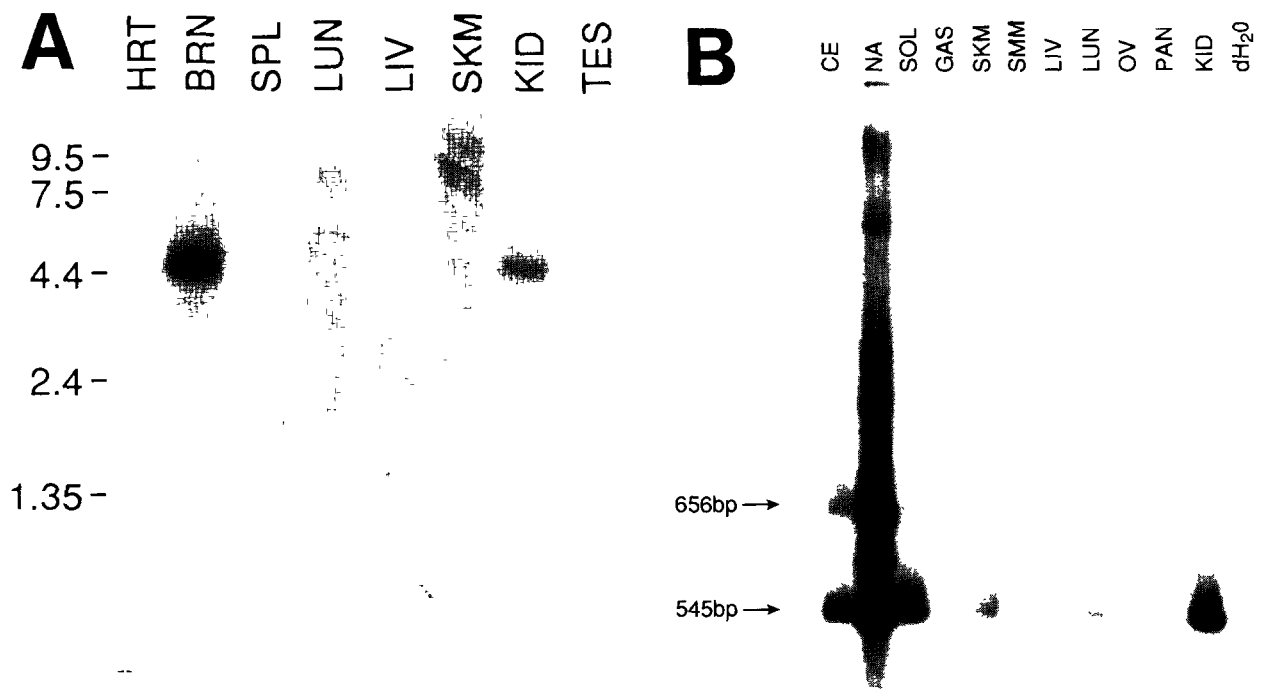

Fig. 3. Tissue distribution of transcripts for C1a and C1b receptors. (A) Northern blot of poly $(A)^+$ RNA from rat tissues hybridized with a receptor coding region probe. RNA size markers (kb) are indicated to the left. The integrity of the RNA samples on this Northern blot was subsequently verified by hybridization with a β-actin probe (data not shown). Abbreviations: HRT, heart; BRN, brain; SPL, spleen; LUN, lung; LIV, liver; SKM, skeletal muscle; KID, kidney; TES, testis. (B) Southern blot of RT-PCR products from a variety of rat tissue poly$(A)^+$ RNA hybridized with a receptor coding region probe. The integrity of the poly$(A)^+$ RNA samples used in these RT-PCR amplifications and successful conversion to cDNA was demonstrated by the PCR amplification of a 983-bp fragment of rat glyceraldehyde- 3-phosphate dehydrogenase cDNA from the same sample used for the above amplification (data not shown). Abbreviations: CE, cerebellum, NA, nucleus accumbens, SOL, soleus muscle; GAS, gastrocnemius muscle; SKM, skeletal muscle; SMM, smooth muscle; LIV, liver; LUN, lung; OV, ovary; PAN, pancreas; KID, kidney; dH_2O, water negative control.

bovine [20], and rat [20,21] brain. The calcitonin receptors described herein appear to be another system in which diversity is achieved by alternative splicing.

An in-frame termination codon at position 173 is followed by three potential in-frame AUG translation initiation codons at positions 191, 212, and 242 (Fig. 1). All three include an A at the +4 position and an A at the −3 position that is sufficient for translation initiation [22]. However, translation from either upstream site results in a protein beginning with 10 or 17 amino acids with a rather hydrophilic nature not typical of a signal sequence. Therefore, the putative translation initiation site was assigned at position 242 based on its similarity to porcine and human calcitonin receptor initiation sites and its hydrophobic profile resembling a more prototypical leader sequence.

The rat C1a and C1b receptors display the cardinal features of the secretin-type peptide receptor family, including a large E1 domain with 14 conserved cysteine residues and several conserved potential N-linked glycosylation sites (Asn-X-Ser/Thr), as well as the seven transmembrane domain topography [23] shared by all G protein-coupled receptors. The rat C1a receptor shares 66% overall sequence identity to the calcitonin receptor cloned from the porcine renal LLC-PK_1 cell line [1] and 78% sequence identity to a human calcitonin receptor cloned from the BIN-67 cell ovarian carcinoma [2] (Fig. 2). They also share a site in the I4 domain (R-R-P-T^{423}) for potential phosphorylation by cAMP-dependent protein kinase [24].

3.2. *Tissue distribution*

Northern blot analysis was performed using RNA from various tissue sources and probing with a nearly complete coding region sequence of the C1a receptor. A single band of ~4.4 kb was identified in rat brain and kidney but could not be detected under the conditions employed in heart, spleen, lung, liver, skeletal muscle, or testis (Fig. 3A). Identical results were obtained using a TM 2–7 probe from the C1b receptor sequence (data not shown).

The tissue-specific distribution of these transcripts was further investigated by qualitative RT-PCR on a variety of rat tissue RNA. Distribution of each of the two receptors was distinguished using primers flanking the 111 bp insert present in the C1b receptor but not in

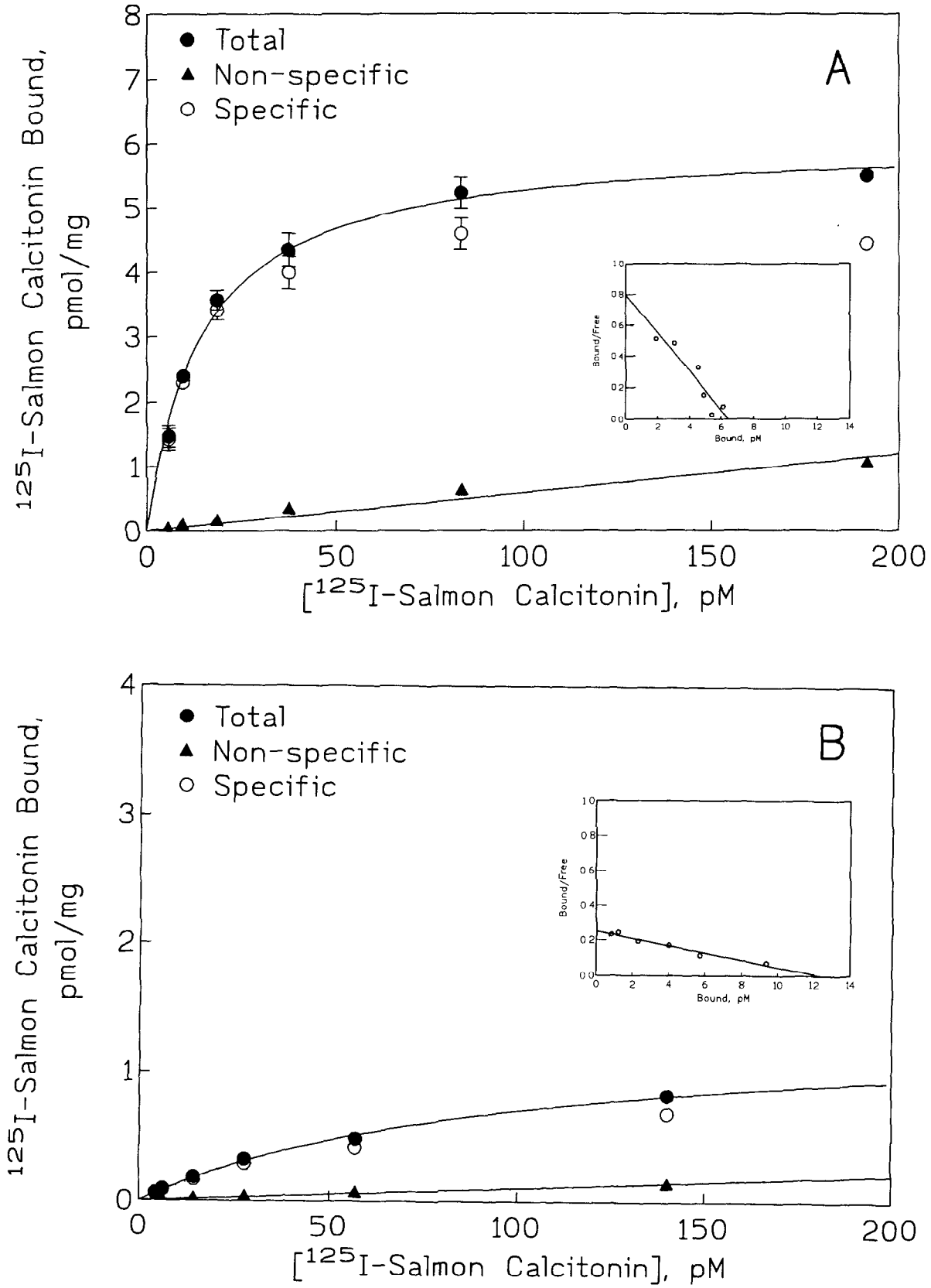

Fig. 4. Binding isotherms for [^{125}I]iodotyrosyl-salmon calcitonin with COS-7 cell membranes transiently expressing rat C1a receptor (A) or rat C1b receptor (B) Inset, Scatchard plot of the specific binding.

the C1a receptor. The predicted 545 bp product corresponding to the C1a receptor was again demonstrated in brain and kidney, and additionally in skeletal muscle and lung (Fig. 3B). In contrast, the predicted 656 bp product corresponding to the C1b receptor was present predominantly in brain, although it could be detected in muscle, lung, and kidney upon longer exposure of the blot. Neither transcript was detected in liver, ovary or pancreas. Sequencing the amplified fragment from rat gastrocnemius muscle indicated that it was identical to the C1a sequence over the 470 bp region between TM2 and TM7, although the possibility of differences outside this region cannot be eliminated. A third product with mobility between the 656 bp and 545 bp fragments is also evident in amplification from nucleus accumbens RNA. We believe it is a heteroduplex of the C1a and C1b products: (i) multiple subclones of this gel isolated fragment were either the 656 bp or 545 bp product; and (ii) it could be formed by subjecting a mixture of the 656 bp and 545 bp products to thermal cycling in the absence of DNA polymerase.

3.3. *Receptor expression and binding studies*

Full-length receptor cDNA constructs were expressed by transient transfection of COS-7 cells in order to determine the ligand binding properties of these unique receptors. Specific and saturable binding of [^{125}I]iodotyrosyl-salmon calcitonin was observed in membranes from COS cells expressing either C1a (Fig. 4A) or C1b receptors (Fig. 4B). Membranes from COS cells transfected with the pcDNAI vector without any cDNA insert did not exhibit specific binding of [^{125}I]salmon calcitonin. Scatchard analysis of C1a saturation data yielded a dissociation constant (K_d) of 8.2 ± 0.3 pM and a binding site density (B_{max}) of 3.9 ± 1.3 pmol/mg protein (mean ± S.D., 2 experiments). Scatchard analysis of C1b saturation data yielded a K_d of 47.8 ± 2.8 pM and a B_{max} of 0.92 ± 0.03 pmol/mg protein. Salmon calcitonin potently competed for [^{125}I]salmon calcitonin binding to both C1a (K_i = 14 ± 2 pM, n = 3) and C1b (K_i=113±6 pM, n=3) receptor-expressing cell membranes. K_is for unlabeled salmon calcitonin measured in competition experiments were somewhat lower affinity than the K_ds for radioiodinated salmon calcitonin measured by saturation binding, but again demonstrated the significant 6- to 8-fold difference in affinity between C1a and C1b receptors. Rat calcitonin was weak at inhibiting [^{125}I]salmon calcitonin binding to either receptor (IC_{50} > 1 μM).

3.4. *Stimulation of cAMP production*

As with other members of the secretin-type receptor family, the C1a and C1b receptors stimulate adenylate cyclase activity upon expression in COS cells (Fig. 5) and contain a motif in the I3 domain (R-X_{11}-K-A-V-K^{343}) postulated to be involved in coupling to Gs_α [25]. Control COS-7 cells transfected with the pcDNAI expression vector alone were unresponsive to salmon calcitonin. All cells showed equivalent cAMP production in response to isoproterenol which activates the adenylate cyclase-linked β adrenergic receptor. Salmon calcitonin stimulated adenylate cyclase activity in membranes from rat kidney but not in membranes from rat spinal cord [26] or hypothalamus [27]. C1a and C1b receptors were capable of stimulating adenylate cyclase activity when expressed in COS-7 cells. Perhaps these receptors are uncoupled from adenylate cyclase during isolation of brain membranes, or they may couple to a different transduction system in brain cells.

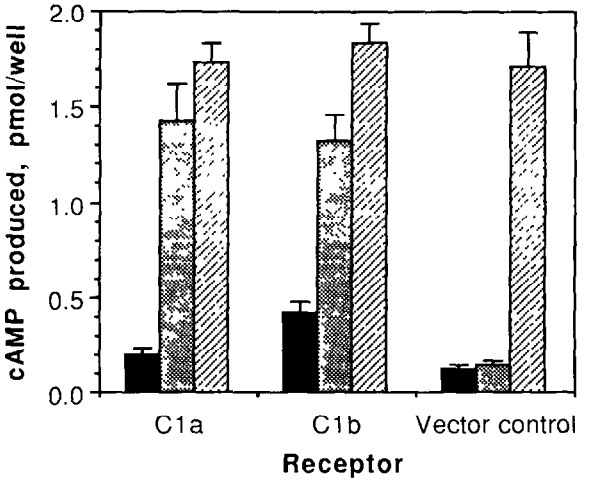

Fig. 5 Cyclic AMP production in COS cells transiently transfected with the C1a or C1b receptor. Cells were incubated with buffer alone (filled bars), 1 μM salmon calcitonin (shaded bars), or 10 μM isoproterenol (cross-hatched bars). Values are means ± S.D. (n = 8).

3.5. *Summary*

We have identified and cloned two receptor isoforms from rat nucleus accumbens that bind salmon calcitonin with high affinity and can couple to stimulation of adenylate cyclase activity. It has come to our attention that two similar calcitonin receptor isoforms have recently been cloned from a rat hypothalamus cDNA library (P. Sexton, personal communication). The role of C1a and C1b receptors in central nervous system function is not known. Intracerebral injection of salmon calcitonin produces analgesia [28], anorexia [29], inhibition of gastric acid secretion [30], inhibition of amphetamine-induced locomotor activity [31] and inhibition of prolactin secretion [32], demonstrating that salmon calcitonin activates functional central receptors. While salmon calcitonin is not present in mammals, substances that display salmon calcitonin-like immunoreactivity have been reported in brain and thyroid [33], lung cancer [34] and prostate [35]. Salmon calcitonin immunoreactive material isolated from rat hypothalamus is reported to be functionally active [36]. These reports point to the existence in the rat of salmon calcitonin-related peptides, one or more of which could

be active at the C1a and/or C1b receptors. The presence of C1a transcript, and presumably C1a receptor, in skeletal muscle may account for the recent findings that salmon calcitonin potently reduces incorporation of glucose into glycogen (K. Beaumont and A. Young, unpublished) and stimulates sodium pump activity [37] in rat soleus muscle, both actions likely mediated by increased cAMP.

The two receptors cloned from nucleus accumbens differ by a 37 amino acid insert in the predicted E2 domain that is associated with significantly lower binding affinity for salmon calcitonin. This is the first example we are aware of where an amino acid insert in a G protein-coupled receptor has been found to alter ligand binding and it may provide a novel mechanism for the modulation of receptor responsiveness. These results also provide structural evidence for intraspecies calcitonin receptor heterogeneity and, together with existing pharmacological data [38–40], indicate that actions of calcitonins may be mediated by distinct receptor subtypes. Studies with receptor-specific pharmacological agents will help in determining the functional significance of the calcitonin receptor isoforms identified in these tissues.

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