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Cloning and functional characterization of a third pituitary adenylate cyclase-activating polypeptide receptor subtype expressed in insulin-secreting cells

(GTP-binding-protein-coupled receptor/gene family/phospholipase C/Ca signal)

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ABSTRACT **Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide belonging to the vasoactive intestinal polypeptide/glucagon/secretin family. It is widely distributed in the body, and a variety of biological actions have been reported. PACAP exerts its biological effects by binding to specific receptors that are coupled to GTP-binding proteins. Recent studies have shown that there is a family of PACAP receptors (PACAPRs), and two members of this family have been identified. We report here the cloning, functional expression, and tissue distribution of a third PACAPR subtype, designated PACAPR-3. The cDNA encoding PACAPR-3 has been isolated from a mouse insulin-secreting β-cell line MIN6 cDNA library. Mouse PACAPR-3 is a protein of 437 amino acids that has 50% and 51% identity with rat PACAP type I and type II receptors, respectively. Expression of recombinant mouse PACAPR-3 in mammalian cells shows that it binds to vasoactive intestinal polypeptide as well as PACAP-38 and -27, with a slightly higher affinity for PACAP-38, and is positively coupled to adenylate cyclase. The expression of PACAPR-3 in *Xenopus* oocytes indicates that calcium-activated chloride currents are evoked by PACAP and vasoactive intestinal polypeptide, suggesting that PACAPR-3 can also be coupled to phospholipase C. RNA blot analysis studies reveal that PACAPR-3 mRNA is expressed at high levels in MIN6, at moderate levels in pancreatic islets and other insulin-secreting cell lines, HIT-T15 and RINm5F, as well as in the lung, brain, stomach, and colon, and at low levels in the heart. Furthermore, insulin secretion from MIN6 cells is significantly stimulated by PACAP-38. These results suggest that the diverse biological effects of PACAP are mediated by a family of structurally related proteins and that PACAPR-3 participates in the regulation of insulin secretion.**

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide of the vasoactive intestinal polypeptide (VIP)/glucagon/secretin family of peptides (1). PACAP is widely distributed, occurring in the central nervous system and peripheral tissues such as pituitary, adrenal medulla, testis, gastrointestinal tract, and pancreas. PACAP has diverse biological effects that are tissue-specific (2). Two forms of PACAP, PACAP-38 and PACAP-27, sharing the same N-terminal 27 peptides, are derived by tissue-specific proteolytic processing of a 176-amino acid precursor protein (3) and are present at various concentrations in different tissues, suggesting different processing in various tissues (4).

PACAP exerts its biological effects by binding to high-affinity receptors that are coupled to GTP-binding (G) proteins (2). Pharmacological studies have indicated that there are at least two types of PACAP receptor (PACAPR) (2). The PACAP type I receptor present in the central nervous system, pituitary, adrenal medulla, and germ cells of the testis binds PACAP with high affinity but binds VIP with 1000-fold lower affinity. In contrast, the PACAP type II receptor present in the lung, liver, and gastrointestinal tract binds PACAP and VIP with similar affinity. Recently, cDNAs encoding PACAP type I (5–9) and type II (10) receptors have been cloned.

Regulation of insulin biosynthesis and secretion is crucial for glucose homeostasis in animals (11). As part of a project characterizing the proteins expressed in pancreatic β cells that may be involved in the regulation of insulin secretion, we have amplified the cDNAs derived from pancreatic islets that could encode G-protein-coupled receptors, by using polymerase chain reaction (PCR). Here we report the cloning, sequence, and functional characterization of a third PACAPR subtype designated PACAPR-3,‖ the tissue distribution of which is distinct from that of PACAP type I and type II receptors. PACAPR-3 has similar binding properties to that of the type II receptor in that it binds both PACAP and VIP with high affinity. Heterologous expression studies have indicated that PACAPR-3 can be coupled to phospholipase C as well as to adenylate cyclase. Interestingly, PACAPR-3 is expressed in pancreatic islets and insulin-secreting cell lines including MIN6 (12), HIT-T15, and RINm5F. Since PACAP-38 stimulates insulin secretion in MIN6 cells, PACAPR-3 may play a physiological role in the regulation of insulin secretion.

MATERIALS AND METHODS

General Methods. Standard methods were carried out as described (13, 14). Total cellular RNA was isolated by the guanidinium isothiocyanate/CsCl procedure. DNA sequencing was done by the dideoxynucleotide chain-termination procedure after subcloning appropriate DNA fragments into M13 mp18 or mp19. Both strands were sequenced. Radiolabeled and unlabeled peptides were purchased from Peninsula Laboratories and the Peptide Institute (Osaka), respectively.

Cloning of cDNA Encoding a G-Protein-Coupled Receptor. First-strand cDNA was prepared using 10 μg of total rat pancreatic islet RNA, reverse transcriptase (Superscript,

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Abbreviations: PACAP, pituitary adenylate cyclase-activating polypeptide; PACAPR, PACAP receptor; VIP, vasoactive intestinal peptide; G protein, GTP-binding protein; PHM, peptide histidine methionine; GIP, gastric inhibitory peptide.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. D28132).

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                                                                  1
                                                                  Met Arg Ala Ser Val
GTTGCTGTCGGACCGTGCTGCTGAGGCGCCAAGGACCGAGGCAGCACGCTGAGCCCAAG ATG AGG GCG TCG GTG
                10                                                20
Val Leu Thr Cys Tyr Cys Trp Leu Leu Val Arg Val Ser Ser Ile His Pro Glu Cys Arg
GTG CTG ACC TGC TAC TGC TGG TTG CTG GTG CGG GTG AGC AGC ATC CAT CCA GAA TGT CGC
                30                                                40
Phe His Leu Glu Ile Gln Glu Glu Glu Thr Lys Cys Ala Glu Leu Leu Ser Ser Gln Thr
TTT CAT CTA GAA ATA CAA GAA GAG GAG ACA AAA TGT GCA GAG CTG CTA AGC AGC CAA ACG
                50                          ▼                     60
Glu Asn Gln Arg Ala Cys Ser Gly Val Trp Asp Asn Ile Thr Cys Trp Arg Pro Ala Asp
GAG AAT CAG AGA GCC TGC AGC GGT GTC TGG GAC AAC ATC ACA TGC TGG CGC CCG GCA GAC
                70                                                80
Val Gly Glu Thr Val Thr Val Pro Cys Pro Lys Val Phe Ser Asn Phe Tyr Ser Arg Pro
GTT GGG GAA ACT GTC ACA GTG CCC TGC CCC AAA GTA TTC AGC AAT TTC TAC AGC AGA CCA
   ▼            90    ▼                                            100
Gly Asn Ile Ser Lys Asn Cys Thr Ser Asp Gly Trp Ser Glu Thr Phe Pro Asp Phe Ile
GGA AAC ATA AGC AAA AAC TGC ACT AGC GAT GGA TGG TCA GAG ACA TTT CCA GAT TTC ATA
                110                                               120
Asp Ala Cys Gly Tyr Asn Asp Pro Glu Asp Glu Ser Lys Ile Ser Phe Tyr Ile Leu Val
GAT GCG TGT GGC TAC AAC GAC CCC GAG GAT GAG AGT AAG ATC TCG TTT TAT ATT TTG GTG
                130                                               140
Lys Ala Ile Tyr Thr Leu Gly Tyr Ser Val Ser Leu Met Ser Leu Thr Thr Gly Ser Ile
AAG GCC ATT TAT ACC TTG GGC TAC AGT GTT TCT CTG ATG TCT CTT ACA ACA GGA AGC ATA
                150                                               160
Ile Ile Cys Leu Phe Arg Lys Leu His Cys Thr Arg Asn Tyr Ile His Leu Asn Leu Phe
ATT ATC TGC CTC TTC AGG AAG CTG CAC TGC ACA AGG AAC TAC ATC CAC CTA AAC CTC TTC
                170                                               180
Leu Ser Phe Met Leu Arg Ala Ile Ser Val Leu Val Lys Asp Ser Val Leu Tyr Ser Ser
CTC TCC TTC ATG CTG AGA GCC ATC TCT GTG CTG GTC AAG GAC AGC GTG CTC TAC TCC AGC
                190                                               200
Ser Gly Leu Leu Arg Cys His Asp Gln Pro Ala Ser Trp Val Gly Cys Lys Leu Ser Leu
TCA GGT CTA CTG CGC TGC CAC GAC CAG CCA GCC TCC TGG GTT GGC TGC AAG CTC AGC CTG
                210                                               220
Val Phe Phe Gln Tyr Cys Ile Met Ala Asn Phe Tyr Trp Leu Val Glu Gly Leu Tyr
GTA TTC TTC CAG TAC TGT ATC ATG GCA AAC TTC TAC TGG CTT GTG GAG GGT CTC TAC
                230                                               240
Leu His Thr Leu Leu Val Ala Ile Leu Pro Pro Ser Arg Cys Phe Leu Ala Tyr Leu Leu
CTG CAC ACC CTC CTG GTA GCC ATC CTT CCT CCC AGC AGG TGC TTC CTG GCC TAC CTT CTG
                250                                               260
Ile Gly Trp Gly Ile Pro Ser Val Cys Ile Gly Ala Trp Thr Ala Thr Arg Leu Ser Leu
ATC GGA TGG GGC ATC CCC AGT GTG TGT ATA GGT GCA TGG ACA GCA ACT CGC CTC TCT TTA
                270                                               280
Glu Asp Thr Gly Cys Trp Asp Thr Asn Asp His Ser Ile Pro Trp Trp Val Ile Arg Met
GAA GAC ACA GGT TGC TGG GAC ACA AAC GAC CAC AGC ATC CCC TGG TGG GTC ATT CGG ATG
                290                                               300
Pro Ile Leu Ile Ser Ile Val Val Asn Phe Ala Leu Phe Ile Ser Ile Val Arg Ile Leu
CCC ATT CTA ATT TCT ATT GTA GTC AAC TTT GCC CTC TTC ATC AGC ATT GTA AGG ATC TTA
                310                                               320
Leu Gln Lys Leu Thr Ser Pro Asp Val Gly Gly Asn Asp Gln Ser Gln Tyr Lys Arg Leu
CTT CAG AAA CTA ACT TCT CCA GAT GTT GGT GGC AAT GAC CAG TCA CAG TAC AAG AGG CTT
                330                                               340
Ala Lys Ser Thr Leu Leu Leu Ile Pro Leu Phe Gly Val His Tyr Met Val Phe Ala Ala
GCC AAG TCC ACA CTG CTG CTA ATC CCC CTG TTT GGC GTC CAC TAC ATG GTG TTT GCT GCC
                350                                               360
Phe Pro Ile Gly Ile Ser Ser Thr Tyr Gln Ile Leu Phe Glu Leu Cys Val Gly Ser Phe
TTC CCT ATT GGC ATC TCA TCC ACA TAC CAG ATC CTG TTT GAG TTA TGT GTT GGT TCC TTC
                370                                               380
Gln Gly Leu Val Val Ala Val Leu Tyr Cys Phe Leu Asn Ser Glu Val Gln Cys Glu Leu
CAG GGC CTG GTG GTA GCA GTT CTA TAC TGC TTC CTG AAC AGT GAG GTA CAG TGT GAA CTG
                390                                               400
Lys Arg Arg Trp Arg Gly Leu Cys Leu Thr Gln Ala Gly Ser Arg Asp Tyr Arg Leu His
AAA AGA AGA TGG CGA GGC CTG TGC CTG ACC CAA GCT GGG AGC CGG GAC TAC CGG CTG CAC
                410                                               420
Ser Trp Ser Met Ser Arg Asn Gly Ser Glu Ser Ala Leu Gln Ile His Arg Gly Ser Arg
AGC TGG TCC ATG TCC CGG AAT GGC TCA GAA AGT GCC CTA CAG ATA CAC CGT GGC TCC CGC
                430                         437
Thr Gln Ser Phe Leu Gln Ser Glu Thr Ser Val Ile AM
ACC CAG TCC TTC CTG CAG TCA GAG ACT TCA GTC ATT TAG CTGTGTCCCTTGTACAGAGCTGTCAGT

GCTGCTGGGTTTGACATATGTTTGCTGGATTCCTCTGCTGCCCCAGTGTCTGGTGCCTTATTGGTTCAGCCCTGGTCCT
TAACTCTGATCCTCATGTGTAACTTGATTGAAACACCAATTATTATTGTCAAACTCTAGCCTTTAAGCCATCTTCTTCA
TAATATGGCTCAGCCATATTCTACTTTCAAAGAGAGCAAGGAAGCCAGGTGGCTGTGAACATCAAAACTGGATTCTAGT
ACCTATGAAGAGAACAAGGAGGAAGATTCCAGTTCCCCTACTGCCTCCTGTCAGGGGGAACCAGGTCCAGAAACAGCTG
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FIG. 1. Nucleotide and predicted amino acid sequence of mouse PACAPR-3 cDNA. Numbers above each line refer to the amino acid positions. Solid triangles indicate three potential sites of N-linked glycosylation.

GIBCO/BRL), and a random hexamer primer (Pharmacia). The cDNAs were amplified by PCR. The primers for PCR were selected from a region of homology among G-protein-coupled receptors for the VIP/glucagon/secretin family (15–20). The sense and antisense primers used were GS-1 [5′-CA(T/C)TG(T/C)AC(G/A/T/C)CG(G/A/T/C)AA(T/C)TA(T/C)AT-3′] and GS-2 [5′-GC(G/A/T/C)AC(G/A/T/C)A(A/T/C)(G/A/T/C)A(G/A)(G/A/T/C)CC(T/C)TG(G/A)AA-3′], respectively. The PCR products were further amplified using the sense and antisense primers GS-3 [5′-TGG(A/T/C)T(G/A/T/C)(T/C)T(G/A/T/C)GT(G/A/T/C)GA(G/A)GG-3′] and GS-2, respectively. The PCR was performed for 40 cycles under the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 45°C, and extension for 1 min at 72°C. The PCR products of ≈480 bp were gel-purified and cloned into M13 mp18 and sequenced. One of the PCR products, rGLR66, encoded a putative receptor for VIP/glucagon/secretin family. A MIN6 cDNA library has been made from a mouse insulin-secreting cell line, MIN6 cells (12), in the vector λZAP II (Stratagene), and 5×10^5 plaques were screened by hybridization with a ^{32}P-labeled rGLR66 DNA fragment as a probe. A full-length cDNA encoding a λ mouse GLR66 (termed λmGLR66) was isolated from this library and sequenced.

Identification of the Ligand for mGLR66. Receptors for the VIP/glucagon/secretin family are known to mediate intracellular cAMP formation. To identify the ligand for mGLR66, we examined changes in cAMP levels in response to various peptides in COSGs1 cells transfected with a mGLR66 expression vector. COSGs1 cells (a gift from S. Nagata, Osaka Bioscience Institute, Osaka) constitutively express the stimulatory G protein Gs (15). A 2.4-kb *Eco*RI fragment of mGLR66 was inserted into pCMV6c (21). The resulting construct (20 μg) was transfected into the COSGs1 cells in 10-cm plates by the calcium phosphate method (22). The transfected COSGs1 cells were split into 6-well plates 48 hr after transfection and further incubated in Dulbecco's modified Eagle's medium (DMEM) for 24 hr at 37°C. The cells were washed with incubation buffer (DMEM containing bovine serum albumin at 1 mg/ml) and incubated for 45 min at 37°C in 2 ml of the same buffer containing 0.2 mM 1-methyl-3-isobutylxanthine with various peptides at the indicated concentrations. After aspirating the buffer, the reaction was terminated by the addition of 1 ml of iced ethanol to the cells, and the suspension was centrifuged. The supernatants were dried under vacuum, and the cAMP levels were quantified by an enzyme immunoassay system (Amersham).

Expression of Mouse PACAPR-3 in Chinese Hamster Ovary (CHO) Cells and Binding Experiments. The mGLR66 expression plasmid that carries a full-length cDNA encoding a third PACAPR subtype (PACAPR-3) was cotransfected with pSV2neo into CHO cells by using the Lipofectin reagent (GIBCO/BRL). Stable transformants were selected in α minimal essential medium containing 10% (vol/vol) fetal calf serum (Hyclone) and G418 at 400 μg/ml. The cells were grown to confluency in 12-well plates at 37°C. The cells were washed twice with buffer containing 50 mM Tris·HCl (pH 7.4), 200 mM sucrose, 5 mM $MgCl_2$, and bovine serum

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PACAPR-3  MRASVVLTCYCWL----------LVRVSSI----HPECRFHLEIQEEETKCAEL------LSSQTENQRACSGVWDWITCWRPADVG  67
PACAPR-1  MRPPSPPHVR-WLCVLAGALACALRPAGSQAASPQHECEYLQLIEIQRQQCLEEAQ------LENETT-GCSKMWDWLTCWPTTPRG  79
PACAPR-2  MAR----------VLQLSLTALLLPV---AIAMHSDCIFKKEQAM----CLERIQRANDLMGLNESSPGCPGMWDWITCWKPAQVG  69

PACAPR-3  ETVTVPCPKVFSNFYSRP----------------------GNISKNCTSDGWSETFPD-FIDACGYND----PEDESKISFYILVK  126
PACAPR-1  QAVVLDCPLIFQLFAPIHGY----------------------NISRSCTEEGWSQLEPGPYHIACGLNDRASSLDEQQQTKFYNTVK  144
PACAPR-2  EMVLVSCPEVFRIFNPDQVWMTETIGDSGFADSNSLEITDMGVVGRNCTEDGWSEPFPH-YFDACGFDDYE--PESGDQDYYYLSVK  153

          <---------- M1 --------->          <-------- M2 ------>                              <----------
PACAPR-3  AIYTLGYSVSLMSLTTGSIIICLFRKLHCTRNYIHLNLFLSFMLRAISVLVKDSVLYSSSGLLRCHDQPASWVGCKLSLVFFQYCIM  213
PACAPR-1  TGYTIGYSLSLASLLVAMAILSLFRKLHCTRNYIHMHLFMSFILRATAVFIKDMALFNSGEIDHCSEAS---VGCKAAVVFFQYCVM  228
PACAPR-2  ALYTVGYSTSLATLTTAMVILCRFRKLHCTRNFIHMNLFVSFMLRAISVFIKDWILYAEQDSSHCFVST---VECKAVMVFFHYCVV  237

          --- M3 --------->                <------ M4 ----->                <---------- M5 ------
PACAPR-3  AWFYWLLVEGLYLHTLLVAILPPSRC-FLA-YLLIGWGIPSVCIGAWTATRLSLEDTGCWDTNDHSIPWWVIRMPILISIVVNFALF  298
PACAPR-1  AWFFWLLVEGLYLYTLLAVSFFSERKYF-WGYILIGWGVPSVFITIWTVVRIYFEDFGCWDTIINSSLWWIIKAPILLSILVNFVLF  314
PACAPR-2  SWYFWLFIEGLYLFTLLVETFFPERRYFYW-YTIIGWGTPTVCVTVWAVLRLYFDDAGCWDMNDSTALWWVIKGPVVGSIMVNFVLF  323

          ------->                                                <-------- M6 ------->
PACAPR-3  ISIVRILLQKLTSPDVGGNDQSQYK----------------------------RLAKSTLLLIPLFGVHYMVFAAFPIGISSTYQIL  357
PACAPR-1  ICIIRILVQKLRPPDIGKNDSSPYS----------------------------RLAKSTLLLIPLFGIEYVMFAFFPDNFKAQVKMV  373
PACAPR-2  IGIIIILVQKLQSPDMGGWESSIYFSCVQKCYCKPQRAQQHSCKMSELSTITLRLARSTLLLIPLFGIHYTVFAFSPENVSKRERLV  410

            <------- M7 ------->
PACAPR-3  FELCVGSFQGLVVAVLYCFLNSEVQCELKRRWRGLCLTQAGSRDYRLHSWSMSRNGSESALQIHRGSRTQ------SFLQSETSVI  437
PACAPR-1  FELVVGSFQGFVVAILYCFLNGEVQAELRRKWRRWHLQGVLGWSSKSQHPWGGSNGATCSTQVSMLTRVSPSARRSSSFQAEVSLV  459
PACAPR-2  FELGLGSFQGLVVAVLYCFLNGEVQAEIKRKWRSWKVNRYFTMDFKHRHPSLASSGVNGGTQLSILSKSS-SQLRMSSLPADNLAT  495
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FIG. 2. Comparison of the amino acid sequences of three PACAPR subtypes: mouse PACAPR-3, rat PACAPR-1 (type II receptor), and rat PACAPR-2 (type I receptor). The seven predicted transmembrane domains (M1–M7) are noted. Identical amino acid residues are shown in boldface type. Solid circles indicate the seven cysteine residues conserved in the extracellular regions among all three receptors.

albumin (10 mg/ml). For saturation experiments, the cells were incubated in triplicate with 0.8 ml of the same buffer containing bacitracin (1 mg/ml), 0.1 mM *p*-amidinophenylmethanesulfonyl fluoride hydrochloride, and various concentrations of ^{125}I-labeled PACAP-27 in the presence or absence of 1 μM unlabeled PACAP-27. For competition experiments, the cells were incubated in triplicate with 50 pM ^{125}I-labeled PACAP-27 in the presence of the various peptides at the indicated concentrations. After incubation for 1 hr at 22°C, the cells were washed twice with the ice-cold phosphate-buffered saline and solubilized in 1 ml of 1 M NaOH, and the radioactivity was measured in a γ-counter.

***In Vitro* Transcription of Mouse PACAPR-3 and Expression in *Xenopus* Oocytes.** pGEM-3Z (Promega) (10 μg) containing a full-length cDNA encoding the mouse PACAPR-3 was linearized with *Bam*HI and transcribed *in vitro* using T7 RNA polymerase in the presence of cap analog m^{7}G(5′)ppp(5′)G, according to the manufacturer's recommendations. *Xenopus* oocytes were injected with 70 nl (≈20 ng) of transcribed RNA. After 2–3 days, electrophysiological measurements were performed under a two-electrode voltage clamp with a holding potential of −60 mV. Various peptides [PACAP-38, PACAP-27, VIP, peptide histidine methionine (PHM), glucagon, and gastric inhibitory polypeptide (GIP)] were applied independently and directly to the constantly perfused bath, and ligand-dependent chloride currents were measured as described (23).

Effect of PACAP-38 on Insulin Secretion from MIN6 Cells. MIN6 cells were seeded at 70% confluency in 48-well plates in the medium as described (12). After preincubation in Krebs–Ringer bicarbonate buffer (24) containing 0.2% bovine serum albumin and 3.3 mM glucose for 30 min at 37°C, the

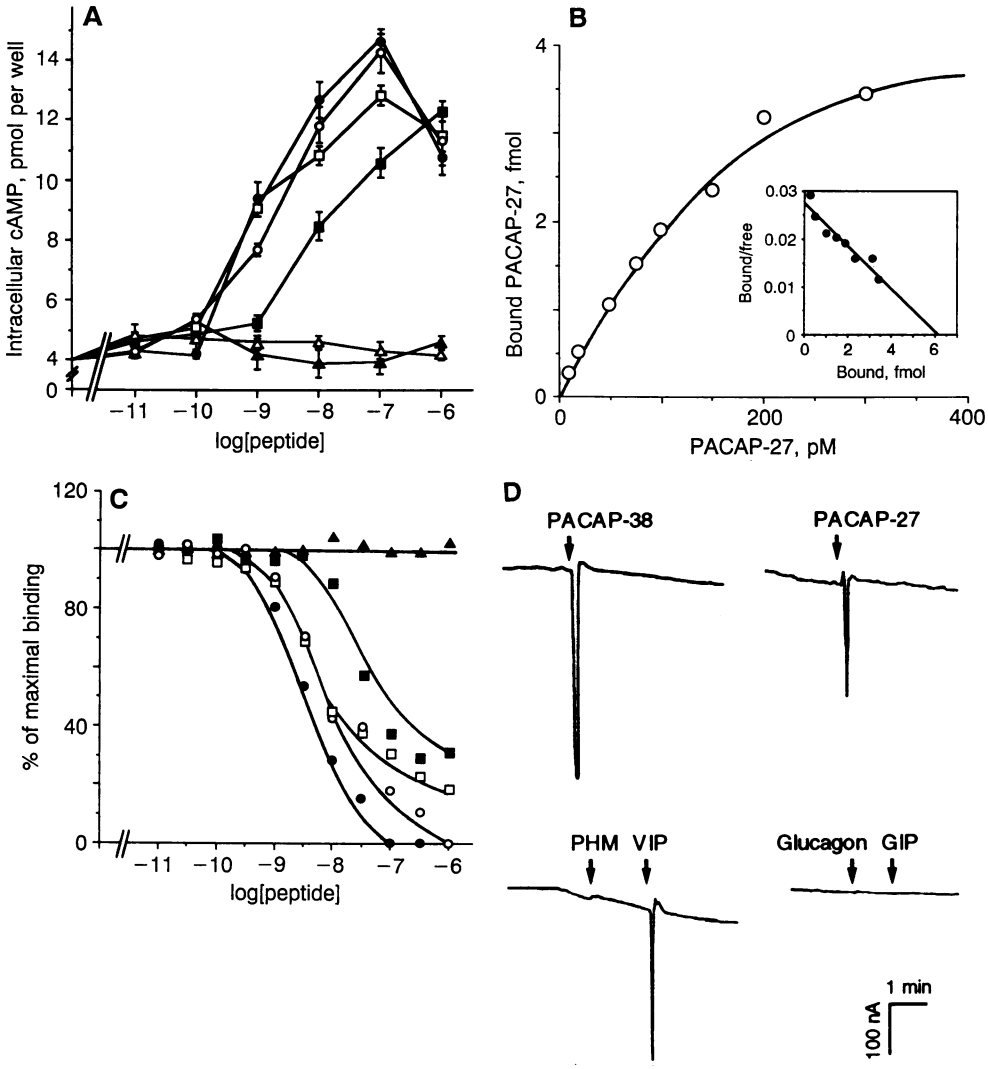

FIG. 3. Pharmacological properties of PACAPR-3. Representative examples of two or three experiments are shown in *A–D*. (*A*) Accumulation of intracellular cAMP in COSGs1 cells transiently expressing PACAPR-3. The COSGs1 cells were incubated for 45 min with various concentrations of PACAP-38 (solid circles), PACAP-27 (open circles), VIP (open squares), PHM-27 (solid squares), glucagon (solid triangles), and GIP (open triangles). The assays were done in triplicate, and the values are the mean ± SEM. (*B*) Saturation and Scatchard analyses of ^{125}I-labeled PACAP-27 in CHO cells stably expressing PACAPR-3. The CHO cells were incubated for 1 hr with various concentrations of ^{125}I-labeled PACAP-27 with or without an excess (1 μM) of unlabeled PACAP-27. (*Inset*) Scatchard analysis of the binding data. The assays were done in triplicate, and the mean values are plotted. (*C*) Displacement of ^{125}I-labeled PACAP-27 by various peptides in CHO cells stably expressing PACAPR-3. The CHO cells were incubated for 1 hr with 50 pM ^{125}I-labeled PACAP-27 alone or in the presence of various concentrations of unlabeled PACAP-38 (solid circles), PACAP-27 (open circles), VIP (open squares), PHM-27 (solid squares), or glucagon (solid triangles). The assays were done in triplicate, and the mean values are plotted. (*D*) Calcium-activated chloride currents evoked by PACAP-38, PACAP-27, and VIP in *Xenopus* oocytes expressing PACAPR-3. PACAP-38, PACAP-27, VIP, PHM, glucagon, and GIP (each at 0.1 nM) were applied rapidly (5–7 sec) and independently to the constantly perfused bath. Chloride currents were recorded at a holding potential of −60 mV.

cells were incubated with the same buffer in the absence or presence of various concentrations of PACAP-38 for 1 hr. The insulin released into the medium was measured by radioimmunoassay (24).

RESULTS AND DISCUSSION

Cloning of PACAPR-3 cDNA. mRNA sequences from rat pancreatic islet cells that encode receptors for the VIP/glucagon/secretin family were amplified by PCR. Of the PCR products sequenced, clones encoding two putative receptors were obtained, termed rGLR61 and rGLR66. The remaining clones encoded either glucagon (25) or glucagon-like polypeptide 1 receptors (18). Screening of 5×10^5 clones from a MIN6 cDNA library with a ^{32}P-labeled rGLR66 DNA fragment resulted in the isolation of a full-length cDNA clone, designated λmGLR66. A 2.4-kb insert was obtained and sequenced. The sequence of the cDNA revealed a 1311-bp open reading frame (Fig. 1) encoding a 437-amino acid protein with a predicted molecular mass of 49.5 kDa. A hydropathy plot of this protein showed seven hydrophobic domains separated by hydrophilic amino acids, a feature characteristic of G-protein-coupled receptors. An alignment of the amino acid sequences among all known G-protein-coupled receptors showed that the mGLR66 had the highest degree of homology with the rat PACAP type II (designated PACAPR-1 in this report) (51% identity) (10) and type I (designated PACAPR-2) (50% identity) (5–9) receptors (Fig. 2). mGLR66 is also homologous to the rat secretin (47% identity) (15), glucagon-like polypeptide 1 (40% identity) (18), glucagon (39% identity) (25), and growth hormone-releasing hormone (38% identity) (16, 17) receptors. This comparison of the amino acid sequences suggests that mGLR66 encodes a receptor related to receptors for the VIP/glucagon/secretin family and is most homologous to PACAPRs.

Functional Properties of PACAPR-3. Receptors for the VIP/glucagon/secretin family are known to be positively coupled to adenylate cyclase. Accordingly, to identify the ligand for mGLR66, we examined changes in cAMP levels in response to various peptides in COSGs1 cells transiently expressing mGLR66 (Fig. 3*A*). As shown in Fig. 3*A*, PACAP-38 (EC_{50} = 1.3 nM), PACAP-27 (EC_{50} = 2.4 nM), and VIP (EC_{50} = 3.2 nM) stimulate the accumulation of cAMP in COSGs1 cells with similar efficiency. PACAP-38 (1 μM) did not change cAMP levels in nontransfected COSGs1 cells. PHM-27 (EC_{50} = 10 nM) is less potent than these peptides. However, no changes in cAMP levels in response to glucagon or GIP were observed. These results suggested that mGLR66 was a PACAPR-like protein.

We further characterized the pharmacological properties of the cloned putative PACAPR. ^{125}I-labeled PACAP-27 bound to CHO cells stably expressing the mGLR66 in a saturating manner (Fig. 3*B*), whereas untransfected CHO cells did not exhibit specific binding of ^{125}I-labeled PACAP-27. A Scatchard analysis of ^{125}I-labeled PACAP-27 binding data revealed a high-affinity (K_d = 0.22 nM) binding site of PACAP-27. This dissociation constant is in reasonable agreement with the value for binding of PACAP-27 to native and cloned PACAPRs (2, 5–10). The binding specificities of the cloned receptor were then examined. The CHO cells stably expressing the mGLR66 were incubated with ^{125}I-labeled PACAP-27 alone or in the presence of various concentrations of unlabeled PACAP-38, PACAP-27, VIP, PHM-27, glucagon, or GIP (Fig. 3*C*). PACAP-38 (IC_{50} = 3 nM) is most potent in displacing ^{125}I-labeled PACAP-27 binding. PACAP-27 (IC_{50} = 8 nM) and VIP (IC_{50} = 8 nM) are equally potent in displacing ^{125}I-labeled PACAP-27 binding. PHM-27 (IC_{50} = 50 nM) is much less potent. These results demonstrate that GLR66 is a third PACAPR subtype that we have designated PACAPR-3. Since some peptides of the VIP/glucagon/secretin family are known to stimulate intracellular calcium release as well as cAMP production, we next determined whether PACAP-38, PACAP-27, VIP, and PHM could evoke calcium-activated chloride currents in the *Xenopus* oocytes expressing the mouse PACAPR-3. PACAP-38 (0.1 nM), PACAP-27 (0.1 nM), and VIP (0.1 nM) elicited chloride currents in oocytes injected with *in vitro*-transcribed PACAPR-3 complementary RNA, whereas PHM, glucagon, and GIP did not (Fig. 3*D*). Neither PACAP-38 nor VIP evoked any current in oocytes injected with water.

Tissue Distribution of PACAPR-3 mRNA. The expression of PACAPR-3 mRNA was examined by RNA blot analysis on various rat tissues and insulin-secreting cell lines of mouse (MIN6), hamster (HIT-T15), and rat (RINm5F) (Fig. 4). A 3.7-kb PACAPR-3 mRNA was expressed at high levels in MIN6 cells. A transcript of the same size was expressed at moderate levels in the pancreatic islets and RINm5F cells, as well as in the lung, brain, stomach, and colon, and expressed at low levels in the heart. A larger 3.8-kb transcript was expressed at moderate levels in HIT-T15 cells. Low but detectable levels of an additional 1.8-kb transcript were also observed in MIN6 cells and the lung and brain. However, PACAPR-3 mRNA was not present in the kidney, skeletal muscle, liver, and jejunum. The tissue distribution of PACAPR-3 is different from that of PACAPR-2. PACAPR-2 mRNA is expressed at high levels in the brain and at low levels in the adrenal gland but is not expressed in the lung, heart, stomach, and colon (6, 7). The tissue distributions of PACAPR-1 and PACAPR-3 mRNAs overlap but are distinct: PACAPR-1 mRNA is expressed in the lung, brain, liver, jejunum, and colon, but it is not expressed in the heart, kidney, and stomach (10).

Stimulating Effect of PACAP-38 on Insulin Secretion from MIN6 Cells. Since PACAPR-3 mRNA is expressed in pancreatic islets and insulin-secreting cell lines and a recent study has shown that PACAP-27 stimulates insulin secretion from isolated perfused pancreas (26), we examined the effect of PACAP-38 on insulin secretion from MIN6 cells. As shown in Table 1, PACAP-38 (0.1–10 nM) in the presence of 3.3 mM glucose stimulates insulin secretion.

It has been suggested that the biological actions of PACAP are mediated by a family of structurally related proteins (2). Recently, two PACAPR subtypes have been cloned (5–10). PACAPR-2 has high affinity for PACAP but very low affinity for VIP, and PACAPR-1 does not discriminate between PACAP and VIP. We have determined the full sequence of the third member of the PACAPR family (PACAPR-3) and have characterized the functional properties of this protein in heterologous expression systems. The sequences of the three PACAPR subtypes show the greatest similarity in the membrane-spanning regions and diverge the most at their N and C termini (Fig. 2). Analysis of the sequence of mouse PACAPR-3 shows that there are three potential N-linked glycosylation sites located in the extracellular N-terminal

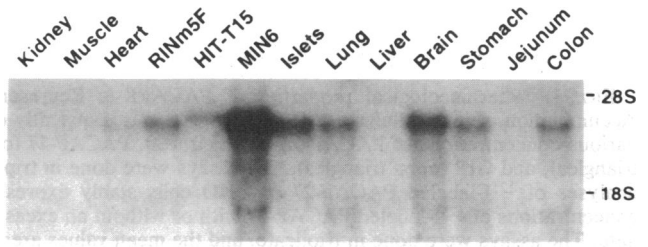

FIG. 4. RNA blot analysis of PACAPR-3 mRNA in insulin-secreting cell lines (MIN6, HIT-T15, and RINm5F) and rat tissues as indicated. Total RNA (20 μg) was denatured with formaldehyde, electrophoresed in a 1% agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled mouse PACAPR-3 cDNA probe.

Table 1. Stimulation of insulin secretion by PACAP-38 from MIN6 cells

Medium addition(s)	Insulin release, ng per hr per well
Glucose (3.3 mM)	65.0 ± 6.2
Glucose (3.3 mM) + PACAP-38 (0.1 nM)	81.2 ± 3.4*
Glucose (3.3 mM) + PACAP-38 (1 nM)	91.8 ± 3.4†
Glucose (3.3 mM) + PACAP-38 (10 nM)	115.9 ± 5.6‡

MIN6 cells in 48-well plates were cultured for 1 hr with 3.3 mM glucose with or without various concentrations of PACAP-38, and the insulin released into the medium was measured by radioimmunoassay (24). Values are the mean ± SEM ($n = 7$). Statistical analysis was conducted by an unpaired Student's t test. *, $P < 0.05$; †, $P < 0.005$; ‡, $P < 0.001$ vs. 3.3 mM glucose alone.

domain (Asn-57, Asn-87, and Asn-91) (Fig. 1). PACAPR-1, -2, and -3 have conserved cysteines in the extracellular domains (seven in the N terminus, two in the second extracellular region, and one in the third extracellular region) (Fig. 2), some of which may be important for the tertiary structure (16).

Although all PACAPR subtypes identified to date bind PACAP with high affinity and are positively coupled to adenylate cyclase, the binding specificity of PACAPR-3 for VIP is different from that of PACAPR-2, which has very low affinity to VIP, and is rather similar to that of PACAPR-1 in that both PACAPR-1 and -3 bind VIP with high affinity. In a study using *Xenopus* oocytes, we have found that PACAP and VIP elicit calcium-activated chloride currents, suggesting that PACAPR-3 can be coupled to phospholipase C. This property is similar to that observed in some splice variants of PACAPR-2 recently reported (8). It is, therefore, possible that as does PACAPR-2, PACAPR-3 mediates different intracellular signaling systems, as has been reported for other G-protein-coupled receptors (27). Interestingly, PACAPR-3 is expressed in pancreatic islets and insulin-secreting cell lines. Furthermore, we have found that PACAP-38 stimulates insulin secretion from MIN6 cells. A recent study has demonstrated the presence of PACAP-like immunoreactivity in nerve fibers innervating pancreatic islets (28). Thus these findings indicate that PACAP may play a physiological role in insulin secretion through PACAPR-3, probably by regulating cAMP levels and/or phosphatidylinositol hydrolysis that could affect the activities of the proteins involved in calcium signaling in pancreatic β cells (29, 30).

Note Added in Proof. After the submission of this manuscript for review, a VIP receptor subtype (VIP_2) cDNA that is a rat homolog of PACAPR-3 was reported (31).

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