Identification of a protein that confers calcitonin gene-related peptide responsiveness to oocytes by using a cystic fibrosis transmembrane conductance regulator assay

(guinea pig/cochlea/expression cloning)

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ABSTRACT   An expression-cloning strategy was used to isolate a cDNA that encodes a protein that confers calcitonin gene-related peptide (CGRP) responsiveness to *Xenopus laevis* oocytes. A guinea pig organ of Corti (the mammalian hearing organ) cDNA library was screened by using an assay based on the cystic fibrosis transmembrane conductance regulator (CFTR). The CFTR is a chloride channel that is activated upon phosphorylation; this channel activity was used as a sensor for CGRP-induced activation of intracellular kinases. A cDNA library from guinea pig organ of Corti was screened by using this oocyte–CFTR assay. A cDNA was identified that contained an open reading frame coding for a small hydrophilic protein that is presumed to be either a CGRP receptor or a component of a CGRP receptor complex. This CGRP receptor component protein confers CGRP-specific activation to the CFTR assay, as no activation was detected upon application of calcitonin, amylin, neuropeptide Y, vasoactive intestinal peptide, or β-endorphin. *In situ* hybridization demonstrated that the CGRP receptor component protein is expressed in outer hair cells of the organ of Corti and is colocalized with CGRP-containing efferent nerve terminals.

Calcitonin gene-related peptide (CGRP) is a 37-amino acid carboxyl-amidated neuropeptide secreted by nerves of the central and peripheral nervous systems (1, 2). CGRP, one of the most potent vasodilators known (3), may also be a neuromodulator (4, 5). CGRP has a plethora of functions in the body. Aside from its vasodilatory and neuromodulatory actions, CGRP has been proposed to increase acetylcholine receptor synthesis at the neuromuscular junction (6) and to desensitize the acetylcholine receptor at the neuromuscular junction and the efferent synapses of the inner ear (5, 7). CGRP-containing efferent fibers from the brainstem synapse at the inner ear end-organ sites, perhaps contributing to the processes responsible for the detection of auditory signals in the presence of background noise (8, 9). Additionally, CGRP has been implicated as a growth factor for human endothelial cells (10) and shown to inhibit antigen-presentation by Langerhans cells in epidermal cell culture (11).

Protein purification strategies based on cross-linking cell extracts to CGRP followed by SDS/PAGE analysis have identified candidate proteins for the CGRP receptor and its complex, with molecular masses of 17–70 kDa (12–16). CGRP activation results in increased levels of intracellular cAMP in endothelial cells and aorta (17, 18). An increase in cAMP can be monitored in oocytes by using the cystic fibrosis transmembrane conductance regulator (CFTR) as a reporter (19). We have discovered a protein from the guinea pig organ of Corti that confers CGRP responsiveness to oocytes by using a CFTR-based assay. This newly identified protein has no homology to any known receptor and is a candidate to be a CGRP receptor or a part of a CGRP receptor complex.‡

MATERIALS AND METHODS

Library Screening. A cDNA library made from guinea pig cochlea (organ of Corti) was obtained from E. R. Wilcox (20). In this library, cDNAs were directionally inserted into the pSport cloning vector (GIBCO BRL) between the *Sal* I and *Not* I restriction sites. For expression screening, ≈100,000 colonies from this library were plated into 20 pools (each of ≈5000 colonies). Cesium-purified DNA was prepared from each pool, linearized by restriction enzyme digestion, and transcribed *in vitro* into capped complementary RNA (cRNA) with T7 RNA polymerase (Promega). The CFTR-containing plasmid (pACF23) obtained from J. Riordan (Mayo Clinic, Scottsdale, AZ) was linearized and transcribed *in vitro* into capped cRNA with SP6 RNA polymerase (Promega). The CFTR cRNA (20 ng) was coinjected into oocytes with the cRNA (20 ng) from individual pools of the guinea pig organ of Corti library. Oocytes were prepared for injection as described (21). Injected oocytes were incubated for 24–72 hr at 19°C in OR2 medium (22) to allow for protein synthesis and for transport of the receptor protein to the cell surface. Oocytes were then voltage-clamped at −50 mV, and 10^{-7} M rat αCGRP (Bachem) was applied (rat αCGRP was used for all experiments). The pools were subdivided and retested until a candidate for the CGRP receptor was obtained. Candidate cDNAs were sequenced by using synthetic oligonucleotide primers with the dsCycle sequencing kit (GIBCO BRL).

Oocyte–CFTR Dose–Response Analysis. Oocytes were coinjected with 20 ng of CGRP receptor component protein (CGRP-RCP) cRNA and 20 ng of CFTR cRNA, and after 24 hr individual oocytes were voltage-clamped at −50 mV and exposed to increasing concentrations of CGRP (10^{-9} M through 10^{-7} M), with sequential applications to injected oocytes. All sequential applications were carried out at ≥30-min intervals to avoid desensitization effects (19, 23). In other experiments, injected oocytes were separated into pools, and all oocytes of each pool were subjected to the same concentration of CGRP (10^{-10} M through 10^{-5} M).

Antisense Injections. An antisense oligonucleotide containing a thiol-substituted backbone was synthesized to the 3′ untranslated region of the CGRP-RCP cDNA (Fig. 4A, double-underlined sequence). This antisense oligonucleotide was mixed with either receptor cRNA or guinea pig cerebellar mRNA before injection. Oocytes were incubated for 48 hr at

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19°C and then tested with 10^{-7} M CGRP. Cerebellar mRNA or cRNA (diluted 1:100) was mixed with equal volumes of CFTR cRNA and either antisense oligonucleotide or water.

Northern Blot Analysis. Twenty micrograms of guinea pig cerebellar RNA was separated by denaturing agarose gel electrophoresis, transferred to a Nytran membrane (Schleicher & Schuell), and hybridized with a ^{32}P-labeled random-primed probe made to the guinea pig cochlear CGRP-RCP (24).

In Vitro Translation. Capped cRNA was transcribed *in vitro* from CGRP-RCP cDNA by using the mMessage mMachine kit (Ambion). One microgram of cRNA was used for *in vitro* translation in rabbit reticulocyte extracts (Promega), with or without canine pancreatic microsomes, in the presence of [^{35}S]methionine (1000 Ci/mmol; 1 Ci = 37 GBq; Amersham, Inc.). Samples were boiled in Laemmli sample buffer and separated by SDS/PAGE on a 15% polyacrylamide gel. The gel was fixed, incubated with Amplify (Amersham), and exposed to Kodak XAR-5 film.

In Situ Hybridization. Twelve female guinea pigs (250 g) were anesthetized and perfused with formalin. Twenty-four cochleas were removed and decalcified in 0.1 M EDTA (25). This animal protocol has been approved by the University of Miami's Animal Care and Use Committee. A midmodiolar cut was made in the cochlea, and individual half-turns of the organ of Corti were microdissected and subjected to *in situ* hybridization with sense and antisense digoxigenin-labeled RNA probes transcribed *in vitro* from the cochlear CGRP-RCP (24).

Immunocytochemistry. Individual half-turns of the organ of Corti were microdissected from cochleas prepared as described for *in situ* hybridization experiments. These half-turns were preincubated in 5% (vol/vol) inactivated horse serum/0.01% Triton in PBS for 1 hr. The primary antibody MU33 (I.M.D., unpublished results) was added (1:500 dilution), and sections were incubated for 16 hr at 25°C, after which time the sections were washed three times with PBS. The sections were then incubated with donkey anti-rabbit antibody conjugated to biotin (1:1000 dilution; Jackson Immuno Research Laboratories) for 1 hr. The sections were washed three times in PBS and incubated with ABC reagent (Vector Laboratories), which binds streptavidin molecules conjugated to horseradish peroxidase to each biotinylated site on the secondary antibody. This horseradish peroxidase was reacted with diaminobenzidine to yield a brown reaction product and visualized by using bright-field microscopy.

RESULTS AND DISCUSSION

To isolate the CGRP-RCP, an expression-cloning strategy was used that was based on an assay described by Uezono *et al.* (19). This assay used the CFTR as a sensor for cAMP levels when
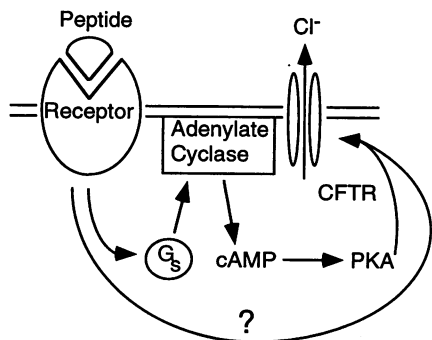

FIG. 1. Oocyte–CFTR assay. Peptide binds to receptor, resulting in activation of G_S. G_S stimulates adenylate cyclase, which activates PKA. PKA phosphorylates the CFTR, resulting in an inward current. An alternative pathway (?) that bypasses cAMP production is also shown.

expressed in *Xenopus* oocytes. The CFTR is a protein kinase A (PKA)-activated chloride channel. Thus, increased intracellular cAMP, which activates PKA, results in a CFTR Cl$^-$ current in cells expressing the CFTR (19, 23, 26–29). The oocyte–CFTR assay is shown in Fig. 1: when the receptor of interest is coexpressed with the CFTR in the oocyte, binding of ligand to the receptor is expected to activate G_S (the stimulatory G-protein α subunit), thus stimulating adenylate cyclase and increasing cAMP levels, which produce a ligand-inducible CFTR Cl$^-$ current. CGRP binding has been reported to result in increased levels of intracellular cAMP (17, 18). Thus, binding of CGRP to its receptor was predicted to elicit a Cl$^-$ current. Peak height of the CFTR Cl$^-$ current can then be used as a quantitative indicator of receptor activation.

As a test of the CFTR assay, a CFTR Cl$^-$ current was demonstrated by application of forskolin to oocytes injected
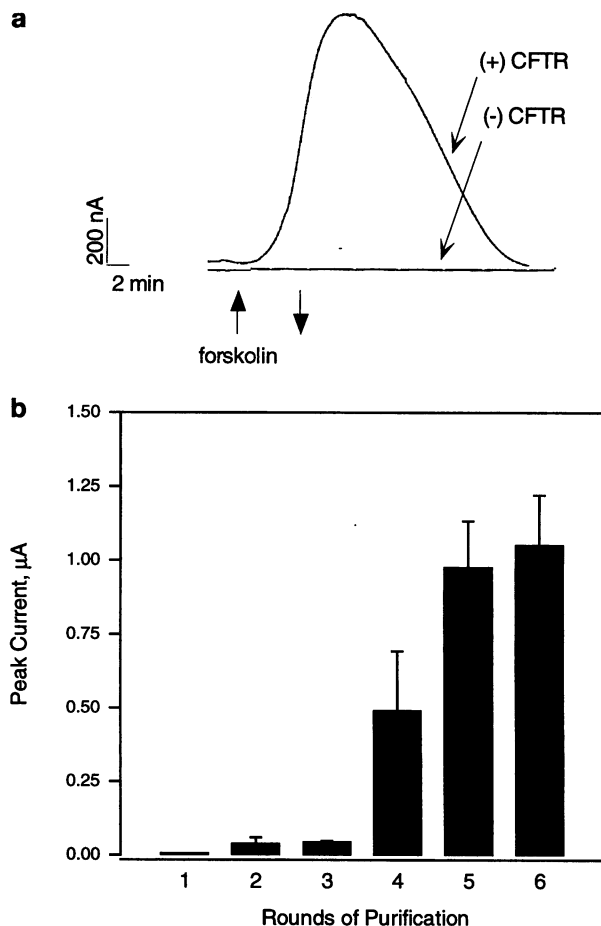

FIG. 2. (*a*) Membrane current from a voltage-clamped oocyte injected with 20 ng of CFTR cRNA and challenged with 20 μM forskolin. Injected oocytes were incubated for 24 hr before recording. The membrane potential of oocytes was clamped at -50 mV for all experiments. Inward currents are depicted as an upward deflection in the current trace in all figures. For all experiments, upward arrows indicate the beginning of the application of reagents and downward arrows indicate beginning of reagent washout. (*b*) CFTR Cl$^-$ currents from successive pools of clones. The number of species per pool is as follows. Bars: 1, 100,000; 2, 5000; 3, 625; 4, 78; 5, 10; 6, 1. To directly compare the activity of the various pools pursued during the expression cloning process (the pools with highest activity in each round), pools were tested for this experiment in one oocyte preparation. Furthermore, in this experiment for round 6 (single species), a PCR product generated by using primer pairs 1 and 4 (Fig. 4*a*) was used as template for cRNA production, demonstrating that the short ORF is sufficient for expression of a fully functional protein. Primer 1 incorporated the T7 promoter sequence at its 5' end to allow transcription of the PCR product with T7 polymerase.
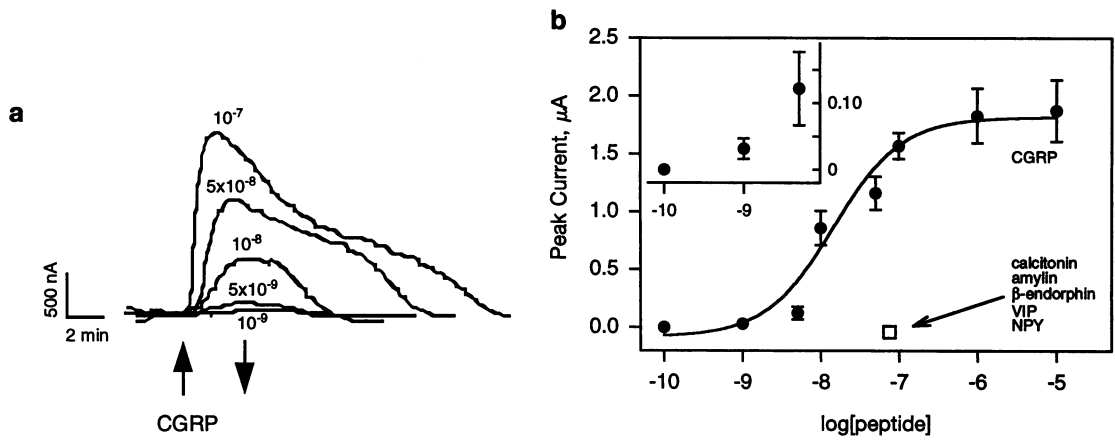
FIG. 3. (*a*) CFTR Cl⁻ currents from an individual oocyte coinjected with 20 ng of CFTR cRNA and 20 ng of CGRP-RCP cRNA and challenged with increasing levels of CGRP (10^{-9} M, 5×10^{-9} M, 10^{-8} M, 5×10^{-8} M, and 10^{-7} M). (*b*) Currents (mean $\pm$ SEM) from eight pools (three to six oocytes per pool) coinjected with 20 ng of CFTR cRNA and 20 ng of CGRP-RCP cRNA, incubated for 24 hr, and then challenged with CGRP at the indicated concentrations. (*Inset*) First three points plotted with an expanded scale. Open square indicates the lack of currents obtained upon application of 10^{-7} M of any other peptide tested, as indicated.

with CFTR cRNA. Forskolin raises intracellular cAMP levels, and as shown in Fig. 2*a*, the addition of 20 μM forskolin resulted in the production of an inward current of ≈ 1.0 μA. In control oocytes that were not injected with CFTR cRNA [(−) CFTR in Fig. 2*a*], incubation with forskolin did not cause a membrane current (19, 27, 28).

The guinea pig organ of Corti library was screened in 20 pools of ≈ 5000 colonies. In the first round of screening, a single pool demonstrated CGRP-induced CFTR currents, generating an average membrane current of 0.105 ± 0.049 μA

($\pm$SEM; $n = 9$ oocytes) upon application of 10^{-7} M CGRP. This effect was reversible and could be repeated by reapplication of CGRP to the bath. No current could be elicited by CGRP in the absence of CFTR. This positive pool was subdivided and retested, until after six subdivisions, a candidate CGRP-RCP was identified. During subsequent rounds of purification, single or multiple positive pools were identified. In each case of multiple positive pools, only one pool demonstrated an increase in current over the parent pool, demonstrating an increase in specific activity, and this pool was
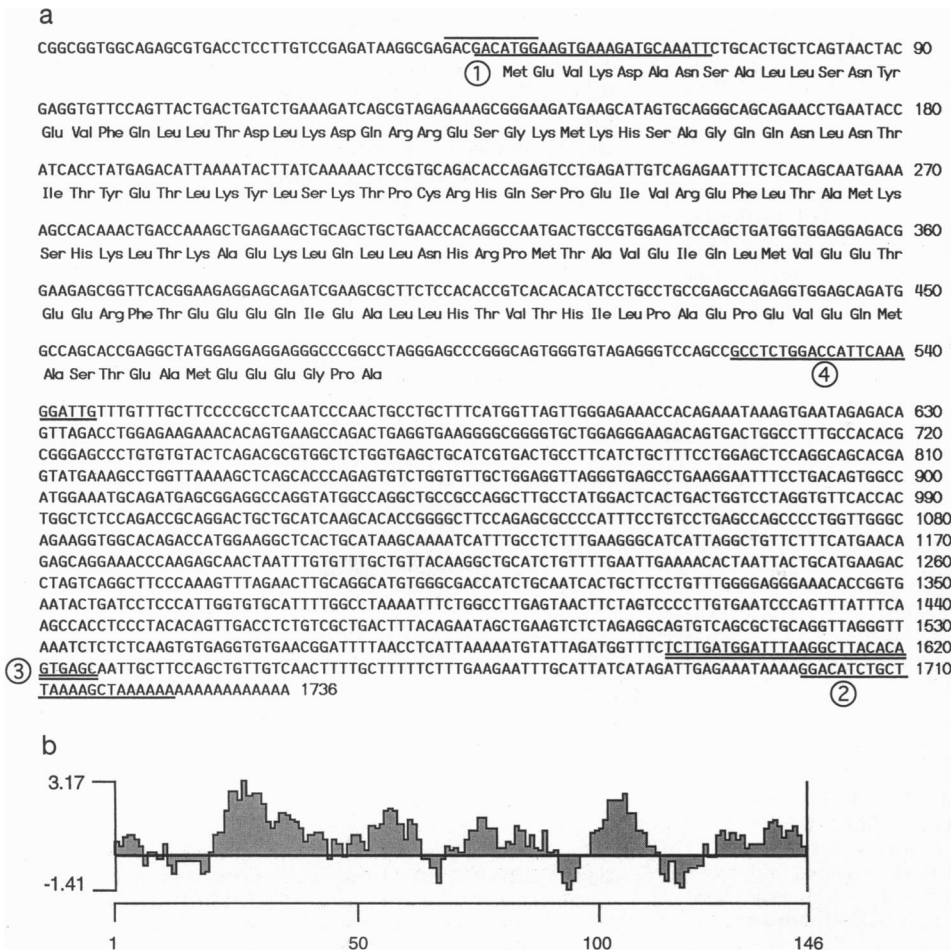

FIG. 4. (*a*) Nucleotide sequence and predicted amino acid sequence for the CGRP-RCP. Single-underlined sequences (numbers 1 and 2) represent the two oligonucleotide primers used for PCR on cerebellar mRNA; double-underlined sequence (number 3) represents antisense oligonucleotide used for experiments shown in Fig. 7, and single-underlined sequence (number 4) represents primer used for direct testing of ORF in Fig. 2. Kozak translational initiation consensus sequence is indicated by bar over the sequence. (*b*) Kyte–Doolittle hydrophilicity plot of the cochlear CGRP-RCP. An amino acid scale is shown at the bottom.

chosen for subsequent analysis. For initial rounds of library screening, oocytes were incubated for 72 hr after injection. As the purification proceeded and the CGRP-induced CFTR current increased, the incubation time was shortened to 24 hr. In the final round of purification with only a 24-hr incubation after injection, application of CGRP resulted in an average current >1.5 μA. A comparison of the CFTR activation by the positive pools 24 hr after injection is shown in Fig. 2b.

A representative set of current traces from a single oocyte is shown in Fig. 3a to illustrate CGRP-RCP activation in response to five doses of CGRP. In this experiment, oocytes were injected with CGRP-RCP cRNA and CFTR cRNA, incubated for 24 hr, and sequentially exposed to increasing doses of CGRP. Desensitization of the system was observed with application intervals shorter than 30 min, so all sequential applications were carried out with ≥30-min intervals. The CFTR is known to become desensitized (19, 23), and it is unclear whether the desensitization observed in our experiments is due to the CFTR alone or whether there is an additional contribution by the CGRP receptor. At higher concentrations of CGRP, a biphasic CFTR current was detected (5×10^{-8} M and 10^{-7} M). The basis for this biphasic response is currently unknown; however, a similar biphasic response to CGRP has been observed in the firing rate of hair cells in the *Xenopus* lateral line organ when exposed to similar doses of CGRP (30).

Fig. 3b shows data from a representative CGRP dose–response experiment. Oocytes were coinjected with CGRP-RCP cRNA and CFTR cRNA and subjected to eight doses of CGRP. Data were fit to a sigmoidal curve by nonlinear regression using the Marquardt method (GRAPHPAD PRIZM, Graphpad Software, Inc., San Diego) with an EC_{50} of 14 nM. The same EC_{50} value has been reported for CGRP's effect on the beat rate of guinea pig left atria (31, 32). CFTR activation was specific to CGRP, as oocytes injected with CGRP-RCP cRNA would produce CFTR Cl$^-$ current upon application of 10^{-9} M CGRP, but no detectable current was observed upon subsequent application of 10^{-7} M calcitonin (rat or salmon), 10^{-7} M amylin, 10^{-7} M vasoactive intestinal peptide, 10^{-7} M neuropeptide Y, and 10^{-7} M β-endorphin (Fig. 3b).

The cochlear CGRP-RCP was identified on a 1.8-kb cDNA that contains a 438-bp open reading frame (ORF), beginning with a Kozak translation initiation consensus sequence (33), GACGACATGG, with the codon for the initiator methionine

underlined (Fig. 4a). The ORF is preceded by a 48-bp 5′ untranslated region containing no upstream ATG codons and is followed by a 1232-bp 3′ untranslated region containing stop codons distributed between all three reading frames. This cochlear cDNA was confirmed by direct sequencing of a reverse transcription-coupled PCR product amplified from cerebellar mRNA; cerebellum has been widely used as a source for CGRP binding studies (14–16, 34). The two oligonucleotide primers used for PCR (labeled 1 and 2) are underlined in Fig. 4a. Two hundred nanograms of cerebellar poly(A)$^+$ RNA was used as template for reverse transcription with the downstream oligonucleotide primer, and the resulting first-strand cDNA was used as template for PCR (24). DNA sequence analysis detected no differences between the cochlear cDNA and the cerebellar reverse transcription-coupled PCR product. Thus, the identical CGRP-RCP is present both in cerebellum and cochlea.

The 146-amino acid protein encoded by the ORF (Fig. 4a) is largely hydrophilic (Fig. 4b) and has no homology to any reported receptor when searched against the GenBank database with BLAST software (35). However, the BLAST search did identify an unpublished partial cDNA from a chicken lens cDNA library (expressed sequence tag, GenBank accession no. D26313). The protein predicted by this chicken cDNA has 70.5% amino acid identity with the guinea pig cochlear CGRP-RCP protein, using MEGALIGN (DNASTAR, Madison, WI) software.

Northern blot analysis from the guinea pig cerebellum revealed the size of the CGRP-RCP mRNA to be ≈1800 bp (Fig. 5), similar in size to the cochlear CGRP-RCP cDNA, indicating that the cDNA contains most of the 5′ untranslated sequence.

The size of the CGRP-RCP protein, as predicted by the nucleotide sequence, is consistent with data obtained by *in vitro* translation. In these experiments, a 20-kDa protein was detected after *in vitro* transcription of the cochlear CGRP-RCP cDNA, and subsequent *in vitro* translation and SDS/PAGE, as shown in Fig. 6. The addition of microsomal membranes did not alter the mobility of the translation product on SDS/PAGE, making post-translational modifications such
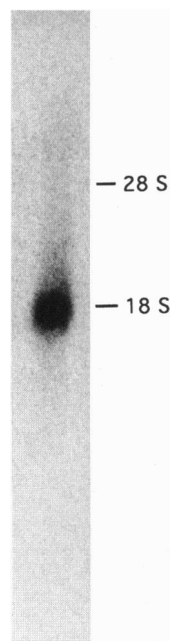

FIG. 5. Northern blot of guinea pig cerebellar RNA. The lane was loaded with 20 μg of cerebellar total RNA and probed with a full-length random-primed CGRP-RCP PCR product. Cerebellar CGRP-RCP RNA migrated with an estimated size of ≈1.8 kb. Positions of guinea pig rRNA bands are indicated.
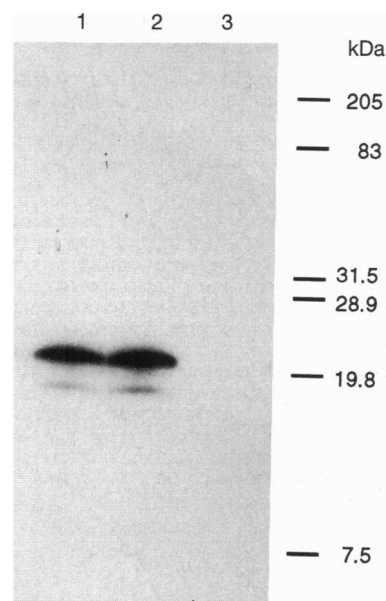

FIG. 6. *In vitro* translation of the cochlear CGRP-RCP. Lane 1 shows CGRP-RCP cRNA translated *in vitro* with canine microsomal pancreatic extracts. Lane 2 shows CGRP-RCP cRNA translated *in vitro* without microsomal extracts. Lane 3 shows control *in vitro* translation with no cRNA added.
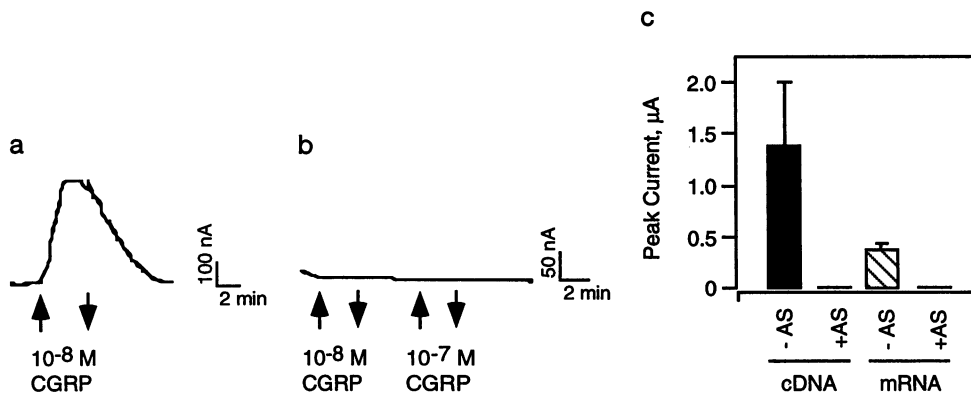
FIG. 7. (*a*) Record of a CFTR Cl⁻ current from an oocyte injected with 20 ng of guinea pig cerebellar mRNA and 20 ng of CFTR cRNA. (*b*) Record of CFTR Cl⁻ current from an oocyte injected with 20 ng of antisense CGRP-RCP oligonucleotide in addition to guinea pig cerebellar mRNA and CFTR cRNA (*c*). CFTR Cl⁻ currents from oocytes coinjected with CFTR cRNA (20 ng) and cRNA from either CGRP-RCP cDNA (200 pg, solid bar) or cerebellar mRNA (20 ng, hatched bar), with (+) or without (−) antisense CGRP-RCP (20 ng) oligonucleotide (AS) and incubated with 10^{-7} M CGRP. Note the absence of currents from oocytes coinjected with the antisense CGRP-RCP oligonucleotide. Data are the mean ± SEM ($n = 4$).

as glycosylation unlikely. An *in vitro* translation product of identical size to the cochlear receptor was detected when the cerebellar reverse transcription-coupled PCR product described above was used as a template for *in vitro* transcription (data not shown). PCR was carried out as described, except a T7 promoter sequence was incorporated into the 5′ end of the upstream primer (Fig. 4*a*, primer 1) to enable subsequent *in vitro* transcription.

To confirm the identity of the cochlear CGRP-RCP, an antisense oligonucleotide was synthesized based on the sequence of the CGRP-RCP cDNA (Fig. 4*a*, primer 3, double-underlined sequence). The antisense oligonucleotide to the cochlear CGRP-RCP eliminated receptor activity from both the cochlear receptor cRNA and cerebellar mRNA in the oocyte–CFTR assay, indicating that the cerebellum contains a CGRP-RCP homologous to that of the cochlear form (Fig. 7). This conclusion agrees with the identical nucleotide sequence obtained from the cochlear cDNA and the cerebellar PCR product.

As a control, oocytes were coinjected with CFTR and CGRP-RCP cRNAs and an antisense oligonucleotide made to the *Xenopus* connexin 38 cDNA. Connexin 38 is a membrane-bound gap-junction protein that has been functionally removed from oocytes in antisense oligonucleotide experiments (36). The connexin 38 antisense oligonucleotide did not alter CGRP-RCP activity contained in the CGRP-RCP cRNA (data not shown). Thus, the presence of thiol-substituted oligonucleotides alone had no effect on CGRP-RCP expression in the oocyte. Additionally, calcitonin-receptor-directed antisense oligonucleotides have been shown to have no effect on CGRP-RCP activity in oocytes injected with guinea pig brain mRNA (37). Furthermore, CGRP-RCP-directed antisense oligonucleotides had no effect on calcitonin receptor activity from brain mRNA (A.E.L., G.P.D., and I.M.D., unpublished observations), indicating that the CGRP-RCP and the calcitonin receptor do not interact.

The CGRP-RCP was shown to be present in cells innervated by CGRP-containing neurons. By using an antibody against CGRP (MU33), the efferent nerve fibers that terminate on outer hair cells of the basal two turns of the guinea pig cochlea were demonstrated to contain CGRP (Fig. 8*A*). *In situ* hybridization with the CGRP-RCP antisense RNA probe showed that the mRNA for the CGRP-RCP is also only present in outer hair cells of the basal two turns of the guinea pig cochlea (Fig. 8*B*). This is in agreement with previous studies of CGRP's effect on high frequency regions (basal turns) of the guinea pig and rat cochleas (4, 8).

In summary, we have identified a cDNA from the cochlea of guinea pig that encodes a protein that confers responsiveness

to CGRP in oocytes. An antisense oligonucleotide made against the cochlear CGRP-RCP eliminates receptor activity
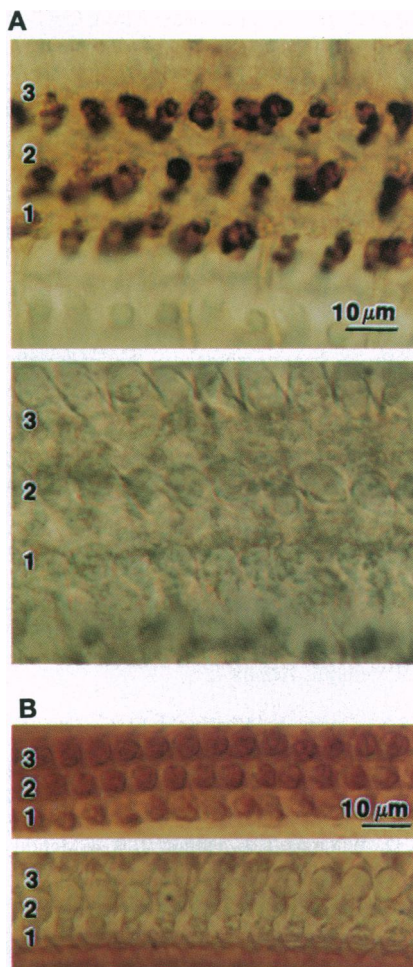

FIG. 8. (*A*) Results of immunocytochemistry using an antibody to CGRP (MU33) on a microdissected basal turn of the guinea pig organ of Corti. (*A Upper*) Immunostaining of CGRP-containing efferent fiber terminals on the three rows of outer hair cells (labeled 1, 2, and 3). (*A Lower*) No staining when the preimmune serum was substituted for the CGRP antibody. (*B*) Results of *in situ* hybridization using an antisense RNA probe to the CGRP-RCP on a microdissected basal turn of the guinea pig organ of Corti. (*B Upper*) Staining of outer hair cells with the digoxigenin-labeled antisense probe. (*B Lower*) Background staining using the digoxigenin-labeled sense probe.

induced in oocytes by cochlear receptor cRNA or by cerebellar mRNA. *In vitro* translation yields a product consistent in size with the 146-amino acid receptor protein as predicted by the nucleotide sequence. In agreement with this size, a 17-kDa protein that cross-links with CGRP has been identified from solubilized cerebellum (14).

Because the cochlear CGRP-RCP protein is short and not obviously hydrophobic, it does not belong to the class of G-protein-coupled receptors that contain seven membrane-spanning helices. Two models could reconcile the primary structure of the CGRP-RCP with reports that CGRP binding is coupled to G-protein activation: (*i*) The CGRP-RCP could represent the complete CGRP receptor, which would contain an atypical membrane-spanning domain, in which case it might resemble the mannose-6-phosphate receptor, which couples to G proteins despite lacking the prototypical seven membrane-spanning helices (38). (*ii*) The CGRP-RCP could be part of a complex of proteins that constitute the CGRP receptor. Such a complex has recently been described for the receptors for ciliary neurotrophic factor, interleukine 6, and type I interferon (39–41). For the ciliary neurotrophic factor and interleukin 6 receptors, a small extracellular membrane-associated protein binds the ligand and interacts with a membrane-spanning protein for signal transduction, conferring specificity to a more generic signaling pathway. The CGRP-RCP may be contributing specificity in a similar manner. In this scenario, binding of CGRP to its receptor may activate the CFTR in the oocyte assay via a membrane-associated kinase, either in conjunction with, or separately from, the adenylate cyclase pathway depicted in Fig 1. Alternatively, the CGRP-RCP may couple the ligand-binding complex to the cellular signal transduction machinery. If the latter case, the CGRP-RCP is specific for only CGRP, as no other tested ligand could activate the oocyte–CFTR assay when tested with the CGRP-RCP. Whether the newly identified CGRP-RCP functions independently or in a complex of proteins remains to be determined.

Note Added in Proof. Since acceptance of this manuscript, it has been reported that RDC-1, an orphan G-protein-coupled transmembrane receptor, is a CGRP receptor (42).

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