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Pharmacological Characterization of the CGRP Receptor in the Lateral Line Organ of *Xenopus laevis*

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ABSTRACT

Calcitonin gene-related peptide (CGRP) is a neurotransmitter candidate colocalized with acetylcholine in efferent fibers innervating hair cell organs. We have used the *Xenopus laevis* lateral line organ to investigate the responses of a hair cell organ to the CGRP family of peptides. Two isoforms of CGRP, rα-CGRP and rβ-CGRP, and a human analog of α-CGRP, h(Tyr°) α–CGRP, produced dose-dependent increases in afferent nerve fiber discharge rate with EC_{50} values of approximately 1 μM. Rate increases were 31.2, 18.9, and 10.3%, respectively. The peptide fragment $rCGRP_{8-37}$ a selective $CGRP_1$ receptor antagonist, competitively inhibited the response to rα-CGRP. Diacetoamidomethyl cysteine CGRP (r[Cys$(ACM)_{2,7}$]α–CGRP), a $CGRP_2$ agonist, did not change discharge rate. Rat amylin did not increase rate until very high concentrations, and then the change was less than 7%. Rat adrenomedullin produced no increase in rate. Responses to rα-CGRP developed after metamorphosis. No change in spontaneous discharge rate was observed until postmetamorphic day 6, and then it was only a fraction of the maximal response. This response progressively increased until postmetamorphic day 28, when it reached its maximal value. The most straightforward interpretation of our results is that the effect of CGRP is mediated by the $CGRP_1$ receptor and that CGRP, of the peptides presently known to exist in the CGRP family, is the most likely endogenous peptide mediating these effects.

Keywords: cochlea, vestibular, hair cell, efferents, CGRP, metamorphic

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is an efferent neurotransmitter candidate in hair cell organs. This peptide is immunolocalized in cholinergic efferent fibers innervating hair cells of the vestibular system (Tanaka et al. 1989a, b; Wackym et al. 1990, 1993; Ohno et al. 1993; Scarfone et al. 1996), organ of Corti (Lu et al. 1987; Takeda et al. 1987; Sliwinska-Kowalska et al. 1989; Kuriyama et al. 1990; Ohno et al. 1993; Safieddine et al. 1997; Reuss et al. 1999), and the lateral line organ (Adams et al. 1987). The strongest case for its role as a neurotransmitter comes from work in the lateral line organ, where CGRP suppresses responses to mechanical stimulation by around 10 dB while increasing spontaneous discharge rate (Bailey and Sewell, 2000). A CGRP-like increase in spontaneous rate is also seen with electrical stimulation of efferent fibers even when the cholinergic component of efferent stimulation has been blocked (Sewell and Starr 1991). In this report, we examine the pharmacology of the CGRP family of peptides and characterize the CGRP receptor present at this synapse.

CGRP is a member of a family of structurally related peptides that are biologically cross-reactive (Muff et al. 1995; Bell and McDermott 1996; Wimalawansa 1996, 1997; Poyner 1997; van Rossum 1997; Martinez and

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Cuttitta 1998 for reviews). Other members of this family include calcitonin, amylin, and adrenomedullin. Although there is appreciable sequence homology between CGRP and amylin, CGRP has less homology to adrenomedullin and calcitonin. However, there is significant conservation of secondary structure within this family. All have in common an *N*-terminally-located disulfide ring, an amphipathic α-helix, and an amidated C-terminus. These regions are important for biological activity, and this level of similarity explains their biological and potential immunohistochemical cross-reactivity.

Specific receptors exist for each member of the CGRP family (Gorn et al. 1992; Kapas and Clark 1995; Kapas et al. 1995; Aiyar et al. 1996; Muff et al. 1999). Two subtypes of CGRP receptors ($CGRP_1$ and $CGRP_2$) have been proposed based on pharmacological properties (Chiba et al. 1989; Dennis et al. 1989, 1990; Mimeault et al. 1991; Quirion et al. 1992; Poyner 1992, 1995; Bell and McDermott 1996; Wisskirchen et al. 1998). The $CGRP_1$ receptor subtype is preferentially activated by α-CGRP. The peptide fragment α-$CGRP_{8-37}$, which lacks the first 7 N-terminal amino acids, is a competitive antagonist for the $CGRP_1$ receptor, but has no action on the $CGRP_2$, calcitonin, amylin, or adrenomedullin receptors. While there are no specific antagonists for the $CGRP_2$ receptor subtype, the linear analog diacetoamidomethyl cysteine CGRP ($Cys[ACM]_{2,7}CGRP$), which lacks the N-terminal region disulfide linkage, has little or no agonist activity at $CGRP_1$ receptors, but is a full agonist with less potency relative to α-CGRP in activating $CGRP_2$ receptors.

These findings raise two questions about the role of CGRP as an efferent neurotransmitter. First, is the peptide present in efferent nerve terminals CGRP, or some other member of the CGRP family with biological and immunological cross-reactivity; and second, what receptor mediates CGRP's action in hair cell organs? To approach these questions we have examined the effects of these peptides, their agonists, and antagonists on the spontaneous discharge rate in the *Xenopus* lateral line organ.

MATERIALS AND METHODS

In experiments that examined the response to members of the CGRP family, *Xenopus laevis* approximately 2 cm from nose to vent were obtained from Nasco (Fort Atkinson, WI) between postmetamorphic days 30–60 and housed at room temperature in deionized water containing 1 mM added calcium chloride. Each frog was anesthetized by chilling to near 0°C, then decapitated. A piece of skin containing the middle-lateral row of stitches was removed and placed inner surface up on a piece of moistened filter paper. The skin was rinsed with an artificial perilymph solution containing sodium chloride (120 mM), potassium chloride (3.5 mM), calcium chloride (1.5 mM), and glucose (5.5 mM), buffered with HEPES (20 mM) and adjusted to pH 7.5 with sodium hydroxide (total Na^+ 130 mM). Perfusion of the inner surface of the skin allowed relatively rapid diffusion (within 20 s) to the basolateral surface of the sensory epithelium.

The nerve branch innervating the middle-lateral row of stitches was dissected from the inner surface of the skin. Extracellular recordings were made using a silver wire electrode (Sewell and Mroz 1987). For these experiments, activity from three adjacent stitches was monitored simultaneously to reduce the variability in discharge rate normally seen in spontaneous afferent fiber discharge over time. The observed monophasic action potentials with positive polarity were amplified 1000-fold and monitored on an oscilloscope. The signal-to-noise ratio was optimized by analog filtering. A Schmitt trigger device was used to determine the occurrence of action potentials, which were counted with a microprocessor. Throughout the experiments, the inner synaptic surface was perfused continuously at a flow rate of 1 μL/s. Peptides were delivered directly to the inner synaptic surface. Only those preparations maintaining a spontaneous discharge rate greater than 100 spikes/min were used. Only those preparations that recovered completely after drug administration were considered in the analysis.

To obtain the dose–response relationships for each peptide, peptides were applied while monitoring spontaneous discharge rates in the afferent fibers. Peptides were divided into three groups for testing. Group 1 consisted of the CGRP family peptides rα-CGRP, rβ-CGRP, and rAmylin. Group 2 consisted of rADM and the synthetic analogs r[Tyr°]α–CGRP and r[$Cys(ACM)_{2,7}$]α–CGRP. Group 3 consisted of the peptide fragments rα-$CGRP_{8-37}$, $rADM_{11-50}$, and $rAmylin_{8-37}$. The peptides were applied in random order within each group. Each preparation was exposed to only one peptide concentration. The preparation was allowed to reverse fully from each peptide application before subsequent applications were made. Each of the Group 1 peptides were tested separately at high concentrations ($>10^{-6}$ M). Sigmoidal curves were fit to the data by a least-squares method. EC_{50} values were determined from the sigmoidal curve fits. Data were obtained from 60 preparations.

In separate experiments, the α-CGRP antagonist, rα-$CGRP_{8-37}$ (1 μM), was perfused for 15 minutes prior to and during the application of rα-CGRP. Each preparation was exposed to only one concentration of rα-CGRP. The dose–response for rα-CGRP was determined (0.1–3 μM) in the presence of rα-$CGRP_{8-37}$. Sigmoidal curves were fit to the data by a least-squares

method. EC_{50} values were determined from the sigmoidal curve fits. Data were obtained from 13 preparations.

In experiments that studied the postmetamorphic (PM) response to CGRP, *Xenopus laevis* tadpoles were obtained from Nasco at stage 65 (~54 days), and housed at room temperature in deionized water as above. Frogs were allowed to grow until adult stage 66 (~58 days), as determined by morphological staging criteria (Nieuwkoop and Faber 1956). Frogs were designated PM day 0 on the day they were categorized as stage 66. Postmetamorphic responsiveness to rα-CGRP (3 μM) was determined by monitoring spontaneous discharge rates during peptide application as above. Response was recorded approximately every other day for 33 days, then one time at day 72. One or two frogs were used each day that the response to rα-CGRP was tested. Sigmoidal curves were fit to the data by a least-squares method. Data were obtained from 18 preparations.

The peptide response of the preparation was evaluated by on-line computer analysis for all experiments. The average number of spikes that occurred between 60 and 220 s after peptide injection determined maximal response. Percent change in rate was calculated by comparing the maximal response with the average baseline spontaneous rate of the preparation taken 160 s prior to peptide injection. Sigmoidal curves were graphically fit to the data using software by KaleidaGraph, Synergy Software, Reading, PA.

All peptides, peptide fragments, and synthetic analogs were purchased from Peninsula Labs (Belmont, CA).

RESULTS

CGRP is more effective than calcitonin, adrenomedullin, or amylin

As previously described (Adams et al. 1987; Sewell and Starr 1991), one effect of rα-CGRP on this preparation was to increase spontaneous discharge rate. We used this rate change as a measure of the functional response to application of these peptides. This increase was observed within tens of seconds of application, and the rate could stay elevated for tens of minutes after washing the peptide from the synapse. An example is shown in Figure 1.

We found CGRP to be the most potent peptide examined. All CGRP isoforms and a synthetic analog produced dose–dependent increases in spontaneous discharge rate, with EC_{50} values of approximately 1 μM (Fig. 2). The rα-CGRP isoform was more effective than rβ-CGRP, and r(Tyr°) α–CGRP was the least effective, producing increases (mean ± s.e.) of 31.2 ± 4.9%, 18.9 ± 5.3%, and 10.3 ± 2.1% respectively.

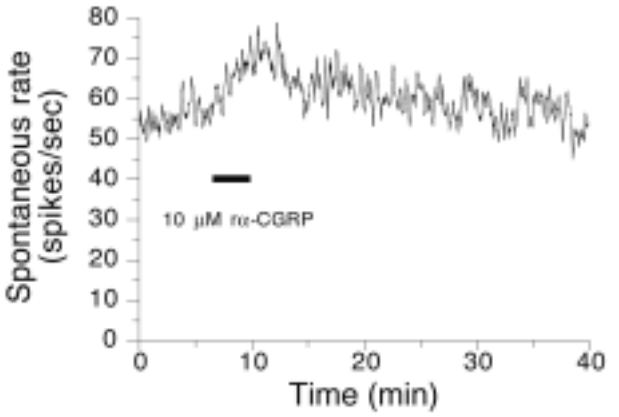

FIG. 1. CGRP increased spontaneous discharge rate, an effect lasting long after CGRP was washed out. The preparation was continually perfused with a balanced salt solution. CGRP was administered during the time indicated by the solid bar.

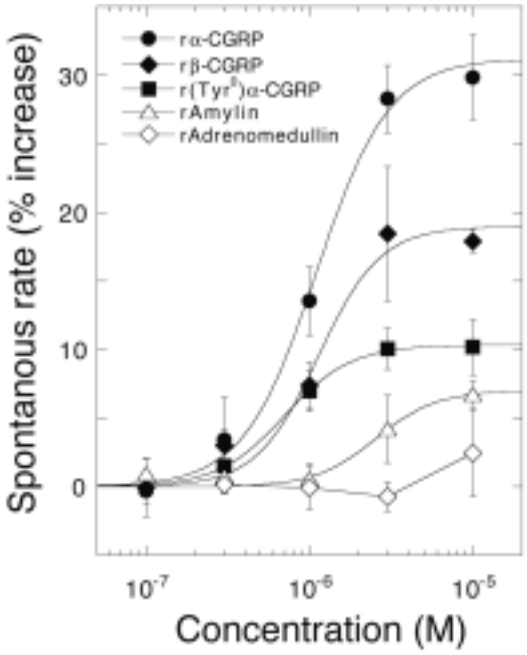

FIG. 2. CGRP was more effective than amylin or adrenomedullin at producing a change in afferent nerve fiber discharge rate. Sigmoidal curves were fit to the data for all peptides except adrenomedullin (open diamonds) by a least-squares algorithm. Each peptide was tested at least three times at each concentration.

Adrenomedullin (ADM) and amylin were relatively ineffective. Rat amylin, with an EC_{50} near 3 μM, was less potent than rα-CGRP and produced a maximal increase in rate of only 7.1 ± 1.2%. Rat ADM essentially had no biological effect at concentrations up to 10 μM (Fig. 3). We have previously shown that salmon calcitonin does not increase spontaneous discharge rate at this synapse (Adams et al. 1987).

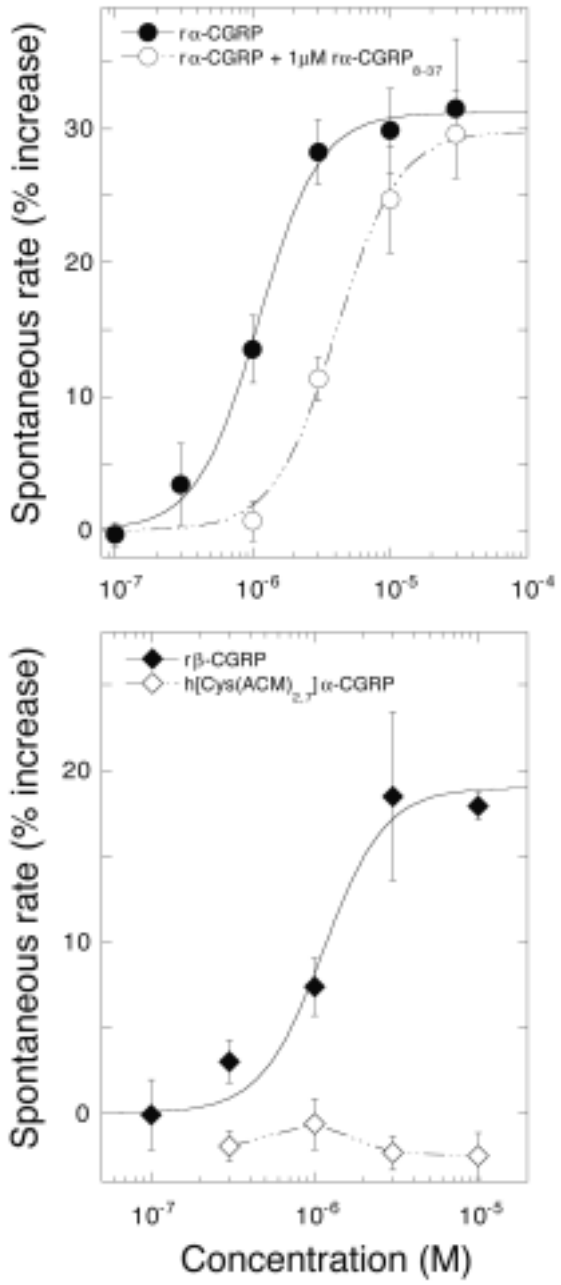

FIG. 3. (**Upper panel**) Responses to CGRP are shifted to the right by preincubation of the preparation with the $CGRP_1$ receptor antagonist, $CGRP_{(8–37)}$. (**Lower panel**) r[Cys $(ACM)_{2,7}$]α–CGRP, a selective agonist for the $CGRP_2$ receptor, had no effect on discharge rate, indicating that the response seen with 3 μM rβ-CGRP was likely due to cross-reactivity with the $CGRP_1$ receptor.

Responses to CGRP are likely produced by the $CGRP_1$ receptor

The truncated peptide fragment rα-$CGRP_{8–37}$ is a selective blocker for the $CGRP_1$ receptor. We first studied whether rα-$CGRP_{8–37}$ had any intrinsic biological activity and found that this antagonist had none at concentrations up to 30 μM (data not shown). We then studied the effect of rα-$CGRP_{8–37}$ on the excitatory response produced by rα-CGRP. The response to rα-CGRP was inhibited competitively by pretreatment with 1 μM rα-$CGRP_{8–37}$. The inhibition shifted the dose–response curve to the right, changing the EC_{50} from 1 to 4 μM (Fig. 3).

The β-CGRP isoform is thought to mediate its effects through the $CGRP_2$ receptor (Jansen 1992; Tomlinson and Poyner 1996; Yoshimoto et al. 1998). Our results showed that rβ-CGRP was similar in potency but 40% less effective than rα-CGRP at increasing spontaneous rate. While no specific antagonist for the $CGRP_2$ receptor exists, we did examine the effects of r[Cys$(ACM)_{2,7}$]α–CGRP, a specific peptide agonist for the $CGRP_2$ receptor. This compound was inactive, producing no effect on the spontaneous discharge rate at doses up to 10 μM (Fig. 3), indicating that the response to rβ-CGRP was not mediated by the $CGRP_2$ receptor but was more likely a result of cross-reactivity with the $CGRP_1$ receptor.

Responses to CGRP develop post-metamorphically

Early in our investigation we recognized that occasionally when new frogs were received they were not immediately responsive to rα-CGRP. The response to rα-CGRP gradually increased over the next few days to weeks. To eliminate variability in the age of the frogs and determine the age most responsive to rα-CGRP, we raised tadpoles to determine the exact day they became adult stage 66 (PM day 0). Responses to rα-CGRP (3 μM) were recorded from PM day 0 through PM day 72. Increases in spontaneous rate were not apparent until PM day 6, and then at only a fraction of the maximal response. Activity continued to increase until PM day 28, at which time it had plateaued with maximal responses of a 28.4% increase in discharge rate (Fig. 4).

DISCUSSION

The most straightforward interpretation of our results is that the effect of CGRP is mediated by the $CGRP_1$ receptor, and that CGRP, of the peptides presently known to exist in the CGRP family, is the most likely endogenous peptide mediating these effects. Calcitonin, amylin, and adrenomedullin were ineffective at increasing afferent discharge, suggesting that exogenously applied CGRP is not acting on calcitonin, adrenomedullin, or amylin receptors. If receptors for other members of the CGRP family do exist, their effects on the hair cell organ are likely different than those for CGRP.

In general, the effects on discharge rate for peptides within the CGRP family was closely related to their

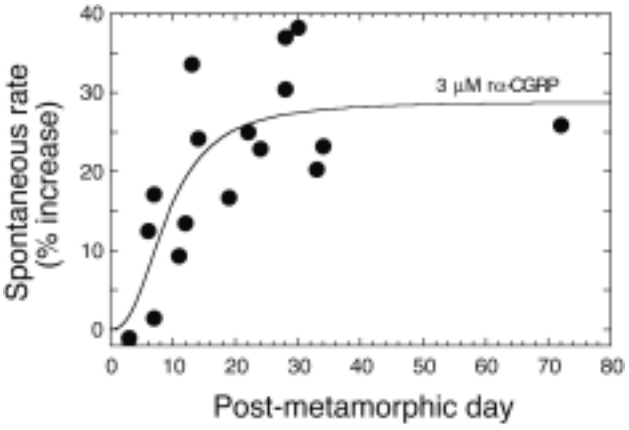

FIG. 4. The response to rα-CGRP increased postmetamorphically. The change in afferent discharge produced by 3 μM rα-CGRP was not apparent until postmetamorphic day 6, and then at only a fraction of the maximal response. This response progressively increased until postmetamorphic day 28, when it began to plateau near its maximal value (28.4% change in rate).

homology to CGRP. The amino acid sequences of rα-CGRP, rβ-CGRP, and r(Tyr°)α–CGRP differ by only 3%, and are approximately 82% homologous with the CGRP sequence of the European green frog *Rana ridibunda* (Conlon et al. 1993). Rat amylin, which had only slight activity, exhibits a 51% homology with frog CGRP. Rat adrenomedullin and salmon calcitonin, which were inactive, have a 22% and 27% homology, respectively, with frog CGRP.

The EC_{50} of 1 μM for rat CGRP in the *Xenopus* lateral line preparation is 10–30-fold higher than that found in *Xenopus* oocytes (Luebke et al. 1996) and in tissue slices of frog adrenal gland (Esneu et al. 1994). This difference is likely attributable to the fact that, in the lateral line preparation, CGRP must diffuse through 20–30 μm of connective tissue to reach the synapse, where it may encounter peptidases, uptake, and nonspecific binding. The EC_{50} for rat CGRP in our preparation is also 100–1000-fold higher than in mammalian preparations (Ohhashi and Jacobowitz 1985; Maton et al. 1990; Cox 1995; Sheykhzade and Nyborg 1998; Wisskirchen et al. 1998, 1999), and may reflect differences between amphibian and mammalian CGRP receptors.

The strongest evidence for our conclusion that CGRP is acting on the $CGRP_1$ receptor is the blockade of CGRP effects by rα-$CGRP_{8–37}$, a selective $CGRP_1$ receptor antagonist. Supporting evidence is the inactivity of r[Cys(ACM)$_{2,7}$]α–CGRP, a $CGRP_2$ receptor agonist. While $CGRP_2$ receptors are known to mediate the biological effects of β-CGRP (Jansen 1992; Tomlinson and Poyner 1996; Yoshimoto et al. 1998), α-CGRP and β-CGRP can both act on $CGRP_1$ receptors in certain tissues (Giuliani et al. 1992; Quirion et al. 1992; Stangl et al. 1993; Yoshimoto et al. 1998).

While amylin had a small effect on spontaneous rate at high concentrations, this action is likely mediated through the CGRP receptor. Because in other systems salmon calcitonin and amylin are equally effective on amylin receptors (Muff et al. 1999; Christopoulos et al. 1999), the biological inactivity of salmon calcitonin in our preparation suggests that an amylin receptor is not mediating this increase in discharge rate.

The functional profile of CGRP activity in the lateral line organ indicates that responsiveness to the peptide develops during the first postmetamorphic month. We do not know specifically what developmental changes are responsible for this observation. Possibilities include activation of the CGRP receptor or an induction of the receptor components (Luebke et al. 1996; McLatchie et al. 1998) necessary for a functional receptor at this synapse. In the lateral line organ, efferent activity is functional during the premetamorphic larval stages of development, (Shelton 1971), but it is during metamorphic transformation that myelinated efferent fibers send branches to each neuromast (Shelton 1970) and establish synaptic contact with receptor cells (Fritzsch 1989; Hellmann and Fritzsch 1996). Postmetamorphically, myelinated efferent fiber diameters increase from 0.3–1.2 to 2–4 μm (Mohr and Görner 1996). It is possible that this temporal sequence has some significance for CGRP receptor maturation. There are likely many biochemical and molecular events associated with these anatomical changes that may lead to an induction or activation of the CGRP receptor.

In summary, the experiments presented here provide further support for the hypothesis that CGRP is an efferent peptide transmitter, characterize the receptor meditating that effect as the $CGRP_1$ receptor, and present evidence for the developmental regulation of this receptor.

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