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Characterization of the receptor mediating the effect of calcitonin gene-related peptide in the frog adrenal gland

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Abstract

We have recently reported the presence of calcitonin gene-related peptide (CGRP)-containing nerve fibers in the frog adrenal gland and we have shown that CGRP is a potent stimulator of corticosterone and aldosterone secretion by adrenocortical cells. The aim of the present study was to characterize the type of receptors mediating the effect of CGRP in the frog adrenal gland. Amylin and adrenomedullin, two members of the CGRP family, induced a weak stimulation of corticosterone and aldosterone secretion from perifused frog adrenal slices. In contrast, salmon and human calcitonin had no effect on corticosteroid secretion. Administration of the type-1 CGRP receptor antagonists human CGRP-(8–37) and human CGRP-(19–37) did not significantly affect the secretory response induced by frog CGRP. Concurrently, the type-2 CGRP receptor agonist [acetamidomethyl-Cys^{2,7}]human CGRP ([Cys(ACM)^{2,7}]human CGRP) provoked a dose-dependent stimulation of corticosterone and aldosterone secretion ($EC_{50} = 1.6 \times 10^{-7}$ M). Both frog CGRP and [Cys(ACM)^{2,7}]human CGRP induced a significant increase in cAMP production by frog adrenal tissue. These data indicate that, in the frog adrenal gland, the stimulatory effect of CGRP is mediated through activation of a type-2 CGRP receptor positively coupled to adenylyl cyclase.

Keywords: Steroidogenesis; CGRP (calcitonin gene-related peptide); Amylin; Adrenomedullin; CGRP receptor, type 2; Corticosteroid secretion; Adenylyl cyclase activity

1. Introduction

Calcitonin gene-related peptide (CGRP) is a cyclic polypeptide containing 37 amino acids, which is generated through an alternate splicing of the calcitonin gene primary transcript (Amara et al., 1982; Rosenfeld et al., 1983). The sequence of CGRP has been determined in a number of mammalian species including rat (Amara et al., 1982), rabbit (Eysselein et al., 1991), sheep (Miyata et al., 1992), cow (Collyear et al., 1991), pig (Kimura et al., 1987) and man (Morris et al., 1984). Two forms of CGRP (α- and β-CGRP) have been characterized in rat and man (Breimer et al., 1988). The sequence of CGRP has also been determined in two representative species of sub-mammalian vertebrates, the chicken (Minvielle et al., 1986) and the frog *Rana ridibunda* (Conlon et al., 1993). These studies have demonstrated that the sequence of CGRP has been highly conserved during evolution. In particular, the two cysteine residues at position 2 and 7 are present in all CGRPs identified so far.

Two other peptides structurally related to CGRP have been characterized. Amylin, a 37-amino acid peptide isolated from pancreatic islet cells (Cooper et al., 1987; Westermark et al., 1987) has about 45% sequence identity with CGRP. Adrenomedullin, a 50-amino acid polypeptide isolated from human pheochromocytoma (Kitamura et al., 1993) exhibits about 20% sequence similarity with CGRP. All three peptides possess in common an N-terminal ring structure and a C-terminal amidated residue.

CGRP exerts a large array of biological activities (Poyner, 1992, for review). Pharmacological studies have shown that the effects of CGRP are mediated by at least three types of receptors. The type-1 CGRP receptor is specifically blocked by N-terminally truncated CGRP analogues (Dennis et al., 1990). The type-2 CGRP receptor is selectively activated by the linear analogue [acetamidomethyl-Cys^{2,7}]human CGRP ([Cys(ACM)^{2,7}]human CGRP) (Dennis et al., 1989). Finally, the type-3 CGRP receptor is unique in that it exhibits high affinity for both

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CGRP and salmon calcitonin (Sexton et al., 1988; Dennis et al., 1991). All three types of receptors exhibit differential distribution in the central nervous system and in peripheral organs (Poyner, 1992).

We have recently shown that the frog adrenal gland is richly innervated by CGRP-contained nerve terminals (Esneu et al., 1994). We have also shown that synthetic CGRP is a potent stimulator of corticosteroid secretion from adrenal tissue in vitro (Esneu et al., 1994). The aim of the present study was to determine the type of receptor involved in the corticotropic effect of CGRP.

2. Materials and methods

2.1. Animals

Adult male frogs (*Rana ridibunda*; body weight 30–40 g) were obtained from a commercial source (Couétard, Saint-Hilaire de Riez, France). Frogs were housed in a temperature-controlled room (8°C) under an established photoperiod of 12 h of light/day (lights on from 06:00–18:00 h). The animals had free access to running water, and were maintained in these conditions for at least one week before use. All surgical procedures were performed according to the recommendations of the French Ethical Committee and under the supervision of authorized investigators.

2.2. Reagents and test substances

Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) and IBMX (3-isobutyl-1-methylxanthine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [1,2,6,7,-^{3}H]Corticosterone and [1,2,6,7,-^{3}H]aldosterone were obtained from Amersham International (Les Ulis, France). Frog CGRP, human CGRP-(8–37) and [Cys(ACM)^{2,7}]human CGRP were synthesized as previously described (Dennis et al., 1989). Synthetic salmon calcitonin was obtained from Sandoz (Basel, Switzerland), human calcitonin was from Ciba-Geigy Laboratories (Rueil-Malmaison, France), rat amylin was from Novabiochem (Meudon, France) and rat adrenomedullin was from Scientific Marketing Associates (Herts, UK). Human CGRP-(19–37) was generously provided by Asahi Chemical Industry (Shizuoka, Japan).

2.3. Perifusion experiments

For each experiment, 12 adrenal glands were dissected free of kidney tissue. The glands were sliced and preincubated in a Ringer's buffer (15 mM Hepes buffer, 100 mM NaCl, 2 mM $CaCl_2$, 2 mM KCl, and 15 mM $NaHCO_3$) supplemented with 2 mg/ml glucose and 0.3 mg/ml BSA. The Ringer's solution was gassed with O_2-CO_2 (95 : 5), and the pH was adjusted to 7.4. The adrenal slices were then transferred into a perifusion chamber and layered between several beds of Bio-Gel P2 (Bio-Rad Laboratories, Richmond, CA, USA). The tissues were continuously perifused with gassed Ringer's solution at constant flow rate (200 μl/min) and temperature (24°C) as previously described (Delarue et al., 1990). The effluent perifusate was collected every 5 min and the fractions were frozen until assay.

2.4. Corticosteroid radioimmunoassays

Corticosterone and aldosterone concentrations were determined directly, without prior extraction, in 200–300 μl samples of effluent perifusate, as previously described (Leroux et al., 1980; Leboulenger et al., 1982). Direct measurement of corticosterone and aldosterone was validated by radioimmunoassay quantification of corticosteroid after high-performance liquid chromatography (HPLC) analysis of the effluent perifusate (Feuilloley et al., 1988). The detection limits of the assays were 20 pg for corticosterone and 5 pg for aldosterone. For both assays, the intra- and the inter-assay reproducibility was 6% and 3%, respectively.

2.5. Measurement of tissue cAMP content

Adrenal glands were dissected, sliced and preincubated for 10 min at 24°C in the Ringer's solution supplemented with the phosphodiesterase inhibitor IBMX (10^{-4} M). The equivalent of two glands were then incubated for 10 min in the absence or presence of test substances (frog CGRP and [Cys(ACM)^{2,7}]human CGRP) as previously described (Yon et al., 1994). To study the effect of a type-1 CGRP receptor antagonist on cAMP formation, tissue slices were preincubated for 40 min with human CGRP-(8–37) just before the incubation with frog CGRP. The reaction was stopped by adding 150 μl of ice-cold 5% perchloric acid. Adrenal slices were then homogenized and centrifuged (13 000 × *g*, 5 min, 4°C). The supernatant was neutralized with 1 M $KHCO_3$, diluted in acetate buffer (0.05 M) and stored at −20°C until assay. The amount of cAMP contained in each sample was determined using a commercial kit (Amersham International, UK) as previously described (Larcher et al., 1992). The detection limit of the assay was 12 fmol/tube. The pellet was used for protein quantification by the Lowry method.

2.6. Calculations

Each perifusion pattern was established as the mean profile of corticosteroid production (±S.E.M.) calculated over at least three independent experiments. The rates of corticosterone and aldosterone secretion were expressed as the mean of eight consecutive samples (40 min) just preceding the administration of the test substances. EC_{50} refers to the agonist concentrations yielding 50% of the maximal corticosteroid response determined for each dose-response curve. To compare the net increase in steroid

production induced by frog CGRP in the absence or in the presence of the CGRP receptor antagonists, the areas under the curves (AUC) were calculated using the trapezoidal rule (Contesse et al., 1996). Results were expressed as means ± S.E.M. and statistical differences were analyzed by using the Student's *t*-test.

3. Results

3.1. Effect of amylin, adrenomedullin and calcitonin on corticosteroid secretion

Administration of increasing concentrations of amylin (from 10^{-8} M to 3×10^{-6} M) over 20 min to perifused adrenal slices induced a dose-related increase in corticosterone (Fig. 1A) and aldosterone secretion (Fig. 1B). The minimum effective dose was 10^{-7} M ($P < 0.05$). At the higher concentration tested (3×10^{-6} M), amylin increased corticosterone and aldosterone secretion by 52% and 55%, respectively.

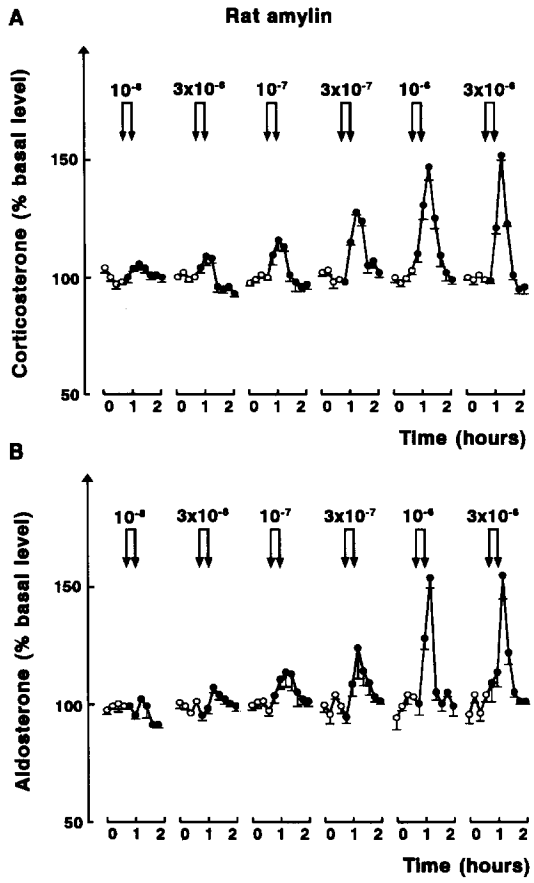

Fig. 1. Effect of increasing concentrations of rat amylin (from 10^{-8} M to 3×10^{-6} M) on corticosterone (A) and aldosterone (B) secretion by perifused frog adrenal slices. After a 120-min equilibration period, amylin was administered for 20 min (arrows), and the tissue was allowed to stabilize for another 90-min period before the next pulse of amylin. The profiles represent the mean secretion patterns (±S.E.M.) of three independent perifusion experiments. Each point is the mean secretion level of corticosteroid in two consecutive fractions collected during 5 min. The spontaneous level of corticosterone and aldosterone release (100% basal level) was calculated as the mean of eight consecutive fractions (40 min; o—o) just preceding the infusion of each dose of amylin. The mean basal levels of corticosterone and aldosterone in these experiments were 21 ± 1.2 and 16 ± 1.8 pg/interrenal gland per min, respectively.

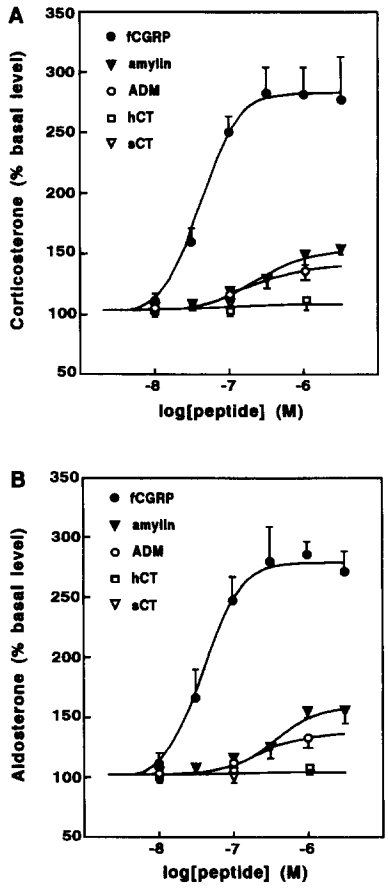

Fig. 2. Semi-logarithmic plot comparing the effects of frog CGRP (fCGRP), rat amylin, rat adrenomedullin (ADM), human calcitonin (hCT) and salmon calcitonin (sCT) on corticosterone (A) and aldosterone (B) secretion by perifused frog adrenal slices. All experimental values were calculated from data similar to those presented in Fig. 1. The mean corticosteroid concentration in two consecutive fractions collected just after each pulse of test substance (peak height) was compared to the mean corticosteroid level observed just prior to the administration of the secretagogue. See legend to Fig. 1 for other details.

A series of experiments similar to those presented in Fig. 1 was conducted with frog CGRP, adrenomedullin, salmon calcitonin and human calcitonin, and the corresponding dose-response curves are presented in Fig. 2. As previously reported (Esneu et al., 1994), frog CGRP gave rise to a dose-related increase in corticosterone (Fig. 2A) and aldosterone secretion (Fig. 2B) with an EC_{50} of 4×10^{-8} M. Amylin and adrenomedullin were less potent ($EC_{50} = 2 \times 10^{-7}$ M and 10^{-7} M, respectively) and far less efficient than frog CGRP (Fig. 2). Salmon and human calcitonins were totally devoid of effect on corticosteroid secretion.

3.2. Effect of type-1 CGRP receptor antagonists

The effect of frog CGRP on corticosteroid secretion was tested in the absence and presence of the type-1 CGRP receptor antagonists human CGRP-(8–37) and human CGRP-(19–37). A 20-min pulse of frog CGRP induced a reproducible increase in corticosterone and aldosterone secretion (Fig. 3). Neither human CGRP-(8–37) nor human CGRP-(19–37) had any effect on the spontaneous release of corticosteroids (Fig. 3). In addition, the secretory response to frog CGRP was not affected during prolonged administration of the type-1 CGRP receptor antagonists (Fig. 3).

3.3. Effect of a type-2 CGRP receptor agonist

The effect of graded concentrations of human CGRP[Cys(ACM)^{2,7}] (from 3×10^{-9} M to 10^{-5} M) on corticosteroid secretion is illustrated in Fig. 4. Administration of 20-min pulses of the type-2 CGRP receptor agonist induced a dose-related increase in corticosterone (Fig. 4A) and aldosterone secretion (Fig. 4B). The EC_{50} of [Cys(ACM)^{2,7}]human CGRP was 1.6×10^{-7} M and the maximum response was obtained at a concentration of 10^{-6} M.

3.4. Effect of CGRP agonists and antagonists on cAMP formation

Incubation of adrenal tissue with frog CGRP (10^{-6} M) provoked a significant increase of cAMP content (Fig. 5). In the presence of the type-1 CGRP receptor antagonist human CGRP-(8–37) (10^{-5} M), the stimulatory effect of frog CGRP on cAMP formation was not affected (Fig. 5). Concurrently, the type-2 CGRP receptor agonist [Cys(ACM)^{2,7}]human CGRP (10^{-6} M) significantly stimulated cAMP production (Fig. 5).

4. Discussion

The adrenal gland of amphibians is innervated by fibers containing various neuropeptides including atrial natriuretic factor (Lihrmann et al., 1988), pituitary adenylate cyclase-activating polypeptide (Yon et al., 1993) and tachykinins (Leboulenger et al., 1993; Kodjo et al., 1995). We have recently found the presence of CGRP-immunoreactive fibers in the frog adrenal gland, we have characterized CGRP by reversed phase HPLC analysis and we have shown that synthetic CGRPs cause stimulation of

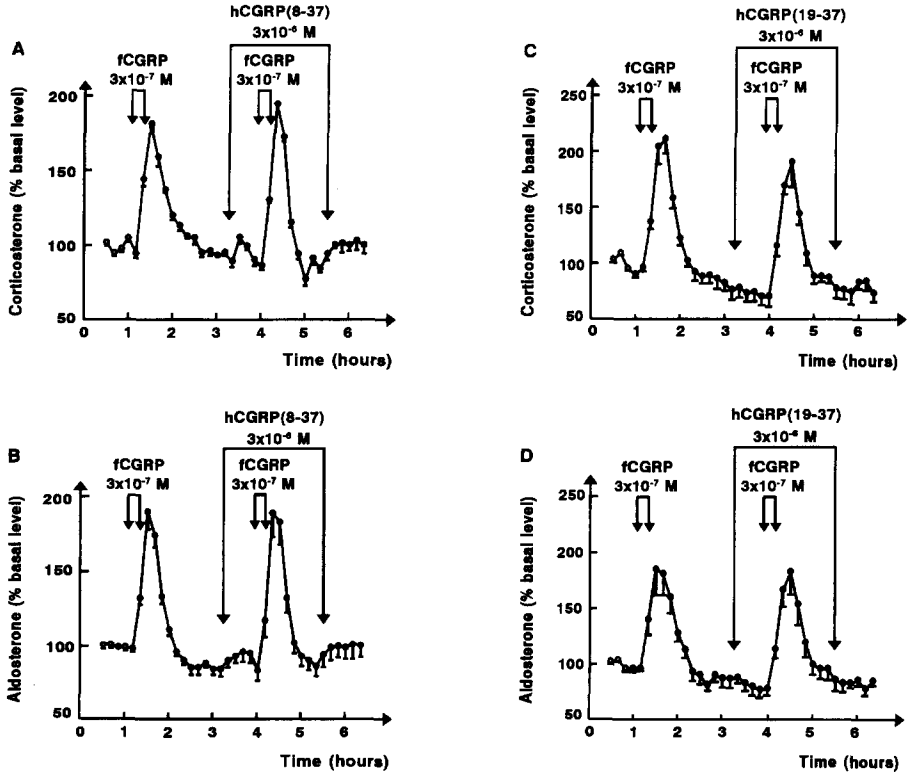

Fig. 3. Effect of frog CGRP (fCGRP) alone or during prolonged infusion of the selective type-1 CGRP receptor antagonists, human CGRP-(8–37) (A,B) or human CGRP-(19–37) (C,D) on corticosterone and aldosterone secretion by frog adrenal slices. After a 120-min equilibration period, a first pulse of frog CGRP (3×10^{-7} M) was administered for 20 min and the interrenal tissue was allowed to stabilize for another 110-min period. Then, human CGRP-(8–37) or human CGRP-(19–37) was infused for 140 min. During infusion of the antagonists, a second dose of frog CGRP (3×10^{-7} M) was added for 20 min. The mean basal levels of corticosterone and aldosterone in these experiments were 39 ± 4 and 13 ± 2.2 pg/interrenal gland per min, respectively. See legend to Fig. 1 for other details.

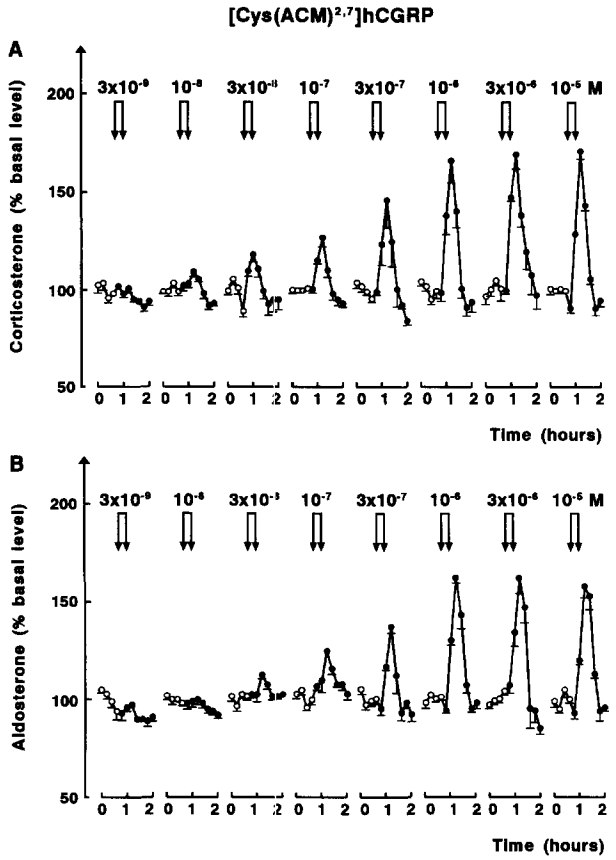

Fig. 4. Effect of increasing concentrations of the type-2 CGRP receptor agonist [Cys(ACM)^{2,7}]human CGRP on corticosterone (A) and aldosterone (B) secretion by frog adrenal slices. The mean basal level of corticosterone and aldosterone in these experiments were 25 ± 1.7 and 19 ± 2.9 pg/interrenal gland per min, respectively. See legend to Fig. 1 for other details.

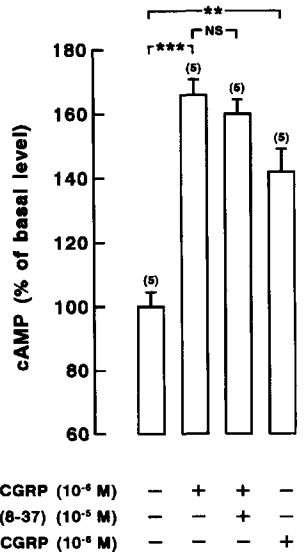

Fig. 5. Effect of frog CGRP (CGRP), the type-1 CGRP receptor antagonist human (h) CGRP-(8–37) and the type-2 CGRP receptor agonist [Cys(ACM)^{2,7}]human CGRP on cAMP formation by frog adrenal slices. The cAMP content in the adrenal tissue was measured after a 10-min incubation with the drug, and calculated as a percentage of the control level. Each value is the mean ($\pm$S.E.M.) of five independent determinations. N.S., non significantly different; ** $P < 0.01$; *** $P < 0.001$.

corticosteroid secretion by frog adrenocortical tissue in vitro (Esneu et al., 1994). The present report describes the characterization of the CGRP receptor expressed by adrenal cells.

We first observed that rat amylin and rat adrenomedullin, two polypeptides which exhibit 54% and 24% sequence similarity with frog CGRP, induced a slight stimulation of corticosteroid secretion. The kinetics of the response of adrenocortical cells to amylin was very similar to that observed with frog or mammalian CGRPs (Esneu et al., 1994). Although amylin and adrenomedullin were about 5 times less potent and 4 times less efficient than frog CGRP, their efficacy was in the same range as those of human α- and β-CGRP and rat β-CGRP (Esneu et al., 1994). Whether the effects of amylin and adrenomedullin on frog adrenocortical cells were mediated through CGRP receptors or whether the peptides acted on distinct receptors remains unknown. The effect of adrenomedullin on rat glomerulosa cells has been described in two recent reports (Yamaguchi et al., 1995; Mazzochi et al., 1996). These studies have shown that adrenomedullin does not affect basal or adrenocorticotropin-stimulated aldosterone secretion, but strongly depresses angiotensin II- and potassium-evoked aldosterone production. It was also shown that the effect of adrenomedullin was blocked by CGRP-(8–37), indicating that the polypeptide acts through type-1 CGRP receptors (Mazzochi et al., 1996). However, an adrenomedullin receptor has recently been identified in the rat adrenal gland (Kapas et al., 1995), suggesting that the effect of adrenomedullin could also be mediated through its proper receptor.

Although calcitonin and CGRP have little sequence similarity, salmon calcitonin exhibits high affinity for CGRP binding sites in the rat nucleus accumbens (Sexton et al., 1988; Dennis et al., 1991) indicating the existence of a novel type of receptor, distinct from the type-1 and type-2 CGRP receptors. The present study showed that salmon calcitonin as well as human calcitonin were totally devoid of activity on corticosteroid secretion. These data indicate that, in the frog adrenal gland, the effect of CGRP is not mediated through the type-3 CGRP receptor previously characterized in the rat nucleus accumbens.

Two series of CGRP analogues are now commonly used to characterize type-1 and type-2 CGRP receptors: human CGRP-(8–37) and, to a lesser-extend, human CGRP-(19–37) act as type-1 CGRP receptor antagonists whereas the linear analogue [Cys(ACM)^{2,7}]human CGRP is a selective agonist for the type-2 CGRP receptor (Dennis et al., 1989, 1990; Donoso et al., 1990; Rovero et al., 1992). The present study showed that human CGRP-(8–37) and human CGRP-(19–37) did not affect the stimulation of corticosteroid secretion evoked by frog CGRP on the adrenal gland. Similarly, human CGRP-(8–37) had no effect on the cAMP formation induced by frog CGRP. These data indicate that the stimulatory effect of CGRP on frog adrenocortical cells is probably not mediated by a

type-1 CGRP receptor. In contrast, the type-2 receptor agonist [Cys(ACM)^{2,7}]human CGRP induced a dose-dependent stimulation of corticosteroid secretion. [Cys(ACM)^{2,7}]human CGRP also mimicked the stimulatory effect of CGRP on cAMP formation. Consistent with previous findings (Dennis et al., 1989; Poyner, 1992; Stangl et al., 1993) we observed that [Cys(ACM)^{2,7}]human CGRP was less potent and slightly less efficient than CGRP in stimulating corticosteroid secretion. Taken together, these data indicate that the stimulatory effect of CGRP on frog adrenocortical cells can be accounted for by activation of a type-2 CGRP receptor.

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