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Functional characteristics of calcitonin gene-related peptide receptors in human Ewing's sarcoma WE-68 cells

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Calcitonin gene-related peptide (CGRP) receptor activity was studied in WE-68 human Ewing's sarcoma cells. ^{125}I-human CGRP bound in a time-dependent, reversible and saturable manner. Scatchard plots were compatible with the presence of a homogenous population of CGRP receptors with high affinity (K_d = 15 pM, and B_{max} = 1.9 fmol/mg protein). The potency order of unlabeled peptides, in the presence of radioligand, was: human CGRP-II > human CGRP = chick CGRP > rat CGRP = rat [Tyr^0]CGRP > human [Tyr^0] CGRP > > salmon calcitonin (CT) > rat [Tyr^0]CGRP-(28–37). Each peptide except CT and [Tyr^0]CGRP-(28–37) stimulated cyclic AMP generation in a concentration-dependent manner, and the relative potencies paralleled their relative ability in inhibiting ^{125}I-human CGRP binding. We conclude that WE-68 Ewing's sarcoma cells express genuine CGRP receptors which upon activation lead to stimulation of cyclic AMP formation.

Calcitonin gene-related peptide; Calcitonin; cyclic AMP; (Human; Ewing's sarcoma cell)

1. INTRODUCTION

Calcitonin gene-related peptide (CGRP; CGRP-I; α-CGRP) is a recently discovered 37-amino-acid polypeptide which is generated by tissue-specific alternative calcitonin gene transcription [1,2]. The mRNA transcripts of the calcitonin gene encode calcitonin (CT) in thyroid C-cells, and CGRP in human ventral spinal cord, thyroid, pituitary, and in medullary thyroid carcinoma cells [3–5]. A structurally related second human gene, which is a pseudogene for CT, generates CGRP-II (β-CGRP) that differs from human CGRP-I in 3 of 37 amino acids [4]. Radioreceptor assay and immunological studies have demonstrated discrete binding sites for CGRP and CT in the human and rat central nervous system and peripheral tissues [5,6]. Nonetheless, because of structural similarities between the two peptides (a disulfide bond in the N-terminus; an amidated C-terminus) CGRP and CT have been shown to cross-react with the high-affinity binding site of the other [6,7]. Although the central nervous system expresses predominantly CGRP receptors no influence of CGRP on the cyclic AMP-generating system was observed [6] whereas the kidney-adenylate cyclase was stimulated by CGRP through interaction with functional CT receptors [6,7].

WE-68 is a human Ewing's bone tumor cell line which expresses morphological features of neuroectodermal differentiation, namely neuron-specific enolase, S-100 protein, Leu-7-antigen and neurofilaments [8]. Furthermore, WE-cells respond to various neurohormones including β-adrenergic agonists, dopamine, vasoactive intestinal peptide, neuropeptide Y, by a modulation of cyclic AMP metabolism [9–12]. On the basis of these data the possibility was examined whether the neuropeptide CGRP might interact with WE-68 cells. Our study shows that WE-68 cells possess specific CGRP receptors and is the first demonstration of functional CGRP receptors in

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cultured cells of human nature, with neural differentiation potential.

2. MATERIALS AND METHODS

Human, chick and rat CGRP, human CGRP-II, human and rat [Tyr^0]CGRP, rat [Tyr^0]CGRP-(28–37), human and salmon CT, human vasoactive intestinal peptide (VIP) and human neuropeptide Y (NPY) were purchased from Peninsula (St. Helens, England). Monoiodinated ^{125}I-His^{10}-human CGRP (^{125}I-human CGRP; 2000 Ci/mmol) was obtained from Amersham (Braunschweig) and 3-isobutyl-1-methylxanthine (IBMX) from Sigma (Munich).

Ewing's sarcoma WE-68 cells were cultured in Dulbecco's modified Eagle's medium/Ham's Formula-12 (1 : 1; DME/F12) with 5% FCS and maintained in fibronectin-coated (0.5 μg/cm^2; Boehringer, Mannheim) Falcon T-25 flasks [9]. ^{125}I-CGRP binding and cyclic AMP production experiments were conducted on fibronectin-coated (0.5 μg/cm^2) Costar 24-well dishes seeded with 4×10^5 cells/well.

To study ^{125}I-CGRP binding, confluent cultures were incubated in DME/F12 (1 : 1) containing 1% (w/v) human serum albumin (HSA) and varying concentrations of ^{125}I-human CGRP (2.3–86.9 pM) in the absence (total binding) and presence (nonspecific binding) of unlabeled human CGRP (200 nM) in a total volume of 300 μl at 37°C for 150 min. Cell-associated radioactivity was determined in an LKB 1270 Rackgamma-II γ-counter after cell lysis with Lubrol-PX (1%, v/v)/HSA (1%) in H_2O as described [10].

Incubation of monolayer cell cultures to study cyclic AMP formation was carried out as in [11]. In short, cells were incubated for 15 min at 37°C with peptide in Hank's buffer with 20 mM Hepes (pH 7.4) after a 15-min preincubation period in this buffer containing 1 mM IBMX. Cyclic AMP samples were treated according to the trichloroacetic acid/Tris protocol [12] and determined with the Amersham assay kit. Cell protein was measured by the Bio-Rad protein assay (Bio-Rad, Munich) using HSA as standard.

Data are expressed as means ± SE of triplicate determinations from three experiments in each case. Binding data were analyzed by the computer COMBICEPT 2000 CA receptor assay program from Canberra-Packard (Frankfurt).

3. RESULTS

The association of ^{125}I-human CGRP with intact human Ewing's sarcoma WE-68 cells reached equilibrium after 120–150 min at 37°C, and was reversed within 100 min in the absence and presence of unlabeled human CGRP (fig.1). Binding of radioligand was concentration-dependent (fig.2). Specific binding averaged 75% of total ^{125}I-human CGRP bound, which represented 4–6% of total radioactivity added to

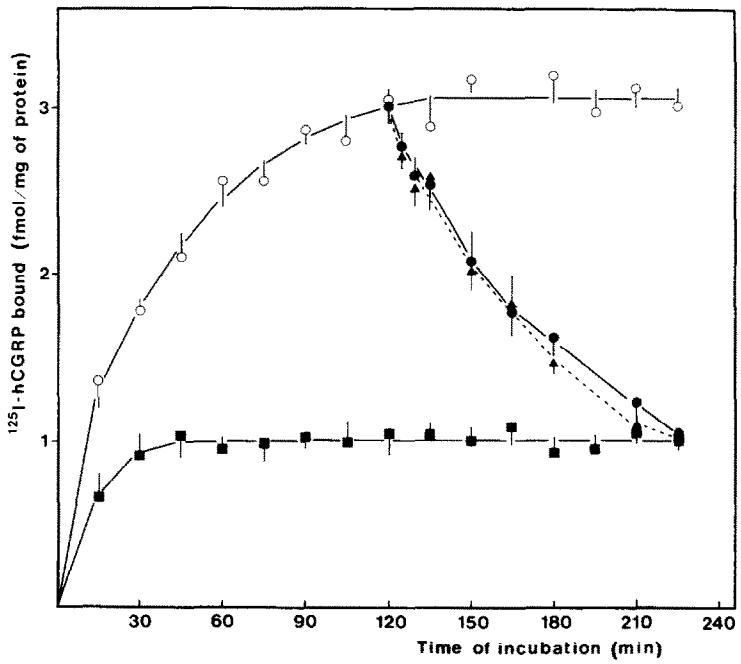

Fig.1. Association-dissociation pattern for the binding of ^{125}I-human CGRP in WE-68 cells. Cells were incubated at 37°C in the presence of 150 pM ^{125}I-human CGRP alone (○, total binding) or with excess human CGRP (200 nM) (■, nonspecific binding); 120 min later cells were reincubated in binding medium alone (●) or supplemented with human CGRP (▲; 200 nM). Values are means ± SE from 3 independent experiments.

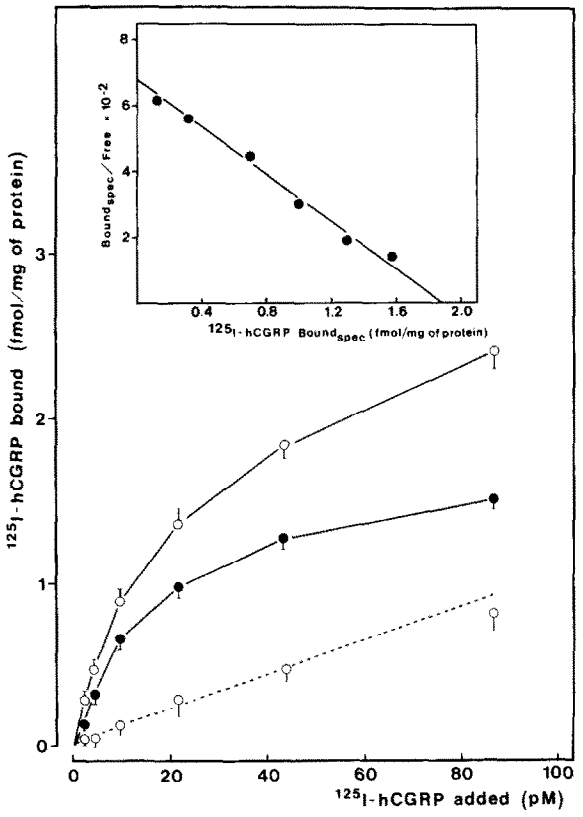

Fig.2. Saturation isotherm obtained from incubating WE-68 cells for 150 min at 37°C with increasing concentrations of ^{125}I-human CGRP alone (○—○) or in the presence of 200 nM human CGRP (○---○). Specific ^{125}I-human CGRP binding (●) was calculated as the difference between the total and nonspecific radioactivity associated to the cells. Values are means ± SE from 3 independent experiments. Inset: Scatchard plot of ^{125}I-human CGRP specific binding data.

the culture wells containing 140–160 μg cell protein. Scatchard plot analysis of ^{125}I-human CGRP steady-state binding data gave a straight line (fig.2, inset). The dissociation constant (K_d) was calculated to be 15 pM, and maximal binding capacity (B_{max}), 1.9 fmol/mg cell protein. As shown in fig.3, radioligand binding was inhibited by human CGRP-II (half-maximal inhibitory concentration [IC_{50}] = 20 pM), human and chick CGRP (IC_{50S} = 100 pM), rat CGRP and [Tyr^0]CGRP (IC_{50S} = 200 pM) and human [Tyr^0]CGRP (IC_{50} = 1 nM). Salmon CT was 50000 times less potent than human CGRP-II, and the C-terminal fragment [Tyr^0]CGRP-(28–37) at 1 μM inhibited binding of ^{125}I-human CGRP by

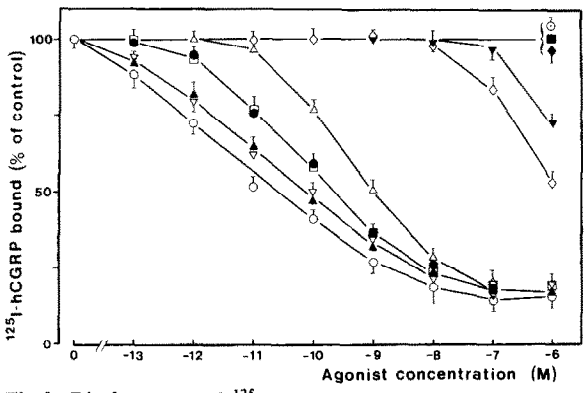

Fig.3. Displacement of ^{125}I-human CGRP binding in WE-68 cells incubated for 150 min at 37°C with 100 pM tracer in the presence of increasing concentrations of human CGRP-II (○), chick CGRP (▲), human CGRP (▽), rat CGRP (□), rat [Tyr^0]CGRP (●), human [Tyr^0]CGRP (△), salmon CT (◇), rat [Tyr^0]CGRP-(28–37) (▼), human CT (⊙), VIP (■), and NPY (◆) expressed as percentage of ^{125}I-human CGRP binding in the absence of added peptide. Values are means ± SE from 3 independent experiments.

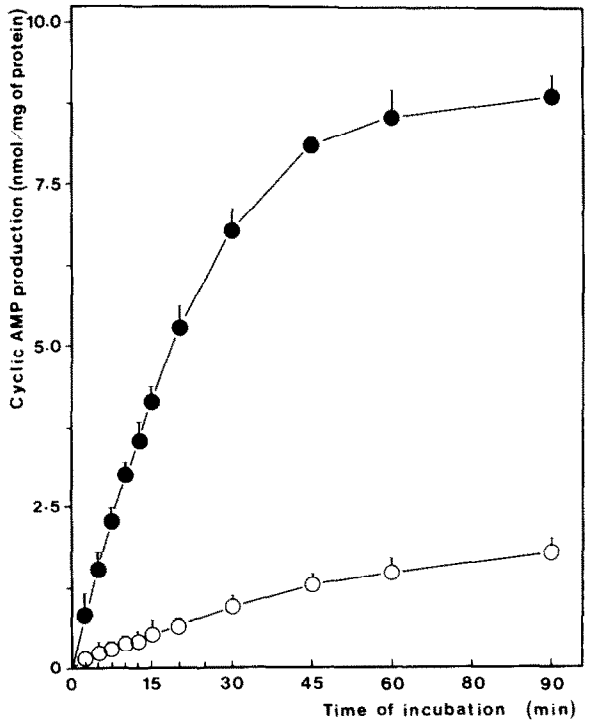

Fig.4. Time course of cyclic AMP production in WE-68 cells incubated in the presence of 200 nM human CGRP without (open symbols) and with (closed symbols) 1 mM IBMX. Basal cyclic AMP levels (nmol/mg of protein) were 0.14 ± 0.03 (in the absence of IBMX) and 0.29 ± 0.02 (in the presence of IBMX) for the indicated time periods, and were subtracted from the appropriate peptide-stimulated cyclic AMP levels. Values are means ± SE from three independent experiments.

30%. Tracer binding was not displaced by human CT, VIP and NPY at up to 1 μM.

Human CGRP provoked a curvilinear increase in the cyclic AMP content in WE-68 cells (fig.4). The stimulation was noticeable as early as 2.5 min after addition of the peptide and peaked between 60 and 90 min which corresponded with a 30-fold increase over the basal. The course of the time curve was the same as that observed in the absence of the phosphodiesterase inhibitor IBMX, but under these conditions the peptide raised cyclic AMP levels by maximally 9-fold. The concentration-effect curves for cyclic AMP generation by CGRPs and CTs are depicted in fig.5. Human CGRP-II was the most potent peptide stimulating cyclic AMP already significant at 0.5 nM (fig.5A) with half-maximal effective concentration (EC_{50}) of 1.5 nM human CGRP-II. Human and chick CGRP were as efficient as human CGRP-II but were each approx. 4-fold less potent (EC_{50}s = 6 nM); CGRP and [Tyr^0]CGRP of rat were each 10-fold less potent than human CGRP-II (EC_{50}s = 15 nM). The potency of human [Tyr^0]CGRP was 40-fold lower than that of human CGRP-II (EC_{50} = 60 nM). Rat [Tyr^0]CGRP-(28–37) as high as 1 μM caused no increase in cellular cyclic AMP content and was unable to modify rat CGRP action (fig.5B). Salmon CT increased cyclic AMP marginally (1.6-fold over the basal) but significantly ($P < 0.05$) only at a very high (1 μM) concentration, and human CT was inactive in raising cyclic AMP levels (fig.5A).

4. DISCUSSION

Our results demonstrate the existence of functional CGRP receptors in WE-68 human Ewing's sarcoma cells. ^{125}I-human CGRP bound in a time-dependent and reversible manner with a single class of receptors that were saturable (B_{max} = 1.9 fmol/mg cell protein) and of high affinity (K_d = 15 pM). The specificity of the CGRP receptor was shown by its selectivity towards a series of CGRPs and CTs tested on ^{125}I-human CGRP binding and cyclic AMP generation: human CGRP-II > chick CGRP = human CGRP > rat CGRP = rat [Tyr^0]CGRP > human [Tyr^0]CGRP ≫ salmon CT, implying CGRP receptors predominate in WE-68 cells and CT most probably interacts with these CGRP receptors. Specific high-affinity CGRP receptors (K_d values ranging from 4 to 85 pM) have been observed with membranes from

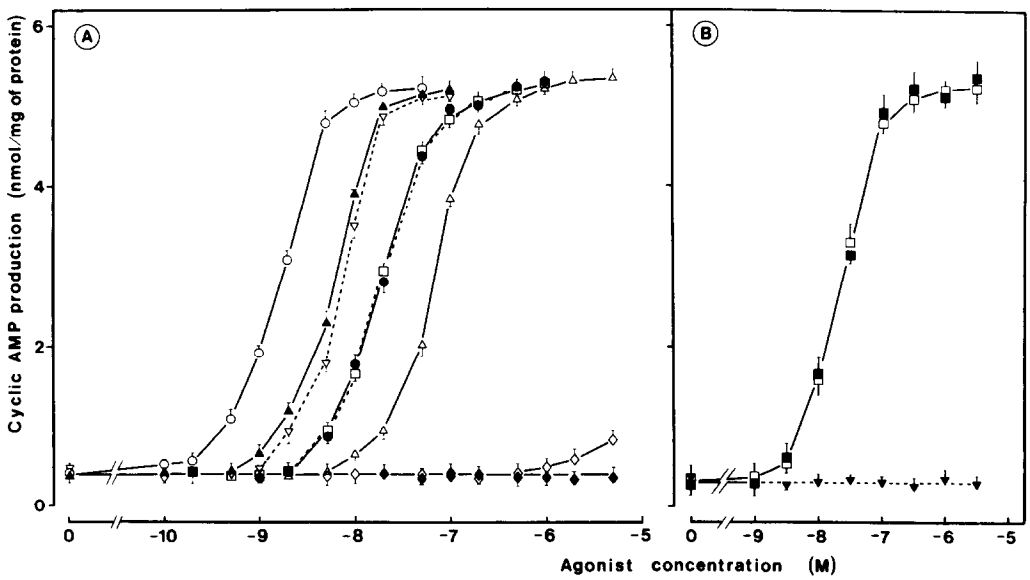

Fig.5. Cyclic AMP production in WE-68 cells incubated with increasing concentrations of human CGRP-II (○), chick CGRP (▲), human CGRP (▽), rat CGRP (□), rat [Tyr^0]CGRP (●), human [Tyr^0]CGRP (△), salmon CT (◇) and human CT (◆) (A), and rat [Tyr^0]CGRP-(28–37) (▼), rat CGRP (□) and rat CGRP plus 1 μM rat [Tyr^0]CGRP-(28–37) (■) (B). Basal cyclic AMP level in (A) and (B) was 0.31 ± 0.03 nmol/mg of protein. Values are means ± SE of independent experiments.

rat brain and heart [13], human cerebellum [14] and porcine spinal cord and heart ventricle [15,16]. CGRP-mediated cyclic AMP stimulation has been reported for rat aortic smooth muscle cells [17], rat osteosarcoma UMR 106-01 cells [18], bovine endothelial cells [19] and in membranes of rat spleen, liver and atrial myocytes [20–22]. In contrast, high-affinity CGRP receptors were reported to be ineffective in activating adenylate cyclase in rat brain and spinal cord membranes [6]. Hence WE-68 is the first human, neural differentiated cell line displaying CGRP receptors functionally coupled to cyclic AMP accumulation. Human CGRP-II was the most potent of CGRPs studied indicating that WE-68 CGRP receptor specifically recognizes CGRP-II. Interestingly, CGRP-II but not CGRP-I mRNA has been detected in four of six Ewing's sarcoma cell lines studied by Höppener et al. [23] and CGRP-II was found to be secreted by one of these cells lines, IARC/EW-1. The C-terminal fragment [Tyr^0]CGRP-(28–37), slightly capable of displacing radioligand, was functionally inactive suggesting that the N-terminal portion of CGRP is essential for adequate binding and eliciting a biological response in WE-68 cells.

In conclusion, CGRP receptors are positively linked to cyclic AMP production in Ewing's sarcoma cells. CGRP must be added to the list of neurohormones that modulate cyclic AMP generation in WE-68, a human bone tumor cell line with neural differentiation characteristics [8,10,11].

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