Characterization of Calcitonin Gene-Related Peptide (CGRP) Receptors in Guinea Pig Lung

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Abstract—Receptors for calcitonin gene-related peptide (CGRP) in the lung membranes of guinea pig were characterized by using a millimeter plate precoated with polyethylenimine. Specific binding of $^{125}$I-CGRP was time-dependent, rapid, and reversible, and the binding increased linearly with increasing concentrations of membrane protein. Scatchard analysis revealed two classes of CGRP binding sites: high affinity sites with a $K_d$ value of $7.17 \times 10^{-11}$ M and a $B_{max}$ of 364 fmol/mg protein and low affinity sites with a $K_d$ value of $1.7 \times 10^{-8}$ M and a $B_{max}$ of 39594 fmol/mg protein. Furthermore, the specific binding of $^{125}$I-CGRP was dissociated in the presence of GTP or Gpp(NH)p, suggesting that CGRP receptors in guinea pig lung membranes were coupled to the guanine nucleotide regulatory protein. Scatchard analysis of CGRP binding in the presence of GTP revealed selective inhibition of the binding to high affinity binding sites. Unlabeled CGRP displaced the binding of $^{125}$I-CGRP to guinea pig lung membranes with an IC50 value of $3.1 \times 10^{-10}$ M. In contrast, salmon calcitonin and human calcitonin displaced the binding at 600-fold higher concentrations. We suggest that both low and high affinity binding sites for CGRP exist in the lung membranes of the guinea pig, and the high affinity binding sites for CGRP may be coupled to GTP binding regulatory protein.

Calcitonin gene-related peptide (CGRP) is a novel 37-residue peptide produced by alternative RNA processing of calcitonin gene transcripts (1, 2). CGRP-like immunoreactivity is widely distributed in both the central and peripheral nervous systems in many species (3, 4). Furthermore, CGRP-like immunoreactivity was found to be distributed in the sensory nerves of the lung of rats, guinea pigs, humans and others (5, 6). Therefore, it has been suggested that CGRP may be involved in the regulation of airway function.

By direct binding techniques, Carstairs found both high and low affinity binding sites for CGRP in rat lung sections (7), and Mak and Barnes found only high affinity binding sites in guinea pig lung sections (8). Since contradictory results were obtained, we decided to study this further by using millimeter plates coated with polyethylenimine for the radio-receptor assay to identify and characterize the receptors for CGRP in the lung membrane of guinea pigs and to determine whether GTP binding regulatory protein couples to the receptor for CGRP.

Materials and Methods

Animals: Male Hartley guinea pigs (250–350 g) were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan).

Chemicals: (2-[$^{125}$I]iodohistidyl$^{110}$) human calcitonin gene-related peptide (Amersham International plc., specific activity ~2000 Ci/mmol); human calcitonin gene-related peptide (Peptide Institute, Inc., Osaka, Japan); bacitracin, polyethylenimine, bovine serum albumin, salmon calcitonin, guanine triphosphate, guanylylimidodiphosphate, guanine monophosphate, adenosine triphosphate and human calcitonin (Sigma, U.S.A.) were obtained as indicated.
Preparation of guinea pig lung membranes: The preparation of the membrane homogenate was carried out by a modification of the method of Hagaboone et al. (9). Lungs were excised from male guinea pigs, placed on ice, minced into small pieces and rinsed in 10 mM Tris HCl. The tissue was placed in six volumes of 10 mM Tris HCl and homogenized at 0°C with a Brinkman PT10-35 polytron (Brinkman Instruments, Westbury, U.S.A.) for 1 min (30 sec pulses at a setting of 6). The homogenate was filtered through a single gauze layer and centrifuged at 1,000 x g at 0°C for 10 min to remove tissue and unbroken cells, nuclei and debris. The supernatant was recentrifuged at 50,000 x g for 10 min to obtain the crude membrane fractions. These fractions were then resuspended in six volumes of 10 mM Tris HCl, homogenized with a polytron and centrifuged again at 50,000 x g at 0°C for 10 min. The resulting pellets were resuspended in 10 mM Tris HCl, homogenized with a polytron, pipetted into cryotubes, and then stored at -196°C. The protein concentration was determined by using the method of Lowry et al. (10).

Binding of 125I-CGRP to guinea pig lung membranes: The CGRP radioligand receptor-binding assay was a modification of the method described by Yoshizaki et al. (11). Lung membranes were suspended in an ice-cold incubating buffer: 50 mM Tris HCl (pH 7.4), 5 mM MgCl₂, 2 mM EGTA, 0.1 mg/ml bacitracin, 1% BSA and 0.24 M sucrose. A binding study was done using a Millititer GV-96-well filtration plate (Millipore) which had been precoated overnight with 0.025% poly-ethylenimine. Lung membranes were incubated with 1.0 x 10⁻¹⁰ M 125I-CGRP at 23°C for 60 min in the absence or presence of unlabeled CGRP in a final volume of 0.25 ml in a millititer plate. After 60-min incubation, the membrane bound tracer was separated from free ligand by rapid filtration through a millititer plate and washed twice with 300 µl of buffer. The filters were transferred to counting vials, and the radioactivity was estimated by using a Packard autogamma counter. The nonspecific binding of 125I-CGRP was determined in the presence of 10⁻¹⁰ M unlabeled CGRP. Scatchard analysis with the nonlinear regression analysis of MLIN Procedure of SAS (12) was used to calculate K₀ and Bₘₐₓ values after the raw data were first processed according to Feldmann’s equations for the one-ligand, two-binding site model.

Results

Time course of the specific binding of 125I-CGRP to guinea pig lung membranes: Specific binding of 125I-CGRP to guinea pig lung membranes was determined as a function of time. The specific binding increased with time, reaching a plateau after 120 min of incubation. The binding was saturable, and Scatchard analysis revealed a single class of binding sites with a dissociation constant (K₀) of 10⁻¹⁰ M and a maximum binding capacity (Bₘₐₓ) of 10⁻⁹ M. The results are shown in Fig. 1.

Fig. 1. Time course of the specific binding of 125I-CGRP to the lung membranes of guinea pig. Membranes (5.2 µg protein) were incubated with 1.0 x 10⁻¹⁰ M 125I-CGRP at 23°C for the time indicated in the absence or presence of 10⁻⁶ M unlabeled CGRP. Each point represents the mean of two experiments.
binding of $^{125}$I-CGRP to guinea pig lung membranes depended on the time (Fig. 1). Apparent equilibrium binding at 23°C was reached during a 60–90 min incubation and was stable after 90 min. Nonspecific binding in the presence of $10^{-6}$ M unlabeled CGRP was stable after 90 min. Nonspecific binding.

Fig. 2. Time course of the dissociation of the specific binding of $^{125}$I-CGRP from the lung membranes of guinea pig. Membranes (5.2 µg protein) were preincubated with $1.0 \times 10^{-10}$ M $^{125}$I-CGRP for 60 min at 23°C, and then the dissociation reaction was initiated with $10^{-6}$ M unlabeled CGRP (○), $10^{-6}$ M GTP (■), $10^{-6}$ M Gpp(NH)p (□) or unlabeled $10^{-6}$ M CGRP and $10^{-5}$ M GTP (▲). Each point represents the mean of two experiments.

Fig. 3. Nucleotide specificity for dissociation of the specific binding of $^{125}$I-CGRP from the lung membranes of guinea pig. Membranes (5.2 µg protein) were preincubated with $1.0 \times 10^{-10}$ M $^{125}$I-CGRP for 60 min at 23°C, and then the dissociation reaction was initiated with various concentrations of GTP (○), Gpp(NH)p (■), GMP (▲) or ATP (△). After bound $^{125}$I-CGRP was incubated for 30 min at 23°C in the presence of various concentrations of nucleotides, $^{125}$I-CGRP binding was assessed.
was approximately 30% of the binding. The specific binding of \(^{125}\text{I}\)-CGRP was linearly related to the amount of lung membrane (3.0–26.1 \(\mu\)g protein/ml) used (data not shown). Subsequent binding experiments were done at 23°C with a 60 min-incubation period and 5.2 \(\mu\)g membrane protein.

Time course of the dissociation of the specific binding of \(^{125}\text{I}\)-CGRP from the lung membranes of guinea pig: The time course of the dissociation of \(^{125}\text{I}\)-CGRP was investigated by preincubating the membrane with \(^{125}\text{I}\)-CGRP for 60 min. The specific binding of \(^{125}\text{I}\)-CGRP was reversible; and at 23°C, the addition of unlabeled \(10^{-6}\) M CGRP displaced approximately 85% of the bound \(^{125}\text{I}\)-CGRP within 20 min (Fig. 2). Dissociation of \(^{125}\text{I}\)-CGRP was dependent on the presence of GTP or Gpp(NH)p because when unlabeled CGRP and GTP were added simultaneously, almost all of the \(^{125}\text{I}\)-CGRP had dissociated within 50 min. The nucleotide specificity for dissociation of \(^{125}\text{I}\)-CGRP was further investigated. GTP was more potent than Gpp(NH)p, whereas GMP was only weakly active. ATP was inactive up to \(10^{-4}\) M (Fig. 3).

Saturation and Scatchard analysis of \(^{125}\text{I}\)-CGRP binding to guinea pig lung membranes: The binding of various concentrations (5.0\(\times10^{-12}\) M–8.0\(\times10^{-9}\) M) of \(^{125}\text{I}\)-CGRP to guinea pig lung membranes is shown in Fig. 4A and B. Scatchard analysis of the binding data revealed two classes of binding sites for CGRP (Fig. 4C). The high affinity binding sites have a dissociation constant (\(K_d\)) of 7.17\(\times10^{-11}\) M and a \(B_{\text{max}}\) of 364 fmol/mg

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 4.** Saturation analysis of \(^{125}\text{I}\)-CGRP binding to guinea pig lung membranes. Membranes (5.2 \(\mu\)g protein) were incubated for 60 min at 23°C with various concentrations of \(^{125}\text{I}\)-CGRP (5.0\(\times10^{-12}\) M–8.0\(\times10^{-9}\) M). The binding data of low concentrations of \(^{125}\text{I}\)-CGRP (5.0\(\times10^{-12}\) M–2.58\(\times10^{-10}\) M) are shown in A, and those of high concentrations of \(^{125}\text{I}\)-CGRP (1.25\(\times10^{-10}\) M–8.0\(\times10^{-9}\) M) are shown in B. Each point represents the mean of duplicate determinations from a typical experiment. Scatchard analysis of the binding data is shown in C. (□) total binding, (●) specific binding, (△) nonspecific binding.
protein, and the low affinity binding sites have a \( K_D \) value of \( 1.7 \times 10^{-8} \) M and a \( B_{\text{max}} \) of 39594 fmol/mg protein. Next, saturation experiment \(^{125}\text{I}-\text{CGRP}\) binding was carried out in the presence of GTP. Scatchard analysis of the data (Fig. 5) revealed that the decreased binding was primarily due to decreased binding to the high affinity sites.

**Competitive inhibition of the binding of \(^{125}\text{I}-\text{CGRP}\) to the lung membranes of guinea pig by CGRP and calcitonin:** Binding of \(^{125}\text{I}-\text{CGRP}\) to guinea pig lung membranes was displaced by unlabeled CGRP with an \( IC_{50} \) value of \( 3.1 \times 10^{-10} \) M (Fig. 6); salmon calcitonin displaced the binding of CGRP at a 600-fold higher concentration or greater (\( IC_{50}=2.0 \times 10^{-7} \) M), and human calcitonin displaced the binding by about 20% at \( 1.0 \times 10^{-6} \) M.

**Discussion**

The results reported here show that specific binding sites for CGRP are present in the lung membranes of the guinea pig. The specific binding of \(^{125}\text{I}-\text{CGRP}\) was time-dependent, saturable, and reversible, and the binding increased linearly with increasing membrane

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**Fig. 5.** Scatchard analysis of \(^{125}\text{I}-\text{CGRP}\) binding to the lung membranes of the guinea pig in the presence of GTP. Membranes were incubated for 60 min at 23°C in the absence (●) or presence of \( 1 \times 10^{-6} \) M (■) or \( 1 \times 10^{-4} \) M (▲) GTP and various concentrations of \(^{125}\text{I}-\text{CGRP}\) (\( 9.0 \times 10^{-12} \) M to \( 1.5 \times 10^{-9} \) M). The specific binding data were analyzed by the method of Scatchard. Each point represents the mean of duplicate determinations from a typical experiment.

**Fig. 6.** Competitive inhibition of CGRP, salmon calcitonin or human calcitonin on the binding of \(^{125}\text{I}-\text{CGRP}\) to the lung membranes of the guinea pig. Membranes (5.2 µg protein) were incubated with \( 1.0 \times 10^{-10} \) M \(^{125}\text{I}-\text{CGRP}\) in the absence or presence of \( 1.0 \times 10^{-6} \) M unlabeled CGRP and various concentrations of CGRP (●), salmon calcitonin (■) or human calcitonin (▲) for 60 min at 23°C. Each point represents the mean±S.E.M. from three experiments.
concentrations. The saturation experiment and Scatchard analysis yielded two apparent classes of CGRP binding sites: high affinity binding sites with a $K_D$ value of $7.17 \times 10^{-11} \text{ M}$ and a $B_{\text{max}}$ of 364 fmol/mg protein and low affinity binding sites with a $K_D$ value of $1.70 \times 10^{-8} \text{ M}$ and a $B_{\text{max}}$ of 39594 fmol/mg protein. Our observations are consistent with the findings of Carstairs (7); however, our $K_D$ values for the high and low affinity binding sites were smaller than those reported by him. This disparity probably existed either because the source of our lung membranes was different from that of Carstairs or because the experimental conditions were different. In contrast, our findings were different from those of Mak and Barnes (8) who reported a single population of binding sites with high affinity even though we both used the same source of lung membrane. For the saturation experiment, we used higher ligand concentrations (5.0x10^{-12} \text{ M}–8.0x10^{-9} \text{ M}) which may explain in part why the conflicting results were obtained. The higher concentrations of 125I-CGRP were necessary for the determination of the low affinity sites.

In addition to the characterization of the two classes of specific binding sites, we have also demonstrated the direct coupling between guinea pig lung CGRP receptors and the guanine nucleotide regulatory binding protein (G protein). This coupling was evidenced by the effect of GTP and Gpp(NH)p on the binding of CGRP. Approximately 50% of the specific binding of CGRP had dissociated with the addition of GTP and the nonhydrolyzable analog of GTP (Gpp(NH)p) in the assay mixture. Furthermore, the dissociation of bound 125I-CGRP by 10^{-6} \text{ M} unlabeled CGRP was accelerated with the addition of GTP. On the other hand, ATP and GMP, nucleotides incapable of binding to guanine nucleotide binding protein did not dissociate bound 125I-CGRP. The nucleotide specificity for the dissociation of 125I-CGRP from lung membrane receptors of the guinea pig was similar to that observed for other hormone receptor systems coupled to adenylate cyclase through Gs (13). Furthermore, saturation and Scatchard analysis of CGRP binding in the presence of GTP indicated that the decreased affinity was primarily due to loss of binding to the high affinity class of sites. These results suggested that the lower affinity sites may not be as tightly coupled to G protein as the high affinity sites, pointing to a more functional role for the latter in lung membranes.

Members of a family of G proteins are responsible for transmission of information from many membrane-bound receptors (14, 15). It has been well-documented that receptors coupled to adenylate cyclase systems are usually associated with the G protein. Specific CGRP receptors linked to the stimulation of adenylate cyclase activity in the pancreas (16), peripheral arteries (17, 18), heart (19) and lymphocytes (20) have also been found, although CGRP receptors are not linked to adenylate cyclase in the central nervous system. Therefore, G protein coupled to the high affinity binding sites for CGRP in the lung membranes of the guinea pig may be Gs, the G protein responsible for stimulation of adenylate cyclase.

In competition experiments, unlabeled CGRP displaced the binding of labeled CGRP with an IC50 value of $3.1 \times 10^{-10} \text{ M}$, while salmon and human calcitonins were less able to replace the 125I-CGRP binding, which shows that CGRP receptors have pharmacologic specificity.

The biological functions of the high affinity and low affinity binding sites for CGRP in the lung are unknown. Marzo et al. and Tippins et al. have reported that CGRP inhibits PAF-stimulated leukotriene release from rat lung and calcium ionophore A23187-stimulated leukotriene release from guinea pig lung (21, 22). Thus, CGRP may have a regulatory role in airway function, because leukotrienes are suggested to be active in the pathogenesis of hypersensitivity bronchial asthma and allergic rhinitis (23).

Our present study suggests that both low and high affinity binding sites for CGRP exist in the lung membranes of the guinea pig, and the high affinity binding sites for CGRP may be coupled to GTP binding regulatory protein.

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