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Multiple Affinity Forms of the Calcitonin Gene-Related Peptide Receptor in Rat Cerebellum

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SUMMARY

Binding of ^{125}I-calcitonin gene-related peptide (^{125}I-CGRP) to rat cerebellum membranes and the sensitivity to guanine nucleotides of binding were investigated. Cerebellum binding sites labeled by ^{125}I-CGRP appear to be highly specific, inasmuch as CGRP inhibited binding with an IC_{50} of 100 pM but other peptides were inactive or much less active in displacing ^{125}I-CGRP from these sites. ^{125}I-CGRP binding sites in cerebellum membranes were saturable and of high affinity. Scatchard analysis of the saturation binding data revealed a homogeneous population of binding sites, with a K_D of 224 ± 28 pM and B_{max} of 131 ± 15 fmol/mg of protein. In the presence of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (100 μM), a single population of binding sites, with a K_D of 464 ± 77 pM and B_{max} of 100 ± 14 fmol/mg of protein, was observed. The kinetics of association of ^{125}I-CGRP with cerebellum membranes were monophasic at all ligand concentrations tested. However, the observed association rate constant (k_{obs}) was not dependent on [^{125}I-CGRP] in a linear fashion in either the absence or the presence of GTPγS (100 μM). The kinetics of dissociation of ^{125}I-CGRP from cerebellum membranes were multiexponential, with fast and slow dissociating components having rate constants of 0.34 ± 0.01 and 0.025 ± 0.001 min^{-1}, respectively. The fast dissociating component represented 60 ± 2% of the total specific binding sites. Dissociation of ^{125}I-CGRP from cerebellum sites was much faster in the presence of GTPγS (100 μM) but still exhibited dissociation from two affinity components. The rate constants for these components of dissociation were 0.67 ± 0.03 and 0.077 ± 0.007 min^{-1}, with the faster dissociating component representing 66 ± 1% of the total specific binding sites. These findings provide the first evidence that CGRP receptors exist in multiple affinity states and that cerebellum CGRP receptors are regulated by guanine nucleotides. Our results also suggest the existence of two affinity states of the CGRP-receptor-guanine nucleotide-binding protein ternary complex.

CGRP is a 37-amino acid peptide whose existence was predicted by analysis of the nucleotide sequence of the calcitonin gene in rats (1). CGRP arises by alternative processing of the RNA transcript of this gene. Immunochemical studies have shown that CGRP is distributed widely in both the central and peripheral nervous system (2–4). Autoradiographic and membrane binding studies have demonstrated the presence of ^{125}I-CGRP binding sites throughout the rat central nervous system, with relatively high levels of binding sites present in cerebellum (3, 5, 6). Although the precise role of CGRP as a neurotransmitter or neuromodulator is far from clear, this peptide possesses neurotrophic properties (7, 8) and has been implicated in the regulation of central sympathetic outflow (9), nociception (10), cerebrovascular tone (11), and neuronal differentiation (12). CGRP exerts diverse biological effects in peripheral tissues, including stimulation of acetylcholine receptor synthesis in muscle (8), inhibition of gastric smooth muscle contraction by carbachol (13), stimulation of endothelial cell proliferation (14), stimulation of calcium current in heart (15), vasodilation (16), and positive chronotropic and inotropic actions in heart (9, 15, 17–20).

Stimulation of CGRP receptors produces increases in cellular cAMP concentration or activation of adenylate cyclase in various tissues and cells, including heart (18, 20), liver (21), smooth and skeletal muscle (13, 22, 23), kidney (24), endothelial cells (14, 25), and pancreatic acinar cells (26). Thus, in these tissues, CGRP receptors may be coupled to G_s and, hence, belong to the G protein-coupled family of receptors. In keeping with this idea, a recent study demonstrated that binding of ^{125}I-Tyr-CGRP to liver membranes was sensitive to GTP (21). Early suggestions were that CGRP stimulates adenylate cyclase activity in tissues by cross-reacting with high affinity calcitonin receptors (24), but this does not appear to be the case in many tissues (13, 18, 20, 22, 26).

Little is known about the molecular basis of CGRP action in

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ABBREVIATIONS: CGRP, calcitonin gene-related peptide; EGTA, [ethylene glycol bis(oxyethylenenitrilo)]tetraacetic acid; BSA, bovine serum albumin; G protein, guanine nucleotide-binding regulatory protein; G_s, stimulatory guanine nucleotide-binding regulatory protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

the central nervous system. However, in contrast to other tissues, CGRP binding to spinal cord membrane preparations is not associated with activation of adenylate cyclase (24). Some evidence exists that suggests that CGRP analogs can distinguish between CGRP receptors in different tissues, including brain, suggesting the possibility of distinct types of CGRP receptors (27, 28). It is unknown whether CGRP receptors in the central nervous system are linked with G protein-coupled effector systems or whether they represent an altogether distinct receptor class. The present study examined the characteristics of binding of CGRP to rat cerebellum receptors and the sensitivity of binding to guanine nucleotides.

Experimental Procedures

Materials. Human CGRP, human calcitonin, and human diabetes-associated peptide were purchased from Bachem. Rat insulin and fatty acid-free BSA (type V) were obtained from Sigma. ^{125}I-CGRP (2000 Ci/mmol) was obtained from Amersham. All other chemicals used were of analytical grade and of the highest purity available.

Preparation of membranes. Cerebella were collected from Harlan Sprague-Dawley rats (250 g) of either sex and homogenized (1:50 wet weight/volume) in 50 mM Tris·HCl, pH 7.4, containing 5 mM $MgCl_2$ and 1 mM EGTA (buffer A), with a Brinkman Polytron for 20 sec at setting 7. The homogenate was centrifuged at 48,000 × *g* for 10 min at 4°, and the resulting pellet was washed twice with buffer A by rehomogenization and centrifugation. The final crude membrane pellet was resuspended in buffer A to a final protein concentration of 0.5 mg/ml. Membranes always were prepared freshly; however, cerebella could be frozen in liquid N_2 and stored at −80° for up to 1 week before membrane preparation with no differences in ^{125}I-CGRP binding.

Binding assay procedure. Standard binding assays were performed at 37° in polypropylene tubes (12 × 75 mm) in a total volume of 100 μl. The assay mixture contained 50 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 2% BSA, 2 mM bacitracin, various concentrations of ^{125}I-CGRP, and 20–25 μg of membrane protein. Under these assay conditions, ^{125}I-CGRP binding to membranes reached equilibrium within 15 min and was stable for at least 1 hr. Binding reactions were initiated by addition of ^{125}I-CGRP and then incubated at 37° for 30 min. Reactions were terminated by addition of 5 ml of ice-cold buffer A, followed by rapid vacuum filtration over GF/B filters (Whatman) that had been presoaked with a solution containing 1% polyethyleneimine and 0.5% BSA. Filters were rinsed by four consecutive additions of 5 ml of ice-cold buffer A, dried, and then counted. Total binding in all assays represented less than 10% of the ^{125}I-CGRP present in the incubation. Nonspecific binding was determined by measurement of ^{125}I-CGRP binding in the presence of 1 μM unlabeled CGRP. Specific binding represents total binding minus nonspecific binding.

Association kinetic studies were performed by incubating cerebellum membranes with different concentrations of ^{125}I-CGRP for various time periods. Incubation volumes were increased so that samples of the reaction mixture could be removed at selected times for these kinetic experiments. Samples of the reaction mixture (0.1 or 0.5 ml) were removed at various times after addition of ^{125}I-CGRP and were filtered over GF/B filters, as described above.

Dissociation kinetic studies were performed after equilibrium binding of ^{125}I-CGRP (100 pM) to cerebellum membranes by incubation at 37° for 30 min. The dissociation reaction was initiated by addition of unlabeled CGRP to a final concentration of 1 μM. Volume changes caused by addition of unlabeled CGRP were less than 1%. Samples of the reaction mixture (0.1 ml) were removed before and at various times after addition of CGRP (1 μM) and were filtered over GF/B filters, as described above.

Protein was determined according to the method of Bradford (29), using BSA as the standard. Binding data were analyzed using the binding programs EBDA, LIGAND, KINETIC, and LOWRY (30). The run test was used to determine the goodness of fit of data to a given curve. The *F* test was applied to compare curve fitting for a one- versus two or more-receptor site model. Data shown represent means ± standard errors. Significance of differences between samples was determined by Student's *t* test for comparisons between two groups and by analysis of variance and Sheffe's *post hoc* analysis for multiple comparisons.

Results

To investigate the specificity of ^{125}I-CGRP binding to cerebellum membranes, the ability of unlabeled CGRP and other peptides to compete with ^{125}I-CGRP binding was investigated. Fig. 1 illustrates that unlabeled CGRP inhibited binding of ^{125}I-CGRP (100 pM) to membranes in a dose-dependent manner, with an IC_{50} of approximately 100 pM. Nearly complete inhibition of ^{125}I-CGRP binding to membranes was observed with 10 nM CGRP, and further increases in CGRP concentration to 1 μM did not affect the level of nonspecific binding that was observed. Calcitonin and insulin did not compete for ^{125}I-CGRP binding when present at concentrations as high as 1 μM. Diabetes-associated peptide, a 37-amino acid peptide with 49% direct sequence homology to CGRP (31), competed for ^{125}I-CGRP binding with an IC_{50} approximately 1000-fold greater than that of unlabeled CGRP. Thus, binding of ^{125}I-CGRP to cerebellum membranes is highly selective.

To determine the saturability, affinity, and number of ^{125}I-CGRP binding sites in cerebellum membranes, saturation binding studies were performed. Fig. 2 illustrates that binding of ^{125}I-CGRP to cerebellum membranes was saturable. Nonspecific binding increased linearly with increasing ^{125}I-CGRP concentration and represented approximately 30% of total binding at 100 pM ^{125}I-CGRP, the concentration of ^{125}I-CGRP used in our standard binding assay. The saturation binding data were analyzed by a nonlinear curve-fitting program (LIGAND), and the data were fitted successively to a one- or two-site binding

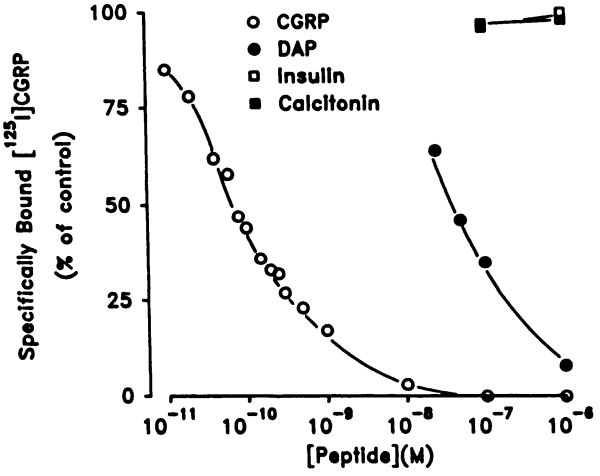

Fig. 1. Competition of specific ^{125}I-CGRP binding by CGRP and other peptides in rat cerebellum membranes. Cerebellum membranes were incubated with ^{125}I-CGRP (100 pM) and varying concentrations of peptides, and the binding assay was performed as described in Experimental Procedures. The data are expressed as percentage of control binding, which refers to specific binding observed in the absence of competing peptides. Specific binding in control incubations averaged 35 fmol of ^{125}I-CGRP bound/mg of protein and approximately 3200 dpm/assay. Nonspecific binding averaged 30% of total binding. Data represent the means of one experiment performed in duplicate and are representative of three separate experiments performed with different cerebellum membrane preparations. *DAP*, diabetes-associated peptide.

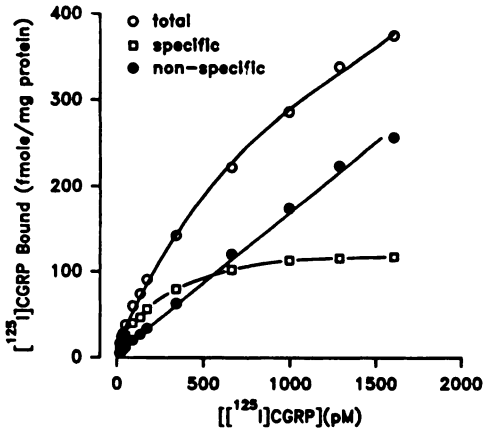

Fig. 2. Saturation binding of ^{125}I-CGRP to rat cerebellum membranes. Cerebellum membranes were incubated with increasing concentrations of ^{125}I-CGRP and total binding, nonspecific binding, and specific binding of ^{125}I-CGRP to membranes were assessed as described in Experimental Procedures. Data represent the means of one experiment performed in duplicate and are representative of three separate experiments performed with different cerebellum membrane preparations.

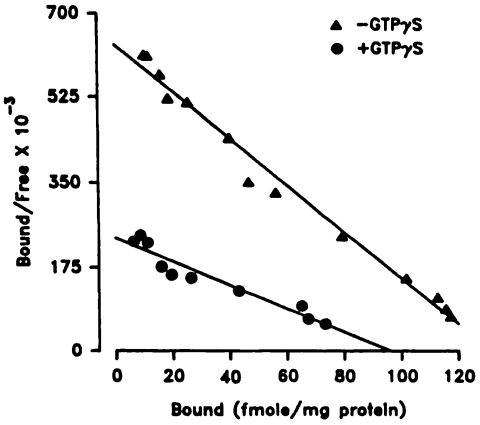

Fig. 3. Scatchard plot of specific binding of ^{125}I-CGRP to rat cerebellum membranes in the absence and presence of GTPγS. Saturation binding of ^{125}I-CGRP to cerebellum membranes was performed as described in Fig. 2, in the presence and absence of 100 μM GTPγS. The control experiment (*−GTPγS*) is the same as the experiment shown in Fig. 2. Data represent the means of one experiment performed in duplicate and are representative of three separate experiments performed with different cerebellum membrane preparations.

model. The one-site model gave the best fit of the data points, as revealed by the statistical superiority of the fit compared with the two-site model. Scatchard transformation of the saturation binding data is shown in Fig. 3. The linear Scatchard plot demonstrates the existence of a single population of ^{125}I-CGRP binding sites in cerebellum membranes, with a K_D of 224 ± 28 pM and a B_{max} of 131 ± 14 fmol/mg of protein (three experiments).

Observations that CGRP increases cAMP concentration in various tissues and that CGRP binding to liver membranes is affected by GTP (21) indicate that CGRP receptors may be coupled to G proteins in some tissues. To investigate the possibility that ^{125}I-CGRP binding sites in cerebellum membranes are associated with G proteins, saturation binding of ^{125}I-CGRP to cerebellum membranes was performed in the presence of 100 μM GTPγS. The Scatchard transformation of these saturation binding data is included in Fig. 3. In the presence of GTPγS, ^{125}I-CGRP bound to a single population of sites, with a K_D of 464 ± 77 pM and a B_{max} of 100 ± 14 fmol/mg of protein (three experiments). Thus, the affinity but not the number of cerebellum binding sites for ^{125}I-CGRP was decreased significantly ($p < 0.05$) by GTPγS. The decrease in the number of cerebellum binding sites for ^{125}I-CGRP observed in the presence of GTPγS was not statistically significant. The effects of various nucleotides on the binding of 100 pM ^{125}I-CGRP to cerebellum membranes are summarized in Fig. 4. As shown, GTPγS was most effective in inhibiting ^{125}I-CGRP binding to cerebellum membranes. Inhibition of ^{125}I-CGRP binding was observed with GTPγS at concentrations ranging from 0.5 to 100 μM, with maximum inhibition of 55% being present at 10 μM GTPγS. With the other nucleotides tested, no inhibition of ^{125}I-CGRP binding was observed at nucleotide concentrations of up to 10 μM. Binding was inhibited approximately 40% by 100 μM GDP but only slightly reduced by 100 μM ATP or GMP.

To determine the rate constant for ^{125}I-CGRP association with cerebellum membranes, the kinetics of association of ^{125}I-CGRP with membranes were examined at different ligand concentrations. The plot of the pseudo-first-order association is shown in Fig. 5. Association was monophasic at all ligand concentrations tested, suggesting association of ^{125}I-CGRP with a single population of sites in cerebellum membranes. The observed rates of association (k_{obs}) at different ligand concentrations were calculated and plotted versus ligand concentration, to determine the association rate constant. However, k_{obs} was not related to ligand concentration in a linear fashion (Fig. 6). Instead, a hyperbolic dependence of k_{obs} on ligand concentration was observed, with a plateau at ^{125}I-CGRP concentrations of approximately 200 pM. The kinetics of association were examined also when ^{125}I-CGRP binding was performed in the presence of GTPγS (100 μM). As observed in the absence of GTPγS, association was monophasic at all ligand concentrations tested (data not shown) but the calculated k_{obs} values

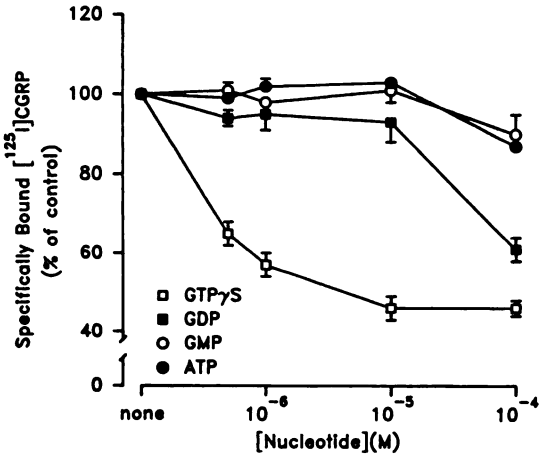

Fig. 4. Effects of nucleotides on ^{125}I-CGRP binding to cerebellum membranes. Cerebellum membranes were incubated with ^{125}I-CGRP (100 pM) and varying concentrations of nucleotides, and the binding assay was performed as described in Experimental Procedures. The data are expressed as percentage of control binding, which refers to specific binding observed in the absence of nucleotides. Specific binding in control incubations averaged 35 fmol of ^{125}I-CGRP bound/mg of protein and approximately 3200 dpm/assay. Nonspecific binding averaged 30% of total binding. Data represent means ± standard errors of three or four separate experiments performed with different cerebellum membrane preparations.

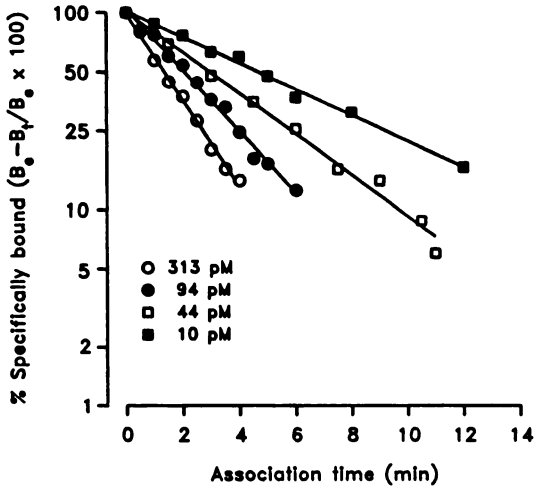

Fig. 5. Association kinetics of specific ^{125}I-CGRP binding to rat cerebellum membranes at various ^{125}I-CGRP concentrations. Cerebellum membranes were incubated for various lengths of time with different concentrations of ^{125}I-CGRP, and binding of ^{125}I-CGRP was assessed as described in Experimental Procedures. The data are presented as a pseudo-first-order kinetic plot, and plots for only four ligand concentrations are shown for purposes of clarity. The slope of each line, as determined by an iterative curve-fitting program (30), is equal to k_{obs}, the observed rate constant for the pseudo-first-order reaction. Nonspecific binding was essentially maximal at the first time point (15 sec). Data represent the means of one experiment performed in duplicate and are representative of three separate experiments performed with different cerebellum membrane preparations.

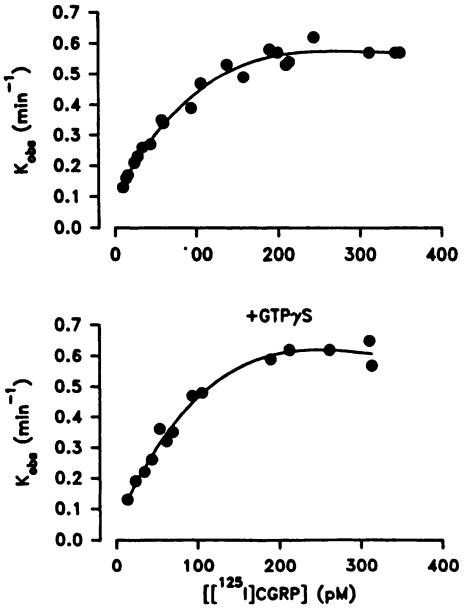

Fig. 6. Relationship between k_{obs} and [^{125}I-CGRP] for association kinetic studies performed in the absence and presence of GTPγS. Association of ^{125}I-CGRP with rat cerebellum membranes was performed at various ^{125}I-CGRP concentrations, in the absence and presence of GTPγS (100 μM), and the k_{obs} of association was determined as described in Fig. 5. The plot of k_{obs} of association of ^{125}I-CGRP with cerebellum membranes versus ^{125}I-CGRP concentration is shown for experiments performed in the absence (*upper*) and presence (*lower*) of GTPγS (100 μM). The values of k_{obs} are derived from several experiments and represent means of duplicate assays. The curves are representative of results obtained in three separate experiments performed with different cerebellum membrane preparations.

were not related linearly to ligand concentration (Fig. 6). These results indicate that ^{125}I-CGRP binding to cerebellum receptor sites may proceed by a multistep reaction process rather than via a simple bimolecular reaction (32, 33).

Fig. 7 illustrates the results of experiments in which dissociation of ^{125}I-CGRP from cerebellum membrane sites was investigated in the absence and presence of GTPγS (100 μM). As shown, curvilinear dissociation plots were observed under both experimental conditions, indicating dissociation of ^{125}I-CGRP from receptors of more than one affinity. Similar results were obtained when dissociation was initiated by infinite dilution rather than by addition of excess unlabeled CGRP (data not shown). In the absence of GTPγS, the dissociation data fit best to a model of dissociation from two affinity sites, in which the fast and slow dissociating components have dissociation rate constants of 0.34 ± 0.01 and 0.025 ± 0.001 min^{-1} (three experiments), respectively. The fast dissociating component represented 60 ± 2% of the sites occupied by ^{125}I-CGRP under these experimental conditions. Dissociation of ^{125}I-CGRP from cerebellum membranes occurred much more rapidly in the presence of GTPγS (100 μM), and the dissociation data fit best to a model of dissociation from two affinity sites with dissociation rate constants of 0.67 ± 0.03 and 0.076 ± 0.007 min^{-1} (three experiments). The fast-dissociating component represented 66 ± 1% of the sites occupied by ^{125}I-CGRP when dissociation was performed in the presence of GTPγS. The dissociation rate constants for the high and low affinity ^{125}I-CGRP binding sites observed in the presence of GTPγS were significantly greater than those observed in the absence of GTPγS ($p < 0.005$). Moreover, ^{125}I-CGRP binding sites with similar dissociation rate constants were not observed under these two experimental conditions.

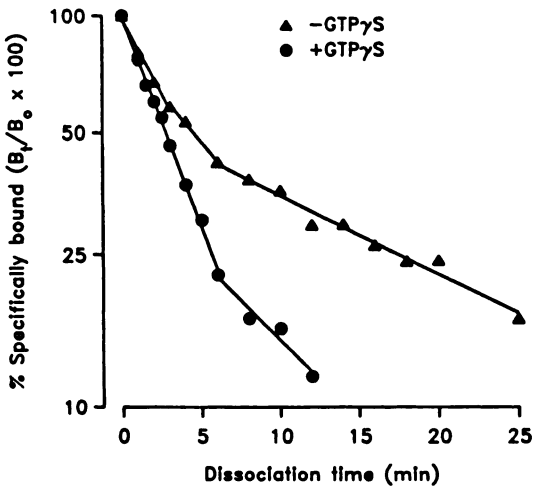

Fig. 7. Effects of GTPγS on dissociation kinetics of specific ^{125}I-CGRP binding to rat cerebellum membranes. Cerebellum membranes were incubated with 100 pM ^{125}I-CGRP for 30 min, in the absence and presence of 100 μM GTPγS. Dissociation was initiated by addition of unlabeled CGRP (1 μM), and incubations were terminated at various times as described in Experimental Procedures. Total specific binding before dissociation averaged 32 and 15 fmol/mg of protein and approximately 3000 and 1400 dpm/assay for binding performed in the absence and presence of 100 μM GTPγS, respectively. Data represent the means of one experiment performed in duplicate and are representative of three separate experiments performed with different cerebellum membrane preparations.

Discussion

The present study investigated binding of ^{125}I-CGRP to cerebellum membranes and the sensitivity to guanine nucleotides of binding. The data obtained demonstrate the existence in cerebellum membranes of saturable high affinity binding sites for ^{125}I-CGRP that are highly selective, i.e., other peptides such as calcitonin and insulin were ineffective in competing with ^{125}I-CGRP for these sites and a closely related peptide, diabetes-associated peptide, was much less effective than CGRP in competing for binding. The binding sites labeled by ^{125}I-CGRP appear homogeneous, as revealed by the linear Scatchard plot of the saturation binding data. Scatchard analysis of ^{125}I-CGRP binding to tissue sections containing rat cerebellum, evaluated by autoradiography, similarly demonstrated a homogeneous receptor population, with a K_D and B_{max} of 0.96 ± 0.36 nM and 76 ± 13 fmol/mg of protein, respectively (5). These findings are in relative agreement with our studies, in which binding was assessed directly in cerebellum membranes. However, it must be acknowledged that binding studies performed in intact tissues may not reveal high affinity ternary complex forms of receptors that are coupled to G proteins. Indeed, we observed that GTPγS decreased significantly the affinity of ^{125}I-CGRP receptor sites in cerebellum membranes. Similar sensitivity to guanine nucleotides of ^{125}I-CGRP binding in rat liver membranes was reported recently (21). As observed for other G protein-coupled receptors (34), GDP also was capable of influencing the binding of ^{125}I-CGRP to cerebellum receptors.

In agreement with the saturation binding data demonstrating a homogeneous population of receptor sites for ^{125}I-CGRP, association of ^{125}I-CGRP to cerebellum receptors displayed monoexponential kinetics at all ligand concentrations tested. The observed rate constant of association (k_{obs}) was calculated from the slopes of these association plots. In a simple pseudo-unimolecular reaction, ligand (L) interaction with its receptor (R) proceeds as:

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} LR$$

In such a reaction, k_{obs} and the association rate constant (k_1) are related by the equation $k_{obs} = k_1[L] - k_{-1}$. Thus, a plot of k_{obs} versus ligand concentration is linear, and the rate constant of association is derived from the slope of the line. However, such plots were not linear for the interaction of ^{125}I-CGRP with cerebellum receptors. Possible explanations for this behavior include ligand-induced receptor isomerization and receptor formation of a ternary complex involving a third component (35–38). In view of the sensitivity of ^{125}I-CGRP binding to GTPγS, ternary complex formation of receptors with G proteins could account for the hyperbolic dependence of k_{obs} on ligand concentration. In such a situation, it could be argued that ^{125}I-CGRP binding to receptors is followed by slow association of the ligand-receptor complex with a G protein, resulting in a high affinity ternary complex. The slow rate-limiting step of complexing with the G protein would produce a nonlinear dependence of k_{obs} on ligand concentration. However, a similar relationship between k_{obs} and ligand concentration was observed when association reactions were performed in the presence of GTPγS. Because GTPγS prevents formation of such ternary complexes, it is not likely that the observed association data result directly from ternary complex formation of receptors with G proteins.

Alternatively, it is possible that binding of ^{125}I-CGRP to receptors initiates a slow receptor isomerization, which is followed subsequently by ternary complex formation with G proteins:

$$L + R \rightleftharpoons LR \rightleftharpoons LR^* \rightleftharpoons LR^*G$$

This scheme assumes that the receptor undergoes a change before formation of the high affinity ternary complex. Accordingly, the ligand-receptor complex may exist in equilibrium in three states, i.e., LR, LR^*, and LR^*G. If these states represent different affinity forms of the CGRP receptor, any accumulation of the intermediate state (LR^*) during equilibrium binding would be apparent by examination of dissociation of ^{125}I-CGRP in membranes in which binding and dissociation were performed in the presence and absence of GTPγS.

Examination of the dissociation of ^{125}I-CGRP from cerebellum receptors indeed revealed a multiexponential dissociation, which could be described by fast and slow dissociating components. Such dissociation supports the concept of multiple affinity forms of CGRP receptors. Although a curvilinear dissociation plot could result from CGRP receptor heterogeneity, the linear Scatchard and association kinetic data argue against such heterogeneity. In experiments performed in the absence of GTPγS, the two components of binding could be explained by suggesting that ligand-occupied receptors exist in equilibrium between the G protein-coupled (LR^*G) and -uncoupled (LR^*) receptor states, with ligand dissociating slowly from the high affinity ternary complex form of the receptor. In fact, dissociation was enhanced markedly in the presence of GTPγS. This sensitivity to GTPγS of the dissociation rate supports the suggestion that receptor sites labeled with ^{125}I-CGRP interact with G proteins, resulting in high affinity states of the receptor. The finding that two affinity components of dissociation were observed also in membranes incubated with GTPγS suggests that CGRP receptors exist in multiple affinity states even before complexing with G proteins. In this case, the two components of binding could be explained by suggesting that the ligand-occupied receptors exist in equilibrium between the LR and LR^* receptor states. Thus, the proposed scheme of ligand-induced receptor isomerization before association with G proteins is consistent with the results of association and dissociation kinetic experiments performed in the presence of GTPγS.

Recently, a three receptor-state model was proposed to describe the interaction of bradykinin with B2 receptors in myometrial membranes (39). Interaction of bradykinin with B2 receptors induced the formation of two intermediate affinity states of the receptor before receptor coupling to G proteins. The present findings with the CGRP receptor system suggest the occurrence of similar ligand-dependent changes in the

$$\begin{array}{ccccc} L+R & \rightleftharpoons & LR & \rightleftharpoons & LRG \\ \uparrow & & \updownarrow & & \updownarrow \\ L+R^* & \rightleftharpoons & LR^* & \rightleftharpoons & LR^*G \end{array}$$

Fig. 8. Proposed scheme for ^{125}I-CGRP binding to rat cerebellum membrane receptors. L, ligand = ^{125}I-CGRP; R, receptor; G, G protein(s). In this model, ligand-induced alterations in receptor affinity are designated as R^*. Both LR and LR^* may form ternary complexes with G proteins.

receptor affinity state before coupling to G proteins. However, in contrast to the B2 receptor studies, we observed evidence for more than one kinetic form of the CGRP receptor in the presence and absence of GTPγS. With the scheme proposed above, in which CGRP receptors undergo ligand-dependent isomerization before G protein coupling, it would be expected that one of the dissociation rate constants would be similar in the presence and absence of GTPγS, i.e., accumulation of *LR** would occur in the presence and absence of GTPγS. However, we did not observe this experimentally.

To explain this finding, it may be argued that CGRP receptors exist precoupled with G proteins and that CGRP induces a change in the receptor so that the ligand-receptor-G protein exists in equilibrium between these two states:

$$L + RG \rightleftharpoons LRG \rightleftharpoons LR^*G$$

However, it is not absolutely necessary to assume receptor precoupling to G proteins to explain our results. For instance, it can be suggested that CGRP binding to receptors induces rapid receptor coupling with G proteins, followed by changes in receptor affinity states as shown:

$$L + R \rightleftharpoons LR \rightleftharpoons LRG \rightleftharpoons LR^*G$$

With either of these models, the accumulation under equilibrium conditions of both *LRG* and *LR*G* will result in a plot demonstrating dissociation from two affinity states. In the presence of GTPγS, ligand binding with the receptor may still induce receptor affinity changes, as observed for B2 receptors in myometrial membranes (39). This would result in accumulation of *LR* and *LR**:

$$L + R \rightleftharpoons LR \rightleftharpoons LR^*$$

Thus, dissociation in the presence of GTPγS would proceed multiexponentially, with ligand dissociating from both *LR* and *LR** states with dissociation rate constants different from those of the *LRG* and *LR*G* states, i.e., the states that would accumulate in the absence of GTPγS. It is not clear in this proposed model whether the ligand induces the same or different conformational modification of the receptor (i.e., *R**) in the presence and absence of GTPγS. A summary of this proposed scheme for interaction of CGRP with cerebellum receptors is presented in Fig. 8.

The possible existence of multiple forms of ligand-receptor-G protein ternary complexes has been proposed recently (40). Multiple forms of the ternary complex may signify a different functional coupling of receptors with G proteins. Indeed, Hausdorff *et al.* (40) showed recently that mutational modification of the β-adrenergic receptor impairs agonist activation of adenylate cyclase without affecting high affinity agonist binding to receptors. It was suggested that agonist binding to β-adrenergic receptors induces receptor coupling to G_s and that this ternary complex undergoes conformational changes, leading to formation of an active ternary complex that stimulates adenylate cyclase. Thus, the mutated receptor is thought to be unable to undergo this change and form the active ternary complex form of the β-adrenergic receptor. This idea of two conformational forms of the ternary complex is quite in agreement with our kinetic observations with the CGRP receptor system. Our results provide, for the first time, evidence in support of this concept of multiple forms of the ternary complex with a receptor existing in its native membrane-bound state.

In summary, the major finding of the present study is the observation that an apparently homogeneous population of CGRP receptors exists in multiple affinity states and that these affinity states are sensitive to the stable guanine nucleotide GTPγS. We interpreted these findings as evidence for multiple affinity forms of the CGRP-receptor-G protein ternary complex. This evidence for the existence of multiple affinity states of the ternary complex is novel and intriguing; however, the functional importance of multiple affinity forms of the ternary complex in transmembrane signaling by G protein-coupled receptors remains to be determined.

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