Limitations in Using Peptide Drugs to Characterize Calcitonin Gene-Related Peptide Receptors

DAVID J. J. WAUGH, CHARLES S. BOCKMAN, D. DAVID SMITH, and PETER W. ABEL

Department of Pharmacology (D.J.J.W., C.S.B., D.D.S., P.W.A.) and Department of Biomedical Sciences (D.J.J.W., D.D.S.), Creighton University School of Medicine, Omaha, Nebraska

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ABSTRACT

Calcitonin gene-related peptide (CGRP) is an endogenous vasodilator peptide that produces its effects by activation of CGRP₁ and CGRP₂ receptor subtypes. These receptor subtypes are characterized in functional studies using the agonist Cys(Acm)²⁻⁷-h-α-calcitonin gene-related peptide (Cys(Acm)²⁻⁷-h-CGRP), which activates CGRP₂ receptors, and the antagonist h-CGRP(8–37) which has a high affinity for CGRP₁ receptors and a low affinity for CGRP₂ receptors. Our aim was to identify factors that may limit the use of these drugs to characterize CGRP receptor subtypes. We studied CGRP receptors using isolated ring segments of pig coronary and basilar arteries studied in vitro. The affinity of the antagonist h-CGRP(8–37) for inhibiting h-CGRP-induced relaxation of coronary arteries (log₁₀ of the antagonist equilibrium dissociation constant = −5.33) was determined from Schild plots that had steep slopes. Therefore, we used capsaicin to investigate the role of endogenous CGRP in confounding affinity measurements for h-CGRP(8–37). After capsaicin treatment, the slopes of the Schild plots were not different from one, and a higher affinity of h-CGRP(8–37) in blocking relaxation was obtained (log₁₀ of the antagonist equilibrium dissociation constant = −6.01). We also investigated the agonist activity of the putative CGRP₂ receptor selective agonist Cys(Acm)²⁻⁷-h-α-CGRP. We found that maximal relaxation of coronary arteries caused by Cys(Acm)²⁻⁷-h-CGRP was dependent upon the level of contractile tone induced by KCl. We also determined the Kₐ for Cys(Acm)²⁻⁷-h-α-CGRP and found that the Kₐ (817 nM) was not significantly different from the EC₅₀ (503 nM) for this drug in causing relaxation, indicating that Cys(Acm)²⁻⁷-h-α-CGRP is a partial agonist. Because experimental conditions affect the actions of h-CGRP(8–37) and Cys(Acm)²⁻⁷-h-α-CGRP, the conditions must be carefully controlled to reliably identify CGRP receptor subtypes.

Human α-calcitonin gene-related peptide (h-αCGRP) is a 37-amino-acid product resulting from the alternative tissue-specific processing of primary messenger ribonucleic acid derived from the α calcitonin gene (Amara et al., 1982; Rosenfeld et al., 1983). Calcitonin gene-related peptide (CGRP) is widely distributed throughout the central and peripheral nervous systems where it has been suggested to function as a neurotransmitter or neuromodulator. In the central nervous system CGRP is concentrated in neurons in the dorsal horn of the spinal cord that receive sensory input and in cell bodies in the thalamus and hypothalamus. Central nervous system effects of CGRP include decreased appetite (Tannenbaum and Goltzmann, 1985), decreased gastric acid secretion (Lenz et al., 1985), and decreased intestinal motility (Fargeas et al., 1985). In the periphery, CGRP-containing nerve fibers are found in both afferent sensory and efferent motor nerves of the autonomic nervous system. Peripheral effects of CGRP include a role in sensory neurotransmission, vasodilation, increases in the rate and force of cardiac contraction, and actions as an inflammatory mediator. CGRP produces its effects by activating CGRP receptors. Two CGRP receptor subtypes have been proposed based on their differential affinities for the competitive antagonist h-αCGRP(8–37). The C-terminal fragment of h-αCGRP, CGRP₁ receptors have a high affinity for h-αCGRP(8–37) whereas CGRP₂ receptors have a low affinity for this antagonist. Another analog of h-αCGRP, the linear agonist Cys(Acm)²⁻⁷-CGRP (Dennis et al., 1990) is also used to distinguish CGRP₁ from CGRP₂ receptors. It has been reported that Cys(Acm)²⁻⁷-CGRP has little or no stimulatory action at CGRP₁ receptors, whereas this drug is a full agonist but is less potent relative to h-αCGRP in activating responses mediated by CGRP₂ receptors (Dennis et al., 1989). A comprehensive review of the criteria for CGRP receptor classifica-

ABBREVIATIONS: CGRP, calcitonin gene-related peptide; h-αCGRP, human α-calcitonin gene-related peptide; Kₐ, antagonist equilibrium dissociation constant; pA₂, log₁₀ of the antagonist equilibrium dissociation constant.
tion using these compounds has been published (Poyner, 1993).

Unfortunately, the use of an agonist drug such as Cys(Acm)²⁷-h-CGRP to differentiate CGRP receptor subtypes has potential flaws. For instance, the response produced by an agonist drug is dependent upon the intrinsic efficacy of the drug, receptor density, and the efficiency of receptor coupling in a given tissue. Therefore, whether or not Cys(Acm)²⁷-h-CGRP acts as an agonist depends upon tissue-specific receptor effector coupling mechanisms that are independent of the drug’s affinity for CGRP receptor subtypes. In addition, the affinity of the antagonist h-CGRP(8–37), measured using the same tissue but in different laboratories, varies considerably. The variability in affinity values coupled with the low selectivity of h-CGRP(8–37) for CGRP₁ over CGRP₂ receptors, which is approximately 10-fold, complicates the use of this antagonist to differentiate CGRP receptor subtypes.

In this study we have identified potential factors that can explain the variability in the response caused by Cys(Acm)²⁷-h-CGRP and in the affinity of h-CGRP(8–37) determined in functional studies. We also show that these factors can limit the usefulness of h-CGRP(8–37) and Cys(Acm)²⁷-h-CGRP for identification of CGRP receptor subtypes. Finally, we also describe some experimental conditions that can be used to optimize the use of these drugs for CGRP receptor characterization.

Materials and Methods

Drugs and Chemicals. h-CGRP, h-CGRP(8–37), and Cys(Acm)²⁷-h-CGRP were purchased from Peninsula Laboratories (Belmont, CA) or synthesized as described below. Isoproterenol bitartrate and indomethacin were obtained from Sigma (St. Louis, MO). Capsaicin (BIOMOL Research Laboratories, Plymouth Meeting, PA) and indomethacin were dissolved in 50 and 100% (v/v) ethanol, respectively. All peptides were dissolved in distilled water and diluted in 0.9% (w/v) saline solution. Sources of chemicals used in solid-phase peptide synthesis are listed in a prior publication (Smith et al., 1993). All other chemicals were obtained from Sigma or Fisher (Pittsburgh, PA).

Peptide Synthesis. h-CGRP, h-CGRP(8–37), and Cys(Acm)²⁷-h-CGRP were synthesized by Merrifield solid-phase methodology using N-acetyl-butyloxycarbonyl amino acids and para-methoxybenzyldihydroxylamine resin. Details of peptide synthesis and purification of crude synthetic peptides by gel-permeation chromatography, cation-exchange, and reverse-phase HPLC are provided in a previous publication (Smith et al., 1993). All synthetic peptides were characterized by amino acid analysis and electrospray mass-spectrometry to confirm their chemical structure. Peptide purity was greater than 98% after analytical reverse-phase HPLC. There were no differences in the potency or affinity of commercially obtained h-CGRP and its analogs compared with those same peptides synthesized in our laboratories (data not shown).

Relaxation of Pig Coronary and Basilar Arteries. The proximal portion of the left circumflex coronary artery (large coronary artery = 3 mm outer diameter) was dissected from pig hearts at a local slaughterhouse and transported in ice-cold Krebs’ solution (composition in mM; NaCl 125, KCl 5.5, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.25, NaHCO₃ 25, dextrose 11.1, Na₂Ca-EDTA 0.029) equilibrated with 95% O₂/5% CO₂. Arteries were cut into 2-mm-long rings. The rings were mounted between two stainless steel pins passed through the lumens of the vessel and placed in water-jacketed organ baths maintained at 37°C which contained Krebs’ solution gassed with 95% O₂/5% CO₂, pH 7.4. One pin was connected to a Grass FT.03 force transducer for measurement of isometric tension with a Grass model 5 polygraph (Quincy, MA). Coronary artery rings were equilibrated at 6 g of resting tension (Bockman et al., 1993) for 30 min, contracted with 45 mM KCl, washed for 40 min, and the entire sequence was then repeated a second time. To measure relaxation, tone was induced in the rings by adding 15 mM KCl. In some experiments different amounts of contractile tone were generated by using different concentrations (8–15 mM) of KCl. When the response reached a stable degree of contractile tone, complete cumulative concentration-response relaxation curves for agonists were generated. Log EC₅₀ (concentration of drug causing 50% of maximal response) values were used to quantify the potency of agonists in causing relaxation and were calculated by nonlinear regression of all data points on the relaxation concentration-response curve. Log EC₅₀ values were compared using Student’s t test with a P < .05 accepted as a significant difference between groups. The log mean ± S.E.M. EC₅₀ values were then converted to their antilogs, which are listed in the text.

Small coronary arteries (diameter < 1 mm outer diameter), which were branches of the left anterior descending coronary artery, were dissected from the apical region of the heart. Basilar arteries were carefully dissected from the surface of the brain. These arteries were then cleaned and cut into 3-mm-long ring segments. The rings were mounted and equilibrated in Krebs’ solution as described above using 2 g of resting tension for small diameter coronary arteries (Foulkes et al., 1991) and 1 g of resting tension for basilar arteries. Relaxation experiments on these rings were conducted using the same procedures described above for large coronary arteries.

In some experiments the endothelium was removed from the rings by gentle rubbing of the vessel lumen with a smoothed wooden probe. The presence or absence of the endothelium was assessed by the ability of the endothelium-dependent vasodilator Substance P to cause relaxation of the rings.

Determination of Antagonist Affinity Values for h-CGRP (8–37). Log₁₀ of the antagonist equilibrium dissociation constant (pA₂) values for h-CGRP (8–37) were determined as described by Arulakshana and Schild (1959). Cumulative concentration-response curves for h-CGRP-induced relaxation were generated and the rings were then washed and re-equilibrated with Krebs’ solution for 60 min. Control rings were then incubated with Krebs’ solution only for an additional 90 min followed by relaxation curves for h-CGRP. No change in the potency of h-CGRP in causing relaxation was observed after the 90-min incubation period in control arteries. Other rings were incubated with the competitive antagonist, h-CGRP(8–37) for 90 min before beginning the second concentration-response curves for h-CGRP-induced relaxation. Three adjacent rings from each animal were treated with different concentrations of the antagonist. In some experiments, endogenous CGRP was depleted by incubating the rings in Krebs’ solution containing 100 μM capsaicin and 10 μM indomethacin for 3 h. Indomethacin was added to prevent capsaicin-induced contraction of coronary arteries mediated through release of prostaglandins from the adventitia (Franco-Cereceda et al., 1987). The rings were then washed extensively for 1 h to remove capsaicin and indomethacin. For each concentration of antagonist used, dose-ratios were calculated by dividing the EC₅₀ value for h-CGRP-induced relaxation in the presence of the antagonist by its EC₅₀ value in the absence of the antagonist. Schild plots were constructed and linear regression used to determine the x-intercept (pA₂ value). Differences in pA₂ values and slopes of Schild plots were determined by analysis of covariance with a P < .05 level of probability accepted as a significant difference. Slopes of the Schild plots were considered to be different from unity if the 95% confidence interval did not include the slope value of 1.0. The slopes of the Schild plots are expressed as the mean ± 95% confidence interval. The individual pA₂ values were averaged and are listed in the text as mean antagonist equilibrium dissociation constant (Kᵦ) ± S.E.M. values by conversion to their antilogs.
**Results**

Mean concentration-response curves for h-α-CGRP in causing relaxation of large pig coronary artery rings in the presence and absence of endothelium are shown in Fig. 1. In rings with endothelium, h-α-CGRP caused 100% relaxation to the baseline level of tone with an EC₅₀ of 3.58 ± 0.49 nM (n = 18). Removal of the endothelium by rubbing did not affect the potency of h-α-CGRP in causing relaxation (EC₅₀ = 3.73 ± 0.59 nM; n = 9). To confirm that rubbing removed the endothelium, we generated concentration-response curves for the endothelium-dependent vasodilator, Substance P, in endothelium-intact and endothelium-denuded rings. As shown in Fig. 1, the EC₅₀ of Substance P for causing relaxation in endothelium-intact rings was 0.22 ± 0.1 nM (n = 3). No response to Substance P was observed in the endothelium-denuded rings. To determine whether the lack of response to Substance P in endothelium-denuded rings was caused by damage to relaxation mechanisms in vascular smooth muscle, we also generated concentration-response curves for the endothelium-independent vasodilator isoproterenol (data not shown). The potency of isoproterenol in causing relaxation of coronary artery rings with endothelium (EC₅₀ = 3.38 ± 0.8 nM; n = 4) was not significantly different from that in rings without endothelium (EC₅₀ = 4.70 ± 1.2 nM; n = 4), suggesting that endothelium removal did not damage the arterial smooth muscle.

The affinity of h-α-CGRP(8–37) for inhibiting CGRP-induced relaxation of large coronary arteries was determined by generating concentration-response curves for h-α-CGRP in the presence and absence of various concentrations of h-α-CGRP(8–37). As shown in Fig. 2A, h-α-CGRP(8–37) inhibited h-α-CGRP-induced relaxation and caused rightward shifts of the concentration-response curve for h-α-CGRP. These data were used to construct Schild plots (Fig. 3A) from which the affinity (pA₂ value) for h-α-CGRP(8–37) in blocking h-α-CGRP-induced relaxation was calculated. Table 1 lists the mean pA₂ value for h-α-CGRP(8–37) in blocking individual Schild regressions which was −5.33 whereas the mean of the slopes of the Schild regressions was 2.44, with individual slopes ranging from 1.96 to 3.36.

In some experiments, addition of h-α-CGRP(8–37) to large coronary artery rings contracted with KCl caused an additional contractile response was due to h-α-CGRP(8–37) blocking the relaxant effect caused by KCl-induced release of endogenous CGRP, endogenous stores of CGRP were depleted by incubating coronary artery rings with 100 μM capsaicin and 10 μM indomethacin for 3 h. After this treatment, addition of h-α-CGRP(8–37) did not cause contraction. The potency of h-α-CGRP in causing relaxation of capsaicin-treated coronary arteries (EC₅₀ = 2.37 ± 0.56 nM; n = 4) was not significantly different from its potency in relaxing untreated coronary arteries (EC₅₀ = 3.81 ± 0.82 nM; n = 4).

The fact that the antagonist h-α-CGRP(8–37) could cause contraction and that the slopes of the Schild regressions were significantly >1 suggests that endogenous CGRP may be released by KCl and that this endogenous CGRP may confound our measurements of the affinity of h-α-CGRP(8–37) for CGRP receptors. To deplete endogenous CGRP we treated large coronary arteries with capsaicin and then generated pA₂ values for h-α-CGRP(8–37) using capsaicin-treated arteries (Fig. 2B). The mean slope of Schild regressions for h-α-CGRP(8–37) in blocking relaxation in capsaicin-treated large coronary arteries was reduced to 1.34, which was not significantly different from 1 (Fig. 3A). The slopes of individ...
nal Schild regressions ranged from 0.90 to 1.49. After treatment with capsaicin, the affinity of h-αCGRP(8–37) for blocking relaxation increased by 5-fold (Table 1). Thus, in large coronary arteries, release of endogenous CGRP appears to cause an underestimation of the true potency of h-αCGRP(8–37) in blocking h-αCGRP-induced relaxation.

We also sought to determine whether this effect of capsaicin depletion of endogenous CGRP would confound affinity measurements for h-αCGRP(8–37) in blood vessels taken from other regions of the circulation. For these studies Schild plots for h-αCGRP(8–37) in blocking h-αCGRP-induced relaxation of untreated and capsaicin-treated pig basilar arteries were constructed (Fig. 3B). In untreated arteries the mean slope and pA2 values were not significantly different by analysis of covariance from slope and pA2 values in capsaicin-treated basilar arteries (Table 1). Thus the effect of capsaicin treatment may be limited to those blood vessels that can release significant amounts of endogenous CGRP.

A previous report has shown that h-αCGRP(8–37) has a higher affinity for CGRP receptors present on small diameter (<1 mm outer diameter) pig coronary arteries when compared with large diameter (>1 mm outer diameter) pig coronary arteries (Foulkes et al., 1991). We found that release of endogenous CGRP can affect measurement of the affinity of h-αCGRP(8–37) in large pig coronary arteries but not in pig basilar arteries. Thus the difference in affinity for h-αCGRP(8–37) in large versus small coronary arteries may be related to whether there is a difference in release of endogenous CGRP between these different sized arteries. Therefore, we treated small diameter coronary artery rings with capsaicin and then determined the pA2 for h-αCGRP(8–37) in blocking CGRP-induced relaxation. Comparison of the Schild regressions after capsaicin treatment between large and small coronary arteries using analysis of covariance revealed no significant difference in the mean slope or pA2 value for h-αCGRP(8–37) (Table 1). However, the mean slope and pA2 values in capsaicin-treated small coronary arteries were significantly different from those values found in large coronary arteries not treated with capsaicin (Table 1). As shown in Fig. 3A, the difference in the affinity of h-αCGRP(8–37) in large compared with small coronary arteries is eliminated by capsaicin-induced depletion of endogenous CGRP.

Cys(Acm)2,7-h-αCGRP has been reported to be a selective, full agonist at CGRP2 receptors but has little or no agonist effects at CGRP1 receptors. We found that the experimental conditions determined whether or not Cys(Acm)2,7-h-αCGRP

Fig. 2. Effects of h-αCGRP(8–37) on concentration-response curves for h-αCGRP-induced relaxation of untreated (A) and capsaicin-treated (B) large pig coronary arteries. Relaxation concentration-response curves are plotted as percent relaxation to the baseline tone present before contraction with KCl. Each concentration-response curve represents mean responses of at least four arteries, each from individual animals.

Fig. 3. Mean Schild plots of the data shown in Fig. 2 (A) and of data obtained using small pig coronary arteries (A) and pig basilar arteries (B). Capsaicin treatment increased the affinity of h-αCGRP(8–37) in blocking relaxation in large coronary arteries but not in small coronary arteries. In basilar arteries capsaicin treatment had no effect on the affinity of h-αCGRP(8–37) in blocking h-αCGRP-induced relaxation. Each Schild regression is the mean of three to five individual experiments.
was an agonist in causing relaxation of large coronary arteries. Figure 4 shows that Cys(Acm)\textsuperscript{2,7}-h-CGRP-induced relaxation of coronary arteries was dependent on the amount of KCl used to contract the rings. For example, when rings were contracted with 8 mM KCl, Cys(Acm)\textsuperscript{2,7}-h-CGRP was a full agonist compared with h-CGRP; however, with higher concentrations of KCl, the maximal relaxation caused by Cys(Acm)\textsuperscript{2,7}-h-CGRP was markedly reduced. For instance, when 15 mM KCl was used to contract coronary arteries, Cys(Acm)\textsuperscript{2,7}-h-CGRP did not cause relaxation; however, h-CGRP still caused complete relaxation of KCl-induced tone. There was little change in the potency of Cys(Acm)\textsuperscript{2,7}-h-CGRP in causing relaxation associated with the decreases in maximal response. In these experiments the EC\textsubscript{50} for Cys(Acm)\textsuperscript{2,7}-h-CGRP in causing complete relaxation in rings contracted to less than 10% of their maximum tone was 389 ± 50 nM.

The relationship between KCl-induced contractile tone and the maximal relaxation caused by Cys(Acm)\textsuperscript{2,7}-h-CGRP and h-CGRP in large coronary arteries is shown in Fig. 5. The percent of maximum relaxation is plotted versus the percent-maximum KCl-induced tone. When large coronary artery rings were contracted to their maximal degree of tone with KCl, h-CGRP caused 100% relaxation to the baseline level of tone. Thus the degree of contractile tone had no effect on CGRP-induced relaxation. In contrast, Cys(Acm)\textsuperscript{2,7}-h-CGRP-induced relaxation was only observed when rings were contracted with KCl to less than 20% of their maximum response. These results suggest that relaxant responses to Cys(Acm)\textsuperscript{2,7}-h-CGRP are sensitive to the concentration of KCl and the level of contractile tone, whereas relaxant responses to h-CGRP are not. Thus Cys(Acm)\textsuperscript{2,7}-h-CGRP could act as either a full or a partial agonist compared with h-CGRP depending upon the experimental conditions.

In contrast, Cys(Acm)\textsuperscript{2,7}-h-CGRP is a partial agonist as our data suggest, then its affinity for the CGRP receptors causing relaxation should be the same as its potency in causing relaxation (Ruffolo, 1982). As shown in Fig. 6A, we compared equiactive concentrations of h-CGRP and Cys(Acm)\textsuperscript{2,7}-h-CGRP in causing relaxation of large coronary arteries. These concentrations were then used to construct a double reciprocal plot (Fig. 6B), from which the affinity of Cys(Acm)\textsuperscript{2,7}-h-CGRP was determined. The $K_A$ value of Cys(Acm)\textsuperscript{2,7}-h-CGRP for the CGRP receptors causing relaxation in these experiments was 817 ± 590 nM (Table 1). The potency (EC\textsubscript{50}) of Cys(Acm)\textsuperscript{2,7}-h-CGRP in causing relaxation in these same rings was 503 ± 130 nM. The functionally determined affinity and potency values were not signif-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Artery + Treatment</th>
<th>pA\textsubscript{2} + S.E.M.</th>
<th>Schild Slope</th>
<th>$K_A$ or $K_B$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys(Acm)\textsuperscript{2,7}-h-CGRP</td>
<td>Large coronary</td>
<td>−5.33 ± 0.11</td>
<td>2.44 (1.70–3.18)</td>
<td>0.82</td>
</tr>
<tr>
<td>h-CGRP(1–37)</td>
<td>Large coronary</td>
<td>−6.01 ± 0.12\textsuperscript{a}</td>
<td>1.34 (1.00–1.62)</td>
<td>4.83</td>
</tr>
<tr>
<td>h-CGRP(8–37)</td>
<td>Large coronary + capsaicin</td>
<td>−5.89 ± 0.06\textsuperscript{b}</td>
<td>1.62 (1.34–1.56)</td>
<td>1.28</td>
</tr>
<tr>
<td>h-CGRP(8–37)</td>
<td>Small coronary + capsaicin</td>
<td>−5.85 ± 0.12</td>
<td>1.78 (1.30–2.25)</td>
<td>1.44</td>
</tr>
<tr>
<td>h-CGRP(8–37)</td>
<td>Basilar + capsaicin</td>
<td>−5.77 ± 0.09\textsuperscript{c}</td>
<td>1.75 (1.46–2.04)</td>
<td>1.70</td>
</tr>
</tbody>
</table>

Each value represents data generated from three to five individual arteries, each from different animals. 95% C.I. = 95% confidence intervals are shown in parentheses.

\textsuperscript{a} Not significantly different from h-CGRP by analysis of covariance ($P = .05$).

\textsuperscript{b} Not significantly different from h-CGRP by analysis of covariance ($P < .05$).

\textsuperscript{c} Not significantly different from h-CGRP by analysis of covariance ($P = .05$).
CGRP is a 37 amino acid peptide that is widely distributed in peripheral nerves including those found in carotid arteries, cerebral arteries (Hanko et al., 1985), renal arteries (Edvinsson et al., 1989), and coronary arteries (Gulbenkian et al., 1993). Many of the cardiovascular effects of CGRP result from its release from perivascular nerves causing vasodilation of blood vessels (Brain et al., 1985). CGRP containing nerves in the heart are associated with the sinoatrial node and atrial muscle and CGRP can increase both the rate and the force of contraction of the right atrium. These and other cardiovascular effects of CGRP suggest several therapeutic roles for CGRP receptor agonists including treatment of subarachnoid hemorrhage, improving hemodynamics in patients with heart failure, and counteracting ischemia in patients with coronary artery disease (Feuerstein et al., 1995). In addition, CGRP receptor antagonists may be useful in the treatment of migraine and septic shock (Feuerstein et al., 1995).

The actions of CGRP are mediated by activation of CGRP receptors that have been identified in numerous tissues. The CGRP receptor is a member of the G protein-coupled, heptahelical receptor superfamily. Other receptors included in the CGRP receptor family include those for the related peptides amylin and adrenomedullin. Two CGRP receptor subtypes have been identified in functional studies using isolated tissues. CGRP1 receptors have a high affinity for h-oCGRP(8–37) in blocking h-oCGRP-induced responses (pA2 > 7) whereas low-affinity pA2 values of less than –6 are reported for h-oCGRP(8–37) in blocking responses mediated by CGRP2 receptors. Another peptide drug used to distinguish CGRP receptor subtypes is the analog Cys(Acm)2,7-CGRP, which is thought to act as an agonist to activate CGRP2 receptors selectively with little or no stimulatory effects at CGRP1 receptors (Dennis et al., 1989). Currently, Cys(Acm)2,7-h-oCGRP and h-oCGRP(8–37) are the only two CGRP receptor-selective drugs available to discriminate CGRP receptor subtypes. Therefore, these two drugs are used routinely to characterize the CGRP receptor subtype mediating various functional effects.

Although Cys(Acm)2,7-h-oCGRP and h-oCGRP(8–37) are widely used, some studies suggest that the ability of these drugs to discriminate CGRP1 from CGRP2 receptor-mediated responses is poor. The use of Cys(Acm)2,7-h-oCGRP as a selective agonist to identify CGRP2 receptors can lead to equivocal results. For example, whether a drug acts as an agonist in a particular tissue depends on the drug’s intrinsic efficacy and the receptor reserve in the tissue. Results obtained using the antagonist h-oCGRP(8–37) can also be difficult to interpret. For instance, Maggi et al. (1991) reported only a 3-fold difference in the affinity of h-oCGRP(8–37) between CGRP1 receptors in guinea pig left atria and CGRP2 receptors in rat vas deferens. In addition, the affinities reported for h-oCGRP(8–37) in the same tissue but in different laboratories vary considerably. For example, in guinea pig heart pA2 values reported for h-oCGRP(8–37) inhibition of h-oCGRP-induced contraction vary by 6-fold (Maggi et al., 1991; Mimeault et al., 1991). Therefore, the effectiveness of h-oCGRP(8–37) in differentiating CGRP receptor subtypes may be limited by the significant variability in its reported affinities (Poyner, 1993). The goal of our study was to identify factors that confound the use of these drugs to characterize CGRP receptor subtypes and to define experimental conditions that allow for accurate receptor characterization.

Because Cys(Acm)2,7-h-oCGRP is proposed to selectively stimulate CGRP2 receptors (Dennis et al., 1989) we used it to relax large pig coronary arteries, an effect reported to be mediated by the CGRP2 receptor subtype (Foulkes et al., 1991). We found that Cys(Acm)2,7-h-oCGRP was 140-fold less potent than h-oCGRP in causing relaxation of this tissue. In contrast to h-oCGRP, Cys(Acm)2,7-h-oCGRP did not cause complete relaxation of precontracted coronary artery rings in all circumstances. For instance, the maximal response of Cys(Acm)2,7-h-oCGRP depended on the degree of contractile tone produced by KCl whereas the level of contractile tone had no effect on the full agonist h-oCGRP. With the greater degree of functional antagonism produced by increased contractile tone, Cys(Acm)2,7-h-oCGRP was found to be a partial agonist compared to h-oCGRP. These results are consistent...
with the idea that functional antagonism has a greater effect on the maximal response to a partial agonist compared to a full agonist (Kenakin, 1993b). If Cys(Acm)<sub>2,7</sub>-h-αCGRP is a partial agonist as we suggest, then its potency in causing a response should be similar to its affinity for CGRP receptors causing relaxation (Ruffolo, 1982). When \( K_A \) (affinity) values for Cys(Acm)<sub>2,7</sub>-h-αCGRP were determined in relaxation experiments, those values were not significantly different from the EC<sub>50</sub> (potency) values of Cys(Acm)<sub>2,7</sub>-h-αCGRP in causing relaxation in the same rings. These results are consistent with the hypothesis that Cys(Acm)<sub>2,7</sub>-h-αCGRP is a partial agonist in large pig coronary arteries.

The partial agonist nature of Cys(Acm)<sub>2,7</sub>-h-αCGRP may explain why this analog is an agonist in some tissues (rat vas deferens; Dennis et al., 1989) but has little or no effect in other tissues (guinea pig atria; Dennis et al., 1989). The ability to observe responses in different tissues depends on the receptor reserve in a tissue and the intrinsic efficacy of the agonist. Thus in tissues without a receptor reserve, receptors are not efficiently coupled to cause an effect and agonists with low intrinsic efficacy will produce little or no response. In contrast, in tissues that have a large receptor reserve, receptors are very efficiently coupled to response and partial agonists with low intrinsic efficacy may produce a maximal response just like a full agonist. Whether Cys(Acm)<sub>2,7</sub>-h-αCGRP is an agonist may not be related to its putative selectivity for CGRP<sub>2</sub> receptors but instead may be a function of tissue-dependent factors such as the presence of a receptor reserve. Thus the agonist activity of Cys(Acm)<sub>2,7</sub>-h-αCGRP may not be a reliable criteria to differentiate CGRP receptor subtypes.

Because the affinity of an antagonist is not related to tissue-dependent factors, comparison of antagonist affinity values is a more useful method for characterizing receptor subtypes. Therefore, we also determined the affinity of h-αCGRP(8–37) in blocking h-αCGRP-induced relaxation in large pig coronary arteries. In our experiments a pA<sub>2</sub> value of −5.33 for h-αCGRP(8–37) was calculated from Schild plots with steep slopes that were significantly greater than one. Our high slope values suggest that the pA<sub>2</sub> value of −5.33 may not be an accurate measurement of the affinity of h-αCGRP(8–37) at CGRP receptors in large pig coronary arteries. Explanations for slopes of Schild plots that are greater than one include failure of the antagonist to reach equilibrium with the receptor, competition for the receptor between the antagonist and an endogenous ligand, and removal or metabolism of the antagonist (Kenakin, 1993c).

Coronary arteries are innervated by peptidergic nerves containing CGRP (Gulbenkian et al., 1993) that can be released from these nerves by depolarizing concentrations of KCl. In our experiments KCl was used to precontract the arteries. Therefore, we hypothesized that KCl-induced release of endogenous h-αCGRP might compete with h-αCGRP(8–37) at the CGRP receptor, resulting in inaccurate estimates of the pA<sub>2</sub> for h-αCGRP(8–37). To test this hypothesis, coronary arteries were treated with capsaicin to deplete endogenous stores of CGRP. Capsaicin treatment significantly increased the affinity of h-αCGRP(8–37) in large pig coronary arteries and reduced the slope of the Schild plot so that it was not significantly different from one. These results are consistent with our hypothesis that CGRP released from the arteries competes with exogenously admin-istered h-αCGRP(8–37) for the CGRP receptor. Competition between endogenous CGRP and exogenous h-αCGRP(8–37) for the CGRP receptor would be greatest when low concentrations of the antagonist h-αCGRP(8–37) are used. This competition would result in a significant decrease in receptor occupancy primarily when low concentrations of the antagonist are used causing a smaller shift of the h-αCGRP concentration-response curve. In fact, capsaicin treatment caused the greatest effect at the lowest concentration of antagonist added. It is unlikely that this effect of capsaicin was due to a toxic effect because the potency of h-αCGRP in causing relaxation was similar in both nontreated and capsaicin-treated arteries. We also found that the increase in tone of the rings after administration of h-αCGRP(8–37) was abolished in capsaicin-treated coronary arteries. These results are consistent with h-αCGRP(8–37)-induced blockade of the relaxant effect caused by basal release of endogenous CGRP. Capsaicin treatment depleted endogenous CGRP and thus prevented the contraction caused by h-αCGRP(8–37).

A previous study has suggested that h-αCGRP(8–37) can differentiate CGRP receptor subtypes in large compared with small diameter coronary arteries (Foulkes et al., 1991). In those studies the pA<sub>2</sub> value for h-αCGRP(8–37) in small coronary arteries was −7.02, and the slope of the Schild plot was significantly less than 1. The pA<sub>2</sub> value for h-αCGRP(8–37) in large coronary arteries was −5.7 and was determined using only a single concentration of antagonist, thus slope values were not obtained. In contrast, in our experiments after capsaicin treatment we found no difference in the affinity of h-αCGRP(8–37) in large compared with small coronary arteries. Possible explanations for the differences reported by Foulkes et al. between large and small coronary arteries may be related to their experimental conditions. In their studies the slopes of the Schild plots were either not ideal or not determined; thus, their pA<sub>2</sub> values may not represent the true affinity of h-αCGRP(8–37) for the CGRP receptors in these tissues.

We also wanted to determine whether the effect of capsaicin treatment to increase the affinity of h-αCGRP(8–37) in blocking relaxation would also be seen in arteries from other regions of the circulation. Cerebral arteries, like coronary arteries, also have a relatively high density of CGRP containing nerves. Therefore, the affinity of h-αCGRP(8–37) in blocking h-αCGRP-induced relaxation was tested in untreated and capsaicin-treated pig basilar arteries. In contrast to large pig coronary arteries, capsaicin treatment had no significant effect on the measured affinity of h-αCGRP(8–37) or on the steep slopes of the Schild plots in basilar arteries. These data suggest that mechanisms other than KCl induced release of endogenous CGRP are responsible for the steep slopes of the Schild plots in basilar arteries.

Although methodological considerations can confound measurements of pA<sub>2</sub> values for h-αCGRP(8–37), the pA<sub>2</sub> values reported by us in pig blood vessels (−5.33 to −6.01) indicate a low affinity of this drug for its receptors. This is consistent with pA<sub>2</sub> values of −6 or less for h-αCGRP(8–37) inhibition of responses caused by activation of the CGRP<sub>2</sub> receptor reported in the literature (Poyner, 1993). These data suggest that the CGRP<sub>2</sub> receptor mediates CGRP-induced relaxation in these pig blood vessels.

In summary, we have used in vitro studies of pig coronary artery relaxation to determine affinities and/or potencies for
h-αCGRP, h-αCGRP(8–37), and Cys(Acm)2,7-h-αCGRP at CGRP receptors. We have shown that Cys(Acm)2,7-h-αCGRP is a partial agonist that may or may not have potent agonist activity depending on the experimental conditions. The affinity of the antagonist h-αCGRP(8–37) may also depend upon the conditions of the experiment. Thus in tissues containing capsaicin-sensitive stores of endogenous CGRP, functional measurements of the affinity of h-αCGRP(8–37) may be inaccurate unless release of endogenous CGRP is controlled. The small number of CGRP receptor-selective drugs, their low receptor subtype selectivity, and their dependence on experimental conditions limit the use of these drugs to discriminate subtypes of CGRP receptors.

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References


Send reprint requests to: Dr. Peter W. Abel, Ph.D., Department of Pharmacology, Creighton University School of Medicine, 2500 California Plaza, Omaha, NE 68178. E-mail: pabel@creighton.edu.