In Vitro Evidence That Carteolol Is a Nonconventional Partial Agonist of Guinea Pig Cardiac $\beta_1$-Adrenoceptors: A Comparison with Xamoterol

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ABSTRACT

The present study was designed to verify our previous hypothesis that carteolol, a $\beta_1$/$\beta_2$-adrenoceptor-blocking agent, is a nonconventional partial agonist of cardiac $\beta_1$-adrenoceptors. To this purpose, we characterized the effects of carteolol in guinea pig myocardial preparations and measured the affinities of carteolol for high- and low-affinity sites of $\beta_1$-adrenoceptors labeled by CGP12177 [(−)-4-(3-tert-butylamino-2-hydroxypropoxy)-2-benzimidazol-2-one]. All experiments were performed in comparison with xamoterol, a cardioselective $\beta_1$-adrenoceptor partial agonist. Both drugs caused cAMP-dependent positive inotropic and chronotropic effects, but carteolol was less effective and less potent than xamoterol, and its cardiac actions were not affected by conventional concentrations of the $\beta$-blocker propranolol. Both carteolol and xamoterol antagonized the cardiac effects of isoprenaline, but although the antagonistic concentrations of xamoterol were almost equal to those producing cardiostimulation, the antagonistic concentrations of carteolol were 3 log units lower than those causing cardiostimulant effects. Both carteolol and xamoterol competed with (−)[$^3$H]CGP12177 for a high-affinity site of $\beta_1$-adrenoceptors, but carteolol showed a higher affinity than xamoterol. Moreover, carteolol, unlike xamoterol, bound also to a low-affinity site of the receptors. The binding affinity constants of the drugs for the high-affinity site correlated well with the respective blocking potencies against isoprenaline, whereas the affinity constant of carteolol for the low-affinity site was well related to its agonist potency. In conclusion, our findings demonstrate that carteolol, unlike xamoterol, is a nonconventional partial agonist, which causes agonistic effects through interaction with the low-affinity propranolol-resistant site of $\beta_1$-adrenoceptors and antagonistic actions through the high-affinity site of the same receptors.

Carteolol is a $\beta_1$/$\beta_2$-adrenoceptor-blocking agent (Yabuochi and Kinoshita, 1974; Chiba, 1979), currently used in the management of patients with cardiovascular and noncardiovascular (e.g., glaucoma) diseases. Like other $\beta$-blockers, it may be classified as a partial agonist since it is endowed with intrinsic sympathomimetic activity (ISA) in several animal species, including humans (for review, see Odenthal, 1983; Frishman and Covey, 1990).

Within the last 20 years, much evidence has been accumulated that partial agonists of the $\beta$-adrenoceptors have two modes of action. Although conventional partial agonists produce stimulant effects at concentrations equal to those causing blockade of $\beta$-adrenoceptors, other compounds, namely the nonconventional partial agonists, produce cardiostimulation at concentrations greater than those blocking the effects of catecholamines (Kaumann and Blinks, 1980; Kaumann, 1989; Takayanagi et al., 1989), their agonistic effects being resistant to blockade by the classic $\beta$-antagonist propranolol (Sarero et al., 1999). Currently, the behavior of these agents is ascribed to the existence of two different active sites or conformations of the $\beta_1$-adrenoceptor: an $H$ site through which the effects of catecholamines are blocked and an $L$ site generally defined as the propranolol-resistant state of the $\beta_1$-adrenoceptor (Bundkirchen et al., 2002) through which the stimulant effects of these partial agonists are mediated (Konkar et al., 2000; Granneman, 2001; Baker et al., 2003; Arch, 2004; Baker, 2005). Various compounds have been recently classified as nonconventional partial agonists of the

**ABBREVIATIONS:** ISA, intrinsic sympathomimetic activity; H, high affinity; L, low affinity; (−)CGP12177, (−)4-(3-tert-butylamino-2-hydroxypropoxy)-2-benzimidazol-2-one; IBMX, 3-isobutyl-1-methylxanthine.
β₁-adrenoceptor (Sarsero et al., 1999; Bundkirchen et al., 2002; Lowe et al., 2002; Joseph et al., 2003). Among them, CGP12177 contains, like carteolol, an aryloxypropanolamine moiety (Fig. 1) (Sarsero et al., 1998, 1999; Konkar et al., 2000; Sarsero et al., 2003; Joseph et al., 2004). Therefore, we wondered whether carteolol also behaves as a nonconventional partial agonist of β₁-adrenoceptors. We recently showed that, in rat-isolated myocardial preparations, the drug antagonized the effects of isoprenaline at concentrations 2 log units lower than those causing positive inotropic and chronotropic effects (Floreani et al., 2004). Moreover, cardiostimulation by carteolol was resistant to a concentration of propranolol (1 μM) greater than that antagonizing the effects of catecholamines in the same preparations. Collectively, these findings indicate that carteolol behaves as a nonconventional partial agonist of rat β₁-adrenoceptors.

The aim of the present work was to verify our hypothesis that ISA of carteolol is mediated through the propranolol-resistant state of the β₁-adrenoceptor. To this purpose, we characterized the effects of carteolol in myocardial preparations isolated from guinea pigs and measured the affinity of carteolol for the high- and low-affinity sites of cardiac β₁-adrenoceptors labeled by (-)³H(CGP12177; xamoterol, a cardioselective β₁-adrenoceptor partial agonist (Hattori et al., 1987; Hicks et al., 1987), was used as a reference drug. In our research, we used myocardial preparations obtained from reserpine-treated animals; the use of catecholamine-depleted tissues, to avoid the underlying sympathetic tone, is crucial because the ISA of β-blockers critically depends on the activation state of the β-adrenoceptors (Maack et al., 2003) and/or on the degree of the underlying sympathetic tone (Lipworth and Groff, 1997).

Our results demonstrate, for the first time, that carteolol binds with different affinities to both the high- and low-affinity sites of β₁-adrenoceptors present in guinea pig heart membranes. Moreover, here we show that carteolol behaves like a nonconventional partial agonist, eliciting agonistic responses through its interaction with the low-affinity propranolol-resistant site of β₁-adrenoceptors while antagonizing the effects of catecholamines through its interaction with the high-affinity site. Therefore, carteolol markedly differs from xamoterol, which behaves as a conventional partial agonist.

**Fig. 1.** Chemical structures of carteolol and CGP12177 showing the common aryloxypropanolamine moiety.

### Materials and Methods

#### Animals

The procedures described below involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies (National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication 85-23, 1985; European Economic Community Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987). Female guinea pigs (300–380 g), obtained from Harlan Italy (S. Pietro al Natisone, Udine, Italy), were kept in controlled environmental conditions (temperature, 23 ± 2°C; light/dark cycle, 7:00 AM to 7:00 PM). Animals had free access to a standard laboratory diet and water. To obtain myocardial tissues depleted in endogenous catecholamines, the animals were treated daily for 2 days with reserpine (2 mg/kg i.p.) before sacrifice. At the time of sacrifice, animals were anesthetized by inhalation of methoxyflurane and then killed by cervical dislocation; the hearts were then rapidly removed.

#### Isolated Myocardial Preparations

**Evaluation of Chronotropic and Inotropic Effects.** The experiments were carried out on both spontaneously beating atria and electrically driven (2 Hz) left atria, as previously described (Floreani et al., 2003). Since myocardial preparations were isolated from reserpine-treated animals, the occurred depletion of cathecolamines was confirmed by the lack of any effect following addition of 1.5 μM tyramine before the beginning of each experiment. Atria responding to tyramine, arrhythmic atria, as well as atria with basal frequency less than 100 and more than 160 beats/min, were discarded. In each atrial preparation, a cumulative concentration-response curve for isoprenaline was obtained before the addition of the drugs to determine the maximum effect (E_{max}) induced by the catecholamine. When three consecutive increasing concentrations of isoprenaline did not significantly modify the functional response of atria, the elicited effect was considered to be the E_{max}. Noncumulative concentration-effect curves were then obtained for increasing concentrations of both carteolol (from 10 nM to 0.1 μM) and xamoterol (from 1 nM to 10 μM). Three minutes after the maximum response to each drug concentration was reached, the atria were washed before the addition of a higher concentration. Chronotropic effects, determined in spontaneously beating atria, were defined as the difference between the heart rate (beats per minute) before and after drug additions and were expressed as percentages of the E_{max} induced by isoprenaline in the same preparation. Inotropic effects, determined both in spontaneously beating atria and in electrically driven (2 Hz) left atria, were defined as the difference between the force of contraction before and after drug addition and were expressed as percentages of the E_{max} induced by isoprenaline in the same preparation. The concentration-response data from each individual curve were evaluated by means of the Prism 3.03 software (GraphPad Software Inc., San Diego, CA) by sigmoidal curve fitting using eq. 1,

\[
\text{Response} = \frac{E_{max}}{1 + 10^{(D - D_{50})/\text{pD}_2}}
\]

where E_{max} is the effect in the absence of the agonist, E_{max} represents the maximum agonist-induced effect, X is the molar concentration of the agonist, D_{50} is the Hill slope, and log EC_{50} is the log of molar concentration of the agonist that produces a half-maximal response. In this way, we obtained the individual values of the negative log of EC_{50}; i.e., the pD_{2} value ([pD_{2} = -\log EC_{50} (molar)]), as well as the Hill slope value. Intrinsic activity (a), defined as the fraction of the maximum effect of the drug compared with the maximum response elicited by isoprenaline in the same preparation, was calculated from individual concentration-effect curves. Thereafter, the individual values of each parameter were averaged and the obtained values were used to fit the curves from the averaged experimental data. Isometric contraction curves were analyzed for time to peak force.
(τ₁), relaxation time (τ₂), mean velocity of force development (S₁), and mean velocity of relaxation (S₂), according to Reiter (1972). In some sets of experiments, propranolol, butoxamine, prazosin, carbachol, or 3-isobutyl-1-methylxanthine (IBMX) was added to the bath medium 20 to 30 min before the addition of carteolol or xamoterol.

Evaluation of pA₂ and Constants of Dissociation (Kᵦ) for Carteolol and Xamoterol. Some experiments evaluated the antagonistic effects of carteolol and xamoterol toward the positive chronotropic and inotropic effects of isoprenaline in spontaneously beating atria and in electrically driven left atria, respectively. To this end, a cumulative concentration-response curve for isoprenaline was first obtained in the presence of increasing concentrations of isoprenaline (from 1 nM to 3 μM). The cardiac tissue was then washed, and the bathing fluid was replaced two to three times until the developed tension was stable. Appropriate concentrations of carteolol or xamoterol were then added to the bathing medium, and the cardiac preparation was allowed to equilibrate for 30 min before the onset of the cumulative concentration-response curve for isoprenaline. To quantify the potency of carteolol and xamoterol as antagonists toward isoprenaline effects, experiments were carried out in the presence of three increasing concentrations of carteolol (from 5 to 50 nM) or xamoterol (0.1, 0.5, and 1 μM). Individual pA₂ values were determined from each experiment by linear regression analysis of log (DR-1) against log [B] plots (Arunlakshana and Schild, 1959), where [B] represents the molar concentration of the antagonist and DR, the dose ratio, is the ratio of the concentrations of isoprenaline required to produce an identical response in the presence and absence of the antagonist. Since the maximum response to isoprenaline was identical both in the absence and presence of the antagonist, DR was calculated as the ratio of EC₅₀ values in the presence and absence of the antagonist. In these experiments, the slope of the Schild plots appeared near to unit for both drugs (1.01 ± 0.03 and 1.18 ± 0.14, n = 3, for carteolol and xamoterol, respectively), indicating that pA₂ was independent from the concentrations of carteolol or xamoterol (MacKay, 1978). Therefore, it was possible to routinely estimate each pA₂ value using the relationship: pA₂ = log(DR-1) - log [B] (MacKay, 1978). Moreover, the dissociation constants Kᵦ for carteolol and xamoterol were obtained using a single antagonist concentration, from the equation: Kᵦ = [B]/(DR-1) (Besse and Furchgott, 1976).

Biochemical Assays

Preparation of Membranes from Guinea Pig Ventricular Tissue. Guinea pig cardiac ventricular tissue was homogenized in 10 volumes (w/v) of ice-cold 50 mM Tris-HCl, pH 7.4, and 10 mM MgCl₂. The homogenate was centrifuged at 48,000g for 10 min. The pellet was suspended in the same buffer, centrifuged again at 48,000g for 10 min, and used for binding assays and adenylate cyclase activity measurement. Protein content was determined according to a Bioanalytical Sciences, Boston, MA. The radioactivity retained on filters was determined in a Beckman Liquid Scintillation Spectrometer. IC₅₀ values for carteolol and xamoterol (where IC₅₀ is the concentration of the drug that displaces 50% of the labeled ligand) were obtained for the binding of (−)³H][CGP12177 to both its high- and low-affinity sites and were calculated for a system of one or two binding site populations by nonlinear curve fitting using the program Ligand (Munson and Rodbard, 1980). pKᵦ values (= −log Kᵦ (molar)), where Kᵦ is the binding inhibition constant, were calculated from the Cheng-Prusoff equation (1973) with Kᵦ = IC₅₀/(1 + Cᵦ₁/Cᵦ₀), where Cᵦ₁ is the radioligand concentration used (1 and 30 nM for the high- and low-affinity sites, respectively) and Kᵦ₀ is the obtained dissociation constant of radioligand from the high- and low-affinity sites (see Results).

Assay of Adenylate Cyclase Activity. Membrane preparations (100 μg/100 μl) obtained from guinea pig heart were preincubated for 10 min in a shaking bath at 30°C in a medium (final volume 0.5 ml) containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM EDTA, pH 7.4, 5 μM GTP, and 0.5 mM IBMX, as a nonspecific phosphodiesterase inhibitor. At the end of the preincubation period, 0.5 mM ATP and increasing concentrations (1 nM to 10 μM) of carteolol or xamoterol were added. The reaction was stopped after 10 min by transferring the tubes to a boiling water bath for 2 min. The samples were then cooled to room temperature and centrifuged at 2000g for 10 min at 4°C, and the supernatants were analyzed for cAMP content by a competition protein binding assay as previously reported (Varani et al., 1998). In brief, [³H]cAMP was incubated with binding protein, previously prepared from beef adrenals, added to the samples, and incubated at 4°C for 150 min. At the end of the incubation time and after the addition of charcoal, the samples were centrifuged at 2000g for 10 min. The clear supernatant was mixed with Atomilight and counted in a LS-1800 Beckman scintillation counter. In our experimental conditions, the basal value of adenylate cyclase activity was 140 ± 15 pmol cAMP/10 min/mg protein. EC₅₀ values were calculated by nonlinear least-squares curve fitting using the Prism 3.03 software.

Drugs. (−)-Carteolol hydrochloride was provided by Società Industria Farmaceutica Italiana S.p.A. (Catania, Italy). Xamoterol hemifumarate, (−)-propranolol hydrochloride, butoxamine hydrochloride, (−)[CGP12177 hydrochloride, reserpine, tyramine hydrochloride, (−)-isoprenaline hydrochloride, carbamoylcholine chloride (carbachol), prazosin hydrochloride, IBMX, and ATP were from Sigma-Aldrich (St. Louis, MO). (−)[³H]CGP12177 was purchased from PerkinElmer Life and Analytical Sciences, and [³H]cAMP was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). All other reagents were of analytical grade and obtained from commercial sources. Reserpine was dissolved in distilled water containing 10% ascorbic acid. Ascorbic acid (0.1 mM) was also added to isoprenaline solution to prevent auto-oxidation of the catecholamine. Carteolol and xamoterol were dissolved in saline.
Analysis of the Data. All data are expressed as arithmetic means ± S.E.M. Statistical analyses were performed using Prism 3.03 software. The evaluated variables were tested for normality with the Kolmogorov-Smirnov test, and all were found to be parametric. Therefore, statistical comparisons of data were performed by one-way analysis of variance followed by Newman-Keuls post hoc test. Bonferroni test was used for multiple comparisons of data sets. Critical values of P < 0.05 were judged statistically significant.

Results

Positive Chronotropic and Inotropic Effects of Carteolol: Comparison with Xamoterol. The effect of carteolol on heart frequency was determined in spontaneously beating atria and was compared with that caused by xamoterol. The positive chronotropic effects of the drugs were expressed as percentages of the maximum effect (E_max) induced by isoprenaline (3 μM) in the same myocardial preparations. Results are shown in Fig. 2 (top panel). Both carteolol and xamoterol elicited a concentration-dependent positive chronotropic effect. Their effects were very rapid in onset and reached their peak within 3 to 4 min after drug additions to the bathing medium. The effect of carteolol peaked at 10 μM (27.24 ± 4.92% of E_max) and decreased at higher concentrations. The effect of xamoterol peaked at 31 μM (6.30 ± 0.11 and 7.18 ± 0.13, respectively) were very similar to those reported for their respective chronotropic effects (Table 1).

Because of the strict relationship existing between heart frequency and developed cardiac tension, the effects of carteolol and xamoterol on the cardiac force of contraction were also studied in preparations of the left atria electrically driven at 2 Hz. We chose this frequency of stimulation, because it was rather similar to the spontaneous rate of our guinea pig heart preparations (134.4 ± 3.7 beats/min, n = 31). In these experimental conditions, the extent of the positive inotropic effect of xamoterol was rather similar to that observed in spontaneously beating atria (Fig. 3). Moreover, its pD2 value (7.41 ± 0.14) was comparable with that calculated from data obtained from spontaneously beating atria. In contrast, the positive inotropic response to carteolol in electrically driven left atria (Fig. 3) was smaller (13.88 ± 2.47% of E_max induced by isoprenaline) than that observed in spontaneously beating atria. Furthermore, pD2 value for carteolol in the left atria (5.91 ± 0.03) was slightly lower than that calculated in spontaneously beating atria (for a comparison, see Table 1).

The Chronotropic Effect of Carteolol Is Resistant to Inhibition by Propranolol. To confirm the involvement of β-adrenoceptor activation in the cardiac actions of carteolol, the chronotropic effect of the drug was studied in spontaneously beating atria in the presence of the pure β-antagonist propranolol. For comparison, we also tested the effects of propranolol on the actions of xamoterol and isoprenaline. The positive chronotropic effect caused by carteolol was not influenced at all by the presence of 0.1 μM propranolol (data not shown) and was slightly decreased by 1 μM propranolol (Fig. 4). The response of atria to carteolol was affected only by a very high concentration (10 μM) of propranolol (data not shown). In contrast, 1 μM propranolol exerted significant antagonistic actions toward xamoterol and, as expected, toward isoprenaline (Fig. 4).

Furthermore, to assess the possible contribution of β2-adrenoceptors to the cardiac actions of carteolol, further experiments were carried out in the presence of the selective β2-blocker butoxamine. Neither the chronotropic nor the inotropic effects of carteolol were influenced by butoxamine concentrations up to 10 μM (data not shown).

Similarly, to exclude any contribution of α1-adrenoceptors to the cardiac actions of carteolol, additional experiments evaluated the effects of the drug in the presence of the α1-antagonist prazosin. The concentration-effect curves of carteolol were not affected at all by the presence of prazosin (10 nM) in the bathing medium (data not shown).
obtained as explained in detail under Materials and Methods. The results are presented as means ± S.E.M. and were obtained from seven experiments carried out on different myocardial preparations.

**Functional Evidence of the Involvement of cAMP in the Cardiac Effects of Carteolol.** To ascertain the involvement of cAMP in the cardiac actions of carteolol, three different evaluations were performed. First, the isometric contraction curves obtained in spontaneously beating atria in the presence of increasing concentrations of carteolol and xamoterol were analyzed for time to peak force ($t_1$), relaxation time ($t_2$), mean velocity of force development ($S_1$), and mean velocity of relaxation ($S_2$). There is general agreement that modifications of such parameters are indicative of variations in cardiac cAMP concentrations (Reiter, 1972). As shown in Table 2, although $t_1$ and $t_2$ values were not significantly modified by carteolol, a clear trend of decrease of these parameters was observed at 1 and 10 μM carteolol. In contrast, $S_1$ values showed a statistically significant increase, indicating an increased velocity of force development. In the presence of xamoterol, a significant shortening of $t_1$ value occurred and increases in $S_1$ and $S_2$ values were observed, indicating increased mean velocities of both force development and relaxation. For comparison, Table 2 also shows the values of the same parameters obtained in the presence of isoprenaline.

Second, we determined the effect of carteolol on the force of contraction of electrically driven left atria in the presence of the phosphodiesterase inhibitor IBMX. The slight positive inotropic effect of carteolol was concentration-dependently increased by IBMX (Fig. 5, top panel), indicating involvement of a cAMP-dependent pathway in the positive inotropic action of the drug. As expected, IBMX addition increased also the positive inotropic effect of xamoterol (Fig. 5, top panel).

Third, experiments were carried out to evaluate the effects of carteolol and xamoterol in the presence of carbachol, which is known to selectively decrease the positive inotropic effects induced by a rise in cAMP levels, as a consequence of either stimulation of adenylate cyclase or inhibition of cAMP-dependent phosphodiesterase (Endoh, 1979; Floreani et al., 2003). Carbachol was used at a concentration (50 nM) that slightly modified basal contractility (10–20% decrease of developed force of contraction with respect to the basal value) but prevented isoprenaline cardiostimulant effects (data not shown). Carbachol significantly decreased the inotropic effects of both carteolol (10 μM) and xamoterol (1 μM) (Fig. 5, bottom panel).

**Carteolol and Xamoterol Differ in Their Antagonistic Action toward Isoprenaline Effects.** In guinea pig myocardial preparations, carteolol, at concentrations (from 5 to 50 nM) not affecting per se the heart rate and the force of contraction, significantly antagonized the chronotropic and inotropic effects of increasing concentrations of the full β-agonist isoprenaline. Results of representative experiments are shown in Fig. 6. Carteolol parallel rightwardly shifted the concentration-response curves of isoprenaline without modifying the maximum responses to isoprenaline. Higher concentrations of carteolol (0.1 μM) completely prevented the effects of conventional concentrations of isoprenaline (data not shown). From this set of data, we calculated both the blocking potency ($pA_2$) of carteolol toward isoprenaline-induced chronotropic and inotropic effects (9.04 ± 0.05 and 9.23 ± 0.18, respectively) (Table 3) and its dissociation constants ($K_b$ values of 0.94 ± 0.04 and 0.68 ± 0.03 nM, n = 6, in spontaneously beating and in left atria, respectively).

An analysis of the effects of xamoterol against the chronotropic and inotropic effects of isoprenaline showed that the antagonistic activity of xamoterol became evident at a concentration (0.1 μM) affecting per se the rate and the force of cardiac contraction. Both the frequency of spontaneously beating atria and the force developed by electrically driven left atrium were increased by xamoterol. This compound shifted to the right the concentration-response curves of isoprenaline, showing a behavior typical of a partial agonist against a full agonist endowed with higher affinity. Results of representative experiments are shown in Fig. 6. The $pA_2$ values for xamoterol against isoprenaline-induced positive chronotropic and inotropic effects were 7.92 ± 0.28 and 7.99 ± 0.39, respectively (Table 3). The $K_b$ values were

**Table 1**

Characteristics of the positive chronotropic and inotropic effects caused by carteolol and xamoterol in spontaneously beating atria. $pD_2$ is the negative log of the molar concentration of agonist that produced 50% of the maximum effect [i.e., $-\log EC_{50}$ (M)]. Intrinsic activity ($\alpha$) was calculated as the fraction of the maximum effect of the drugs compared with the maximal response ($E_{\text{max}}$) caused by isoprenaline in the same preparation. The values were obtained as explained in detail under Materials and Methods. The results are presented as means ± S.E.M. and were obtained from seven experiments carried out on different myocardial preparations.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Potency ($pD_2$)</th>
<th>Intrinsic Activity ($\alpha$)</th>
<th>Potency ($pD_2$)</th>
<th>Intrinsic Activity ($\alpha$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carteolol</td>
<td>6.14 ± 0.12</td>
<td>0.30 ± 0.03</td>
<td>6.30 ± 0.11</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>Xamoterol</td>
<td>7.41 ± 0.20</td>
<td>0.64 ± 0.04</td>
<td>7.18 ± 0.14</td>
<td>0.74 ± 0.10</td>
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*P < 0.001 vs. carteolol.
11.38 ± 0.77 and 10.41 ± 0.48 nM (n = 6) in spontaneously beating and in left atria, respectively.

**Carteolol Binds to Both High- and Low-Affinity Sites in Cardiac Membranes.** To determine the affinities of carteolol and xamoterol for β₁-adrenoceptors of guinea pig cardiac membranes, we measured their ability to specifically displace (−)\[^{3}H\]CGP12177 from its binding sites. Because (−)CGP12177 is a nonconventional partial agonist that binds to both the high- and low-affinity sites of β₁-adrenoceptors in rat (Sarsero et al., 1998), ferret (Lowe et al., 2002), and human (Sarsero et al., 2003) cardiac tissues, as well as in recombinant human β₁-adrenoceptors (Joseph et al., 2004), we first assayed the binding of increasing concentrations (from 0.1 to 100 nM) of (−)\[^{3}H\]CGP12177 in guinea pig cardiac membranes. The data collected from saturation binding experiments are shown in Fig. 7; computer analysis of these data showed a significantly better fit to a two-site than to a one-site binding model. By means of this analysis, we resolved the curve into two sites: H site (pKᵦH = 9.88 ± 0.01; Bₘₐₓ,H = 28 ± 3 fmol/mg protein; n = 3) and L site (pKᵦL = 7.52 ± 0.7; Bₘₐₓ,L = 36 ± 4 fmol/mg protein; n = 3).

These findings let us perform competition binding studies for both carteolol and xamoterol using 1 and 30 nM \[^{3}H\]CGP12177 to evaluate the binding inhibition constants (i.e., the affinities) of the two drugs for the H and L sites, respectively (Table 3). When increasing concentrations of carteolol were tested for their ability to displace 1 nM \[^{3}H\]CGP12177 (Fig. 8A), a pKᵦH value of 9.37 ± 0.92 was obtained. Under the same experimental conditions, the pKᵦL value of xamoterol was markedly lower (7.34 ± 0.80). Carteolol was capable of interacting also with the L site of the receptor, since, in the presence of 30 nM \[^{3}H\]CGP12177, a pKᵦL value of 7.14 ± 0.79 was calculated (Fig. 8B). In contrast, xamoterol (>1 μM) was unable to displace 30 nM \[^{3}H\]CGP12177 from its binding site.

**Carteolol and Xamoterol Increase Adenylate Cyclase Activity in Cardiac Membranes.** Increasing concentrations of carteolol and xamoterol (from 1 nM to 10 μM) were also tested on the β₁-adrenoceptor-related adenylate cyclase activity present in cardiac membranes. Both carteolol and xamoterol caused a concentration-dependent increase in adenylate cyclase activity, the maximum increases over the basal value being 40.7 ± 4.1 and 96.4 ± 8.7% in the presence of 10 μM carteolol and 1 μM xamoterol, respectively (data not shown). Under the same experimental conditions, the maximum increase evoked by 10 μM isoprenaline was about 160% over basal values (data not shown). From the concentration-effect curves, EC₅₀ values of 635 ± 67 nM for carteolol and 187 ± 22 nM for xamoterol were calculated, these values corresponding to pD₂ of 6.19 ± 0.65 and 6.72 ± 0.79, respectively (Table 3).

**Discussion**

In the present study, we demonstrate for the first time that, also in guinea pig cardiac membranes as previously observed in preparations of cardiac tissue from rat (Sarsero et al., 1998), ferret (Lowe et al., 2002), and humans (Sarsero et al., 2003), as well as in recombinant human β₁-adrenoceptors (Joseph et al., 2004), two different sites or conformations of β₁-adrenoceptors exist and bind the aryloxypropanolamine (−)\[^{3}H\]CGP12177. The first one is H site (pKᵦH = 9.88 ± 0.01, Bₘₐₓ,H = 28 ± 3 fmol/mg protein), and the other one is L site (pKᵦL = 7.52 ± 0.7, Bₘₐₓ,L = 36 ± 4 fmol/mg protein), these values being almost equal to those reported by Sarsero et al. (1998) for the rat atrium. Here, we show for the first time that carteolol, whose molecule contains an aryloxypropanamine moiety identical to that of CGP12177, is able to compete with (−)\[^{3}H\]CGP12177 for both sites. When bound to the H site, carteolol behaves as an antagonist of catechol-
amine effects while behaving as a partial agonist when bound to the L site. Therefore, carteolol is a nonconventional partial agonist. Moreover, by comparing the cardiac effects of carteolol with those of xamoterol, a well-known cardioselective β₁-adrenoceptor partial agonist, this study gives new insights into the differences existing among the β-blockers provided with ISA. In fact, in guinea pig myocardial preparations, both carteolol and xamoterol were able to antagonize the positive chronotropic and inotropic effects of isoprenaline, shifting to the right, in a parallel manner, the concentration-effect curves for isoprenaline. However, a marked difference exists in the behavior of the two drugs. Interestingly, carteolol antagonized the effects of isoprenaline at concentrations not affecting per se the heart rate and/or the force of contraction. In contrast, xamoterol behaved as an antagonist toward isoprenaline at the same concentrations that increased per se the heart rate and the force of contraction, thus showing the typical effect of a conventional partial agonist on the action of a full agonist. The fact that the binding inhibition constant of carteolol for the high-affinity site of cardiac membranes (pKᵢᵢ = 9.37 ± 0.92) was very similar to its blocking potency against isoprenaline (pA₂ values of 9.04 ± 0.05 and 9.23 ± 0.18 toward isoprenaline-induced chronotropic and inotropic effects, respectively) is consistent with the hypothesis that carteolol prevents the effect of catecholamine through interaction with an H site of β₁-adrenoceptors labeled with 1 nM ([³H]CGP12177. However, the binding of carteolol to this H site is not able to trigger biological responses, as indicated by lack of any cardiotimulant effect by its antagonistic concentrations. On the contrary, evaluating the potency of xamoterol as an antagonist toward isoprenaline, we obtained pA₂ values (7.92 ± 0.28 and 7.99 ± 0.39 against chronotropic and inotropic effects of isoprenaline, respectively) rather similar to its binding inhibition constant for the high-affinity site (pKᵢᵢ = 7.34 ± 0.80) and in the same range of concentrations causing cardiotimulant effects. Although at present we cannot exclude a priori that neuronal and/or extraneuronal uptake of isoprenaline influenced the above-mentioned results, this seems to be unlikely because the neuronal uptake limits the synaptic concentrations of physiological catecholamines but not that of isoprenaline (Leenen et al., 2005 and references therein); moreover, in guinea pig atria, the extraneuronal uptake of isoprenaline has been found to be poor (Trendelenburg, 1988, and references therein). In this regard, guinea pig heart
seems to be markedly different from rat heart in which Brotto (2003) recently demonstrated a significant uptake of isoprenaline.

In spontaneously beating atria, both carteolol and xamoterol were provided with positive chronotropic and inotropic effects, although they differed in potency and intrinsic activity. The $pD_2$ values of carteolol for the positive chronotropic and inotropic effects ($6.14 \pm 0.12$ and $6.30 \pm 0.11$, respectively) were very similar to its $pD_2$ value ($6.19 \pm 0.65$) for stimulation of adenylate cyclase activity and were also not significantly different from the binding inhibition constant ($pK_i = 7.14 \pm 0.79$) calculated for an L site of cardiac $\beta_1$-adrenoceptors labeled with 30 nM (−)[$^3$H]CGP12177. In contrast, $pD_2$ values of xamoterol for chronotropic and inotropic effects ($7.41 \pm 0.20$ and $7.18 \pm 0.14$, respectively) were quite similar to the above-reported binding inhibition constant ($pK_i = 7.34 \pm 0.80$) for the H site of cardiac membrane preparations, the above-mentioned $pA_2$ values against isoprenaline, and the $pD_2$ value for stimulation of adenylate cyclase activity ($6.72 \pm 0.79$). Xamoterol, unlike carteolol, did not exhibit affinity for the L site of cardiac $\beta_1$-receptors labeled with 30 nM (−)[$^3$H]CGP12177. Therefore, our results

$\begin{array}{cc}
\text{Carteolol} & \text{Xamoterol} \\
\text{Against positive chronotropic effect of isoprenaline} & 9.04 \pm 0.05^{*,a,b} & 7.92 \pm 0.28^{b,d} \\
\text{Against positive inotropic effect of isoprenaline} & 9.23 \pm 0.18^{*,a,b} & 7.99 \pm 0.39^{b,d} \\
\text{H site} & 9.37 \pm 0.92^* & 7.34 \pm 0.80^d \\
\text{L site} & 7.14 \pm 0.73^d & >6.00 \\
\text{pD}_2 \text{ values for stimulation of adenylate cyclase activity} & 6.19 \pm 0.65^c & 6.72 \pm 0.79^p \\
\end{array}$

$^* P < 0.05 \text{ vs. respective } pD_2 \text{ values for stimulation of adenylate cyclase activity.}$

$^{a} P < 0.05 \text{ vs. respective } pK_i \text{ values for L site.}$

$^{b} \text{Not significantly } (P > 0.05) \text{ different from the respective } pK_i \text{ values for H site.}$

$^{c} \text{Not significantly } (P > 0.05) \text{ different from the respective } pK_i \text{ values for L site.}$

$^{d} \text{Not significantly } (P > 0.05) \text{ different from the respective } pD_2 \text{ values for stimulation of adenylate cyclase activity.}$

Fig. 6. Representative concentration-effect curves for the positive chronotropic and inotropic effects of isoprenaline in the presence of increasing antagonistic concentrations of carteolol and xamoterol. The positive chronotropic effect of isoprenaline was determined in spontaneously beating atria, whereas the positive inotropic effect was assessed in left atria electrically driven at 2 Hz. The effects of xamoterol on the basal rate and force of contraction, obtained in the absence of isoprenaline, were expressed as percentage of the maximum chronotropic or inotropic effect ($E_{\text{max}}$) caused by isoprenaline.

TABLE 3
Comparison between carteolol and xamoterol for their blocking potencies ($pA_2$) against isoprenaline effects, their binding inhibition constants ($pK_i$) against (−)[$^3$H]CGP12177, and their potency ($pD_2$) as stimulants of adenylate cyclase activity
Experimental conditions are described under Materials and Methods. $pA_2$, $pK_i$, and $pD_2$ values of carteolol and xamoterol were calculated as specified under Materials and Methods. The results are means ± S.E.M. of three experiments.

![Graphs showing concentration-effect curves for positive chronotropic and inotropic effects of isoprenaline in the presence of increasing antagonist concentrations of carteolol and xamoterol. The graphs illustrate the effects on spontaneously beating atria and left atria driven electrically at 2 Hz.](https://aspetjournals.org/jpet/10.1124/jpet.113.198057)
Cheng-Prusoff (1973) equation, as reported under Materials and Methods. Each data point is the mean ± S.E.M. of three independent experiments.
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