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

Maura Floreani, Guglielmina Froldi, Luigi Quintieri, Katia Varani, Pier Andrea Borea, Maria Teresa Dorigo, and Paola Dorigo

Department of Pharmacology and Anesthesiology, Pharmacology Section (M.F., G.F., L.Q., P.D.) and Eye Clinic (M.T.D.), University of Padova, Padova, Italy; and Department of Clinical and Experimental Medicine, Pharmacology Unit, University of Ferrara, Ferrara, Italy (K.V., P.A.B.)

Received May 6, 2005; accepted September 8, 2005

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-$t$-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 [(−)−3H]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 (Sarsero 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
β₁-adrenoceptor (Sarşero 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) (Sarşero et al., 1998, 1999; Konkar et al., 2000; Sarşero 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 Grove, 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.

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 catecholamines 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 (Eₘₐₓ) 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 Eₘₐₓ. Noncumulative concentration-effect curves were then obtained for increasing concentrations of both carteolol (from 10 nM to 0.1 mM) 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 Eₘₐₓ 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 Eₘₐₓ 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,

\[ \text{Response} = \frac{E_{\text{max}} - E_{\text{min}}}{1 + 10^{(D_{50} - X)/H}} \]  

where Eₘₐₓ is the effect in the absence of the agonist, Eₖₐₜₐ represents the maximum agonist-induced effect, X is the molar concentration of the agonist, H is the Hill slope, and log EC₅₀ 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 EC₅₀; i.e., the pD₂ value (pD₂ = –log EC₅₀ [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.

Figure 1. Chemical structures of carteolol and CGP12177 showing the common aryloxypropanolamine moiety.
(t₁), relaxation time (t₂), 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 (Arumugakshana 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 48,000 g 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 Biochemical method (Bredford, 1976) with bovine serum albumin as standard.

(-)[³H]CGP12177 Saturation Binding Experiments. In human membranes suspension (100–120 μg of proteins/100 μl) were incubated for 120 min at 25°C in a medium (0.25 ml final volume) containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, and 18 to 20 increasing concentrations (from 0.1–100 nM) of (-)[³H]CGP12177. Nonspecific binding, which increased linearly with concentrations, was defined from the Cheng-Prusoff equation (1973) with

\[
P_{nonspecific} = P_{total} \times \frac{([B]/Kᵦ^* + 1)^{-2} \times Kᵦ^*}{([B]/Kᵦ^* + 1)^{-2} + (Kᵦ^*/Cᵦ)}
\]

where Cᵦ is the radioligand concentration used (1 and 30 nM for the high- and low-affinity sites, respectively) and Kᵦ* 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 Atomlight 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, (-)[³H]CGP12177 hydrochloride, reserpine, tyramine hydrochloride, (-)-isoprenaline hydrochloride, carbamylcholine 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\text{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\text{max}) and decreased at higher concentrations. Xamoterol was more effective and more potent than carteolol, its effect being more marked (56.64 ± 7.70% of E\text{max}) than that of carteolol and peaking at 1 μM. As for carteolol, the effect of xamoterol tended to decrease at higher concentrations. The values of intrinsic activity (α) and potency (pD\text{α}_2) of both drugs are summarized in Table 1.

In spontaneously beating atria, both carteolol and xamoterol caused also a concentration-dependent increase in the force of contraction (Fig. 2, bottom panel). The pattern of this effect was similar to that of their chronotropic action, peaking at 10 and 1 μM for carteolol and xamoterol, respectively, and decreasing at higher concentrations. The pD\text{α}_2 values, calculated for the inotropic effect of carteolol and xamoterol (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 pD\text{α}_2 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\text{max} induced by isoprenaline) than that observed in spontaneously beating atria. Furthermore, pD\text{α}_2 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 β\text{2}-adrenoceptors to the cardiac actions of carteolol, further experiments were carried out in the presence of the selective β\text{2}-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 α\text{1}-adrenoceptors to the cardiac actions of carteolol, additional experiments evaluated the effects of the drug in the presence of the α\text{1}-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).
Materials and Methods details under Experimental data in brackets were excluded in the fitting. Results are 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>Positive Chronotropic Effect</th>
<th>Positive Inotropic Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potency (pD₂)</td>
<td>Intrinsic Activity (α)</td>
</tr>
<tr>
<td>Carteolol</td>
<td>6.14 ± 0.12</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>Xamoterol</td>
<td>7.41 ± 0.20*</td>
<td>0.64 ± 0.04*</td>
</tr>
</tbody>
</table>

* P < 0.001 vs. carteolol.

The curves have been obtained as explained in detail under Materials and Methods. The experimental data in brackets were excluded in the fitting. Results are means ± S.E.M. from four assays carried out using four different guinea pig hearts for each drug.

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 (t₁), relaxation time (t₂), mean velocity of force development (S₁), and mean velocity of relaxation (S₂). 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 t₁ and t₂ 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, S₁ values showed a statistically significant increase, indicating an increased velocity of force development. In the presence of xamoterol, a significant shortening of t₁ value occurred and increases in S₁ and S₂ 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 (pA₂) 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 (Kᵦ 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 pA₂ 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 Kᵦ values were
of beating atria were expressed as percentage of the maximum positive
isotropic effect. The effects of the drugs on the rate of contraction of spontaneously
beating and in left atria, respectively.

Fig. 4. Concentration-effect curves for the chronotropic effects of carteolol, xamoterol, and isoprenaline in the absence and presence of propranolol. The effects of the drugs on the rate of contraction of spontaneously beating atria were expressed as percentage of the maximum positive
isotropic effect (E_{max}) caused by 3 μM isoprenaline in the same preparation. Propranolol (1 μM) was added to the bathing medium 30 min before the addition of the tested drugs. For each drug, the results are means ± S.E.M. from three assays carried out using three different guinea pig hearts. * P < 0.05; ** P < 0.01; *** P < 0.001; a, no significant differences were detected (P > 0.05).

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 β_1-adrenoceptors of guinea pig cardiac membranes, we measured their ability to specifically displace (-)[3H]CGP12177 from its binding sites. Because (-)[3H]CGP12177 is a nonconventional partial agonist that binds to both the high- and low-affinity sites of β_1-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 β_1-adrenoceptors (Joseph et al., 2004), we first assayed the binding of increasing concentrations (from 0.1 to 100 nM) of (-)[3H]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 (pK_{d H} 9.88 ± 0.01; B_{max} 28 ± 3 fmol/mg protein; n = 3) and L site (pK_{d H} 7.52 ± 0.7; B_{max} 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 (-)[3H]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 (-)[3H]CGP12177 (Fig. 8A), a pK_{IH} value of 9.37 ± 0.92 was obtained. Under the same experimental conditions, the pK_{IL} 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 (-)[3H]CGP12177, a pK_{d L} value of 7.14 ± 0.79 was calculated (Fig. 8B). In contrast, xamoterol (>1 μM) was unable to displace 30 nM (-)[3H]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 β-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, EC_{50} values of 635 ± 8.7% in carteolol and 187 ± 22 nM for xamoterol were calculated, these values corresponding to pD_{2} 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 β_1-adrenoceptors (Joseph et al., 2004), two different sites or conformations of β_1-adrenoceptors exist and bind the arylxopropanolamine (-)[3H]CGP12177. The first one is H site (pK_{d H} 9.88 ± 0.01; B_{max} 28 ± 3 fmol/mg protein), and the other one is L site (pK_{d L} 7.52 ± 0.7; B_{max} 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 arylxopropanolamine moiety identical to that of CGP12177, is able to compete with (-)[3H]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 car-
teolol with those of xamoterol, a well known cardioselective
β1-adrenoceptor partial agonist, this study gives new in-
sights into the differences existing among the β-blockers
provided with ISA. In fact, in guinea pig myocardial prepara-
tions, both carteolol and xamoterol were able to antagonize
the positive chronotropic and inotropic effects of isoprenal-
line, shifting to the right, in a parallel manner, the con-
centration-effect curves for isoprenaline. However, a marked
difference exists in the behavior of the two drugs. Interest-
ingly, 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 con-
traction, 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 (pKIH = 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. How-
ever, the binding of carteolol to this H site is not able to
trigger biological responses, as indicated by lack of any car-
diostimulant effect by its antagonistic concentrations. On the
contrary, evaluating the potency of xamoterol as an antago-
nist 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 inhibi-
tion constant for the high-affinity site (pKIH = 7.34 ± 0.80)
and in the same range of concentrations causing cardiostimu-
lant 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

### Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>t₁ Value (ms)</th>
<th>t₂ Value (ms)</th>
<th>S₁ Value (mg/ms)</th>
<th>S₂ Value (mg/ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>126.6 ± 4.9</td>
<td>246.3 ± 18.7</td>
<td>6.0 ± 0.5</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Carteolol (0.1 μM)</td>
<td>125.0 ± 2.9</td>
<td>250.0 ± 6.3</td>
<td>5.6 ± 1.0</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>Carteolol (1 μM)</td>
<td>115.0 ± 7.6</td>
<td>226.6 ± 12.0</td>
<td>7.4 ± 0.3*</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>None</td>
<td>117.5 ± 3.2</td>
<td>215.0 ± 18.1</td>
<td>7.7 ± 0.5**</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>Xamoterol (0.01 μM)</td>
<td>108.7 ± 2.5*</td>
<td>216.2 ± 4.8</td>
<td>6.1 ± 0.5</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>Xamoterol (0.1 μM)</td>
<td>107.5 ± 2.8*</td>
<td>218.1 ± 6.4</td>
<td>8.8 ± 0.9*</td>
<td>5.7 ± 0.3***</td>
</tr>
<tr>
<td>Xamoterol (1 μM)</td>
<td>102.5 ± 2.9**</td>
<td>220.0 ± 8.5</td>
<td>11.4 ± 1.2***</td>
<td>7.1 ± 0.3***</td>
</tr>
<tr>
<td>None</td>
<td>121.2 ± 2.5</td>
<td>228.7 ± 5.5</td>
<td>6.2 ± 0.5</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>Isoprenaline (2 μM)</td>
<td>96.2 ± 3.7***</td>
<td>201.2 ± 6.9*</td>
<td>15.9 ± 1.9***</td>
<td>7.7 ± 1.1**</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01; *** P < 0.001 vs. respective control values.

![Fig. 5](image)
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 $([3H]CGP12177$. 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 $([3H]CGP12177$. Therefore, our results

---

**TABLE 3**

Comparison between carteolol and xamoterol for their blocking potencies ($pA_2$) against isoprenaline effects, their binding inhibition constants ($pK_i$) against $([3H]CGP12177$, 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 $\pm$ S.E.M. of three experiments.

<table>
<thead>
<tr>
<th></th>
<th>Carteolol</th>
<th>Xamoterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>$pA_2$ values</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Against positive chronotropic effect of isoprenaline</td>
<td>9.04 ± 0.05$^{a,b}$</td>
<td>7.92 ± 0.28$^{b,d}$</td>
</tr>
<tr>
<td>Against positive inotropic effect of isoprenaline</td>
<td>9.23 ± 0.18$^{a,b}$</td>
<td>7.99 ± 0.39$^{b,d}$</td>
</tr>
<tr>
<td>$pK_i$ values</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H site</td>
<td>9.37 ± 0.92$^a$</td>
<td>7.34 ± 0.80$^d$</td>
</tr>
<tr>
<td>L site</td>
<td>7.14 ± 0.73$^d$</td>
<td>&gt;6.00</td>
</tr>
<tr>
<td>$pD_2$ values for stimulation of adenylate cyclase activity</td>
<td>6.19 ± 0.65$^c$</td>
<td>6.72 ± 0.79$^b$</td>
</tr>
</tbody>
</table>

---

$^a$ $P < 0.05$ vs. respective $pD_2$ values for stimulation of adenylate cyclase activity.

$^b$ $P < 0.05$ vs. respective $pK_i$ values for L site.

$^c$ Not significantly ($P > 0.05$) different from the respective $pK_i$ values for H site.

$^d$ Not significantly ($P > 0.05$) different from the respective $pK_i$ values for L site.

$^e$ Not significantly ($P > 0.05$) different from the respective $pD_2$ values for stimulation of adenylate cyclase activity.
demonstrate the substantial difference existing between carteolol and xamoterol. Whereas the partial agonist activity of carteolol appears at concentrations 3 log units higher than those causing the blockade of \( \beta_1 \)-receptors and is related to the interaction of the drug with the low-affinity site of receptors, xamoterol, as a typical partial agonist, exerts both its agonist and antagonist activity at the same concentrations through the interaction with the H site. Both in spontaneously beating atria and in electrically driven left atria, the intrinsic activity of xamoterol was significantly higher than that of carteolol. An explanation for this difference is certainly provided by the different extents of increase in adenylate cyclase activity triggered by the two drugs in guinea pig cardiac membranes (96.4 ± 8.7 and 40.7 ± 8.7% for xamoterol and carteolol, respectively).

Biochemical and functional data confirm the involvement of cAMP in the cardiostimulant effects of carteolol. First, we found a good relationship between the \( pD_2 \) values for activation of the adenylate cyclase activity in cardiac membranes by carteolol and those for its cardiostimulant effects. Second, in the left atrium, the addition of IBMX, a well established phosphodiesterase inhibitor, increased significantly and concentration-dependently the positive inotropic effect of carteolol. Furthermore, the effect of carteolol was significantly decreased by carbachol, which is known to impair the biological effects of compounds whose activity is mediated through a rise in cAMP levels (Endoh, 1979; Floreani et al., 2003). Third, at concentrations exerting positive inotropic effects (1–10 \( \mu \)M), carteolol caused decreasing trends in the \( t_1 \) and \( t_2 \) values and produced a statistically significant increase in \( S_1 \) values; this pattern of behavior is consistent with a cAMP-mediated effect (Tsien, 1977; Brixius et al., 1997; Floreani et al., 2003). The impact of carteolol on these parameters was less marked than that of xamoterol, probably because of the differences in the intrinsic activity and in the extent of cAMP increases induced by the drugs.

Moreover, the relative resistance of carteolol effects to propranolol further supports the conclusion that it is a nonconventional partial agonist, whose ISA is mediated through its interaction with the L site (namely the propranolol-insensitive site) of \( \beta_1 \)-adrenoceptors, as previously shown for other \( \beta \)-blockers including CGP12177 (Konkar et al., 2000; Joseph et al., 2004).

The results obtained in this study do exclude that carteolol causes its effects on guinea pig heart tissues through its interaction with \( \beta_2 \)-adrenoceptors, because its actions were not affected at all by butoxamine, a selective \( \beta_2 \)-antagonist. A recent study carried out by Bruck et al. (2004) in healthy volunteers suggests that the ISA of carteolol may be due to its interaction with \( \beta_2 \)-adrenoceptors. However, the same authors, referring to our previous paper (Floreani et al., 2003), do not exclude but rather consider the possibility that their results could be explained also by the interaction of carteolol with the propranolol-resistant state of \( \beta_1 \)-adrenoceptors (Bruck et al., 2004).

In conclusion, our results suggest that remarkable differences may exist among the \( \beta \)-blockers endowed with ISA; therefore, the cardiac effects of the \( \beta \)-blockers endowed with ISA cannot be generalized because there does not exist a paradigm of behavior for all partial agonists. These findings may impact the clinical usefulness of nonconventional partial agonists, such as carteolol. Due to the distance existing between their antagonistic and agonistic concentrations, they may be very safe drugs among the \( \beta \)-blocking agents endowed with ISA.

Acknowledgments

We thank Anna Scuderi (Società Industria Farmaceutica Italiana S.p.A., Catania, Italy) for the kind gift of carteolol, Robert Strobl for...
assistance in preparing the manuscript, Roberto Padri for helpful discussions, and Enrico Secchi and Paolo Favero for excellent technical assistance.

References


Address correspondence to: Pro. Paola Dorigo, Department of Pharmacology and Anesthesiology, Pharmacology Section, University of Padova, Largo Meneghetti 2, 35131 Padova, Italy. E-mail: paola.dorigo@unipd.it.