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Recovery of β-adrenoceptors and cyclic AMP response after long term treatment of intact heart cells with β-blockers

Charlotte Becker & Hartmut Porzig

Pharmakologisches Institut der Universität Bern, Friedbühlstrasse 49, CH-3010 Bern, Switzerland

1 We have studied the recovery of receptor binding and of isoprenaline-stimulated cyclic AMP responses after chronic (2–5 days) exposure of tissue-cultured living rat heart cells to several β-adrenoceptor antagonists. Most experiments were performed with [^{3}H]-(±)-carazolol and [^{3}H]-(±)-CGP 12177, as prototypes of high affinity lipophilic and hydrophilic ligands respectively.

2 Chronic antagonist treatment did not alter the total number of receptors nor did it cause intracellular accumulation of the ligands.

3 At the end of the treatment, radiolabelled antagonists were displaced either by 'infinite' dilution of the incubation medium or by competitive displacement with the non-labelled ligand (−)-timolol. In dilution assays dissociation of carazolol from specific sites was biphasic with $t_{1/2}$ values of 41 ± 14 and 219 ± 15 min. Dissociation of CGP 12177 was monophasic with $t_{1/2}$ of 102 ± 2 min.

4 Timolol enhanced the dissociation rates of both radioligands and suppressed the slow phase of carazolol dissociation.

5 Isoprenaline-stimulated cyclic AMP formation did not recover in parallel with the release of the two antagonists from receptor binding sites. To reach about 80% of control values for receptor availability or cyclic AMP response required 3 h and 24 h washout periods, respectively, after carazolol (0.2 nM) treatment, or 1.5 and 12 h washout periods after CGP 12177 (4 nM) treatment. Such a 'decoupling' effect was not observed during recovery from chronic exposure to the antagonists, timolol and propranolol.

6 We conclude that some antagonists cause a novel form of desensitization that is not linked to their partial agonistic potency. Moreover, carazolol-type drugs seem to induce an additional isomeric form of the β-receptor that is not recognized by other antagonists. These observations could explain the well known discrepancy between long duration of action and rapid removal from the circulation of several antagonists in current therapeutic use.

Introduction

β-Adrenoceptor blocking drugs are widely used for chronic treatment of hypertension and for other therapeutic purposes. Nevertheless, comparatively little is known of the detailed pharmacodynamic interactions of these compounds with their target system in the membrane of intact cells during long term application. For some high affinity lipophilic ligands (e.g. carazolol) it has been shown that the apparent rate of decrease in β-receptor blocking activity may outlast the rate of elimination from the plasma. This kinetic behaviour has been ascribed to ligand receptor interactions (Innis *et al.*, 1979; Abshagen & Möllendorff, 1980). Studies in intact, tissue-cultured cardiac cells have suggested that among the specific antagonists carazolol and cyanopindolol, unlike timolol and propranolol, may affect the primary responses of the system to agonist stimulation in addition to occupying receptor binding sites (Porzig *et al.*, 1982). These differences in the effects of individual 'competitive' ligands could contribute to determining the duration of action of a particular compound *in vivo*. We have now treated rat heart cells in tissue culture for prolonged periods of time with (±)-carazolol (Morris *et al.*, 1978) and (±)-CGP 12177 (4-(3-tertiarybutylamino- 2-hydroxypropoxy) -benzimidazole- 2-on hydrochloride, Staehelin *et al.*, 1983) as prototypes of high affinity lipophilic and hydrophilic β-receptor blockers. We have then compared the dissociation kinetics of the radioligands with the rate of recovery of recep-

tor binding and of agonist-stimulated cyclic AMP accumulation.

Our results suggest that after exposure to carazolol and CGP 12177 the loss of receptor-mediated cyclic AMP response survives the actual occupation of the receptor. This effect was not observed with timolol and propranolol. Moreover carazolol appears to induce a second high affinity form of the receptor which does not exist in the presence of other β-adrenoceptor antagonists.

Methods

Rat heart cell culture

Primary cultures were established with cells obtained by tryptic digestion of hearts from 2–4 day old baby rats. For the experiments we used cells which had been cultivated for 2–7 days on glass cover slips in Dulbecco's MEM or Leibowitz L15 medium supplemented with 10% foetal calf serum. A detailed description of the tissue culture methods is given by Porzig *et al.* (1982). Cardiac myoblasts in these cultures appeared to stop dividing by day 3 of culture. Total cell number between day 3 and day 6 increased by 20%, mainly due to the ongoing growth of fibroblasts.

C6 glioma cell cultures

C6 cells were kindly provided by Dr Wiesmann (Dept of Pediatrics, Univ. Bern). The cells were grown to confluency in Dulbecco's MEM (with 10% foetal calf serum) either in petri dishes (for cells used in binding studies) or in glass scintillation vials (for cells used in cyclic AMP assays). During the experimental treatment with β-adrenoceptor antagonists confluent cells were maintained in DMEM containing only 2% serum in order to stop cell multiplication.

Treatment with β-adrenoceptor antagonists

Cover slip cultures were grown for 1.5 to 96 h in the presence of one of the following antagonists; (±)-carazolol (0.2 nM), (±)-CGP 12177 (4 nM), (−)-timolol (9 nM), (±)-propranolol (10 nM). The chosen free ligand concentrations in the culture medium were 5–10 fold higher than the respective K_D values. The antagonist-containing media were changed every 24 h. For the two ^{3}H-labelled compounds carazolol and CGP 12177 we determined the dissociation rate constants by the 'infinite dilution' and by the 'competitive displacement' methods. For the infinite dilution assay, antagonist-exposed single glass cover slip cultures (100–250 μg protein) were rinsed 3 times at 37°C in 100 ml normal drug-free culture medium. Each culture was then immersed in a glass beaker containing 50 ml of the same medium at 37°C and placed in the CO_2-incubator for predetermined time periods. We calculated that after complete equilibration under these conditions the final concentrations of free carazolol and CGP 12177 in the dilution medium corresponded to about 0.5 and 0.2%, respectively, of their K_D values for cardiac β-receptors. Consequently, the radioactivity of the dilution medium at the end of the experiment was not significantly different from background levels. Zero time binding was determined in antagonist-equilibrated cultures after three 2 min washing periods in 50 ml medium at 37°C. In competitive displacement assays an excess ($\sim 10^4 \times K_D$) of nonlabelled antagonist (timolol, 10 μM) or agonist (isoprenaline, 300 μM) was added at zero time to cultures grown in the presence of ^{3}H-labelled carazolol or CGP 12177. The concentrations of the radiolabels were kept constant over the whole assay period. Sample cultures were removed at suitable intervals to follow the time course of the displacement of label from tissue binding sites.

Receptor binding assay

Binding of ^{3}H-labelled antagonists to intact, cover slip-attached heart cells was measured according to the method given in Porzig *et al.* (1982). Recovery of binding sites after a short time or chronic occupancy by a nonlabelled β-receptor ligand was followed by establishing [^{3}H]-CGP 12177 binding curves at various time intervals after the beginning of the washout period. Unspecific binding was always defined as that amount of radiolabel that could not be displaced by 1 μM timolol. Binding assays were generally conducted in triplicate. The incubation media were identical to the standard culture medium. At the end of the 90 min incubation periods with CGP 12177 the cultures were washed 3 × 2 min in 3 ml of Hank's salt solution, at 4°C and digested in 800 μl Protosol (NEN) for 2 h at 60°C. Radioactivity was counted in an Intertechnique SL 4000 liquid scintillation counter. We used a triton/xylol 1:1 (v/v) scintillator containing 4 g l^{-1} Omnifluor (NEN) for the hydrophilic compound CGP 12177 and a toluene based scintillator with 4 g l^{-1} BBOT (2,5-*bis*-2- (5-*t*-butylbenzoxazolyl) thiophene, Ciba-Geigy AG, Basel) for the lipophilic ligand carazolol.

Cyclic AMP assay

Cyclic AMP accumulation in living cells was measured as described previously (Porzig *et al.*, 1982). All assay media (total volume 1 ml per cover slip culture)

contained standard growth medium including serum, supplemented with 1 mM ascorbic acid and 2 mM of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX). Cyclic AMP formation was initiated after a pre-equilibration period of 15 min by adding (−)-isoprenaline (1 nM–10 μM) and was stopped after 1 min by heating the sample to 100°C for 10 min. Total cyclic AMP content i.e. the sum of intra-and extracellular cyclic AMP was measured according to the method of Tovey *et al.* (1974). Control experiments showed that the efficacy of isoprenaline in stimulating cyclic AMP formation depended on the nature of the serum supplement. Thus, the use of newborn instead of foetal calf serum reduced receptor-mediated cyclic AMP formation by about 50%. Therefore, we added 10% of a preselected foetal calf serum to all incubation media.

Desensitization experiments

Desensitization of the cardiac β-adrenergic system was induced by growing cultures for 3–5 days in the presence of 10 μM (−)-isoprenaline. The standard culture medium was supplemented with 10 μM ascorbic acid and was replaced daily. The gradual recovery of receptor binding and receptor-mediated cyclic AMP formation were measured over a 2 day period according to the methods described above.

Protein determination

To estimate the mean protein content of cover slip cultures, we removed 3 sample cultures from each group of 12 cultures growing together in a Petri dish. The cell layer of each culture was detached from the substratum by a freeze-thawing cycle, suspended in 1 ml 30 mM NaCl solution and homogenized by a 5 s sonification. The protein content of the suspension was then measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Data analysis

Apparent radioligand dissociation constants (K_D values), maximal binding capacities (B_{max} values) and their respective standard deviations were calculated from the untransformed binding data by nonlinear least square regression analysis. The calculation (computers HP 85 or HP 9816) used a BASIC version of the 'Modfit' programme published by McIntosh & McIntosh (1980). The programme was also suitable to estimate dissociation rate constants and compartment sizes from radioligand washout- and displacement curves. The programme 'Design' published by the same authors was used to discriminate between different kinetic models on the basis of statistical criteria.

Materials and sources

[^{3}H]-(±)-carazolol (0.74–1.48 TBq) was purchased from NEN (Dreieich, FRG). [^{3}H]-(±)-CGP 12177 (1.48 TBq) and nonlabelled CGP were generous gifts of Prof. M. Staehelin, Friedrich-Miescher-Institute, Basel, Switzerland. (±)-Carazolol and (−)-timolol were gifts of Boehringer Mannheim Corp. (F.R.G.) and of Merck, Sharp & Dohme, Rahway, NJ (U.S.A.), respectively. (−)-Isoprenaline and isobutylmethylxanthine were obtained from Sigma, St. Louis, MO (U.S.A.). All tissue culture reagents, media and sera were purchased from Boehringer Mannheim Corp. Rotkreuz, Switzerland.

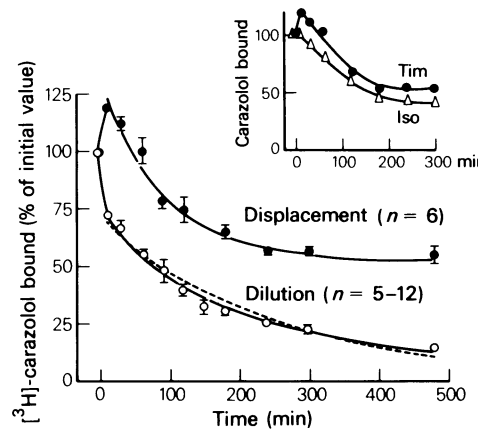


Figure 1 Time course of [^{3}H]-carazolol dissociation when initiated by 'infinite' dilution (○) or by addition of timolol 1 μM (●) after 1.5–120 h equilibration periods with [^{3}H]-carazolol 0.2 nM. Experimental points were normalized with respect to initial total binding. Between the 10th and the 480th min, dissociation by displacement could be fitted with a single exponential of the form $y = 77.8e^{-0.0113t} + 52.4$. For the same period of time, dissociation by dilution followed a double exponential time course of the form $y = 26.5e^{-0.017t} + 53.9e^{-0.0032t}$. The parameters for the dilution curve were calculated including an additional time point at 720 min (not shown). The broken line gives the best fit of a single exponential to the same data. Experimental points give the mean of 5–12 cover slip cultures from 5 independent dilution experiments and the mean of 6 cultures from 2 independent displacement assays; vertical lines show s.e.mean. Two other experiments with fewer time points gave similar results. The inset shows the result of a single experiment where the dissociation of carazolol after a 5 h equilibration period was induced by the addition of either timolol 10 μM or isoprenaline 300 μM. The curves are fitted by eye.

Table 1 Total and unspecific binding of [^{3}H]-carazolol (0,2 nm) to intact cardiac cells after short and long term exposure

Duration of exposure (h)	*Total binding* (fmol mg^{-1} protein)	*Unspecific binding* (fmol mg^{-1} protein)	(% of total)	*Specific binding* (fmol mg^{-1} protein)	(% of total)
1.5	65.8 ± 2.7	24.2 ± 0.9	36.8	41.6	63.2
3	68.2 ± 1.8	22.7 ± 0.7	33.3	45.5	66.7
12	66.2 ± 3.5	14.5 ± 0.6	21.3	51.7	75.8
24	53.0 ± 3.1	14.0 ± 0.3	26.4	39.0	73.6
120	59.0 ± 2.0	17.9 ± 0.3	30.3	41.1	69.7

Cover slip cultures were prepared from a large pool of cells. Values for total and unspecific binding (in the presence of 1 μM timolol) are the mean, each, of 8 cultures. Specific binding is the difference between these two mean values. Binding was always measured on day 5 of culture. Consequently, the data in the first line were obtained from cultures which were exposed to carazolol only for 1.5 h on day 5 of culture, whereas the data in line 5 originate from cells which were exposed to carazolol for the whole cultivation period.

Results

Dissociation of carazolol and CGP 12177 from cardiac β-receptors

In a first set of experiments we studied the dissociation kinetics of ^{3}H-labelled carazolol and CGP 12177 in intact heart cells by the infinite dilution or the competitive displacement technique after chronic (2–5 days) exposure to the antagonist (Figure 1 and 2).

In dilution experiments with carazolol we monitored the loss of radioactivity from the cultures for 12 h. Total binding of carazolol decreased to 73 ± 3.2% of the equilibrium value within the first 10 min of incubation. Most of this rapid initial release probably corresponds to the fraction of ligand which is bound unspecifically to the cell surface. An additional amount of unspecifically bound carazolol may have been lost during the 6 min washing period at 37°C which always preceded the zero time measurement of bound radioactivity. In control experiments (Table 1) we have measured repeatedly total and unspecific binding of carazolol (free concentration 0.2 nM) during chronic exposure for time periods ranging between 90 min and 120 h. Mean unspecific binding reached 29.7 ± 2.7% of total binding and did not change with time. This value is compatible with the amount of ligand that was rapidly lost during the initial phase of washout.

The dissociation of carazolol from the 10th to the 720th min is well described by a double exponential time course (Figure 1) but poorly by a single exponential fit (broken line in Figure 1; the point at 720 min has been omitted in the graph but was included in the calculation). Computer-assisted comparison between the two fitting procedures yielded a 99.6% likelihood in favour of the double exponential fit. These data suggest that dissociation of carazolol from high affinity binding sites involves two reaction steps corresponding to dissociation half times of 41 ± 14.5 and 219 ± 15.3 min (rate constants of 0.017 and 0.0032 min^{-1}). When extrapolated to zero time of the experiment, the fast and the slowly dissociating population of sites comprised 33 and 67%, respectively, of all high affinity sites. A similar kinetic pattern for the release of carazolol was observed when the preceding equilibration period was reduced from 4 days to 90 min. When the dissociation of the antagonist was initiated by the addition of timolol, we observed a significant transient increase in total binding by 19 ± 2.5%. This effect reached its maximum within the first 10 min of incubation. From the 10th to the 480th min the radiolabel was released monophasically with a half time of 61 ± 11.3 min. This value was not significantly different from the corresponding figure for the fast component of specific loss in dilution experiments. The dissociation kinetics were similar for all experiments in which the displacing ligand was added after carazolol had reached binding equilibrium, irrespectively of whether the equilibration period had been 90 min or 5 days. The time constant for the release of carazolol did not change when (−)-isoprenaline (0.1 M) rather than timolol was used as the displacing ligand, but the agonist failed to produce an initial increase of total binding (see inset to Figure 1). The two dissociation curves run essentially parallel to each other such that the total amount of carazolol that was displaced did not depend on the nature of the competing ligand. In absolute terms, the mean total amount of ligand displaced by timolol between the 10th and the 480th min (56.5 fmol mg^{-1} prot.) was somewhat smaller than the amount dissociating upon dilution after the 10th min, i.e. after subtraction of the unspecific initial loss (71.4 fmol mg^{-1} prot.). The mean

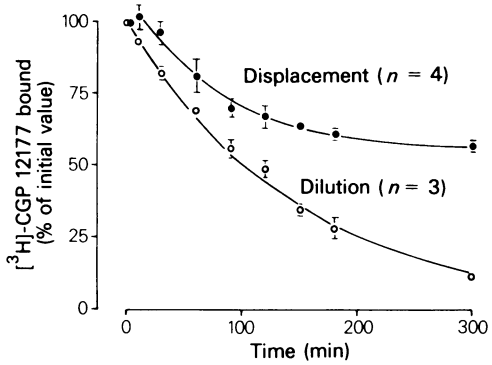


Figure 2 Time course of [^{3}H]-CGP 12177 dissociation when initiated by 'infinite' dilution or by addition of timolol 1 μM after a 120 h equilibration period with [^{3}H]-CGP 12177 4 nM. The experimental points between the 10th and the 300th min were fitted by single exponentials according to the equations $y = 100e^{-0.0068t}$ (dilution) or $y = 56.3e^{-0.0129t} + 53.2$ (displacement). All data were normalized with respect to initial total binding. Inclusion of the zero time value had no significant effect on the parameter estimates. Data points give the mean of 3 or 4 coverslip cultures from one large batch of cells; s.e.mean shown by vertical lines. Two other experiments yielded similar results but were terminated after 150 min.

non-displaceable binding in displacement experiments was significantly larger than the fraction of carazolol that dissociated within the first 10 min of dilution experiments (52.4 versus 26.7% of initial total binding, see Figure 1).

Dissociation of the hydrophilic antagonist [^{3}H]-CGP 12177 following 'infinite' dilution after a 2–4 day equilibration in the presence of 4 nM free ligand was well described by a single exponential time course with a half time of 102 ± 1.8 min (Figure 2). Assuming a double exponential time course did not improve the fit but increased significantly the standard deviations of the parameter estimates. In contrast to carazolol, there was no initial rapid loss of CGP 12177 from unspecific low affinity binding sites. Consequently, the kinetic parameters estimated from dilution experiments were the same irrespective of whether or not the fitting procedure included the zero time value.

In the competitive displacement assay, [^{3}H]-CGP 12177 also dissociated monoexponentially. The addition of timolol as the displacing ligand caused no significant transient increase in CGP 12177 binding. We calculated a dissociation half time of 54 ± 9.6 min when the curve fit was started 10 min after addition of timolol in strict analogy to the procedure with carazolol. However, inclusion of the zero time value caused no significant change in any of the parameter estimates. The apparent non-displaceable fraction of [^{3}H]-CGP 12177 binding at infinite time made up 56 ± 2.4% of the initial total binding. In independent binding experiments with simultaneous equilibration of timolol and CGP 12177 using the same concentrations as above, we found a mean non-displaceable fraction of 42.7 ± 2.9% ($n = 6$). Total binding in the presence of 4 nM [^{3}H]-CGP 12177 at zero time of the displacement assay was not different from the corresponding value for the dilution experiment (151.3 ± 6.1 and 150.6 ± 12.8 fmol mg^{-1} prot., respectively).

Isoprenaline-stimulated cyclic AMP formation after chronic exposure to antagonists

Is the kinetic pattern of antagonist-receptor dissociation reflected in the rate of recovery of the cyclic AMP response after chronic receptor blockade? To study this problem, we have equilibrated cover slip cultures for 5 days with either timolol (9 nM) or carazolol (0.2 nM) or CGP 12177 (2 nM). The cultures were then carefully washed for 90 min to remove the different ligands. The washout was followed by a 1 min stimulation period of the β-adrenoceptor system in the presence of isoprenaline. Figure 3 shows the corresponding concentration-response curves for agonist-induced cyclic AMP for-

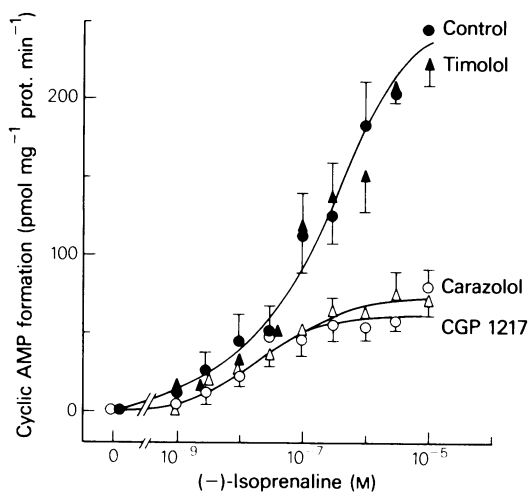


Figure 3 Concentration-response curves for isoprenaline-stimulated cyclic AMP formation during recovery from a 5 day exposure to timolol (9 nM, ▲), CGP 12177 (2 nM, ○) or carazolol (0.2 nM, △). Cover slip cultures were washed for 105 min in antagonist-free culture medium. After equilibration for 15 min with isobutylmethylxanthine 2 mM they were stimulated for 1 min with isoprenaline. Data points give mean values of 4–5 cultures from 2 different batches of cells; s.e.mean shown by vertical lines.

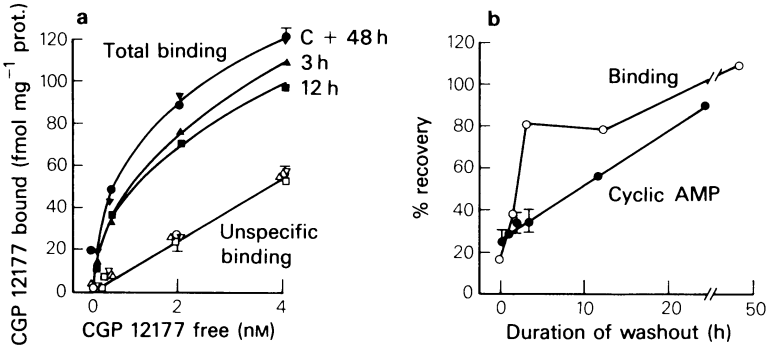


Figure 4 Recovery of receptor binding and of isoprenaline-stimulated cyclic AMP formation after a 4–5 day incubation period with carazolol 0.2 nM. (a) Total and unspecific binding of [^{3}H]-CGP 12177 (filled and open symbols, respectively) was determined after washout periods of 3 h (▲, △), 12 h (■, □) or 48 h (▼, ▽) in parallel with controls (●, ○) to monitor the gradual increase in receptors available to a second ligand. The data points are mean values of 2 cultures from one batch of cells. Control curves for total and unspecific binding give means of 4 cultures from 2 separate cell preparations; s.e. mean shown by vertical lines. Similar results were obtained in another independent experiment. (b) The data for specific [^{3}H]-CGP 12177 binding shown in (a) and similar results from 2 other experiments were fitted by nonlinear least square regression lines using the equation $BL = B_{max}\,L/(L + K_{DL})$ where BL is the amount of ligand bound, B_{max} the maximal β-receptor binding capacity, L the free ligand concentration and K_{DL} its dissociation constant. The resulting B_{max} estimates were then used to construct the curve designated 'Binding'. All values were normalized with respect to specific binding under control conditions and give the mean of 2 independent estimates. Maximal receptor-mediated cyclic AMP formation during removal of carazolol (●) was tested by stimulating cultures with isoprenaline 10 μM in the presence of isobutylmethylxanthine after washout periods ranging between 15 min and 24 h. Results are plotted as % of controls and give means of 2–6 cultures form 4 different experiments; s.e.mean is given where more than 2 cultures have been measured.

mation. In the controls and in timolol-treated cultures isoprenaline was equally effective, indicating full functional recovery of the β-adrenoceptor system within 90 min after the removal of this antagonist. By contrast, in carazolol or CGP 12177-pretreated cells, the maximal effect of isoprenaline was reduced to about one third of the control response. For carazolol this result was to be expected from its slow dissocation rate. However, with CGP 12177 recovery of the system should have been significant after a 90 min dissociation period. This apparent discrepancy prompted us to analyze in more detail the relation between the recovery of receptor binding properties and the cyclic AMP response after chronic treatment with the three antagonists carazolol, CGP 12177 and timolol. Figure 4 summarizes the results of such experiments with carazolol. Cardiac cell cultures were exposed to 0.2 nM free non-labelled carazolol for 2–3 days. The cells were then transferred to drug-free medium and incubated for an additional period of 48 h (including regular medium changes). Binding curves for CGP 12177 obtained after removal of carazolol are shown in Figure 4a. Figure 4b shows the relation between B_{max} expressed as % of the control value and the duration of the washout period. The fraction of receptors not occupied in the presence of a given concentration of antagonist was calculated from the known equilibrium binding constants for the ligand in rat heart cell cultures (Porzig *et al.*, 1982). With 0.2 nM carazolol, 17% of the receptors were estimated to be free at time zero. At the end of a 3-h washing period 80% of the specific receptors were available again for binding to CGP 12177. The remaining 20% of β-adrenoceptor binding recovered at a much slower rate. The receptor populations which became successively available to the second ligand after prolongation of the washout period differed only in number but not in their affinity for CGP 12177. Unspecific binding of the second ligand in the presence of timolol did not change during the whole course of carazolol washout.

The antagonist-induced inhibition of the isoprenaline-stimulated cyclic AMP response outlasted the apparent β-receptor blockade (see Figure 4). The restoration of this response followed a slow linear time course and was complete within 48 h. For the earliest time, i.e. 15 min after removal of carazolol from the culture medium, maximal β-receptor-mediated cyclic AMP formation reached 26.3 ± 3.9% of the corresponding control value. After 3 h when 80% of the receptors were free, maximal cyclic AMP accumulation had increased to only 34% of its control value.

This action of carazolol was not restricted to cardiac β-adrenoceptors. We have made analogous observations in C6 rat glioma cells. The cells were

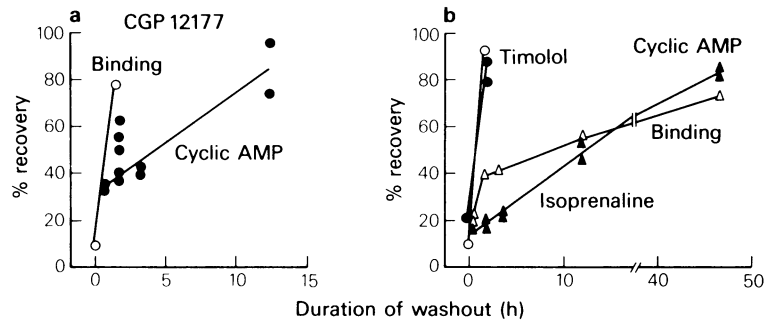


Figure 5 Recovery of β-adrenoceptor binding (open symbols) and of isoprenaline-stimulated cyclic AMP response (filled symbols) after 3–5 days exposures to nonlabelled CGP 12177 (2 nM), timolol (9 nM) or isoprenaline (10 μM). All data are normalized with respect to control values from similarly treated cultures which were not exposed to β-receptor ligands. Experimental procedure and analysis as described in the legend to Figure 4b. (a) Recovery of binding after CGP 12177 treatment was measured in one experiment. Recovery of cyclic AMP formation was measured in 2–5 cultures from 2 different cell preparations. (b) [^{3}H]-CGP 12177 binding after washout of timolol was studied in one experiment, the return of isoprenaline-stimulated cyclic AMP formation was measured in 2 cultures 5 and 105 min after removal of timolol. B_{max} values for [^{3}H]-CGP 12177 binding after chronic exposure to isoprenaline were estimated after washout periods ranging between 15 min and 48 h using one batch of cells. Receptor-mediated cyclic AMP formation was studied in 2–5 cultures per time point over the same period.

grown for 2 days in the presence of 0.2 nM carazolol, and then washed free of the antagonist. After 3 h, 68% of the total number of receptors were available for CGP 12177 binding whereas maximal cyclic AMP formation remained at 10.9% of the value for untreated cells. Similarly, after 8 h washing the relative recovery was 81% for receptor binding, but only 28.9% for isoprenaline-stimulated cyclic AMP accumulation.

The recovery of β-receptor binding and receptor-mediated cyclic AMP response after 3–4 days of treatment with nonradioactive CGP 12177 is illustrated in Figure 5a. We calculated that 19% of the receptor population were free after binding equilibrium had been reached in the presence of 2 nM CGP 12177. A 90 min washing period was sufficient to raise the fraction of free receptors to 80%. But again, the isoprenaline-stimulated cyclic AMP response was restored with considerable delay. Within 105 min after removal of CGP 12177, mean maximal cyclic AMP accumulation reached 48.9 ± 4.8% of the control value. A washing period of 12.5 h was needed to bring the cyclic AMP response back to 84.8%. Discrepant recovery rates for receptor binding and receptor-mediated cyclic AMP formation were not a common property of all β-adrenoceptor antagonists in the rat heart system. After exposure of cardiac cell cultures to timolol for 5 days and subsequent washout, binding sites and β-adrenoceptor cyclic AMP response reappeared in parallel (Figure 5b). By the time receptor availability had increased from 19 to 85.4%, maximal cyclic AMP formation had risen from 22.8 to 83.7%. Similar results were obtained with (±)-propranolol (data not shown).

Isoprenaline-induced desensitization in heart cell cultures

The 'desensitizing' effect of antagonists described above has some analogy with the well-known agonist-induced down-regulation of β-adrenoceptor responses. For a direct comparison of the two processes in our system, we exposed heart cell cultures for 3–5 days to 10 μM isoprenaline. After washout of isoprenaline, we followed the recovery of receptor binding and the reappearance of a receptor-coupled cyclic AMP response for up to 46 h. In the experiments plotted in Figure 6a we have measured specific binding of [^{3}H]-CGP 12177 and agonist-stimulated cyclic AMP formation 15 min after removal of isoprenaline. Compared to controls, maximal specific binding was reduced to 33.7 ± 4.2% ($n = 3$) whereas the affinity for the antagonist remained unchanged. Under the same conditions cyclic AMP formation in the presence of 10 μM isoprenaline had decreased to 16.4 ± 2.6% ($n = 5$, Figure 6b). The β-adrenoceptor system recovered slowly from this agonist-induced desensitization (Figure 5b). Availability of β-receptors for antagonist binding and maximal cyclic AMP formation both reached about 80% of the control values after an incubation period of 46 h in the absence of isoprenaline. Similar results have been obtained earlier in other systems (see Perkins *et al.*, 1982). However, the rate of reappearance of receptors and of hormone-sensitive adenylase cyclase activity were similar during recovery from chronic agonist-dependent desensitization. This is in marked contrast to the behaviour of the system following chronic treatment with antagonists.

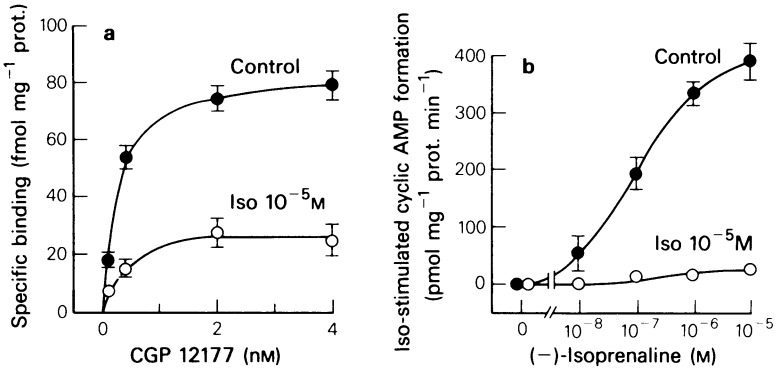


Figure 6 Loss of apparent receptor sites and of receptor-mediated cyclic AMP response during a 4 day exposure of heart cells to isoprenaline 10 μM. (a) Equilibrium binding curves for [^{3}H]-CGP 12177 under control conditions and after isoprenaline (Iso) treatment. The data are mean values of 6 cultures from 2 independent cell preparations; s.e.mean indicated by vertical lines. (b) Concentration-response curves for isoprenaline-stimulated cyclic AMP formation under control conditions and after chronic agonist exposure. Stimulation of desensitized cells was started after a 15 min isoprenaline washout period. Data points are the mean of 8 cultures from 2 independent cell preparations; s.e.mean indicated by vertical lines. Mean maximal stimulation with isoprenaline 10 μM reached 16.4 ± 2.6% of control values ($n = 5$). Points without bars in desensitized cells: s.e.mean smaller than symbol.

Discussion

An important aspect of our results is the surprising difference in the kinetics of carazolol dissociation when initiated by competitive displacement or infinite dilution. The dilution, but not the displacement experiments indicate the presence of at least two high affinity binding sites for carazolol. Assuming a mean association rate constant of $4 \times 10^8 M^{-1}min^{-1}$ for carazolol in cardiac tissue (Manalan *et al.*, 1981) and dissociation rate constants of 0.017 and 0.0032 min^{-1} (this study) yields K_D values of 42 and 8 pM, respectively, for the two sites. The larger number corresponds to the K_D value which we have measured earlier in short term equilibrium binding studies where carazolol binding sites appeared as a homogeneous population (Porzig *et al.*, 1982), and is somewhat higher than the value which can be derived from our competitive displacement data (28 pM). However, in dilution experiments only 26.5% of totally bound carazolol dissociated with the faster rate, accounting for not more than 37–50% of the amount which is specifically bound under equilibrium conditions or which could be displaced by a competitive ligand. Hence, the slowly dissociating component of carazolol binding must also represent, at least partially, loss of ligand from specific sites. The combination of single K_D values in equilibrium binding studies and two or more dissociation rate constants in kinetic experiments is compatible with a situation where the ratio of isomer receptor states is fixed and does not depend on the ligand concentration. Such models have been carefully analyzed by Prinz (1983) and Prinz & Maelicke (1983) for the nicotinic acetylcholine receptor. Biphasic dissociation curves for high affinity receptor ligands have been repeatedly observed in the β-adrenoceptor as well as in the muscarinic cholinoceptor system (Ross, *et al.*, 1977; Galper *et al.*, 1977; Klein, 1980). Their interpretation by a receptor isomerization model has been questioned by Bürgisser *et al.*, (1981). These authors showed that in some cases biphasic dissociation kinetics of racemic ligands can be explained plausibly by the large differences between the dissociation rates of the (+)- and (−)- isomers. For the following reasons it is very unlikely that this explanation holds for our results with carazolol. (1) With increasing equilibration time the contribution of the low affinity (+)-isomer to overall dissociation rates becomes very small because, under equilibrium conditions, it will occupy less than 5% of the available receptor sites (Bürgisser *et al.*, 1981). The equilibrium condition is met in our study. (2) Any significant contribution of the (+)- isomer would have affected equally the dissociation kinetics under competitive displacement and infinite dilution conditions (compare Manalan *et al.*, 1981). This was not observed.

Alternatively, the slow component of carazolol dissociation may be ascribed to the release from an intracellular compartment (e.g., internalised receptor-ligand complexes) rather than from isomeric receptor sites on the membrane surface. The fact that apparent unspecific binding was indeed larger in competitive displacement than in dilution experiments (52 versus 26% of initial total binding, see Figure 1), points to a non-displaceable fraction of specifically bound carazolol which may be located intracellularly. Quantitatively, this fraction could

contribute about 60% of the slowly dissociating ligand.

On the other hand, several of our findings argue against a substantial specific or unspecific intracellular accumulation of carazolol. (1) Total binding was constant during 5 days of continuous exposure (see Table 1). (2) Dissociation kinetics of carazolol in dilution or displacement assays were essentially the same after a 90 min or a 5 day equilibration period. (3) If the lipophilic ligand carazolol labelled intracellular and extracellular β-receptors alike, as suggested for the antagonist [^{3}H]-dihydroalprenolol by Staehelin & Simons (1982), specific binding of carazolol should have exceeded specific binding of the hydrophilic ligand CGP 12177. This was never observed.

We conclude that receptor isomerization is the most likely explanation for the biphasic dissociation kinetics of carazolol in dilution experiments. Furthermore one has to assume that isomer formation is reversed by keeping the overall receptor saturation high in the presence of the inert antagonist timolol or of the agonist isoprenaline. With both ligands, displacement curves became monophasic. The stimulating effect of timolol on total carazolol binding was not shared by isoprenaline, nor did it occur during displacement of [^{3}H]-CGP 12177 and hence, may represent an independent manifestation of some cooperative interaction between the two ligands.

Dissociation of CGP 12177

The dissociation kinetics of this hydrophilic antagonist were monoexponential in both, the displacement and the dilution assay. Consequently, the receptor population appeared homogeneous with respect to its affinity for CGP 12177. Assuming an association rate constant for CGP 12177 of $2.9 \times 10^7\,\text{M}^{-1}\text{min}^{-1}$ in intact cardiac cells (Porzig *et al.*, 1982), the present dissociation rates would correspond to K_D values ranging between 0.23 and 0.44 nM. This agrees with our previous estimate of 0.35 nM for K_D from equilibrium binding studies. Nevertheless, the kinetic parameters under the two conditions differed in important aspects: (1) The doubling of the dissociation rate constant from $0.0068\,\text{min}^{-1}$ in dilution to $0.0128\,\text{min}^{-1}$ in displacement assays indicates that cooperative enhancement of the dissociation rate by a second ligand seems to be a common property of CGP 12177– and of carazolol-liganded receptors. In our earlier study we failed to observe this effect of timolol on CGP 12177 dissociation because we terminated our measurements after 150 min, a time sufficient to estimate displacement, but too short to estimate dilution rate constants. (2) Under our conditions almost 50% of total CGP 12177 binding was not displaceable in the competitive assay. If all of this was simply unspecific binding to the membrane surface, then a rapid loss should have preceded the slow monoexponential decrease of bound ligand.

Since such rapid loss of label has not been observed and in addition, intracellular uptake of this compound is probably small (Staehelin & Simons, 1982), we have to conclude that a considerable fraction of high affinity CGP 12177 binding is not accessible for the displacing ligand. The nature and the significance of these 'concealed' sites remain completely unknown.

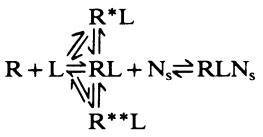
Antagonist-induced 'uncoupling' of receptors

Our results indicate that chronic exposure of living cells to carazolol or CGP 12177 produces some kind of 'desensitization' that is characterized by a striking time delay between the early recovery of receptor binding capacity and the later reappearance of receptor-mediated adenylate cyclase stimulation. It is tempting to attribute this loss of cyclic AMP response to the weak agonistic activity of these ligands, since partial agonists are known to have strong desensitizing effects (Franklin & Twose, 1979; Su *et al.*, 1980; Perkins *et al.*, 1982). However, closer inspection of the data suggests that this interpretation is not valid. Typically, the availability of receptors and the cyclase response reappeared in parallel rather than successively during recovery from long-term agonist-induced desensitization (Su *et al.*, 1980; Perkins, 1983, see also Figure 6). In the most sensitive tests (positive chronotropic effect in spontaneously beating rat or guinea-pig atria) half maximal partial agonistic activity was observed with 32 nM CGP 12177 (own unpublished results) or 200 nM carazolol (Kaumann *et al.*, 1979). In C6 rat glioma cells half maximal stimulation of cyclic AMP formation by CGP 12177 required about 100 nM (Staehelin & Simons, 1982). In our experiments, long term incubations were maintained with 0.2 nM free carazolol and 4 nM CGP corresponding to 1/1000$^{\text{th}}$ and 1/8$^{\text{th}}$ of their respective K_a values. Since the strength of the desensitizing effect of partial agonists parallels their agonistic potency (see Perkins *et al.*, 1982) CGP 12177 should have been much more efficient than carazolol. The contrary has been observed. Therefore, we suggest that the decoupling action of the two antagonists is caused by a mechanism different from typical desensitization. Most likely these ligands disturb the interaction of β-receptors with the guanylnucleotide binding protein (N-protein). The known effects of guanylnucleotides on the binding properties of several high affinity lipophilic pindolol derivatives (Wolfe & Harden, 1981) support this view.

In summary the results of the present study confirm our previous suggestion that competitive β-adrenoceptor antagonists in current clinical or experimental use cannot be considered a homogeneous

group of drugs, even if equilibrium binding data for all of them seem to show reversible interaction with a single class of sites.

Our data are not sufficiently complete to allow the construction of detailed kinetic models. One of several possible reaction schemes is shown below and can account for the observed interactions of different antagonists with the cardiac β-adrenoceptor system. In this scheme R, R* and R** represent the native receptor and two of its isomeric forms, L is an agonist or antagonist ligand and N_s the stimulatory nucleotide binding protein. Within the very limited sample of six β-receptor blockers which we have tested in this and an earlier study (Porzig *et al.*, 1982), three different patterns of interaction emerged.



Carazolol-type ligands will bind to R* and R**, the latter form having a high affinity for carazolol, will show a low apparent affinity for isoprenaline (see Porzig *et al.*, 1982). Low agonist affinity has been observed repeatedly in intact cell systems with different ligands (Terasaki & Brooker, 1978; Pittman & Molinoff, 1980; 1983; Toews *et al.*, 1983; Insel *et al.*, 1983). Most of the authors suggested that a rapid agonist-induced desensitization was the reason for the apparent decrease in agonist affinity. However, all of these studies used high affinity lipophilic antagonists as radiolabels. Most likely these drugs classify as carazolol-type ligands (see Porzig *et al.*, 1982 for data on iodocyanopindolol). We have shown that no decrease in agonist affinity occurred in the presence of the hydrophilic label CGP 12177. Therefore we conclude that R** is inducible by carazolol-type antagonists.

Both, R* and R** are supposed to have a reduced capacity of interacting with N_s. The slow recovery of the receptor-mediated cAMP response may reflect slow R* → R and R** → R transition rates.

CGP 12177-type ligands will induce the R* form of the receptor which differs from R only in its failure to interact properly with N_s.

Timolol-type ligands will bind to R without changing any of its properties. These compounds, propranolol among them, fulfil the criteria of pure competitive antagonists.

It is obvious from this listing that precise knowledge of the pharmacodynamic properties of individual β-blockers is a prerequisite for using a particular compound as a tool in β-adrenoceptor research. Moreover, these findings may explain the long duration of action of some rapidly excreted β-blockers (see Abshagen & Möllendorff, 1980; Antonaccio & Evans, 1980) and provide a rational basis for the selection of β-antagonists for therapeutic purposes.

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