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CHARACTERIZATION OF β-ADRENOCEPTORS ON RAT SKELETAL MUSCLE CELLS GROWN *IN VITRO*

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Abstract—The binding properties of an hydrophilic β-adrenergic receptor radioligand, (−)[^{3}H](4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazolo-2-one); ([^{3}H]CGP-12177), were investigated in rat skeletal muscle cells in culture. The binding of [^{3}H]CGP-12177 at 25° was saturable, reversible and of high affinity ($K_d = 1.3 \pm 0.3$ nM). The maximal number of [^{3}H]CGP-12177 binding sites was 30.6 ± 3.2 fmol/dish (34 ± 3.5 fmol/mg protein). β-Adrenergic agonists and antagonists inhibited [^{3}H]CGP-12177 binding. The competing ligand inhibition binding is a typical one for β_2-adrenoceptors. The increase in β-adrenoceptors was independent of cell fusion. Amiodarone (10^{-5} M) decreased the β-adrenoceptor number in skeletal muscle cells differentiated *in vitro* by 48%, while the affinity for [^{3}H]CGP-12177 was not affected.

Catecholamines have long been known to act on skeletal muscle and to modify its response to both direct and indirect (nerve) stimulation. These biogenic amines affect biochemical and physiological properties of the muscle [1, 2]. The interaction of adrenoceptors with their ligands and the mechanism underlying the resulting changes in cellular activities provide important areas of research for the understanding of normal and abnormal cell function. Therefore, the aim of this work is to further characterize the β-adrenoceptors of rat muscle differentiated in cell cultures using the hydrophilic β-antagonist radioligand, [^{3}H]CGP-12177* [3]. Amiodarone has been shown to antagonize the effects of catecholamines but the mechanism of this action is unknown [4, 5]. In the present investigation we evaluated the effects of amiodarone on the amount of β-adrenoceptors. In view of discrepant reports in the literature on the number of adrenoceptors in myogenic cells [6, 7], it was also pertinent to correlate the level of the receptors with myogenesis.

MATERIALS AND METHODS

Preparation of cultures. Skeletal muscle cell cultures were prepared from thigh muscles of 1–2-day-old rats as already described [8, 9]. The limbs were removed, washed in phosphate-buffered saline (PBS) to remove excess blood cells, and then transferred to a Ca-free, 0.25% trypsin solution containing EDTA (1 mM) for incubation with continuous stirring at 37°. Cells were collected after serial trypsinizations (successive 10 min periods) until all tissue was dispersed, and then centrifuged for 5 min at 500 *g*. Pellets were resuspended in growth medium and preplated for 20–30 min to reduce the number of fibroblasts. The supernatant was collected and the remaining myoblasts were diluted with growth medium to a concentration of 0.8×10^6 cells/mL for plating in 35 mm collagen-coated plastic tissue culture dishes (Nunc, Roskilde, Denmark, 1.5 ml/dish). Cultures were grown in a water-saturated atmosphere of 90% air, 10% CO_2 at 37°. The composition of the growth medium was as follows: Dulbecco's Minimal Essential Medium (Gibco, Uxbridge, U.K.), 83%; horse serum (Biolab, Jerusalem, Israel), 15%; chick embryo extract, 2%. Unless otherwise indicated, experiments were done on cultures of 6–10 days *in vitro* (DIV).

Ligand binding. Intact cells were incubated at room temperature (22–25°) for 45 min, with various concentrations of [^{3}H]CGP-12177, in PBS, pH 7.4. Incubation was stopped by rinsing the cells 10 times with cold (4–10°) PBS. The cells were solubilized with 0.3 mL Triton X-100 (1%) and radioactivity determined by scintillation counting. Non-specific binding of [^{3}H]CGP-12177 was defined as the amount of radioactivity remaining after incubation with L-alprenolol (10^{-4} M). Specific [^{3}H]CGP-12177 binding was calculated as the total radioactivity bound minus the non-specific binding (less than 20%). For calculation of association rate constant, k_1, specifically bound [^{3}H]CGP-12177 (3 nM) was determined as a function of time indicated in the graph. In dissociation of specific [^{3}H]CGP-12177 binding experiment, cells were incubated at 25° with 3 nM [^{3}H]CGP-12177 to equilibrium (about 45 min). At time zero, the binding mixture was replaced by PBS (1 mL) containing L-alprenolol (10^{-4} M). The experiment was terminated like in ligand binding method. From the slope of the plot, k_2 was calculated. For competition experiments, cells were incubated in a mixture containing the competing drug and [^{3}H]CGP-12177 at a concentration of 3 nM for 45 min at room temperature. In the decreased [^{3}H]CGP-12177 binding experiment, cells were preincubated

* Abbreviations: [^{3}H]CGP-12177, (−)[^{3}H](4-(3-tert-butylamino-2-hydroxypropoxy)-benzimidazolo-2-one); EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; alp, alprenolol; prop, propranolol; isop, isoproterenol; meto, metoprolol; clenb, clenbuterol.

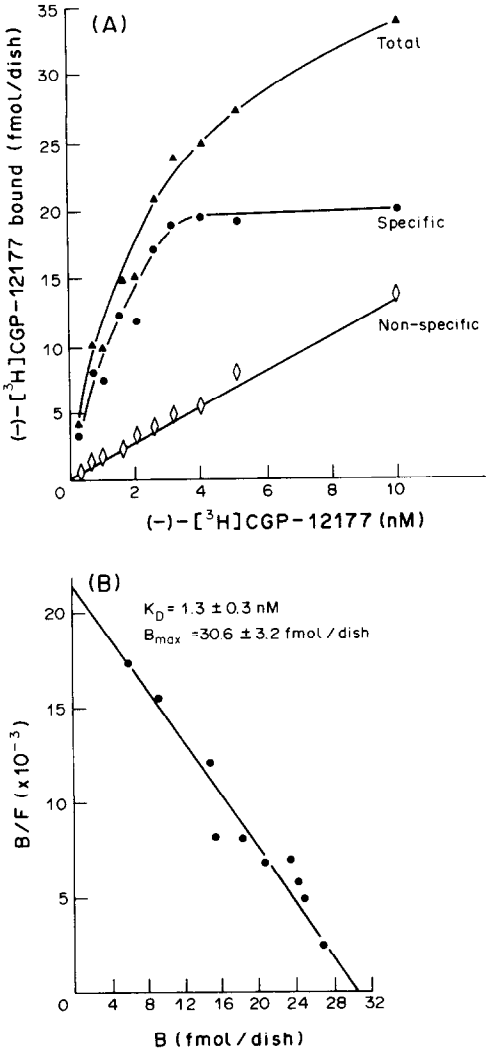

Fig. 1. Specific binding of [^{3}H]CGP-12177 to skeletal muscle cells. (A) Seven-day-old rat muscle cultures were exposed to the indicated concentrations of the radioligand as described in Materials and Methods. Specific binding was defined as the [^{3}H]CGP-12177 binding displaceable by 10^{-4} M L-alprenolol. Data points represent means of duplicate determinations from a representative experiment of sister cultures. (B) Scatchard plot of specific [^{3}H]CGP-12177 binding of Fig. 1A. The K_d for [^{3}H]CGP-12177 was 1.3 ± 0.3 nM and the maximal binding capacity was 30.6 ± 3.2 fmol/dish. (The average amount of protein per dish was 0.9 mg.)

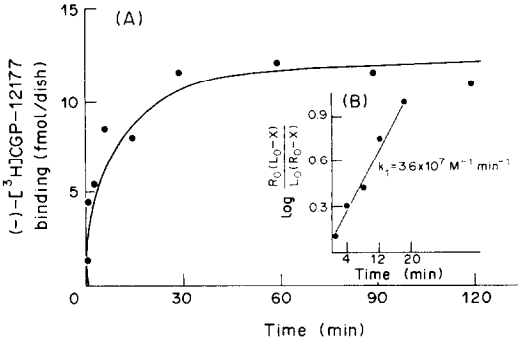

Fig. 2. Time course of [^{3}H]CGP-12177 binding to skeletal muscle cells. (A) Seven-day-old myotubes were incubated in the presence or absence of 10^{-4} M alprenolol at 25°. Specific binding was determined as described in Materials and Methods. Each point is the average of triplicate sister cultures. (B) Regression line of association curve of Fig. 2A determined by least squares fit of log $[R_0(L_0\text{-}X)/L_0(R_0\text{-}X)]$ at different times of the initial phase of a second order reaction. $[R_0]$ and $[L_0]$ are unoccupied receptor and free ligand concentrations respectively, at the beginning of the association reaction, and $[X]$ equals the concentration of bound ligand at time t. The association rate constant $k_1 = 3.6 \times 10^7$ M^{-1} min^{-1}.

with amiodarone (10^{-5} M) 2 hr in PBS and binding assay was performed during 45 min. Amiodarone–HCl was dissolved in 95% ethanol at a concentration of 10^{-2} M and diluted at 10^{-3} M in 2 × distilled water. The final solution was obtained by adequate dilution with PBS. The final concentration of ethanol in the dish is 0.1%. This concentration of ethanol did not change the [^{3}H]CGP-12177 binding. Protein determination was performed according to the Lowry method using bovine serum albumin as standard [10].

The following drugs were used: L-isoproterenol, DL-propranolol, L-alprenolol, epinephrine, norepinephrine, phenylephrine, DL-metoprolol(+)-tartrate and amiodarone–HCl were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Clenbuterol was a gift from J. Etlinger. (−)[^{3}H]CGP-12177, sp. act. 45–55 Ci/mmol, was purchased from Amersham (Little Chalfont, Bucks, U.K.).

RESULTS

Characterization of the β-adrenergic receptors

Intact rat muscle cultures were incubated with various concentrations of [^{3}H]CGP 12177 in the presence or absence of alprenolol. Figure 1A shows the relationship between the radioligand concentrations and the number of specific binding sites in the cultured myotubes. The maximal saturation of the antagonist occurred at a concentration of 3 nM. Scatchard analysis [11] indicates that the maximum number of binding sites is 30.6 fmol/dish (34 fmol/mg protein) and the K_d for [^{3}H]CGP-12177 is 1.3 nM (Fig. 1B).

Specific binding reached equilibrium levels within 30 min at 25°. Association rate constant, calculated according to Engel *et al.* [12], was found to be $k_1 = 3.6 \times 10^7$ M^{-1} min^{-1} (Fig. 2).

Figure 3 shows dissociation of [^{3}H]CGP-12177 from its binding sites that was measured as described in Materials and Methods. At 25° the dissociation rate constant k_2 was found to be 0.03 min^{-1}. At 37°, 60% of the specifically bound [^{3}H]CGP-12177 were dissociated within 30 min. The ratio $k_2/k_1 = 0.83$ nM provides an independent measurement of the K_d for the interaction of [^{3}H]CGP-12177 with its receptor, which is in good agreement with the values obtained by steady state analysis (1.3 nM).

In order to study whether [^{3}H]CGP-12177 is inter-

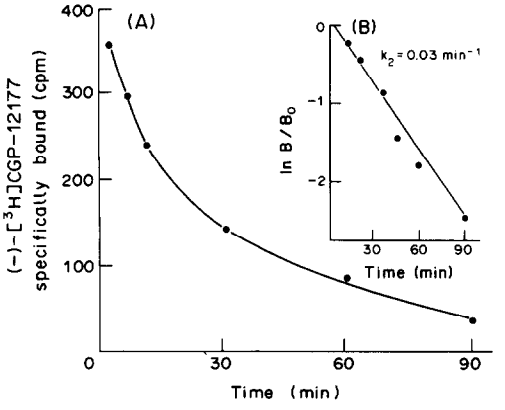

Fig. 3. Dissociation curve of [^{3}H]CGP-12177 binding from skeletal muscle cells. (A) Reverse kinetic of dissociation of [^{3}H]CGP-12177 from skeletal muscle cells (7-day-old). Specific binding at 25° was determined at subsequent time intervals after that the binding mixture was removed and PBS containing alprenolol (10^{-4} M) was added. Each value is the mean of duplicate determinations. (B) First order kinetic plot of dissociation of [^{3}H]CGP-12177 binding shown in Fig. 3A. B = amount of [^{3}H]CGP-12177 bound at each time after dilution of the binding mixture. B_0 = amount of [^{3}H]CGP-12177 bound at time 0. The dissociation constant $k_2 = 0.03\ min^{-1}$.

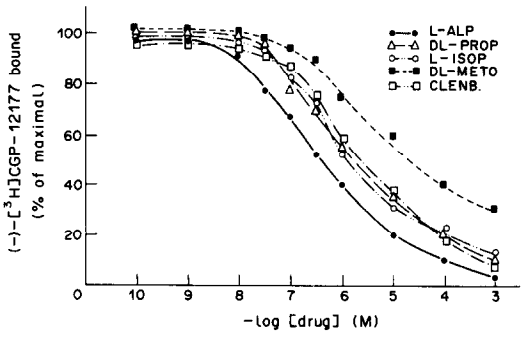

Fig. 4. Inhibition of [^{3}H]CGP-12177 binding to skeletal muscle cells. Seven-day-old myotubes were incubated in the presence of [^{3}H]CGP-12177 (3 nM) and increasing concentrations of different drugs. After 45 min of incubations, at room temperature, specific binding was estimated as described in Materials and Methods. The results are expressed as the percentage of [^{3}H]CGP-12177 specifically bound. Data points represent means of duplicate determinations from a representative experiment.

nalized by the myotubes, we used the Mahan *et al.* method [13] to remove the ligand from its receptors. In this method the myotubes, following ligand binding, were washed with acetic acid pH 2.5. The residual radioactivity of the cells (approx. 10%) indicated the amount of internalized ligand.

The number of adrenoceptors in rat skeletal muscle cells in culture increased progressively from the onset of cell fusion until it reached a plateau at maximum value, when cells became aged 6–7-day-old *in vitro* (Table 1). This pattern of receptors increase was unchanged when cell fusion was inhibited by reducing Ca^{2+} concentration in the medium (Table 1). Thus, the appearing of β-adrenoceptors is independent of muscle differentiation, confirming previous finding on muscle cell lines [6].

Inhibition of [^{3}H]*CGP*-12177 *binding*

We examined effects of several adrenoceptor agonists and antagonists on specific inhibition of [^{3}H]CGP-12177 binding to cultured myotubes. As shown in Fig. 4, the β-agonists, isoproterenol and clenbuterol and the β-antagonists, alprenolol, propranolol and metoprolol effectively competed for binding sites with [^{3}H]CGP-12177. The inhibition constants (K_i) and IC_{50} of each drug were calculated by the method of Cheng and Prusoff [14] (Table 2). In contrast, L-norepinephrine and L-epinephrine at concentrations of 0.1 mM, reduced [^{3}H]CGP-12177 binding by only 5% and 20%, respectively (not illustrated). Phentolamine, a potent alpha receptor antagonist, did not inhibit specific binding of [^{3}H]CGP-12177 even at 10^{-5} M, confirming previous results [15].

Decrease of CGP-12177 *binding by amiodarone*

To determine whether amiodarone exerts its effect through β-adrenoceptors we measured its influence on [^{3}H]CGP-12177 binding (Fig. 5A). Scatchard analysis of this experiment shows a decrease of 47.5% in receptor number in amiodarone-treated cells, without a significant change in its affinity to the ligand (Fig. 5B).

DISCUSSION

[^{3}H]CGP-12177 has a chemical structure, similar

Table 1. The effect of EGTA on the development of β-receptors in skeletal muscle cells

	Days in culture				
	2	3	4	6	7
β-Receptor number in EGTA-treated cells (fmol/mg protein)	6.0 ± 0.8	11.8 ± 2.2	14.8 ± 2.0	18.0 ± 1.5	17.3 ± 2.3
β-Receptor number in control non-treated cells (fmol/mg protein)	7.5 ± 0.9	9.5 ± 1.9	13.2 ± 2.1	17.3 ± 1.3	17.6 ± 1.8

EGTA (1.7 mM) was added after one day in culture, to prevent cell fusion. The results are means of triplicate determinations ± SE.

Table 2. Competitive inhibition of [^{3}H]CGP-12177 by β-adrenergic agonists and antagonists

Competitive ligand	Receptor	K_i (μM)	IC_{50} (μM)
Antagonists			
L-Alprenolol	$\beta_2 > \beta_1$	0.18 ± 0.06	0.6 ± 0.07
DL-Propranolol	$\beta_2 = \beta_1$	0.54 ± 0.15	1.8 ± 0.1
DL-Metoprolol	β_1	7.6 ± 2.9	25 ± 4.0
Agonists			
L-Isoproterenol	$\beta_2 = \beta_1$	0.36 ± 0.14	1.2 ± 0.2
Clenbuterol	β_2	0.67 ± 0.27	2.2 ± 0.4

Values (±SE) of the inhibition constant (K_i) for each of the competitive ligands were calculated according to the following equation:

$$K_i = \frac{IC_{50}}{(1 + L/K_d)},$$

where K_i = inhibition constant of a non-radioactive drug for the radioactive drug binding site; IC_{50} = the concentration of non-radioactive drugs that inhibits 50% of the radioactive drug binding; L = free concentration of the radioactive drug (3 nM) in the incubation solution; and K_d = equilibrium dissociation constant (1.3 nM) of the radioactive drug for the binding site.

to those of other β-adrenergic ligands. The side chain is of the *N*-tertiary butyl type, which is reported to be of higher affinity than the corresponding *N*-isopropyl analogues [16]. In addition, [^{3}H]CGP-12177 blocks both *in vivo* and *in vitro* responses to the β-adrenergic agonist isoproterenol.

Since we were working with intact cells, it was important to use a ligand with low non-specific binding sites and with a high affinity for the beta-receptor. The reason for the low non-specific binding of [^{3}H]CGP-12177 is probably due to its hydrophilicity [3]. Thus, it binds mainly to the cell surface receptors [17]. In support of this, we demonstrate that this ligand is almost not internalized into the cells in comparison to dihydroalprenolol, a lipophilic ligand.

In previous studies it was shown that the K_d of [^{3}H]CGP-12177, in different systems varied from 0.2–0.5 nM [6, 17, 18]. In these experiments we showed a K_d = 1.3–2.9 nM, a little higher than in other systems examined with the same ligand. These values could result from different membrane preparations and cell types.

It has not been clear whether the increased number of β-adrenoceptors in primary rat muscle cultures is a function of cell growth or a specific activation of the genes associated with β-receptors during muscle differentiation. To address this problem, Schonberg *et al.* [6] used L_6E_9 muscle cell line which can be distinguished by conditions of cell growth or cell differentiation. Using [^{125}I]ICYP as a probe for β-adrenoceptors it was shown that both differentiating and non-differentiating cells exhibited an increase in β-adrenoceptors. Similar results were shown in this study for primary rat muscle cultures, suggesting that the process of myogenesis and the process of receptor appearance are not linked.

Competitive binding experiments showed that [^{3}H]CGP-12177 displaced by series of β-adrenergic ligands in an order of potency corresponding to their known potencies on β-adrenergic systems. The β-antagonist ligand, L-alprenolol, had the highest potency to displace [^{3}H]CGP-12177. At a concentration of 0.6 μM it displaced 50% of [^{3}H]CGP-12177 binding. L-Alprenolol is the most potent competitive ligand of those that inhibited [^{3}H]CGP-12177 binding site. Propranolol and the two other agonists, isoproterenol and clenbuterol have a very similar

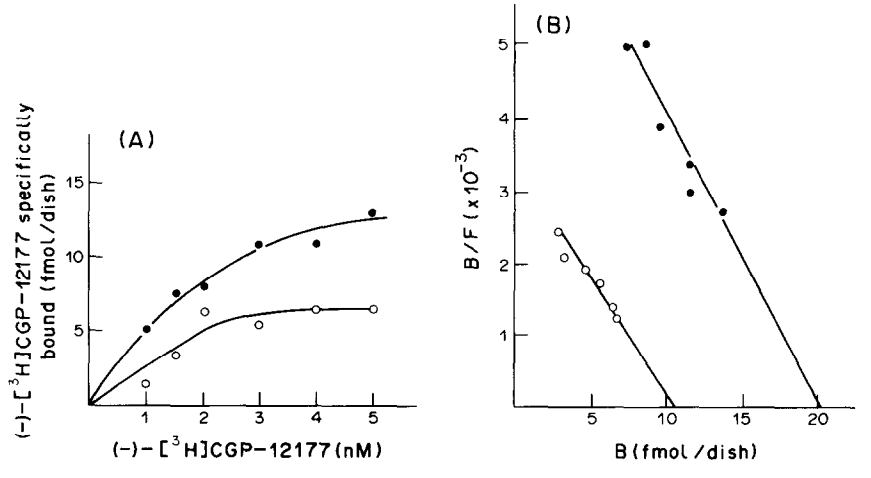

Fig. 5. Effect of amiodarone on adrenergic receptors. (A) Specific binding of [^{3}H]CGP-12177 to amiodarone-treated skeletal muscle cells as a function of [^{3}H]CGP-12177 concentrations. Binding was carried out as described in Fig. 1. Six-day-old myotubes were treated with amiodarone 10^{-5} M (open symbols) for 2 hr, and binding was performed during 45 min. Data points represent means of duplicate determinations from three experiments. (B) Scatchard analysis of the Fig. 5A. The K_d for [^{3}H]CGP-12177 from control (closed symbols) and amiodarone-treated cells (open symbols) are respectively 2.5 ± 0.8 nM and 2.9 ± 0.6 nM. The B_{max} are 20 fmol/dish and 10.5 fmol/dish respectively (amount of protein/dish = 0.6 mg).

IC_{50}; 1.8, 1.2 and 2.2 μM respectively. Metoprolol, a β_1-selective antagonist, inhibits [^{3}H]CGP-12177 binding only at a high concentration (IC_{50} = 25 μM). These results indicate that the adrenoceptors on skeletal muscle membrane seem to be of the β_2 subtype [19], with a higher affinity for the β_2-competitive ligand than for β_1-competitive ligand like metoprolol. In our system, L-norepinephrine almost did not inhibit the [^{3}H]CGP-12177 binding, whereas, L-epinephrine displaced the [^{3}H]CGP-12177 binding only at a concentration of 0.1 mM and above (20% inhibition). A possible explanation for the above results is that the affinity of [^{3}H]CGP-12177 for β-adrenoceptors on muscle cells is much higher than β-adrenergic agonist such as epinephrine and norepinephrine. The α-adrenergic ligand phentolamine did not displace the [^{3}H]CGP-12177 bound to muscle cells, which proved that the radioligand was not bound to α-adrenoceptors. However, the existence of α-adrenoceptors on the myotubes is not ruled out.

In recent years, it was found that regulation of the adrenergic response occurs through variations in the number of adrenoceptors. Studies have shown that an anti-arrythmic drug, amiodarone, can regulate the β-adrenoceptors by reducing their numbers [4, 5]. The amiodarone action on the β-receptors has been explained by a non-competitive inhibition which occurs only at high dosages [20]. According to this explanation, our data strongly suggest that amiodarone decreases the density of β-adrenoceptors on the surface of skeletal muscle cells without changing the affinity of [^{3}H]CGP-12177 to the receptors. Two possible mechanisms may underly the action of amiodarone: the first one is a non-competitive inhibition where amiodarone binds to a regulatory site on the receptor and alters [^{3}H]CGP-12177 binding. The second is that amiodarone provokes the internalization of the receptors into the cytoplasm and the hydrophilic ligand [^{3}H]CGP-12177 cannot bind to the masked receptors. According to our results we cannot differentiate between these two mechanisms. Whether this decrease caused by amiodarone affects later events is under current investigation.

In conclusion, [^{3}H]CGP-12177 is a hydrophilic β-adrenoceptor ligand, which gives low non-specific binding with intact cells, and which probably binds mainly to cell surface receptors [3, 17, 21]. In this paper we show that the number of β-adrenoceptors on the muscle cells *in vitro* is similar to those reported in previous studies performed in other systems [22, 23]. According to these results, it is now important to extend this study and to learn the regulation phenomenon and biosynthesis of β-adrenoceptors on the plasma membrane of muscle cells.

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REFERENCES

1. Stiles GL, Caron MG and Lefkowitz RJ, Beta adrenergic receptors: biochemical mechanisms of physiological regulation. *Physiol Rev* **64**: 661–743, 1984.
2. Bowman WC and Nott MW, Actions of sympathomimetic amines and their antagonists on skeletal muscle. *Pharmacol Rev* **21**: 27–72, 1969.
3. Staehelin M, Simons P, Jaeggi K and Wigger N, CGP-12177, A hydrophilic β-adrenergic receptor radioligand reveals high affinity binding of agonist to intact cells. *J Biol Chem* **258**: 3496–3502, 1983.
4. Nokin P, Clinet M and Schoenfeld P, Cardiac β-adrenoceptor modulation by amiodarone. *Biochem Pharmacol* **32**: 2473–2477, 1983.
5. Polster P and Broekhuysen J, The adrenergic antagonism of amiodarone. *Biochem Pharmacol* **25**: 131–134, 1976.
6. Schonberg M, Morris SA, Krichevsky A and Bilezikian JP, The use of [^{125}I]iodocyanopindolol as a specific probe for beta-adrenergic receptors in differentiating cultured rat skeletal muscle. *Cell Diff* **12**: 321–327, 1983.
7. Schonberg M, Bilezikian JP, Apfelbaum M and Been RC, Beta-adrenergic receptors and myogenesis. *J Cyclic Nucleotide Res* **4**: 55–69, 1978.
8. Shainberg A, Yagil G and Yaffe D, Alterations of enzymatic activities during muscle differentiation *in vitro*. *Dev Biol* **25**: 1–29, 1971.
9. Shainberg A and Brik H, The appearance of acetylcholine receptors triggered by fusion of myoblast *in vitro*. *FEBS Lett* **88**: 327–331, 1978.
10. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
11. Scatchard G, The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
12. Engel G, Houer D, Berthold R and Wagner H, [^{125}I]Cyanopindolol, a new ligand for β-adrenoceptors. Identification and quantitation of subclasses of β-adrenoceptors in guinea pig. *Naunyn Schmiedebergs Arch Pharmacol* **317**: 277–285, 1981.
13. Mahan LC, Motulsky HJ and Insel PA, Do agonists promote rapid internalization of β-adrenergic receptors? *Proc Natl Acad Sci USA* **82**: 6566–6570, 1985.
14. Cheng YC and Prusoff WH, Relationship between the inhibition constant K_i and the concentration of inhibition which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
15. Furchgott RF, The pharmacological differentiation of adrenergic receptors. *Ann NY Acad Sci* **139**: 553–563, 1967.
16. Kaumann AJ, Morris TH and Birnbaumer L, A comparison of the influence of *N*-isopropyl and *N*-tert butyl substituents on the affinity of ligands for sinoatrial β-adrenoceptors in rat atria and β-adrenoceptors coupled to the adenylyl cyclase in kitten ventricle. *Naunyn Schmiedebergs Arch Pharmacol* **307**: 1–8, 1979.
17. Portenier M, Hertel C, Muller P and Staehelin M, Some unique properties of CGP-12177. *J Receptor Res* **4**: 103–111, 1984.
18. Hertel C and Staehelin M, Reappearance of β-adrenergic receptors after Isoproterenol treatment in intact C6-cells. *J Receptor Res* **3**: 35–43, 1983.
19. Reddy NB and Engel WK, *In vitro* characterization of skeletal muscle β-adrenergic receptors coupled to adenylate cyclase. *Biochim Biophys Acta* **585**: 343–359, 1979.
20. Charlier R, Cardiac actions in the dog of a new antagonist of adrenergic excitation which does not produce competitive blockage of beta-adrenoceptors. *Br J Pharmacol* **39**: 668–674, 1970.
21. Staehelin M and Hertel C, [^{3}H]CGP-12177, a β-adrenergic ligand suitable for measuring cell surface receptors. *J Receptor Res* **3**: 35–43, 1983.
22. Pochet R and Schmitt H, Re-evaluation of the number of specific β-adrenergic receptors on muscle cells. Atlas

D, Hanski E and Levitzki A, Reply. *Nature* **277**: 58–60, 1979.
23. Law YH, Robinson RB, Rosen MR and Bilezikian JP, Subclassification of β-adrenergic receptors in cultured rat cardiac myoblasts and fibroblasts. *Circ Res* **47**: 41–48, 1980.