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Functional Studies of the First Selective β_3-Adrenergic Receptor Antagonist SR 59230A in Rat Brown Adipocytes

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SUMMARY

The SS-enantiomer 3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2S)-2-propanol oxalate (SR 59230A) is proposed to be the first β_3-adrenergic receptor antagonist. The present work shows that SR 59230A, unlike its inactive *RR*-enantiomer (SR 59483), antagonized a typical β_3-adrenergic response in vitro, i.e., SR 58611A, the ethyl-[(7s)-7-[[(2*R*)-2-(3-chlorophenyl)-2-hydroxethyl]amino]-5,6,7,8-tetrahydronaphth-2-yl]oxyacetate hydrochloride- or (−)-4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one (CGP 12177)-stimulated synthesis of cAMP in rat brown adipose tissue membranes, with pK_B values of 8.87 ± 0.12 and 8.20 ± 0.15. In addition, SR 59230A had no antagonistic effect on forskolin-induced cAMP accumulation in rat interscapular brown adipose tissue. SR 59230A, in contrast to the selective β_1- and β_2-adrenoceptor antagonists (±)[2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-2 propanol and erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol-hydrochloride did not counteract the cAMP production induced by (−)-isoprenaline or norepinephrine (NE) in rat brain areas rich in β_1- or β_2-adrenoceptors, such as frontal cortex and cerebellum. Moreover, in proliferating brown fat cells, in which the β_1-adrenoceptor is the only β-adrenergic subtype coupled to cAMP production, SR 59230A did not modify the production of cAMP induced by NE, whereas CGP 12177 did. In confluent brown fat cells, in which the β_3-adrenoceptor is the functional β-adrenergic subtype coupled to adenylyl cyclase, SR 59230A antagonized the NE-induced cAMP accumulation and glycerol release without affecting their basal values, whereas CGP 12177, which per se stimulated cAMP accumulation and glycerol release, did not change the NE-induced increase of either parameter. Finally, SR 59230A concentration-dependently counteracted the NE-stimulated synthesis of uncoupling protein gene in confluent brown fat cells, which is considered mainly a result of selective stimulation of β_3-adrenoceptors. These results provide evidence that the new selective β_3-adrenoceptor antagonist can contribute considerably to functional characterization of the β_3-adrenoceptors.

An atypical β-adrenoceptor called the β_3-adrenoceptor has been cloned, starting from human and mouse genomic DNA (1, 2) and rat cDNA (3–5) libraries. It is becoming widely accepted that this receptor mediates the non-β_1- and non-β_2-adrenergic responses of rat BAT and WAT (see review in Ref. 6). It has also been suggested that β_3-adrenoceptors mediate a variety of functions in different tissues and organ systems such as intestine (7), skeletal muscle (8), and possibly heart (9). The BAT β_3-adrenoceptor has been pharmacologically characterized as having a high affinity for selective agonists such as BRL 37344 and SR 58611A and a low affinity and stereoselectivity for the classic β-adrenoceptor antagonists such as propranolol (10, 11); also, it is stimulated by high concentrations of certain antagonists of β_1- and β_2-adrenoceptors such as CGP 12177 (10).

Both functional and binding studies have often been interpreted as showing that only β_1-adrenoceptors are relevant in brown adipocytes (12, 13), without defining a function for a novel β-adrenoceptor subtype. More recent works have demonstrated that the β_3-adrenoceptor is the functionally relevant β-adrenoceptor subtype in BAT (see review and references in Ref. 10). In our experience, β_1- and β_2-adrenoceptor antagonists have shown very low pA_2 values for inhibiting NE-, (−)-isoprenaline-, or varying β_3-adrenoceptor agonist-induced cAMP accumulation, lipolysis, or respiration in rat brown adipocytes (11). The most important problem in de-

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ABBREVIATIONS: BAT, interscapular brown adipose tissue; NE, norepinephrine; UCP, uncoupling protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction; SR 59230A, 3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronaphth-1-ylamino]-(2S)-2-propanol oxalate; SR 58611A, ethyl-[(7s)-7-[[(2*R*)-2-(3-chlorophenyl)-2-hydroxethyl]amino]-5,6,7,8-tetrahydronaphth-2-yl]oxyacetate hydrochloride; CGP 12177, (−)-4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one; CGP 20712A, (±)-[2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-2 propanol; ICI 118,551, erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol-hydrochloride.

tecting and pharmacologically characterizing the β_3-adrenoceptor may be the lack of a selective β_3-adrenoceptor antagonist. The present report describes for the first time the β_3-adrenoceptor antagonistic activity in rat brown adipocytes of a newly synthesized compound, SR 59230A (see Fig. 1 for chemical structure), which belongs to the aryloxypropanolaminotetralin family (14). The affinity and selectivity of SR 59230A for the β_3-adrenoceptor have been investigated in BAT membranes, frontal cortex and cerebellum homogenates, and cultured brown fat cells, challenging this compound against NE, (−)-isoprenaline, and the more selective β_3-adrenoceptor agonists SR 58611A and CGP 12177. The results suggest that SR 59230A is a powerful and selective β_3-adrenoceptor antagonist and that it may be a useful tool for further studies.

Experimental Procedures

Animals. Male Sprague-Dawley rats (180–200 g body weight) were obtained from Charles River (Como, Italy). The animals were housed with lights on from 7:00 a.m. to 7:00 p.m., with free access to standard laboratory chow and water. All animal experiments were conducted in accord with the highest standards of humane animal care.

cAMP determination in BAT membranes and in frontal cortex or cerebellum homogenates. Interscapular BAT pads were homogenized by Ultraturax in 5 volumes 10 mM Tris·HCl buffer, pH 7.4, containing 0.25 M sucrose and 2 mM EGTA. After filtration through a fine gauze, the homogenate was centrifuged at 1000 × *g* for 15 min at 10°. The infranatant was removed with a syringe and ultracentrifuged at 100,000 × *g* for 30 min at 4°. The resulting pellet was resuspended in fresh buffer and then centrifuged and resuspended twice, as described above. The final pellet was resuspended in homogenization buffer (100 mM Tris-maleate, pH 7.5, containing 1.2 mM EGTA). cAMP levels in BAT membranes and frontal cortex and cerebellum homogenates were measured as previously described (15, 16).

Isolation of brown adipocytes. Brown fat precursor cells were isolated as described previously (17). The BAT fragments (12–15% w/v) were carefully dissected under sterile conditions and placed in the HEPES-buffered solution, pH 7.4, described by Néchad *et al.* (17), containing 0.2% (w/v) type II collagenase. After 30 min of enzyme treatment in a shaking water bath with vortexing of the tube every 5 min for 10 sec at 37°, the tissue remnants were removed by filtration through a 250-μm nylon screen. The mature adipocytes and the fat droplets resulting from broken cells were then allowed to float to the surface on the cell suspension in ice for ~30 min. The infranatant, containing adipocyte precursor cells, was then collected with a 2-mm metal syringe, filtered through a 25-μm nylon screen, pelleted by centrifugation for 10 min at 700 × *g* in 10 ml of culture medium (see below), and diluted to 20 ml. Samples of both precursor and mature cells were counted in Burker's chambers.

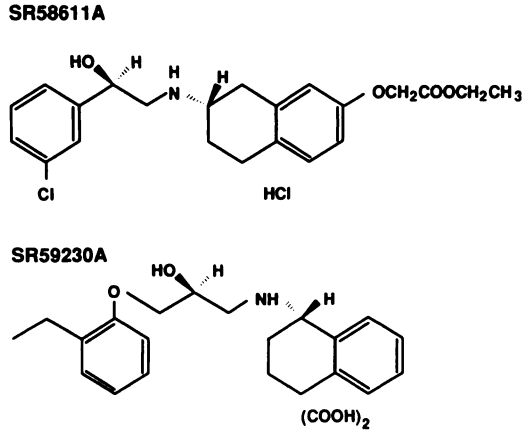

Fig. 1. Structural formulas of SR 58611A and SR 59230A.

Adipose cell culture and treatment. We added 2 million cells to collagen-coated glass Petri dishes (50-mm diameter). The glass Petri dishes were used to avoid binding of SR 59230A to the walls, as it does to plastic culture dishes. The cells were cultivated in 2.5 ml of a culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine, 10% newborn calf serum, 4 nM insulin, and 10 mM HEPES with 50 IU of penicillin, 50 μg of streptomycin, and 25 μg of sodium ascorbate per milliliter (all from Flow Laboratories, Milan, Italy) at 37° in a water-saturated atmosphere of 6% CO_2 in air in a NAPCO 5430 model incubator. The medium was completely exchanged with fresh prewarmed medium on day 1 (when the cultures were first washed with 5 ml of prewarmed Dulbecco's modified Eagle's medium) and on days 3 and 9 (without washing). In experiments for cAMP and glycerol determination, the cells were exposed to NE (freshly made aqueous solution for each treatment) and/or CGP 12177 for 30 min and harvested.

Determination of cAMP and lipolysis in culture cells. Cells were grown for 4 or 8 days (i.e., preconfluent or confluent cells) in the glass Petri dishes. At 2 hr before each experiment, the medium was replaced by the modified Krebs-Ringer bicarbonate buffer, containing 5 mM 3-isobutyl-1-methylxanthine, without bovine serum albumin (which readily binds SR 59230A[1]), in the presence or absence of the tested drugs. Fatty acid-free bovine serum albumin is normally used to prevent extracellular accumulation of unbound fatty acids during enzymatic dispersion of adipocytes and during the period preceding the experiment (18). Under our experimental conditions, i.e., measurements of the glycerol release from cultured fat cells, this was not essential, probably because the cultured cells contain fewer lipids than the dispersed mature brown fat cells.

Cell cultures were incubated at 37° for 30 min with agonists, and the antagonists were added 15 min before the agonists. The reaction was stopped in ice by removal of the incubation medium, which was then frozen and later analyzed fluorometrically in duplicate for glycerol using reagents supplied by Boehringer-Mannheim (Mannheim, Germany). The cells were harvested with ice-cold phosphate-buffered saline containing 5 mM 3-isobutyl-1-methylxanthine and, after sonication (100 W for 10 sec), centrifuged for 5 min; the resulting infranatants were stored until cAMP determination. Protein content was measured by the BCA Protein Assay (Pierce, Milan, Italy).

PCR assay. Total RNA was isolated from rat cultured brown cells by the RNAzol method (TM Cinna Scientific, Friendswood, TX). For PCR analysis, RNAs were treated for 1 hr at 37° with 6 units of RNase-free Dnase I/μg RNA in 100 mM Tris·HCl, pH 7.5, and 50 mM $MgCl_2$ in the presence of 2 units/μl of placenta RNase inhibitor. One microgram of total RNA was reverse- transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) in 20 μl of buffer (Promega, Madison, WI) containing 0.4 mM dNTP, 2 units/ml RNase inhibitor, 0.8 μg oligo$(dT)_{15}$ primer (Promega), and [^{32}P]dCTP. The resulting cDNA was quantified through determination of the amount of radioactivity incorporated into trichloroacetic acid-precipitable nucleic acid. A control without reverse transcriptase was run for each sample to verify that amplification did not proceed from residual genomic DNA. PCR was performed with truncated *Taq* DNA polymerase (Biotaq, Bioprobe Systems, Milan, Italy) in 50 μl of standard buffer (20 mM Tris·HCl, pH 8.55, 16 mM $(NH_4)_2{\cdot}SO_4$, 2.5 mM $MgCl_2$, 150 μg/ml bovine serum albumin, and 200 μM dNTP) containing 10 or 20 ng of cDNA from the preparations and 1 μM of each sense- and antisense-specific oligonucleotide primers in the presence of trace amounts of radioactive dCTP. All oligonucleotide primers for PCR were synthesized by phosphoramidite chemistry with an Applied Biosystem synthesizer with reagents purchased from Applied Biosystem (Foster City, CA). The specific sequence of primers for UCP was 5′-GTGAGTTCGACAACTTC-

[1] L. Manara, T. Croci, M. Landi, personal communication.

CGAAGTG-3′ and 5′-CATGAGGTCATATGTCACCAGCTC-3′ (amino acids 1–197) (19). Primers for β-actin were as described by Gaudette and Crain (20) and correspond to nucleotides 861–884 and 1086–1107 in the published rat sequence (21). Thirty cycles at 94° for 30 sec, at 60° for 30 sec, and at 72° for 60 sec, followed by a 10-min final extension at 72°, were performed. After amplification, 10 μl of the reaction mixture were separated by electrophoresis (1.2% agarose gel in Tris-borate-EDTA buffer) and visualized by ethidium bromide staining. UCP and β-actin cDNAs were coamplified, and the bands visualized by ethidium bromide were cut out. The radioactivity incorporated into the DNA-amplified fragments was counted by Cerenkov counting and expressed as UCP/β-actin ratio.

In addition, gels were blotted onto nitrocellulose filters that were hybridized to probes obtained by PCR amplification of cloned UCP and β-actin genes with the specific primers given above. Nitrocellulose membranes were exposed for 8 hr to NIF RX-100 films (Fuji, Milan, Italy).

Data analysis. Data were analyzed statistically by analysis of variance with Newman-Keuls multiple-comparisons posthoc test. The significance of differences between cAMP accumulation and lipolytic activities under basal or stimulating conditions was determined with the nonparametric Mann-Whitney *U* test.

The IC_{50} and EC_{50} values were obtained by using the four-parameter logistic model according to Ratkowsky and Reedy (22). The adjustment was obtained by nonlinear regression analysis using the Levenberg-Marquandt algorithm in RS/1 software (B.B.N., Cambridge, MA). None of the pseudo-Hill slopes obtained (range, 0.8–1.2) were statistically different than 1. The negative logarithms of the

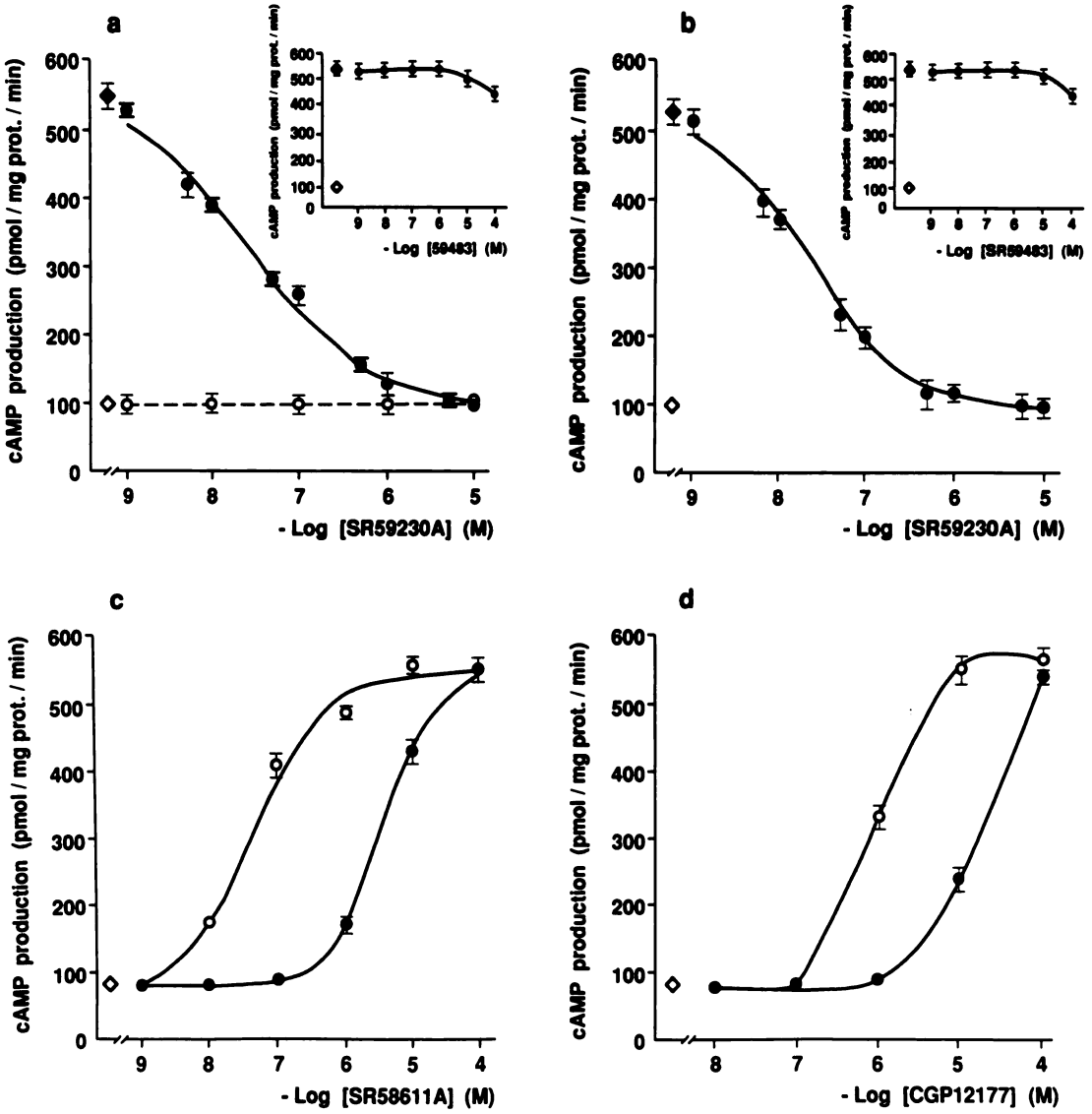

Fig. 2. Antagonistic effects of SR 59230A on SR 58611A- or CGP 12177-increased cAMP production in rat BAT membranes. SR 58611A-stimulated (1 μM [a]) or CGP 12177-stimulated (10 μM [b]) cAMP synthesis (♦) over the basal value (◇) was concentration-dependently antagonized by SR 59230A (●) (a: IC_{50}, 26.7 nM; 95% confidence limits, 23.4–30.5; pK_B, 8.90 ± 0.18; b: IC_{50}, 21.1 nM; 95% confidence limits, 16.2–27.2; pK_B, 8.73 ± 0.16) but not by SR 59483 (●, *insets*). SR 59230A alone was unable to induce changes of cAMP levels (○, a). SR 59230A (100 nM, ●) induced considerable rightward shifts of the concentration-response curves of SR 58611A-induced (○, c) or CGP 12177-induced (○, d) cAMP accumulation (EC_{50} without and with the antagonist were 52 [42–69] and 3891 [3176–4882] nM for SR 58611A and 974 [804–1116] and 15,210 [13,300–17,960] nM for CGP 12177). Antagonists were added 15 min before agonists. Reactions were performed in the presence of the β-adrenoceptor agonists at 30° for 20 min and stopped by placing the tubes in a boiling water bath for 5 min. Then, the tubes were centrifuged at 1000 × *g* for 10 min, and cAMP was measured in the supernatants by radioimmunoassay (DuPont-NEN, Boston, MA). Each point represents the mean ± 95% confidence limits for three independent experiments performed in triplicate. Where 95% confidence limits are not reported, they were less than the size of the symbol.

antagonist dissociation constant K_B were calculated either from a derivative of the Cheng-Prusoff equation (23):

$$K_B = \frac{IC_{50}}{1 + (A_f/EC_{50})}$$

in which $[A_f]$ was the fixed agonist concentration used in the competitive assay, or from the Schild equation: log(dr − 1) = log[B] − log K_B, in which [B] was the concentration of antagonist used in the agonist concentration-response curve rightward shifts (24).

Materials. NE hydrochloride (Sigma, Milan, Italy) was diluted in buffer containing 0.1% ascorbic acid to prevent its possible oxidation. ATP and 3-isobutyl-1-methylxanthine were purchased from Sigma. Type II collagenase, fatty acid-free bovine serum albumin, DNase I, creatine kinase, and creatine phosphate were obtained from Boehringer (Mannheim, Germany). SR 59230A and SR 58611A were synthesized in the Chemistry Section of the Sanofi Midy S.p.A. Research Centre (Milan, Italy). CGP 12177 and CGP 20712A were from CIBA-GEIGY (Basel, Switzerland). ICI 118,551 was from Zeneca (Macclefield, Cheshire, UK).

Results

The fact that SR 59230A (Fig. 1) but not its *RR*-enantiomer SR 59483 has β_3-adrenoceptor-antagonistic properties in rat BAT membranes is suggested by the observation that it concentration-dependently antagonized the cAMP accumulation induced by SR 58611A or CGP 12177, both compounds that have recently been demonstrated to be β_3-adrenoceptor agonists (25, 26). Fig. 2 shows that 1 μM SR 58611A-stimulated cAMP production was blocked by SR 59230A and the inhibition was 100% at 10 μM of the antagonist. Fig. 2b also shows that cAMP synthesis stimulated by 10 μM CGP 12177 was blocked by SR 59230A with 100% inhibition at 7.8 μM. The lack of intrinsic efficacy of SR 59230A alone is shown in Fig. 2a. Unlike the *SS*-enantiomer, the *RR*-enantiomer SR 59483 did not antagonize cAMP accumulation stimulated by SR 58611A or CGP 12177 up to 100 μM. Fig. 2, c and d, also shows that 100 nM of SR 59230A induced notable rightward shifts of the concentration-response curves of SR 58611A- or CGP 12177-induced cAMP accumulation, with calculated pK_B values of 8.87 ± 0.12 and 8.20 ± 0.15, respectively. The inhibitory potencies of SR 59230A against cAMP production induced by the two selective β_3-adrenergic receptor agonists were much higher than those for CGP 20712A and ICI 118,551 (pK_B, 6.41 ± 0.10 and 6.21 ± 0.11 or 5.99 ± 0.18 and 5.88 ± 0.12, respectively; data not reported in Fig. 2).

To investigate the selectivity of SR 59230A as an antagonist of β_3-adrenergic receptors, the cAMP production stimulated by selective and nonselective β_1- and/or β_2-adrenergic agonists was measured in rat frontal cortex and cerebellum homogenates. In rat cerebral cortex, the density of β_1-adrenoceptors is approximately four times that of β_2-adrenoceptors, whereas in the cerebellum, the relative amounts of the two receptors are reversed (27).

Furthermore, our previous work demonstrated that there are no β_3-adrenoceptors in adult rat brain and that the β_3-adrenergic agonist SR 58611A acts as a β_1-adrenoceptor agonist in cerebral cortex (28). Fig. 3 shows the effects of the β-adrenoceptor antagonists CGP 20712A, ICI 118,551, and SR 59230A on the cAMP levels induced by 10 μM (−)-isoprenaline in both frontal cortex and cerebellum. The IC_{50} values of SR 59230A were 27,320 (850–61,050) and 23,430 (10,760–48,980) nM in frontal cortex and cerebellum, respectively.

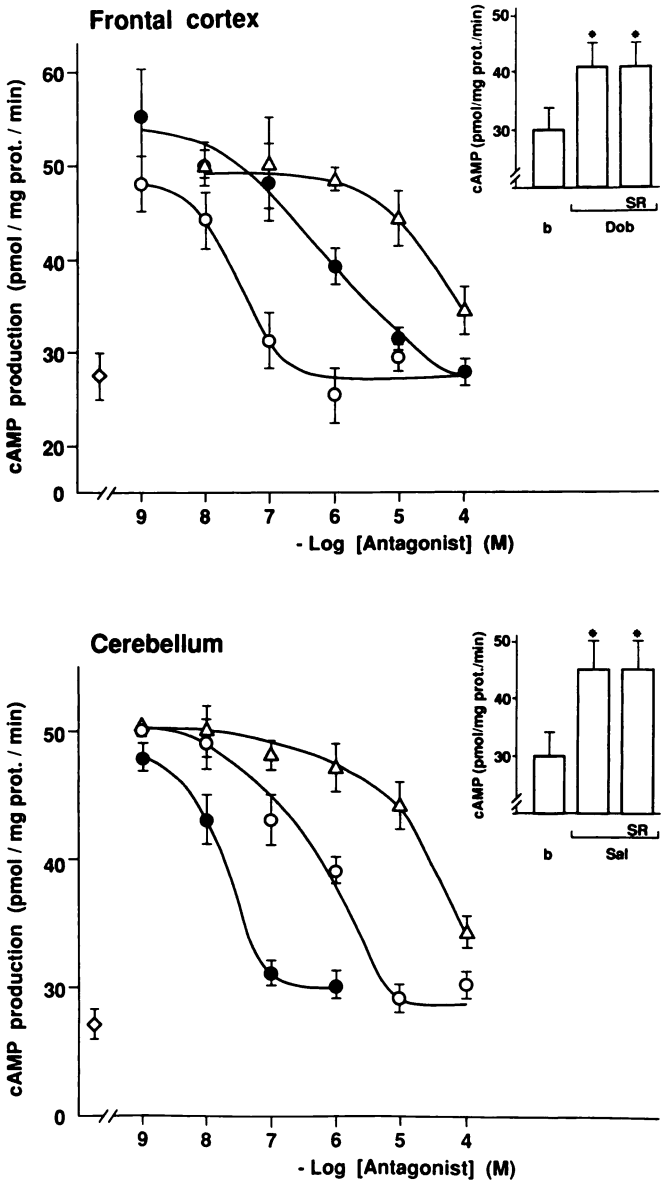

Fig. 3. Effects of CGP 20712A (○), ICI 118,551 (●), or SR 59230A (△) on the increases in cAMP induced by 10 μM (−)-isoprenaline in both frontal cortex and cerebellum homogenates. ◇, Basal value of the cAMP levels. *Insets*, lack of antagonistic effects of 1 μM SR 59230A (*SR*) on 1 μM dobutamine (*Dob*)- or salbutamol (*Sal*)-increased cAMP over the basal value (b). Each point represents the mean ± 95% confidence limits of three independent experiments performed in triplicate. The significance of differences between cAMP accumulation under basal or stimulating conditions was determined with the nonparametric Mann-Whitney *U* test. *, $p < 0.01$ vs. basal value.

Under the same experimental conditions, CGP 20712A and ICI 118,551 potently antagonized the (−)-isoprenaline stimulation in both frontal cortex (IC_{50} = 43 [11–160] and 602 [123–2950] nM, respectively) and cerebellum (IC_{50} = 592 [157–1489] and 30 [11–67] nM, respectively). Similar results were obtained with 10 μM NE, which was used to increase cAMP accumulation (data not shown). In addition, Fig. 3 shows that 1 μM SR 59230A did not modify the cAMP accumulation stimulated by either the selective β_2-adrenoceptor agonist salbutamol in cerebellum or the selective β_1-adrenoceptor agonist dobutamine in frontal cortex. SR 59230A also did not change the cAMP levels increased by SR 58611A (10

μM) in this brain area (data not shown), in which the latter activates cAMP production through β_1-adrenergic receptors (28).

To further confirm the selectivity of SR 59230A for the β_3-adrenoceptor, studies were performed on immature and mature brown fat cells in culture. In preconfluent (i.e., immature) brown fat cells, the β-adrenoceptor that coupled to cAMP production was β_1, whereas in confluent (i.e., mature) cells, it was β_3 (29), the stimulation of which also gives rise to glycerol release (30). As shown in Fig. 4, 1 μM NE induced an increase in cAMP levels in proliferating cells. The addition of 1 μM CGP 12177 produced inhibition, but SR 59230A (at the same concentration) did not affect the increase. CGP 12177 or SR 59230A per se had no or only modest effects on cAMP production, like SR 58611A (Fig. 4). In addition, no significant increases in glycerol release were obtained with these drugs. Taken together, these data suggest that NE acts on β_1-adrenergic receptors to stimulate cAMP production, without antagonism by SR 59230A of this stimulation. On the other hand, both increases in cAMP and glycerol release induced by 1 μM NE or SR 58611A in confluent cells were antagonized by 1 μM SR 59230A (Fig. 4), whereas CGP 12177 did not counteract the NE-induced increases but rather, like SR 58611A, led to effects quantitatively identical to those of NE when added alone to the cell cultures (Fig. 4). SR 59230A (up to 10 μM) did not change the basal level of either parameter (Fig. 4). These results confirm both that NE, CGP 12177, and SR 58611A work as agonists and that SR 59230A works as a selective antagonist on the β_3-adrenergic receptor in mature brown fat cells in culture.

To determine whether the antagonistic action of SR 59230A on cAMP production induced by the β-adrenoceptor agonists was due to a selective receptor binding, its activity was studied on forskolin-stimulated second messenger production in BAT membranes. Forskolin is an ubiquitous activator of cAMP-generating systems in both broken and intact cells. In BAT homogenate, 1 μM forskolin induced up to a 4-fold increase in cAMP formation (Fig. 5) (see also Ref. 16). Fig. 5 clearly shows the lack of any inhibitory effect of SR

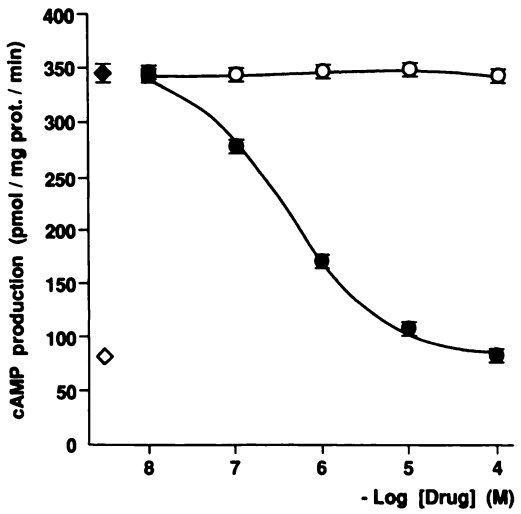

Fig. 5. Effects of SR 59230A (○) or clonidine (●) on 1 μM forskolin-induced (◆) cAMP accumulation in BAT membranes. ◇, Basal value of the cAMP levels. Each point represents the mean ± 95% confidence limits for three independent experiments performed in triplicate.

59230A on this forskolin-induced cAMP accumulation, whereas the selective α_2-adrenoceptor agonist clonidine, used as an internal control, was active (IC_{50} = 414 [355–483] nM) as expected (31).

All these findings are consistent with a selective β_3-adrenoceptor antagonistic property of SR 59230A. Thus, to demonstrate its usefulness in studying the functional relevance of the different β-adrenoceptor subtypes in controlling the metabolism of brown adipocytes, the effects of different concentrations of SR 59230A on UCP mRNA production induced by NE were analyzed in cultured confluent fat cells. Since we previously developed a PCR approach that enabled us to study the expression of the UCP gene in cultured brown fat cells (30) without particular pharmacological treatments, in the present work the effects of SR 59230A were analyzed by PCR. As previously reported (30, 32), NE (10 μM for 4 h) induced an intense expression of the UCP gene in confluent

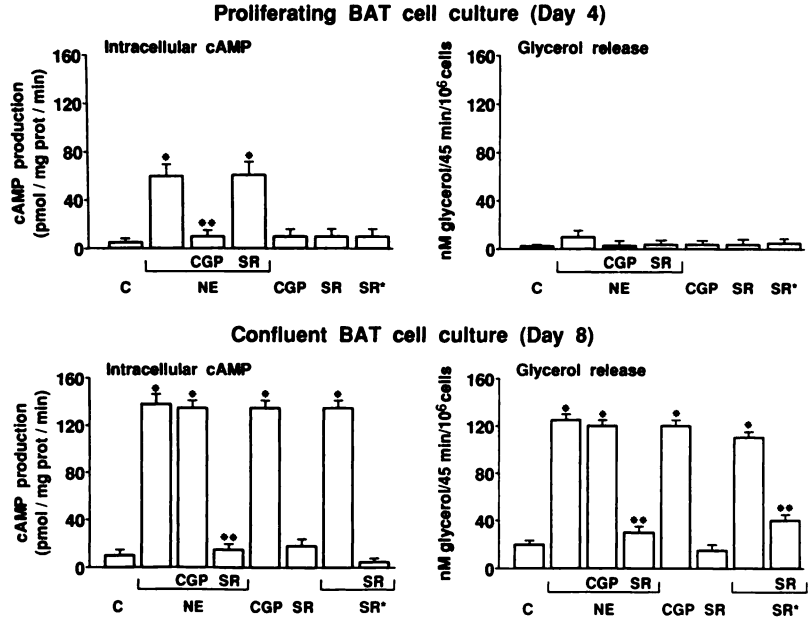

Fig. 4. Effects of SR 59230A on cAMP production and glycerol release in proliferating and confluent cell cultures. On day 4 or day 8 of culture, 1 μM NE, CGP 12177 (*CGP*), SR 59230A (*SR*), SR 58611A (*SR**), or a combination of these drugs was added to the cell cultures. The cell cultures were incubated at 37° for 30 min with agonists (SR 59230A and CGP 12177 [when the latter used as antagonist] were added 15 min before NE or SR 58611A). The incubation medium was removed, frozen, and later analyzed in duplicate fluorometrically for glycerol. The cells were harvested, homogenized by sonication, and centrifuged. The resulting infranatants were stored for cAMP determination. Values are mean ± standard error for three independent experiments run in triplicate. *, $p < 0.01$ vs. basal value; **, $p < 0.001$ vs. stimulated cAMP or glycerol production.

brown fat cells that had developed in culture from undifferentiated precursor cells. A representative agarose gel of PCR-derived products corresponding to UCP mRNA is illustrated in Fig. 6a. A pair of primers designed on the basis of the published β-actin sequence were added to each PCR tube to allow co-amplification of the structural protein mRNA, which generated a fragment of 241 bp. The identity of the bands was confirmed by Southern analysis with ^{32}P-labelled probes complementary to the expected amplified fragments on the basis of the published sequences (data not shown).

Several investigators have demonstrated that NE does not affect the levels of β-actin mRNA (32). Therefore, measurement of β-actin mRNA was used as an internal control of the amount of starting cDNA template in the different experimental samples. Recent papers (33) have reported that in selected experimental conditions, the yield of the reverse-transcribed, PCR amplified DNA fragment is linearly correlated with the amount of original RNA template. Thus, the radioactivity content of the different DNA fragments obtained after PCR amplification of cDNA with primers selective for each target gene can be taken as an index of mRNA steady-state levels. It is also to be stressed that the UCP to β-actin ratios have been calculated to normalize the amount of starting template and, due to the differences of efficiency of the amplification reaction with different pairs of primers, they do not reflect the relative levels of the two transcripts actually expressed in the cells. Fig. 6b reports the cpm values for UCP- and β-actin-corresponding bands as detected by PCR, and it shows that SR 59230A was able to concentration-dependently antagonize the NE-induced UCP mRNA increase. Fig. 6c and d also report the lack of any consistent antagonistic effect of CGP 20712A on the NE-increased UCP mRNA levels. These findings clearly demonstrate that the β_3-adrenoceptor is the functionally active β-adrenoceptor subtype through which NE modulates the BAT thermogenetic functions.

Discussion

SR 59230A has been synthesized, studied, and developed as a putative β_3-adrenoceptor antagonist for the gut (14). Here we report that in vitro SR 59230A selectively blocks the effects of the β_3-adrenergic agonists, SR 58611A and CGP 12177 in BAT. Under the experimental conditions used in the present study, SR 59230A had IC_{50} and pK_B values in antagonizing SR 58611A- or CGP 12177-induced cAMP accumulation in rat BAT membranes consistent with the idea that the compound is a potent β_3-adrenergic antagonist. The potency obtained was much higher than those obtained with CGP 20712A and ICI 118,551, which had been previously described as selective β_1- and β_2-adrenergic receptor antagonists, but more recently reported to possess weak β_3-antagonistic properties in CHO cells transfected with the human β_3-adrenergic receptor gene (34). In addition, the lack of any antagonistic effect of SR 59230A on cAMP production induced by non-selective or selective β_1- and/or β_2-adrenoceptor agonists in rat brain regions rich in β_1- or β_2-adrenoceptors, such as frontal cortex and cerebellum, showed that this compound has no β_1- or β_2-adrenergic antagonistic properties.

Under our experimental conditions, although SR 59230A had IC_{50} values of the same order of magnitude as those observed in rat colon (14), it showed greater selectivity than in guinea-pig atrium (β_1-adrenoceptors) and trachea (β_2-adrenoceptors), in which the compound had affinity values ~<10-fold lower than in the rat proximal colon (β_3-adrenoceptors) (14). It remains to be established whether the much higher selectivity of SR 59230A observed in the present study is due to the peculiar characteristics of the in vitro experimental model or to the different pattern of transducing systems coupled to β-adrenoceptor subtypes concomitantly expressed in the various tissues or organs. Nevertheless, the findings of different selectivities under differing experimen-

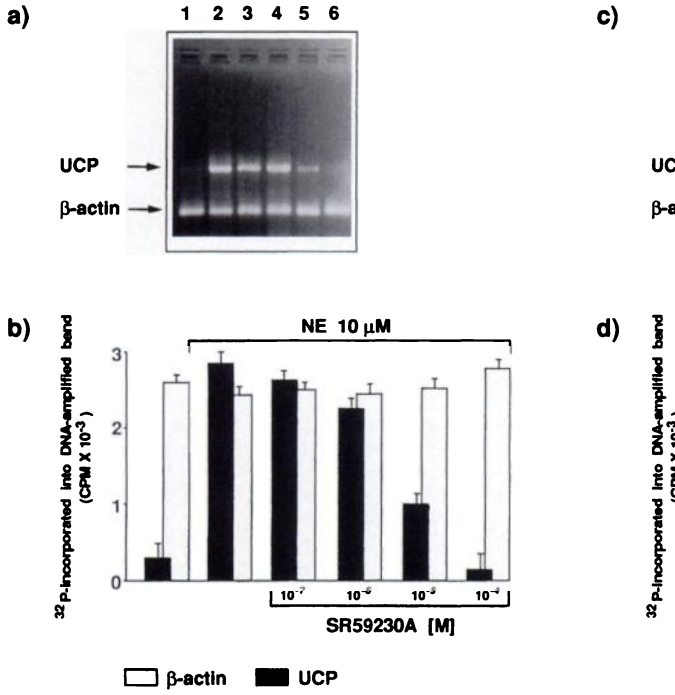

Fig. 6. Effects of β_1- or β_3-adrenoceptor antagonists on NE-stimulated UCP mRNA synthesis in confluent brown fat cells. Representative agarose gels showing PCR analysis for the UCP and β-actin mRNA content in confluent brown fat cells untreated or treated with 10 μM NE alone or by 10 μM NE plus different concentrations of SR 59230A (a) or CGP 20712A (c) are reported. cDNA was amplified in the presence of both UCP and β-actin primers. b and d, Relative quantifications of the drug-induced changes in UCP mRNA. Radiolabeled PCR products were excised from the gels and counted. For UCP mRNA determination, 30 cycles (94° for 30 sec, 60° for 30 sec, 72° for 60 sec) were performed, followed by a 10-min final extension at 72°.

tal conditions are not surprising considering that SR 58611A, unlike other β_3-adrenoceptor agonists such as BRL 37344, ICI 215,001, and CGP 12177, behaves as a β_1-agonist in central nervous system but not in a peripheral tissue also rich in β_1-adrenoceptors, such as atrium, or in Chinese hamster ovary cells transfected with the β_1-adrenoceptor (35). It is worth emphasizing that SR 59230A was very effective in antagonizing SR 58611A acting as selective β_3-agonist in BAT but ineffective against the same SR 58611A acting as a β_1-agonist in frontal cortex (28).

In addition, the considerable inhibition by SR 59230A of the cAMP accumulation induced by NE in cultured confluent BAT cells and the lack of any effect on NE-stimulated cAMP production in preconfluent cells confirmed its selectivity for the β_3-adrenoceptors. As demonstrated by Bronnikov *et al.* (29) in preconfluent young cells, the β_1-adrenoceptor is the only expressed and coupled β-adrenoceptor, whereas in the confluent/mature cells, only the β_3-adrenoceptor is coupled to a response.

SR 59230A, acting on the β-adrenoceptor and not directly on adenylyl cyclase, left the forskolin-stimulated cAMP synthesis in BAT preparations completely unchanged even at the highest concentrations.

Some investigators have speculated about a possible difference among β_3-, β_2-, and, especially, β_1-adrenoceptors in the site that interacts with the β-hydroxy group of aryloxypropanolamine antagonists and about the possibility that β_3 represents the most primitive adrenoceptor subtype in evolutionary terms on the basis of the alleged limited ability to distinguish between the (−)-enantiomers of various standard antagonists and their less potent (+)-enantiomers (10). The present results clearly demonstrate that β_3-adrenoceptors are endowed with high stereoselectivity. SR 59230A was >10,000 times as potent as SR 59483 in antagonizing the increases in cAMP induced by different β_3-adrenoceptor agonists. These findings do not support the above speculations.

Taken together, the present results suggest that SR 59230A is a suitable and useful tool to study the functional role of the β_3-adrenoceptors in various tissues. We used SR 59230A to examine the β-adrenoceptor subtype involved in the control of the expression of the gene coding for the brown fat-specific UCP. BAT mitochondria possess this unique protein in the inner mitochondrial membrane. Its main function is to dissipate the proton gradient generated by respiration activity and thus uncouple mitochondrial respiration from oxidative phosphorylation, giving rise to dissipation of energy as heat (19). As previously reported (32), the addition of NE to brown fat cells, isolated as undifferentiated precursors from BAT and differentiated in culture, induced the expression of the UCP gene, and this ability was maximal in cells near the stage of confluence. Under our experimental conditions, the β_3-adrenoceptor antagonist SR 59230A, but not the β_1-adrenoceptor antagonist CGP 20712A, concentration-dependently and fully inhibited the NE-induced increase of the UCP mRNA in confluent culture cells. This demonstrates that the β_3-adrenoceptor is the functionally relevant β-adrenoceptor subtype in the control of the thermogenetic signalling pathways stimulated by NE in BAT. Finally, because our findings also show that expression of the UCP gene is low in nonstimulated cells, i.e., in control cells not exposed to NE, it is worth noting that an apparently differentiated state is reached in these cells without previous exposure to NE.

In conclusion, SR 59230A is a selective and potent β_3-adrenoceptor antagonist and a useful pharmacological tool with which to study the function of rat β_3-adrenoceptors. Further in vitro studies with this compound on human tissue can contribute to better understanding of the species-specific differences in β_3-adrenoceptor subtypes, and future in vivo experiments should take into account its significant plasma protein binding characteristic.

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