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Biochemical and Functional Characterization of 1-Benzyl Substituted Trimetoquinol Affinity Analogs on Rat and Human β-Adrenoceptors

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ABSTRACT. The site of interaction for the 1-(3′,4′,5′-trimethoxybenzyl) group of trimetoquinol (TMQ) with β-adrenoceptors (β-ARs) is important for the rational design of highly potent and β_3-AR-selective analogs. 1-Benzyl ring-substituted TMQ analogs were evaluated for binding affinities and biochemical activities (cyclic AMP accumulations) in Chinese hamster ovary (CHO) cells expressing the rat and human β_3-AR, and for functional activities on isolated rat tissues. Binding affinities ($K_i \approx 0.055$ to 1.5 μM) for the rat β_3-AR and potencies for adenylyl cyclase activation ($K_{act} \approx 0.43$ to 2.5 nM) of the 3′-monoiodo or 3′,5′-diiodo derivatives with 4′-isothiocyanato-, 4′-amino, 4′-acetamido, or 4′-α-haloacetamido substitutions were higher than those of (-)-isoproterenol, and comparable to those of BRL 37344 [(±)-(*R**,*R**-[4-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]-acetic acid sodium]. A similar rank order of binding affinities ($K_i \approx 0.11$ to 2.5 μM) and potencies ($K_{act} \approx 0.45$ to 9.5 nM) was obtained for TMQ analogs on the human β_3-AR. The 4′-acetamido and 4′-α-chloroacetamido analogs of 3′,5′-diiodoTMQ were more potent than (-)-isoproterenol in rat atria (β_1-AR) and rat trachea (β_2-AR) and exhibited partial agonist activities, whereas full agonist activities were observed in rat esophageal smooth muscle ($EC_{50} \approx 2$–8 nM, β_3-AR). 4′-α-Chloroacetamido-3′,5′-diiodoTMQ-mediated chronotropic responses in atria were sustained and resistant to washout. Further, the 4′-α-chloroacetamido and 4′-α-bromoacetamido analogs of 3′,5′-diiodoTMQ demonstrated significant concentration-dependent irreversible binding to the rat β_3-AR. Reversible β-AR agonists such as (-)-isoproterenol, BRL 37344, and 4′-acetamido-3′,5′-diiodoTMQ or nucleophilic l-amino acids (lysine, glutathione, cysteine) did not protect against this irreversible binding. Thus, the lipophilic 1-benzyl ring of TMQ analogs interacts with a hydrophobic region of the β-AR that may represent an exo-site or an allosteric binding site. BIOCHEM PHARMACOL **59**;5:517–529, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. β_3-adrenoceptor; tetrahydroisoquinolines; Chinese hamster ovary cells; adenylyl cyclase; radioligand binding; affinity labels

β-ARs** are known to mediate several important physiological functions. These receptors belong to the superfamily of G-protein-coupled receptors, and are single-chain membrane-bound proteins with characteristic seven transmembrane-spanning domains that form the ligand-binding pocket [1]. It is well established that the endogenous ligands norepinephrine and epinephrine as well as synthetic β-AR agonists interact with critical amino acid residues in the transmembrane (tm) regions of the receptor to produce conformational changes leading to G_s-protein-mediated activation of adenylyl cyclase and production of cAMP [2]. Major sites of ligand binding within the β-AR are proposed for the catechol hydroxyls and amine group of catecholamines with aspartic acid and serine residues in tm 3 and 5, respectively. The β-ARs were classified initially into β_1- and β_2-subtypes [3], and, more recently, a β_3-subtype [4–6]. Since then, the β-AR subtypes obtained from

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**Abbreviations: β-ARs, β-adrenoceptors; cAMP, cyclic AMP; TMQ, trimetoquinol; DITMQ, 3′,5′-diiodoTMQ; aminoDITMQ, 4′-amino-3′,5′-diiodoTMQ; acetamidoDITMQ, 4′-acetamido-3′,5′-diiodoTMQ; chloroacetamidoDITMQ, 4′-α-chloroacetamido-3′,5′-diiodoTMQ; bromoacetamidoDITMQ, 4′-α-bromoacetamido-3′,5′-diiodoTMQ; demethoxyDITMQ, 4′-demethoxy-3′,5′-diiodoTMQ; isothiocyanatoITMQ, 4′-isothiocyanato-3′-iodo, 5′-demethoxyTMQ; CHO cells, Chinese hamster ovary cells; and [^{125}I]ICYP, [^{125}I](-)-3-iodocyanopindolol.

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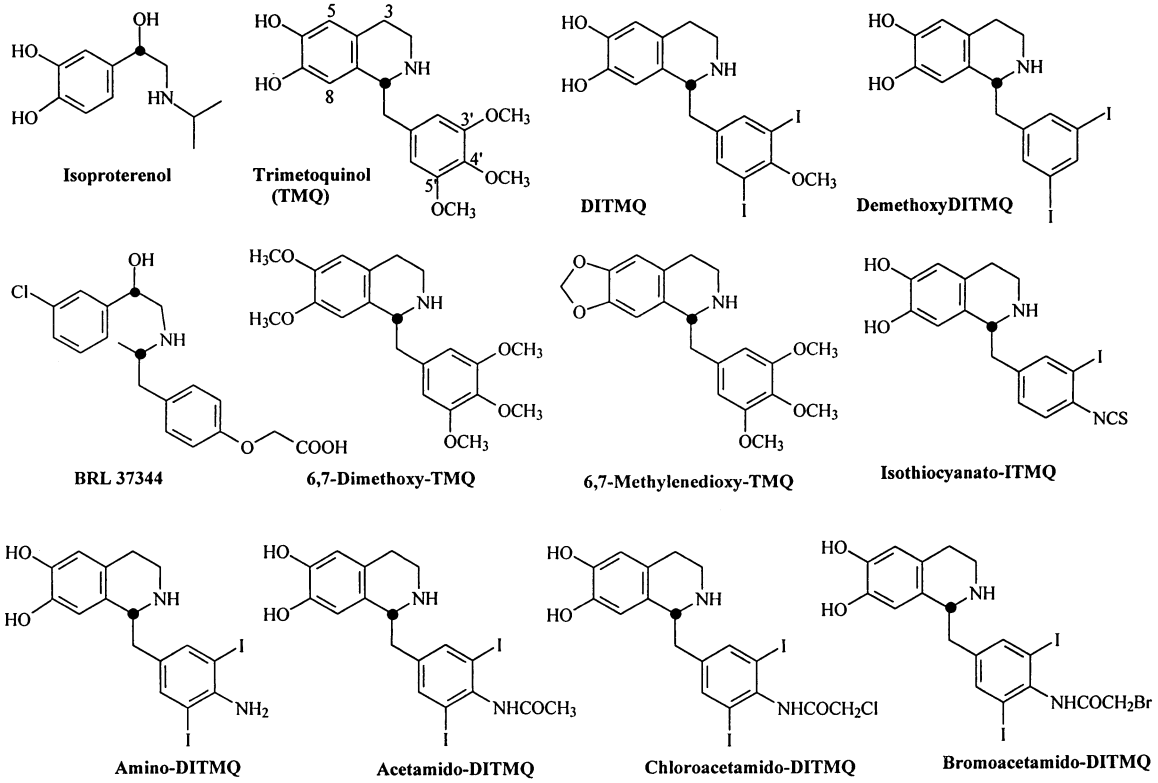

FIG. 1. Chemical structures of isoproterenol, BRL 37344, trimetoquinol (TMQ), and its analogs, including abbreviations of the TMQ derivatives used in the studies. The ● within each structure indicates the location of a chiral carbon atom.

different species have been cloned and expressed as recombinants in cell lines including CHO cells [1, 5, 7–9]. The existence of a β_4-AR subtype has also been proposed [10], but conclusive evidence of its existence is yet to be established.

Traditionally, the therapeutic usefulness of β-AR drugs has been limited to cardiovascular (β_1-AR-selective) and bronchorelaxant (β_2-AR-selective) applications. The more recent identification and characterization of the β_3-AR from different species including rodents and humans generate the potential for new therapeutic applications of selective agonists. In several species including rodents and humans, the β_3-AR is co-expressed with β_1- and/or β_2-AR subtypes in tissues such as adipose and heart, and mediates important metabolic functions [4, 10–14]. Activation of the β_3-AR is known to mediate catecholamine-stimulated lipolysis in white adipocytes, and both lipolysis and thermogenesis in brown adipocytes. More importantly, β_3-AR selective agonists produce a decrease in body fat and an improvement in insulin sensitivity in a variety of animal models of obesity [15–17]. Obese animals and human subjects treated with β_3-AR-selective agonists such as BRL 35135 (a prodrug of BRL 37344) and CL 316,243 show an improvement in the rate of fat metabolism and protein accretion without affecting their food intake [4, 15–18]. In rhesus monkeys, β_3-AR agonist treatment increases lipolysis, basal metabolic rate, and expression of uncoupling protein-1 in brown adipocytes [18]. Taken collectively, the results of these studies suggest that β_3-adrenoceptor selective agonists are promising candidates for the management of human obesity and type II diabetes. In addition, the β_3-AR has also been identified in the smooth muscle of the gastrointestinal tract of several species and found to mediate relaxation and secretory functions in these tissues [19, 20]. Selective agonists of the β_3-AR, therefore, may be useful for the treatment of gastrointestinal hypermotility disorders [21].

TMQ is chemically described as 1-(3′,4′,5′-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline. The *S*(-)-isomer of TMQ is functionally more active on the β_2-AR and is used in Japan as a bronchodilatory agent [22]. The tetrahydroisoquinoline nucleus of this compound has important structural similarities to endogenous catecholamines and to several β_1-/β_2-AR agonists, e.g. the presence of a protonated secondary amine at physiological pH and catechol hydroxyl groups at the 6,7-positions, which presumably interact with aspartic acid in tm 3 and two serine residues in tm 5, respectively, and are important for β-AR-mediated functional activity [23–28]. On the other hand, the 3′,4′,5′-trimethoxybenzyl ring provides a bulky, aromatic side-chain, which is a common structural feature of β_3-AR selective agonists. However, the interactions of this aromatic substituted 1-benzyl side-chain of TMQ with the β-ARs are not well characterized. In functional studies on rat atria and trachea, the 3′,5′-diiodo analog of TMQ (DITMQ, see Fig. 1) was a partial agonist with potencies significantly lower than those of TMQ, whereas it was a full and potent agonist for β_3-AR-

mediated relaxation of rat distal colon and lipolysis in rat brown adipose tissue [29]. Additionally, in CHO cells expressing the rat β_3-AR, the diiodo analog exhibited a higher potency for cAMP accumulation than did TMQ and (-)-isoproterenol, and these values paralleled those reported earlier for the selective β_3-AR agonist BRL 37344 [7]. These results are in agreement with other investigations which indicated that β-AR ligands with large substituents on the amino nitrogen exhibit antagonist or partial agonist activities at β_1- and β_2-AR subtypes, but are potent agonists at the β_3-AR [30–32].

Characterization of the receptor interactions of the hydrophobic 1-benzyl ring-substituted side-chain of TMQ with β-AR subtypes, and especially the β_3-AR, will enable rational design of TMQ analogs with highly potent and selective agonist activity for the β_3-AR. Towards this goal, several 1-benzyl ring substituted analogs of TMQ including those possessing chemically reactive electrophilic groups (α-chloroacetamido, α-bromoacetamido, and isothiocyanato moieties) were synthesized (Fig. 1), and their binding affinities and functional activities on the rat β_3-AR expressed in CHO cells were determined. These cells express a homogeneous population of β_3-AR, and overcome the disadvantage in interpreting results of agonists in native tissues, which are complicated by the presence of competing β-AR. The pharmacological properties of agonists on rat β_3-AR expressed in CHO cells are reported to be similar to those associated with lipolysis in rat adipocytes [7]. However, differences have been reported in the pharmacological interactions of agonists with the rat and human β_3-AR [16]. Hence, we additionally determined the pharmacological properties of selected TMQ analogs on human β_3-AR expressed in CHO cells for comparative evaluation with the results obtained with the rat β_3-AR. The affinity-labeling properties of 4′-α-haloacetamido derivatives of DITMQ also were evaluated on rat β_3-AR in CHO cells. Additionally, functional studies with acetamidoDITMQ and chloroacetamidoDITMQ were conducted using isolated rat tissues containing representative α- and β-ARs to elucidate the pharmacological properties of these compounds in native receptor systems. The functional potencies of these analogs for the β_3-AR-mediated relaxation of the rat esophageal smooth muscle [33] were compared with their biochemical potencies for activation of adenylyl cyclase in CHO cells expressing the rat β_3-AR. The rat right atrium was used as the model for the functional evaluation of the irreversible binding properties of chloroacetamidoDITMQ.

MATERIALS AND METHODS

Chemicals

The chemicals used (and their sources) were: Trizma-HCl, (-)-isoproterenol bitartrate, 3-isobutyl-1-methylxanthine (IBMX), forskolin, and phentolamine HCl (Sigma Chemical Co.); L-ascorbic acid (J. T. Baker Chemical Co.); (±)-propranolol (Ayerst Laboratories Inc.); [^{125}I]ICYP (2000 Ci/mmol) (Amersham); Ham's F-12 cell culture medium, Hanks' phosphate-buffered saline, L-glutamine, penicillin-streptomycin solution, trypsin-EDTA solution, geneticin, and fetal bovine serum (Gibco); hydrocortisone sodium succinate (Abbott Laboratories); carbamyl choline chloride (carbachol, Aldrich Chemical Co.); cocaine HCl (Mallinckrodt Chemical Works); and (±)-pindolol (Receptor Research Biochemicals Inc.). U-0521 (3′,4′-dihydroxy-2-methylpropiophenone) was provided by Dr. Popat N. Patil (College of Pharmacy, The Ohio State University). BRL 37344 [(±)-(*R**,*R**-[4-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]-acetic acid sodium] was a gift from SmithKline Beecham Pharmaceuticals. TMQ and the following analogs were provided by Dr. Duane Miller (University of Tennessee): DITMQ, aminoDITMQ, acetamidoDITMQ, chloroacetamidoDITMQ, bromoacetamidoDITMQ, 6,7-dimethoxy-TMQ, 6,7-methylenedioxyTMQ, demethoxyDITMQ, and isothiocyanatoITMQ. The chemical structures of these TMQ analogs are shown in Fig. 1. All other chemicals used were of reagent grade. Tris buffer (75 mM Trizma-HCl, 154 mM NaCl, and 20 mM EDTA, pH 7.4) was used for binding studies with CHO cells, whereas modified Krebs buffer was used in functional studies [29]. Hydrocortisone (10^{-4} M), cocaine HCl (3×10^{-5} M), and U-0521 (10^{-5} M) were added to Krebs buffer to inhibit extraneuronal uptake, adrenergic neuronal uptake, and the enzyme catechol-*O*-methyltransferase, respectively.

CHO Cells: Source and Culture Conditions

CHO cells expressing the rat β_3-AR were provided by Dr. Claire Fraser (National Institutes of Health). CHO cells expressing the human β_3-AR (truncated β_3-AR, 402 amino acids) were obtained from Dr. Stephen Liggett, Department of Pulmonary and Critical Care Medicine, University of Cincinnati). Culture medium for these cells consisted of Ham's F-12 nutrient medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL of penicillin and 50 μg/mL of streptomycin, and 500 μg/mL of geneticin. Cells were rinsed with Hanks' phosphate-buffered saline and incubated at 37° in a humidified atmosphere of 5% CO_2:95% air in a water-jacketed incubator.

Competitive Radioligand Binding Assays

Competitive radioligand binding assays were performed on rat and human β_3-AR-expressing CHO cells, using the method of Engel *et al.* [34]. The following concentrations of the radioligand [^{125}I]ICYP were used in the assays with the rat and human β_3-AR subtypes: 0.8 to 1.25 nM for rat β_3-AR (dissociation constant, K_d = 1.3 nM [7]), and 0.2 to 0.3 nM for human β_3-AR (dissociation constant, K_d = 0.44 nM).* The radioligand was used at these fixed concentra-

* Personal communication from Dr. Stephen Liggett, University of Cincinnati, 1995. Cited with permission.

tions in the absence and presence of various concentrations (usually the range was 10^{-12} to 10^{-3} M) of competing drugs. The drugs were added to the cells (~500,000) in 75 mM Tris buffer (pH 7.4) to a total volume of 250 μL and incubated at 37° for 1 hr. Nonspecific binding was determined in the presence of 10^{-4} M (±)-propranolol. Reactions were terminated by rapid filtration through Whatman GF/B filters using a Brandel model 12-R cell harvester followed by rapid washes with ice-cold buffer. Radioactivity on the dried filter discs was measured in a Beckman model 8000 gamma counter.

β_3-AR-Mediated cAMP Accumulation Studies in CHO Cells

Conditions of drug treatment of the CHO cells expressing rat and human β_3-AR, isolation of cAMP from these cells, and quantitation of cAMP levels by the radioimmunoassay method of Brooker *et al.* [35] have been described previously [36]. Protein content of the samples was determined by the method of Lowry *et al.* [37]. Briefly, confluent cultures of the CHO cells were incubated with the compounds in the presence of 1 mM IBMX, a phosphodiesterase inhibitor, for 20 min at 37°. The content of cAMP in each sample was determined by radioimmunoassay using [^{125}I]-labeled succinyl-cAMP-tyrosine methyl ester as the competing radioligand. The cAMP amount was determined as picomoles per milligram of protein, and expressed as a percentage of the maximal (-)-isoproterenol-stimulated response. Functional viability for each assay was assessed by measuring basal and 25 μM forskolin-stimulated cAMP levels.

Time- and Concentration-Dependent Affinity Binding Studies in CHO Cells

CHO cells expressing the rat β_3-AR (about 3.5 to 4.0 × 10^6) were suspended in 50 mM Tris buffer, pH 7.4, and incubated with various concentrations of selected TMQ analogs for time periods ranging from 1 to 45 min at 25°, in a total volume of 1.2 mL. Incubations were stopped by centrifugation of the cell suspension at about 1500 *g* using an Eppendorf microcentrifuge (model 5415C). Cell pellets were resuspended in 1.2 mL of fresh Tris buffer for 10–15 min and recentrifuged. Washings were repeated 3 times, and protein content of the final reconstituted suspension was determined by the method of Lowry *et al.* [37]. Aliquots of normalized suspensions (~400,000 cells/250 μL) were incubated with 0.8 to 1.25 nM [^{125}I]ICYP for 1 hr at 37°. Binding reactions were terminated and radioactivity was measured as described in the section for competitive radioligand binding.

Binding Protection Studies in CHO Cells

The rat β_3-AR CHO cells suspended in Tris buffer (3.5 to 4 × 10^6 cells/1.2 mL) were incubated for 20 min with various molar concentrations (30 and 100 K_i) of either (-)-isoproterenol, BRL 37344, or acetamidoDITMQ. Then the affinity ligand chloroacetamidoDITMQ was added to the suspension at a molar concentration of 3 or 10 K_i for a period of 2 min. Competitive binding reactions were stopped by centrifugation of the cell suspensions at 1500 *g*, and then cells were subjected to washout protocol and radioligand binding with [^{125}I]ICYP as described previously. Protection assays were carried out similarly with 10–100 mM glutathione, L-cysteine, or L-lysine at pH 7.4 as protecting agents. In all protection experiments, appropriate controls were included to demonstrate complete washout of the protecting agents by the protocol.

Functional Activity of TMQ Analogs in Isolated Rat Tissues

Male Sprague-Dawley rats (Harlan Industries) housed under a 12-hr light/dark cycle and fed Purina Rodent Laboratory Chow (Ralston Purina Co.) and water *ad lib.* were used for the studies. On the day of the experiment, rats weighing 200–430 g were killed by cervical dislocation and tissues were removed quickly. Chronotropic responses of spontaneously beating right atria were used as a model for measuring β_1-AR-mediated activity [29]. Relaxations of spirally cut tracheal strips precontracted with 3 x 10^{-7} M carbachol, and of longitudinal segments of the esophageal smooth muscle precontracted with 10^{-6} M carbachol (in the presence of 1 μM pindolol and 10 μM phentolamine), were used to measure β_2- and β_3-AR-mediated activity, respectively [29, 33]. Contractions of spirally cut aortal strips [38] and inhibition of phenylephrine-induced contraction of the tissue were used to measure α-AR-mediated agonist or antagonist activity of the compounds, respectively. The tissues were isolated and prepared for measurement of functional activity as per protocols described earlier [29, 38]. All tissues were suspended and equilibrated in modified Krebs buffer in water-jacketed baths at 37°. Resting tensions of 1 g for right atria, trachea, and aorta and of 200 mg for esophageal smooth muscle were used. All tissue responses were measured on a Grass Polygraph model 7C with a Grass FT-03C isometric force-displacement transducer. Cumulative concentration-response curves for each drug were constructed by the method of van Rossum [39]. Increasing concentrations of compounds were added every 2–3 min with (-)-isoproterenol and every 10–15 min with TMQ analogs, or until no further change in response was observed.

In the right atrium, the concentration-response curve for (-)-isoproterenol was followed by complete washout of the drug, after which a curve for either acetamido- or chloroacetamidoDITMQ was constructed. The tissue was washed again 6–7 times with 10 mL of buffer, followed by repeated washes every 10–15 min. Changes observed in the duration of chronotropic effect following repeated washes were used as an indicator of irreversible binding of the compound to the atrial tissue. A second concentration-response curve

TABLE 1. Inhibition constants (-log K_i or pK_i) for binding and functional activity constants (-log EC_{50} or pK_{act}) for cAMP accumulation in CHO cells expressing rat β_3-adrenoceptors

Compound	pK_i	pK_{act}	% E_{max}*
(−)-Isoproterenol	4.45 ± 0.06 (14)	7.90 ± 0.12 (16)	100
BRL 37344	6.96 ± 0.08† (5)	8.90 ± 0.12† (9)	103 ± 6.8
S(−)TMQ	5.67 ± 0.03† (%)	8.19 ± 0.19 (7)	87.6 ± 6.9
DITMQ	6.34 ± 0.03† (5)	9.40 ± 0.08† (9)	96.9 ± 10
AminoDITMQ	6.14 ± 0.08† (5)	ND‡	ND
AcetamidoDITMQ	7.28 ± 0.06† (5)	9.34 ± 0.12† (6)	89.6 ± 6.9
ChloroacetamidoDITMQ	6.49 ± 0.05† (5)	9.05 ± 0.16† (6)	92.5 ± 6.6
BromoacetamidoDITMQ	6.70 ± 0.05† (6)	9.36 ± 0.39† (5)	87.3 ± 12
6,7-DimethoxyTMQ	3.88 ± 0.07† (4)	No activity up to 3×10^{-5} M	
6,7-MethylenedioxyTMQ	4.29 ± 0.05 (5)	5.92 ± 0.12† (8)	70.9 ± 4.4†§
DemethoxyDITMQ	5.80 ± 0.03† (5)	8.74 ± 0.10† (4)	97.7 ± 5.9
IsothiocyanatoITMQ	5.83 ± 0.12† (4)	8.61 ± 0.15†	104 ± 7.1

Values are means ± SEM of the number of experiments indicated in parentheses. The structures and abbreviations of compounds are given in Fig. 1.

*% E_{max} is the maximal cAMP accumulation stimulated by the compounds relative to that of (−)-isoproterenol × 100.

†Indicates a significant difference in the value of each compound compared with the corresponding value of (−)-isoproterenol ($P < 0.05$).

‡ND = value not determined.

§Highest concentration used was 3×10^{-5} M.

with (-)-isoproterenol was constructed in atria to determine desensitization effects.

The concentration-response curves of the drugs in tracheal and esophageal smooth muscle culminated with the addition of 10^{-5} M (-)-isoproterenol to determine the maximal relaxation induced in the tissue and to express functional responses of the TMQ analogs as a percentage of maximal (-)-isoproterenol-induced response. Carbachol-contracted tissues were included as controls through the duration of relaxation studies.

Studies with aorta were performed in the presence of 1 μM pindolol to block β-AR-mediated effects. Concentration-response curves to phenylephrine were followed by washout of the drug and a 30-min incubation with 10^{-5} M acetamido- or chloroacetamidoDITMQ. Then a second phenylephrine concentration-response curve was constructed to determine α-AR blocking activity of the TMQ analogs. In control experiments, the second concentration-response curves of phenylephrine were constructed in the absence of treatment with the compounds.

Duration of Response Following Washout of Compounds

Following construction of each concentration-response curve in rat right atria, the tissue was washed immediately (7 times) with the buffer solution, followed by subsequent washings every 10–15 min until the heart rate returned to baseline levels. An identical washing procedure was followed subsequent to treatment with (-)-isoproterenol and the TMQ analogs. Responses were measured periodically to determine the average washout time required for the heart rate to return to basal levels.

Data Analyses

GraphPAD Inplot version 3.14 (GraphPAD Software Inc.) was used for data analysis. Binding data yielded IC_{50} values, from which dissociation constants (K_i) were determined by the equation of Cheng and Prusoff [40]. The concentration of drug producing half-maximal stimulation of cAMP accumulation (K_{act}) or functional response in rat tissues (EC_{50}), and maximal cAMP levels or maximal response produced (E_{max}), were obtained from nonlinear regression analysis of concentration-response curves. Relative efficacies (e_r) were calculated from plots of cAMP accumulation versus percent receptor occupancy as described by Furchgott and Bursztyn [41]. Statistical significance was determined by Student's *t*-test at a significance level of 5%.

RESULTS

Competitive Binding and cAMP Accumulation in CHO Cells Expressing the Rat β_3-AR

Table 1 summarizes the binding affinities (pK_i or -log K_i values), biochemical potencies (pK_{act} or -log K_{act} values), and relative maximal activities (E_{max}) for (-)isoproterenol, BRL 37344, and selected TMQ analogs on the rat β_3-AR. Specific binding of [^{125}I]ICYP ranged from 70 to 90%. Each compound displaced [^{125}I]ICYP from rat β_3-AR binding sites in a concentration-dependent competitive manner. Figure 2 displays the competitive displacement curves of the standards and of selected TMQ analogs. It should be noted that, based upon subsequent studies with affinity TMQ compounds (bromoacetamidoDITMQ, chloroacetamidoDITMQ, and isothiocyanatoITMQ), their displacement may not be competitive with the radioligand. Therefore, experimentally determined displacement potencies for these affinity TMQ analogs should be considered as pseudo-K_i values. The rank order of potency for inhibition of ICYP binding was as follows (pK_i values in parentheses): acetamidoDITMQ (7.28) > BRL 37344 (6.96) > bromoacetamidoDITMQ (6.70) > chloroacetamidoDITMQ (6.49) > DITMQ (6.34) > aminoDITMQ (6.14) ≥ isothiocyanatoITMQ (5.83) ≥ demethoxyDITMQ (5.80) > (-)-TMQ (5.67) > (-)-isoprotere-

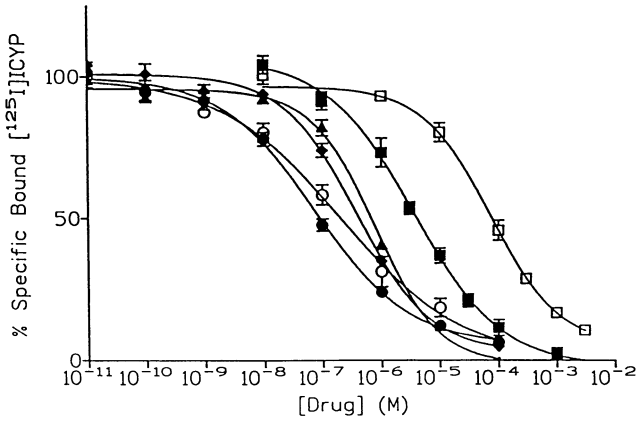

FIG. 2. Concentration-dependent inhibition of specific [^{125}I]ICYP binding to rat β_3-AR in CHO cells by β-AR ligands and TMQ analogs. Values are expressed as the means ± SEM of N = 5–14 experiments. Key: (-)-isoproterenol (□); *S*(-)TMQ (■); BRL 37344 (○); DITMQ (▲); acetamidoDITMQ (●); and bromoacetamidoDITMQ (♦).

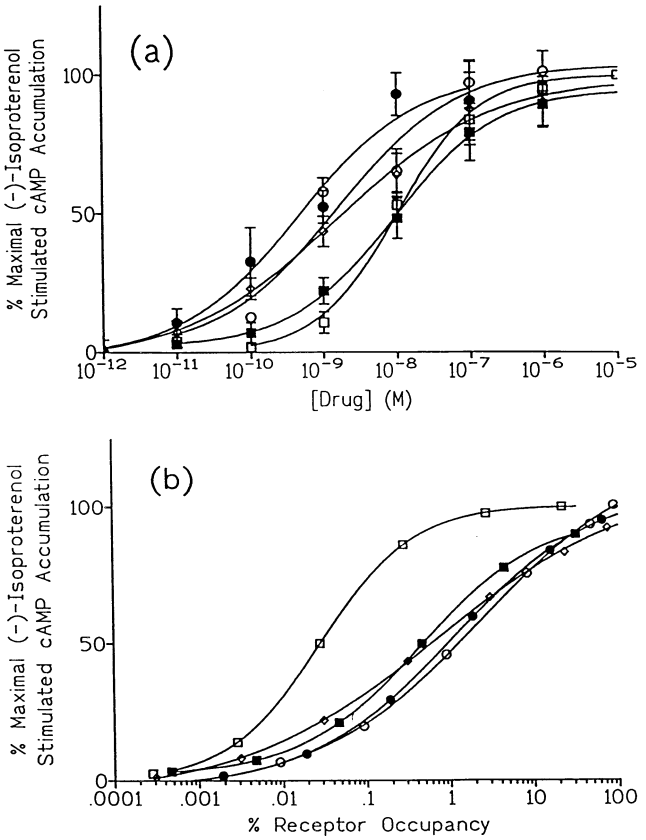

FIG. 3. Rat β_3-AR-mediated increases in cAMP accumulation in CHO cells as a function of (a) agonist concentration and (b) receptor occupancy by agonist. The data are expressed as the means ± SEM of 4–14 experiments. Key: (-)-isoproterenol (□); *S*(-)TMQ (■); BRL 37344 (○); acetamidoDITMQ (●); and chloroacetamidoDITMQ (◇).

nol (4.45) = 6,7-methylenedioxyTMQ (4.29) > 6,7-dimethoxyTMQ (3.88). Substitutions on the 1-benzyl ring of TMQ increased binding affinities for the β_3-AR, whereas modification of the catechol function (6,7-hydroxyls) of TMQ with a methylenedioxy or a dimethoxy group decreased affinity. Except for the 6,7-dimethoxy or methylenedioxy analogs, the remaining TMQ compounds possessed significantly higher affinities as compared with that of (-)-isoproterenol. In general, the β_3-AR affinity increased with increasing lipophilicity and bulk at the 3′, 4′-, or 5′-positions on the 1-benzyl ring of TMQ.

Biochemical potencies and maximal activities of selected TMQ analogs and the standard compounds are also summarized in Table 1. The maximal activity (E_{max}) values of the compounds were determined relative to that of (-)-isoproterenol. The basal levels of cAMP and the amounts accumulated in the cells by 25 μM forskolin and maximal (-)-isoproterenol stimulation were 103 ± 22, 5458 ± 1054, and 2258 ± 387 pmol/mg protein, respectively (means ± SEM, N = 16). Figure 3a shows concentration-dependent cAMP generation by selected TMQ analogs, which is expressed relative to the maximal (-)-isoproterenol response. The rank order of potencies of the compounds for β_3-AR-mediated activation of adenylyl cyclase was as follows (pK_{act} value in parentheses): DITMQ (9.40) ≥ bromoacetamidoDITMQ (9.36) ≥ acetamidoDITMQ (9.34) ≥ chloroacetamidoDITMQ (9.05) ≥ BRL 37344 (8.90) ≥ demethoxyDITMQ (8.74) ≥ isothiocyanatoITMQ (8.61) ≥ (-)-TMQ (8.19) ≥ (-)-isoproterenol (7.90) > 6,7-methylenedioxyTMQ (5.92). 6,7-MethylenedioxyTMQ was a partial agonist, while 6,7-dimethoxyTMQ exhibited no activity at a concentration of 3×10^{-5} M. These data indicate that the masking of the catechol function of TMQ with dimethoxy or methylenedioxy groups markedly reduced the potency and agonist activity of the parent compound. By contrast, the 4′-acetamido or 4′-α-haloacetamido substituents retained a high potency comparable with that of DITMQ, and were at least 10-fold greater than that of TMQ. These diiodo analogs of TMQ were also more potent than the standard β_3-AR-selective agonist BRL 37344. IsothiocyanatoITMQ was found to be a full agonist, and although its potency was lower than those of the diiodoTMQ derivatives, it was comparable to that of the parent compound, TMQ. In addition, the potencies of all iodinated TMQ analogs tested were greater than that of (-)-isoproterenol.

The concentration-dependent biochemical responses (cAMP accumulations) of selected compounds were plotted as a function of receptor occupancy (Fig. 3b). The efficacies of the compounds relative to (-)-isoproterenol (e_r = 1) were significantly lower and were ordered as follows: (-)-TMQ, 0.059; acetamidoDITMQ, 0.20; chloroacetamidoDITMQ, 0.047; bromoacetamidoDITMQ, 0.11; isothiocyanatoITMQ, 0.20; and BRL 37344, 0.022.

Competitive Binding and cAMP Accumulation in CHO Cells Expressing the Human β_3-AR

Selected TMQ analogs (acetamido, chloroacetamido, bromoacetamido, and isothiocyanato) with high affinities and biochemical potencies at the rat β_3-AR were tested for

TABLE 2. Inhibition constants (-log K_i or pK_i) for binding and functional activity constants (-log EC_{50} or pK_{act}) for cAMP accumulation in CHO cells expressing human β_3-adrenoceptors

Compound	pK_i	pK_{act}	% E_{max}*
(−)-Isoproterenol	4.55 ± 0.14 (6)	7.99 ± 0.23 (4)	100
BRL 37344	5.79 ± 0.12† (4)	7.64 ± 0.19 (4)	65 ± 4.2
AcetamidoDITMQ	6.63 ± 0.14† (5)	8.96 ± 0.15† (5)	101 ± 8.3
ChloroacetamidoDITMQ	6.77 ± 0.13† (5)	8.68 ± 0.14† (6)	111 ± 7.7
BromoacetamidoDITMQ	6.94 ± 0.06† (6)	9.35 ± 0.16† (5)	108 ± 4.0
IsothiocyanatoITMQ	5.60 ± 0.15† (5)	8.02 ± 0.24 (5)	114 ± 7.4

Values are means ± SEM of the number of experiments indicated in parentheses. The structures and abbreviations of compounds are given in Fig. 1.

*% E_{max} is the maximal cAMP accumulation stimulated by the compounds relative to that of (−)-isoproterenol × 100.

†Indicates a significant difference in the value of each compound compared with the corresponding value of (−)-isoproterenol ($P < 0.05$).

comparative affinities and activities at the human β_3-AR. (-)-Isoproterenol and BRL 37344 also were included as standards for the study. Table 2 summarizes the binding affinities (pK_i or -log K_i values), biochemical potencies (pK_{act} or -log K_{act} values), and relative maximal activities (E_{max}) of these compounds at the human β_3-AR. Specific binding of [^{125}I]ICYP ranged from 70 to 75%. Each compound displaced [^{125}I]ICYP from human β_3-AR binding sites in a concentration-dependent competitive manner. The binding affinities of the compounds for the human β_3-AR in decreasing order were as follows (pK_i values in parentheses): bromoacetamidoDITMQ (6.94) ≥ chloroacetamidoDITMQ (6.77) ≥ acetamidoDITMQ (6.63) > BRL 37344 (5.79) ≥ isothiocyanatoITMQ (5.60) > (-)-isoproterenol (4.55). With the exception of the acetamido analog, the selected TMQ analogs demonstrated very similar affinities for the rat and human β_3-AR. Among the standards used, (-)-isoproterenol demonstrated nearly identical affinities for the β_3-AR of the two species, whereas BRL 37344 had a much lower affinity (<10-fold) for the human β_3-AR.

Biochemical potencies and maximal activities of the selected TMQ analogs and the standard compounds are also summarized in Table 2. The maximal activity (E_{max}) values of the compounds were determined relative to that of (-)-isoproterenol. The basal levels of cAMP and the amounts accumulated in the cells by 25 μM forskolin and maximal (-)-isoproterenol stimulation were 126 ± 23, 10,573 ± 924, and 3,603 ± 364 pmol/mg protein, respectively (means ± SEM, N = 6). The TMQ analogs exhibited greater biochemical potencies compared with the standards, (-)-isoproterenol and BRL37344, and the rank order of potencies of the compounds for human β_3-AR-mediated activation of adenylyl cyclase was as follows (pK_{act} value in parentheses): bromoacetamidoDITMQ (9.35) ≥ acetamidoDITMQ (8.96) ≥ chloroacetamidoDITMQ (8.68) > isothiocyanatoITMQ (8.02) ≥ (-)-isoproterenol (7.99) > BRL 37344 (7.64). BRL 37344 was a partial agonist with maximal activity (means ± SEM) of 65 ± 4.2% that of (-)-isoproterenol-mediated maximal activity, whereas the TMQ analogs were full agonists with E_{max} values ranging from 101 ± 8.3 to 114 ± 7.4% of maximal (-)-isoproterenol-mediated activity. The rank order and values of biochemical potencies and maximal activities of the TMQ analogs and (-)-isoproterenol at the human β_3-AR were very closely comparable with those at the rat β_3-AR. Unlike its properties at the rat β_3-AR, the lower potency and partial agonist activity of BRL 37344 observed at the human β_3-AR are in agreement with previously documented results [15, 16].

Functional Activities in Rat Tissues Representing β-AR Subtypes

AcetamidoDITMQ and chloroacetamidoDITMQ were equally or more potent than (-)-isoproterenol for increasing the chronotropy of right atria, and for producing relaxation of trachea and esophageal smooth muscle that were precontracted with carbachol (see Fig. 4). The potency (pEC_{50} value) and relative intrinsic activity of each compound are summarized in Table 3. Potency values for the compounds in atria were corrected for tissue desensitization effects. Whereas the three compounds were equipotent in right atria ($pEC_{50} \approx 8.95$), the TMQ analogs were significantly more potent than (-)-isoproterenol in both trachea and esophagus. The pEC_{50} values were 8.00, 9.22, and 8.90 in trachea and 7.34, 8.68, and 8.08 in esophagus for (-)-isoproterenol, acetamidoDITMQ, and chloroacetamidoDITMQ, respectively. In these tissues, acetamidoDITMQ was the most potent compound among the three analogs. However, the TMQ analogs were partial agonists on the β_1- and β_2-AR systems giving intrinsic activities (I.A.) of 0.93 and 0.81 in right atria, and 0.84 and 0.83 in trachea for acetamidoDITMQ and chloroacetamidoDITMQ, respectively. In contrast, these analogs exhibited full agonist activity in the β_3-AR system (I.A. = 0.99).

Functional Activity in Rat Atria Following Washout of Compounds

The time-course profile of changes in chronotropic responses to fixed concentrations of isoproterenol and the two TMQ analogs following a series of repeated buffer washes (every 10–15 min) is shown in Fig. 5. In the atria treated with (-)-isoproterenol and acetamidoDITMQ, the chronotropic responses lasted for an average of 70 and 75 min, respectively. In comparison, the duration of the atrial response to chloroacetamidoDITMQ treatment was signif-

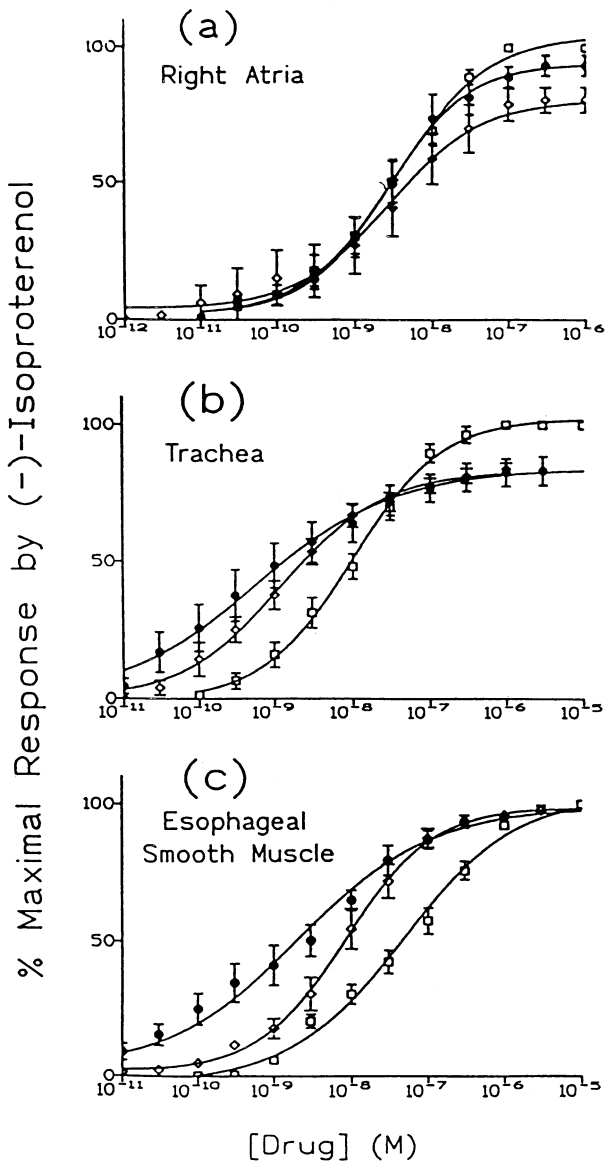

FIG. 4. Concentration-dependent functional responses of (-)-isoproterenol (□), acetamidoDITMQ (●), and chloroacetamidoDITMQ (◇) mediated by the different β-AR subtypes in isolated rat tissues. The data are expressed as the means ± SEM of 4–12 experiments. Key: (a) chronotropic response in right atria (β_1-AR); (b) relaxation of partially contracted tracheal strips (β_2-AR); and (c) relaxation of partially contracted esophageal smooth muscle (β_3-AR).

icantly longer; a response equal to 10% that of the initial maximal response was still present after 200 min. The average time required for a reduction in the chronotropic response to 50% of the maximal drug effect was 14, 17, and 95 min for (-)-isoproterenol, acetamidoDITMQ, and chloroacetamidoDITMQ, respectively.

Functional Studies of AcetamidoDITMQ and ChloroacetamidoDITMQ in Rat Aorta

These two TMQ analogs did not produce a contraction of the rat aortic strips at concentrations ranging from 10^{-9} to 10^{-6} M, or significantly shift ($P > 0.05$) the phenylephrine concentration-response curve following preincubation of tissues with 10^{-6} M of each analog (data not shown). The results of these studies indicate that the TMQ analogs were not α-AR agonists or antagonists in rat aorta.

Irreversible Binding of the TMQ Derivatives to Rat β_3-AR CHO

Our results on binding indicated that the comparative affinities of the acetamidoDITMQ, and its halogenated analogs, chloro- and bromoacetamidoDITMQ, were nearly the same in human versus rat β_3-AR CHO cells. Subsequent binding studies were initiated with the β_3-AR CHO cells to provide correlations with the functional tissue experiments in this same species. When the CHO cells were incubated for 40–45 min with the TMQ analogs with a washout, a concentration-dependent decrease in [^{125}I]ICYP binding to the receptors was observed (see Fig. 6, top panel). In contrast to acetamidoDITMQ-treated cells, specific ICYP binding to the rat β_3-AR was inhibited significantly following washout of cells treated with either chloro- or bromoacetamidoDITMQ. Specific binding was inhibited to the extent of 80–90% of the total ICYP-bound receptor fraction in the presence of 30- and 100-fold the K_i concentrations of chloroacetamidoDITMQ, whereas the bromoacetamido derivative inhibited specific ICYP binding to receptors by only 50% at the highest concentration (100 K_i value). Thus, the chloroacetamidoDITMQ analog displayed the properties of an affinity-labeling agent and was used in the subsequent experiments.

The kinetics of this irreversible binding of chloroacetamidoDITMQ to the rat β_3-AR was studied at drug concentrations of 3 and 10 K_i, and the results are shown in Fig. 6, bottom panel. No significant relationship could be established between the period of incubation (1–40 min) and the degree of reduction in the specific ICYP binding to the receptors. The irreversible binding reaction was nearly complete within 1 min, indicating the high reactivity and rapid binding kinetics of chloroacetamidoDITMQ as an affinity ligand.

Binding Protection Assays

Reversibly acting agonists, (-)-isoproterenol, BRL 37344, and acetamidoDITMQ (a close structural analog), and nucleophilic amino acids were tested on the rat β_3-AR for their ability to protect against the reduction in specific [^{125}I]ICYP binding by chloroacetamidoDITMQ. AcetamidoDITMQ, at concentrations of 30–100 times its K_i value, did not protect receptor sites from the binding of the chloroacetamidoDITMQ (Fig. 7). Similar findings were obtained when (-)-isoproterenol and BRL 37344 were used as protecting agents (data not shown). Additionally, the nucleophiles, glutathione, L-cysteine and L-lysine, at concentrations of 10–100 mM, also failed to interfere with the

TABLE 3. Agonist activities of (−)-isoproterenol, acetamidoDITMQ and chloroacetamidoDITMQ on β-adrenoceptors in isolated rat tissues

Compound	Right atria (β_1-AR)	Trachea (β_2-AR)	Esophageal smooth muscle (atypical-β/β_3-AR)
(−)-Isoproterenol			
pEC_{50}	8.95 ± 0.06	8.00 ± 0.05	7.34 ± 0.08
I.A.	1.00	1.00	1.00
(*N*)	(5)	(8)	(12)
AcetamidoDITMQ			
pEC_{50}	8.96 ± 0.04	9.22 ± 0.07*	8.68 ± 0.12*
I.A.	0.93 ± 0.04	0.84 ± 0.02*	0.99 ± 0.03
(*N*)	(4)	(4)	(7)
ChloroacetamidoDITMQ			
pEC_{50}	8.94 ± 0.07	8.90 ± 0.05*	8.08 ± 0.03*
I.A.	0.81 ± 0.05*	0.83 ± 0.02*	0.99 ± 0.01
(*N*)	(4)	(4)	(6)

Data are calculated as pEC_{50} ($-\log EC_{50}$, concentration required to produce a response equal to 50% of the maximal response elicited by the drug) and I.A. (intrinsic activity, maximal drug-induced response relative to the maximal response elicited by (−)-isoproterenol). Values are means ± SEM of the number of experiments indicated in parentheses. Structures and abbreviations are given in Fig. 1.

*Indicates a significant difference in the value of the TMQ analog compared with the corresponding value of (−)-isoproterenol ($P < 0.05$).

reactivity of chloroacetamidoDITMQ for binding to the receptors (data not shown).

DISCUSSION

The major goal of our studies was to determine the influence of chemical modifications on the lipophilic 1-benzyl ring of TMQ on the profile of β_3-AR activity. The binding affinities and biochemical potencies of the 1-benzyl substituted TMQ analogs on rat β_3-ARs in CHO cells were higher than those of the standard β-AR agonist (-)-isoproterenol and were comparable to those of the highly potent β_3-AR-selective agonist BRL 37344. In comparison with the parent compound, TMQ, these 1-benzyl substituted analogs of TMQ possessed higher binding affinities and biochemical potencies on the rat β-AR, and these properties improved significantly with the addition of lipophilic and bulky substituents on the 3′,4′,5′-portions of the 1-benzyl group. Diiodo substitution at the 3′- and 5′-positions of the 1-benzyl ring of TMQ adds considerable bulk and increases the lipophilicity of the compound. Acetamido substitutions at the 4′-position of the 1-benzyl ring further translated into improved binding affinities. These results are in agreement with the hypothesis of Blin *et al.* [32] that the bulky, aromatic side-chains of β_3-AR agonists may be contributing significantly to their agonist activities on this receptor, and that this group interacts at a binding site that is different from that of the catechol hydroxyls.

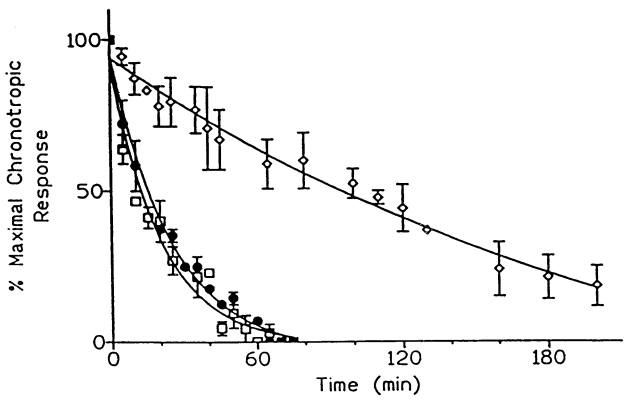

FIG. 5. Duration of the chronotropic response after washout in rat right atria treated with (-)-isoproterenol (□), acetamidoDITMQ (●), and chloroacetamidoDITMQ (◇). The data are means ± SEM of 3–6 experiments.

Modification of the 6,7-hydroxyl groups (catechol function) of TMQ with either a 6,7-dimethoxy or a 6,7-methylenedioxy moiety greatly reduced the affinity and agonist activity for the rat β_3-AR. In this regard, it is well known that the affinity and efficacy of catecholamine agonists on β-AR subtypes are greatly affected by removal of the catechol hydroxyls, thereby interrupting the hydrogen bonding between these groups and serine residues in tm 5 [2, 28]. Accordingly, we propose that these substitutions may be perturbing the spatial and/or electronic aspects of the hydrogen bonding with the serine residues in tm 5. It is of some interest that weak agonist activity was retained by methylenedioxyTMQ, and that β_3-AR agonists, as compared to β_1- or β_2-AR agonists, are less dependent upon the presence of a catechol nucleus [32]. For β_3-AR agonists, the catechol hydroxyls are generally replaced with chloro atoms (as in BRL 37344) or by bioisosteres for the catechol or phenolic groups. In this regard, we have reported recently that bioisosteric 2-aminothiazole derivatives of TMQ are selective agonists on the β_3-AR [42].

The biochemical potencies of (-)-isoproterenol, acetamidoDITMQ, and chloroacetamidoDITMQ correlated fairly well with their functional potencies for relaxing the esophageal smooth muscle (pK_{act} values of 7.90, 9.34, and 9.05, and pEC_{50} values of 7.34, 8.68, and 8.08, for the three compounds, respectively), indicating that the expressed recombinant receptors provided a convenient and fairly good substitute for the native receptors under physiological

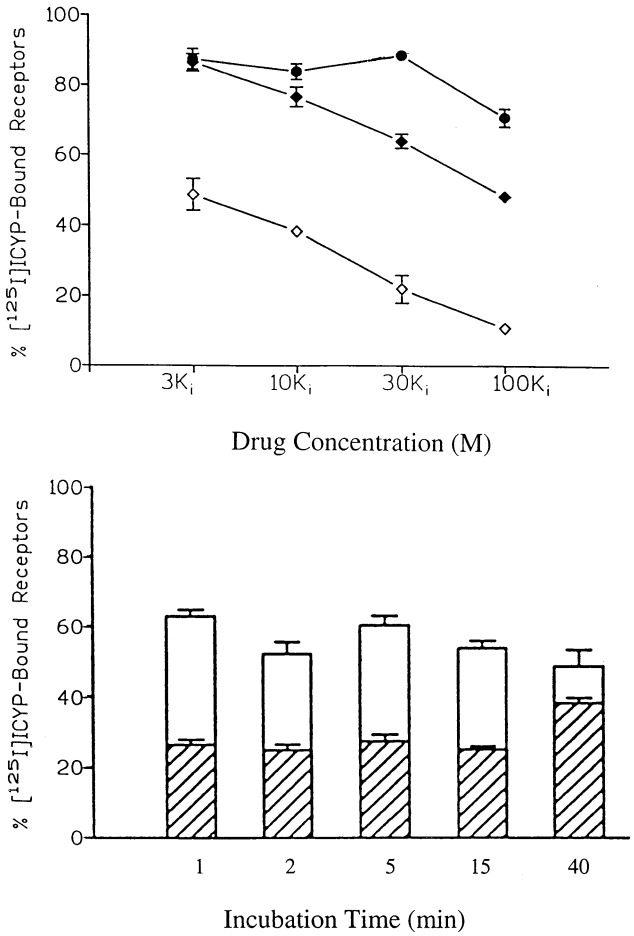

FIG. 6. (Top panel) Concentration-dependent inhibition of [^{125}I]ICYP binding to rat β_3-AR in CHO cells in washout experiments after incubation for 40–45 min with acetamidoDITMQ (●), chloroacetamidoDITMQ (◇), or bromoacetamidoDITMQ (◆). Values are expressed as the means ± SEM of 4 experiments, each in triplicate. (Bottom panel) Effect of time of incubation with chloroacetamidoDITMQ at molar concentrations of 3 K_i (open bars) and 10 K_i (hatched bars) in washout experiments, upon the inhibition of [^{125}I]ICYP binding to rat β_3-AR in CHO cells. The data are expressed as the means ± SEM of 2–4 experiments, each in triplicate.

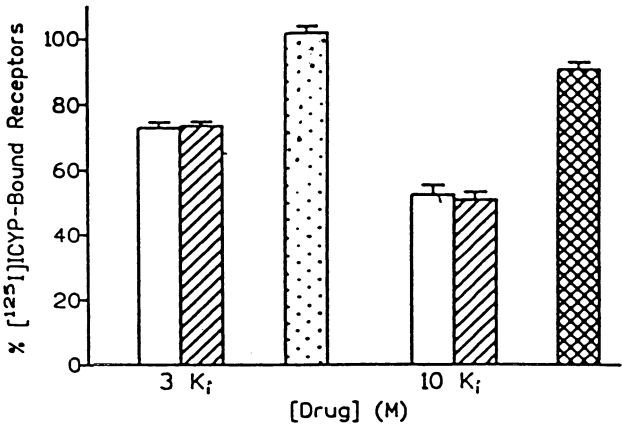

FIG. 7. Concentration-dependent inhibition of [^{125}I]ICYP binding by chloroacetamidoDITMQ to rat β_3-AR in CHO cells in the presence of acetamidoDITMQ as the protecting agent. The data are expressed as the means ± SEM of 3 experiments, each in triplicate. Key: chloroacetamidoDITMQ (open bars, 3 K_i and 10 K_i molar concentrations), chloroacetamidoDITMQ plus acetamidoDITMQ (forward hatched bars, 3 K_i plus 30 K_i, respectively; and 10 K_i and 100 K_i, respectively) molar concentrations; and acetamidoDITMQ only at 33 K_i (dotted bar) and 110 K_i (cross-hatched bar).

conditions. Although acetamido- and chloroacetamido-DITMQ had lower relative efficacies than (-)-isoproterenol in the recombinant system (as seen from the receptor occupancy plots), these were higher than that of BRL 37344. In addition, the two TMQ compounds exhibited full agonist activities in the functional studies with this β_3-AR-containing tissue [33]. The differences in biochemical and functional potencies of the compounds, as well as the differences in maximal activities, may be explained by differences in amounts of spare receptors in the two systems as well as by different end-points of response measurement in the signal transduction pathway. In summary, the acetamido- and haloacetamidoDITMQ analogs were more potent agonists on the rat and human β_3-AR than (-)-isoproterenol, and comparable to or greater than BRL 37344. However, these analogs did not exhibit any β_3-AR subtype selectivity in functional studies on rat tissues.

Rational structural modifications based on an understanding of the ligand-receptor interactions may lead to more selective analogs. The haloacetamido derivatives of DITMQ were synthesized to serve as tools for this purpose, and another objective of this research was to examine the irreversible binding of the haloacetamidoDITMQ analogs to rat β_3-AR in CHO cells. In reversible competition experiments, the chloroacetamido and bromoacetamido derivatives of DITMQ inhibited the binding of [^{125}I]ICYP to rat β_3-AR with affinities of 323 and 200 nM, respectively, which are comparable to that of BRL 37344 (110 nM). In washout experiments, indirect evidence for covalent interaction of these compounds was presented by the fact that [^{125}I]ICYP binding activity was not restored to control levels despite extensive washing of the cells. More importantly, the degree of receptor blockade by these compounds was concentration dependent. In these studies, the chloroacetamido derivative was a more effective affinity agent than the bromoacetamido analog. Since stability studies (data not shown) indicated that both of these compounds were stable in aqueous or methanolic solutions, it is possible that the bromoacetamido derivative could be reacting additionally to a greater extent than the chloroacetamido TMQ analog with nonspecific sites on the receptor protein. Moreover, time-dependence studies suggested that the kinetics for affinity binding of these analogs to the receptor were very rapid (<2 min). This is not surprising, considering that the nucleophilic attack by the haloacetamido moiety is a one-step reaction versus covalent binding of 2-haloethylamines (i.e. phenoxybenzamine), which involves generation of intermediate reactive aziridinium cations [43, 44]. In the latter case, incuba-

tions of phenoxybenzamine for longer than 10 min are required to achieve maximal inactivation of α-ARs. The inability of exogenous nucleophilic reagents such as glutathione, L-cysteine, and L-lysine to quench the reactivity of the TMQ affinity analog in protection assays, failed to offer convincing evidence for the covalent binding of the compound to the receptors via nucleophilic substitution. However, in several similar studies involving iodonaphthylazide labeling of intrinsic membrane proteins such as cytochrome oxidase, Ca-ATPase, (Na,K)-ATPase, and glycophorin, the presence of glutathione in the aqueous phase failed to offer any protection, in contrast to the findings with the much less hydrophobic phenylazide label [45]. By analogy, it is possible that the nucleophiles, being relatively polar, were poor competitors for the highly reactive lipophilic affinity compound that we postulate to be occupying a hydrophobic binding site on the β_3-AR. The absence of protection by competing agonists also strongly suggests that the affinity interaction of our compound may be located at a site outside of the agonist binding pocket, but at a site that may be more in common with the occupation site of ICYP. In this regard, the β_2-/β_3-AR agonist salmeterol is proposed to bind to an exo-receptor site in a non-competitive manner [46]. Salmeterol possesses an extended *N*-alkyl substituent that is predicted to be located deep into a hydrophobic core domain of the receptor that represents the specific exo-site, while the saligenin head interacts with the receptor in a manner analogous to salbutamol and other conventional β-AR agonists. The high-affinity binding of the side-chain to the exo-site allows the saligenin head to activate the receptor in a continuous manner, enabling salmeterol to exhibit a long-acting pharmacological effect. TMQ, like salmeterol, possesses an extended *N*-alkyl side-chain that may be binding to a hydrophobic exo-site on the β_3-AR in a non-competitive manner. The persistent chronotropic response of chloroacetamidoDITMQ in atria following extensive washout procedures provides evidence for the irreversible interaction of this compound with the β_3-AR.

Blin and co-workers [32] have postulated that the bulky, aromatic substituent of β_3-AR agonists interacts with amino acid side-chains in tm 1, 2, and 7 of the receptor. The three-dimensional view of the β_2- and β_3-AR sites showing docking of β-AR subtype selective ligands has confirmed this difference in steric space occupation of the site [47, 48]. Thus, in addition to the established involvement of aspartic acid and serine residues in tm 3 and 5, respectively [2, 28], several amino acids present in tm 1, 2, and 7 are implicated in catecholamine agonist binding and signal transduction [27, 32]. Recently, Gros and coworkers [49] have demonstrated through site-directed mutagenesis of the human β_3-AR that aspartate 117 and asparagine 312 on tm 3 and 6, respectively, are essential for ligand binding and signal transduction. Previous studies in our laboratory indicate that the catechol and secondary amino groups of the TMQ molecule interact with aspartate in tm 3 and serines in tm 5 [25]. Thus, we hypothesize that the trimethoxybenzyl side-chain of agonist analogs of TMQ interacts with amino acids in a hydrophobic ligand binding pocket that may involve tm 1, 2, 6, and 7, and facilitates receptor activation. In our studies, we have demonstrated a concentration-dependent irreversible binding of chloroacetamidoDITMQ with rat β_3-AR in CHO cells, and a persistence of functional activity in the washout studies on rat atria. Thus, we propose that the high-affinity interactions of the reactive 4′-chloroacetamido group of DITMQ is with some amino acid(s) located at a site external (an exo-site) to those of reversibly acting TMQ and β_3-AR agonists within the hydrophobic pocket of the receptor.

Photoaffinity antagonist labeling of the β_2-AR with compounds possessing photo-activatable groups on the aryloxy or the amino ends of the molecule showed that the aryloxy portion of β-AR antagonists is highly constrained within tm 6 and 7 of the receptor, whereas the amino terminus is much less constrained and able to assume multiple conformations [50]. The antagonist derivatives containing a photo-activatable group on the amino end derivatized amino acids to a greater extent on tm 1, 2, 6, and 7, indicating that a folded conformation of the molecule is favored. Several β_1-/β_2-AR antagonists including ICYP are known to behave as partial agonists on the β_3-AR [1]. The proposed model of antagonist conformation and interaction with tm 6 and 7 of the β-AR may provide an explanation for the partial agonist behavior of several β_1-/β_2-AR agonists on the β_3-AR, as well as for the non-conventional interactions of the ether-containing alkylamine side-chain of salmeterol. It also provides added credibility to the hypothesis of Blin *et al.* of a hydrophobic binding pocket on tm 1, 2, and 7 of the β_3-AR [32]. Likewise, the affinity binding profile of chloroacetamidoDITMQ seems to favor the explanation that the compound interacts with amino acids on tm 1, 2, 6, and 7 of the β-AR and thereby interferes with the binding of ICYP, but this site is not competed for by classical agonists. The rapid kinetics of affinity binding of the compound may provide additional explanation for the lack of protection observed with the β_3-AR agonist BRL 37344 as well as by the close structural analog acetamidoDITMQ. Notably, the acetamido- and chloroacetamidoDITMQ analogs did not interact with α-AR in rat aorta, suggesting that the interactions are selective for the ligand binding pocket at the rat β_3-AR.

Finally, the significantly longer duration of response in rat atria after periodic washing of the tissue treated with the chloroacetamido analog versus those treated with (-)-isoproterenol or acetamidoDITMQ provides substantial evidence of the pharmacologically irreversible nature of the former compound. Although this response in the isolated tissue may be a combined result of several phenomena, it is reasonable to infer from the above studies that the nucleophilic chemical moiety present in chloroacetamidoDITMQ, as opposed to its closest analog, acetamidoDITMQ, imparts affinity properties to the former in its interactions with the rat β_3-AR. Of course, conclusive evidence for the covalent nature of binding and site-specificity of affinity binding of

our compound to the β_3-AR will be demonstrated only when we isolate and purify the receptors, and label and identify peptide fragments carrying the TMQ analog. Currently, we have reported on newer related analogs of DITMQ with catechol ring and 1-benzyl ring modifications on human β-AR subtypes [42]. These studies have provided important leads for rational modifications of the TMQ molecule for the long-term goal of designing drugs for the possible treatment of obesity, non-insulin-dependent diabetes, and gastrointestinal hypermotility disorders.

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