Antagonism of Thyroid Hormone Action by Amiodarone in Rat Pituitary Tumor Cells

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Abstract

A thyroid hormone antagonist has not been previously described. A number of thyroid hormone analogues have been shown to compete with [^{125}I]triiodothyronine ([^{125}I]T$_3$) for binding to the intranuclear thyroid hormone receptor and to have agonist activity proportional to their affinities for the receptors. We report that the benzofuran amiodarone acts as a competitive antagonist to thyroid hormone action as defined by its dose-dependent ability to (*a*) bind to the thyroid hormone receptor and (*b*) inhibit T$_3$-induced increases in growth hormone mRNA levels in a cultured rat pituitary cell line, GC cells. Like T$_3$ itself, amiodarone also decreases transport of [^{125}I]T$_3$ across GC cell membranes. An analysis of the amiodarone structure suggests that this compound has certain similarities to T$_3$. These findings hold promise for the development of other thyroid hormone antagonists for clinical use and for understanding thyroid hormone action.

Introduction

Most cell receptors for small organic molecules have a developed pharmacology of agents that act as agonists or antagonists. For example, suitable antagonists can block the actions of dopaminergic, alpha and beta adrenergic, and cholinergic agonists (1). Antagonists have been described that interact with the mineralocorticoid, glucocorticoid, estrogen, and progesterone receptors (1). It is surprising, however, despite extensive structure activity studies conducted over the past 20 years (2, 3), no antagonist for thyroid hormone receptor action has been identified.

Amiodarone is a benzofuran (2-butyl-3-[3,5-diiodo-4-(β-diethylaminoethoxy)-benzoyl]benzofuran) widely prescribed for the treatment of ventricular and supraventricular arrhythmias (4, 5). Chronic administration of amiodarone has been associated with both hyper- and hypothyroid-like side effects. The drug has also been reported to cause changes in the concentrations of serum thyroxine (T$_4$) and triiodothyronine (T$_3$) levels, which have been attributed to an inhibition of peripheral T$_4$ monodeiodination, and to iodine-induced changes in glandular hormonogenesis (6, 7). However, cases of clinical hypothyroidism have occurred, often with mildly elevated thyroid-stimulating hormone (TSH) and normal T$_4$ and T$_3$ levels, and in vitro studies have suggested an amiodarone-induced

increase in pituitary TSH release, together with a decrease in [^{125}I]T$_3$ binding to pituitary nuclei (8); these observations raise the suspicion that amiodarone could act as a thyroid hormone antagonist at the receptor level.

In this study we report that amiodarone fulfills the criteria of a thyroid hormone antagonist. The drug behaves as a competitive inhibitor of T$_3$ binding to solubilized thyroid hormone receptors, and it blocks both receptor binding and the biological effect of thyroid hormone when administered to hormone-responsive cells in culture.

Methods

Cell culture. GC cells were obtained from the cell culture facility, University of California at San Francisco (UCSF). They were plated at 2–4 × 10^6 cells/100 mm tissue culture dish (Falcon Labware, Oxnard, CA) and were maintained at 37°C, 95% air, 5% CO$_2$, in DME H-21 medium containing 10% fetal calf serum (FCS; J. R. Scientific; Woodland, CA), 100 U/ml penicillin and streptomycin, and 2 mM glutamine.

Removal of thyroid hormones from FCS. Thyroid hormones were removed from serum with AG 1-X8 ion exchange resin (Bio-Rad Laboratories, Richmond, CA) as described (9). Before treatment, T$_4$ and T$_3$ concentrations in whole serum were 1.9 × 10^{-7} M and 1.9 × 10^{-9} M, respectively. In 100% thyroid hormone-depleted (stripped) serum they were 1.7 × 10^{-8} M and nondetectable (< 300 pM). Actual concentrations in 2% media were therefore 1/50 of these values.

Preparation of solubilized T$_3$ nuclear receptors. Rat liver nuclear receptors were prepared according to Apriletti et al. (10). Solubilized nuclear receptors from whole rat brain were prepared according to Schwartz and Oppenheimer (11) except that receptors were solubilized with 0.4 M KCl. Livers and brains were from euthyroid rats, which were obtained from Rockland, Inc. (Gilbertsville, PA). Solubilized receptors from GC cells were prepared as follows: confluent 150-cm^2 culture plates were rinsed for 5 min with PBS (37°C), then scraped with a rubber policeman in 5 ml PBS (4°C). The 1,000 *g* cell pellet was suspended in 10 ml solution A, on ice [20 mM KPO$_4$, pH = 7.6; 4 mM EGTA; 4 mM MgCl$_2$; 0.25 M sucrose; 0.5% NP-40; 0.5 mM PMSF; 0.1% monothioglycerol (MTG)^1]. Cells sat on ice for 3 min, followed by a second spin. The resulting cell lysate was washed with solution A minus NP-40, pelleted again, and resuspended in 10 ml of solution B (20 mM KPO$_4$, pH = 7.2; 10 mM EDTA; 2 mM MgCl$_2$). The nuclei were gently mixed and allowed to sit at 4°C for 30 min. This suspension was then centrifuged (300 *g*, 10 min), and the resulting chromatin pellet was washed twice in 10 ml of solution C (10 mM KPO$_4$, pH = 7.2; 1 mM MgCl$_2$; 0.1% MTG). The washed pellet was then solubilized in 2 ml of solution D (10 mM Tris; 1 mM MgCl$_2$; 0.5 mM EDTA; 400 mM KCl; 10% glycerol; 0.1% MTG; pH = 7.6). The chromatin was then sonicated in this solution (30 1-s pulses). After a 30-min incubation on ice, polyethyleneimine (PEI) was added to a final concentration of 0.01% and the solution stirred gently for 5 min. Following centrifugation at 15,000 *g* for 15 min the supernatant was adjusted to 3% PEG, 0.02% PEI, and 20 mM KPO$_4$. After 20 min on ice this

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1. *Abbreviations used in this paper:* MTG, monothioglycerol; PEI, polyethyleneimine.

suspension was centrifuged again (15,000 g, 30 min) and aliquots were frozen in liquid N_2 and stored at $-70°C$. Treatment with PEI removed any contaminating DNA. Yield for 900×10^6 cells = 7.5 pmol; sp act, 3.1 pmol/mg protein = 5,000 sites/cell.

In vitro binding assays. [^{125}I]T_3 and thyroid hormone receptor preparations were incubated with varying concentrations of nonradioactive T_3 or amiodarone for 18 h at 4°C in a buffer consisting of 20 mM KPO_4, 0.5 mM EDTA, 1 mM $MgCl_2$, 400 mM KCl, 8% glycerol, and 0.1% MTG. Bound and free [^{125}I]T_3 were separated with G-25 Sephadex. Receptor concentrations were 150–300 pM. L-T_3 was from Aldrich Chemicals (Milwaukee, WI). A 1-mM stock was prepared in MeOH containing 1% NH_4OH. This was diluted in 0.1 mM NaOH and added directly to binding and culture assays. Amiodarone-HCl was obtained in liquid form (Cordarone; Labaz, France) or as a purified powder (Sanofi Chemicals, Montpellier, France). The liquid form was supplied at 73 mM in a vehicle consisting of (vol/vol) 88.8% H_2O, 9.3% Tween 80, 1.9% benzyl alcohol. The powder was prepared fresh at 14 mM in EtOH:10 mM HCl (1:1). Dilutions from both solutions were prepared in 1 mM HCl. Control experiments were carried out in the presence of appropriate vehicles, which had no effect on binding reactions. Preliminary experiments suggested that the powdered form, prepared and diluted as described, did not affect intact, cultured GC cells in a manner identical to the liquid form, probably because of solubility problems. For this reason, all data reported herein are based upon experiments utilizing the liquid stock. L-[^{125}I]T_3 was from New England Nuclear (Boston, MA; NEX-110X: 2,200 Ci/mmol).

Binding of [^{125}I]T_3 to intact GC cells and cell fractions. 2 million cells were plated per well of 6-well tissue culture plates (Falcon; 9.3 cm^2/well) in 3 ml of media. After 18 h cells were rinsed with PBS (1 ml, 10 min, 37°C) and incubated for 4 h in DME containing 10% serum substitute solution (12) and 2% stripped FCS. For competition assays, cells were incubated in 1 ml of stripped media containing 300 pM [^{125}I]T_3 and various doses of amiodarone. Incubations were carried out at 37°C on a tilt table. After 3 h, during which time equilibrium was established, cells were washed thoroughly in PBS, and whole-cell radioactivity was determined. Cell nuclei were then prepared by lysis in situ and specific nuclear binding determined according to established methods for pituitary tumor cells (13). Binding in each nuclear pellet was normalized to DNA content (14). [^{125}I]T_3 radioactivity was also determined in the supernatant of the nuclear pellet. For the purposes of this study the supernatant, which contained cytosol and lysed cellular membranes, was termed cytosol. In separate experiments, GC cells were cultured as described except in the presence of 1.5 nM [^{125}I]T_3. Under these conditions, binding capacity was $\sim$ 85 fmol/100 μg DNA. Nuclear radioactivity was 5% or less of total cellular radioactivity.

Analysis of receptor binding parameters. Binding data were analyzed with the Scatfit and Allfit computer programs. These programs perform nonlinear, least squares model fitting utilizing untransformed data by sequential iteration of binding parameters and generate K_d, maximum bound (B_{max}), EC_{50}, and pseudo-Hill slope constants (b). Slopes greater than unity indicate positive cooperativity or nonequilibrium conditions. Slopes less than unity indicate heterogeneity of binding sites and/or negative cooperativity. Slopes indistinguishable from one are consistent with a single site, mass action binding model (15). The ability of amiodarone to compete with T_3 for binding to the thyroid hormone receptor was assessed by comparison of K_d and EC_{50} values generated by the Scatfit and Allfit programs, respectively (15, 16).

Preparation and quantitation of messenger RNA. After plating and attachment (12–18 h), GC cells were cultured for 2–4 d in 20 ml of DME containing 10% serum substitute solution plus 2% stripped serum (9). Media were replaced after the second day of culture. It has been demonstrated previously (17, 18) that 2–4 d of culture under "hypothyroid" conditions ("deinduction") produce baseline levels of rGH mRNA in pituitary tumor cells. In one set of experiments, cells were deinduced for 4 d, then cultured for an additional 2 d in stripped media containing: (a) 300 pM T_3 plus varying concentrations of amiodarone, or (b) no additional treatment (Fig. 4). In a second set of experiments, they were deinduced for 48 h, then cultured for an additional 48 h in stripped media containing 3.2 μM amiodarone plus varying concentrations of T_3 (Fig. 5). Total cytoplasmic (19) or total cellular (20) mRNA were prepared as described. The amount of rGH message present in aliquots of GC cell cytoplasm was determined by Northern hybridization using glyoxylated RNA (21). Equal amounts of cytoplasmic RNA were examined per lane (15 or 20 μg). HeLa cell RNA also was tested as a control for specificity and background binding. The rGH message was detected by hybridization with the 0.8 kb pRGH-1 rat growth hormone cDNA (22). Some blots were also probed with a 1.6 kb Xho I fragment of the pHF1 human γ-actin gene (23). Both probes were labeled by nick translation with [^{32}P]dCTP (Amersham Corp., Arlington Heights, IL) to 3–5 $\times 10^8$ cpm/μg (24). Hybridizations were carried out for 18–48 h at 42°C. Blots were autoradiographed with Kodak XAR-2 film using Cronex Lightning Plus intensifying screens (E. I. DuPont de Nemours Co., Wilmington, DE), and then quantitative densitometry was performed with a Zeineh soft laser scanning densitometer. Alternatively, filter strips were dissolved in Filtron-X (National Diagnostics, Inc., Somerville, NJ) and counted.

Amiodarone and T_3 were added separately to each 100-mm culture dish (250 μl of each drug or vehicle in a total volume of 20 ml). It was essential that this procedure be followed: amiodarone was ineffective when added in advance to a large volume of serum-containing media in a glass beaker.

Computer modeling of T_3 and amiodarone structures. Computer modeling was performed by Teri Klein and George Seibel at the Computer Graphics Lab, UCSF. The compounds were modeled in extended conformation using standard geometries. Coordinates were displayed and manipulated in real time with the MIDAS program on an Evans and Sutherland graphics system. Very small torsion angle adjustments were required to superimpose the molecular structures.

Results

The characteristics of binding by T_3 to its nuclear receptor were first determined using crude preparations of KCl-solubilized rat liver nuclei. In these assays, T_3 bound to a single class of sites with high affinity (weighted mean of six determinations (K_d = 359 pM; range 189–630 pM). The K_d value of 359 pM was derived from nonlinear least-squares regression analysis of experimental data (15, 16) and was used as an initial estimate of receptor affinity in subsequent analyses of binding assays. Affinities were similar for receptors from solubilized GC cell nuclei and from cultured GC cells (560 and 470 pM, respectively). The results for solubilized hepatic receptors are similar to those reported previously (25, 26).

In a variety of solubilized receptor preparations, amiodarone competed with T_3 for binding to a single class of sites in a dose-dependent fashion at concentrations from 0.1 to 100 mM (Fig. 1 a). For rat liver-derived receptor, the 50% inhibition (EC_{50}) occurred at 3 μM amiodarone, and 80% inhibition was at 50 μM. Amiodarone rarely produced > 80% inhibition of [^{125}I]T_3 binding in these preparations. This is probably due to the poor solubility of the drug in high ionic strength buffers (27), which results in precipitates at higher concentrations. The calculated affinity of the receptor for amiodarone in this experiment expressed as the K_d was 1.4 μM. Amiodarone also competed with [^{125}I]T_3 for binding to solubilized receptors from pituitary tumor cells of the GC cell line with a K_d of 1.6 μM. The affinity for receptors from rat brain was somewhat less (K_d = 13.7 μM; n = 3; see Table I).

The competitive nature of amiodarone binding was further investigated by examining the effect of 10 μM amiodarone on the binding of increasing doses of [^{125}I]T_3 to its receptor
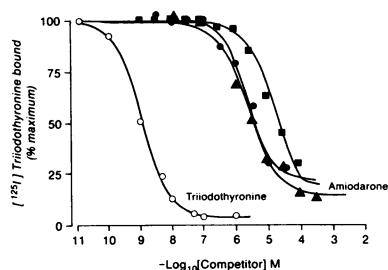
Figure 1. Competition by unlabeled L-T₃ and amiodarone for binding of L-[^{125}I]T₃ to rat nuclear thyroid hormone receptors solubilized from various tissues. Pictured are competition curves for T₃ in liver receptors (*open circles*); and for amiodarone in liver (*closed circles*), whole brain (*closed boxes*), and GC cell receptors (*closed triangles*). The affinity (K_d) for T₃ in liver nuclear receptors was 3.7×10^{-10} M. In the individual experiments shown, affinities for amiodarone were 1.4, 13.7, and 2.9 μM in solubilized receptors from rat liver, brain, and pituitary tumor (GC) cells, respectively. [r] = 160–280 pM. [^{125}I]T₃ = 100–200 pM.

(Fig. 2 *A*). Computer modeling of these curves revealed an amiodarone K_i of 9.3 μM for a single class of sites, not statistically different from 7.2 μM obtained from the studies with a fixed amount of [^{125}I]T₃ and varying amiodarone concentrations. Increasing concentrations of T₃ reversed amiodarone's effect on T₃ binding, characteristic of a competitive binding inhibitor. The data are redrawn as a Lineweaver-Burk plot in Fig. 2 *B*. The slopes (±95% C.I.) were 0.07±0.00 and 0.13±0.00 in the absence or presence of 10 μM amiodarone, respectively. The y-intercepts did not differ significantly. They were (±standard error of estimate) 6.6±0.2 and 6.8±0.7, respectively, indicative of competitive binding.

We next addressed the issue of whether amiodarone could compete with [^{125}I]T₃ for thyroid hormone receptor binding when incubated with intact cells. The nuclear T₃ receptors in cultured rat pituitary tumor cells (GC and related sublines) have been well characterized (26). Both T₃ and amiodarone competed with [^{125}I]T₃ for binding to the thyroid hormone receptor in intact GC cells (Table I). Because 2% serum was included in the incubations for these experiments, the free concentrations of drug or hormone present within the media may not be exactly equal to the concentration of drug or hormone added to the media. In addition, the free intracellular concentration cannot be easily determined. For these reasons

it is more accurate to compare the EC₅₀ values for the blockade of receptor binding and the blockade of receptor function. Competition binding isotherms determined in this system at 300 pM [^{125}I]T₃ displayed an EC₅₀ value of 3.3 μM for amiodarone (Table I). Since these numbers were in close agreement to those obtained with solubilized receptors, the effects of 2% serum were slight. Computer-derived (15, 16) slope constants (pseudo-Hill coefficients) were not significantly different than 1.0, suggesting a competitive interaction to a single class of sites for both T₃ and amiodarone.

The effects of T₃ and amiodarone on cellular equilibrium levels of [^{125}I]T₃ were examined in cultured GC cells (Fig. 3). Both competitors caused parallel, dose-dependent decreases in radioactivity in whole cells and cytosol. In addition, the maximum decrease in both cellular and cytosolic radioactivity was ~ 45–50% in response to either competitor. This result is in contrast to those obtained from GC cell nuclei, where radioactivity decreased by ~ 75 and 95% in response to increasing concentrations of amiodarone and nonradioactive T₃, respectively.

Cultured GC cells were utilized to assess the effect of amiodarone on T₃ action. These cells respond to T₃ by increasing their synthesis of rat growth hormone (rGH) due to increases in rGH mRNA resulting from transcriptional activation of the growth hormone gene (18, 28). As shown in Fig. 4 *A*, amiodarone blocked the induction of rGH mRNA by 300 pM T₃ as analyzed by RNA blot (Northern) analysis. In three experiments RNA was normalized by loading equal amounts (15 or 20 μg) of cytoplasmic RNA per lane, and equivalency of loading was verified by visualization of 18S and 5S rRNA by ultraviolet shadowing. As a further control, RNA content was normalized to human gamma-actin mRNA in an additional experiment with identical results. Autoradiographs were analyzed by densitometry, and arbitrary densitometric units were used to construct amiodarone competition curves. Fig. 4 *B* (*open circles*) displays an amiodarone mRNA competition curve that represents a composite of four experiments. The EC₅₀ for blockade of rGH mRNA by amiodarone under these conditions was 3.1 μM, very similar to the EC₅₀ for binding to the thyroid hormone receptor of these cells in the presence of 300 pM T₃ (3.3 μM) and is consistent with the K_d for binding

Table I. Binding Affinities (K_d) of T_3 and Amiodarone to Solubilized Nuclear Receptors of Rat Liver, Brain, Pituitary Tumor Cells or in Intact Cultured Cells

Receptor source	Triiodothyronine		Amiodarone	
	K_d	*b	K_d	b
Liver	$3.7\pm0.1 \times 10^{-10}$ M	1.0 ± 0.1	$7.2\pm2.1 \times 10^{-6}$ M	0.9 ± 0.1
Brain			$13.7\pm1.6 \times 10^{-6}$ M	1.0 ± 0.1
GC cell	$5.6\pm1.7 \times 10^{-10}$ M		$1.6\pm0.2 \times 10^{-6}$ M	0.7 ± 0.1
	‡EC₅₀		EC₅₀	
GC nuclear binding (intact cells)	$8.7\pm0.2 \times 10^{-10}$ M	1.0 ± 0.1	$3.3\pm0.2 \times 10^{-6}$ M	0.9 ± 0.4
Cytoplasmic rGH mRNA accumulation			$3.1\pm0.3 \times 10^{-6}$ M	0.8 ± 0.1

K_d values determined by competition binding studies and analyzed by weighted, nonlinear least squares regression with the aid of the Scatfit computer program (15, 16). Affinities derived from liver receptor preparations represent the weighted means from six experiments. Other values represent the mean of at least two experiments. (*b) slope factor. Determined with Allfit (15, 16). Values for "b" did not differ significantly from 1.0, indicating that both competitors interact with a single class of binding sites. ‡(EC₅₀) concentration of agonist (T₃) or antagonist (amiodarone) needed to decrease binding of [^{125}I]T₃ (300 pM) to 50% of maximum values.

A

B
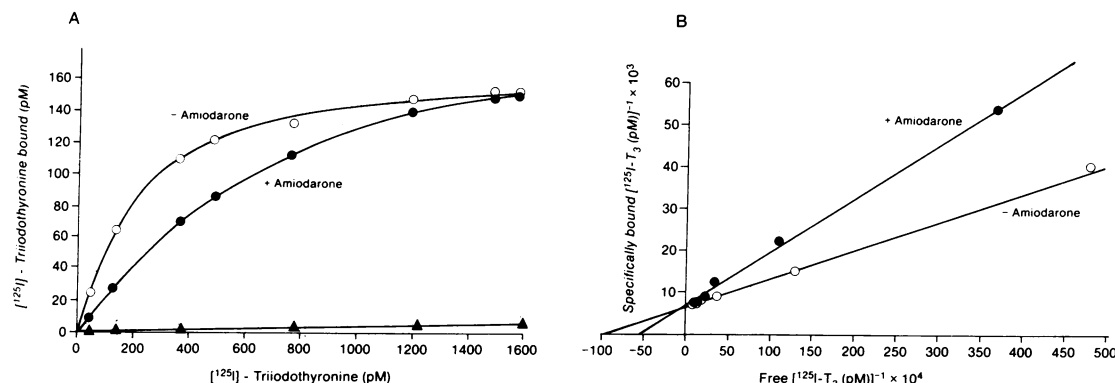
Figure 2. Effects of amiodarone on saturation binding of T_3 to soluble nuclear receptors. (a) Saturation binding isotherms: 150 pM of soluble rat liver nuclear receptor (3 pmol/mg) was incubated with increasing amounts of $[^{125}I]T_3$ in the absence (K_d) and presence (K_d') of inhibitor, and these values were used to determine a K_i for amiodarone. In these experiments more pure preparations of rat liver receptor were utilized than before. The following values were obtained from computer modeling: $K_d = 108$ pM, $K_d' = 224$ pM, and $K_i = 9.3$ μM. The lower line (*closed triangles*) indicates nonspecific binding (average of both curves), which represented 2–4% of total binding. Binding of $[^{125}I]T_3$ in the presence of 1 μM unlabeled T_3 yields

values for nonspecific binding (NSB). Separation of bound from free T_3 was achieved with G-25 Sephadex. (b) Lineweaver-Burk transformation of the data. The abscissa (x) and ordinate (y) represent inverse of free pM $[^{125}I]T_3$ and pM $[^{125}I]T_3$ specifically bound, respectively. The slopes (b) of the regressions in the absence and presence of amiodarone were 0.07 ± 0.00 and 0.13 ± 0.00, respectively ($b\pm95\%$ C.I.). The calculated K_i from these slopes using the less accurate graphical methods was 11.6 μM. The y-intercepts (a) were 6.6 ± 0.2 and 6.8 ± 0.7 ($a\pm$SEE), respectively, indicating that maximum binding did not differ in the absence or presence of amiodarone. $r^2 = 0.99$ for each regression line.

to GC cell solubilized receptor (1.6 μM). The binding and functional curves are essentially identical as illustrated by the open and closed circles in Fig. 4 B. The slope factor of the composite curve did not differ significantly from 1.0, again suggesting that amiodarone's effects on rGH mRNA are directly mediated by its interaction with a single class of nuclear receptor binding sites (see Table I).
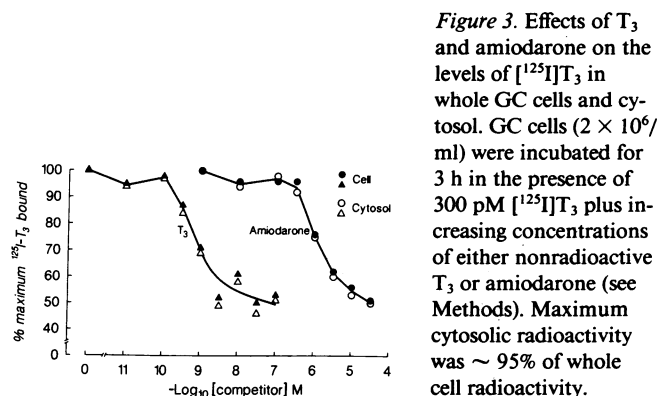
Since amiodarone displayed competitive binding to solubilized T_3 receptors (Fig. 2), we tested whether its inhibitory effects on rGH mRNA could be overcome by T_3. GC cells were deinduced 2 d, then treated with 3.2 μM amiodarone plus varying concentrations of L-T_3. After 24 h of culture, total cellular mRNA was quantified as described previously. In the absence of T_3, amiodarone produced no effect on rGH mRNA (Fig. 5). GC cells exposed to 300 pM T_3 in the absence of amiodarone had rGH mRNA levels about threefold greater than those in untreated cells, while amiodarone caused a reduction in mRNA levels under these conditions. Fig. 5 also demonstrates that a saturating dose of T_3 (5 nM) was able to overcome the inhibitory effects of amiodarone.

Figure 3. Effects of T_3 and amiodarone on the levels of $[^{125}I]T_3$ in whole GC cells and cytosol. GC cells (2 × 10^6/ml) were incubated for 3 h in the presence of 300 pM $[^{125}I]T_3$ plus increasing concentrations of either nonradioactive T_3 or amiodarone (see Methods). Maximum cytosolic radioactivity was $\sim 95\%$ of whole cell radioactivity.
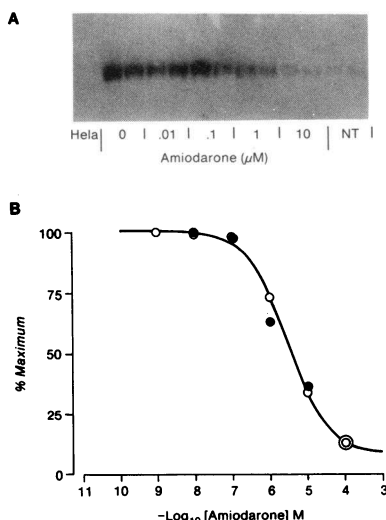
Discussion

These results are consistent with the concept that amiodarone acts as an antagonist to thyroid hormone action. This conclusion is supported by our observation that amiodarone (a) binds to thyroid hormone receptors from a variety of tissues and (b) inhibits T_3-induced increases in growth hormone mRNA levels in GC cells in a similar dose-dependent manner.

Previous binding studies utilizing amiodarone have yielded conflicting results. For example, an abstract by Wiersinga and Broenik (29) reported that amiodarone acts as a competitive inhibitor to T_3 binding in solubilized rat liver nuclear receptors. However, a more recent study (30) reported that amiodarone possessed no competitor activity in solubilized rat liver receptors. In cell culture studies, amiodarone did not inhibit hormone binding to rat hepatic nuclei (31) or to nuclei from cultured human fibroblasts (32), whereas it did compete for $[^{125}I]T_3$ binding to isolated rat anterior pituitary nuclei (8). The reason for this disparity is unknown. However, potentially important methodological differences exist among our studies. For example, two of the groups reporting negative results (30, 32) prepared amiodarone stocks from powder. We have found that amiodarone prepared in this way may gradually go out of solution, especially under tissue culture conditions. It is noteworthy that liquid amiodarone is supplied in a vehicle containing both benzyl alcohol and detergent (Tween 80), which may be essential for its solubility under assay conditions (see Methods for exact formulation). In addition, three of the groups reporting negative results (references 30–32) used ethanol as their diluent, whereas we used 1 mM HCl. Finally, one group (32) utilized glass tubes for their culture experiments. Our experience indicates that amiodarone adheres to glass (see Methods).

Previous functional studies have suggested that amiodarone exhibits thyroid hormone antagonist-like effects. In physiologic studies, for example, amiodarone administration caused

A

Hela| 0 | .01 | .1 | 1 | 10 | NT |
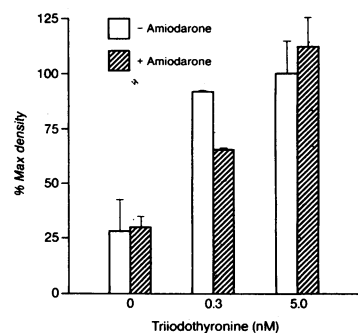Amiodarone (µM)

B

%Maximum

-Log₁₀ [Amiodarone] M

Figure 4. Effects of amiodarone on accumulation of rGH mRNA and on binding by T₃ to nuclear fractions of cultured GC cells. (*A*) Autoradiogram of a representative Northern blot of rGH mRNA from GC cell cytoplasm. GC cells were plated at 2–4 × 10⁶ cells/100 mm tissue culture dish (Falcon) and then deinduced as described in Methods. After 4 d of deinduction, cells were cultured for an additional 2 d in the presence of stripped media containing (*1*) 300 pM T₃ plus one of the following concentrations of amiodarone: 0, 10 nM, 100 nM, 1 µM or 10 µM; or (*2*) no additional treatment (NT). (*B*) (*Open circles*) composite mRNA response curve constructed from four amiodarone experiments with cultured GC cells. After treatment with T₃ and graded doses of amiodarone, cytoplasmic RNA was prepared and analyzed by Northern hybridization as described in Methods. After hybridization, blots were autoradiographed and analyzed using a Zeineh scanning densitometer. Arbitrary density units were used to construct competition curves and to derive EC₅₀ values and slope factors for amiodarone's effect on rGH mRNA accumulation (15, 16). The "100%" point on the curve represents data from cells treated with 300 pM T₃ without amiodarone. The circled point represents data from nontreated cells. (*Closed circles*) amiodarone inhibition of binding of [¹²⁵I]T₃ to nuclei of intact GC cells.

increases in basal TSH release (7), decreases in malic enzyme and α-glycerol-phosphate dehydrogenase in rat liver (33), decreased rat serum triglycerides (34), and decreased cardiac myosin activity, an enzyme that is known to be induced by thyroid hormone (35). In cell culture studies amiodarone reduced T₃-induced increases in [³H]thymidine incorporation into GH₃ cells (36) and increased TSH release from cultured rat anterior pituitary cells (8).

Franklyn et al. (37) observed conflicting effects of in vivo administered amiodarone on mRNA levels for T₃-responsive

% Max density

Triiodothyronine (nM)

Figure 5. Effects of amiodarone plus increasing concentrations of T₃ on rGH mRNA accumulation in cultured GC cells. GC cells were deinduced 2 d, then incubated in the presence of 3.2 µM amiodarone plus increasing concentrations of T₃. Total cellular mRNA was analyzed by hybridization to the rGH or actin cDNAs (see Methods), autoradiographed and quantitated by scanning multiple exposures. Results are normalized to γ-actin. Data are expressed as percent maximum densitometry units. The bars indicate the mean±range of two determinations.

genes in rats. Thus, amiodarone caused increases in pituitary TSH α and β subunit mRNAs, consistent with a direct antagonistic effect of amiodarone to thyroid hormone action at the receptor level. However, no effect was observed on rGH mRNA levels. It is surprising that in hypothyroid rats, amiodarone exhibited T₃ agonist-like activity, causing decreased levels of TSH mRNA and increases in rGH mRNA. The reason for these disparate results was not fully resolved by the authors. However, hormonal interactions in vivo are much more complex than those observed under cell culture conditions, and comparisons between these approaches must be made with caution. Nevertheless, in our cell culture studies, we observed no evidence for T₃ agonist-like effects of amiodarone. Under "hypothyroid" cell culture conditions, amiodarone produced no significant effect on rGH mRNA.

Prolonged (24 h or more) incubation of GC cells with 10⁻⁴ M amiodarone, an amount that precipitates under culture conditions, reduced cell viability, which is an effect observed by others (36). However, decreases in rGH mRNA levels are not due to nonspecific effects of amiodarone since the antagonist was selective for rGH mRNA, and amiodarone at 10 µM was unable to reduce rGH mRNA levels below baseline. Further experiments revealed that 5 nM T₃ abolished the effects of amiodarone (Fig. 5). Finally, when amiodarone-treated cells are cultured in fresh media containing FCS, growth and rGH mRNA levels are restored (data not shown); an effect that is to be expected of a hormone antagonist (38).

Cellular and nuclear transport systems for thyroid hormones have been described, but studies have not yielded consistent data in the hands of all investigators. A nonsaturable, stereo-specific, T₃ uptake system has been described for rat hepatocytes by Oppenheimer (39). Krenning et al. have described a different low affinity, energy-dependent transport system for T₃ in rat hepatocytes that, in contrast to that described by Oppenheimer, appears to be inhibited by L-T₃ (40) or, when tested at a single dosage and time point, by amiodarone (41). In addition, rat pituitary tumor (GH₁) cells are able to concentrate T₃ across the cellular and especially the nuclear membranes (42) and transport into GH₁ cells appears to be saturable, similar to the effects observed in the present study.

In any case, it might be predicted that a substance that recognizes the nuclear thyroid hormone receptor would interact at other hormone binding sites on or within the cell. Therefore, we asked whether amiodarone altered [¹²⁵I]T₃ levels in the nonnuclear compartment of GC cells. In our study, both T₃ and amiodarone caused a maximum 45 to 50% reduction in total cellular or cytosolic radioactivity in GC cells when assayed at equilibrium with 300 pM [¹²⁵I]T₃. However, a relatively greater reduction in nuclear counts was seen with both amiodarone (75%) and L-T₃ (95%). If the competitors were acting solely to inhibit [¹²⁵I]T₃ transport, exactly parallel relative decreases in cellular, cytosolic and nuclear radioactivity would be observed. We also found that the affinity of amiodarone for binding to the thyroid hormone receptor is very similar in solubilized receptor from GC cell nuclei and in nuclear fractions from intact GC cells (Table I), a result that would be unexpected if amiodarone's blockade of the nuclear thyroid hormone receptor were occurring by different mechanisms in the intact cells and solubilized preparations. In addition, the affinity of T₃ for its receptor is essentially the same in whole cells and in solubilized nuclear extracts. Finally, the slope factors (*b*) for amiodarone's interactions with thyroid

hormone receptor preparations from both solubilized tissues and intact cells do not differ from 1.0, consistent with the interpretation that amiodarone interacts with a single class of binding sites in the receptor preparations. Thus, we conclude that both competitors affect equilibrium levels of nonnuclear [^{125}I]T$_3$ in a parallel manner by altering hormone transport across the cell surface, but both T$_3$ and amiodarone are likely to have additional direct effects on the nuclear thyroid hormone receptor. Indirect support for a nuclear site of action for amiodarone is found in a study by Hostetler et al. (43), who observed that the cell nucleus contained most of the amiodarone recovered from fractionated lung proteins of drug-treated rats.

Our findings raise the question as to whether the cardiac effects of amiodarone could be due to direct interactions with the T$_3$ receptor. For example, chronic amiodarone treatment causes decreases in both growth rate and cardiac weight in rabbits, and these changes are reversible by T$_4$ administration (44). In rats, thyroidectomy causes a reduction in heart weight, cardiac CO_2 production and myosin Ca^{2+}-ATPase activity. Parallel, although less pronounced, changes are induced by amiodarone. Notably, the effects of both thyroidectomy as well as amiodarone treatment are reversible by thyroid hormone (35).

Serum levels of amiodarone in humans and rats receiving long-term treatment vary from ~ 1 to 6 μM (45, 46), which is in the range of K_d values observed in this study. Perhaps of more importance, however, is the fact that amiodarone is concentrated preferentially in certain tissues over time, and it could conceivably accumulate at levels that would allow it to work as a full antagonist (5, 45). For example, necropsy studies have revealed that liver, lung, and heart preferentially accumulate extraordinarily high concentrations of the drug, containing between 2,600 mg/kg and 200 mg/kg of amiodarone and its metabolite desethylamiodarone; this is far more (40- to 3.5-fold) than that contained by brain or thyroid gland (60 mg/kg) (46). Pituitary accumulation is unknown. Therefore amiodarone might produce variable in vivo effects on differing tissues based on varying concentrations within the tissue. This could explain why obvious hypothyroidism does not occur in most amiodarone-treated patients, since tissue levels depend on individual patient idiosyncrasy and variation of dose and duration of therapy. Similarly, because of the weak affinity of the drug and its varying tissue absorption, some patients even have symptoms of thyrotoxicosis when iodine induced hyperthyroidism overwhelms the antagonistic properties of amiodarone.

Numerous structure-activity studies have suggested the necessity of bulky iodine or propyl substituents on the outer ring 3′ position and the presence of a carboxylic acid group for effective receptor binding and agonist function (2, 47). Amiodarone possesses neither of these moieties, and indeed comparison of the two-dimensional chemical structures reveals no obvious structural similarities between the drugs. To explore a possible structural similarity between T$_3$ and amiodarone, extended conformation molecular models, using standard data sets, were constructed with the MIDAS program at the Computer Graphics Laboratory, University of California, San Francisco (Fig. 6). Fig. 6 A displays triiodothyronine (left) and amiodarone (right), each with computer-generated van der Waals surfaces displayed around their respective iodine atoms. When the diiodophenyl rings of each compound are juxta-
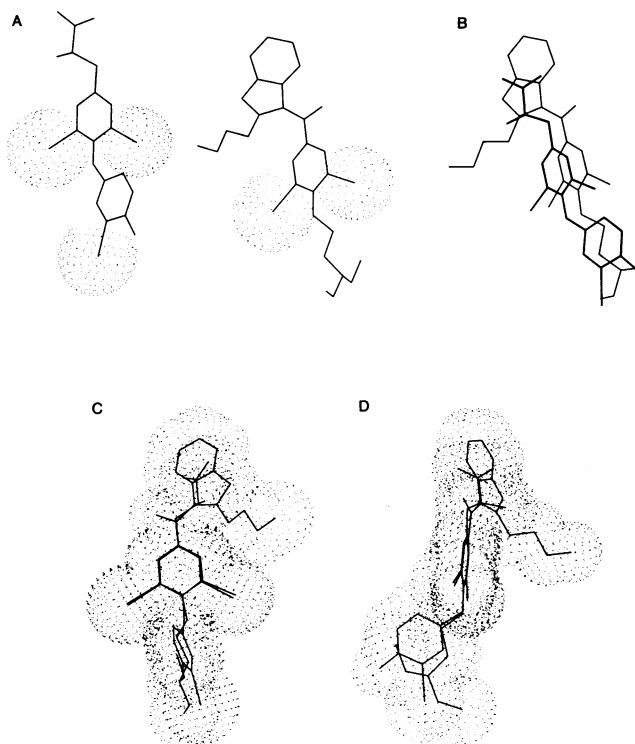

Figure 6. Computer-generated models of T$_3$ and amiodarone. (A) van der Waals forces associated with the iodine atoms are illustrated for each molecule. T$_3$ is on the left, amiodarone on the right. (B) Hypothetical alignment of molecules along "vertical" axes. (C) Superimposition of phenol rings showing similarity of spacefilling by surface van der Waals forces. (D) As in c, with 90° rotation along vertical axes. Slight changes in amiodarone torsional angles would increase the similarity of the superimposition of amiodarone and the "lower" ring of triiodothyronine.

posed a remarkable degree of similarity can be seen along their entire "vertical" axes (Fig. 6 B). Complete superimposition of the diiodophenyl rings leads to the structures shown in Fig. 6 C and, rotated 90° along their "vertical" axes, Fig. 6 D. A striking homology exists between the van der Waals surfaces of each drug. The "lower" portion of the superimposed structures containing the inner and outer rings of triiodothyronine displays the greatest degree of similarity, and has been proposed by Jorgensen (48) to interact with the receptor protein. The "upper" portion of the structures, containing the benzofuran and its 2-butyl adduct, is most dissimilar, suggesting a structural basis for antagonism.

The utility of amiodarone as a thyroid hormone antagonist is limited for several reasons. First, the drug has a low affinity for the thyroid hormone receptor. Second, numerous amiodarone-induced toxic side effects have been reported (4, 5). Third, amiodarone probably exerts effects that are not related to thyroid hormone activity. For example, amiodarone inhibits in vitro phospholipase A$_1$ production in rat alveolar macrophages, J-774 macrophages and rat liver (43) when used at concentrations 5–10 times greater than that required to inhibit T$_3$ receptor binding by 50%. Finally, the drug has a long half-life and accumulates extensively in tissues; this could hinder the ability to regulate the actions of the drug.

Nevertheless, this study demonstrates that amiodarone interacts directly with the nuclear thyroid hormone receptor,

and that it antagonizes the effects of thyroid hormone on rGH message. In addition, it indicates that other amiodarone-like derivatives have the potential to be of considerable practical value both clinically as cardiotherapeutic drugs, and as tools for further investigating the molecular mechanisms of thyroid hormone action.

Acknowledgments

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