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The Class III Antiarrhythmic Drug Amiodarone Directly Activates Pertussis Toxin-Sensitive G Proteins

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SUMMARY

The class III antiarrhythmic drugs amiodarone and bretylium tosylate are cationic/amphiphilic, and various substances with these physico-chemical properties are known to directly activate heterotrimeric regulatory G proteins. We asked the question of whether class III antiarrhythmic drugs are also direct G protein activators, using HL-60 leukemic cells and purified bovine brain G proteins as model systems. In HL-60 cell membranes, amiodarone increased high affinity GTP hydrolysis with an EC_{50} of 7.5 μM. The stimulatory effect of amiodarone on GTP hydrolysis was inhibited by pertussis toxin. Amiodarone stimulated binding of guanosine-5′-*O*-(3-thio)triphosphate to, and incorporation of GTP azidoanilide into, G_i protein α subunits in HL-60 membranes. The drug increased the cytosolic Ca^{2+} concentration in HL-60 cells in the presence but not in the absence of extracellular Ca^{2+}. Amiodarone-induced increases in the cytosolic Ca^{2+} concentration were reduced by pertussis toxin and by a blocker of nonselective cation channels, SK&F 96365. Amiodarone activated the GTPase of reconstituted G_i/G_o proteins and G_{i2} with EC_{50} values of 20 μM and 50 μM, respectively. Bretylium tosylate did not increase GTP hydrolysis in HL-60 membranes or with G_i/G_o proteins. Our data suggest that amiodarone but not bretylium tosylate is a direct activator of G_i and G_o proteins and that amiodarone activates nonselective cation channels in HL-60 cells via G_i proteins and independently of Ca^{2+} mobilization from intracellular stores. Future studies will have to test the hypothesis that direct G protein activation by amiodarone contributes to its toxic and/or therapeutic effects.

The class III antiarrhythmic drug amiodarone prolongs the repolarization phase of the cardiac action potential (1–3). Unlike class Ic antiarrhythmic drugs, amiodarone produces beneficial effects in the treatment of patients with ventricular arrhythmias after myocardial infarction (4–6). Unfortunately, however, amiodarone possesses a broad spectrum of unwanted effects, such as thyroid dysfunction, hepatitis, neuropathy, impaired vision, photosensitivity of the skin, and pneumonitis (1–3). Pulmonary toxicity is associated with alterations in leukocyte functions (7–9). The molecular mechanism by which amiodarone exerts its toxic and therapeutic effects is unknown (1–3).

Amiodarone is a cationic/amphiphilic drug. Its lipophilic domain consists of an alkyl-substituted benzofuran that, via a keto group, is linked to diiodobenzene. The latter group bears an *N*,*N*-diethylaminoethoxy function as the cationic domain (Fig. 1). It is well known that various cationic/amphiphilic substances, such as mastoparan, compound 48/80, local anesthetics, and β-adrenoceptor antagonists, are direct activators of G proteins, which mediate signal transfer from heptahelical membrane receptors to effectors (10–13). Interestingly, bretylium tosylate, another class III antiarrhythmic drug that is structurally unrelated to amiodarone, is also cationic/amphiphilic (Fig. 1). From these findings the question arises of whether amiodarone and bretylium tosylate are direct G protein activators.

To answer this question, we studied the effects of amiodarone and bretylium tosylate on the activation of G proteins in the human leukemia cell line HL-60 and with purified bovine brain G proteins. In HL-60 cells, formyl peptide receptors interact with PTX-sensitive G proteins of the G_i family (originally termed G_i proteins for their ability to inhibit adenylyl cyclase), resulting in activation of phospholipase C, of nonselective cation channels mediating Ca^{2+} influx, and of the O_2^--forming NADPH oxidase (14–19). Here, we report on direct G protein activation by amiodarone.

Experimental Procedures

Materials. SK&F 96365 was kindly donated by Dr. J. E. Merritt (SmithKline Beecham, Welwyn, Herts., UK). Amiodarone and CTX

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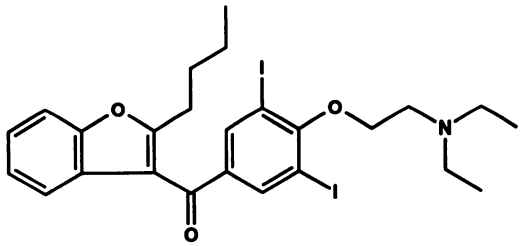
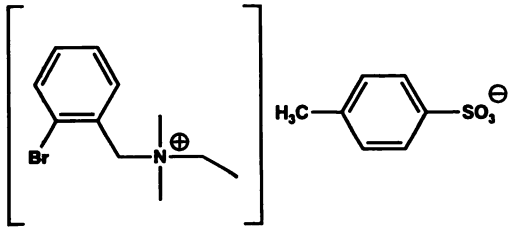
ABBREVIATIONS: PTX, pertussis toxin; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CTX, cholera toxin; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; O_2^-, superoxide anion; SK&F 96365, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole hydrochloride; Me_2SO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.



Amiodarone



Bretylium tosylate

Fig. 1. Structural formulae of amiodarone and bretylium tosylate.

were obtained from Sigma Chemie (Deisenhofen, Germany). Bretylium tosylate was from Research Biochemicals (Natick, MA). For experiments with HL-60 membranes or intact HL-60 cells, a stock solution of amiodarone (30 mM) in Me_2SO was prepared. Dilutions of amiodarone were prepared in distilled water. As a control, Me_2SO was used at the same concentrations present in assays containing amiodarone. The stimulatory effects of amiodarone are with respect to these control values. To avoid interference with liposome formation, Me_2SO was not used in experiments with reconstituted G proteins. Hence, amiodarone was dissolved in distilled water according to the method of Bonati *et al.* (20), with modifications. In brief, approximately 50 mg of amiodarone were suspended in 5 ml of distilled water and heated to 70° with constant stirring. After cooling to room temperature, the suspension was centrifuged for 5 min at 15,000 × *g*. The concentration of amiodarone in the supernatant fluid was determined by measuring the absorbance at 241 nm. For spectrophotometry, the amiodarone solution was diluted with methanol (final methanol concentration, 90%, v/v). The concentration of the amiodarone solution in distilled water was 12–16 mM, and the solution could be stored at room temperature for at least 3 weeks (longer periods were not tested). Bretylium tosylate (30 mM) was dissolved in distilled water and was stored at −20°. PTX was from List Biological Laboratories (Campbell, CA). [^{35}S]GTPγS (1200–1500 Ci/mmol) and [^{32}P]NAD (800 Ci/mmol) were obtained from New England Nuclear (Bad Homburg, Germany). Sources of other materials have been described elsewhere (13, 16–19, 21–23).

Cell culture and membrane preparation. HL-60 cells were purchased from the American Type Culture Collection (Rockville, MD). HL-60 cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% (v/v) horse serum, 1% (v/v) nonessential amino acids, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin, in a humidified atmosphere with 7% CO_2 at 37°, and were differentiated towards neutrophil-like cells with dibutyryl-cAMP (0.2 mM) for 48 hr (16). PTX (100 ng/ml) or its carrier was added to cell cultures 24 hr before experiments or membrane preparation. Under these conditions, virtually all G_i protein α subunits in HL-60 membranes were ADP-ribosylated, as assessed by *in vitro* ADP-ribosylation with activated PTX and [^{32}P]NAD (data not shown). For measurement of GTP hydrolysis, GTPγS binding, and photolabeling, HL-60 membranes were prepared as described by Seifert and Schultz (21). For measurement of CTX-catalyzed ADP-ribosylation, HL-60 membranes were prepared as described by Klinker *et al.* (19).

Purification of G_i/G_o proteins and G_{i2}. Heterotrimeric G_i/G_o proteins were isolated from bovine brain membranes (22). Briefly, cholate extracts of membranes were subjected to chromatography on a DEAE-Sepharose Fast Flow column (Pharmacia, Freiburg, Germany), followed by an AcA 34 gel filtration column (Serva, Heidelberg, Germany) and a heptylamine-Sepharose column. The preparation of G_i/G_o proteins (purity, >90%) contained predominantly G_{o1}, significant amounts of G_{o2} and another, as yet unidentified, G_o subtype ("G_{o3}"), G_{i1}, G_{i2}, and traces of G_{i3}. Heterotrimeric G_{i2} was resolved from other G_i and G_o proteins by repetitive fast protein liquid chromatography on Mono Q columns, according to the method of Codina *et al.* (24). The purity of the G_{i2} preparation was >90%.

Reconstitution of G_i/G_o proteins and G_{i2} into phospholipid vesicles. Reconstitution of G proteins was performed as described (13), with several modifications. Compared with our recently described procedure (13), the modified reconstitution method resulted in an up to 2-fold increase in the effectiveness of amiodarone to activate G_i/G_o proteins and G_{i2}, without a change in the potency of the drug (data not shown). In detail, 100 mg of azolectin were suspended in 10 ml of distilled water and were gently stirred at 4° for 2 hr. The resulting suspension was carefully overlaid with 1 g of sodium cholate. The suspension was kept at 4° without stirring or shaking. After about 2 hr, the suspension had clarified. G proteins (25–30 pmol) were mixed with 60 μl of the azolectin/sodium cholate suspension and brought to 600 μl with a buffer consisting of 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 20 mM HEPES/NaOH, pH 7.4. The mixture was loaded onto a G-50 gel filtration column (18.0 × 1.5 cm). Vesicles containing G proteins were formed by rinsing the column with the buffer described above. Reconstituted G proteins eluted in the void volume and were immediately used for further experiments. Association of G proteins with liposomes was quantitated by GTPγS binding (see below).

GTPase assay. GTP hydrolysis was determined as described (19). For determination of GTP hydrolysis in HL-60 membranes, reaction mixtures (100 μl) contained 3.0–7.0 μg of membrane protein/tube, 0.5 μM [γ-^{32}P]GTP (0.1 μCi/tube), 0.5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 1 mM adenosine 5′-(β,γ-imido)triphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, 1 mM dithiothreitol, and 0.2% (w/v) bovine serum albumin in 50 mM triethanolamine/HCl, pH 7.4. Reactions were conducted for 15 min at 25°. Low affinity GTPase activity was determined in the presence of 50 μM GTP and amounted to <5% of total GTP hydrolysis measured in the presence of 0.5 μM GTP. For determination of the GTPase activity of reconstituted G proteins, reaction mixtures (100 μl) contained 0.4–0.6 pmol of G_i/G_o proteins or G_{i2}, 0.1 μM [γ-^{32}P]GTP (0.1 μCi/tube), 2.0 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 5 mM creatine phosphate, 40 μg of creatine kinase, and 1 mM dithiothreitol in 50 mM triethanolamine/HCl, pH 7.4. Reactions were conducted for 15 min at 25°.

Assay for GTPγS binding. [^{35}S]GTPγS binding in HL-60 membranes was assessed as described (13). In brief, reaction mixtures (100 μl) contained HL-60 membranes (3.0–5.0 μg of protein/tube), 0.4 nM [^{35}S]GTPγS, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 0.2% (w/v) bovine serum albumin in 50 mM triethanolamine/HCl, pH 7.4. Reactions were conducted for 60 min at 0°. Reactions were terminated by rapid filtration through nitrocellulose BA 85 filters (Schleicher & Schuell, Dassel, Germany), followed by two washes with 5 ml of buffer (0°) consisting of 5 mM $MgCl_2$ and 50 mM triethanolamine/HCl, pH 7.4. Filter-bound radioactivity was determined in a liquid scintillation counter. Nonspecific binding was determined in the presence of 10 μM GTPγS and was <1% of total binding. G proteins were quantitated by binding of [^{35}S]GTPγS (500 nM) as described (22). For measurement of amiodarone-stimulated [^{35}S]GTPγS binding to purified G_i/G_o proteins and G_{i2}, reconstituted G proteins (0.15–0.50 pmol, in a total volume of 100 μl) were incubated for various periods of time at 25° in

the presence of 100 nM [^{35}S]GTPγS, in a buffer consisting of 100 μM $MgCl_2$, 80 μM EDTA, and 20 mM HEPES/NaOH, pH 7.4. Reactions were terminated by addition of 900 μl of a buffer consisting of 100 mM $MgCl_2$, 20 μM GTP, and 20 mM Tris/HCl, pH 8.0 (0°), and filtration through nitrocellulose BA 85 filters, followed by two washes with 5 ml of the latter buffer (0°).

Assay for photolabeling of membrane proteins. HL-60 membranes (50 μg of protein in 60 μl) were incubated at 30° in a buffer consisting of 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM benzamidine, 10 μM GDP, and 30 mM HEPES/NaOH, pH 7.4. After exposure to amiodarone for 3 min, samples were incubated for another 3 min with 10 nM [α-^{32}P]GTP azidoanilide (1 μCi/tube). Stopping of reactions and irradiation of samples were performed as described (23).

Assay for CTX-catalyzed ADP-ribosylation of membrane proteins. Reaction mixtures (50 μl) contained HL-60 membranes (50 μg/tube), 3 μM [^{32}P]NAD (1 μCi/tube), 2.5 mM $MgCl_2$, 1 mM ATP, 0.2% (w/v) bovine serum albumin, 2 μg of activated CTX, 0.1 M potassium phosphate, pH 7.4, and various substances. Reactions were conducted for 60 min at 30° (19).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined using the fluorescent dye fura-2, as described (17). Fluorescence of HL-60 cells (1.0×10^6 cells in 2 ml) was determined at 37°, with constant stirring at 10^3 rpm, using a Ratio II spectrofluorometer (Aminco, Silver Spring, MD). Cells were incubated for 3 min, in the absence or presence of SK&F 96365, before the addition of stimuli. The excitation and emission wavelengths were 340 and 500 nm, respectively. Experiments were performed in the presence of 1 mM $CaCl_2$ or 1 mM EGTA.

Assay for O_2^- formation in HL-60 cells. O_2^- formation was monitored at 550 nm by continuous measurement of superoxide dismutase-inhibitable ferricytochrome *c* reduction, using an Uvikon 810 dual-beam spectrophotometer (Kontron, Eching, Germany) (16). HL-60 cells (2.5×10^6 cells in 500 μl) were incubated for 3 min at 37°, in the absence or presence of cytochalasin B (1 μg/ml), before the addition of stimuli.

Miscellaneous methods. Protein was determined according to the method of Lowry *et al.* (25). [γ-^{32}P]GTP was prepared as described (26). [α-^{32}P]GTP azidoanilide was synthesized according to the method of Offermanns *et al.* (23).

Results

First, we studied the effects of amiodarone and bretylium tosylate on high affinity GTPase, i.e., the enzymatic activity of G protein α subunits, in HL-60 membranes. In membranes of dibutyryl-cAMP-differentiated HL-60 cells, amiodarone activated GTPase with an EC_{50} of 7.5 μM and a maximum at 30 μM (Fig. 2). At concentrations above 100 μM, the stimulatory effect of amiodarone decreased. In contrast to amiodarone, bretylium tosylate at up to 3 mM did not activate GTP hydrolysis in HL-60 membranes (Fig. 2). As is the case for the formyl peptide receptor agonist fMLP (19), the stimulatory effect of amiodarone on GTPase was due to an increase in V_{max} without an effect on K_m (data not shown). Table 1 shows that amiodarone and fMLP were similarly effective activators of GTPase in HL-60 membranes and that the stimulatory effects of both substances on GTP hydrolysis were PTX sensitive.

Stimulation of GTPγS binding is another parameter used to assess G protein activation (13, 14). Amiodarone stimulated GTPγS binding in HL-60 membranes with an EC_{50} of 15 μM and a maximum at 300 μM (Fig. 3). At concentrations above 300 μM, amiodarone was less effective. In membranes from PTX-treated HL-60 cells, amiodarone did not activate GTPγS binding.

Labeling of G_i protein α subunits in HL-60 membranes by the photoreactive GTP analogue GTP azidoanilide (23) was

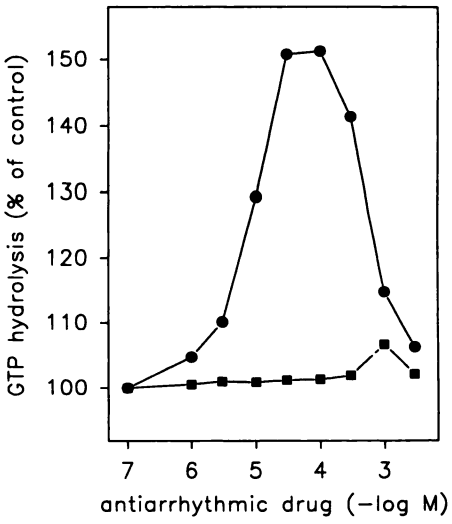


Fig. 2. Concentration-response curves for the effects of amiodarone and bretylium tosylate on GTP hydrolysis in HL-60 membranes. GTP hydrolysis in HL-60 membranes was determined in the presence of amiodarone and bretylium tosylate at the indicated concentrations, as described in Experimental Procedures. Data shown are the means of assay quadruplicates. ●, Amiodarone; ■, bretylium tosylate. The standard deviation values were generally <5% of the means. Similar results were obtained in three experiments. Basal GTP hydrolysis (control) was 17.4 ± 0.6 pmol/mg/min.

TABLE 1

Amiodarone- and fMLP-stimulated GTP hydrolysis in HL-60 membranes and effects of PTX

Pretreatment of HL-60 cells with PTX or carrier (control) was performed as described in Experimental Procedures. GTP hydrolysis in HL-60 membranes was determined in the presence of amiodarone (30 μM), fMLP (10 μM), or solvent (basal), as described in Experimental Procedures. Data shown are the means ± standard deviations of assay quadruplicates. Similar results were obtained in three experiments.

Addition	GTP hydrolysis	
	Control	PTX
	pmol/mg/min	
Solvent (basal)	15.2 ± 0.6	7.0 ± 0.3
Amiodarone	24.2 ± 0.1	6.8 ± 0.4
fMLP	23.2 ± 0.2	7.2 ± 0.5

studied. Similarly to fMLP (19), amiodarone increased incorporation of GTP azidoanilide into 40/41-kDa proteins, corresponding to G_i protein α subunits (Fig. 4A). fMLP also stimulated CTX-catalyzed ADP-ribosylation of G_i protein α subunits in HL-60 membranes (Fig. 4B) (15, 19). In contrast, amiodarone had no stimulatory effect on CTX-catalyzed ADP-ribosylation of G_i protein α subunits.

Next, we studied the effects of amiodarone on $[Ca^{2+}]_i$ in HL-60 cells. In the presence of extracellular Ca^{2+}, 1 μM amiodarone induced a rise in $[Ca^{2+}]_i$ that was delayed in onset and sustained (Fig. 5). The effects of higher concentrations of amiodarone on $[Ca^{2+}]_i$ could not be assessed, due to strong autofluorescence of the drug (data not shown). Compared with the amiodarone-induced rise in $[Ca^{2+}]_i$, that induced by fMLP was considerably more rapid in onset. In the absence of extracellular Ca^{2+}, amiodarone did not increase $[Ca^{2+}]_i$, whereas fMLP did so. SK&F 96365, a blocker of nonselective cation channels (18, 27), reduced the stimulatory effects of amiodarone and fMLP on $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. SK&F 96365

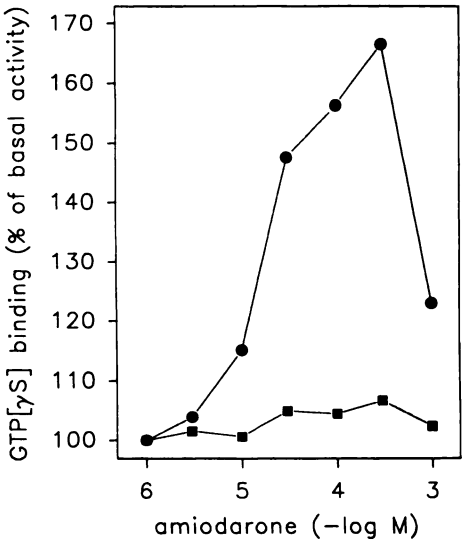


Fig. 3. Concentration-response curves for the effect of amiodarone on GTPγS binding in membranes of carrier- and PTX-treated HL-60 cells. Pretreatment of HL-60 cells with PTX or carrier (control) was performed as described in Experimental Procedures. GTPγS binding in HL-60 membranes was determined in the presence of amiodarone at the indicated concentrations. Data shown are the means of assay quadruplicates. ●, Control; ■, PTX. The standard deviation values were generally <5% of the means. Similar results were obtained in three experiments. Basal GTPγS binding in membranes of carrier- and PTX-treated HL-60 cells was 1.14 ± 0.03 and 0.86 ± 0.4 pmol/mg, respectively.

did not affect the rise in $[Ca^{2+}]_i$ induced by fMLP in the absence of extracellular Ca^{2+}.

Table 2 shows the effect of PTX on amiodarone- and fMLP-induced rises in $[Ca^{2+}]_i$. In agreement with recently published data (17), PTX partially reduced the stimulatory effect of fMLP on $[Ca^{2+}]_i$. In PTX-treated cells, the stimulatory effect of amiodarone on $[Ca^{2+}]_i$ was reduced by about 50%.

fMLP effectively activates O_2^- formation in dibutyryl-cAMP-differentiated HL-60 cells (16–18). In contrast, amiodarone (1 μM to 1 mM) did not activate O_2^- formation in these cells, regardless of whether cytochalasin B (1 μg/ml) was present. In addition, amiodarone did not potentiate O_2^- formation induced by fMLP (1 μM) (data not shown).

We also studied the effects of amiodarone and bretylium tosylate on activation of purified G proteins. Amiodarone activated GTP hydrolysis by reconstituted bovine brain G_i/G_o proteins with an EC_{50} of about 20 μM and a maximum at 0.3 mM (Fig. 6). Similarly to the results obtained with HL-60 membranes (Fig. 2), bretylium tosylate (1 μM to 1 mM) had no stimulatory effect on GTP hydrolysis by G_i/G_o proteins (data not shown). Amiodarone activated the GTPase of reconstituted G_{i2} with an EC_{50} of about 50 μM and a plateau at 0.5–1.0 mM (Fig. 6).

Finally, we evaluated the effect of amiodarone on GTPγS binding to reconstituted G proteins. Fig. 7 shows the kinetics of GTPγS binding to G_i/G_o proteins in the absence and presence of amiodarone (250 μM). At early time points (e.g., at 1 and 2 min), the stimulatory effect of amiodarone was >100%. At later time points (e.g., at 30 and 60 min), the effect of amiodarone was smaller but still amounted to about 20%. When GTPγS binding to G_{i2} was studied, the stimulatory effect of amiodarone (250 μM) at an incubation time of 10 min was about 60% (data not shown), compared with 35% for G_i/G_o proteins (Fig. 7).

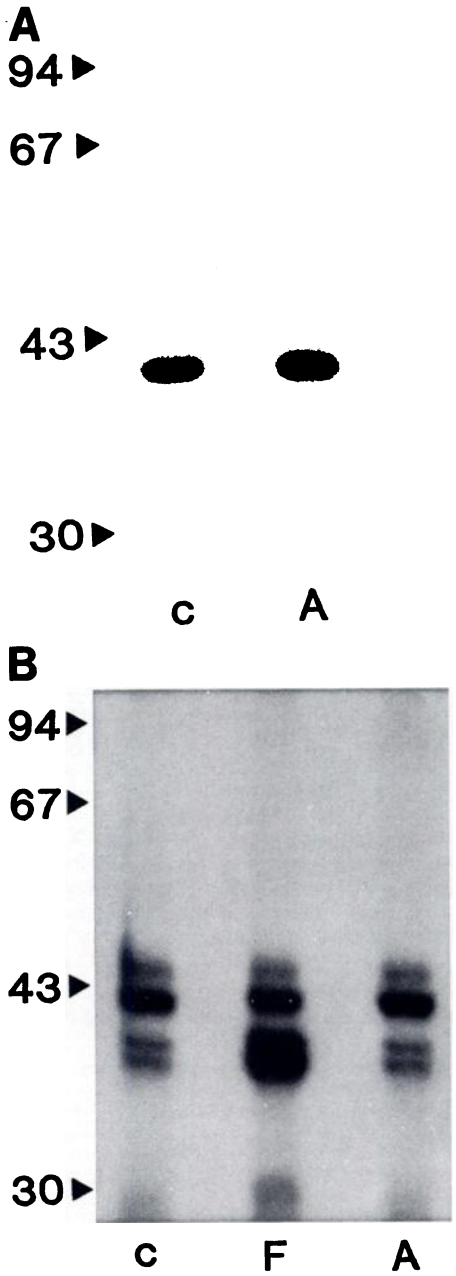


Fig. 4. Effect of amiodarone on incorporation of GTP azidoanilide into, and CTX-catalyzed ADP-ribosylation of, G_i protein α subunits in HL-60 membranes. A, Photolabeling was performed as described in Experimental Procedures. The autoradiogram of an sodium dodecyl sulfate gel containing 4 M urea and 9% (w/v) acrylamide is shown. Similar results were obtained in three experiments. *Lane c*, control; *lane A*, amiodarone (30 μM). B, ADP-ribosylation was performed as described in Experimental Procedures. The autoradiogram of a sodium dodecyl sulfate gel containing 4 M urea and 9% (w/v) acrylamide is shown. Similar results were obtained in three experiments. *Lane c*, control; *lane F*, fMLP (10 μM); *lane A*, amiodarone (30 μM). *Numbers on the left*, molecular masses of marker proteins (kDa).

Discussion

The present study was undertaken to answer the question of whether class III antiarrhythmic drugs, particularly amiodarone, are direct G protein activators. Amiodarone was found to activate GTP hydrolysis and GTPγS binding in HL-60 membranes in a PTX-sensitive manner (Fig. 3; Table 1). In addition,

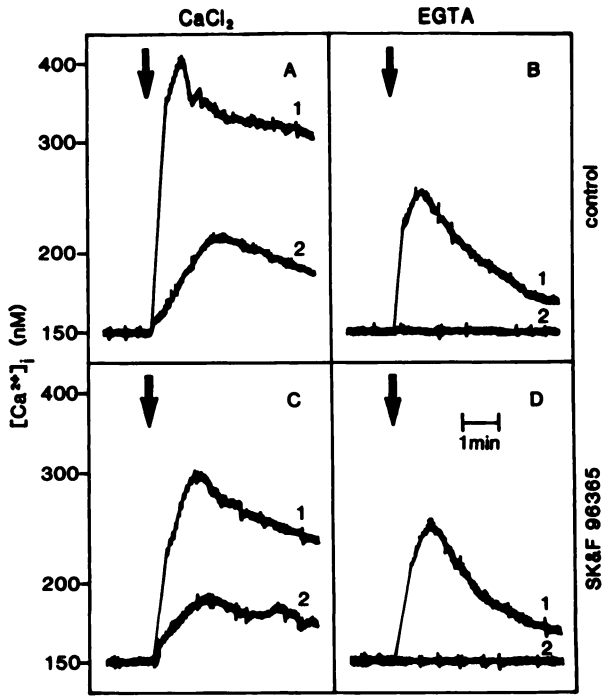


Fig. 5. Amiodarone- and fMLP-induced rises in $[Ca^{2+}]_i$ in HL-60 cells and effects of extracellular Ca^{2+} and SK&F 96365. HL-60 cells were harvested and loaded with fura-2/acetoxymethyl ester, and the effects of amiodarone (1 μM) and fMLP (10 nM) on $[Ca^{2+}]_i$ were assessed in the presence of 1 mM extracellular $CaCl_2$ (A and C) or 1 mM EGTA (B and D), with solvent (control) (A and B) or SK&F 96365 (30 μM) (C and D), as described in Experimental Procedures. *Arrows*, addition of stimuli. *Traces 1*, fMLP; *traces 2*, amiodarone. Superimposed original fluorescence traces are shown. Similar results were obtained in four experiments with different preparations of HL-60 cells.

TABLE 2

Amiodarone- and fMLP-induced rises in $[Ca^{2+}]_i$ in HL-60 cells and effects of PTX

Pretreatment of HL-60 cells with PTX or carrier (control) was performed as described in Experimental Procedures. Cells were then harvested and loaded with fura-2- acetoxymethyl ester, and the effects of amiodarone (1 μM) and fMLP (10 nM) on $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} were assessed as described in Experimental Procedures. Data shown are the means ± standard deviation of three experiments performed in quadruplicate. PTX had no effect on basal $[Ca^{2+}]_i$ in HL-60 cells.

Addition	Increase in $[Ca^{2+}]_i$	
	Control	PTX
	nM	
Amiodarone	61 ± 17	31 ± 9
fMLP	242 ± 8	134 ± 10

amiodarone-induced rises in $[Ca^{2+}]_i$ in HL-60 cells were at least partially PTX sensitive (Table 2). Moreover, the antiarrhythmic drug increased photolabeling of 40/41-kDa proteins in HL-60 membranes (Fig. 4A). Taken together, these findings indicate that amiodarone activates G_i proteins in HL-60 cells. The differences in the concentration-response curves for amiodarone effects on GTP hydrolysis and GTPγS binding in HL-60 membranes (compare Figs. 2 and 3) may be due to differences in the assay conditions (e.g., differences in $MgCl_2$ concentrations and incubation temperatures) (see Experimental Procedures).

If amiodarone increases $[Ca^{2+}]_i$ in HL-60 cells through direct interaction with G proteins, then it has to penetrate the plasma membrane before reaching its target. In addition, amiodarone is a very bulky drug (Fig. 1), and this property may delay membrane penetration (28). Thus, amiodarone-induced rises in $[Ca^{2+}]_i$ would be expected to be delayed in onset. This was in fact the case (Fig. 5). Although it is very unlikely, one cannot completely exclude the possibility that amiodarone exerted its effects in HL-60 cells and membranes through activation of an orphan receptor (29). Therefore, we assessed the effects of amiodarone on activation of purified G proteins. Amiodarone was found to effectively activate GTP hydrolysis by reconstituted G_i/G_o proteins and G_{i2} (Fig. 6). Moreover, amiodarone stimulated GTPγS binding to reconstituted G_i/G_o proteins (Fig. 7) and G_{i2}. Thus, amiodarone is, indeed, a direct G protein activator. Amiodarone belongs to the group of heterocyclic direct G protein activators. Other heterocyclic direct G protein activators are the β-adrenoceptor antagonists pindolol and timolol (13). Noteworthy is the fact that, in many cases, amiodarone showed biphasic effects on G protein activation, both in HL-60 membranes and with purified proteins (Figs. 2, 3, and 6). This phenomenon possibly reflects G protein denaturation by amiodarone.

For comparison with amiodarone, we assessed the effects of bretylium tosylate on G protein activation. Interestingly, this drug, unlike amiodarone, does not activate G_i and G_o proteins (Fig. 2). These findings show that cationic/amphiphilic properties *per se* (Fig. 1) are not sufficient for a substance to be a direct G protein activator. Future studies will have to determine which structural properties of the antiarrhythmic drugs account for this differential G protein activation.

To determine by which mechanism amiodarone increases $[Ca^{2+}]_i$ in HL-60 cells, we studied the effects of extracellular Ca^{2+} and SK&F 96365 on rises in $[Ca^{2+}]_i$. The stimulatory effects of amiodarone on $[Ca^{2+}]_i$ were completely dependent on the presence of extracellular Ca^{2+} and were reduced by SK&F 96365 (Fig. 5). These findings indicate that amiodarone activates Ca^{2+} influx through nonselective cation channels. The partial PTX insensitivity of the amiodarone-mediated Ca^{2+} influx raises the intriguing possibility that, in addition to G_i proteins, as yet unidentified PTX-insensitive G proteins (e.g., members of the G_q family, G_{12}, or G_{13}) are involved in the activation of nonselective cation channels (Table 2). The involvement of both PTX-sensitive and -insensitive G proteins in the activation of nonselective cation channels is not without precedence (30). The fact that amiodarone did not induce Ca^{2+} mobilization suggests that phospholipase C is not activated and that activation of Ca^{2+} influx is independent of Ca^{2+} mobilization from intracellular stores. Dissociation of the activation of phospholipase C and nonselective cation channels has been repeatedly reported (30–32).

Amiodarone activates only Ca^{2+} influx, whereas fMLP induces both Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx through nonselective cation channels (Fig. 5) (17, 18). In addition, amiodarone, unlike fMLP, does not show stimulatory effects on O_2^- formation or CTX-catalyzed ADP-ribosylation of G_i protein α subunits (Fig. 4B) (15, 16, 19). Thus, amiodarone may induce a state of G_i protein activation that differs from that induced by fMLP. By analogy to histamine (acting via H_1 receptors) (17) and leukotriene B_4 (14), amiodarone may be classified as an incomplete secretagogue in dibutyryl-cAMP-differentiated HL-60 cells, as defined by its ability to induce rises in $[Ca^{2+}]_i$ and its inability to activate O_2^- formation (Fig. 5).

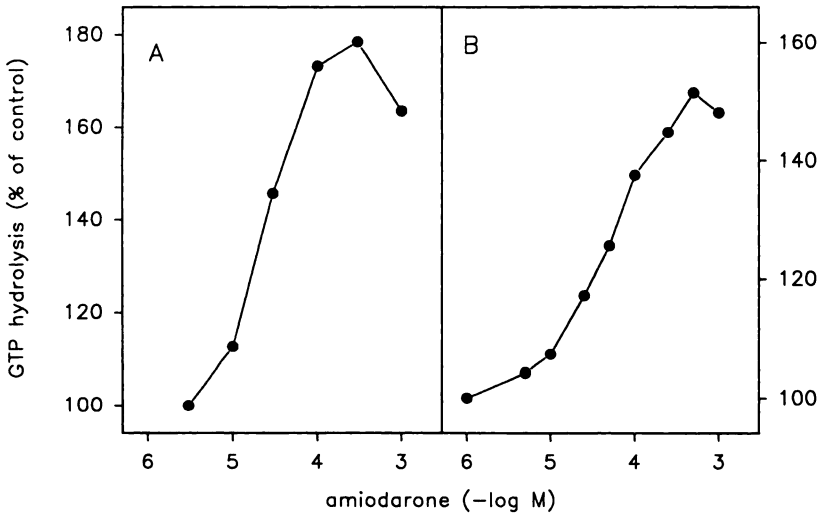


Fig. 6. Concentration-response curves for the effects of amiodarone on GTP hydrolysis by reconstituted G_i/G_o proteins and G_{i2}. GTP hydrolysis by G_i/G_o proteins (A) and G_{i2} (B) was determined in the presence of amiodarone at the indicated concentrations, as described in Experimental Procedures. Data shown are the means of assay triplicates. The standard deviation values were generally <5% of the means. Similar results were obtained in three experiments. Basal GTP hydrolyses of G_i/G_o proteins and G_{i2} were 0.101 ± 0.006 min^{-1} and 0.207 ± 0.003 min^{-1}, respectively.

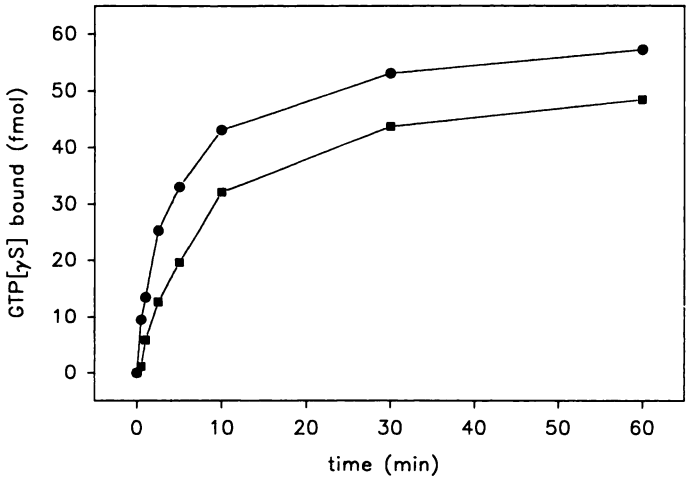


Fig. 7. Kinetics of GTPγS binding to reconstituted G_i/G_o proteins. GTPγS binding to reconstituted G_i/G_o proteins was determined for the indicated periods of time as described in Experimental Procedures. ■, Control; ●, amiodarone (250 μM). Data shown are the means of assay triplicates. The standard deviation values were generally <5% of the means. Similar results were obtained in three experiments.

Amiodarone accumulates extensively in tissues (2, 3, 33). For example, the total concentration of amiodarone in human myocardium may be as high as 70 μmol/kg and in adipose tissue, lung, and liver the total concentrations of amiodarone may reach 160–530 μmol/kg (33). G protein activation by amiodarone was evident at concentrations as low as 1–10 μM, depending on the parameter studied (Figs. 2, 3, 5, and 6; Table 2). Thus, even if one takes into consideration the possibility that, due to its high lipophilicity, the free amiodarone concentration in tissues is much lower than the total drug concentration, the former could well be high enough for direct G protein activation to occur *in vivo*. Moreover, amiodarone induces pneumonitis, and this disorder may be due to alterations in leukocyte functions (1–3, 7–9). Most interestingly, we found that amiodarone activates G_i proteins in the human leukemia cell line HL-60 (Figs. 2–5; Tables 1 and 2).

In conclusion, we have shown that amiodarone but not bretylium tosylate is a direct activator of G_i and G_o proteins. Amiodarone, via G_i proteins, induces activation of nonselective cation channels in HL-60 cells without concomitant Ca^{2+} mobilization from intracellular stores. Future studies will have to test the hypothesis that direct G protein activation by amiodarone contributes to its toxic and/or therapeutic effects.

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