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The New Positive Inotrope Sulmazole Inhibits the Function of Guanine Nucleotide Regulatory Proteins by Affecting GTP Turnover

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SUMMARY

The effect of the new cardiotonic agent sulmazole on the guanine nucleotide regulatory protein-adenylate cyclase system was studied in rat adipocyte membranes. The inotrope enhanced basal adenylate cyclase activity by 46%. This stimulation occurred only at GTP concentrations (5 μM) sufficient to activate G_i. This stimulatory effect of sulmazole was abolished after functional inactivation of G_i, either by pertussis toxin or by using 10 nM GTP in the assay mixture, suggesting an important role of an active G_i in this process. Similarly, sulmazole enhanced isoproterenol-, forskolin-, and fluoride-stimulated adenylate cyclase activity by 33, 34, and 45%, respectively. However, when these latter experiments were performed after inactivation of G_i, sulmazole actually inhibited by ~25% adenylate cyclase activity stimulated by 1 and 10 μM isoproterenol. Under similar treatment conditions, enhancement of forskolin- and fluoride-stimulated activity by sulmazole was abolished. Sulmazole inhibited in a dose-dependent manner pertussis toxin- and cholera toxin-catalyzed labeling of G_i and G_s, respectively, with the respective inhibition observed at 100 μM of the inotrope being 29% and 56% of control. In addition, sulmazole inhibited PGE_1 and isoproterenol-stimulated [^{3}H]GDP release from G_i and G_s to 32% and 64% of control, respectively. Finally, the inotrope completely abolished PGE_1-stimulated [^{3}H]Gpp(NH)p binding with IC_{50} in the low micromolar range. These findings suggest that, whereas sulmazole inhibits the functioning of G_i and (to a lesser extent) G_s at low micromolar concentrations, expression of these effects on adenylate cyclase activity requires high micromolar to low millimolar concentrations of the drug. Thus, it appears sulmazole inhibits the function of G_i by decreasing its activation process, i.e., GTP-GDP exchange. Effects on G_s are manifested (at least in terms of adenylate cyclase activity) only after inactivation of G_i.

G proteins transmit signals from cell surface receptors to a variety of effector systems. These proteins include G_s and G_i, which mediate activation and inhibition of adenylate cyclase, respectively; G_o, whose function is not clearly defined; transducin, which couples rhodopsin to activation of cyclic GMP phosphodiesterase in the visual system; and a growing list of other G proteins (1–4). G proteins are heterotrimers, consisting of similar β and γ subunits but different α subunits (1–4). Recently, two distinct forms of the β subunit have been observed (5). Activation of receptors by specific agonists facilitates the exchange of GTP for GDP at the α subunits of the G proteins, followed by dissociation of the βγ subunits. The α_s·GTP complex can then activate adenylate cyclase directly. Several pieces of evidence suggest that inhibition of adenylate cyclase results from the binding of free α_s by βγ subunits released consequent to dissociation of G_i, thereby preventing its interaction with adenylate cyclase (1–4). Termination of the activation is brought about by GTPase activity intrinsic to the α subunit, which cleaves α·GTP to inactivate α·GDP. The α·GDP complex then rebinds the βγ subunit to reform the heterotrimer (1–4).

The discovery of two toxins, cholera and pertussis toxins, has greatly expanded our understanding of the functioning of the G proteins. For example, pertussis toxin catalyzes the transfer the ADP ribose from NAD to a cysteine residue on the carboxy terminus of α_i and α_o, resulting in a loss of receptor- and GTP-dependent activation of these proteins (1–3). In addition, cholera toxin catalyzes the ADP ribosylation of an arginine residue of G_s, leading to persistent activation of adenylate cyclase (1–3).

Recent evidence from our laboratory suggests that another

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; G_i, inhibitory guanine nucleotide regulatory protein; α_i, α subunit of G_i; G_s, stimulatory guanine nucleotide regulatory protein; α_s, α subunit of G_s; (*R*)-PIA, (−)-*N*^{6}-(R)-phenylisopropyl) adenosine; DTT, dithiothreitol; IBMX, 3-isobutyl-1-methylxanthine; PGE_1, prostaglandin E_1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; App(NH)p, adenosine 5′-(β,γ-imido)triphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N,N*′-tetraacetic acid.

group of compounds, the newer positive inotropes, can inhibit the function of G_i, albeit in a reversible manner (6). By inhibiting G_i function, these drugs (e.g., sulmazole) elevate basal cyclic AMP levels, which might then contribute to enhanced inotropic actions. In this report, we present data showing that sulmazole inhibits the activity of both G_i and G_s in rat adipocyte membranes even though its net effect is to elevate cyclic AMP. Furthermore, we provide evidence that the inhibitory effect of sulmazole is mediated by attenuating agonist-induced GTP turnover.

Methods

Materials. Adenosine deaminase, cyclic AMP, GTP, ATP, dATP, App(NH)p, creatine phosphokinase, and papaverine were from Sigma Chemical Co. (St. Louis, MO). Creatine phosphate and (*R*)-PIA were from Boehringer Mannheim (Indianapolis, IN). [2,8,-^{3}H]cAMP (40 Ci/mmol), [^{3}H]GTP (36 Ci/mmol), and [α-^{32}P]ATP (27 Ci/mmol) were from New England Nuclear (Boston, MA). [^{32}P]NAD (250 Ci/mmol) was from ICN (Plainview, NY). [^{3}H]Gpp(NH)p (36.1 Ci/mmol) was from Amersham (Arlington Heights, IL). Crude collegenase were from Cooper Biomedical (Malvern, PA). Bovine serum albumin (fraction 4) was from Armour (Tarrytown, NY). Sulmazole (AR-L 115 BS) was generously donated by Boehringer Ingelheim Pharmaceuticals, Inc. Cholera and pertussis toxins were from List Biological Laboratories. Pertussis vaccine concentrate (153 opacity units/ml, strain 1302) was from Lederle (Wayne, NJ). All other chemicals and reagents were of analytical grade.

Membrane preparation. Pertussis vaccine (~300 opacity units/kg; 0.3–0.5 ml) was administered intraperitoneally to rats (male Sprague-Dawley, 250–350 g; Charles River Breeding Laboratories) 3 days before sacrifice, as described previously (6, 7). This treatment effectively abolishes *in vitro* labeling of the α_i substrate of pertussis toxin without effect on cholera toxin substrates. In addition, administration of the vaccine for 3 days eliminates (*R*)-PIA-mediated inhibition of adenylate cyclase (data not shown). Control and pertussis vaccine-treated rats were sacrificed by decapitation and adipocyte membranes were prepared from epididymal fat pads as previously described (8) with the following modifications: leupeptin and soybean trypsin inhibitor were omitted during the collagenase digestion; both of these and phenylmethylsulfonyl fluoride were omitted from the hypotonic buffer. The membrane pellet obtained from centrifugation was suspended in 75 mM Tris (pH 7.4 at 30°), 12.5 mM $MgCl_2$, 200 mM NaCl, and 2.5 mM DTT to give an approximate protein concentration of 1–2 mg/ml. Adenosine deaminase (4.0 units/ml) was then added and the membrane suspension was preincubated at 30° for 15 min. Membranes prepared as described above were used immediately for adenylate cyclase assay. For toxin-catalyzed labeling, membrane pellets were resuspended in 25 mM HEPES buffer (pH 7.4) containing 2.5 mM $MgCl_2$, 0.3 mM EDTA, and 1 mM DTT. Membranes used in [^{3}H]GTP loading experiments were resuspended in 25 mM Tris·HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 1 mM EDTA, and 100 mM NaCl and further supplemented with 1 mM ATP, 6.7 mM phosphocreatine, and 30 units/ml creatine phosphokinase. Protein concentrations were determined by the method of Bradford (9), using bovine serum albumin as standard.

Adenylate cyclase assay. Adenylate cyclase activity in adipocyte membranes was determined as described previously (10). Briefly, 20 μl of membranes suspended in 75 mM Tris·HCl (pH 7.4 at 30°) containing 12.5 mM $MgCl_2$, 200 mM NaCl, and 2.5 mM DTT were incubated with 20 μl of a mixture containing 0.14 mM dATP, 5 mM phosphocreatine, 1 μM cAMP, 30 units/ml creatine phosphokinase, and ~1.5 μCi of [^{32}P]ATP, and 10 μl of H_2O or drug. Papaverine (0.1 mM) was used in all experiments to provide full phosphodiesterase inhibition. Addition of sulmazole to 0.1 mM papaverine provides no further inhibition of the low K_m cAMP phosphodiesterase (6). Experiments were performed at low (10 nM) or inhibitory (5 μM) GTP concentrations at 30° for the times indicated (see Results). Cyclic AMP assays were terminated by addition of 1 ml of ice-cold stop solution containing ~20,000 [^{3}H]cAMP (internal standard), 0.3 mM cAMP, and 0.4 mM ATP. Cyclic AMP was isolated by the method of Salomon *et al.* (11).

Toxin labeling. Bacterial toxin-catalyzed labeling of membranes were performed, with modifications, according to the method of Owens *et al.* (12). Membranes, prepared as described above, were incubated with 25 mM HEPES buffer (pH 7.4) containing the following constituents: 2.5 mM $MgCl_2$, 0.3 mM EDTA, 10 mM thymidine, 1 mM ATP, 1 mM DTT, and 5 μM [^{32}P]NAD, with or without sulmazole (0.001–3.3 mM). Bacterial toxins were activated in buffer containing 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, and 50 mM DTT for 15 min at 37° just before use. The final concentration of pertussis toxin was 30 μg/ml and of cholera toxin was 200 μg/ml. Incubations were for 15 min at 30° containing ~1 mg/ml membrane protein.

After incubation, membranes were washed twice with ice-cold buffer containing 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, and 1 mM EDTA and pelleted in a microfuge. Membrane pellets were then solubilized in buffer (10% SDS, 10% glycerol, 20 mM Tris·HCl, 6% β-mercaptoethanol, pH 6.5) for 1 hr and then subjected to SDS-PAGE.

SDS-PAGE. Electrophoresis was performed according to the method of Laemmli (13) using homogeneous slab gels containing 12% acrylamide. After electrophoresis, the gels were dried and exposed to Kodak XAR-5 X-ray film with intensifying screens for 24–72 hr. The relative labeling of α_i and α_s was determined by densitometric scanning of the autoradiographs.

Loading of membranes with [^{3}H]GTP. Loading of [^{3}H]GTP and [^{3}H]GDP release experiments was performed according to those described previously (14, 15). Briefly, rat adipocyte membranes were prepared as described previously and pretreated with adenosine deaminase (4 units/ml). Membranes (1–1.5 mg of protein/ml) were incubated in 25 mM Tris·HCl buffer (pH 7.4), containing 5 mM $MgCl_2$, 1 mM EDTA, and 100 mM NaCl (buffer A) and further supplemented with 1 mM ATP, 6.7 mM phosphocreatine, and 30 units/ml creatine phosphokinase, in the presence of 0.12 μM [^{3}H]GTP, 1 mM DTT, and the appropriate agonist (see Table 3). After a 3-min incubation period, the [^{3}H]GTP labeling reaction was terminated by addition of the appropriate receptor antagonist (100 μM betaxolol or 100 μM IBMX) and 100 μM unlabeled GTP, followed by cooling the mixture at 4°. Labeled membranes were centrifuged at 10,000 × *g* for 5 min and the resulting pellet was washed three times with ice-cold 25 mM Tris·HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ (buffer B). The final pellet was resuspended in 200 μl of buffer A, containing 1 mM ATP, 6.7 mM phosphocreatine, 30 units/ml creatine phosphokinase, and 100 μM GTP, and incubated for 10 min at 37°. Incubation was stopped by addition of 1 ml of ice-cold buffer B and centrifuged for 5 min at 10,000 × *g*. The resulting pellet was washed once in buffer B and then resuspended in 1 ml of buffer A containing 1 mM DTT.

[^{3}H]GDP release assay. For [^{3}H]GDP release assay, 50 μl of labeled membranes (50–75 μg of protein/assay tube) was incubated in a total volume of 200 μl of buffer A containing 0.3 mM GTP, isoproterenol (10 and 100 μM), or PGE_1, (30 μM) in the absence or presence of sulmazole (3.3 mM). Incubation was performed at 30° for 5 min and terminated by addition of 1 ml of ice-cold buffer B with 100 μM betaxolol (for release induced by isoproterenol) or 1 ml of ice-cold buffer B alone (for release induced by PGE_1). The reaction mixture was centrifuged at 10,000 × *g* for 5 min and 1 ml of the supernatant was counted for tritium.

[^{3}H]Gpp(NH)p binding assay. The binding reaction was performed as described previously (16). Membranes (~30 μg/assay tube) were incubated in 20 mM Tris·HCl buffer (pH 7.5) containing 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1.14 mM ATP, 0.5 mM App(NH)p, and 0.1 μM [^{3}H]Gpp(NH)p under basal conditions (no additions) or after addition of 10 μM PGE_1. Incubations were performed in the absence and presence of sulmazole (1 nM–2 mM) for 7 min at room temperature. After incubation, the reaction was terminated by rapid vacuum filtration using glass fiber filters (Schleicher & Schuell, no. 31), which were then washed three times with 3 ml of ice-cold 20 mM

Tris·HCl buffer (pH 7.5), containing 0.25 mM $MgCl_2$ and 50 μM EGTA. Filters were then extracted in 8 ml of toluene-based scintillation fluid for at least 12 hr before counting. Specific binding was determined as the difference between total binding and binding in the presence of 100 μM GTP. Stimulated binding was taken as the difference in [^{3}H] Gpp(NH)p binding observed in the absence and presence of PGE_1 (10 μM).

Results

Effect of Sulmazole on the Time Course of Basal Accumulation of cAMP in Adipocyte Membranes

The time course of cAMP formation as shown in Fig. 1 was linear with time to approximately 15 min, under basal conditions (5 μM GTP and in absence of stimulatory agonist). Thus, all cAMP experiments were performed for 15 min. Sulmazole (2 mM) enhanced cAMP accumulation in a time-dependent manner. This effect, after an initial lag phase, was linear and increased in magnitude with time. The stimulatory effect of sulmazole averaged 146% of control (range, 132–167%, seven experiments) at 15 min.

Effect of Sulmazole on Adenylate Cyclase Activity Levels in Control Adipocyte Membranes at Low GTP Concentrations and in Pertussis-Intoxicated Membranes at Physiologic GTP Concentrations

These experiments were conducted to assess the contribution of an active G_i in mediating the stimulation of adenylate cyclase levels by sulmazole. At low GTP concentrations (10 nM) and after pertussis toxin treatment the inhibitory guanine nucleotide regulatory protein (G_i) is inactive (15, 17). Fig. 1 shows that the time course of basal cAMP production was linear with time and, at low GTP, the addition of sulmazole produced no change in adenylate cyclase activity. In addition, sulmazole failed to increase cAMP above control levels after *in vivo* treatment with pertussis vaccine (Fig. 2). Inactivation of G_i by this treatment was reflected by the inability of (*R*)-PIA to

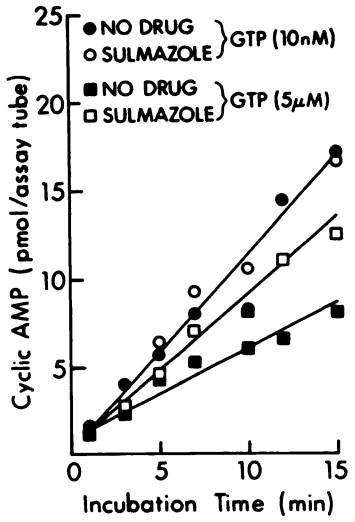

Fig. 1. Effect of GTP concentration on the enhancement of cAMP formation by sulmazole. Rat adipocyte membranes (22.4 μg of protein) were incubated at 30° with ~1.5 μCi of [^{32}P]ATP for different time periods in buffer containing 10 nM or 5 μM GTP and in the absence or presence of 2 mM sulmazole. Adenylate cyclase assays were performed as described under Methods. The figure is a representative experiment, which was replicated at least three times with similar results.

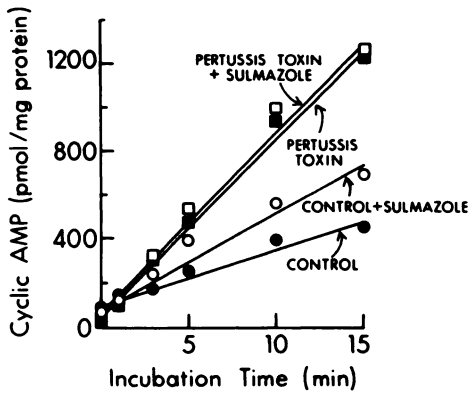

Fig. 2. Attenuation of the stimulatory action of sulmazole on cAMP formation in rat adipocyte membranes after treatment with pertussis vaccine. Adipocyte membranes from control and pertussis-intoxicated rats (36.9 and 30.1 μg of protein, respectively) were incubated for different time periods with ~1.5 μCi of [^{32}P]ATP and 5 μM GTP in the absence or presence of 2 mM sulmazole. This is representative of at least three experiments showing similar results.

mediate inhibition of adenylate cyclase in pertussis-intoxicated membranes (not shown). Furthermore, *in vitro* labeling of G_i in these membranes by pertussis toxin was completely abolished, with no change in cholera toxin-mediated labeling of the G_s protein (not shown). Because the latter experiments were performed at inhibitory GTP concentration (5 μM), it is concluded that the inability of sulmazole to increase cAMP under these conditions is due to prior inactivation of G_i *in vivo* by pertussis vaccine.

The levels of cyclic AMP obtained under the different conditions described above vary considerably. For example, basal adenylate cyclase activities were (mean ± standard error, three to four experiments) 57.3 ± 10.4, 77.1 ± 18.8, and 98.5 ± 9.1 pmol/mg/min in control membranes using 5 μM and 10 nM GTP and in membranes from pertussis-intoxicated rats, respectively. The addition of sulmazole (2 mM) enhanced cyclic AMP levels measured at 5 μM GTP by 46.2%, whereas stimulation in the presence of 10 nM GTP and in pertussis toxin-treated membranes were 3.9 and 3.1%, respectively. Thus, the stimulatory effects of sulmazole on adenylate cyclase activity is manifested only in the presence of an active G_i.

Effect of Sulmazole on cAMP Formation in Presence of Stimulatory Effectors

Isoproterenol. Isoproterenol stimulation of cAMP formation was linear with time to 15 min (Fig. 3A). In the presence of 5 μM GTP, sulmazole potentiated adenylate cyclase activity by an additional 33% of that obtained in the presence of isoproterenol alone. However, in the presence of low GTP concentration (10 nM), isoproterenol-stimulated adenylate cyclase activity was inhibited by sulmazole (Fig. 3B) by approximately 24% in the presence of 10 μM isoproterenol (Table 1). This inhibitory effect of the inotrope was also evident in pertussis-intoxicated membranes, in which it averaged approximately 21% (Fig. 3C; Table 1).

Cyclic AMP levels in the absence of sulmazole were 285.9 and 715.1 pmol/min/mg of protein at 10 nM and 5 μM GTP, respectively, after isoproterenol (10 μM) stimulation (mean of three to five experiments). Adenylate cyclase activity in pertussis-intoxicated membranes (at 5 μM GTP) and in the presence of isoproterenol was 998.9 pmol/min/mg of protein (mean

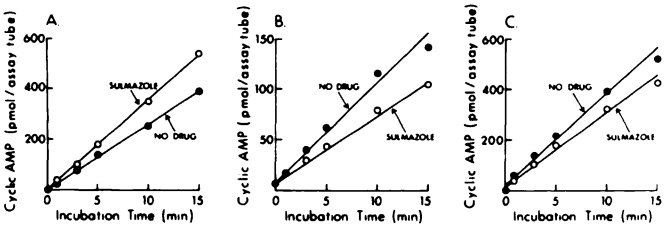

Fig. 3. Differential effects of sulmazole on isoproterenol-stimulated cAMP formation. Adipocyte membranes obtained from control (31.1 μg of protein) and pertussis-intoxicated (38.5 μg of protein) rats were exposed to 10 μM isoproterenol in the absence or presence of 2 mM sulmazole. A, Stimulatory effects of sulmazole in control membranes observed at 5 μM GTP concentration. B, Inhibition of cAMP formation by sulmazole in control membranes in presence of 10 nM GTP. C, Inhibitory effect of sulmazole in pertussis-intoxicated membranes observed at 5 μM GTP concentration. Each figure is a representative experiment of an experimental condition tested. Experiments were replicated at least three times with similar results.

TABLE 1

Effect of sulmazole on isoproterenol-, forskolin-, and fluoride-stimulated adenylate cyclase activity

Membranes (~30 μg of protein/assay tube) obtained from control and pertussis-intoxicated rats were incubated with [^{32}P]ATP (~1.5 μCi) under the experimental conditions described. Incubations were carried out for 15 min (for isoproterenol and fluoride) or 10 min (for forskolin) at 30°. Stimulation of cAMP formation mediated by sulmazole (2 mM) is presented as the mean ± standard error of three to five independent experiments performed in duplicate.

Treatment	cAMP with Sulmazole (2 mM)		
	Isoproterenol (10 μM)	Forskolin (5 μM)	Fluoride (10 mM)
	% of control		
None			
GTP (5 μM)	133.2 ± 6.1	133.6 ± 5.7	145.4 ± 3.4
GTP (10 nM)	75.6 ± 4.6	100.3 ± 5.6	111.7 ± 0.6
Pertussis toxin			
GTP (5 μM)	78.9 ± 1.7		

of three experiments). The difference in activity observed at low and at high GTP concentrations is apparently due to the GTP dependence of isoproterenol-stimulated cAMP formation in rat adipocyte membranes (15, 18). Furthermore, the increased isoproterenol-stimulated adenylate cyclase activity in control versus pertussis toxin-treated membranes is due to abolition of a tonic G_i inhibition normally manifested in this tissue.

The inhibitory effect of sulmazole on isoproterenol-stimulated cAMP formation observed in pertussis-intoxicated adipocyte membranes is reversible at the maximal stimulatory concentration of isoproterenol. Fig. 4 shows isoproterenol dose-response curves for the activation of adenylate cyclase in the absence and presence of sulmazole. Isoproterenol increased adenylate cyclase activity in a dose-dependent manner. Addition of sulmazole (2 mM) resulted in reductions in isoproterenol-stimulated activity averaging 23 ± 2% and 20 ± 2% (mean ± standard error of three or four experiments) at 1 and 10 μM isoproterenol, respectively (significantly different from control, $p < 0.05$). However, this inhibitory action of the drug was abolished after activation of G_s via β-adrenergic receptors using 100 μM isoproterenol (Fig. 4). Thus, after inactivation of G_i, partial activation of G_s is required to unmask the inhibitory action of sulmazole on cAMP production, whereas full agonist activation can overcome this inhibition.

Forskolin. These experiments were terminated after 10 min

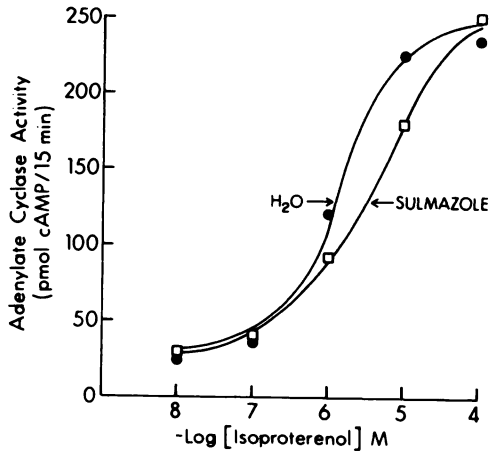

Fig. 4. Reversal of the inhibitory action of sulmazole in pertussis-intoxicated membranes by isoproterenol. Adipocyte membranes (16.2 μg of protein/assay tube) were incubated for 15 min with varying concentrations of isoproterenol and 5 μM GTP. Adenylate cyclase assay was performed as described in Methods, in the absence and presence of sulmazole (2 mM). The figure represents a single experiment performed in duplicate. A total of three experiments were performed with similar results.

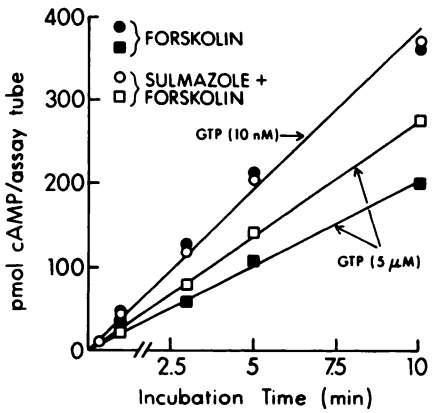

Fig. 5. GTP dependence of sulmazole-mediated stimulation of cAMP formation in the presence of 10 μM forskolin. Membranes (36.1 μg of protein/assay tube) were incubated for different time periods with 10 nM or 5 μM GTP. The effects of sulmazole (2 mM) on cAMP formation at both GTP concentrations were assessed as described in Methods. This is representative of three similar experiments performed in duplicate.

of incubation because initial results indicated that the time course of cAMP formation was not linear beyond this point. Fig. 5 and Table 1 show that sulmazole potentiated forskolin-stimulated cAMP levels by about 34% over control in the presence of 5 μM GTP. At 10 nM GTP, the stimulatory effect of sulmazole was lost (Fig. 5; Table 1). Similarly, the potentiating effect of sulmazole on forskolin-stimulated cAMP formation was attenuated in pertussis-intoxicated membranes (data not shown). However, sulmazole did not inhibit forskolin-stimulated adenylate cyclase activity when assays were performed at 10 nM GTP concentration, as observed with isoproterenol (Table 1). This suggests that sulmazole lacks direct inhibitory action on the catalytic unit, in contrast to its inhibitory effect on G_s.

Forskolin-stimulated adenylate cyclase activity determined at low and high GTP concentrations was remarkably different. For example, activity at low GTP and in the presence of 5 μM forskolin averaged 1082 pmol/min/mg of protein (three exper-

iments), whereas at 5 μM GTP the activity was reduced by about 50%. Because G_i is activated at the latter GTP concentration (6, 18), it is likely that activation of G_i mediates the reduction in adenylate cyclase activity observed under this condition. The fact that sulmazole enhances adenylate cyclase activity over that observed at 5 μM GTP concentration further supports our contention that it inhibits the activity of G_i.

Fluoride. Fluoride-stimulated adenylate cyclase activity was linear with time up to 15 min. Therefore, these experiments were terminated at this time point. As shown in Table 1, sulmazole (2 mM) potentiated fluoride-stimulated adenylate cyclase activity by 45.4 ± 3.4% when experiments were performed at 5 μM GTP. However, this stimulatory effect of sulmazole was significantly reduced at 10 nM GTP (under this latter condition G_i is functionally inactivated). Adenylate cyclase activity in the presence of fluoride was 166.2 pmol/min/mg of protein at 5 μM GTP and 341.5 pmol/min/mg of protein at 10 nM GTP. The difference in activity likely reflects activation of G_i at the higher GTP concentration. Importantly, in these membranes no inhibitory effect of sulmazole on fluoride-stimulated adenylate cyclase activity was observed at low GTP, unlike inhibition of isoproterenol-stimulated activity evident under this latter condition (Fig. 3B). Thus, inhibition of G_s activity by the inotrope is produced by mechanism independent of that involved in fluoride-mediated activation of this protein.

Effect of Sulmazole on ADP-Ribosylation of G_i and G_s by Pertussis Toxin and Cholera Toxin, Respectively

From the above results, it appears that sulmazole inhibits both G_i and G_s, depending on the assay conditions employed. The following experiments were conducted to test whether sulmazole interacts directly with the G proteins G_i and G_s, labeled by pertussis toxin and cholera toxin, respectively. As shown in Fig. 6A, the predominant substrate of pertussis toxin was a protein of M_r 41,000 (α_i). Very little M_r 39,000 substrate (α_o) was labeled under the assay conditions employed. The incorporation of [^{32}P]NAD into α_i was inhibited by sulmazole in a dose-dependent manner (Fig. 6A). Pertussis toxin-catalyzed labeling of G_i (α_i) averaged 69.0, 54.8, 28.6, and 11.6% of control in the presence of 0.001, 0.01, 0.1, and 2 mM sulmazole, respectively.

Under the conditions used in our assay, cholera toxin catalyzed the labeling of two proteins (M_r 47,000 and M_r 42,000) (Fig. 6B). On a few occasions, labeling of a protein of M_r 41,000 was observed (not shown). Labeling of this latter protein was abolished by increasing the GTP concentrations in the incubation mixture (not shown). GTP increased the extent of cholera toxin-promoted labeling of the M_r 47,000 and the M_r 42,000 proteins (12) by about 25% over control (no GTP). Fig. 6B indicates that sulmazole decreased the labeling of G_s in a dose-dependent manner. Labeling (of the M_r 42,000 protein) was decreased to 56%, 43%, and 30% of control in the presence of 0.1, 1, and 3.3 mM sulmazole, respectively. Inhibition of labeling of the M_r 47,000 protein was also observed and this was, respectively, reduced by 50%, 37.5, and 25% of control (Fig. 6B). Thus, it appears that sulmazole can interact directly with both G_i and G_s in this *in vitro* assay at concentrations in the micromolar range.

Effect of Sulmazole on [^{3}H]GDP Release

Labeling of membranes with [^{3}H]GTP was initiated with isoproterenol (for G_s) and (*R*)-PIA (for G_i). [^{3}H]GDP release from G_s and G_i was subsequently initiated by isoproterenol and PGE_1, respectively. Specific [^{3}H]GDP release elicited by 10 and 100 μM isoproterenol averaged 380 cpm per assay volume (range, 249–462 cpm from four experiments, averaging ~0.12 pmol/mg of protein) and 412 cpm per assay volume (range, 388–429 cpm from three experiments; averaging ~0.14 pmol/mg of protein), respectively. These values are consistent with those observed by others (15). In all experiments, the addition of sulmazole resulted in the inhibition of agonist-induced [^{3}H]GDP release. Inhibition was maximal when release was initiated by 10 μM isoproterenol and averaged 63.9% (three experiments) of control obtained in the presence of isoproterenol alone (Table 2). [^{3}H]GDP release initiated by 100 μM isoproterenol was less susceptible to inhibition by sulmazole. In the

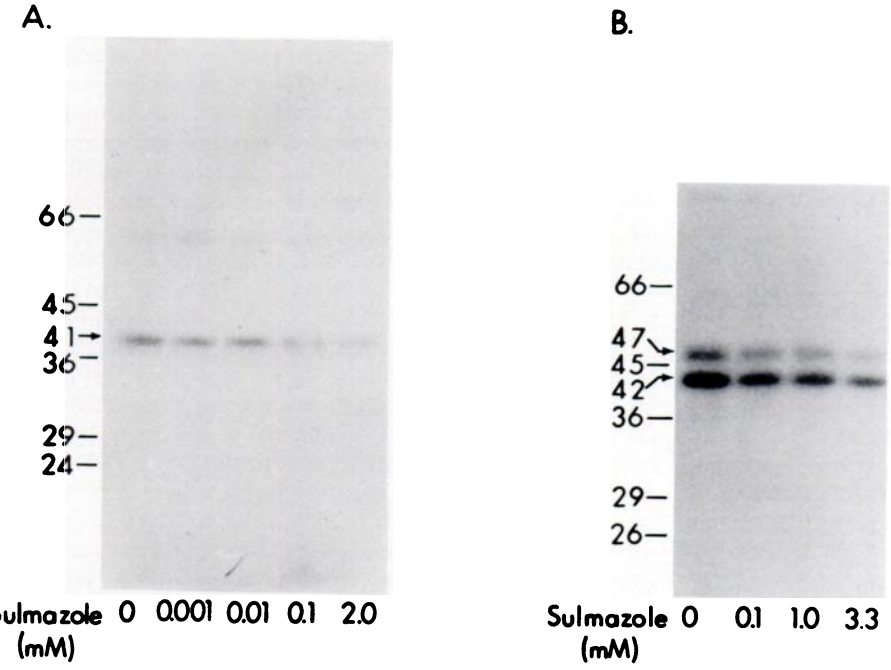

Fig. 6. Inhibition of toxin-catalyzed [^{32}P]NAD labeling of G_i and G_s by sulmazole. [^{32}P]NAD labeling was carried out as described in Methods in the absence and presence of sulmazole (0.001–3.3 mM) and pertussis toxin (A) or cholera toxin (B). The figures depict autoradiograms obtained from exposure of the dried gels to Kodak XAR-5 X-ray film for 24–72 hr.

TABLE 2

Inhibition of agonist-promoted [^{3}H]GDP release from rat adipocyte membranes by sulmazole

Adipocyte membranes (~50–75 μg of protein) were loaded with [^{3}H]GTP (~0.12 μM) in the presence of isoproterenol or (*R*)-PIA. [^{3}H]GDP release was initiated in buffer A containing isoproterenol or PGE_1, in the absence or presence of 3.3 mM sulmazole. Control release (100%) represents [^{3}H]GDP release obtained in the absence of sulmazole. Values are presented as the mean ± standard error of three experiments performed in duplicate.

[^{3}H]GTP loading with isoproterenol (50 μM)	Effect of sulmazole on [^{3}H]GDP release initiated by	
	Isoproterenol (10 μM)	Isoproterenol (100 μM)
	% of control	
	63.9 ± 5.2	84.5 ± 5.7
[^{3}H]GTP loading with (*R*)-PIA (1 μM)	Effect of sulmazole on [^{3}H]GDP release initiated by PGE_1 (30 μM)	
	% of control	
	31.9 ± 12.2	

presence of sulmazole, [^{3}H]GDP release under the latter condition averaged 84.5% of control (Table 2). In three of four experiments performed, sulmazole also displayed inhibition of basal release (not shown).

PGE_1-mediated [^{3}H]GDP release after loading of membranes with 1 μM (*R*)-PIA averaged 113 cpm/assay volume (range, 86–161 cpm from three experiments, averaging 0.04 pmol/mg of protein). In all experiments performed, the addition of sulmazole in the release mixture resulted in significant reductions in the inhibitory agonist-promoted release. In the presence of sulmazole, [^{3}H]GDP release averaged 31.9% of control (range, 9.9–52%; see Table 2).

Effect of Sulmazole on [^{3}H]Gpp(NH)p Binding

Additional experiments were performed to test the effect of sulmazole on [^{3}H]Gpp(NH)p binding to G_i, stimulated by PGE_1. These studies were based on the premise that by reducing agonist-promoted GDP release, sulmazole might inhibit the ability of Gpp(NH)p to bind to the guanine nucleotide binding sites. As shown in Fig. 7, the inotrope inhibited PGE_1-stimulated [^{3}H]Gpp(NH)p binding completely and basal (unstimulated) [^{3}H]Gpp(NH)p binding to a lesser degree. Stimulated binding (binding above *dashed line*) under the conditions of the assay averaged 656 cpm per assay tube (~1.4 pmol/mg of protein) and comprised 22.1 ± 2.3% of total specific binding (mean ± standard error of six experiments). As observed, inhibition of stimulated [^{3}H]Gpp(NH)p binding was evident at micromolar concentrations of sulmazole (IC_{50} of about 1 μM). Inhibition of basal guanine nucleotide binding by sulmazole probably reflects its inhibitory action on basal G_i activity and at GTP binding proteins not activated via PGE_1 receptors.

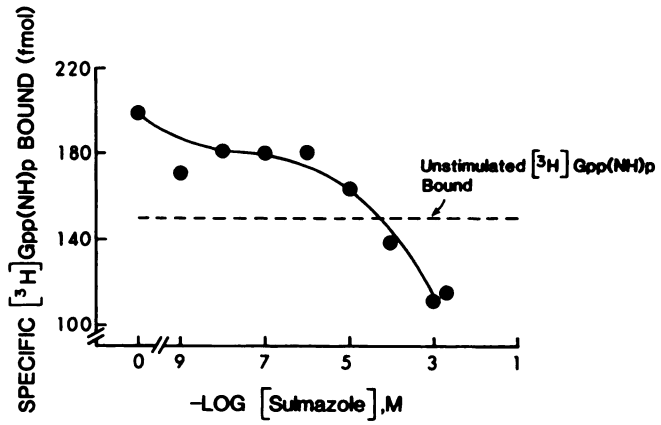

Fig. 7. Inhibition of PGE_1-stimulated binding of [^{3}H]Gpp(NH)p to G_i by sulmazole. Membranes (~30 μg of protein/assay tube) were incubated with 0.1 μM [^{3}H]Gpp(NH)p in the absence (unstimulated) or presence of 10 μM PGE_1, as described in Methods. Nonspecific binding was determined as binding remaining in the presence of 100 μM unlabeled GTP and was subtracted from total binding to yield specific [^{3}H]Gpp(NH)p binding. This is a representative experiment of six experiments showing similar results.

Discussion

The newer positive inotropes increase cellular cAMP levels and this may contribute to their positive inotropic actions. Increases in cAMP are mediated, in part, by the inhibition of the low K_m phosphodiesterase in myocardial cells (19, 20). We have recently demonstrated that a number of these drugs compete directly with A_1 adenosine receptors (6), thereby blocking the reduction in cAMP produced by A_1 adenosine agonists. We now provide evidence for a direct interaction of these inotropic drugs on both the inhibitory guanine nucleotide protein, G_i, and the stimulatory guanine nucleotide regulatory protein, G_s. These agents directly impair the function of G_i and, in addition, after inactivation of G_i by either lowering the concentration of GTP or covalently modifying G_i with pertussis toxin, an inhibition of G_s function is manifested. Inactivation of G_i and G_s by the inotrope is mediated, at least in part, by the inhibition of GTP turnover.

Various pieces of evidence support a direct inhibitory action of sulmazole at the level of G_i. First, the inotrope increases cAMP levels in adipocyte membranes by a mechanism that does not involve inhibition of the low K_m cAMP phosphodiesterase, inasmuch as complete inhibition of this enzyme was attained with papaverine. Furthermore, the concentrations of sulmazole used inhibited the low K_m cAMP phosphodiesterase to only about 50% of that provided by papaverine (not shown). Antagonism of the inhibitory effect of adenosine at A_1 adenosine receptors also appears to be an unlikely explanation for enhancement in cAMP by sulmazole because endogenous adenosine was eliminated by the addition of adenosine deaminase. Increasing the concentration of adenosine deaminase by 2–3-fold resulted in no further elevation in cAMP (data not shown). Because this agent does not activate β-adrenergic receptors (21, 22) or the catalytic unit (the present data), the only likely means of increasing cAMP is to inhibit G_i activity. Rat adipocyte membranes are tonically inhibited by G_i under basal conditions (18). By blocking G_i activity, therefore, the inotrope produces a net stimulation of adenylate cyclase activity.

Second, conditions that result in the functional inactivation of G_i also lead to the loss of the stimulatory effect of sulmazole. In rat adipocyte membranes, micromolar concentrations of GTP are required before inhibition of adenylate cyclase is evident (6, 17, 18). Accordingly, stimulation of cAMP accumulation by sulmazole is maximal at the higher concentrations rather than at lower GTP concentrations. Activation of G_i at micromolar GTP concentration is evidenced by the lower basal adenylate cyclase activity under these conditions compared with higher activity of this enzyme observed at nanomolar GTP concentration (Fig. 1). By offsetting the inhibitory effect of G_i manifested at micromolar GTP concentrations, sulmazole pro-

duces a net stimulation of adenylate cyclase activity equivalent to that observed when G_i is functionally inactive. Thus, sulmazole reversibly transforms G_i from an active to an inactive state. Pertussis toxin catalyzes the ADP-ribosylation of G_i in membranes (2, 4, 10, 15). As might be expected, therefore, the stimulatory effect of sulmazole is abolished in pertussis-intoxicated membranes (Fig. 3). This finding adds further support to the contention that sulmazole inhibition of G_i mediates its enhancement of adenylate cyclase activity. In accordance with its inhibitory effect on G_i, pertussis-intoxicated membranes show a 2–3-fold elevation in basal adenylate cyclase activity, similar to that observed at low GTP concentration (Fig. 2). In addition, we were able to restore the stimulatory effects of sulmazole in these membranes by reconstituting them with purified G_i or a mixture of G_i and G_o (6). Interestingly, reconstitution with purified G_i resulted in a 50% reduction in basal cAMP level (6), indicating an efficient coupling of this protein with the catalytic unit.

Third, we demonstrate a direct effect of sulmazole on G_i by its ability to inhibit pertussis toxin-catalyzed ADP ribosylation of this protein in adipocyte membranes (Fig. 6A). This finding provides direct evidence for an interaction of the positive inotrope with G_i. Reduction in the labeling of G_i was evident with 1 μM sulmazole (31% inhibition) and increased in a dose-dependent fashion. The dose-response relationship for inhibition of labeling differs from that observed for the stimulation of cAMP by sulmazole; effects of sulmazole on cAMP were evident at high micromolar to low millimolar concentrations. This disparity suggests that adenylate cyclase activity might not be a sensitive indicator of the interaction of sulmazole with G_i inasmuch as a significant portion of G_i has to be inactivated before loss of inhibition of adenylate cyclase is evident (23). Presumably, direct determinations of G_i functioning (see below) are more sensitive indicators. The disparity likely relates to the stoichiometric excess of G_i over receptors and the catalytic unit in membranes (1–3).

In addition to potentiating basal adenylate cyclase activity, sulmazole increases both isoproterenol-stimulated and forskolin-stimulated adenylate cyclase activity at inhibitory GTP concentrations (Figs. 3A and 5). The inotrope was, however, less effective in this respect (33% and 34% stimulation versus 49% stimulation under basal conditions; Table 1). A similar effect on fluoride-stimulated cAMP formation was also observed (Table 1). In this case, the stimulatory effects of sulmazole were comparable to those seen under basal conditions (Table 1). The ability of sulmazole to increase cAMP levels above that obtained in the presence of isoproterenol, forskolin, and fluoride suggests that tonic activation of G_i still exists under these conditions.

Inhibition of G_s by sulmazole appears to be unmasked after inactivation of G_i by low GTP or by *in vivo* treatment with pertussis vaccine. However, such inhibition is evident after stimulation only by isoproterenol (Fig. 3, B and C; Table 1) and not by forskolin or fluoride (Table 1). We, and others, have ruled out the possibility of direct inhibition of sulmazole on β-adrenergic receptors by radioligand binding and adenylate cyclase assays[1] (21, 22). Furthermore, the lack of inhibitory effect by sulmazole on forskolin-stimulated cAMP formation observed at low GTP concentration suggests that the inotrope does not interact directly with the catalytic unit to inhibit cAMP. Thus, it is likely that sulmazole acts directly on G_s to produce its inhibitory effect. This contention is supported by the finding that the inotrope inhibited cholera toxin-catalyzed ADP-ribosylation of G_s in a dose-dependent fashion (Fig. 6B). As with G_i, a modest reduction (25%) in adenylate cyclase activity was observed (Table 1) after a significant (70%) loss in cholera toxin-mediated labeling (Fig. 6B). This difference probably relates to the need for a significant loss of G_s before loss of the stimulatory effect of the protein is manifested, owing to the excess of G_s over other components of the stimulatory system (24).

In pertussis toxin-treated membranes, activation of adenylate cyclase at 5 μM GTP concentration by both forskolin and fluoride was not inhibited by sulmazole (not shown). The inability of the inotrope to reduce forskolin-stimulated cAMP can readily be explained by the action of the latter drug primarily with the catalytic unit (25). The ineffectiveness of sulmazole in inhibited fluoride-stimulated cAMP formation suggests the need for GTP turnover before the effect of the inotrope is manifested. Unlike stimulatory agonists (15), fluoride-mediated activation of G_s is not dependent on guanine nucleotides (26). Furthermore, fluoride competes with GTP for activation of adenylate cyclase (27) and is unable to promote [^{3}H]GDP release from rat adipocyte membranes labeled with [^{3}H]GTP in presence of isoproterenol (15). These findings are consistent with the contention that the inhibition of G_s and G_i by sulmazole involves GTP turnover in adipocyte membranes. In accordance with this premise, we reasoned that the inhibitory effect of sulmazole at both G_s and G_i might be attenuated by stimulating GTP turnover maximally with the respective receptor agonists. As shown in Fig. 4, sulmazole-mediated inhibition of G_s is attenuated by 100 μM isoproterenol. Accordingly, activation of G_s by maximally effective concentrations of isoproterenol can overcome the inhibition produced by sulmazole. We have similarly observed loss of sulmazole-mediated inhibition of G_i with increasing concentrations of (*R*)-PIA (6).

To determine the mechanism(s) of inhibition of the G proteins, the effects of sulmazole on GTP turnover mechanisms were assessed. Initially, using purified G_i protein, experiments indicated that the inotrope did not affect GTPase activity (data not shown). However, profound inhibition of agonist-stimulated GDP release and [^{3}H]Gpp(NH)p binding were observed. For example, sulmazole inhibited [^{3}H]GDP release initiated by both the stimulatory agonist (isoproterenol) and the inhibitory agonist (PGE_1). This correlates well with the inhibition of the function of both G_i and G_s as determined from adenylate cyclase data. In accordance with these data, sulmazole was more effective at inhibiting [^{3}H]GDP release initiated by PGE_1 (via G_i) than release initiated by isoproterenol (via G_s). Furthermore, inhibition of isoproterenol-mediated [^{3}H]GDP release by sulmazole was reduced at the higher concentration of isoproterenol (Table 2). This is reminiscent of the reversal of the inhibitory effect of sulmazole on G_s activity observed at the highest isoproterenol concentration used (Fig. 4). Thus, it is reasonable to conclude that reduction of [^{3}H]GDP release is involved in the inhibitory action of sulmazole at both G_i and G_s. The concentration of sulmazole (3.3 mM) used for these assays significantly reduced [^{3}H]GDP release initiated by PGE_1. Although it is likely that lower concentrations of sulmazole would produce significant inhibition of release, lower concentrations

[1] W. J. Parsons and G. L. Stiles, unpublished observations.

of the drug were not used because of the low release signal obtained (see Results).

The ability of sulmazole to decrease agonist-promoted GDP release suggests, indirectly, that the inotrope should also attenuate the binding of guanine nucleotides to G proteins induced by agonists. In order to test this proposal, we assessed the effect of sulmazole on PGE_1-stimulated [^{3}H]Gpp(NH)p binding. As expected, sulmazole inhibited [^{3}H]Gpp(NH)p binding in a dose-dependent fashion. The IC_{50} of sulmazole, in this respect, was approximately 1 μM, similar to concentrations of the inotrope required to produce significant inhibition of G_i labeling. Furthermore, sulmazole inhibited [^{3}H]Gpp(NH)p binding even in the absence of PGE_1 (basal), probably reflecting its inhibitory effects on basal guanine nucleotide binding to G_i and other G proteins. As evident in Fig. 7, lower concentrations of sulmazole are required to inhibit the binding of the guanine nucleotide to G_i than are required to enhance adenylate cyclase activity. This apparent difference in potency might reflect the need for considerable inactivation of G_i before effects on adenylate cyclase inhibition are manifested (23), as described above, owing to the stoichometric excess of G_i over other components of the adenylate cyclase system in membranes (1–3).

Both sulmazole and GTP possess methylxanthine-like ring structures. This might explain the observed interaction of the inotrope at the GTP binding site. Interestingly, other methylxanthines such as IBMX (28) and caffeine[1] and the xanthine amine congener (29) also attenuate the effect of GTP on G_i. However, owing to the only partial structural similarity between these agents and GTP, the observed interaction at the GTP binding site has been characterized as noncompetitive (6, 28, 29), suggesting that these agents do not bind directly to the latter site.

In conclusion, we have demonstrated that sulmazole inhibits the function of both G_i and G_s. Inhibition of G_i activity augments basal and stimulated adenylate cyclase activity by blocking tonic inhibition of the enzyme activity. Whereas inhibition of G_s is also evident, it is unmasked only after inactivation of G_i. Thus, the net stimulation of sulmazole observed in control membranes is a difference between the inotrope inhibition of G_i and G_s activity. Direct interaction at the level of G_i is evident at micromolar concentrations of sulmazole whereas considerably higher concentrations are required to produce stimulation of adenylate cyclase, reflecting the low sensitivity of this parameter to changes in the function of G_i. Although the clinical relevance of G_i blockade by sulmazole as it relates to its cardiotonic actions remains to be determined, the inotropes should prove useful as biochemical tools for studying G_i function.

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