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The New Cardiotonic Agent Sulmazole Is an A_1 Adenosine Receptor Antagonist and Functionally Blocks the Inhibitory Regulator, G_i

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SUMMARY

Although many of the new cardiotonic agents are known to increase cAMP and to inhibit with variable potency a low K_m cAMP phosphodiesterase, there is still debate as to the mechanism(s) by which these agents act. In a rat adipocyte membrane model we demonstrate that only ~50% of the effect of the new cardiotonic agent sulmazole on cAMP accumulation can be attributed to phosphodiesterase inhibition and that the remaining production of cAMP involves stimulation of adenylate cyclase activity. Two distinct pathways for stimulation of adenylate cyclase are herein reported. Sulmazole, UD-CG 212 CL, enoximone, piroximone, amrinone, and milrinone are all shown to be competitive antagonists of inhibitory A_1 adenosine receptors, with EC_{50} values of 11–909 μM. Elimination of the effects of endogenous adenosine with adenosine deaminase reveals a third distinct mechanism for activation of adenylate cyclase. This mechanism appears to involve G_i, the inhibitory guanine nucleotide-regulatory protein, in that sulmazole attenuates the capacity of GTP to inhibit adenylate cyclase activity, and covalent modification of G_i by pertussis toxin treatment abolishes the capacity of sulmazole to mediate stimulation. Thus, functional blockade of G_i activity is the likely mode of action. Restoration of sulmazole's stimulatory effect on adenylate cyclase activity in pertussis toxin-treated membranes can be accomplished by reconstituting purified preparations of either G_i or mixtures of G_i/G_o into treated adipocyte membranes. Of note, this stimulatory effect is completely reversed by inhibitory receptor agonists. Thus, the new cardiotonic agent sulmazole mediates increases in cAMP accumulation by mechanisms other than phosphodiesterase inhibition, including A_1 adenosine receptor antagonism and inhibition of G_i function.

The search for a more effective pharmacologic treatment for heart failure has lead to the recent development of several novel cardiotonic agents (1). Unlike the cardiac glycosides which inhibit Na^+/K^+ ATPase, the new cardiotonic agents increase cAMP levels in cardiac cells. Furthermore, the inotropic and vasodilatory actions of these agents correlate well with their effects on cAMP accumulation (2–4), although other mechanisms independent of cAMP have been suggested (5). A low K_m cAMP phosphodiesterase has been characterized in cardiac tissue which is selectively inhibited by the new cardiotonic agents. Accordingly, it has been proposed that a primary mechanism by which these agents increase cellular cAMP is via selective phosphodiesterase inhibition (6). However, despite substantial investigation, the relationship between phosphodiesterase inhibition and cardiotonic efficacy remains unclear (7, 8).

The effects of the new cardiotonic agents on the components of membrane-bound adenylate cyclase systems have not been thoroughly investigated. These "non-glycoside, non-sympathomimetic" drugs do not appear to mediate stimulation of adenylate cyclase activity by activating stimulatory-coupled adrenergic receptors, or by blocking inhibitory-coupled muscarinic receptors (1, 9). Yet, almost paradoxically, the stimulatory effects of these agents are dramatically attenuated by inhibitory receptor agonists (3, 4, 10). In contrast, isoproterenol-stimulated cAMP production is only modestly inhibited by inhibitory receptor agonists in cardiac tissue (3, 9). Thus, our current understanding is incomplete as to the biochemical mechanisms by which the new cardiotonic agents mediate stimulation of cAMP accumulation and regarding why this stimulation is so markedly attenuated by inhibitory receptor agonists.

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ABBREVIATIONS: G_s, stimulatory guanine nucleotide-regulatory protein; G_i, inhibitory guanine nucleotide-regulatory protein; α_i, α subunit of G_i; A_1AR, A_1 adenosine receptor; *R*-PIA, (−)-N^6-(*R*-phenylisopropyl)adenosine; [^{125}I]HPIA, (−)-N^6-(3-[^{125}I]iodo-4-hydroxyphenylisopropyl)adenosine; RO 20-1724, DL-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone: PGE_1, prostaglandin E_1; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetate; G_o, guanine nucleotide-regulatory protein of unknown function.

In most tissues, the accumulation of cAMP results from stimulation of the enzyme adenylate cyclase and/or from inhibition of one or more cAMP phosphodiesterases. The enzyme adenylate cyclase is coupled to cell surface receptors via guanine nucleotide-binding proteins which mediate stimulation (G_s) or inhibition (G_i) of adenylate cyclase activity. "Stimulatory" adenylate cyclase-coupled receptor systems are comprised of surface receptors which, in response to agonists, activate G_s, leading to an increase in adenylate cyclase activity. Conversely, "inhibitory" receptor systems are comprised of receptors which, in response to agonists, activate G_i, leading to a decrease in adenylate cyclase activity (11).

Like the heart (12), rat adipocyte membranes contain a hormone-sensitive adenylate cyclase system. In addition to the enzyme adenylate cyclase, this system includes G_s and G_i, as well as β-adrenergic receptors which stimulate, and A_1-ARs and prostaglandin receptors which inhibit, adenylate cyclase activity (13, 14). Furthermore, adipocyte membranes are particularly responsive to both stimulatory and inhibitory effectors such as isoproterenol and adenosine analogues (15). Rat adipocytes also contain a low K_m cAMP phosphodiesterase which is antigenically and kinetically similar to the purified cardiac enzyme (16). For these reasons, rat adipocyte membranes were used in the present study to probe potential mechanisms by which the new cardiotonic agents affect cAMP accumulation and, specifically, how these agents mediate cAMP production via the adenylate cyclase system. For the purposes of this report, we particularly emphasized the new cardiotonic agent sulmazole (AR-L 115 BS), a benzimidazole derivative with phosphodiesterase-inhibiting properties and stimulatory effects on cAMP accumulation which are common to most of the new cardiotonic agents (4, 17). We demonstrate that sulmazole can mediate cAMP accumulation by mechanisms other than phosphodiesterase inhibition, including inhibition of A_1-adenosine receptors and by a previously unappreciated mechanism functionally blocking the inhibitory guanine nucleotide-regulatory protein, G_i.

Experimental Procedures

Materials. Male Sprague-Dawley rats (250–350 g) were from Charles River Breeding Laboratories. Adenosine deaminase, cAMP, cGMP, GTP, ATP, dATP, creatine phosphokinase, and papaverine were from Sigma. Creatine phosphate and *R*-PIA were from Boehringer Mannheim. [2,8 $-^3$H]cAMP (40 Ci/mmol) and [$\alpha - ^{32}$P]ATP (27 Ci/mmol) were from New England Nuclear. [^{125}I]HPIA (2000 Ci/mmol) was from Amersham. Crude collagenase was from Cooper Biomedical. Bovine serum albumin (fraction V) was from Armour. Inotropic agents were the kind gifts of Sterling-Winthrop Research Institute (Milrinone and Amrinone), Merrell Dow Research Institute [Piroximone (MDL 19,205) and Enoximone (MDL 17,043)], and Boehringer Ingelheim Pharmaceuticals, Inc. [Sulmazole (AR-L 115 BS) and UD-CG 212 CL]. RO 20-1724 was a gift of Dr. F. Sorter at Hoffman-LaRoche. Rolipram was a gift of Dr. J. W. Daly at National Institutes of Health, Bethesda, MD. Pertussis vaccine concentrate (153 opacity units/ml; strain 1302) was from Lederle. All other chemicals and reagents were of analytical grade.

Membrane preparation. When indicated, pertussis vaccine (~300 opacity units/kg) was given intraperitoneally to rats 3 days before sacrifice by decapitation. Adipocyte membranes were prepared from epididymal fat pads as previously described (18), with the following modifications: leupeptin and soybean trypsin inhibitor were omitted during collagenase digestion; both of these and phenylmethylsulfonyl fluoride were omitted from the hypotonic buffer. For adenylate cyclase assays, the initial membrane pellet was suspended at 1 mg of protein/ml in 75 mM Tris (pH 7.4, 30°), 12.5 mM $MgCl_2$, 200 mM NaCl, 2.5 mM DTT, and 4.0 units/ml adenosine deaminase, and preincubated at 30° for 10 min. For receptor ligand binding studies, the initial membrane pellet was suspended in 50 mM Tris (pH 7.4, 5°) and centrifuged (15,000 × *g* for 15 min, 4°). The washed pellet was then suspended at 0.25 mg of protein/ml in 50 mM Tris (pH 7.4, 37°), 10 mM $MgCl_2$, 1 mM EDTA, and 1.0 unit/ml adenosine deaminase and was preincubated at 37° for 10 min. For all assays, membranes were prepared concurrently from control and pertussis-treated rats and were immediately used. Protein concentrations were determined by the Bradford method (19) using bovine serum albumin as standard.

Adenylate cyclase assays. Assays were performed using rat adipocyte membranes as described previously (20). Papaverine (10^{-4} M) was substituted for RO 20-1724 to provide adequate phosphodiesterase inhibition unless otherwise noted, and adenosine deaminase at 5 units/ml was used to eliminate endogenous adenosine (see Results); incubation was for 15 min at 30° (20). All assays were shown to be linear with respect to time. GTP (5×10^{-6} M) was used except as indicated for GTP dose response experiments. This concentration of GTP was used because it is sufficient to produce maximal *R*-PIA-mediated inhibition of adenylate cyclase (data not shown). The method of Salomon *et al.* (21) was used for isolation of cAMP.

[125]I]HPIA competition experiments. Assays were performed in duplicate in a 250-μl volume consisting of 150 μl of membranes in 50 mM Tris-HCl, pH 7.4, 37°, 10 mM $MgCl_2$, and 1 mM EDTA (~40 μg of protein), 50 μl of radioligand (~0.3 nM), and 50 μl of H_2O or competing ligand. Membranes were treated with adenosine deaminase (1 unit/ml) for 10 min at 37°, prior to assay. Binding was carried out for 1 hr at 37° and was terminated by rapid vacuum filtration as previously described (18). Nonspecific binding was determined with *R*-PIA at 10^{-5} M and was ~10% of total binding. [^{125}I]HPIA was present at a concentration of ~0.2 nM for competition binding studies. To optimize solubility, milrinone and amrinone were dissolved in 8 mM lactate. Piroximone and enoximone were dissolved in 10 mM NaOH. These concentrations of acid or base did not alter [^{125}I]HPIA binding (data not shown). ^{125}I activity was counted in a Packard gamma counter with an efficiency of 75%.

Reconstitution of purified G proteins into adipocyte membranes. Rats were treated with pertussis toxin and adipocyte membranes were prepared as described above. Purified G proteins were generously provided by Drs. J. Benovic and S. E. Senogles of Duke University. Functional reconstitution of G proteins was performed using the method of Katada *et al.* (22) with the following modifications. The G_i/G_o mixture was 95% pure and consisted of a G_i/G_o ratio of 45:55. The purified G_i preparation was 85% pure and had approximately a 10% contamination with G_o. Purification of G_i and G_o from bovine brains was performed as recently described by Cerione *et al.* (23) and is a slight modification of the method of Codina *et al.* (24). These purified G proteins are heterotrimeric in structure and contain the α, β, and γ subunits. Representative sodium dodecyl sulfate-polyacrylamide gels of these preparations are illustrated in Ref. 23. The G proteins were stored in a stock solution of 20 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.6 mM cholate, 100 mM NaCl at a protein concentration of 0.4 mg/ml (G_i/G_o) or ~0.1 mg/ml (G_i). Purified G proteins (mixture of G_i and G_o, and G_i alone) were diluted (>1000-fold) in buffer containing 20 mM Tris-HCl, 1 mM $MgCl_2$, and 1 mM DTT (pH 7.4, 30°). Aliquots (100 μl) of diluted G proteins or dilution buffer (this buffer produced basal levels the same as stock buffer diluted 1000-fold) were mixed with 300 μl of adipocyte membranes (pretreated with 5.0 unit/ml adenosine deaminase for 15 min at 30°) in buffer (75 mM Tris, pH 7.4 at 30°, 12.5 mM $MgCl_2$, 200 mM NaCl, and 2.5 mM DTT, and 5.0 unit/ml adenosine deaminase), and reconstitution was initiated by incubating the mixtures for 15 min at 30°. Adenylate cyclase assays were performed as described above, using approximately 10 ng of G_i/G_o or 5 ng of G_i per assay tube.

Data analysis. Competition curves were analyzed using a nonlinear least squares curve-fitting technique with statistical analysis, as pre-

viously described, and validated (25, 26). Dose response curves were analyzed according to a four-parameter logistic equation to determine EC_{50} values, including a statistical package allowing determination of statistical differences between curves (25, 26).

Assay for the low K_m cAMP phosphodiesterase. Assays were performed according to the method described by De Mazancourt and Guidicelli (27) for rat adipocytes. The reaction mixture contained 0.02 μM [^{3}H]cAMP, 0.5 μM cAMP, 0.04% bovine serum albumin, 0.5 unit/ml adenosine deaminase in a total volume of 250 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$. The reaction mixture was further supplemented with GTP (10^{-7} M) and various concentrations of the cardiotonic agents. Activation of the low K_m cAMP phosphodiesterase was initiated by the addition of 15–40 μg of membrane protein for 7.5 min at 30°. Incubations were terminated by boiling the tubes for 90 sec, after which precipitation of 5′-AMP was accomplished by addition of 200 μl of $Ba(OH)_2$ (0.3 N) and 200 μl of $ZnSo_4$ (0.3 M). The total volume was then brought up to 1 ml by addition of 350 μl of ice-cold deionized water to each tube. The tubes were vortexed, centrifuged for 10 min at 5000 × *g*, and the resulting supernatant was collected and poured over Dowex 50 W-X2 columns (200–400 mesh). The first eluate and subsequent 1.5-ml wash were discarded. The columns were eluted twice with 3 ml each of H_2O, and the eluates were collected and counted. Values obtained from tubes boiled without prior incubation of the reaction mixture were used to establish the maximal (100%) substrate level.

Results

In Fig. 1A, a representative dose response curve illustrates the dramatic effect of sulmazole on cAMP production in adipocyte membranes. An analogous dose response curve is also shown for the potent phosphodiesterase inhibitor papaverine. Maximal cAMP levels are demonstrated for papaverine at a concentration of 10^{-4} M, and half-maximal stimulation occurs at 9.3 ± 1.9 μM ($N = 3$). In contrast, the effect of sulmazole on cAMP stimulation is far greater and does not appear to be maximal even at 10^{-2} M (higher concentrations were limited by solubility). Since, as demonstrated in the bar graphs in Fig. 1B, the stimulatory effects of papaverine (10^{-4} M) and sulmazole (3.3×10^{-3} M) are not fully additive, they may share a common mechanism of action. Phosphodiesterase inhibition is clearly a mechanism common to both sulmazole and papaverine (17, 28), although this would account for only about half of sulmazole's effect on cAMP accumulation. To document that papaverine was producing full inhibition of phosphodiesterase activity in this model system, phosphodiesterase activity was determined as described under Experimental Procedures. Papaverine (10^{-4} M) was found to completely inhibit cAMP hydrolysis, and the IC_{50} for inhibition was found to be 3.8 ± 1.3 μM ($n = 3$). The addition of sulmazole to 10^{-4} M papaverine neither increased nor decreased the capacity of papaverine to inhibit phosphodiesterase activity. Sulmazole inhibited phosphodiesterase activity with an IC_{50} of 100–200 μM. In addition, sulmazole was also found to stimulate greater cAMP production than other phosphodiesterase inhibitors such as RO 20-1724, rolipram, and cGMP or combinations of these (data not shown). Therefore, a mechanism(s) other than phosphodiesterase inhibition must be considered to account for the additional cAMP production induced by this new cardiotonic agent. To probe what mechanisms might be involved, the effects of inhibitory agonists on sulmazole-mediated stimulation of cAMP accumulation were examined. Interestingly, the addition of the A_1AR agonist *R*-PIA to either sulmazole or papaverine inhibits cAMP production to essentially the same level (Fig. 1B). Stated in another way, the "additional" stimulatory effect of sulmazole on cAMP accumulation (above that produced by papaverine) can be completely ablated by the inhibitory receptor agonist *R*-PIA.

Since adenosine has been shown to exert negative inotropic effects in atrial tissue (29) and clearly decreases adenylate cyclase activity in cardiac and adipose tissues (30) by interacting with A_1ARs, we assessed whether sulmazole and several other new cardiotonic agents specifically block A_1ARs. Pertinently, another group of positive inotropic agents, the methylxanthines, mediate many of their physiologic effects through antagonism of A_1ARs (31, 32).

The capacity of each of the new cardiotonic agents to compete for A_1AR-binding sites was compared using the A_1AR agonist radioligand [^{125}I]HPIA (Fig. 2). All of the new cardiotonic agents compete for occupancy of A_1ARs. Those agents with

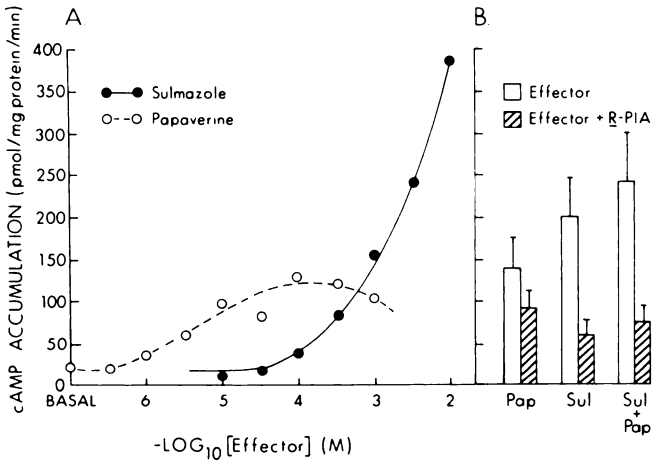

Fig. 1. Effector-mediated stimulation of cAMP accumulation in rat adipocyte membranes. A. Representative dose response curves for sulmazole and papaverine. B. cAMP accumulation induced by sulmazole (3.3×10^{-3} M) versus a maximally stimulatory concentration of papaverine (10^{-4} M). The effect of papaverine is exceeded by sulmazole ($p < 0.04$) or sulmazole plus papaverine ($p < .02$). *R*-PIA (10^{-5} M) significantly ($p < 0.01$) inhibits cAMP accumulation in each case to essentially the same level. Data are presented as mean ± standard error for four experiments. *Pap*, papaverine; *Sul*, sulmazole.

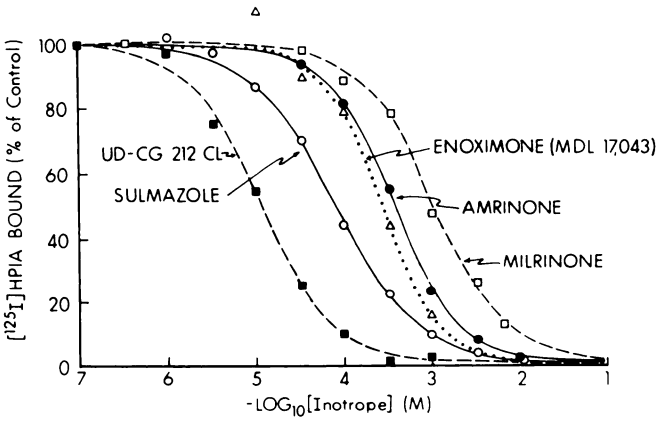

Fig. 2. [^{125}I]HPIA competition curves in rat adipocyte membranes. All of the new inotropes are shown to compete for A_1AR-binding sites with EC_{50} values ranging from 11 to 909 μM. The data points are means of duplicate determinations from a representative experiment. The curves were drawn with the aid of a computer modeling program as described under Experimental Procedures. The concentration of receptor in the absence of competitor is ~40 pM.

methylxanthine-like structures, such as UD-CG 212 CL and sulmazole, had the highest affinity for A_1ARs, whereas the other cardiotonic agents competed with lower affinity for A_1AR-binding sites. Papaverine did not compete for A_1ARs at a concentration of 100 μM (data not shown). The IC_{50} values (μM) for each of the inotropic agents are: UD-CG 212 CL, 11.3 ± 1.5; sulmazole, 78.1 ± 2.6; enoximone, 221.8 ± 41.1; amrinone, 376.3 ± 36.8; piroximone, 561.8 ± 30.5; and milrinone, 909 ± 128.

To further define how the new cardiotonic agents interact with A_1AR, the effects of sulmazole on *R*-PIA-mediated inhibition of adenylate cyclase activity were assessed. To eliminate the effects of endogenous adenosine on A_1ARs, adenosine deaminase was added before and during all assays. Preliminary experiments demonstrated that further increases in the concentration of adenosine deaminase did not increase cAMP accumulation (data not shown). Furthermore, dATP was utilized in all adenylate cyclase assays to prevent adenosine from accumulating. A 15-fold rightward IC_{50} shift (from 4.4 ± 1.5 to 65.1 ± 11.1 nM, $p = 0.006$, $N = 3$) in *R*-PIA-induced inhibition of adenylate cyclase activity by sulmazole is demonstrated in Fig. 3. Sulmazole may therefore be considered an antagonist of A_1ARs. Thus, the blockade of adenosine receptors, which mediate inhibition of adenylate cyclase activity in cardiac tissue, could lead to enhanced accumulation of cAMP *in vivo*.

As demonstrated in Fig. 1, the capacity of *R*-PIA to inhibit sulmazole-mediated stimulation of cAMP accumulation was not adversely affected by the addition of papaverine. Accordingly, papaverine was included in all adenylate cyclase assays after the experiments described in Fig. 1 to provide adequate phosphodiesterase inhibition (17) and, thus, to permit investigation of the stimulatory effect of sulmazole on cAMP accumulation above that which might be attributed to phosphodiesterase inhibition.

In Fig. 4A, the capacity of sulmazole to stimulate adenylate cyclase activity is compared with that of isoproterenol. First, as is shown, the degree of stimulation induced by sulmazole (3.3 mM) is quite modest (~2-fold) when compared with maximal isoproterenol (10^{-5} M) stimulation (~10-fold), which mediates its affects via β-adrenergic receptor activation of G_s. Second, sulmazole appears to weakly potentiate maximal isoproterenol-mediated stimulation, although this effect did not reach statistical significance for $n = 6$ ($p < 0.08$). Furthermore, the effect of sulmazole is not blocked by the β-adrenergic antagonist propranolol (data not shown). Finally, the stimulatory effect of sulmazole above basal adenylate cyclase activity is completely abolished by the addition of *R*-PIA and is attenuated ~75% by PGE_1 (Fig. 4A). Expressed as percentage inhibition (Fig. 4B), the effects of these inhibitory agonists on sulmazole-stimulated adenylate cyclase activity far exceed their effects on basal and isoproterenol-stimulated adenylate cyclase activities. *R*-PIA was never able to abolish isoproterenol-stimulated adenylate cyclase activity even at submaximal doses of isoproterenol. Of note, when isoproterenol concentrations were

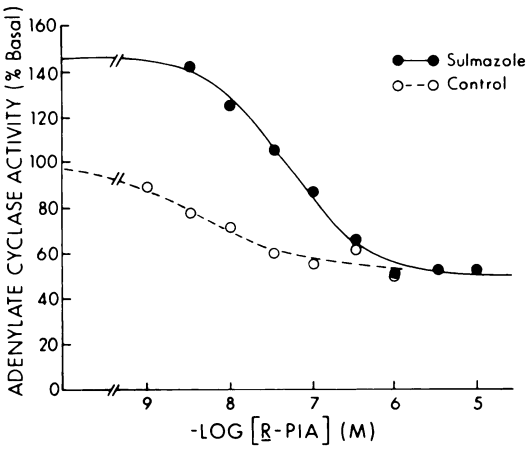

Fig. 3. *R*-PIA-mediated inhibition of sulmazole-stimulated adenylate cyclase activity in rat adipocyte membranes. Sulmazole (3.3×10^{-3} M) is shown to induce a 15-fold rightward EC_{50} shift in *R*-PIA-mediated inhibition of adenylate cyclase activity. Also, the stimulatory effect of sulmazole is totally ablated by *R*-PIA. The concentrations of *R*-PIA are indicated on the *abscissa*. The data points are means of triplicate determinations from a representative experiment which was replicated three times. Basal adenylate cyclase activity was 54.2 pmol of cAMP/mg of protein/min.

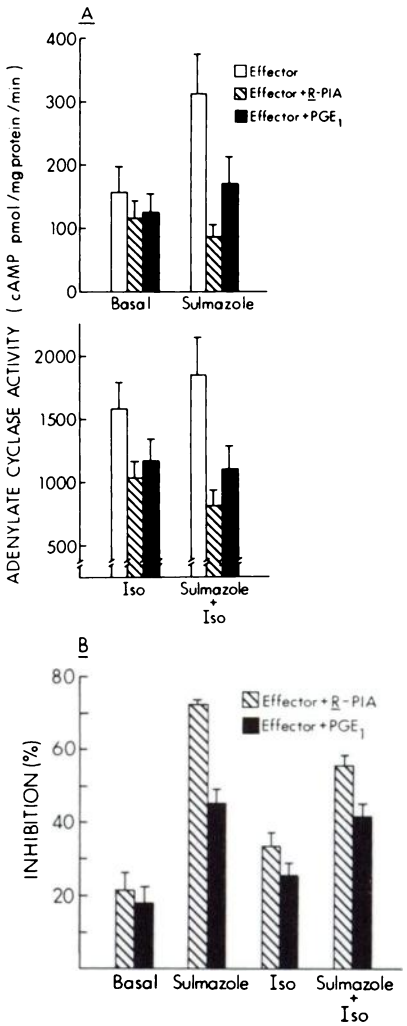

Fig. 4. Effects of inhibitory agonists on basal and effector-stimulated adenylate cyclase activities in rat adipocyte membranes. Data are presented as the mean ± standard error for six experiments. A. Sulmazole (3.3×10^{-3} M) is shown to stimulate adenylate cyclase activity above basal levels ($p < 0.005$). This effect is completely ablated by *R*-PIA (10^{-5} M) and is attenuated ~75% by PGE_1 (10^{-7} M). Sulmazole appeared to weakly potentiate maximal isoproterenol (10^{-5} M)-mediated stimulation ($p < 0.08$). Please note the broken *ordinate* scale. *Iso*, isoproterenol. B. Expressed as percentage inhibition, the effects of the inhibitory agonists *R*-PIA and PGE_1 on sulmazole-stimulated adenylate cyclase activity far exceed their effects on basal ($p < 0.002$) and isoproterenol-stimulated adenylate cyclase activities ($p < 0.03$).

used that produced 2- to 3-fold stimulation (like that seen with sulmazole), *R*-PIA still only inhibited 30–40% of that increase.

Thus, the nature of the stimulatory effect of sulmazole on adenylate cyclase activity is quite different from that of isoproterenol in that it is relatively modest in degree and weakly augments isoproterenol-mediated stimulation of G_s. The stimulatory effect is not blocked by a β-adrenergic receptor antagonist, and it is virtually abolished (reversed) by agonist-mediated activation of G_i using either *R*-PIA or PGE_1, which act via distinct membrane-bound receptors. Importantly, we have recently observed similar stimulatory properties among several other new cardiotonic agents. For example, in a representative adenylate cyclase experiment in which several cardiotonic agents were compared at 2 mM, sulmazole produced an 89.5% stimulation above basal, whereas the stimulation by amrinone, milrinone, and piroximone were 67%, 98% and 51%, respectively. Thus, these other new cardiotonic agents share certain of sulmazole's effects.

One mechanism that produces a modest stimulation of adenylate cyclase activity is the direct blockade of the inhibitory guanine nucleotide regulatory protein, G_i, using pertussis toxin (14). Accordingly, we reasoned that if the stimulatory effect of the new cardiotonic agent sulmazole is mediated by impairing the function (basal activity) of G_i, then the blockade of G_i by covalent modification of the α_i subunit with pertussis toxin should abolish sulmazole-mediated stimulation of adenylate cyclase activity. In Fig. 5, the adenylate cyclase activity in adipocyte membranes from control and pertussis-intoxicated rats was compared with and without sulmazole. Adequate pertussis intoxication is reflected in the inability of *R*-PIA to mediate inhibition as well as the relatively modest trend ($n = 4$, $p = 0.05$) in *R*-PIA-mediated stimulation of basal adenylate cyclase activity which is observed in pertussis-intoxicated membranes due to activation of stimulatory A_2 adenosine receptors, as previously reported (14). Importantly, although sulmazole mediates stimulation of adenylate cyclase activity in control membranes, it has no stimulatory effect above the basal level in pertussis-intoxicated membranes. Thus, we demonstrate that sulmazole's stimulatory effect on adenylate cyclase activity is abolished by pertussis intoxication, implying a role of G_i (or G_o) in mediating stimulation.

To further probe the effect of sulmazole on G_i-mediated inhibition of adenylate cyclase activity, GTP dose response curves were constructed with and without sulmazole (Fig. 6A). With increasing concentrations of GTP, there is a dose-dependent inhibition of adenylate cyclase activity in control membranes, as has been previously reported (33). In the presence of sulmazole, the GTP-dependent inhibition of adenylate cyclase activity is markedly attenuated. Of interest is the fact that, at lower concentrations of GTP, where there is no inhibition of adenylate cyclase in control membranes, there is also no enhancement of cAMP production in the presence of sulmazole. Thus, the stimulatory effects of sulmazole are observed only at "inhibitory" concentrations of GTP (concentrations of GTP sufficient to activate G_i).

Under identical assay conditions, *R*-PIA promotes prominent inhibition of adenylate cyclase activity at inhibitory concentrations of GTP (Fig. 6B). Importantly, the inclusion of the inhibitory agonist completely abolishes the ability of sulmazole to attenuate inhibition of adenylate cyclase activity. This suggests that sulmazole's effect on G_i is quite different from that of pertussis toxin which, as mentioned previously, irreversibly abolishes *R*-PIA-mediated inhibition of adenylate cyclase activity.

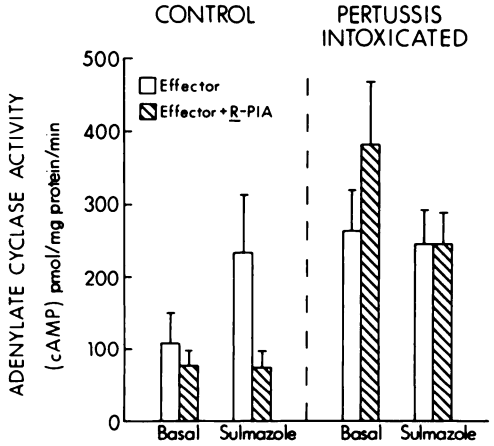

Fig. 5. Comparison of adenylate cyclase activities in control and pertussis toxin-treated rat adipocyte membranes. Adipocyte membranes were concurrently prepared from control and pertussis-intoxicated rats as described under Experimental Procedures. Data are presented as the mean ± standard error for four experiments. For the basal state the effector is water. As in Fig. 4, sulmazole (3.3×10^{-3} M) is shown to stimulate adenylate cyclase activity above basal levels in control membranes. However, in membranes from pertussis-intoxicated rats, sulmazole has no stimulatory effect on adenylate cyclase activity. *R*-PIA was included as indicated at a concentration of 10^{-5} M.

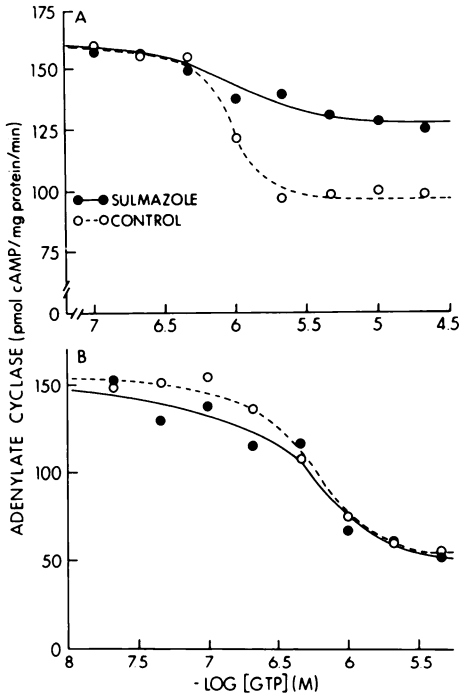

Fig. 6. Effect of sulmazole on inhibition of adenylate cyclase activity in rat adipocyte membranes. A. GTP dose response curve. GTP was included at the concentrations indicated on the *abscissa*. Sulmazole was present at a concentration of 3.3×10^{-3} M as indicated. This representative experiment was replicated five times. B. GTP dose response curve in the presence of *R*-PIA. GTP was included at the concentrations indicated on the *abscissa*. *R*-PIA was also included at a concentration of 3.3×10^{-6} M. Sulmazole was present at 3.3×10^{-3} M as indicated. This representative experiment was replicated three times. Please note the different *ordinate* and *abscissa* scales compared to A. All data points are means of duplicate determinations.

Finally, to demonstrate the specificity of the effect of sulmazole on G proteins, "purified" G proteins were reconstituted into pertussis-intoxicated adipocyte membranes to restore sulmazole-mediated activation of adenylate cyclase. Pertussis toxin treatment enhances basal cAMP formation by approximately 2-fold and abolishes both the inhibitory effect of *R*-PIA and the stimulatory effect of sulmazole as described above. Separate aliquots of membranes were then reconstituted with either buffer alone, a purified preparation of G_i (85% pure), or a purified (95%) mixture of G_i/G_o (~45:55). In control membranes (not pertussis intoxicated) sulmazole produced a dose-dependent (~35% over basal) stimulation of adenylate cyclase activity (Fig. 7). In pertussis-intoxicated membranes which were reconstituted with buffer alone, sulmazole had no stimulatory effect. However, reconstitution of pertussis-intoxicated membranes with G_i or a G_i/G_o mixture restored the dose-dependent stimulatory effect by sulmazole (see Fig. 7). Of note, in "treated" membranes reconstituted with G_i or G_i/G_o and assayed with 5×10^{-6} M GTP, there was pronounced inhibition of basal adenylate cyclase activity (~50%) compared to "treated" membranes reconstituted with buffer alone. In addition, the basal activity of the reconstituted membranes was similar to the basal activity of control (nonintoxicated) membranes. This implies that G_i was reinserted into the membranes in a functional manner. It can also be appreciated from Fig. 7 that concentrations of sulmazole as low as 2×10^{-4} M produce significant stimulation in both control and reconstituted membranes.

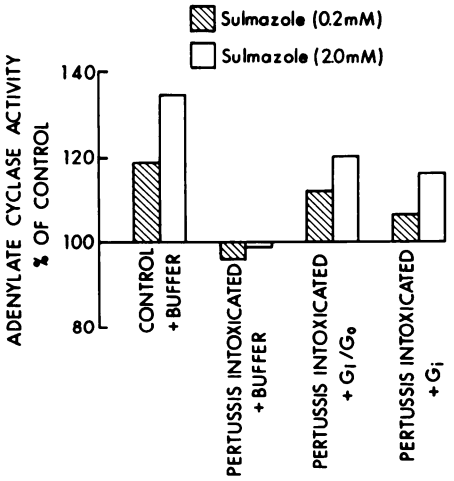

Fig. 7. Reconstitution of sulmazole-stimulated adenylate cyclase activity in pertussis-intoxicated rat adipocyte membranes. Adipocyte membranes were prepared from control and pertussis-intoxicated rats and reconstituted with buffer (control and pertussis-intoxicated membranes) or purified G proteins (either a G_i/G_o mixture for G_i), as described under Experimental Procedures. Adenylate cyclase activity was performed as described under Experimental Procedures. The final concentration of pertinent additives includes GTP (5 μM), papaverine (100 μM), creatine phosphokinase (2.0 μg/ml), creatine phosphate (10 mM), dATP (0.3 mM), [^{32}P]ATP (1 μCi/tube), and adenosine deaminase (4 units/ml), in the absence and presence of 0.2 and 2.0 mM sulmazole. Data are presented as percentage of stimulation above that obtained in the absence of sulmazole (100%) for each group. This figure is a representative experiment from the following data: percentage stimulation obtained by 0.2 mM sulmazole was 117 ± 2 (control plus buffer) and 99 ± 5 (pertussis toxin plus buffer), $n = 3$, $p < 0.007$. Reconstitution of G_i/G_o to pertussis-intoxicated membranes permitted 112.1 ± 1% stimulation by 0.2 mM sulmazole ($n = 3$, $p = 0.03$). Percentage stimulation obtained by 2 mM sulmazole was 142 ± 13 (control plus buffer) and 104 ± 7 (pertussis toxin plus buffer), $n = 3$, $p < 0.03$. Reconstitution of G_i/G_o to pertussis-intoxicated membranes permitted 119 ± 5% stimulation by 2 mM sulmazole ($n = 5$, $p < 0.001$). Reconstitution of G_i to pertussis-intoxicated membranes permitted 114 ± 4% stimulation by 2 mM sulmazole ($n = 4$, $p = 0.02$).

Discussion

In rat adipocyte membranes, the new cardiotonic agent sulmazole induces a marked increase in cAMP accumulation. Although this effect is clearly contributed to by sulmazole's capacity to inhibit the low K_m cAMP phosphodiesterase, the inotropic agent also appears to block the function of the inhibitory guanine nucleotide regulatory protein, G_i, thereby increasing cAMP production.

We demonstrate in Fig. 1 that, although the new cardiotonic agent sulmazole and the nonselective phosphodiesterase inhibitor papaverine each have potent stimulatory effects on cAMP accumulation in rat adipocyte membranes, the degree of stimulation is markedly greater in the former case. The majority of the new inotropic agents, including sulmazole, are believed to mediate an increase in cAMP accumulation in part by selectively blocking a low K_m cAMP phosphodiesterase (1, 4). Since papaverine has also been shown to maximally inhibit this enzyme and other associated phosphodiesterases at the concentration employed in the present study (8, 17), another mechanism must be considered to account for a major portion of sulmazole's stimulatory action.

One possible mechanism by which an inotropic agent might increase cAMP accumulation is via blockade of receptor-mediated inhibition of the enzyme adenylate cyclase. We demonstrate in Fig. 2 that sulmazole and its congener UD-CG 212 CL interact with inhibitory A_1ARs with high affinity and in a reversible manner. Furthermore, since sulmazole induces a marked rightward shift in *R*-PIA-mediated inhibition of adenylate cyclase activity (Fig. 3), it may be considered an antagonist of A_1ARs. Yet, it is unlikely that A_1AR antagonism could produce the observed increase in adenylate cyclase activity (Fig. 4A), since adenosine deaminase was used in all assays to eliminate endogenous adenosine and dATP was utilized to prevent accumulation of adenosine (deoxyadenosine is not an A_1AR agonist). Thus, the blockade by sulmazole of adenosine-mediated inhibition of adenylate cyclase activity is unlikely in our *in vitro* system. However, this does not preclude the possibility that a portion of sulmazole's effect on cAMP accumulation *in vivo* could be attributed to A_1AR antagonism, since adenosine would of course be present *in vivo*. It is less likely that the other cardiotonic agents examined (Fig. 2), which do not have methylxanthine-like structures, mediate their effects on cAMP accumulation via A_1AR antagonism in light of their low affinities for the receptor (9).

In further support of the assertion that sulmazole's stimulatory effect on adenylate cyclase is not mediated exclusively via A_1AR blockade, we demonstrate in Fig. 4A that sulmazole's effect can be virtually abolished with PGF_1, which mediates inhibition of adenylate cyclase in adipocytes via a separate receptor system (34). In addition, sulmazole does not shift the PGE_1 dose response curve for inhibition of adenylate cyclase, suggesting that antagonism of PGE_1 receptors is unlikely (data not shown). The mechanism of sulmazole-induced stimulation of adenylate cyclase activity is thus not likely related to blockade of two separate receptors, but rather a common converging component.

In Fig. 5, pertussis intoxication is shown to abolish sulmazole's stimulatory effect on adenylate cyclase. Similarly, the stimulatory effects of amrinone, milrinone, and piroximone are attenuated by pertussis intoxication (data not shown). This ablation by pertussis toxin is important in elucidating the mechanism of action of agents. The majority of commonly used effectors mediating stimulation of adenylate cyclase (i.e., isoproterenol via β-adrenergic receptors, sodium fluoride via the stimulatory regulator G_s, and the diterpine forskolin principally via direct activation of the catalytic unit) are not blocked by pertussis intoxication (35). It is, therefore, unlikely that sulmazole and the other mentioned cardiotonic agents share their stimulatory mechanism of action with any of these effectors.

It is known that pertussis toxin-catalyzed ADP ribosylation of the α_i subunit of G_i directly impairs the function of G_i and "uncouples" receptor-mediated inhibition of adenylate cyclase (35). Since pertussis intoxication abolishes sulmazole's stimulatory effect on adenylate cyclase, the mechanism of action is apparently dependent upon G_i. Furthermore, since sulmazole's G_i-dependent mechanism mediates stimulation, rather than inhibition of adenylate cyclase, functional blockade of G_i is likely. As expected for a G_i-mediated process, the stimulatory effect of sulmazole not only is GTP dependent but requires GTP concentrations high enough to activate G_i (Fig. 6A). In addition, reconstitution of G_i or a mixture of G_i/G_o into pertussis-intoxicated membranes results in the restoration of sulmazole's capacity to stimulate adenylate cyclase activity (Fig. 7). These data suggest that sulmazole may have a direct effect on G proteins and that its stimulatory effect in the absence of endogenous adenosine is likely related to inhibition of G_i function. Finally, since this G_i-blocking effect is completely overcome by the addition of an inhibitory agonist such as *R*-PIA or PGE_1, a reversible (noncovalent) form of G_i blockade is likely. Thus, we propose a novel molecular mechanism by which the cardiotonic agent sulmazole stimulates adenylate cyclase activity. What contribution each of the effects of sulmazole (phosphodiesterase inhibition, A_1AR antagonism, and G_i blockade) has on producing inotropy remains to be determined. *In vitro*, the IC_{50} for inhibition of A_i binding is ~78 μM, and the IC_{50} for inhibition of phosphodiesterase is ~289 μM (37); the IC_{50} for blockade of G_i is difficult to determine, but effects are seen at 200 μM (see Fig. 7), while positive inotropic effects occur at sulmazole concentrations of ~500 μM (36). *In vivo* serum levels of sulmazole have been reported in the range of 10 μM and at these concentrations there is a positive inotropic effect (37). Since this drug concentration is considerably lower than is required to demonstrate any of the proposed *in vitro* mechanisms, it is difficult to be sure which mechanism plays the major role in producing the inotropic effect.

The seemingly paradoxic capacity of sulmazole to inhibit the activity of G_i and yet, in turn, to be totally overcome by inhibitory receptor agonists may, in part, be understood in light of the current model for adenylate cyclase regulation. There is much evidence that G_i can suppress the activity of G_s and the catalytic unit of adenylate cyclase under basal conditions (38). The basal activity of G_i, in the absence of agonists, is known to involve a relatively slow displacement of GDP by GTP. This slow displacement by GTP (on G_i) may be functionally blocked by sulmazole. If so, the sulmazole-mediated release of tonic inhibition would permit adenylate cyclase activity to increase, as we have observed in the present study. With the addition of an inhibitory agonist such as *R*-PIA, the activation of G_i is fully facilitated (23). Based on our data, this agonist-mediated activation of G_i appears to dramatically overcome the reversible blockade of basal G_i function induced by sulmazole. Thus, stimulation of adenylate cyclase activity by sulmazole may be completely ablated by inhibitory receptor agonists. To our knowledge this is the first demonstration of a drug which inhibits G_i activity and yet is completely reversible. Other agents have recently been shown to attenuate tonic inhibition of adenylate cyclase activity, including phorbol esters and divalent cations such as Mg^{2+} and Mn^{2+} (39, 40). However, in contrast to the new inotropic agents, the blocking effects of these agents are not fully reversed by inhibitory agonists (39, 40). Since there is considerable variation in the concentration of the endogenous inhibitory agonist adenosine *in vivo*, depending on the specific tissue and the presence or absence of ischemia, etc., it is reasonable to propose that inhibition of G_i may play a significant mechanistic role in those regions where adenosine levels are low. Conversely, it is possible that when tissue adenosine levels are high, the capacity of sulmazole to block A_1ARs may be mechanistically more important.

In conclusion, the new cardiotonic agent sulmazole appears to stimulate cAMP accumulation by at least three mechanisms: 1) phosphodiesterase inhibition, 2) A_1AR antagonism, and 3) a novel mechanism mediating stimulation of adenylate cyclase by functionally blocking the basal activity of G_i. The extent to which each of these mechanisms relate to the inotropic effects of sulmazole remains to be determined. Clearly, the fact that sulmazole reversibly blocks the functionality of the G_i protein makes it an important biochemical tool. Furthermore, based on preliminary data from our laboratory, G_i blockade may not be unique to sulmazole. However, the extension of this mechanism to other structurally diverse cardiotonic agents will await further study.

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