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Isobutylmethylxanthine Stimulates Adenylate Cyclase by Blocking the Inhibitory Regulatory Protein, G_i

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SUMMARY

The methylxanthines, such as caffeine and theophylline, are an important and widely used class of drugs, which are believed to mediate many of their physiological effects by increasing intracellular concentrations of cAMP. These agents are known to inhibit phosphodiesterases and to block inhibitory A_1 adenosine receptors in a competitive manner. Thus, the methylxanthines may increase cAMP accumulation by slowing its inactivation or by enhancing its production. Using a rat adipocyte membrane model we demonstrate that isobutylmethylxanthine (IBMX) induces a dose-dependent 34% increase in cAMP production above that produced by complete phosphodiesterase inhibition with papaverine. This stimulatory effect is dependent upon the inhibitory guanine nucleotide regulatory protein G_i, in that inactivation of G_i by pertussis intoxication ablates IBMX-mediated stimulation of adenylate cyclase activity. Because the G_i-dependent effect of IBMX results in increased cAMP production, the mode of action is likely blockade of G_i activity. Accordingly, the capacity of GTP itself to inhibit adenylate cyclase activity is attenuated by IBMX. In contrast to G_i blockade induced by pertussis toxin, this heretofore unappreciated stimulatory mechanism is completely reversed by inhibitory receptor agonists. This mechanism of action may be responsible for certain physiological effects of methylxanthines, which are not easily explained by phosphodiesterase inhibition or antagonism of A_1 adenosine receptors.

For nearly three decades the capacity of the methylxanthines to increase intracellular cAMP has been mechanistically correlated with their inhibitory effect on cAMP phosphodiesterases (1, 2). More recently these agents have been shown to antagonize membrane-bound adenosine receptors in a competitive manner. Accordingly, it has been proposed that certain of the physiological effects of the methylxanthines are mediated via antagonism of inhibitory A_1 and stimulatory A_2 adenosine receptors (3–6). However, the relevance of these biochemical effects to *in vivo* pharmacology is not yet clear (2–4, 7). Furthermore, certain physiolbgical effects, such as bronchodiliatation, may not be related to phosphodiesterase inhibition or antagonism of A_1 adenosine receptors (8, 9).

In this report we demonstrate that the methylxanthine isobutylmethylxanthine directly stimulates adenylate cyclase activity by blocking the function of the inhibitory guanine nucleotide regulatory protein, G_i. G_i blockade may be mechanistically important in understanding the biochemical and physiological effects of isobutylmethylxanthine.

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Experimental Procedures

Materials. Male Sprague-Dawley rats (250–300 g) were from Charles River. Adenosine deaminase, cAMP, GTP, cGMP, ATP, dATP, creatine phosphokinase, papaverine, theophylline, and caffeine were from Sigma Chemical Co. (St. Louis, MO). IBMX was from Calbiochem (La Jolla, CA). Creatine phosphate and *R*-PIA were from Boehringer Mannheim (Indianapolis, IN). [2,8-^{3}H]cAMP (40 Ci/mmol) and [α-^{32}P]ATP (27 Ci/mmol) were from New England Nuclear (Boston, MA). Crude collagenase was from Cooper Biomedical (Malvern, PA). Bovine serum albumin (fraction V) was from Armour (Tuckahoe, NY) RO-20-1724 was a gift of Dr. Peter F. Sorter at Hoffman-LaRoche (Nutley, NJ). Rolipram was a gift of Dr. John W. Daly at the National Institutes of Health, Bethesda, MD. 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 (~100 opacity units/300 g) was given intraperitoneally to rats, when indicated, 3 days before sacrifice by decapitation. Adipocyte membranes were prepared from epididymal fat pads as previously described (10, 11) with the following modifications: the crude collagenase concentration was 1.0 mg/ml, and leupeptin and soybean trypsin inhibitor were omitted from the hypotonic buffer. The initial membrane pellet was suspended at 1 mg of protein/ml in 75 mM Tris (pH 7.5 at 30°), 12.5 mM $MgCl_2$, 200 mM NaCl, 2.5 mM dithiothreitol, and 4.0 U/ml adenosine deaminase and preincubated at 30° for 10 min. Membranes were prepared concurrently from control and pertussis treated rats, as indicated, and were used

ABBREVIATIONS: IBMX, isobutylmethylxanthine; G_i, inhibitory guanine nucleotide regulatory protein; G_s, stimulatory guanine nucleotide regulatory protein; α_i, α subunit of G_i; RO-20-1724, DL-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinine; *R*-PIA, (−)-N^6-(*R*-phenylisopropyl) adenosine.

immediately. Protein concentrations were determined by the method of Bradford (12) using bovine serum albumin as standard.

Assay for the low K_m cAMP phosphodiesterase. Assays were performed according to the method described by DeMazancourt and Guidicelli (13) for rat adipocytes. The reaction mixture contained 0.02 μM cyclic [^{3}H]AMP, 0.5 μM cAMP, 0.04% bovine serum albumin, and 0.5 units/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 phosphodiesterase inhibitors. Activation of the low K_m cyclic AMP 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 and 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 of H_2O each 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.

Adenylate cyclase assays. Assays were performed using rat adipocyte membranes as previously described (10, 11). Papaverine (10^{-4} M) was substituted for RO-20-1724 to provide adequate phosphodiesterase inhibition, unless otherwise noted. GTP (5×10^{-6} M) was used except for GTP dose-response experiments, as indicated. cAMP was isolated according to the method of Salomon *et al.* (14).

Data analysis. Dose-response curves of adenylate cyclase activity were analyzed according to a four-parameter logistic equation to determine EC_{50} values, also allowing determination of statistical differences between curves (15, 16).

Results

In Fig. 1A, a representative dose response curve illustrates the striking effect of IBMX on cAMP production. An analogous dose-response curve is shown for the potent phosphodiesterase inhibitor papaverine. Although both agents mediate saturable stimulation at 10^{-4} M, IBMX induces greater cAMP production ($p < 0.001$) than does papaverine, especially at concentrations greater than 10^{-5} M. This discrepancy in maximal stimulation is particularly striking because it is known that these agents are essentially equipotent and equally effective in their capacity to inhibit phosphodiesterases in several purified preparations (2, 17). We confirmed this in our rat adipocyte membrane model by measuring the inhibition of cAMP hydrolysis. IBMX (10^{-4} M) was found to completely inhibit cAMP hydrolysis with an IC_{50} of 3.4 ± 0.5 μM (three experiments). Similarly, papaverine (10^{-4} M) was found to inhibit cAMP hydrolysis completely with an IC_{50} of 2.0 ± 0.3 μM (three experiments). The addition of 10^{-4} M IBMX to 10^{-4} M papaverine neither increased nor decreased the capacity of papaverine to inhibit phosphodiesterase activity (Fig. 1B). In addition, IBMX promotes greater cAMP production in our system than do other commonly used phosphodiesterase inhibitors such as RO-20-1724, rolipram, and cGMP (data not shown). Thus, in rat adipocyte membranes the additional stimulatory effect of IBMX is not likely due to more complete phosphodiesterase inhibition. Furthermore, this effect is not likely due to antagonism of A_1 adenosine receptors, because all endogenous adenosine was removed by inclusion of adenosine deaminase in the assays. Therefore, the blockade by IBMX of adenosine-mediated inhibition would be unlikely in this *in vitro* system. Dose response curves with adenosine

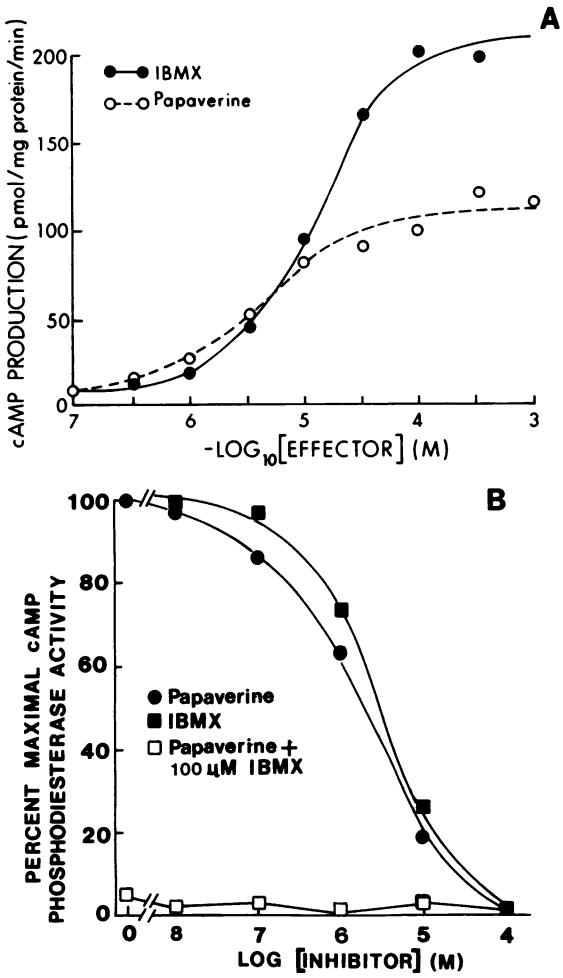

Fig. 1. A, Effector-mediated stimulation of cAMP accumulation in rat adipocyte membranes. Representative dose-response curves for IBMX and papaverine are shown. This experiment was replicated four times. B, Inhibition of the low K_m cAMP phosphodiesterase activity in adipocyte membranes by papaverine and IBMX. Activation of the phosphodiesterase was initiated by the addition of ~25 μg of membrane protein to the incubation mixture (see Materials and Methods) containing 0.02 μM cyclic [^{3}H]AMP for 7.5 min at 30° in the absence and presence of the indicated concentrations of drugs. Activity in the absence of inhibitors was 19.2 pmol of cAMP/min/mg of protein. This is a representative experiment with the different drugs tested under identical experimental conditions. Similar findings were observed in three independent experiments whose standard errors of the data points were less than 5% of the means.

deaminase revealed no change in cAMP generation when higher concentrations of adenosine deaminase were used (data not shown). Accordingly, another mechanism must be sought to account for a substantial portion of the stimulatory action of IBMX.

Recent investigation concerning the transduction of information across cell membranes has led to the recognition of guanine nucleotide regulatory proteins (G proteins) as important intermediaries between cell surface receptors and effector molecules such as adenylate cyclase (18). In this context, an increase in cAMP production could potentially be induced by agents that activate G_S or that block the function of G_i. Pertinently, pertussis toxin, phorbol esters, and divalent cations such as Mn^{2+} have been shown to mediate stimulation of adenylate cyclase activity by blocking G_i function (19–21). Given this precedent in the literature, we questioned whether a portion of the stimulatory effect of IBMX could be due to

blockade of G_i. Accordingly, we reasoned that if this were the case then inactivation of G_i by covalent modification of the α_i subunit with pertussis toxin should ablate methylxanthine-induced stimulation of adenylate cyclase activity. In Fig. 2, the adenylate cyclase activity of rat adipocyte membranes from control and pertussis-intoxicated rats were compared, with and without IBMX. Papavarine (10^{-4} M) was included in all adenylate cyclase assays to block phosphodiesterase activity. In control membranes, IBMX mediates 34% stimulation over basal adenylate cyclase activity. Similarly, IBMX potentiates maximal isoproterenol (10^{-4} M)-mediated stimulation by 22%. This effect is also demonstrable using caffeine or theophylline and is not blocked by the β-adrenergic antagonist propronolol (data not shown). In striking contrast, IBMX does not enhance basal or isoproterenol-stimulated adenylate cyclase levels in pertussis-intoxicated membranes. Although not shown, it should be noted that the A_1 adenosine receptor agonist *R*-PIA (3.3×10^{-6} M) is no longer capable of inhibiting adenylate cyclase activity after pertussis toxin treatment, reflecting complete intoxication (19). Thus, we demonstrate that stimulation of adenylate cyclase activity by IBMX is abolished by pertussis intoxication. In contrast, other stimulatory effectors such as isoproterenol, sodium fluoride, or forskolin are not blocked by pertussis intoxication (22). This implies that the stimulatory effect of IBMX is dependent upon a pertussis toxin substrate, which is likely G_i. Further, because IBMX mediates stimulation rather than inhibition of adenylate cyclase activity, functional blockade of G_i is probable.

We next examined the effect of inhibitory agonists on IBMX-mediated blockade of G_i. Whereas in the absence of inhibitory agonists IBMX stimulates adenylate cyclase activity (Fig. 2), micromolar concentrations of the A_1 adenosine receptor agonist *R*-PIA inhibit control and IBMX-potentiated adenylate cyclase activities to essentially the same level (Fig. 3). We have previously demonstrated (23) that another inhibitory receptor agonist, prostaglandin E_1, blocks the stimulatory effects of the methylxanthine-like agent sulmazole. Similar blockade of IBMX-mediated stimulation may be induced using 10^{-7} M prostaglandin E_1 (data not shown). Otherwise stated, the stimulatory effect of IBMX-mediated blockade of G_i is ablated by the inhibitory agonist *R*-PIA or prostaglandin E_1. Because IBMX is also an A_1 adenosine receptor antagonist (24), it promotes a ~23-fold rightward IC_{50} shift in *R*-PIA-induced inhibition of adenylate cyclase activity (from 3 to 77 nM, $p = 0.04$, three experiments).

To further support a blocking effect of IBMX on G_i-mediated inhibition of adenylate cyclase activity, GTP dose-response curves were constructed with and without IBMX. As previously reported (25) there is a dose-dependent inhibition of adenylate cyclase activity in control membranes with increasing concentrations of GTP (Fig. 4A). However, in the presence of IBMX, this GTP-dependent inhibition is attenuated. Of note, it is only at "inhibitory" concentrations of GTP ($>5 \times 10^{-7}$ M), at which G_i is being activated (25, 26), that IBMX enhances adenylate cyclase activity relative to basal levels. Thus, as expected for a G_i-mediated process, the stimulatory effect of IBMX demonstrated in Fig. 2 is not only GTP dependent but requires GTP concentrations that are high enough to activate G_i. The finding that IBMX attenuates rather than ablates GTP inhibition is also compatible with our data in Fig. 2, wherein IBMX is shown

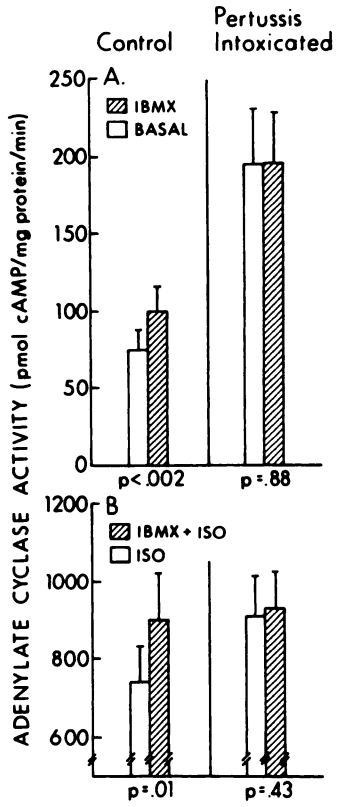

Fig. 2. Adenylate cyclase activities in control and pertussis toxin-treated rat adipocyte membranes. Adipocyte membranes were concurrently prepared from control and pertussis-intoxicated rats and assays were performed as described in Materials and Methods. Papaverine (10^{-4} M) was included to block phosphodiesterase activity. Data are presented as mean ± standard error for eight experiments. For the basal state the effector is water. IBMX (10^{-4} M) and isoproterenol (*ISO*, 10^{-4} M) were included as noted.

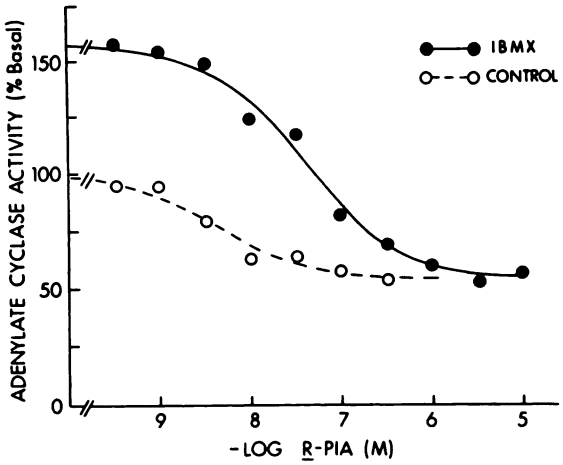

Fig. 3. *R*-PIA-mediated inhibition of IBMX-stimulated adenylate cyclase activity. Rat adipocyte membranes were prepared and assays were performed as described in Materials and Methods. Papaverine (10^{-4} M) was included to block phosphodiesterase activity. The stimulatory effect of IBMX (10^{-4} M) is ablated completely 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. Within each experiment the triplicate determinations were within 5% of each other. The standard error of the data points between experiments was 8% or less. Basal adenylate cyclase activity was 75.8 pmol of cAMP/mg of protein/min.

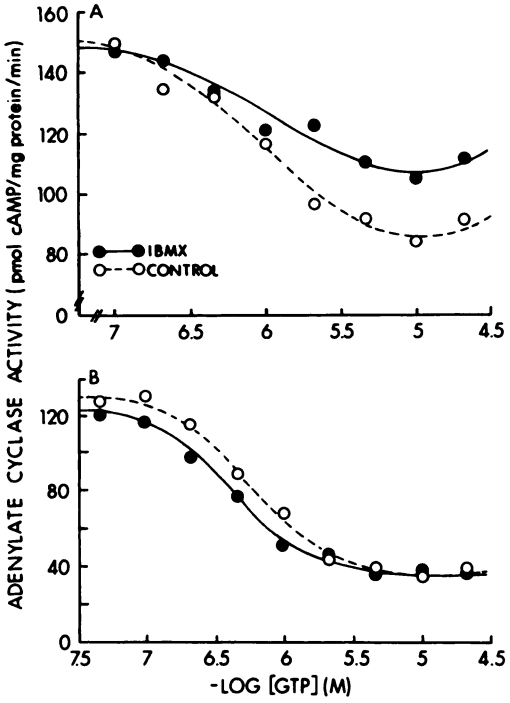

Fig. 4. Effect of IBMX on inhibition of adenylate cyclase activity in rat adipocyte membranes. Membranes were prepared and assays were performed as described in Materials and Methods. However, the final GTP concentration was varied as indicated on the *abscissa*. Papaverine (10^{-4} M) was included to block phosphodiesterase activity. Please note the different *ordinate* and *abscissa* scales in A and B. All data points are means of duplicate determinations. A, GTP dose response curve. IBMX (10^{-4} M) was included as indicated. This representative experiment was replicated four times. The variation of the data points among the different experiments was less than 10%. B, GTP dose-response curve in presence of *R*-PIA. IBMX (10^{-4} M) was included as indicated. *R*-PIA (3.3×10^{-6} M) was also included. This representative experiment was replicated three times. The variation of the data points among the different experiments was less than 10%.

to increase cAMP but not to the level induced by complete G_i blockade with pertussis toxin.

To confirm that inhibitory agonists ablate the methylxanthine-induced blockade of G_i (see Fig. 3), GTP dose-response curves were constructed with and without *R*-PIA. Importantly, the capacity of IBMX to attenuate inhibition of adenylate cyclase activity at GTP concentrations sufficient to activate G_i (Fig. 4A) is abolished by *R*-PIA (Fig. 4B). Thus, the G_i-blocking effect of IBMX is reversed completely by the inhibitory agonist *R*-PIA.

Discussion

The methylxanthines are an important class of drugs, which are known to increase cAMP production in many systems, including rat adipocyte membranes (25). Although these agents have been shown to block phosphodiesterases and act as A_1 adenosine receptor antagonists, we report herein that the methylxanthine IBMX also increases cAMP production (Fig. 1A) by blocking the function of G_i.

It is known that pertussis toxin-catalyzed ADP ribosylation of the α_i subunit of G_i blocks the function of G_i and uncouples receptor-mediated inhibition of adenylate cyclase (19, 26). Because the stimulatory effect of IBMX on adenylate cyclase is abolished by pertussis intoxication (Fig. 2), the mechanism of action of the methylxanthine is apparently dependent upon G_i. Although pertussis toxin can promote the ADP ribosylation of other proteins such as G_o, there is currently no evidence that these proteins play an important role in the regulation of adenylate cyclase activity in intact membranes. Further, because IBMX produces stimulation rather than inhibition of adenylate cyclase, functional blockade of G_i is likely. As would be expected for a G_i-mediated process (20), the stimulatory effect of IBMX is GTP dependent and is only observed at GTP concentrations high enough to activate G_i (Fig. 4A). Thus, IBMX attenuates basal GTP-mediated inhibition of adenylate cyclase. Accordingly, it is by withdrawing GTP-dependent inhibition that IBMX induces stimulation of adenylate cyclase activity.

In further characterizing this mechanism we demonstrate in Fig. 3 that the G_i-blocking effect of IBMX is overcome completely by the addition of the inhibitory agonist *R*-PIA. This effect is not specific for A_1 adenosine receptors inasmuch as prostaglandin E_1 also overcomes IBMX-mediated stimulation, but via distinct inhibitory receptors (data not shown). Therefore, it appears that agonist-mediated activation of G_i reverses the stimulatory action of the methylxanthine. Accordingly, the effect of IBMX on G_i is quite distinct from that of other agents known to block G_i, such as pertussis toxin and phorbol esters, inasmuch as the G_i-blocking action of these agents is not ablated by inhibitory agonists (19, 21). Thus, we demonstrate that IBMX attenuates the basal function of G_i, resulting in stimulation of adenylate cyclase activity by a heretofore unappreciated mechanism. Of note, this stimulatory effect is completely reversed by inhibitory agonists.

At a molecular level, it is known that G_i under basal conditions is capable of suppressing the activity of G_S and the catalytic unit of adenylate cyclase (27). Based on the data presented, the basal activity of G_i, which involves a slow displacement of GDP by GTP and subsequent dissociation of the α and $\beta\gamma$ subunits of G_i, is likely attenuated by IBMX. The precise step in the activation of G_i that is attenuated by IBMX remains to be determined. Inhibitory agonists, such as *R*-PIA, that facilitate activation of G_i (28) would therefore be expected to overcome a reversible blockade of basal G_i activity. Thus, the paradoxical capacity of IBMX to block G_i and yet for this blockade to be totally overcome by inhibitory receptor agonists may, in part, be understood in light of the current model of the adenylate cyclase system.

Initially, the idea that the methylxanthine IBMX should have important effects on both adenosine receptors and G proteins seemed perplexing, considering the marked specificity of hormone-receptor and ligand-G protein interactions. However, because adenosine and guanosine are both purines that share a methylxanthine-like ring structure, the capacity of IBMX to interact at both adenosine receptors and G proteins is not surprising. This is corroborated by our finding that caffeine and theophylline produce similar effects in rat adipocyte membranes. The capacity of IBMX to block stimulatory A_2 adenosine receptors would be expected to lead to a decrease rather than an increase in cAMP production. Accordingly, A_2 adenosine receptor antagonism would not explain the stimulatory effect of IBMX in this system (Fig. 1A).

The potential importance of methylxanthine-induced G_i blockade may be substantial, given the broad use of this class of agents *in vivo* for their stimulant and bronchodilator effects

and the fact that phosphodiesterase inhibition does not adequately explain their mode of action (8, 9). In addition, methylxanthines are widely used *in vitro* as biochemical tools. For example, IBMX (10^{-4} M) has, for over a decade, been routinely used to block phosphodiesterases in the study of stimulatory and inhibitory adenylate cyclase-coupled receptor systems (25). These studies may need to be reinterpreted in light of the blocking effect of IBMX at G_i, as this may increase adenylate cyclase activity or otherwise alter the functionality of G_i and thus change experimental conclusions. Finally, we have very recently shown that direct blockade of basal G_i function is not unique to the methylxanthines. Rather, they are prototypical of an expanding and diverse group of "G_i blockers," which includes certain new inotropic agents with methylxanthine-like structures used to treat congestive heart failure (23). We are currently probing the molecular mechanism(s) by which these agents inhibit G_i function.

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