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ACCELERATED COMMUNICATION

Chronic Caffeine or Theophylline Exposure Reduces γ-Aminobutyric Acid/Benzodiazepine Receptor Site Interactions

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SUMMARY

Methylxanthines, such as caffeine and theophylline, are adenosine receptor antagonists that exert dramatic effects upon the behavior of vertebrate animals by increasing attentiveness, anxiety, and convulsive activity. Benzodiazepines, such as flunitrazepam, generally exert behavioral effects that are opposite to those of methylxanthines. We report the finding that chronic exposure of embryonic brain neurons to caffeine or theophylline reduces the ability of γ-aminobutyric acid (GABA) to potentiate the binding of [^{3}H]flunitrazepam to the GABA/benzodiazepine receptor. This theophylline-induced "uncoupling" of GABA- and benzodiazepine-binding site allosteric interactions is blocked by chloroadenosine, an adenosine receptor agonist, indicating that the chronic effects of theophylline are mediated by a site that resembles an adenosine receptor. We speculate that adverse central nervous system effects of long-term exposure to methylxanthines such as in caffeine-containing beverages or theophylline-containing medications may be exerted by a cell-mediated modification of the $GABA_A$ receptor.

Methylxanthines, such as caffeine and theophylline, exert dramatic effects upon the behavior of vertebrate animals by increasing attentiveness, anxiety, and convulsive activity (1). In contrast, benzodiazepines, such as diazepam and flunitrazepam, have behavioral effects that are opposite to those of methylxanthines. While it is thought that methylxanthines exert these diverse actions by inhibiting adenosine binding to adenosine receptors (2, 3), methylxanthines, albeit at relatively high concentrations, have been shown to inhibit benzodiazepine binding *in vitro* (4), to antagonize several central effects of diazepam (5), and to alter the activity of the inhibitory neurotransmitter GABA (6).

Benzodiazepines enhance GABA action by binding to a site that is allosterically coupled to the GABA receptor (7, 8). Biochemical and electrophysiological studies indicate that benzodiazepines increase the affinity with which GABA binds to its receptor (9, 10) and increase the potency of GABA as an activator of chloride conductance (7, 8, 11–13). Similarly, as expected for allosterically coupled sites, GABA increases the affinity of benzodiazepines for the benzodiazepine-sensitive modulator site of the GABA receptor complex (14, 15).

Chronic use of methylxanthines and benzodiazepines is prevalent. Long-term exposure to caffeine and theophylline is common in coffee drinkers and asthmatics, respectively, and long-term exposure to benzodiazepines is common during treatment for anxiety. Chronic exposure to benzodiazepines has been shown to have a variety of effects on the GABA/benzodiazepine receptor complex (16). In the present study, we have examined the effects of chronic methylxanthine treatment on the modulatory interactions of the GABA/benzodiazepine receptor of embryonic chick brain neurons in cell culture (17).

Materials and Methods

[^{3}H]Flunitrazepam (85 Ci/mmol) and [^{35}S]methionine (1000 Ci/mmol) were from Amersham. Chemicals were obtained from commercial sources. The benzodiazepine flurazepam was a gift of Hoffman-La Roche. Dialysis tubing (15,000 molecular weight cutoff, Spectrapor) was prepared by rinsing with deionized water. Radioactivity was determined by scintillation counting using Liquiscint (National Diagnostics).

Tissue preparation. Brain cell cultures and membrane homogenates derived from 20-day chick embryos (20 day P_2) were prepared as previously described (18). To prepare cell homogenate, 7-day cell cultures were washed once with ice-cold PBSS (17), scraped, and centrifuged (1000 rpm, 5 min), and the pellet was resuspended in either EDTA (1 mM)/PMSF (1 mM), 0.5 ml/100-mm culture dish (for dialysis), or PBSS, 1.25 ml/100-mm culture dish (for immediate use).

To remove endogenous GABA and/or drugs added during treatment,

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ABBREVIATIONS: GABA, γ-aminobutyric acid; PBSS, phosphate-buffered salt solution; EDTA, ethylenediaminetetraacetate; TCA, trichloroacetic acid; PMSF, phenylmethylsulfonyl fluoride.

membrane and cell homogenates were dialyzed. Prior to dialysis, all tissue preparations were suspended in EDTA (1 mM) and PMSF (1 mM), to a final protein concentration of 5 mg/ml. The homogenate was then dialyzed against 25 mM potassium phosphate buffer (pH 7.0, 4°) for 24 hr with four changes of buffer yielding an effective dilution of 3600. Following dialysis, the homogenate was diluted with PBSS to a final concentration of 1.5 mg protein/ml and used immediately.

Reversible binding. Samples (150 μg of protein) of membrane or cell homogenate, [^{3}H]flunitrazepam, and unlabeled drugs were combined in a total volume of 1 ml. The reaction mixture was incubated on ice for 1 hr and filtered rapidly over Whatman GF/B filters. Filters were washed four times with 5 ml of PBSS and counted. "Nonspecific" binding (10% of total) was determined in 100 μM flurazepam and was subtracted from total binding to yield specific binding (all determinations were performed in triplicate). For computer-assisted curve fitting, data were analyzed by nonlinear regression as previously described (12). Data (weighted according to the inverse of the standard error from triplicates) were fitted to one-site and two-site models, and the adjusted sums-of-squares were compared using the F test to determine significance. K_I values were calculated from competition binding experiments according to the equation $K_I = IC_{50}/(1 + L^*/K_D^*)$, where IC_{50} is the concentration of competitor that inhibits 50% of the binding of radioligand, L^* is the concentration of radioligand, and K_D^* is the dissociation constant.

As stated above, GABA increases the affinity of benzodiazepines for the benzodiazepine receptor, without affecting the number of receptors (B_{max}) (Refs. 14, and 15; see also Fig. 1 under Results). The ability of GABA (10 μM, unless stated otherwise) to increase [^{3}H]flunitrazepam binding (% potentiation) was used as an indicator for the degree to which the GABA and benzodiazepine sites are "allosterically coupled." Percentage of potentiation was defined as

$$\frac{\text{Specific binding in the presence of GABA}}{\text{Specific binding in the absence of GABA}} - 1 \times 100$$

To evaluate the relative reduction in allosteric coupling following chronic exposure to drug, data were expressed as "percentage of uncoupling" which equals:

$$\frac{(\%\text{ potentiation}) - (\%\text{ potentiation in treated cells})}{\%\text{ potentiation}} \times 100$$

Protein synthesis. To measure the synthesis of cellular protein, cultures were incubated with [^{35}S]methionine (30 min, 37°) and then washed thoroughly to remove nonincorporated radioactivity. Sodium dodecyl sulfate/Nonidet P-40/urea (0.2%/2%/8 M) was added (2 ml/dish), aliquots were removed, and TCA was added to the aliquots to a final concentration of 10%. TCA-precipitable radioactivity was determined by filtration through Whatman GF/B filters, which were washed under vacuum with a PBSS containing 10% TCA. Radioactivity retained on the filters was determined by liquid scintillation counting.

Protein degradation. To examine the degradation of cellular protein, cultures were labeled with [^{35}S]methionine (24 hr, 37°), washed with complete medium containing 2 mM L-methionine (to prevent further incorporation of label), and incubated at 37° following the addition of fresh medium (containing methionine). At each time point a small aliquot of medium was removed and replaced with fresh medium. TCA was added (final concentration of 10%), and radioactivity in the supernatant was determined after the samples were centrifuged for 5 min in a Beckman Microfuge. Cell-associated radioactivity was determined in washed cells after solubilizing in 0.2% sodium dodecyl sulfate, 2% Nonidet P-40, and 8 M urea.

Stock solutions. Drugs were made up in PBSS, except for isobutylmethylxanthine (0.1 M in 100% ethanol), and flunitrazepam or 2-chloroadenosine which were dissolved in 100% dimethyl sulfoxide. The final concentration of dimethyl sufoxide or ethanol in the binding assay was generally 0.05–0.5%. Neither dimethyl sulfoxide nor ethanol at these concentrations affected specific binding.

Results and Discussion

GABA increases the affinity of [^{3}H]flunitrazepam for the benzodiazepine-sensitive modulator site without affecting receptor number (Fig. 1), indicating that the GABA- and benzodiazepine-binding sites are allosterically "coupled." Significantly, chronic treatment of cultures with 1 μM theophylline for 36 hr reduces GABA potentiation of 1 nM [^{3}H]flunitrazepam binding from 73 ± 3% (n = 37) to 53 ± 3% (n = 22), a 28% decrease. This effect of chronic exposure to methylxanthines demonstrates that there is a relative reduction in the ability of GABA to enhance [^{3}H]flunitrazepam binding. We refer to this decrease in potentiation of [^{3}H]flunitrazepam binding by GABA as allosteric "uncoupling." Our previous results have shown that there is a good correlation between the ability of muscimol, which is a GABA agonist, to potentiate the binding affinity of a series of benzodiazepines, and the ability of the same benzodiazepines to potentiate GABA-induced conductance increases (13). This suggests that allosteric uncoupling is a valid indicator of functional uncoupling.

The dose response curve for the potentiation of [^{3}H]flunitrazepam binding (Fig. 2) by GABA demonstrates that chronic treatment with flurazepam (19, 20) or theophylline results in a decrease in the maximum ability, or efficacy, of GABA to potentiate [^{3}H]flunitrazepam binding, but no change in its half-maximum (EC_{50}) or potency. Furthermore, nonlinear regression analysis of saturation binding data (n = 3) indicates that, in the absence of GABA, [^{3}H]flunitrazepam binding to untreated cells (K_D = 2.48 ± 0.3 nM, B_{MAX} = 0.12 ± 0.01 pmol/mg) and theophylline-treated cells (K_D = 2.68 ± 0.6 nM, B_{MAX} = 0.12 ± 0.02 pmol/mg) is not different (Fig. 3). Thus, uncoupling is not due to a change in the number or affinity of benzodiazepine-binding sites. Instead, it appears that the modulatory interac-

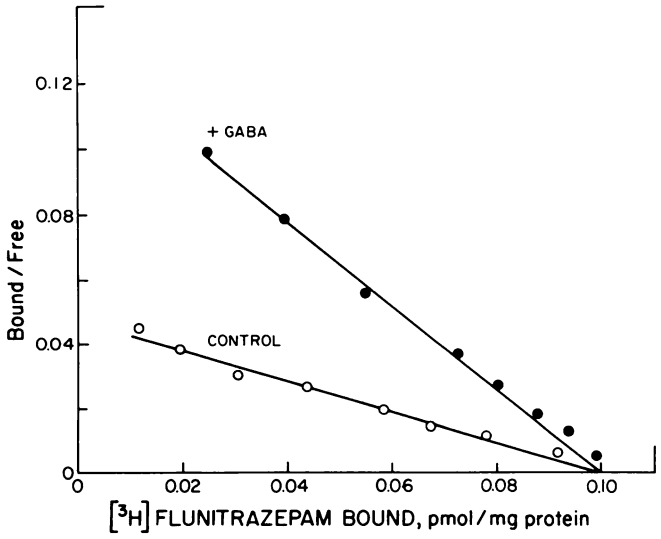

Fig. 1. GABA potentiates [^{3}H]flunitrazepam binding to the benzodiazepine-binding site. Saturation binding of [^{3}H]flunitrazepam was determined in the absence and presence of 10 μM GABA. K_D and B_{MAX} values were determined using nonlinear regression analysis of the data. The lines drawn represent the computer-generated one-site fit to the data. The results indicate that GABA potentiates [^{3}H]flunitrazepam binding but does not alter the number of binding sites (K_D = 0.78 nM and B_{MAX} = 0.10 pmol/mg in the presence of GABA; in the absence of GABA K_D = 2.09 nM and B_{max} = 0.098 pmol/mg). Homogenate was derived from 9-day-old cell cultures. Protein concentration was determined by the method of Lowry *et al.* (30).

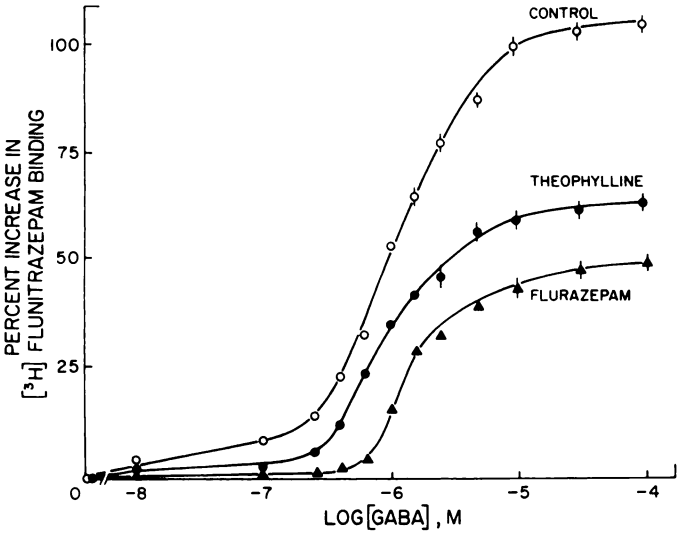

Fig. 2. Chronic theophylline or flurazepam decreases the ability of GABA to potentiate [^{3}H]flunitrazepam binding. GABA potentiation of [^{3}H]flunitrazepam binding to homogenates derived from control cultures (○), or from cultures exposed for 36 hr to either 1 μM theophylline (●) or 100 μM flurazepam (▲) was determined by filtration. Each point is the mean ± standard error of triplicate determinations. The EC_{50} values were 1.07 μM for control, 1 μM for theophylline-treated, and 1.25 μM for flurazepam-treated cells. This experiment was repeated three times with similar results.

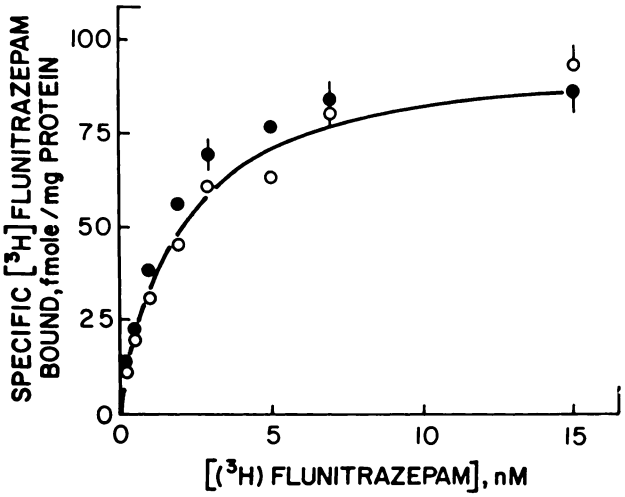

Fig. 3. Chronic theophylline treatment does not alter benzodiazepine receptor affinity or number. [^{3}H]Flunitrazepam binding was determined using cell homogenates derived from control (○) or theophylline-treated (1 μM, 36 hr, ●) cultures. K_D and B_{max} values were determined using nonlinear regression analysis of data. The line drawn represents a computer-generated one-site fit to the control data. The pooled results from three independent experiments indicate that [^{3}H]flunitrazepam binding to untreated cells (K_D = 2.48 ± 0.3 nM, B_{MAX} = 0.12 ± 0.01 pmol/mg) and theophylline-treated cells (K_D = 2.68 ± 0.6 nM, B_{MAX} = 0.12 ± 0.02 pmol/mg) is not altered. Protein concentration was determined by the method of Lowry *et al. (30)*.

tions among the components of the GABA/benzodiazepine receptor complex change and thereby reduce potentiation. Whether reduced potentiation reflects a decrease in the allosteric interactions between all sites or whether some sites become completely uncoupled while others remain coupled remains unknown.

The half-maximal concentration for this effect of chronic methylxanthine treatment is 1 nM for theophylline or caffeine (pooled data from 25 and 18 independent experiments, respectively; Fig. 4). Similarly, isobutylmethylxanthine (10 μM and 100 μM) treatment decreases the enhancement of [^{3}H]flunitrazepam binding caused by GABA (Table 1). In contrast, enhancement of [^{3}H]flunitrazepam binding by GABA is unchanged after treatment with carbachol, a cholinergic receptor agonist, or chloroadenosine, an adenosine receptor agonist, indicating that the phenomenon displays pharmacological specificity.

To investigate whether or not chronic theophylline exposure alters the viability of neurons in cell culture we utilized protein synthesis and protein degradation (21) as indicators for the

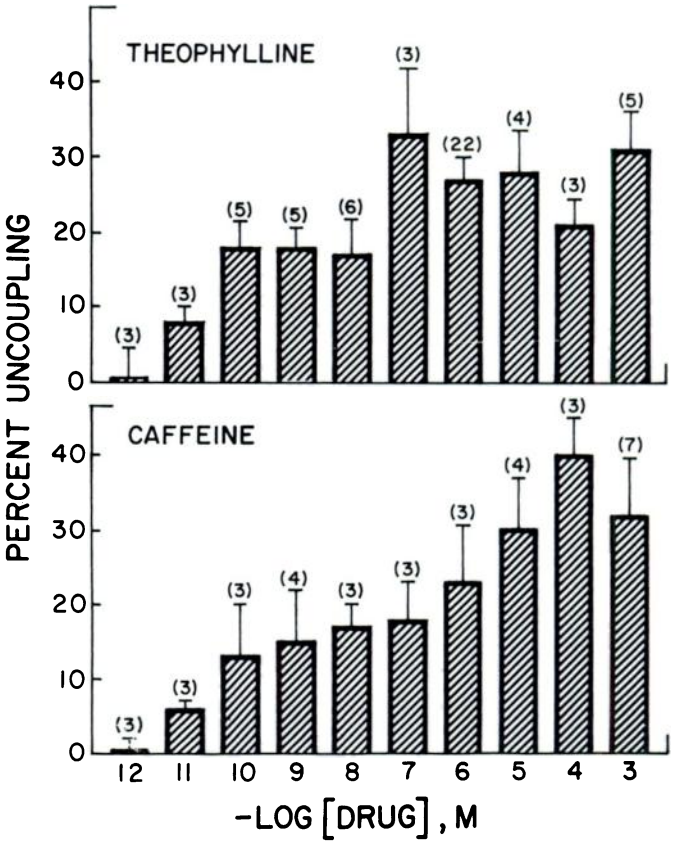

Fig. 4. Chronic theophylline and caffeine induce uncoupling in a dose-dependent manner. Cultures were treated for 36 hr with the indicated concentrations of theophylline or caffeine. GABA potentiation of [^{3}H] flunitrazepam binding was determined in triplicate by filtration. Uncoupling was determined as discussed under Materials and Methods. Values represent the means ± standard errors. *Numbers in parenthesis* indicate the number of independent experiments.

TABLE 1

Uncoupling displays pharmacological specificity

Cultures were treated with the indicated concentrations of drugs for 36 hr. GABA potentiation of [^{3}H]flunitrazepam binding was measured as described under Materials and Methods.

Compound	Concentration M	% Uncoupling	n
Isobutylmethylxanthine	10^{-5}	25 ± 10	3
	10^{-4}	22 ± 6	9
Flurazepam	10^{-5}	39 ± 4	8
	10^{-4}	52 ± 4	23
Carbachol	10^{-4}	7 ± 6	4
Chloroadenosine	10^{-8}	3 ± 4	4
	10^{-5}	1 ± 2	4

overall health of the cells. Theophylline (1 μM) does not change either protein synthesis (108 ± 15% of control cultures, $n = 3$), as determined by [^{35}S]methionine incorporation into TCA-precipitable material, or protein degradation, as determined by the release into the medium of TCA-soluble radioactivity from cells previously labeled with [^{35}S]methionine. These results indicate that chronic treatment with theophylline is not cytotoxic.

To attempt to determine whether uncoupling requires intact cells we chronically treated cellular homogenates and intact cells with theophylline or flurazepam. When intact cells are treated chronically with theophylline (100 μM) or flurazepam (10 μM), uncoupling is 24.5 ± 0.5% ($n = 2$) and 44 ± 9% ($n = 3$), respectively. However, when cell homogenates are treated in a similar manner, no uncoupling occurs (Fig. 5). This result supports the argument that uncoupling does not result from a passive conformational change or from the presence of tightly bound lipophilic drug but rather that the uncoupling process requires one or more intermediary steps mediated by intact cells.

It seems unlikely that theophylline or caffeine induces uncoupling by stimulating calcium mobilization and catecholamine turnover, or by inhibiting phosphodiesterase activity, since these effects require high concentrations of drugs (22–24). Furthermore, uncoupling cannot be accounted for by retention of drug following dialysis. Methylxanthines have low affinity for the benzodiazepine receptor (IC_{50}~400 μM) (25). Therefore, even if there is any residual drug following 1 μM theophylline treatment, it should not affect benzodiazepine binding. Chronic treatment with flurazepam does not alter the affinity or the number of benzodiazepine-binding sites, suggesting that dialysis removes flurazepam efficiently. In addition, as stated earlier, chronic treatment of homogenates is without effect on coupling, providing further evidence that drug sequestration does not cause uncoupling.

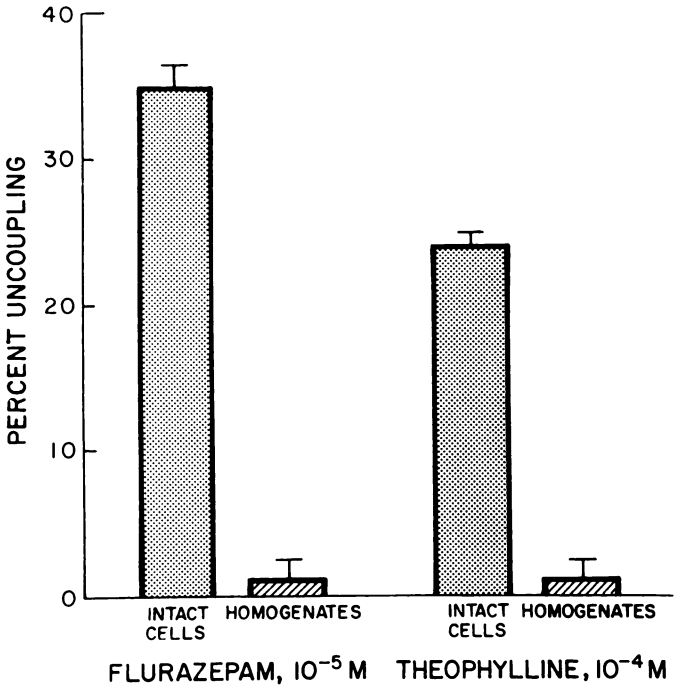

Fig. 5. Chronic treatment of intact cells but not homogenates results in uncoupling. Intact cell cultures and homogenates from sister cultures were incubated in the absence (control) or presence of 100 μM theophylline or 10 μM flurazepam. Data shown are per cent uncoupling, mean ± standard error of triplicate determinations (uncoupling calculated as described under Materials and Methods). This experiment was repeated with similar results.

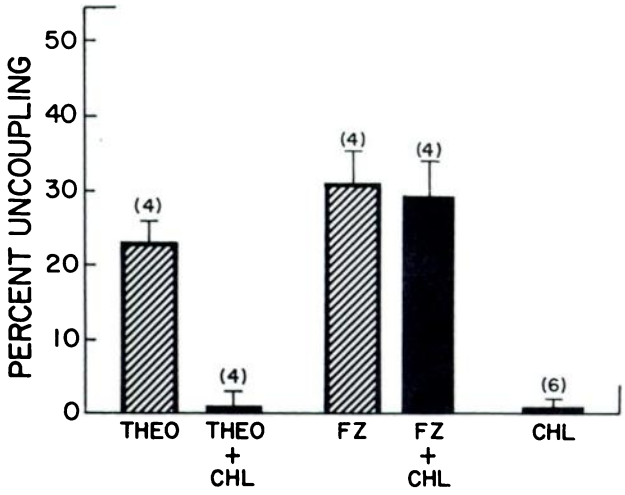

Fig. 6. Chronic theophylline and flurazepam act through different sites to uncouple the GABA/benzodiazepine receptor. Cultures were treated with 1 μM theophylline (*THEO*), 10 μM flurazepam (*FZ*), or 10 μM chloroadenosine (*CHL*). [^{3}H]Flunitrazepam binding was determined with and without 10 μM GABA. Uncoupling was determined as described under Materials and Methods. Similar results were observed with 10 nM chloroadenosine ($n = 4$).

It is generally accepted that methylxanthines exert many of their effects by inhibiting the binding of adenosine to its receptor (2, 3), and adenosine-binding sites have been demonstrated in embryonic chick brain cell cultures (26). Chloroadenosine (a stable adenosine receptor agonist) has no effect on % potentiation by itself, but it blocks the ability of chronic theophylline to uncouple the GABA/benzodiazepine receptors. In contrast, the uncoupling induced by chronic flurazepam treatment is not affected by chloroadenosine (Fig. 6). While the precise mechanism by which theophylline and caffeine uncouple GABA and benzodiazepine binding remains to be elucidated, this finding suggests that uncoupling by theophylline, but not by flurazepam, may be mediated by a site that resembles an adenosine receptor. Furthermore, it raises the intriguing possibility that the GABA/benzodiazepine receptor is subject to trans-receptor regulation.

Although the physiological significance of the results remains to be elucidated, the concentrations required for uncoupling are reached both following consumption of coffee [a single cup of coffee results in serum caffeine levels of approximately 5 μM (1)] and during the use of theophylline in the treatment of asthma, apnea of preterm infants, and chronic obstructive pulmonary disease (1, 27). It seems possible that some of the effects of chronic exposure to theophylline and caffeine, including their proconvulsant actions (28) and adverse effects upon behavior in children (29), may result from such an effect upon the GABA/benzodiazepine receptor.

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References

1. Rall, T. W. Central nervous system stimulants, in *The Pharmacological Basis of Therapeutics* (A. G. Gilman, L. S. Goodman, T. W. Rall, and F. Murad, eds.). Macmillan Publishing Co., New York, 589–603 (1985).

2. Snyder, S. H., J. J. Katims, Z. Annau, R. F. Bruns, and J. W. Daley. Adenosine receptors and behavioral actions of methylxanthines. *Proc. Natl. Acad. Sci. USA* **78**:3260–3264 (1981).
3. Sattin, A., and T. W. Rall. The effect of adenosine and adenine nucleotides on the cyclic adenosine 3′,5′-phosphate content of guinea pig cerebral cortex slices. *Mol. Pharmacol.* **6**:13–23 (1970).
4. Marangos, P. J., S. M. Paul, A. M. Parma, F. K. Goodwin, P. Syapin, and P. Skolnick. Purinergic inhibition of diazepam binding to rat brain (*in vitro*). *Life Sci.* **24**:851–858 (1979).
5. Polc, P., E. P. Bonetti, L. Pieri, R. Cumin, R. M. Angioi, H. Mohler, and W. E. Haefely. Caffeine antagonizes several central effects of diazepam. *Life Sci* **28**:2265–2275 (1981).
6. Nistri, A., and C. Berti. Caffeine-induced potentiation of GABA effects on frog spinal cord: an electrophysiological study. *Brain Res.* **258**:263–270 (1983).
7. Haefely, W., and P. Polc. Electrophysiological studies on the interaction of anxiolytic drugs with GABAergic mechanisms, in *Anxiolytics: Neurochemical, Behavioral, and Clinical Perspectives* (J. B. Malick, S. J. Enna, and H. I. Yamamura, eds.). Raven Press, New York, 113–145 (1983).
8. Tallman, J. F., and D. W. Gallager. The GABA-ergic system: a locus of benzodiazepine action. *Annu. Rev. Neurosci.* **8**:21–44 (1985).
9. Guidotti, A., G. Toffano, and E. Costa. An endogenous protein modulates the affinity of GABA and benzodiazepine receptors in rat brain. *Nature (Lond.)* **275**:553–555 (1978).
10. Skerritt, J. H., M. Willow, and G. A. R. Johnston. Diazepam enhancement of low affinity GABA binding to rat brain membranes. *Neurosci. Lett.* **29**:63–66 (1982).
11. Choi, D. W., D. H. Farb, and G. D. Fischbach. Chlordiazepoxide selectively augments GABA action in spinal cord cultures. *Nature (Lond.)* **269**:342–344 (1977).
12. Chan, C. Y., T. T. Gibbs, L. A. Borden, and D. H. Farb. Multiple embryonic benzodiazepine binding sites: evidence for functionality. *Life Sci.* 33:2061–2069 (1983).
13. Chan, C. Y., and D. H. Farb. Modulation of neurotransmitter action: control of the γ-aminobutyric acid response through the benzodiazepine receptor. *J. Neurosci.* **5**:2365–2373 (1985).
14. Tallman, J. F., J. W. Thomas, and D. W. Gallager. GABAerig modulation of benzodiazepine binding site sensitivity. *Nature (Lond.)* **274**:383–385 (1978).
15. Wastek G. J., R. C. Speth, T. D. Reisine, and H. I. Yamamura. The effect of γ-aminobutyric acid on ^{3}H-flunitrazepam binding in rat brain. *Eur. J. Pharmacol.* **50**:445–447 (1978).
16. Peterson, E. N., and L. H. Jensen. Chronic treatment with lorazepam and FG 7142 may change the effects of benzodiazepine receptor agonists, antagonists and inverse agonists by different mechanisms. *Eur. J. Pharmacol.* **133**:309–317 (1987).
17. Farb, D. H., L. A. Borden, C. Y. Chan, C. M. Czajkowski, T. T. Gibbs, and G. D. Schiller. Modulation of neuronal function through benzodiazepine receptors: biochemical and electrophysiological studies of neurons in primary monolayer cell culture. *Ann. N. Y. Acad. Sci.* **435**:1–31 (1984).
18. Farb, D. H., C. M. Czajkowski, L. A. Borden, G. D. Schiller, and T. T. Gibbs. Turnover of the benzodiazepine/γ-aminobutyric acid receptor complex determined *in situ*, in *GABAergic Transmission and Anxiety* (G. Biggio and E. Costa, eds.). Raven Press, New York, 51–66 (1986).
19. Gallager, D. W., J. M. Lakoski, S. F. Gonsalves, and S. L. Rauch. Chronic benzodiazepine treatment decreases postsynaptic GABA sensitivity. *Nature (Lond.)* **308**:74–77 (1984).
20. Schiller, G. D., and D. H. Farb. Enhancement of benzodiazepine binding by GABA is reduced rapidly during chronic exposure to flurazepam. *Ann. N. Y. Acad. Sci.* **463**:221–223, (1986).
21. Borden, L. A., C. Czajkowski, C. Y. Chan, and D. H. Farb. Benzodiazepine receptor synthesis and degradation by neurons in culture. *Science (Wash. D. C.)* **226**:857–860 (1984).
22. Katz, A. M., D. I. Repke, and W. Hasselbach. Dependence of ionophore- and caffeine-induced calcium release from sarcoplasmic reticulum vesicles on external and internal calcium ion concentrations. *J. Biol. Chem.* **252**:1938–1949 (1977).
23. Smellie, F. W., C. W. Davis, J. W. Daley, and J. N. Wells. Alkylxanthines: inhibition of adenosine-elicited accumulation of cyclic AMP in brain slices and brain phosphodiesterase activity. *Life Sci.* **24**:2475–2482 (1979).
24. Fredholm, B. B., K. Fuxe, and L. Agnati. Effect of some phosphodiesterase inhibitors on central dopamine mechanisms. *Eur. J. Pharmacol.* **38**:31–38 (1976).
25. Asano, T., and S. Spector. Identification of inosine and hypoxanthine as endogenous ligands for the brain benzodiazepine-binding sites. *Proc. Natl. Acad. USA* **76**:977–981 (1979).
26. Barnes, E. M., and K. G. Thampy. Subclasses of adenosine receptors in brain membranes from adult tissue and from primary cultures of chick embryo. *J. Neurochem.* **39**:647–652 (1982).
27. Hirsh, K. Central nervous system pharmacology of the dietary methylxanthines, in *Progress in Clinical and Biological Chemistry*, Vol. 158: *The Methylxanthine Beverages and Foods: Chemistry, Consumption, and Health Effects* (G. A. Spiller, ed.). Alan R. Liss Inc., New York, 235–302 (1984).
28. Ellis, E. F. Theophylline toxicity. *J. Allergy Clin. Immunol.* **76**:297–301 (1985).
29. Furukawa, C. T., G. G. Shapiro, T. DuHamel, L. Weimer, W. E. Pierson, and C. W. Bierman. Learning and behaviour problems associated with theophylline therapy. *Lancet* **1**:621 (1984).
30. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275 (1951).

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