Cyclic Adenosine Monophosphate Phosphodiesterase in Brain: Effect on Anxiety

Abstract. *Drugs that reduce anxiety may be mediated by cyclic adenosine monophosphate in the brain because (i) potent anxiety-reducing drugs are also potent inhibitors of brain phosphodiesterase activity; (ii) dibutyryl cyclic adenosine monophosphate has the ability to reduce anxiety; (iii) the methylxanthines show significant anxiety-reducing effects; (iv) theophylline and chlordiazepoxide produce additive anxiety-reducing activity; and (v) there is a significant correlation between the anxiety-reducing property of drugs and their ability to inhibit phosphodiesterase activity in the brain.*

Cyclic AMP (adenosine 3′,5′-monophosphate), adenylate cyclase [the enzyme that converts adenosine triphosphate (ATP) into cyclic AMP], and cyclic 3′,5′-nucleotide phosphodiesterase (the enzyme that hydrolyzes cyclic AMP to 5′ AMP) are found in greater abundance in the central nervous system than they are in any other tissue (*1*). The effects of hormones and transmitter substances in the brain may thus be mediated by cyclic AMP (*2*).

Although a great deal of biochemistry of cyclic AMP in brain has been reported, comparatively little is known about the role of cyclic AMP in controlling behavior. It has, however, been implicated in the activity of various transmitter substances in the brain; for example, the concentrations of cyclic AMP in brain slices are raised by norepinephrine and histamine (*3*). Such neurotransmitter substances exert a strong influence on behavior and have been implicated in the etiology of various mental disorders (*4*).

Several psychotropic drugs have been reported to affect the cyclic AMP system. For example, tricyclic antidepressive agents are competitive inhibitors of cyclic AMP phosphodiesterase (*5*), and phenothiazines, reserpine, and caffeine inhibit cyclic AMP phosphodiesterase in vitro (*6*).

The tricyclic antidepressive drugs, the phenothiazines, reserpine, and caffeine have significant, but generally dissimilar, effects on behavior. Although these drugs differ in their behavioral effects, they all have the ability to inhibit phosphodiesterase activity in the brain (*5*, *7*, *8*). In addition, a number of these compounds may inhibit adenylate cyclase (*5*, *8*).

Because of this seeming paradox and the scarcity of available information concerned with the behavioral correlates of cyclic AMP activity, we sought to correlate some behavioral effects of psychotropic agents with their effects on cyclic AMP phosphodiesterase activity (*9*). A simple, valid, and reliable behavioral test has been designed to predict anxiety-reducing effects of drugs (*10*). Earlier work with this procedure had implicated cyclic AMP activity in anxiety; consequently, we chose this behavioral state for possible correlation with cyclic AMP phosphodiesterase activity.

The activity of cyclic AMP phosphodiesterase was determined by the method of Brooker *et al.* (*11*), with a cyclic AMP concentration of $1.6 \times 10^{-7} M$ (*12*). Results are expressed in terms of I_{50}, the concentration of drug required to inhibit by 50 percent the cyclic AMP phosphodiesterase activity of rat brain in vitro.

A new compound, SQ 20,009 (Squibb) [which is 1-ethyl-4-(isopropylidene-hydrazino)-1H-pyrazolo[3,-4-b]pyridine-5-carboxylic acid, ethyl ester, hydrochloride], has approximately 60 times the potency of theophylline as an inhibitor of cyclic AMP phosphodiesterase activity in rat brain (*12*). When administered to rats, cats, and monkeys, SQ 20,009 produced behavioral effects similar to those seen after the administration of the anxiety-reducing drugs, chlordiazepoxide and diazepam. In the behavioral test (*10*), SQ 20,009 was more potent than chlordiazepoxide in reducing the anxiety effects of punishment, but had approximately the same potency as does diazepam in this situation (Fig. 1). Diazepam and chlordiazepoxide were also potent inhibitors of cyclic AMP phosphodiesterase activity. Diazepam had an I_{50} of 33 μM; chlordiazepoxide (CDP), 110 μM (approximately equipotent with theophylline); and SQ 20,009, 2 μM. Unlike theophylline, SQ 20,009, chlordiazepoxide, and diazepam had much greater specificity for phosphodiesterase activity in brain than in heart, lipocyte, or adrenal preparations. This specificity appears to be more predictive than are the I_{50} values for determining the potency of drugs in this behavioral situation.

These data seemed to indicate a relation between the effect of a drug on conflict behavior and its ability to inhibit cyclic AMP phosphodiesterase activity in the brain. Figure 2 shows the effects of intraperitoneal administration of dibutyryl cyclic AMP, and of such phosphodiesterase inhibitors as caffeine, theophylline, and theobromine on punished behavior. These agents had qualitatively similar effects, resembling those of chlordiazepoxide and diazepam, in lessening the anxiety caused by punishment.

Although all six compounds have similar attenuation effects on the behavior patterns after punishment and five of them have similar effects on cyclic AMP phosphodiesterase activity in the brain, their actions on other types of behavior are not identical. The compounds SQ 20,009, chlordiazepoxide, and dibutyryl cyclic AMP cause

Table 1. Relation between the effectiveness of a drug in the conflict test and its ability to inhibit cyclic AMP phosphodiesterase activity in the brain.

Drug	I_{50}	Conflict score*	P
	Section A		
SQ 20,009	2	8.0	<.002
Diazepam	33	12.5	<.006
Fluphenazine	48	2.7	<.01
Chlordiazepoxide	110	8.3	<.001
Theophylline	120	3.0	<.02
Caffeine	150	4.8	<.003
Theobromine	150	2.7	<.02
Chlorpromazine	170	2.5	<.02
Reserpine	170	0.6	>.05
Thioridizine	180	4.6	<.05
Nortriptyline	200	0.9	>.05
Triflupromazine	300	1.9	<.02
Amitriptyline	460	0.8	>.05
Diphenylhydantoin	480	0.7	>.05
Imipramine	700	1.8	<.05
	Section B		
Meprobamate	>1000	36.0	<.001
Pentobarbital	>1000	13.5	<.001
Parachloro-phenylalanine	>1000	1.9	>.05
Phenobarbital	>1000	1.9	<.02
Haloperidol	>1000	1.6	<.04
Morphine	>1000	1.4	>.05
Atropine	>1000	1.1	>.05
Chloral hydrate	>1000	1.1	>.05
Amphetamine	>1000	0.9	>.05
Doxepin	>1000	0.7	<.01
Procaine amide	>1000	0.5	>.05
Codeine	>1000	0.3	>.05
Methylphenidate	>1000	0.1	>.05
Scopolamine	>1000	0.1	>.05

* Conflict score $= (N_{max}/N_{CDP}) \times (N_{max}/N_{control})$; where N_{max} is the maximum number of shocks after drug administration; N_{CDP} is the number of shocks after chlordiazepoxide administration; $N_{control}$ is the number of shocks after the control injection. All numbers are expressed as means.

generalized depressant effects. At the doses tested, the methylxanthines produced a variety of behavioral effects that ranged from overt stimulation (for caffeine), through a combination of mild depression and stimulation (for theophylline), to no clear effect on behavior (for theobromine).

Attenuation of reactions to punishment by these compounds may be explained by a single chemical substance or by a single inhibitory or stimulatory process in the central nervous system, but it cannot be explained by the depressant properties of drugs. One might predict that (i) behaviorally dissimilar drugs (such as theophylline and chlordiazepoxide) would have additive or synergistic effects, as measured by the conflict test, and (ii) there should be a correlation between a drug's activity in the conflict test and its inhibition of cyclic AMP phosphodiesterase activity, regardless of its other effects on behavior.

Figure 3 demonstrates the additive effects of chlordiazepoxide and theophylline, and of chlordiazepoxide and SQ 20,009. Doses of theophylline (25 mg/kg) and chlordiazepoxide (4 mg/kg) that were ineffective when administered individually, produced significant anxiety-reducing activity when administered in combination. Doses of SQ 20,009 and chlordiazepoxide that were ineffective when administered separately produced anxiety-reducing effects when given together.

Table 1 is a summary of the activity of various drugs in the conflict test in relation to their capacity to inhibit cyclic AMP phosphodiesterase. The drugs in section A inhibit phosphodiesterase activity in the brain. With few exceptions, these compounds fall into categories according to their effects on behavior when they are ranked according to their ability to inhibit cyclic AMP phosphodiesterase. The most potent inhibitors are the anxiety-reducing drugs (minor tranquilizers), followed by the methylxanthines, the major tranquilizers, and last, the antidepressant drugs. The correlation coefficient between the I_{50} values and the conflict scores (magnitude of the effect compared with control value) for section A was $-.77$, and was significant at $P \leq .001$ (*13*). A statistical comparison (*14*) of the anxiety-reducing potential of drugs that inhibit brain phosphodiesterase activity [I_{50} values less than 1000 μM (section A in Table 1)], and those that were ineffective as inhibitors

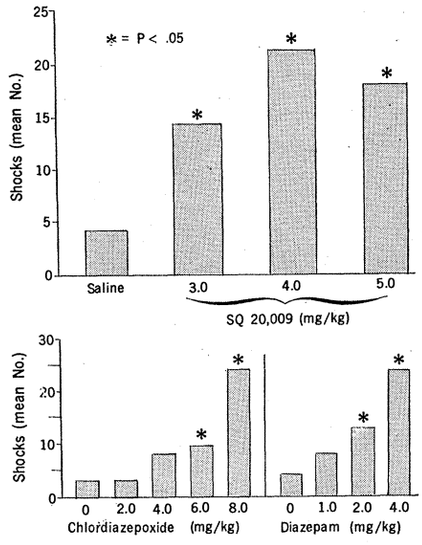

Fig. 1. Mean number of shocks received during the 3-minute test session, after intraperitoneal administration of SQ 20,009, chlordiazepoxide, and diazepam.

of cyclic AMP phosphodiesterase (section B in Table 1), showed a significance by the Wilcoxon rank sum test ($P < .05$). In the group of drugs that were potent inhibitors of cyclic AMP phosphodiesterase activity, only reserpine lacked any appreciable effect in reducing the behavioral effects of punishment. Because the effect on behavior was studied only at 30 minutes after drug administration, the time for effective reserpine activity may have been passed over. Phenobarbital, pentobarbital, and meprobamate did not inhibit the activity of cyclic AMP phosphodiesterase activity in the brain. These agents, currently used for behavioral disorders that involve anxiety, are also effective in our animal anxiety test. Problems of solubility that occur with meprobamate, may account, in part, for this discrepancy. It is also possible that these drugs influence cyclic AMP activity by other means, for example, by effects on adenylate cyclase. Nevertheless, the relation between activity in the conflict test and inhibition of cyclic AMP phosphodiesterase activity in the brain is significant.

Because cyclic AMP phosphodiesterase activity from brain tissue was only measured in vitro in these experiments, our data, which seem to link anxiety-reducing effects of drugs with activity related to cyclic AMP, must be viewed with caution until more data is available on the effects of "anti-anxiety" drugs on brain cyclic AMP, as measured in vivo.

In summary, we have found that (i) some potent anxiety-reducing drugs, such as diazepam and chlordiazepoxide, are also potent inhibitors of cyclic

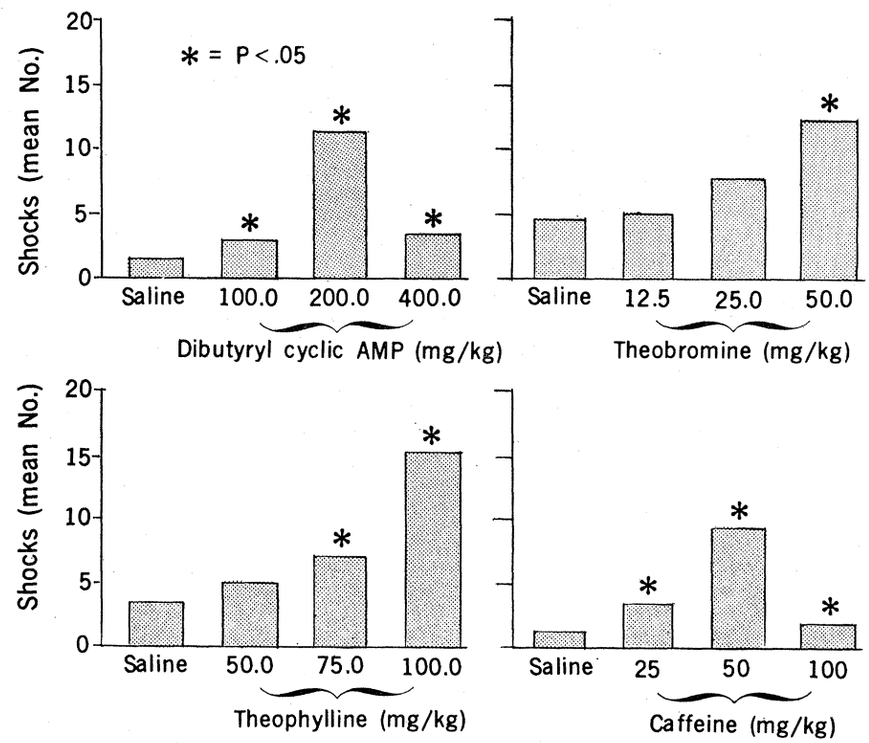

Fig. 2. Mean number of shocks in the 3-minute test session, after intraperitoneal administration of dibutyryl cyclic AMP, theobromine, theophylline, and caffeine.

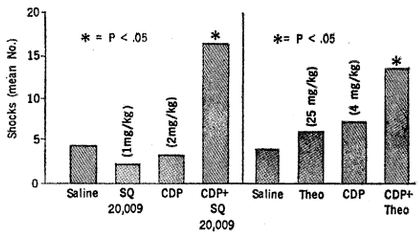

Fig. 3. Mean number of shocks received during the 3-minute test session after intraperitoneal administration of ineffective doses of SQ 20,009, chlordiazepoxide (*CDP*), theophylline (*Theo*), and a combination of the individual doses of chlordiazepoxide and SQ 20,009, and chlordiazepoxide and theophylline.

AMP phosphodiesterase activity in the brain; (ii) dibutyryl cyclic AMP has anxiety-reducing properties, as measured by the conflict test in rats; (iii) the methylxanthines (caffeine, theophylline, and theobromine), known inhibitors of cyclic AMP phosphodiesterase activity, also have significant anxiety-reducing ability in the conflict test; (iv) combinations of theophylline and chlordiazepoxide, or SQ 20,009 and chlordiazepoxide, have at least additive effects in the conflict test; and (v) a significant correlation exists between the activity of a drug in the conflict test and its potency in inhibiting cyclic AMP phosphodiesterase activity in the brain. These results indicate that anxiety-reducing properties of drugs may either involve, or be mediated by, the cyclic AMP system in the brain.

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9. While studying possible effects of pharmacological agents on systems related to cyclic AMP, it was noted that agents active in the central nervous system tended to inhibit cyclic AMP phosphodiesterase activity [I. Weinryb, M. Chasin, C. A. Free, D. Harris, H. Goldenberg, I. Michel, V. Paik, M. Phillips, S. Samaniego, S. Hess, in preparation].
10. Details of this conflict procedure, and evidence that drugs with demonstrated efficacy in the treatment of clinical anxiety states are active in this conflict procedure, have been published elsewhere [J. R. Vogel, B. Beer, D. E. Clody, *Psychopharmacologia* **21**, 1 (1970)]. The rat was placed in the behavior testing apparatus 30 minutes after drug or saline had been administered intraperitoneally. He was allowed to find the drinking tube and complete 20 licks before shocks were administered at maximum durations of 2 seconds. The animal could terminate shock by withdrawing from the tube. The session ended 3 minutes after the first shock. During this 3-minute period, shocks (punishment) were delivered after each 20th lick. The number of shocks delivered during the 3-minute session was recorded for each animal. Each experiment included one group of rats injected intraperitoneally with 8 mg of chlordiazepoxide per kilogram of body weight and a control group that received an intraperitoneal injection of 1.0 ml of distilled water per kilogram of body weight. All statistical comparisons were made using the Mann-Whitney U test (two-tailed).
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12. The enzyme preparation was obtained from a homogenate of rat brain. The homogenate was centrifuged (40,000*g*), the supernatant was mixed with 50 percent saturated ammonium sulfate, and the redissolved precipitate was dialyzed. This cyclic AMP phosphodiesterase preparation has two Michaelis constant (K_m) values for cyclic AMP [(*10*); M. Chasin, *Fed. Proc.* **30**, 1268 (1971)]. If assays are done at very low concentrations of cyclic AMP, the contribution of the enzyme with high K_m may be ignored; the data then represent only inhibition of the activity of cyclic AMP phosphodiesterase with low K_m.
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Avoidance Sessions as Aversive Events

Abstract. *Rats living continuously in conditioning chambers were permitted to work for food before and after their daily avoidance sessions. The avoidance procedure disrupted this responding reinforced by food, a result that indicates conditioned suppression on a time scale much greater than that previously studied in nonhuman animals.*

In their well-known "conditioned anxiety" experiment, Estes and Skinner (*1*) tested animals during training sessions when their responses were being reinforced with food and found that if a 3-minute stimulus terminated with electric shock was presented twice per session in repeated 1-hour sessions, responding was eventually suppressed in the presence of the stimulus. Their procedure is effective with nonhuman animals, and hence is readily used for pharmacological and physiological research on emotion and psychosomatic diseases. The suppression it yields is commonly offered as a laboratory analog of the "fear" or "anxiety" observed in clinical situations with humans. However, the Estes-Skinner procedure uses brief stimuli, seldom more than 5 minutes in duration, followed by brief shocks typically lasting less than a second. Many anxiety-producing situations for humans are not restricted to such brief stimuli and aversive events.

However, the laboratory analog need not be so limited. Laboratory rats respond differentially to different overall shock frequencies, even when brief shocks are distributed over extended periods of time within experimental sessions (*2*). This integration over time may extend beyond the confines of experimental sessions as well as within them. For example, animals in avoidance experiments are usually studied only during the avoidance sessions. Yet, those avoidance sessions may affect behavior in situations that routinely precede or follow them, by periods of minutes or perhaps even hours. That is, extra-session effects of avoidance conditioning may occur on a time scale more like that of anxiety reported from human experience. In the experiments reported here, these effects were examined in rats that lived continuously in conditioning chambers and were permitted to work for food before and after their avoidance sessions.

First, three Long-Evans hooded male rats were housed in identical operant conditioning chambers (Lehigh Valley Electronics), each with the usual response lever, pilot light, grid floor, and dipper. The chambers were modified to include a drinking tube providing free access to water and a ball chain sus-