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INHIBITION OF CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE PHOSPHODIESTERASE BY SUBSTITUTED IMIDAZOPYRAZINES

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Abstract—5-Chloro-6-(ethylamino)-1,3-dihydro-2H-imidazo[4,5-*b*]pyrazin-2-one (CEIP), a new compound with bronchodilator, cardiac stimulant and peripheral vasodilatory properties was found to be approximately two to five times more potent than theophylline as an inhibitor of cyclic AMP phosphodiesterase from several sources. CEIP stimulated lipolysis in isolated fat cells and was synergistic with norepinephrine in increasing glycerol release and the accumulation of [^{14}C] cyclic AMP from adenine-8-^{14}C pre-labeled fat cells. CEIP *in vivo* produced up to a 10-fold increase in the cyclic AMP content of rat heart.

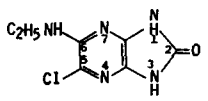
While imidazopyrazines with a secondary amine substituent on position 6 were in general relatively potent phosphodiesterase inhibitors, substituents with a more basic character resulted in decreased enzyme inhibitory activity. 5-Chloro-6-[[2-(dimethylamino)ethyl]amino]-1,3-dihydro-2H-imidazo[4,5-*b*]pyrazin-2-one (CDIP) was less active than theophylline as a phosphodiesterase inhibitor but was nearly as active as theophylline in potentiating norepinephrine-stimulated lipolysis and cyclic AMP accumulation in isolated fat cells. CDIP *in vivo* has peripheral vasodilatory and bronchodilatory activity but is distinct from theophylline and CEIP in that it does not cause tachycardia.

THERE is extensive information on the enzymes which regulate the cellular levels of cyclic AMP.[1–3] Increased concentrations of the cyclic nucleotide may be caused either by enhancement of adenyl cyclase, the enzyme catalysing the conversion of ATP to cyclic AMP,[4] or by inhibition of cyclic 3′,5′-AMP phosphodiesterase, the enzyme which catalyzes its hydrolysis to 5′-AMP.[5,6] Like adenyl cyclase, phosphodiesterase is present in virtually all sources examined. Its distribution and properties have been more fully studied in heart[5,7] and brain[8–11] and reports on its activity in several other mammalian and non-mammalian tissues have appeared.[1]

A variety of agents with established pharmacologic activity has been found to inhibit cyclic AMP phosphodiesterase activity *in vitro*. The methylxanthines are the class of inhibitors most fully studied thus far;[1,5] these compounds have been used clinically for the treatment of bronchial asthma, as central nervous system and cardiac stimulants, and as diuretic agents.[12] Other therapeutic agents found to inhibit phosphodiesterase include phenothiazine and reserpine derivatives,[13] papaverine,[14,15] diazoxide and other benzothiadiazines,[16] triiodothyronine,[17] tricyclic antidepressants,[18] and carbochromen.[19] While it is possible that in some cases the pharmacologic activity of these agents *in vivo* may be related to phosphodiesterase inhibition, direct evidence for such relationships remains to be established.

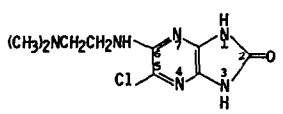
This report will describe studies with representative structures of a new class of phosphodiesterase inhibitors—substituted imidazopyrazines. These compounds possess

a rather broad spectrum of biological properties; they are hypotensive agents, peripheral vasodilators and bronchodilators.* Several of these compounds also increase the force and rate of myocardial contraction and inhibit gastric secretion. *In vitro* they inhibit the conversion of cyclic AMP to 5'-AMP and are lipolytic agents. Two broad classes of imidazopyrazines have been synthesized.[20] Those compounds with a secondary amine substituent, such as an alkylamino group, on position 6 are generally more potent than theophylline as phosphodiesterase inhibitors and are cardiac stimulants. Imidazopyrazines with a more basic substituent on position 6 (such as an aminoalkylamino side-chain) are in general less active than theophylline as diesterase inhibitors and in certain cases are essentially free of central autonomic or direct myocardial augmentation. 5-Chloro-6-(ethylamino)-1,3-dihydro-2H-imidazo[4,5-*b*]pyrazin-2-one (CEIP) and 5-chloro-6-[[2-(dimethylamino)ethyl]amino]-1,3-dihydro-2H-imidazo[4,5-*b*]pyrazin-2-one (CDIP) were the representative compounds selected for the present studies (Fig. 1).



CEIP

5-Chloro-6-(ethylamino)-1,3-dihydro-2H-imidazo [4,5-b] pyrazin-2-one



CDIP

5-Chloro-6-[[2-(dimethylamino) ethyl] amino] 1,3-dihydro-2H-imidazo [4,5-b] pyrazin-2-one

FIG. 1. Structures and nomenclature of CEIP and CDIP.

MATERIALS AND METHODS

Steer heart phosphodiesterase (20,000 *g* supernatant fraction) was prepared by the method of Butcher and Sutherland.[5] The enzyme from steer pancreas and rabbit uterus was purified by a similar procedure while the enzymes from rat lung and heart were the 2000 *g* precipitate and the 15,000 *g* supernatant fractions respectively. Phosphodiesterase activity was measured by the conversion of ^{3}H cyclic AMP to 5'-AMP (and adenosine with crude enzyme preparations) by minor modifications of a previously published procedure.[17] The degradation of cyclic AMP (0·83 mM) was linear with time and enzyme concentration.

Adenyl cyclase (2000 *g* washed precipitate) was prepared from steer brain cerebral cortex by the procedure of Klainer *et al.*[21] and assayed by measuring the conversion of ATP-α-^{32}P to cyclic AM^{32}P. Reaction mixtures (2·0 ml) contained $2{\cdot}5 \times 10^{-3}$ M ATP-α-^{32}P (0·5–5 μc), $2{\cdot}3 \times 10^{-3}$ M $MgSO_4$, $7{\cdot}5 \times 10^{-3}$ M NaF, 4×10^{-2} M tris buffer pH 8, 10^{-2} M theophylline, 10^{-3} M CEIP or CDIP (where indicated), and enzyme. Following incubation for 25 min at 37°, carrier cyclic AMP (0·5 μmoles) was

* L. S. Watson, M. L. Torchiana and N. N. Share, unpublished results.

added and the mixtures heated for 5 min at 100°. After centrifugation the supernatant fluid was passed over a 1-ml column of Dowex-2 chloride. The column was washed with 12 ml of water and cyclic AMP eluted with 15 ml of 0·05 N HCl. Five ml of the cyclic AMP fraction was concentrated to 0·05–0·1 ml in a Buchler rotary evapomix and the entire concentrate chromatographed on Whatmann 3 MM paper in isopropanol–ammonia–0·1 M boric acid (7:1:2). The cyclic AMP area on the chromatograms was placed in a toluene phosphor and the radioactivity determined in a liquid scintillation counter. In later studies with rat lung, 2000 *g* sediment fractions were assayed for adenyl cyclase activity by a procedure similar to ones described by Krishna *et al.*[22] and Melson *et al.*[23] Accumulation of cyclic AM^{32}P was linear with time and enzyme concentration.

Cyclic AMP measurements in rat heart were performed by a previously published method[24] and cyclic AMP formation in fat cells was measured by the radiometric assay of Humes *et al.*[25] The preparation of isolated fat cells from rat epididymal fat pads was based on the Rodbell procedure[26] and the lipolysis experiments *in vitro* were performed as described earlier.[17,24]

ATP-α-^{32}P was obtained from the I.C.N. Corp., adenine-8-^{14}C and ^{3}H cyclic AMP were purchased from Schwarz Bioresearch. Dowex-2 (AG2-X8) was a product of Bio-Rad Corp., norepinephrine was purchased from Calbiochem, theophylline from Mann Research Laboratories or Merck & Co., CEIP and CDIP were obtained from Dr. J. H. Jones, Department of Medicinal Chemistry, Merck Sharp & Dohme Research Laboratories, West Point, Pa. All other chemicals and enzymes used were of the highest quality commercially available products. Bovine organs were obtained from the Allen Meat Packing Co., Elizabeth, N.J. within 2 hr after the animals were killed. Male Holtzman rats were used for the studies *in vivo* and male Charles River rats were used in all experiments *in vitro*.

RESULTS

In Table 1 data are presented which compare the inhibitory effects of 1×10^{-3} M CEIP and CDIP to that of theophylline on cyclic AMP phosphodiesterase prepared from several tissues. In all cases CEIP was more potent than theophylline while CDIP was less active than theophylline as a phosphodiesterase inhibitor. CEIP was nearly

TABLE 1. INHIBITION OF CYCLIC AMP PHOSPHODIESTERASE BY CEIP, CDIP AND THEOPHYLLINE

Enzyme source	Per cent inhibition*		
	CEIP	CDIP	Theophylline
Steer heart	60	19	23
Rabbit uterus	52	7	33
Steer pancreas	62		12
Rat lung	52	0	40
Rat heart	54	8	26

* The compounds were tested at a final concentration of 1×10^{-3} M; the concentration of cyclic AMP was $8{\cdot}3 \times 10^{-4}$ M. Data represent averages of two to three experiments.

equally active in blocking enzyme activity from all sources while CDIP and theophylline produced more variable inhibition depending upon the enzyme source. CEIP was found to produce a competitive type inhibition of the steer heart phosphodiesterase; the K_i is of the order of 5×10^{-4} M (Fig. 2). Other imidazopyrazines were also found to produce a competitive type inhibition.*

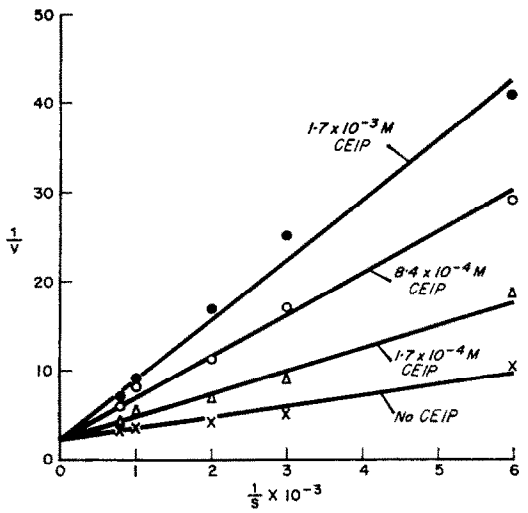


FIG. 2. The nature of the inhibition *in vitro* of partially purified steer heart phosphodiesterase by CEIP.

TABLE 2. EFFECT OF CEIP AND CDIP ON THE ACCUMULATION OF CYCLIC AM^{32}P FROM ATP-α-^{32}P BY ADENYL CYCLASE *in vitro*

Expt. no.	Enzyme source	Counts/min in cyclic AMP*		
		Without CEIP or CDIP	With 10^{-3} M CEIP	With 10^{-3} M CDIP
1	Steer brain	1419	2570	1511
2	Steer brain	1684	2536	
3	Steer brain	2560	4180	
4	Rat lung	7591	11,456	6864
5	Rat lung	9860	10,740	
6	Rat lung	5129		5189

* Theophylline (10^{-2} M) present in all reaction mixtures.

The effects of CEIP and CDIP on the accumulation of cyclic AM^{32}P from ATP-α-^{32}P by adenyl cyclase *in vitro* are presented in Table 2. CEIP (10^{-3} M) produced a marked (50–90 per cent) increase in cyclic AM^{32}P with enzyme preparations from steer brain cerebral cortex; only a small increase was observed with cyclase preparations from rat lung. Other imidazopyrazines of the CEIP-type produced a similar effect with brain adenyl cyclase while CDIP and other CDIP-type structures were

* L. R. Mandel, unpublished results.

inactive.* The mechanism of the increase in cyclic AM^{32}P by CEIP is unknown and may be due to an artifact of the assay. Alternatively it could be attributed to inhibition of phosphodiesterase even though 10^{-2} M theophylline was present in all reaction mixtures. Inhibition by CEIP of phosphodiesterase-activating factors[27,28] or a direct stimulatory effect by CEIP on the brain adenyl cyclase enzyme are also possible explanations.

On the basis of the previously described experiments *in vitro*, it was anticipated that CEIP would increase the intracellular content of cyclic AMP under appropriate experimental conditions *in vivo*. It had been found earlier that CEIP, like theophylline, produces a positive chronotropic effect on the rat heart.* Accordingly, preliminary experiments were performed to determine whether CEIP alters the cyclic AMP content of rat heart. Male rats were anesthetized with vinbarbital, placed on artificial respiration and the thorax was opened in mid-line. CEIP, dissolved in 40% dimethylsulfoxide, was injected directly into a jugular vein. The heart was removed from the animals at various times (0·5–10 min) following the intravenous injection of 10 mg/kg of CEIP and immersed in liquid nitrogen at once. Cyclic AMP was extracted from the frozen tissue by the method of Posner *et al.*,[29] partially separated from other contaminants by chromatography on a Dowex-2 column, and assayed by the modified muscle phosphorylase assay system.[17,24] As shown in Fig. 3, CEIP produced a marked increase

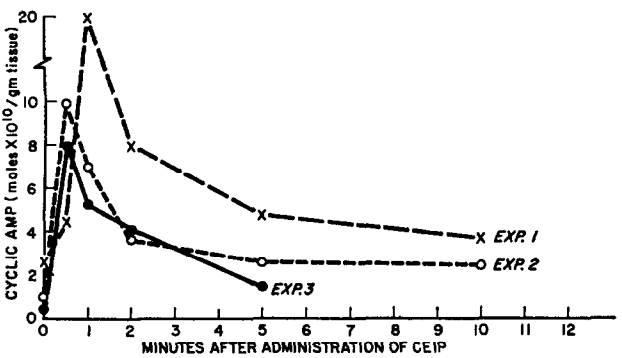


FIG. 3. Effect of intravenous administration of 10 mg/kg CEIP on the cyclic AMP content of rat heart.

in the cyclic AMP content of rat heart within 1 min following intravenous administration. The increment in cyclic AMP was variable from one experiment to another but about a 10-fold increase was always observed. Cyclic AMP levels returned to near control values (1×10^{-10} moles/g tissue) within 5 min. In similar experiments, theophylline (10 mg/kg, i.v.) produced about a 2-fold increase in cyclic AMP. Injection of the 40% DMSO vehicle produced no changes in cyclic AMP. While both cyclic AMP and heart rate (measured from the electrocardiogram) increased under the experimental conditions with CEIP, there was no correlation between the magnitude of these two events. This may be related in part to the variation in the control heart rate in the rats, to the limited number of experiments, and to the variability in the cyclic AMP measurements. Moreover, since CEIP, like other cardiac stimulatory agents, produces changes in cyclic AMP and in the rate and force of myocardial contraction

* M. L. Torchiana, unpublished results.

so rapidly, analyses of tissue biopsy samples for cyclic AMP using a frozen needle technique is necessary in order to correlate these two effects.[30] CDIP had no effect on heart rate or on the force of myocardial contraction and was not evaluated for its effect on altering cyclic AMP levels in the heart.

One of the first hormone responses studied for the involvement of cyclic AMP was the lipolytic response of adipose tissue. It is now well established that the catecholamines, glucagon, ACTH and TSH produce their effects in adipose tissue by stimulating adenyl cyclase.[31] Phosphodiesterase inhibitors such as theophylline and caffeine have been found to act synergistically with the lipolytic hormones in stimulating lipolysis and cyclic AMP accumulation.[32,33] CEIP and CDIP were compared with theophylline for their effects on both lipolysis and cyclic AMP formation in rat isolated fat cells *in vitro*.

As shown in Table 3, CEIP and CDIP, like theophylline, stimulated glycerol release 2 to 7-fold from isolated fat cells when tested at 5×10^{-5} M and 1×10^{-4} M respectively. The activity of the three compounds as lipolytic agents paralleled their order of

TABLE 3. EFFECT OF CEIP, CDIP AND THEOPHYLLINE ON GLYCEROL RELEASE IN ISOLATED FAT CELLS

Compound	Concn(M)	Glycerol release (mμmoles/ml/hr ± S.E.M.) Without norepinephrine	With $5{\cdot}0 \times 10^{-8}$ M DL-norepinephrine
Control		98 ± 9	171 ± 18
CEIP	1×10^{-4}	536 ± 52	816 ± 56
	5×10^{-5}	273 ± 55	786 ± 90
	1×10^{-5}	124 ± 7	735 ± 30
CDIP	1×10^{-4}	239 ± 15	590 ± 71
	5×10^{-5}	200 ± 19	673 ± 35
	1×10^{-5}	130 ± 6	530 ± 11
Theophylline	1×10^{-4}	382 ± 38	583 ± 32
	5×10^{-5}	192 ± 36	580 ± 40
	1×10^{-5}	141 ± 5	510 ± 53

activity as phosphodiesterase inhibitors (cf. Table 1); CEIP was more potent than theophylline, CDIP somewhat less active than theophylline. While the compounds were inactive when tested alone at 1×10^{-5} M, they markedly potentiated lipolysis produced by 5×10^{-8} M DL-norepinephrine. CEIP appeared to be the most active compound in these experiments as well. However, it was not possible to distinguish between CDIP and theophylline in terms of their potency as potentiators of norepinephrine lipolysis. Based upon the results of previous experiments it was anticipated that CEIP and theophylline would be active as lipolytic agents in the isolated fat cell system. On the other hand, the relatively high activity of CDIP was not expected since it was a comparatively weak phosphodiesterase inhibitor. Phosphodiesterase has two distinct K_m values for cyclic AMP[34,35] and it is not yet clear whether the physiological responses elicited by phosphodiesterase inhibitors are primarily due to inhibition of enzyme activity at low or high cyclic AMP concentrations. Since the present studies used cyclic AMP in the millimolar range only (cf. Table 1), the discrepancy between lipolytic efficacy of CDIP and potency as a phosphodiesterase inhibitor may be explained

partly on this basis. A relatively greater permeability of CDIP to adipose tissue as opposed to other tissues could also account for its lipolytic activity.

For further studies on the mechanism of the lipolytic action of CEIP and CDIP, the effect of these compounds on cyclic AMP accumulation in isolated fat cells was measured. Fat cells were preincubated with adenine-8-^{14}C (1 μc/ml cell suspension) for 2 hr at 37° to form an intracellular pool of ^{14}C ATP. Additions of test compounds and norepinephrine followed and the incubations were continued for 5 min at 37°. The reactions were terminated with trichloroacetic acid and the radioactivity in the cyclic AMP-8-^{14}C fraction determined by the method of Humes *et al.*[25] As shown in Table 4, 5×10^{-3} M theophylline and CDIP had marginal effects on cyclic AMP in

TABLE 4. EFFECT OF CEIP AND CDIP ON CYCLIC AMP-8-^{14}C ACCUMULATION IN ISOLATED FAT CELLS

Compound	Concn(M)	Counts/min in cyclic AMP	
		Without norepinephrine	With 5×10^{-7} M DL-norepinephrine
None		318	400
CEIP	$5{\cdot}0 \times 10^{-3}$	3440	45,300
	$2{\cdot}4 \times 10^{-3}$		30,822
	$5{\cdot}0 \times 10^{-4}$		16,533
CDIP	$5{\cdot}0 \times 10^{-3}$	668	8128
	$2{\cdot}4 \times 10^{-3}$		5102
	$5{\cdot}0 \times 10^{-4}$		1571
Theophylline	$5{\cdot}0 \times 10^{-3}$	488	7840
	$5{\cdot}0 \times 10^{-3}$	594	9500

the absence of added norepinephrine. CEIP produced about a 10-fold increase in the ^{14}C-cyclic AMP fraction in a similar experiment. When tested in combination with 5×10^{-7} M DL-norepinephrine, theophylline and CDIP (5×10^{-3} M) produced similar, approximately 20-fold increases in ^{14}C-cyclic AMP; CEIP produced an even greater effect (ca. 100-fold increase). Upon titration to $5{\cdot}0 \times 10^{-4}$ M, CEIP caused a 40-fold increase in radioactivity in the cyclic AMP-8-^{14}C fraction while CDIP was much less active (ca. 4-fold increase). Theophylline was not evaluated at lower levels. The results of these experiments are in accord with the data from the lipolysis measurements and the effects of CEIP and CDIP on phosphodiesterase.

DISCUSSION

The level of cyclic AMP in tissues is the result of a balance between its rate of synthesis from ATP catalyzed by adenyl cyclase and its rate of degradation to 5'-AMP, catalyzed by a specific phosphodiesterase. The phosphodiesterase inhibitors, caffeine and theophylline, potentiate hormonal-induced cyclic AMP formation in several systems, adipose tissue and cardiac muscle among those studied more extensively. These compounds potentiate the action of lipolytic hormones on lipolysis and cyclic AMP formation in rat epididymal fat pads and isolated fat cells and are inhibitors of the adipose tissue phosphodiesterase.[32,33] Close agreement between lipolytic activity

and phosphodiesterase inhibitory activity has also been found for several other xanthine derivatives.[36] There is some evidence that the positive inotropic effect of the methylxanthines is related in part to elevated cyclic AMP levels resulting from phosphodiesterase inhibition.[37] No systematic study *in vivo* of the effects of theophylline or caffeine on cardiac cyclic AMP levels has been described, however.

Chemically, theophylline and caffeine may be considered as substituted imidazopyrimidine derivatives. This report describes studies with a related group of structures, substituted imidazopyrazines. Like the methylxanthines, the imidazopyrazines produce many effects *in vivo*, and *in vitro* those structures of the CEIP type (an alkylamino substituent on position 6) have pronounced effects on the enzymes associated with cyclic AMP. CEIP is more potent than the methylxanthines as a competitive inhibitor of the cyclic nucleotide phosphodiesterase and stimulates both glycerol release and cyclic AMP accumulation in isolated fat cells. *In vivo* it produces a marked elevation in the cyclic AMP content of rat heart. However, unequivocal evidence that the pharmacodynamic effects of CEIP is the result of elevated cyclic AMP levels remains to be established.

CDIP and other imidazopyrazines with a more basic substituent on position 6 (such as an aminoalkylamino group) are less active than theophylline as phosphodiesterase inhibitors. CDIP potentiates cyclic AMP accumulation and lipolysis in isolated fat cells and in these measurements is as active as the methylxanthines. However, CDIP is distinctly different from theophylline and CEIP *in vivo*, particularly in that it does not cause tachycardia. This raises the question of whether the results obtained with CDIP *in vitro* are relevant to the pharmacologic effects observed *in vivo*. Measurements of cyclic AMP in tissues of CDIP-treated animals would be of value in attempting to relate the pharmacologic and biochemical properties of CDIP and the other imidazopyrazines.

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