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4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711): a Potent Inhibitor of Adenosine Cyclic 3',5'-Monophosphate Phosphodiesterases in Homogenates and Tissue Slices from Rat Brain

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SUMMARY

SCHWABE, U., MIYAKE, M., OHGA, Y. & DALY, J. W. (1976) 4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711): a potent inhibitor of adenosine cyclic 3',5'-monophosphate phosphodiesterases in homogenates and tissue slices from rat brain. *Mol. Pharmacol.*, 12, 900-910.

A new class of inhibitor of phosphodiesterases is represented by 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711). This compound enhances the magnitude of accumulations of cyclic 3',5'-AMP elicited by norepinephrine, isoproterenol, histamine, and adenosine in rat cerebral cortical slices and by veratridine in rat cerebellar slices and, in addition, increases basal levels of cyclic AMP in both cortical and cerebellar slices. Increases in intracellular levels of cyclic AMP in brain slices elicited by ZK 62711 do not appear to involve enhanced "release" of adenosine, since both basal and norepinephrine-elicited accumulations of cyclic AMP are increased by ZK 62711 in the presence of exogenous adenosine deaminase. ZK 62711 has little effect on levels of cyclic GMP in cortical or cerebellar slices. In rat cerebral homogenates ZK 62711 inhibits soluble and particulate cyclic AMP phosphodiesterases but is less potent with respect to cyclic GMP phosphodiesterases. At low concentrations it is 100 times more potent than a structurally related phosphodiesterase inhibitor, Ro 20-1724, with respect to the calcium-dependent cyclic AMP phosphodiesterase, and in brain slices it is similarly 100 times more potent in enhancing accumulations of cyclic AMP elicited by norepinephrine.

INTRODUCTION

Phosphodiesterase inhibitors have proven useful tools for the elucidation of the role of cyclic nucleotides in the control of cellular physiology in a variety of tissues and cell types. However, in brain tissue the usefulness of the established phosphodiesterase inhibitors is severely limited because of side effects. These phosphodiesterase inhibitors include theophylline, 3-isobutyl-1-methylxanthine, papaverine, dipyridamole, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), diazepam, phenothiazines, and 1-ethyl-4-isopropylidene hydrazino-14-pyrazolo(3,4)pyridine-5-carboxylate ethyl ester (SQ 20,009) (1-5). Since adenosine appears to play a unique and pivotal role in the control of

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cyclic 3',5'-AMP-generating systems in brain tissue (6, 7), any phosphodiesterase inhibitor which inhibits or augments adenosine mechanisms possesses limited usefulness in delineating the role of phosphodiesterases in intact brain cells. Theophylline and isobutylmethylxanthine are potent adenosine antagonists (8, 9). Other phosphodiesterase inhibitors, such as papaverine and dipyridamole, are potent inhibitors of uptake of adenosine and thus prevent reuptake of the endogenous adenosine which is continually released from brain slices (10). Much of the effect of these compounds on cyclic AMP levels is due not to inhibition of phosphodiesterase, but rather to enhanced activation of adenosine-sensitive cyclases. Ro 20-1724 has been found to inhibit uptake of adenosine into brain slices (8) and appears to have a strong adenosine component in its potentiative effects on biogenic amine-elicited accumulations of cyclic AMP in brain slices (8, 11–13). In addition, Ro 20-1724 has activity as a monoamine oxidase inhibitor (14). Diazepam inhibits uptake of adenosine into brain slices (8), and thus the effects of this component on cyclic AMP levels in brain slices must be considered (8, 15). Phenothiazines, such as chlorpromazine and trifluoperazine, are moderately active phosphodiesterase inhibitors with rather selective effects on the calcium-dependent brain phosphodiesterases (16). In brain slice preparations, phenothiazines usually inhibit rather than augment accumulations of cyclic AMP, probably through inhibition of adenylate cyclases (17–19). SQ 20,009, a very potent inhibitor of brain phosphodiesterases, has little or no effect on accumulations of cyclic AMP in brain slices (8, 11, 12), although 1 mM SQ 20,009 has been reported to enhance dopamine-elicited accumulation of cyclic AMP in rat striatal slices (20). SQ 20,009 inhibits uptake of adenosine into brain slices (8). It is readily apparent that no selective and potent inhibitor of phosphodiesterases in brain tissue is available. It is therefore of interest to investigate other compounds as inhibitors of phosphodiesterases in brain slices and cell-free preparations. A centrally active 4-(phenyl)-2-pyrrolidone (ZK 62711, Fig. 1A) has been found to potentiate prostaglandin E_1-elicited accumulations of cyclic AMP in thrombocytes at low concentrations.[3] In view of the potency of ZK 62711 in thrombocytes, its structural resemblance to the 4-(benzyl)-2-imidazolidinone, Ro 20-1724 (Fig. 1B), and the central depressant activity of both ZK 62711 and RO 20-1724, an investigation of the effects of this pyrrolidinone on cyclic AMP levels in brain slices was undertaken. ZK 62711 was found to be a potent agent with respect to enhancement of amine- or adenosine-elicited accumulations of cyclic AMP in rat brain slices and with respect to inhibition of calcium-dependent cyclic AMP phosphodiesterases from rat brain.

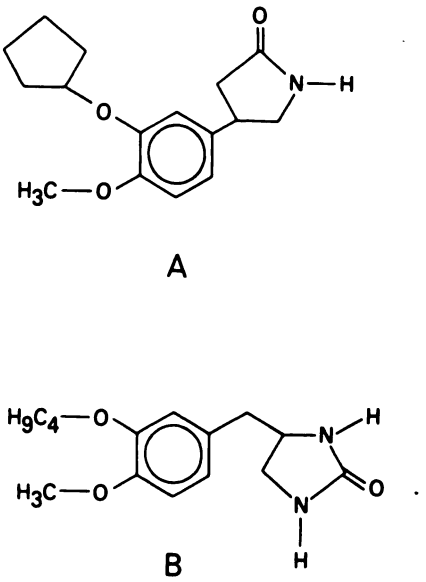

FIG. 1. *Structures of 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62771) (A) and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) (B)*

MATERIALS AND METHODS

Tissue preparation. Male Sprague-Dawley rats (180–220 g) were decapitated, and their brains were removed and chilled in ice-cold Krebs-Ringer-bicarbonate-glucose (21). Longitudinal strips of cerebral cortical gray matter or pieces of cerebellar gray matter from two or three rats were chopped on a McIlwain tissue chopper set at 260 μm. The cerebral cortical slices

[3] W. Kehr, personal communication.

were incubated for 40 min in 20 ml of Krebs-Ringer-bicarbonate-glucose gassed with 95% 0_2–5% CO_2 throughout all incubations. The slices then were washed, collected on fine nylon mesh, and divided into portions which were transferred into 12–22 incubation beakers for an additional incubation period of 30 min in order to reduce the cyclic AMP content to a constant low basal level (13). Each beaker contained 20–40 mg of tissue per 10 ml of medium. Cerebellar slices were incubated for 40 min, divided, transferred to 12–22 separate beakers, and incubated for an additional 60 min. Phosphodiesterase inhibitors were added 2 min prior to addition of other agents (see legends to figures and tables). The final incubation with agents was carried out for 10 min. The incubations were terminated by collection of slices on fine nylon mesh and rapid transfer to ground glass homogenizers containing 1 ml of 5% trichloroacetic acid, followed by immediate homogenization and centrifugation. Cyclic AMP and cyclic GMP were measured in trichloroacetic acid supernatants after addition of 1 N HCl, extraction with ether, and lyophilization (13). Cyclic AMP was measured by the protein binding method of Gilman (22). Cyclic GMP was determined by the radioimmunoassay of Steiner *et al.* (23), using a Schwarz/Mann assay kit. Further purification of the cyclic AMP or cyclic GMP by colmn chromatography did not affect the results. Previous experiments had demonstrated virtually complete reduction of cyclic AMP and cyclic GMP levels by incubation of supernatants with phosphodiesterases. The trichloroacetic acid precipitates were solubilized with 1 N NaOH and assayed for protein according to the method of Lowry *et al.* (24) as modified by Miller (25).

Preparation of rat brain phosphodiesterases. Soluble and particulate phosphodiesterases of rat brain were prepared according to the methods of Russell *et al.* (26). Tissue from rat cerebrum was homogenized in 9 volumes of 0.32 M sucrose, 5 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 0.1 mM dithiothreitol with a Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 100,000 × *g* for 60 min, and the supernatant solution was dialyzed overnight against 20 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$ and 0.1 mM dithiothreitol. Aliquots of the dialyzed solution were used as the source of soluble phosphodiesterases. The pellet was resuspended in sucrose solution equal to the original volume of homogenate, and resuspended by homogenization. After a second 100,000 × *g* centrifugation, the pellet was resuspended in sucrose solution. This washed particulate preparation was solubilized by sonication with a Branson Sonifier (50 W, 30 sec/ml), and the residue was removed by centrifugation at 30,000 × *g* for 30 min. The solubilized solution was dialyzed as described above. Aliquots of the dialysate were used as the source of particulate phosphodiesterases Calcium-dependent activator protein for phosphodiesterase was prepared from rat cerebrum through heat treatment, DEAE-cellulose chromatography, and Sephadex G-75 gel filtration according to Lin *et al.* (27), with slight modifications.

Phosphodiesterase assays. The enzyme activity was measured by the procedure of Boudreau and Drummond (28). An appropriate dilution of enzyme (1.3–5.6 μg of protein per tube) was incubated in 50 mM Tris-HCl buffer (pH 8.0) containing 3 mM $MgCl_2$, 0.1 mM dithiothreitol, 1 μM cyclic AMP or cyclic GMP, and 2 × 10^5 cpm of cyclic [^{3}H]AMP or cyclic [^{3}H]GMP. Either 0.5 mM EGTA[4] or 0.04 mM $CaCl_2$ plus 1 μg of activator protein was present. The total volume was 0.5 ml. Following incubation for 20 min at 30°, the reaction was terminated by placing the tubes in a Dry Ice–acetone bath. The tubes were then placed in a boiling water bath for 3 min, followed by chilling in ice. The solutions were then incubated with 0.1 ml of a snake venom solution (1 mg/ml) for 20 min at 30°. The reaction was terminated by the addition of 1 ml of AG1-X2 ion-exchange resin (1:3 slurry of resin in H_2O containing 15 mM acetic acid for the cyclic AMP phosphodiesterase assay, or in 80 mM formic acid for the cyclic GMP phosphodiesterase assay).

[4] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

After centrifugation, 0.5 ml of supernatant solution was counted in 10 ml of scintillation fluid.

Materials. Adenosine, *l*-norepinephrine HCl, *l*-isoproterenol bitartrate, dopamine HCl, adenosine cyclic 3',5'-monophosphate, guanosine cyclic 3',5'-monophosphate, ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid, adenosine deaminase, and snake venom (*Ophiophages hannah*) were puchased from Sigma Chemical Company. The adenosine deaminase was diluted 10-fold with a 1% solution of bovine serum albumin the day before use and dialyzed overnight against 100 volumes of distilled water to remove the high content of ammonium sulfate. Histamine 2HCl and veratridine were purchased from Aldrich Chemical Company. Anion-exchange resin AG1-X2, chloride form (200–400 mesh), was purchased from Bio-Rad Laboratories. 4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711) was kindly provided by Dr. Wolfgang Kehr of Schering, Berlin, and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), by Dr. W. E. Scott of Hoffmann-La Roche. [8-^{3}H]Adenosine 3',5'-monophosphate (specific activity, 27 Ci/mmole) and [8-^{3}H]guanosine 3',5'-monophosphate (specific activity, 19 Ci/mmole) were purchased from Amersham/Searle.

RESULTS

The effect of ZK 62711 on basal levels of cyclic AMP and on accumulations of cyclic AMP elicited by norepinephrine and adenosine were investigated in rat cerebral cortical slices (Fig. 2). ZK 62711 elevated basal levels of cyclic AMP by about 4–5-fold, with a maximal effect at approximately 30 μM concentration. ZK 62711 enhanced the responses to maximal stimulatory concentrations of norepinephrine and adenosine approximately 2-fold, with an EC_{50} of about 0.4 μM for the norepinephrine response and about 10 μM for the adenosine response. In view of the structural resemblance of ZK 62711 and Ro 20-1724, the two compounds were compared with regard to effects on norepinephrine-elicited accumulations of cyclic AMP in rat cerebral cortical slices (Fig. 3). Both compounds enhanced the response to maximal stimulatory concentrations of norepinephrine by about 2-fold, but ZK 62711 was nearly 100 times more potent than Ro 20-1724.

The effect of Ro 20-1724 on accumula-

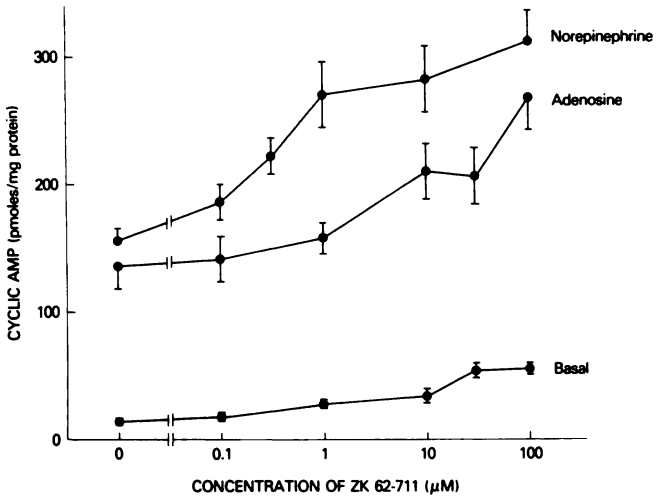

FIG. 2. *Effect of ZK 62771 on accumulations of cyclic AMP elicited by norepinephrine and adenosine in rat cerebral cortical slices*

Slices were first incubated for a total of 70 min (see MATERIALS AND METHODS). Various concentrations of ZK 62771 were added 2 min prior to 100 μM norepinephrine or 100 μM adenosine. Final incubations were then carried out for 10 min. Values represent means ± standard errors for four separate experiments.

tions of cyclic AMP in brain slices has been shown to involve not only inhibition of phosphodiesterase, but also enhanced activation of cyclases by endogenous adenosine (8). In order to investigate possible involvement of endogenous adenosine in the potentiative effect of ZK 62711 on responses of cyclic AMP-generating systems to norepinephrine in brain slices, studies were carried out in the presence of adenosine deaminase. Adenosine deaminase eliminates contributions of adenosine "released" from cells into extracellular space by converting adenosine to the inactive metabolite inosine (8, 29). The presence of adenosine deaminase largely prevents the effect of Ro 20-1724 on levels of cyclic AMP (results not shown). In the presence of adenosine deaminase ZK 62711 still increased basal levels of cyclic AMP by about 4-fold (Fig. 4), indicating that, in contrast to various other phosphodiester-

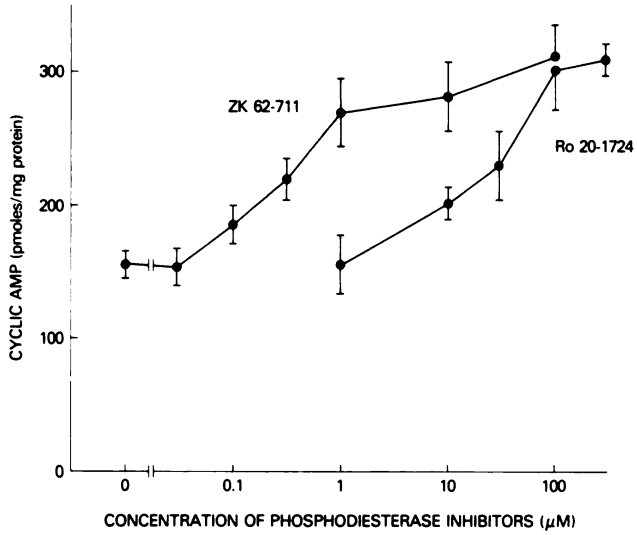

FIG. 3. *Accumulation of cyclic AMP elicited by norepinephrine in rat cerebral cortical slices: potentiation by ZK 62711 and Ro 20-1724*

Slices were first incubated for a total of 70 min (see MATERIALS AND METHODS). Either ZK 62711 or Ro 20-1724 at various concentrations was added 2 min prior to 100 μM norepinephrine. Final incubations were then carried out for 10 min. Values represent means ± standard errors for four or five separate experiments.

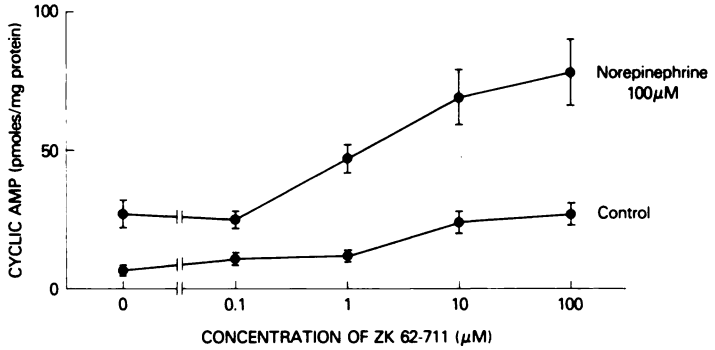

FIG. 4. *Effect of ZK 62711 on levels of cyclic AMP in rat cerebral cortical slices in the presence of adenosine deaminase*

Slices were first incubated for a total of 70 min (see MATERIALS AND METHODS). Adenosine deaminase (10 μg/ml; see ref. 13) and various concentrations of ZK 62771 were added 2 min prior to 100 μM norepinephrine. Final incubations were then carried out for 10 min. Values represent means ± standard errors for five separate experiments.

ase inhibitors (8, 10), ZK 62711 increased cyclic AMP levels in brain slices primarily by mechanisms not involving adenosine. In the presence of 0.5 mM theophylline, the accumulation of cyclic AMP elicited in rat cerebellar slices by 100 μM ZK 62711 was reduced by only about 25% (results not shown), while the accumulation elicited by 100 μM Ro 20-1724 was completely blocked. ZK 62711 enhanced the accumulation of cyclic AMP elicited by norepinephrine by about 3-fold in the presence of adenosine deaminase. The EC_{50} for ZK 62711-elicited enhancement of the response to norepinephrine was about 2 μM, which would appear somewhat greater that its EC_{50} in the absence of adenosine deaminase. The reduction in the response to norepinephrine in the presence of adenosine deaminase presumably is due in large part to elimination of synergistic interactions of the catecholamine with endogenous adenosine.

The accumulations of cyclic AMP elicited in rat cerebral cortical slices by 1-100 μM norepinephrine were enhanced by 1 μM ZK 62711 (Fig. 5). The most marked increases occurred at the lowest concentrations of norepinephrine, where accumulations of cyclic AMP were quite low. The EC_{50} for norepinephrine, about 3 μM, was, however, not significantly altered by the presence of ZK 62711.

The effects of ZK 62711 and Ro 20-1724 on the accumulations of cyclic AMP elicited by various stimulatory agents in rat cerebral cortical slices were compared (Table 1). ZK 62711 at 1 and 100 μM enhanced the response to norepinephrine, isoproterenol, and histamine, as did 100 μM Ro 20-1724. Interestingly, ZK 62711 at 100 μM did not appear to enhance the response to 100 μM dopamine. It has been suggested that the accumulation of cyclic AMP elicited by dopamine in rat cortical slices was slightly enhanced by 250 μM Ro 20-1724 (30). The enhancement of the response to adenosine by ZK 62711 in rat cerebral cortical slices was significant only at 100 μM ZK 62711 (Table 1). Ro 20-1724 at 100 μM had no effect on the adenosine response. Ro 20-1724 at higher concentrations does enhance adenosine responses (9, 11, 12).

Levels of cyclic GMP were measured concurrently in four of the experiments of Table 1. Norepinephrine and adenosine slightly increased cyclic GMP levels in rat cerebral cortical slices (Table 2). Similar responses of cyclic GMP-generating systems to norepinephrine and adenosine have been observed in guinea pig cerebral cortical slices (13). Basal levels of cyclic

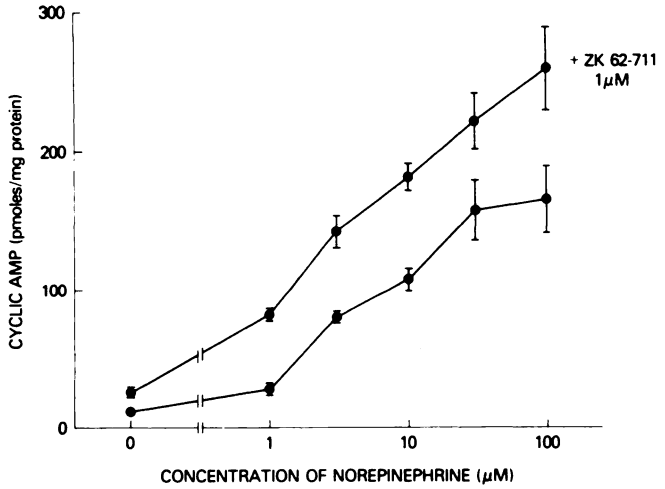

FIG. 5. *Dose-response relationship for norepinephrine-stimulated accumulation of cyclic AMP in rat cerebral cortical slices in the presence and absence of ZK 62711*

Slices were first incubated for a total of 70 min (see MATERIALS AND METHODS). ZK 62711 (1 μM) was added 2 min prior to norepinephrine. The final incubations with various concentrations of norepinephrine were carried out for 10 min. Values represent means ± standard errors for four separate experiments.

TABLE 1

Effects of ZK 62711 and Ro 20-1724 on accumulation of cyclic AMP elicited by amines and adenosine in rat cerebral cortical slices

Slices were first incubated for a total of 70 min (see MATERIALS AND METHODS). Either ZK 62711 or Ro 20-1724 was added 2 min prior to the amine or adenosine. The final incubations were then carried out for 10 min. Values represent the means ± standard errors for five separate experiments.

Additions	Cyclic AMP			
	Control	ZK 62711		Ro 20-1724 (100 μM)
		1 μM	100 μM	
	pmoles/mg protein			
Basal (no additions)	12.1 ± 1.8	28.0 ± 4.5	68.0 ± 6.7	48.9 ± 4.0
Norepinephrine (100 μM)	166.0 ± 25.2	264.0 ± 29.2	325.1 ± 40.3	242.7 ± 30.6
Adenosine (100 μM)	188.6 ± 22.2	224.0 ± 24.8	297.0 ± 27.8	183.0 ± 15.0
Isoproterenol (10 μM)	59.1 ± 3.9	131.7 ± 23.4	163.9 ± 9.7	131.9 ± 18.1
Histamine (100 μM)	24.2 ± 3.5	65.9 ± 5.0	104.2 ± 11.4	86.6 ± 18.9
Dopamine (100 μM)	21.8 ± 2.4		82.2 ± 7.4	

TABLE 2

Effects of ZK 62711 and Ro 20-1724 on accumulation of cyclic GMP elicited by norepinephrine and adenosine in rat cerebral cortical slices

Slices from four of the experiments of Table 1 were assayed for cyclic GMP. Values are means ± standard errors.

Additions	Cyclic GMP		
	Control	ZK 62711 (100 μM)	Ro 20-1724 (100 μM)
	pmoles/mg protein		
Basal (no additions)	0.29 ± 0.07	0.41 ± 0.09	0.39 ± 0.05
Norepinephrine (100 μM)	0.61 ± 0.12	0.53 ± 0.07	0.53 ± 0.08
Adenosine (100 μM)	0.54 ± 0.07	0.51 ± 0.07	0.48 ± 0.11

GMP in rat cerebral cortical slices after incubation for a shorter period were reported by Palmer and Duszynski (31) to be many times higher than the present results. In the presence of 100 μM ZK 62711 or Ro 20-1724, basal levels of cyclic GMP in cortical slices tended to increase, but the increases were not statistically significant (Table 2). In the presence of the phosphodiesterase inhibitors, neither norepinephrine nor adenosine had any significant effect on cyclic GMP levels.

In rat cerebellar slices, 10 and 100 μM ZK 62711 increased basal levels of cyclic AMP by 2- and 5-fold, respectively, over basal values. Ro 20-1724 at 10 μM caused a 3-fold increase in basal levels. Neither compound had any effect on basal levels of cyclic GMP.

The depolarizing agent veratridine elicits significant accumulations of both cyclic AMP and cyclic GMP in cerebellar slices from mouse (23, 32) and guinea pig (13). The accumulations of cyclic AMP elicited by veratridine in brain slices appear to involve depolarization and a resultant increase in release of adenosine (29, 33, 34). The accumulation of cyclic GMP elicited by veratridine in cerebellar slices does not appear to involve depolarization-evoked "release" of adenosine, and the mechanism remains unknown (32, 35). Because of the marked effects of veratridine on both cyclic AMP and cyclic GMP levels in cerebellar slices, it was the stimulatory agent of choice for studies of the effects of ZK 62711 and Ro 20-1724 on stimulation of both cyclic AMP- and cyclic GMP-generating systems.

ZK 62711 at 0.1–10μM elicited about a 2-fold increase in the accumulation of cyclic AMP produced by veratridine, while at 100 μM ZK 62711 an even larger accumulation of cyclic AMP occurred (Table 3). The response of cyclic AMP-generating systems to a combination of 100 μM Ro 20-1724 and veratridine was much smaller than the response to a combination of 100 μM ZK

TABLE 3

Effects of ZK 62711 and Ro 20-1724 on accumulations of cyclic nucleotides elicited by veratridine in rat cerebellar slices

Slices were first incubated for 40 min, divided into separate portions, and incubated for an additional 60 min (see MATERIALS AND METHODS). ZK 62711 or Ro 20-1724 was added 2 min prior to veratridine. The final incubations were carried out for 10 min. Values represent means ± standard errors for four separate experiments.

Additions	Cyclic AMP	Cyclic GMP
	pmoles/mg protein	
Basal (no additions)	62.8 ± 2.7	18.7 ± 3.8
ZK 62711 (0.1 μM)	66.6 ± 14.2	14.6 ± 3.9
ZK 62711 (1 μM)	75.4 ± 10.5	16.7 ± 2.3
ZK 62711 (10 μM)	128.2 ± 13.3	19.5 ± 4.6
ZK 62711 (100 μM)	317.6 ± 42.4	16.7 ± 3.6
Ro 20-1724 (100 μM)	192.3 ± 42.6	21.9 ± 6.7
Veratridine (50 μM)	191.8 ± 13.3	51.3 ± 8.0
ZK 62711 (0.1 μM)	370.2 ± 19.3	66.1 ± 14.7
ZK 62711 (1 μM)	360.2 ± 30.6	59.9 ± 6.7
ZK 62711 (10 μM)	373.6 ± 38.3	66.3 ± 6.7
ZK 62711 (100 μM)	711.6 ± 64.0	101.5 ± 17.0
Ro 20-1724 (100 μM)	458.3 ± 78.4	78.1 ± 12.1

62711 and veratridine, and indeed was only slightly greater than additive; i.e., no significant enhancement of the veratridine component by Ro 20-1724 was found.

Concentrations of ZK 62711 of 0.1–10 μM had no significant effect on veratridine-elicited accumulations of cyclic GMP in rat cerebellar slices (Table 3). Only at 100 μM did ZK 62711 significantly enhance the response to veratridine. Ro 20-1724 at 100 μM slightly enhanced the accumulation of cyclic GMP elicited by veratridine.

The results with brain slices suggested that ZK 62711 had potent inhibitory effects on cyclic AMP phosphodiesterases, perhaps greater at low levels of cyclic AMP, and, in addition, had weaker inhibitory effects on cyclic GMP phosphodiesterases. The effects of ZK 62711 and Ro 20-1724 on soluble and particulate phoshodiesterases from rat cerebrum were therefore investigated (Table 4). The calcium-dependent activator protein and calcium ions were added to ensure that the major phosphodiesterase activity measured in the preparations would be the calcium-dependent phosphodiesterase (see refs. 2, 27, 36, 37). In both rat cerebral cortex and rat cerebellum the major phosphodiesterase isozyme is calcium-dependent (2, 16, 38, 39).

Both ZK 62711 and Ro 20-1724 at 1 μM inhibited the hydrolysis of cyclic AMP by soluble and particulate phosphodiesterases from rat cerebrum, while having virtually no effect on the hydrolysis of cyclic GMP (Table 4). At 100 μM both ZK 62711 and Ro 20-1724 inhibited the hydrolysis of cyclic AMP by soluble and particulate brain phosphodiesterases by about 50%, and inhibition of cyclic GMP hydrolysis was significant. Ro 20-1724 has been reported to inhibit the hydrolysis of cyclic AMP but not cyclic GMP in homogenates from neuroblastoma cells and brain tissue (40, 41).

In view of the apparent somewhat similar potency of ZK 62711 and Ro 20-1724 toward the inhibition of phosphosphodiesterases in cell-free preparations (Table 4) and the nearly 100-fold greater potency of

TABLE 4

Effects of ZK 62711 and Ro 20-1724 on hydrolysis of cyclic AMP and cyclic GMP by phosphodiesterases of soluble and particulate preparations of rat brain homogenates

Phosphodiesterase activities were assayed at 1 μM substrate concentration in the presence of 1 μg of activator protein and 0.04 mM calcium ions (see MATERIALS AND METHODS). The control activities of the cyclic AMP phosphodiesterase were 2.0 nmoles/mg of protein per minute for the soluble preparation and 1.5 nmoles/mg of protein per minute for the particulate preparation. The control activities of the cyclic GMP phosphodiesterase were 8.9 nmoles/mg of protein per minute for the soluble preparation and 3.2 nmoles/mg of protein per minute for the particulate preparation. The enzyme activities are expressed as percentage inhibition relative to the control. Each value is the mean of triplicate determinations.

Additions	Inhibition	
	Cyclic AMP	Cyclic GMP
	%	%
Soluble preparation		
ZK 62711 (1 μM)	35.8	5.0
ZK 62711 (100 μM)	56.0	30.7
Ro 20-1724 (1 μM)	12.0	5.9
Ro 20-1724 (100 μM)	50.0	23.4
Particulate preparation		
ZK 62711 (1 μM)	23.1	5.5
ZK 62711 (100 μM)	55.0	28.4
Ro 20-1724 (1 μM)	32.8	5.9
Ro 20-1724 (100 μM)	56.0	45.3

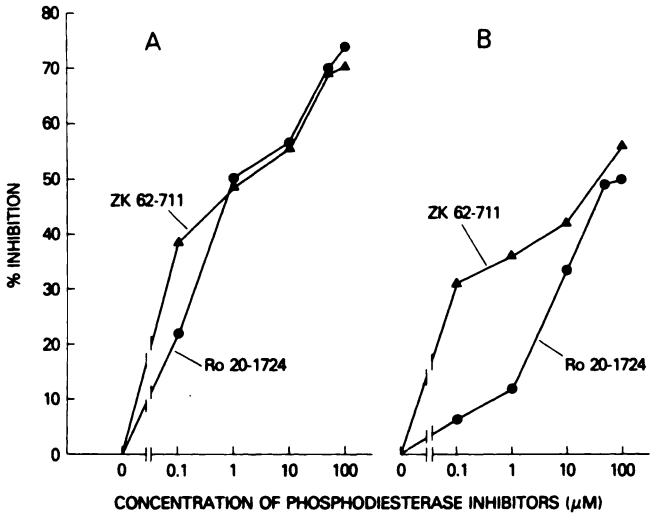

FIG. 6. *Effects of ZK 62711 and Ro 230-1724 on hydrolysis of cyclic AMP by phosphodiesterases from rat cerebrum in the presence of EGTA (A) or calcium ions and calcium-dependent activator protein (B)*

Phosphodiesterase activities of the soluble fraction from rat cerebrum (see MATERIALS AND METHODS) were assayed at 1 μM cyclic AMP in the presence of either 2 mM EGTA (A) or 1 μg of activator protein and 40 μM calcium ions (B). The control activity with EGTA was 1.27 nmoles/mg of protein per minute, while the control activity in the presence of activator and calcium ions was 2.01 nmoles/mg of protein per minute. The enzyme activities are expressed as percentage inhibition relative to the control. Each value is the mean of triplicate determinations.

ZK 62711 toward the potentiation of accumulations of cyclic AMP in rat cortical slices (Fig. 3), further experiments on inhibition of phosphodiesterases by the two compounds were performed (Fig. 6). With soluble rat cerebral phosphodiesterase, in the absence of added calcium-dependent activator protein, and with EGTA added, ZK 62711 appeared only marginally more potent than Ro 20-1724 as a phosphodiesterase inhibitor (Fig. 6A). However, in the presence of excess calcium-dependent activator protein and calcium ions, dose-response relationships revealed that ZK 62711 at low concentrations was nearly 100 times more potent than Ro 20-1724 as a phosphodiesterase inhibitor (Fig. 6B). Neither Ro 20-1724 nor ZK 62711 has structural features necessary for chelation of calcium ions.

DISCUSSION

The phenyl-2-pyrrolidone ZK 62711 is closely related in structure to the phosphodiesterase inhibitor Ro 20-1724 (Fig. 1). Both compounds have central depressant activity, and, as shown in the present paper, in brain preparations ZK 62711 shares the phosphodiesterase inhibitor activity of Ro 20-1724. In brain slices ZK 62711, based on marked enhancement of amine- and adenosine-elicited accumulations of cyclic AMP, appeared to be a potent phosphodiesterase inhibitor (Fig. 2 and Table 1). Indeed, ZK 62711 was nearly 100 times more potent than Ro 20-1724 in enhancing the response of cyclic AMP-generating systems to norepinephrine in rat cerebral cortical slices (Fig. 3). Unlike Ro 20-1724 (13), an enhanced adenosine component of cyclase activation appeared to be relatively unimportant to the effects of ZK 62711 on cyclic AMP levels, since ZK 62711 markedly increased basal and norepinephrine-stimulated levels of cyclic AMP even in the presence of theophylline or adenosine deaminase (Fig. 4). ZK 62711 appeared more potent in enhancing accumulations of cyclic AMP elicited by amines such as norepinephrine, isoproterenol, and histamine than in enhancing adenosine-elicited accumulations of cyclic AMP (Fig. 2 and Table 1).

In rat cerebral cortical slices, neither ZK 62711 nor Ro 20-1724 had a significant effect on basal levels of cyclic GMP, and actually reduced the norepinephrine- and adenosine-elicited accumulations of cyclic GMP (Table 2). Clearly, both compounds appeared to have selective effects on cyclic AMP- rather than cyclic GMP-generating systems.

In rat cerebellar slices both compounds increased basal levels of cyclic AMP, but neither compound had any effect on basal levels of cyclic GMP (Table 3). Somewhat higher concentrations of ZK 62711 appeared to be required to elevate basal levels of cyclic AMP in cerebellar slices as compared to cerebral cortical slices. Perhaps this is a reflection of the already high basal levels of cyclic AMP in cerebellar slices.

The responses of cyclic AMP-generating systems in rat cerebellar slices to veratridine were markedly potentiated by ZK 62711 at concentrations of 0.1–100 μM (Table 3). The accumulations of cyclic AMP elicited in brain slices by this depolarizing agent have been shown to involve, at least in part, depolarization-elicited release of adenosine (8, 29, 33, 34), but release of other factors or direct effects of depolarization cannot be discounted. Clearly, however, ZK 62711, even at 100 μM, does not have sufficient local anesthetic activity to decrease the requisite depolarizing action of veratridine. Local anesthetics can completely block the response to veratridine (34). The response of cyclic AMP-generating systems to combinations of veratridine and 100 μM Ro 20-1724 were not significantly greater than additive, while the responses to combinations of veratridine and ZK 62711 were in all cases greater than additive. At 100 μM, both ZK 62711 and, to a lesser extent, Ro 20-1724 enhanced veratridine-elicited accumulations of cyclic GMP in rat cerebellar slices. Clearly, in cerebellum ZK 62711 was relatively impotent with regard to enhancement of responses of cyclic GMP-generating systems.

The results with brain slices provided indirect evidence that ZK 62711 was a potent inhibitor of phosphodiesterases associated with amine- and adenosine-responsive cyclic AMP-generating systems in intact cells of brain slices. Confirmation of this conclusion required an investigation of the effect of ZK 62711 on brain phosphodiesterases in cell-free preparations. The initial investigation of ZK 62711 with phosphodiesterases revealed it to be an inhibitor that resembled Ro 20-1724 in its potency and selectivity toward cyclic AMP phosphodiesterases rather than cyclic GMP phosphodiesterases (Table 4; cf. refs. 41, 40). Soluble phosphodiesterase from rat cerebrum did appear to be inhibited somewhat more effectively by low concentrations of ZK 62711 than by Ro 20-1724, but this was not true of the particulate enzyme. Dose-response relationships for inhibition of phosphodiesterases from rat brain by ZK 62711 and Ro 20-1724 revealed that in the presence of EGTA ZK 62711 was only marginally more potent than Ro 20-1724 (Fig. 6A). However, in the presence of added calcium-dependent activator protein and calcium ions, ZK 62711 at low concentrations was nearly 100 times more potent as a phosphodiesterase inhibitor than Ro 20-1724 (Fig. 6B). The 100-fold difference in potencies of the two compounds as inhibitors of calcium-dependent cyclic AMP phosphodiesterase mirrors the 100-fold difference in potencies toward enhancement of norepinephrine-stimulated accumulation of cyclic AMP in brain slices. The parallelism between the potencies of ZK 62711 with cell-free, calcium-dependent phosphodiesterases and with intact cell preparations strongly suggests that the potentiation of amine responses of cyclic AMP-generating systems by ZK 62711 is due to inhibition of a calcium-dependent phosphodiesterase associated with amine-responsive cyclases. It is also tempting to speculate that the apparent lower potency of ZK 62711 in brain slices in the presence of adenosine is due to the involvement of another phosphodiesterase with adenosine-activated systems. Further studies with ZK 62711 on high- and low-K_m phosphodiesterase activity and on isozymes from brain are clearly needed. The present data, however, reveal ZK 62711 to be the first member of a new class of phosphodiesterase inhibitors related in

structure to Ro 20-1724, but, at least with respect to calcium-dependent cyclic AMP phosphodiesterase activity from brain, much more potent. The similar high potencies of ZK 62711 with both cell-free and intact cell systems from brain and its lack of apparent effects on "release" of adenosine or inhibition of responses to adenosine suggest it to be the agent of choice for study of the role of phosphodiesterases in control of accumulations of cyclic AMP in the central nervous system.

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