Biochemical Pharmacology, Vol. 33, No. 8, pp. 1205–1212, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

1-(4-AMINOPHENYL)ISOQUINOLINE DERIVATIVES

POTENT INHIBITORS OF CALCIUM-INDEPENDENT AND CALCIUM-DEPENDENT PHOSPHODIESTERASES FROM RAT CEREBRAL CORTEX

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(*Received* 15 *February* 1983; *accepted* 21 *September* 1983)

Abstract—The effects of a series of 1-(4-aminophenyl)isoquinoline derivatives on the activity of calcium-independent and calcium-dependent phosphodiesterases purified from rat cerebral cortex were examined. Agents were approximately equipotent (IC$_{50}$ values, 0.2 to 25 μM) in inhibiting the calcium-dependent hydrolysis of either cyclic AMP or cyclic GMP, while they were 6–35 times more effective as inhibitors of cyclic AMP hydrolysis when compared to cyclic GMP hydrolysis using the calcium-independent enzyme. The diastereomers of 3-(carbomethoxy)propenamido demonstrated a marked difference in specificity. The *cis*-isomer was very potent in inhibiting cyclic AMP or cyclic GMP hydrolysis by either enzyme (IC$_{50}$ values, 0.2 to 8 μM) while the *trans*-isomer was only effective in inhibiting calcium-independent cyclic AMP hydrolysis (IC$_{50}$ values, 2.5 μM). Kinetic analyses of the type of inhibition of the calcium-dependent enzyme revealed that the various agents were competitive inhibitors of cyclic GMP hydrolysis and noncompetitive inhibitors of cyclic AMP hydrolysis. A reverse pattern of inhibition by the isoquinoline derivatives was found using the calcium-independent phosphodiesterase, i.e. noncompetitive inhibition of cyclic GMP while competitive inhibition of cyclic AMP. Inhibition of phosphodiesterases by these agents was also manifest using intact brain slices prepared from rat cerebral cortex. Thus, the agents were found to potentiate forskolin-elicited accumulations of cyclic AMP by 100–700% and increased the half-time for the decline in cyclic AMP following forskolin stimulation from 3 to 6 min.

The presence of both cyclic AMP and cyclic GMP in nervous tissue, and the fact that intracellular levels of each can be altered by putative neurotransmitters and by conditions that modify overt behavior, strongly suggest a functional role for cyclic nucleotides in the central nervous system (for review see Ref. 1). The hydrolytic cleavage of the cyclic nucleotides to their corresponding 5′-nucleotides by 3′,5′-cyclic nucleotide phosphodiesterases is generally accepted as the most important physiological mechanism for terminating their actions. Multiple forms of the enzyme have been described in brain [2–6], as well as other tissues [2, 7]. Both calcium-dependent and calcium-independent enzymes are present in rat brain [6, 8]. The calcium-dependent phosphodiesterases are stimulated by calcium ions only in the presence of a thermostable, low molecular weight protein termed calmodulin [9–12].

In view of the central importance of cyclic nucleotide phosphodiesterases and their potential for selective regulation which may more precisely define the biological role of cyclic nucleotides, extensive investigations into the selective alteration of cyclic nucleotide phosphodiesterase activity by numerous agents have been conducted [13–16]. Among these agents, the alkaloid papaverine has been shown to be relatively potent and more selective for inhibition of the calcium-independent enzyme from brain than the calcium-dependent phosphodiesterase [6, 13, 17, 18]. Recently, a series of papaverine analogues which incorporate alkylating moieties on the molecule have been synthesized [19]. The present investigation examines the effects of a series of these 1-(4-aminophenyl)isoquinolines on the activity of calcium-independent and calcium-dependent phosphodiesterases from rat cerebral cortex.

EXPERIMENTAL

Materials. Cyclic [8-^{3}H]GMP (19.7 Ci/mmole) and cyclic [8-^{3}H]AMP (28 Ci/mmole) were from Amersham, Arlington Heights, IL; polyacrylamide-boronate affinity gel (Affi-Gel 601, Lot No. 22470) was from Bio-Rad, Rockville Centre, NY; and papaverine was from the Sigma Chemical Co., St. Louis, MO. The various 1-(4-aminophenyl)isoquinolines (structures depicted in Fig. 1) were synthesized by

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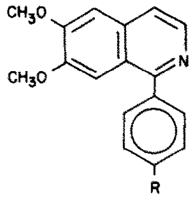

	R=
KW 1:	NH_2
KW 2:	$NHCH_2CH_2Cl$
KW 3:	$N(CH_2CH_2Cl)_2$
KW 4:	$NHCOCH_2Cl$
KW 5:	$NHCOCH{=}CH_2$
KW 6:	$NHCOCH{=}CHCOOCH_3$ (cis)
KW 7:	$NHCOCH{=}CHCOOCH_3$ (trans)

Fig. 1. Structures of -phenyl-6,7-dimethoxyisoquinolines.

Ms. Kathleen Walker as described previously [19]. Other reagents were analytical grade.

Enzyme preparations. All experiments were performed using mature male Sprague–Dawley rats (250–300 g). The procedure for separation of the Ca^{2+}-independent and Ca^{2+}-dependent phosphodiesterases from rat cerebral cortex was essentially that described by Kakiuchi *et al.* [6]. Briefly, animals were decaptitated, and the brains were immediately excised and rinsed in ice-cold 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM $MgCl_2$/3 mM 2-mercaptoethanol/0.1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*-tetracetic acid (EGTA) (extraction buffer). The tissue (3–5 g) was homogenized in 2 vol. of the same buffer using a motor-driven glass–Teflon homogenizer. The homogenate was centrifuged at 105,000 *g* for 60 min, and the supernatant fluid (crude extract) was filtered through glass wool to remove the fat.

An aliquot (3–6 ml) of the crude extract was applied to a Sephadex G-200 column (2.5 × 40 cm) previously equilibrated with extraction buffer containing 0.1 M NaCl. The column was eluted with the same buffer. The enzyme activity eluting at a V_e/V_0 of 1.0 represented a high K_m Ca^{2+}-independent phosphodiesterase. Peak enzyme activity eluting at a V_e/V_0 of 1.5 was used as the source of the Ca^{2+}-dependent phosphodiesterase. This enzyme was not inhibited by EGTA, indicating the absence of significant amounts of calmodulin, and could be stimulated 4- to 6-fold in the presence of both calcium ions and calmodulin as described below.

Further purification of the Ca^{2+}-dependent enzyme was achieved using calmodulin-Sepharose affinity chromatography as described by Miyake *et al.* [20]. The Ca^{2+}-dependent enzyme was applied to the affinity column (1.5 × 11 cm) previously equilibrated with 20 mM Tris–HCl buffer (pH 7.5), containing 1 mM $MgCl_2$/3 mM 2-mercaptoethanol/1 mM $CaCl_2$. The column was washed with 2.5 to 3 vol. of the same buffer and then eluted with 3 column vol. of extraction buffer. An aliquot of each fraction was assayed for phosphodiesterase activity in the absence and presence of calmodulin as described below.

The Ca^{2+}-independent phosphodiesterase was similarly chromatographed on the calmodulin-Sepharose affinity column for the purposes of removal of trace amounts (less than 0.5%) of contamination by the Ca^{2+}-dependent enzyme. Enzyme activity eluted by 1 mM $CaCl_2$ was unaffected by EGTA or calmodulin and was used as the source of the Ca^{2+}-independent phosphodiesterase for most of the experiments in the present investigation.

Phosphodiesterase assay. Phosphodiesterase activity was assayed by the method of Davis and Daly [21], using polyacrylamide-boronate affinity chromatography. The standard reaction mixture contained in a final volume of 0.1 ml: 5 μmoles Tris–HCl buffer (pH 7.5); 0.5 μmole $MgCl_2$; and appropriate concentrations of either cyclic [8-^{3}H]AMP or cyclic [8-^{3}H]GMP. The reaction was carried out at 37° and terminated by heating the mixture at 95–100° for 2 min. Unreacted cyclic nucleotides were separated from 5′-nucleotides using polyacrylamide-boronate affinity gel chromatography. Briefly, an aliquot (0.4 ml) of 0.1 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer (pH 8.5) containing 0.1 M NaCl (Hepes–NaCl) was added to each assay tube. For the purpose of determining recovery, 200 nmoles of the appropriate nonradioactive 5′-nucleotide was included in the 0.4-ml aliquot of Hepes–NaCl. The contents of the assay tube were applied onto polyacrylamide boronate gel columns (0.7 × 1.5 cm), and the unreacted cyclic nucleotides were eluted using 4.5 ml Hepes–NaCl. The 5′-nucleotides were eluted with 7 ml of 0.05 M sodium acetate buffer (pH 4.8), and an aliquot (1 ml) of the eluate was counted in a liquid scintillation spectrometer. Recovery (70–80%) of the product was determined by measuring the absorbance at 258 nm of the remaining (6 ml) sodium acetate eluate. Appropriate amounts of enzyme and appropriate incubation times were used so as to limit hydrolysis of substrate to less than 25% under the assay conditions. In experiments where calmodulin was used, Ca^{2+} (0.01 μmole) was also included in the assay mixture. The amount of partially purified calmodulin included was the lowest amount (100 ng) producing maximum stimulation (4- to 6-fold) of calcium-dependent activity under the assay conditions. All assays were performed in triplicate with a variation of less than 8%. Kinetic analyses were performed using double-reciprocal plots with the slope and intercept determined by linear least-squares analysis. One unit of enzyme activity is defined as that amount of activity that hydrolyzes 1 pmole of cyclic nucleotide/min under the assay conditions. All agents were dissolved in water, except the diastereomers of 1-[4′-(3-carbomethoxy)-propenamido]isoquinoline which were dissolved in 10% ethanol. The final concentration of ethanol (0.1%) in the assay did not affect significantly the activity of the enzymes. None of the agents was found to alter the elution profile of nucleotides and nucleosides using the polyacrylamide-boronate resin.

Preparation of brain slices. Rats were decapitated, and the brains were immediately excised, chilled in ice-cold Krebs–Ringer bicarbonate glucose buffer (KRBG), and place on a chilled plate; longitudinal strips of cerebral cortical grey matter were dissected free with a razor blade. Slices were prepared using a McIlwain tissue chopper set at 260 μm, and the slices were rapidly transferred to KRBG buffer gassed with 95% O_2–5% CO_2 at 37°. The slices were incubated in the buffer (30–50 ml/g slice) for 15 min, washed three times with fresh KRBG buffer, and were collected on polyester mesh and transferred to 15 ml of fresh KRBG buffer. After a 50-min incubation the slices were washed three times with 25-ml portions of buffer, collected on polyester mesh and transferred to 10 ml of incubation medium. Forskolin (20 μM) was added 5 min later, and the slices were allowed to incubate for an additional 15 min. In experiments where the time course for the degradation of cyclic AMP was assessed, the stimulated slices were washed under vacuum with 30–50 ml of prewarmed and pregassed KRBG buffer and then divided into four to six equal portions in separate beakers containing 10 ml of KRBG buffer and 8-phenyltheophylline (100 μM), and incubated in either the absence or presence of the various phosphodiesterase inhibitors. The slices were then collected on polyester mesh at various time points following the washing procedure, and the incubation was terminated by immersing the slices in liquid nitrogen. The first time point (time 0) was taken following the wash procedures which took 1 min. The frozen slices were then rapidly homogenized in 0.5 ml of 5 mM potassium phosphate buffer (pH 6.8) containing 320 mM NaCl, 5 mM EDTA, and 0.05 mM 3-isobutyl-1-methylxanthine (IBMX). The homogenate was centrifuged at 12,000 g using a Sorvall RC-5B refrigerated centrifuge for 15 min. The supernatant fraction was adjusted to 5% with trichloroacetic acid, and the total endogenous cyclic AMP was measured using the radioimmunoassay procedure of Steiner *et al.* [22].

Other methods. Calmodulin was prepared from rat cortex through the DEAE-cellulose step by the method of Lin *et al.* [23]. Protein was determined by the method of Lowry *et al.* [24] using bovine serum albumin as standard. Student's *t*-test was used for the statistical analysis of the data.

RESULTS

The effects of the *cis* and *trans* isomers of 1-[4′-(3-carbomethoxy)propenamido]phenyl-6,7-dimethoxyisoquinoline (KW-6 and KW-7 respectively) on the activity of the calcium-independent and calcium-dependent phosphodiesterases from rat cerebral cortex are depicted in Fig. 2. The *cis* isomer was 3 and 40 times more potent in inhibiting the calcium-dependent enzyme when compared to the calcium-independent phosphodiesterase using cyclic AMP and cyclic GMP respectively (Fig. 2A). In marked contrast, the *trans* isomer inhibited cyclic AMP hydrolysis by the calcium-independent enzyme while having little or no effect on the catalytic activities of either the calcium-independent phosphodiesterase using cyclic GMP or the calcium-dependent enzyme using either substrate (Fig. 2B). The inhibition of the calcium-dependent enzyme was rapid, occurring within 1 min, and remained constant for incubation times up to 7 min. When the *cis* isomer was added to an ongoing reaction, the inhibition was

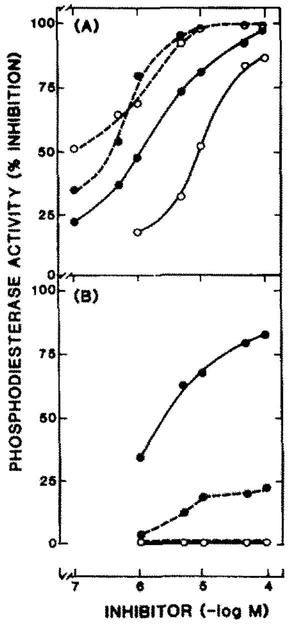

Fig. 2. Inhibition by the diastereomers of 1-[4′-(3-carbomethoxy)propenamido]-phenyl-6,7-dimethoxyisoquinoline (panel A, KW-6; and panel B, KW-7) of the calcium-independent (——) and calcium-dependent (– – –) phosphodiesterases from rat cerebral cortex. The enzymes were assayed using either 1 μM cyclic AMP (●) or 1 μM cyclic GMP (○) as described under Experimental Procedures. The calcium-dependent enzyme was assayed in the absence of Ca^{2+}/calmodulin. The activities and amounts of enzyme were as follows. Calcium-independent: cyclic AMP hydrolysis (2.16 units, 2.1 μg); cyclic GMP hydrolysis (2.41 units, 3 μg). Calcium-dependent: cyclic AMP hydrolysis (1.76 units, 0.9 μg); cyclic GMP hydrolysis (2.27 units, 0.1 μg).

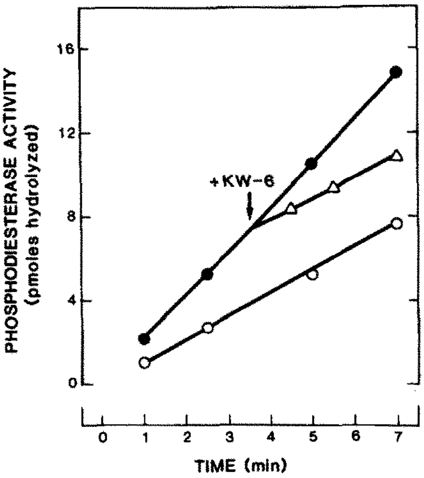

Fig. 3. Effect of KW-6 (1-[4′-*cis*(3-carbomethoxy)propenamido]-phenyl-6,7-dimethoxyisoquinoline on phosphodiesterase reaction rate. The calcium-dependent enzyme (0.09 μg) was assayed using 1 μM cyclic GMP as described under Experimental Procedures. The incubation was carried out in the absence (●) or the presence of 0.5 μM KW-6 which was added either at time zero (○) or 3.5 min subsequent to initiation of the reaction (△).

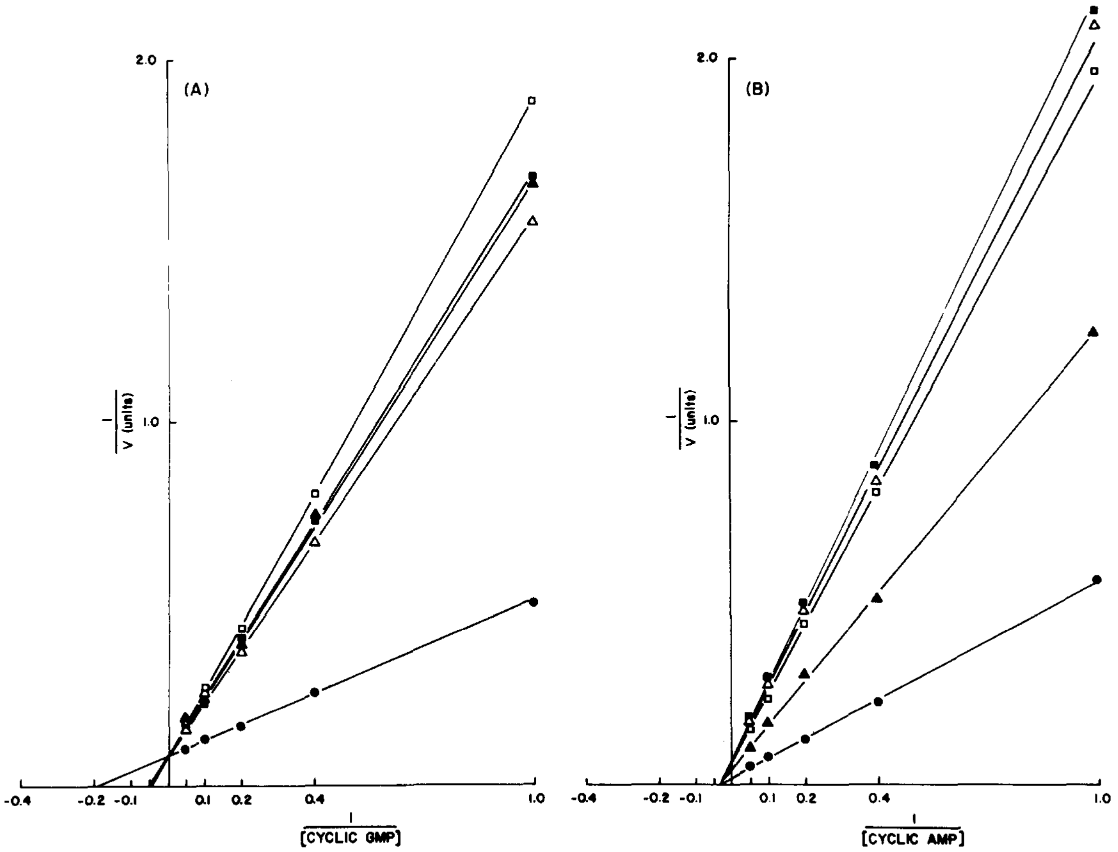

Fig. 4. Double-reciprocal plots of the hydrolysis of cyclic GMP (A) or cyclic AMP (B) by the calcium-dependent phosphodiesterase. Assay conditions and enzyme amounts were as shown in Fig. 2. The substrate concentrations ranged from 1 to 20 μM. Reaction velocity and K_m for enzymes in the absence of added agents (●) were: (A) V_{max}, 11.1 units; K_m, 4.8 μM, and (B) V_{max}, 48 units; K_m, 56 μM. Key: KW-1, 1 μM (▲); KW-5, 40 μM (△); KW-6, 0.5 μM (■); and papaverine (□), 40 μM.

immediate and the reaction proceeded at a rate comparable to that observed when the agent was included at the initiation of hydrolysis (Fig. 3). Similar results were observed for the inhibition of the calcium-independent enzyme (data not shown).

Table 1 summarizes the effect of a series of -phenyl-6,7-dimethoxyisoquinolines on the hydrolysis of cyclic AMP or cyclic GMP by the calcium-independent phosphodiesterase. All the agents were approximately equipotent (IC_{50} values, 1 to 2.5 μM) in inhibiting the hydrolysis of cyclic AMP by the calcium-independent phosphodiesterase, while the relative potency for inhibiting cyclic GMP hydrolysis varied considerably (IC_{50} values, 8 to $\gg$100 μM). In all instances the agents were found to be much more selective for inhibiting the hydrolysis of cyclic AMP when compared to cyclic GMP with the 1-[4′-*bis*(2-chloroethyl)amino] (KW-3) and 1-[4′-*trans* (3-carbomethoxy)propenamido] (KW-7) derivatives at least 33 times more effective in this regard.

With the exception of the *trans* isomer of 1-[4′-(3-carbomethoxy)propenamido], the 6,7-dimethoxyisoquinoline derivatives were 2–40 times more potent in inhibiting the hydrolysis of cyclic GMP by the calcium-dependent phosphodiesterase when compared to the calcium-independent enzyme (Table 1). Little or no differential sensitivity for either cyclic AMP or cyclic GMP hydrolysis by the calcium-dependent enzyme was observed. However, within the series of agents a 200- to 400-fold variation in order of potency was exhibited. No significant difference in the degree of enzyme inhibition was detected when the calcium-dependent activity was assessed in the presence of maximally activating concentrations of Ca^{2+} and calmodulin (data not shown).

Kinetic analyses of the inhibition of either cyclic AMP or cyclic GMP hydrolysis by the calcium-dependent enzyme are shown in Fig. 4 and summarized in Table 2. Each of the agents was found to inhibit the calcium-dependent enzyme noncompetitively using cyclic AMP or competitively using cyclic GMP as substrate. The K_i values were qualitatively and quantitatively identical to the IC_{50} values (Table 1). The inverse type of inhibition was observed using the calcium-independent phosphodiesterase. Thus, cyclic GMP hydrolysis was inhibited in a noncompetitive fashion, while cyclic AMP hydrolysis was inhibited in a competitive manner (Table 2).

Because these agents contain alkylating moieties which may enable the formation of a covalent bond with a nucleophilic group adjacent to the site where the inhibitor may be reversibly complexed, a series

Table 1. Summary of the IC_{50} values for various derivatives of -phenyl-6,7-dimethoxyisoquinolines on the calcium-independent and calcium-dependent phosphodiesterases from rat cerebral cortex*

	IC_{50} (μM)					
	Calcium-independent phosphodiesterase			Calcium-dependent phosphodiesterase		
Isoquinoline derivative	Cyclic GMP	Cyclic AMP	Ratio	Cyclic GMP	Cyclic AMP	Ratio
KW-1: 1-[4′-amino]-	14	2.5	6	0.4	0.35	1
KW-2: 1-[4′-(2-chloroethyl)amino]-	28	1.6	17	2	1.8	1
KW-3: 1-[4′-bis(2-chloroethyl)amino]-	56	1.6	33	16	10	2
KW-4: 1-[4′-(2-chloroacetyl)amino]-	13	1.8	7	0.5	0.2	2.5
KW-5: 1-[4′-ethyleneamido]-	50	1.8	25	24	14	2
KW-6: 1-[4′-*cis*(3-carbomethoxy)propenamido]-	8	1.3	6	0.2	0.4	0.5
KW-7: 1-[4′-*trans*(3-carbomethoxy)propenamido]-	≫100	2.5	≫33	≫100	≫100	1
Papaverine	35	2.6	14	18	20	0.9

* Assay conditions were described in the legend of Fig. 2 except for the variation in the agent used. IC_{50} is defined as the concentrations (μM) of the compounds that inhibit 50% of the phosphodiesterase activity. Ratio: IC_{50} value for inhibition of cyclic GMP/IC_{50} value for inhibition of cyclic AMP.

Table 2. Summary of inhibition by various 1-phenyl-6,7-dimethoxyisoquinoline derivatives of the calcium-independent and calcium-dependent enzymes from rat cerebral cortex*

	Ca^{2+}-independent phosphodiesterase				Ca^{2+}-dependent phosphodiesterase			
	Cyclic GMP		Cyclic AMP		Cyclic GMP		Cyclic AMP	
Isoqunoline derivative	K_i	Type of inhibition	K_i	Type of inhibition	K_i	Type of inhibition	K_i	Type of inhibition
KW-1: 1-[4′-amino]-	14	NC	1.1	C	0.4	C	0.9	NC
KW-2: 1-[4′-(2-chloroethyl)amino]-	ND		ND		ND		ND	
KW-3: 1-[4′-bis(2-chloroethyl)amino]-	ND		ND		ND		ND	
KW-4: 1-[4′-(2-chloroacetyl)amino]-	14	NC	2.1	C	0.6	C	0.5	NC
KW-5: 1-[4′-ethyleneamido]-	35	NC	3.0	C	16	C	28	NC
KW-6: 1-[4′-*cis*(3-carbomethoxy)propenamido]-	9	NC	5.5	C	0.1	C	0.3	NC
KW-7: 1-[4′-*trans*(3-carbomethyoxy)propenamido]-	ND		4.6	C	ND		ND	
Papaverine	19	NC	6.0	C	10.5	C	31	NC

* Enzymes were assayed in the absence or presence of various derivatives in concentrations which inhibit 30–70% of the enzyme activity. Analysis of the data was made using double-reciprocal plots. The concentration of either cyclic GMP or cyclic AMP ranged from 1 to 20 μM. The amounts of the enzymes using either substrate were as indicated in the legend of Fig. 2. Key: C, competitive inhibition; NC, noncompetitive inhibition; and ND, not determined.

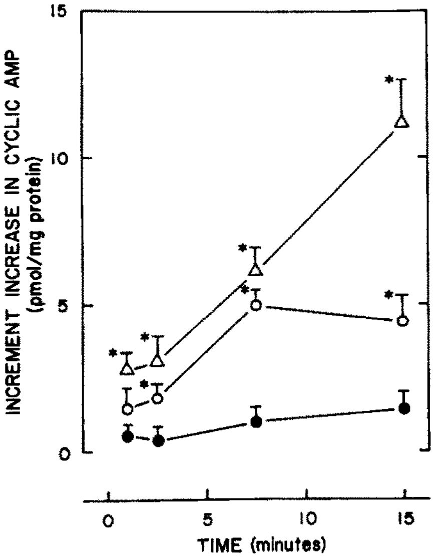

Fig. 5. Effects of 1-phenyl-6,7-dimethoxyisoquinolines on the accumulation of cyclic AMP elicited by forskolin in rat cerebral cortical slices. Rat cerebral slices were prepared as described under Experimental Procedures. Prior to stimulation of cyclic AMP accumulations by forskolin (5 μM), the slices were preincubated for 10 min with 8-phenyltheophylline (100 μM) either in the absence (●) or the presence of KW-4 (△, 20 μM) or KW-6 (○, 20 μM). Increment increase in cyclic AMP was calculated by taking the difference between cyclic AMP levels immediately prior to addition of forskolin and the value obtained at subsequent time points. The data shown are the means (±S.E.) of the values from four to eight separate experiments. Basal cyclic AMP values (pmoles/mg protein) in the absence or presence of KW-4 or KW-6 were 5.3, 4.8, and 5.7 respectively. Key: (*) significantly different from control ($P < 0.05$).

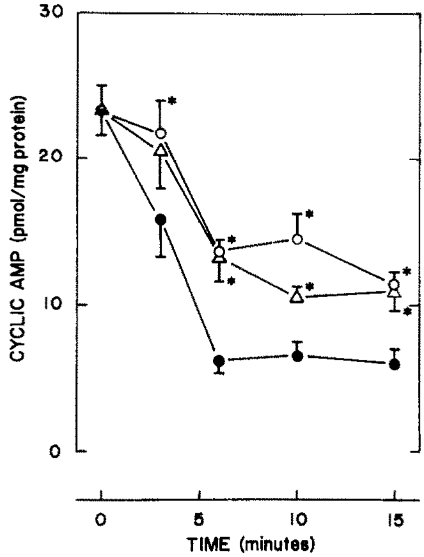

Fig. 6. Effects of 1-phenyl-6,7-dimethoxyisoquinolines on the rate of disappearance of cyclic AMP in rat cerebral cortical slices. Slices were prepared as described in Experimental Procedures and were incubated in the presence of forskolin (20 μM). The slices were collected on polyester mesh and washed under vacuum with KRBG buffer. Postincubation of these slices in 10 ml of KRBG buffer containing 8-phenyltheophylline (100 μM) was then carried out for various periods of time in the absence (●) or the presence of KW-1 (△, 20 μM) or KW-6 (○, 20 μM). Values represent the means ± S.E.M. from four to seven separate experiments. Basal cyclic AMP values (pmoles/mg protein) were 5.8 ± 0.3 for all time points. Key: (*) significantly different from control ($P < 0.05$).

of experiments were performed to determine the extent of reversibility of the inhibition by the agents. Agents (KW-4; KW-6; KW-7; IC_{50} concentrations) were preincubated with the calcium-dependent enzyme for 5–60 min at 37° and were dialyzed overnight against homogenization buffer. After dialysis, the enzyme preparation was assayed for activity, and no significant inhibition of activity was observed. Furthermore, enzymes preincubated with the agents under ultraviolet irradiation for 60 min and subsequently diluted to concentrations at which the inhibitors should have no reversible effect on enzyme activity displayed identical activity to that found for control preparations (data not shown).

The effects of these agents on forskolin-elicited accumulations of cyclic AMP using *in vitro* brain slices prepared from rat cerebral cortex are shown in Fig. 5. Forskolin (5 μM) increased cyclic AMP levels only slightly above basal (5.3 pmoles/mg protein) levels after 15 min. At any time point the 4'-(2-chloroacetyl)amino (KW-4) and 4'-*cis*(3-carbomethoxy)propenamido (KW-6) derivatives potentiated forskolin-elicited accumulation of cyclic AMP by 100–700%, with the latter being more potent in this regard. No significant effect on basal levels of cyclic AMP in brain slices preincubated for 20–25 min with either derivative in the absence of forskolin was observed.

The time course for disappearance of cyclic AMP which had been elevated previously by forskolin 20 μM) using rat cerebral cortical slices was examined in the presence and absence of these agents. Both the 4'-amino (KW-1) and 4'-*cis*(3-carbomethoxy)-propenamido (KW-6) derivatives retarded the subsequent return of total cyclic AMP toward basal levels (5.8 pmoles/mg protein) with the half-time for decline doubling from 3 to 6 min (Fig. 6). The levels of cyclic AMP were found to plateau in the presence of either agent with values at 10–15 min about 2 to 2.5 times higher than in their absence.

DISCUSSION

It has been proposed that active-site directed inhibition of an enzyme may be possible by the addition of a moiety which could form a covalent bond with a nucleophilic group on the enzyme adjacent to the site where the inhibitor is reversibly complexed [25]. Papaverine has been shown to be an inhibitor of cyclic nucleotide phosphodiesterase activity in brain [6, 17, 18] as well as in other tissues [26–28]. In the present investigation, chemical homologs of papaverine displayed a differential sensitivity for the calcium-independent and calcium-dependent enzymes from rat cerebral cortex between both the substrate, using the same enzyme preparation, and the two enzymes using the same substrate. While the agents were approximately equipotent in inhibiting the calcium-dependent hydrolysis of either cyclic AMP or cyclic GMP, they were 6–35 times more effective as inhibitors of cyclic AMP hydrolysis when compared to cyclic GMP hydrolysis by the calcium-independent phosphodiesterase. With the exception of the *trans* isomer of 3-(carbomethoxy)propenamido (KW-7), all the agents were 2–40 times more potent as inhibitors of cyclic GMP hydrolysis by the calcium-dependent enzyme when compared to the calcium-independent phosphodiesterase. Weiss *et al.* [13] has shown that papaverine was much more potent as an inhibitor of calcium-independent isozymes from rat cerebrum than the calcium-dependent phosphodiesterase. Some of the agents in the present study were found to display a similar selectivity with the specificity in increasing degree being: *trans*-(3-carbomethoxy)propenamido (KW-7) $\gg$ ethyleneamido (KW-5) > papaverine > *bis*-(2-chloroethyl)amino (KW-3).

A striking difference in inhibitory potency between the 3,4-dimethoxy-1-phenyl diastereomers of 4-(3-carbomethoxy)propenamido (KW-6, KW-7) for cyclic AMP and cyclic GMP hydrolyzing activity in crude extracts from rat cerebral cortex has been reported [19]. These results can be explained by the marked selectivity of the fumarinilate (*trans*) moiety (KW-7) when compared to the maleanilate (*cis*) moiety (KW-6) for the calcium-independent isozyme. While the *cis* structure binds effectively to an active site(s) on either enzyme, thereby inhibiting hydrolysis of both cyclic AMP and cyclic GMP, the *trans* structure would appear to interfere with the binding to all but the active site on the calcium-independent enzyme hydrolyzing cyclic AMP. A similar specificity for the catalytic activity hydrolyzing cyclic AMP using the calcium-independent enzyme has been observed for 2′-deoxy cyclic AMP [29].

Kinetic analysis of the type of inhibition of the calcium-dependent enzyme revealed that all the agents examined inhibited the hydrolysis of cyclic GMP in a competitive manner, while inhibiting the hydrolysis of cyclic AMP in a noncompetitive fashion. A reverse pattern of inhibition by the isoquinoline derivatives was found using the calcium-independent phosphodiesterase, i.e. noncompetitive inhibition of cyclic GMP while competitive inhibition of cyclic AMP. These observations might be expected if the catalytic sites for each nucleotide were distinct. Multiple binding sites have been suggested for the calcium-dependent enzyme from bovine heart [30], a calcium-independent uterine phosphodiesterase [31], and the calcium-independent enzyme from rat cerebral cortex [29]. In contrast, single catalytic sites have been proposed for a high affinity cyclic AMP enzyme from dog kidney [32] and a calcium-dependent enzyme from bovine brain [33]. Klee *et al.* [34], using a homogeneous calcium-dependent phosphodiesterase from bovine brain, have shown that cyclic GMP competitively inhibits the hydrolysis of cyclic AMP but with a K_i value 4–8 times higher than its apparent K_m. Likewise the hydrolysis of cyclic GMP by the calcium-dependent enzyme from rat cerebral cortex was competitively inhibited by cyclic AMP with a K_i value (5.7 μM) approximately 5–10 times lower than its apparent K_m (C. W. Davis, unpublished observation). While the results of the present investigation, coupled with the discrepancies between apparent K_i and K_m values in alternate substrate experiments, tend to support the existence of multiple catalytic sites, further studies using more direct approaches for ascertaining binding site arrangements are required.

In addition to their potent inhibition of cyclic nucleotide phosphodiesterase activity in cell-free preparations, inhibition of phosphodiesterases in intact brain slices was manifest with respect to both the maximum accumulations elicited by forskolin and the subsequent rate of degradation of cyclic AMP in the absence of forskolin. Papaverine has been shown to be a potent inhibitor of the reuptake of adenosine into brain slices [35]. While the present investigation did not examine their effect on adenosine "metabolism", it seems unlikely that the result obtained could be accounted for by some potentiation of adenosine-mediated cyclic AMP formation since: (i) no significant alteration in basal levels of cyclic AMP in the absence of the isoquinoline derivatives was evident, and (ii) the concentration of 8-phenyltheophylline blocked completely the cyclic AMP generation by addition of exogenous adenosine (50 μM). It was noted that the rate of disappearance of cyclic AMP in the presence of the agents had a biphasic decline occurring more rapidly during the initial 5 min. A similar observation was reported for isobutylmethylxanthine, and it was suggested that this may represent an interaction with a two-compartment system [36]. Since the present investigation did not preincubate the slice preparations with the inhibitor during the initial stimulation with forskolin and all subsequent washing procedures, it seems possible that the biphasic decline may instead represent the time it takes for the isoquinolines to diffuse into the slices.

The various 1-(4-aminophenyl)isoquinolines represent a group of potent phosphodiesterase inhibitors. The marked selectivity of some of these agents, particularly the *cis* and *trans* isomers of 3-(carbomethoxy)propenamido, suggests their potential utility in assessing the physiological role(s) of cyclic nucleotides in the central nervous system. Furthermore, their access to intact brain slices, may allow for more precisely defining the functionally important cyclic nucleotide phosphodiesterases which regulate intracellular levels of cyclic AMP and cyclic GMP.

Acknowledgements—This research was supported by NSF Grant BNS 81-17808 and a grant from the Pharmaceutical Manufacturers. The authors wish to thank Kathy Long and Richard Daughenbaugh for their expert technical help, Cathy Cato and Jeri McClain for the typing of the manuscript, and Dr. Thomas Lincoln for constructive criticism and helpful discussions.

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