0026-895X/00/050967-09$3.00/0
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 58:967–975, 2000

Vol. 58, No. 5
152/861328
Printed in U.S.A.

Structure-Related Inhibition of Calmodulin-Dependent Neuronal Nitric-Oxide Synthase Activity by Melatonin and Synthetic Kynurenines

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Received March 15, 2000; accepted July 10, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

We recently described that melatonin and some kynurenines modulate the *N*-methyl-D-aspartate-dependent excitatory response in rat striatal neurons, an effect that could be related to their inhibition of nNOS. In this report, we studied the effect of melatonin and these kynurenines on nNOS activity in both rat striatal homogenate and purified rat brain nNOS. In homogenates of rat striatum, melatonin inhibits nNOS activity, whereas synthetic kynurenines act in a structure-related manner. Kynurenines carrying an NH_2 group in their benzenic ring (NH_2-kynurenines) inhibit nNOS activity more strongly than melatonin itself. However, kynurenines lacking the NH_2 group or with this group blocked do not affect enzyme activity. Kinetic analysis shows that melatonin and NH_2-kynurenines behave as noncompetitive inhibitors of nNOS. Using purified rat brain nNOS, we show that the inhibitory effect of melatonin and NH_2-kynurenines on the enzyme activity diminishes with increasing amounts of calmodulin in the incubation medium. However, changes in other nNOS cofactors such as FAD or H_4-biopterin, do not modify the drugs' response. These data suggest that calmodulin may be involved in the nNOS inhibition by these compounds. Studies with urea-polyacrylamide gel electrophoresis further support an interaction between melatonin and NH_2-kynurenines, but not kynurenines lacking the NH_2 group, with Ca^{2+}-calmodulin yielding Ca^{2+}-calmodulin-drug complexes that prevent nNOS activation. The results show that calmodulin is a target involved in the intracellular effects of melatonin and some melatonin-related kynurenines that may account, at least in part, for the neuroprotective properties of these compounds.

Activation of the *N*-methyl-D-aspartate (NMDA) receptor by glutamate causes a Ca^{2+} influx into cells, resulting in the formation of nitric oxide (NO) from L-arginine (Bredt et al., 1992). The enzyme involved in this pathway is a calmodulin (CaM)/Ca^{2+}/O_2^- and NADPH-dependent cytosolic nitric-oxide synthase (nNOS), which requires tetrahydrobiopterin (H_4-biopterin) for the expression of its activity (Knowles et al., 1989). The NO may act intracellularly or it may diffuse extracellularly to adjacent neurons, acting as a retrograde messenger (Garthwaite and Boulton, 1993).

NO modulates several processes in the central nervous system (CNS) such as pain perception, long term potentiation and memory, and cerebral blood flow (Garthwaite and Boulton, 1993). In addition, NO has been specifically implicated in the glutamate-dependent excitotoxicity and neuronal death (Dawson et al., 1991). Excessive activation of NMDA has been associated with a wide range of neurological disorders and neurodegenerative diseases, including hypoxic-ischemic brain injury, trauma, epilepsy, Parkinson's disease, Huntington's disease and AIDS dementia (Herrling, 1994). Thus, selective inhibition of nNOS may provide a novel therapeutic approach to these pathological situations.

Melatonin (aMT; *N*-acetyl-5-methoxytryptamine) exerts

This work was partially supported by grants from the Ministerio de Educación y Cultura (SAF98-0156), and from the Junta de Andalucía (CTS-101). J.L. and Ma.M. are a fellows from the Programa de Formación de Personal Investigador, Ministerio de Educación y Cultura, Spain; H.K. is a fellow from the Ministerio de Asuntos Exteriores, Spain; Mi.M. is a fellow from the Universidad de Granada.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; nNOS, neuronal nitric-oxide synthase; H_4-biopterin, 5,6,7,8-tetrahydro-L-biopterin dihydrocloride; CaM, calmodulin; aMT, melatonin; CNS, central nervous system; aMK, *N*-acetyl-5-methoxy-kynurenamine; DTT, DL-dithiothreitol; PAGE, polyacrylamide gel electrophoresis; inosine, hypoxantine-9-β-D-ribofuranosid; serotonin, 5-hydroxytryptamine; aM, 2-acetamide-4-(3-methoxyphenyl)-4-oxobutiric acid; aAM, 2-acetamide-4-(2-amine-5-methoxyphenyl)-4-oxobutiric acid; aDAM, 2-acetamide-4-(2-dimethylamine-5-methoxyphenyl)-4-oxobutiric acid; aBAM, 2-acetamide-4-(2-benzylamine-5-methoxyphenyl)-4-oxobutiric acid; bAM, 2-butyramide-4-(2-amine-5-methoxyphenyl)-4-oxobutiric acid; bM, 2-butyramide-4-(3-methoxyphenyl)-4-oxobutiric acid; CaCaM, calcio-calmodulin complex.

neuroprotective properties reflecting both antioxidant (Reiter et al., 1995, 1997, 1998) and inhibitory effects on the CNS (Acuña-Castroviejo et al., 1986, 1995; Gomar et al., 1994). Electrophysiological experiments have shown that in rats, the iontophoretic ejection of aMT attenuates the excitatory response of striatal neurons to sensorimotor cortex stimulation (Castillo-Romero et al., 1995; Escames et al., 1996). This excitatory response is mainly mediated by glutamate acting on the NMDA receptors (Alexander et al., 1986; Escames et al., 1996). It was suggested that some effects of aMT may be derived from some endogenous brain metabolites of the hormone such as *N*-acetyl-5-methoxy-kynurenamine (aMK; Hirata et al., 1974; Acuña-Castroviejo et al., 1994). Therefore, we showed that some synthetic kynurenines affect the excitatory response of striatal neurons in a structure-related manner. Further experiments proved that the NMDA-subtype of glutamate receptor is involved in these effects of aMT and related kynurenines (León et al., 1998a,b).

aMT binds CaM with high affinity (Benítez-King et al., 1993) and the binding is saturable, reversible, and Ca^{2+}-dependent. It was suggested that the inhibitory effect of aMT on nNOS activity in some brain areas (Bettahi et al., 1996; Pozo et al., 1997) might be produced by removing free cytosolic CaM through a CaM-aMT interaction (Pozo et al., 1997). Thus, we consider it worthwhile to investigate the effect and the mechanism of action of aMT and four synthetic kynurenines on nNOS activity in homogenates of rat striatum and in a commercially available purified nNOS from rat brain. Besides, experiments with urea-polyacrylamide gel electrophoresis (PAGE) were carried out to further assess the possible interaction of these compounds with CaM.

Experimental Procedures

Materials. L-Arginine, L-citrulline, HEPES, DL-dithiothreitol (DTT), leupeptin, aprotinin, pepstatin, phenylmethylsulfonyl fluoride, hypoxantine-9-β-D-ribofuranosid (inosine), EGTA, BSA, Dowex-50W (50 × 8–200) resin, FAD, NADPH, 5,6,7,8-tetrahydro-L-biopterin dihydrocloride (H_4-biopterin), bovine brain CaM (>98% SDS-PAGE), trifluoperazine, calmidazolium, 5-hydroxytryptamine (serotonin), and aMT were obtained from Sigma-Aldrich (Madrid, Spain). L-[^{3}H]Arginine (58 Ci/mmol) was obtained from Amersham (Amersham Pharmacia Biotech GmbH, Barcelona, Spain). Tris·HCl and calcium chloride were obtained from Merck. Purified rat brain nNOS (>98% SDS-PAGE) was obtained from Alexis Biochemicals (San Diego, CA). Six kynurenine derivatives were used: 2-acetamide-4-(3-methoxyphenyl)-4-oxobutiric acid (aM); 2-acetamide-4-(2-amine-5-methoxyphenyl)-4-oxobutiric acid (aAM); 2-acetamide-4-(2-dimethylamine-5-methoxyphenyl)-4-oxobutiric acid (aDAM); 2-acetamide-4-(2-benzylamine-5-methoxyphenyl)-4-oxobutiric acid (aBAM); 2-butyramide-4-(3-methoxyphenyl)-4-oxobutiric acid (bM), and 2-butyramide-4-(2-amine-5-methoxyphenyl)-4-oxobutiric acid (bAM). They were synthesized in the Department of Pharmacological Chemistry, University of Granada; their preparation and purification will be described elsewhere.

nNOS Activity Determination in Rat Striatum. Male Wistar rats (200–250 g) housed under a 12-h:12-h light/dark cycle and given free access to food and water were used. Animals were sacrificed by cervical dislocation and striata were quickly collected and immediately used to measure nNOS activity. Upon removal, tissues were cooled in an ice-cold buffer (25 mM Tris, 0.5 mM DTT, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, pH 7.6). Two striata were placed in 1.25 ml of the same buffer and sonicated (10 s × 6). The crude homogenate was centrifuged 5 min at 1000*g*, and an aliquot of the supernatant was frozen at −20°C for total protein determination with the Folin phenol reagent.

NOS activity was measured by the method of Bredt and Snyder (1989), monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. The final incubation volume was 100 μl and consisted of 10 μl of crude homogenate added to buffer to give a final concentration of 25 mM Tris, 1 mM DTT, 30 μM H_4-biopterin, 10 μM FAD, 0.5 mM inosine, 1 mg/ml BSA, 1 mM $CaCl_2$, 10 μM L-arginine, 50 nM L-[^{3}H]citrulline, at pH 7.6. When required, increasing concentrations of arginine (up to 10 μM) were also added to the incubation medium. The reaction was started by the addition of 10 μl of NADPH (0.75 mM final) and continued for 30 min at 37°C. Control incubations were done by omission of NADPH. The reaction was stopped by the addition of 400 μl of cold 0.1 M HEPES, 10 mM EGTA, 0.175 mg/ml L-citrulline, pH 5.5. The reaction mixture was decanted into a 2-ml column packed with Dowex-50W ion change resin (Na^+ form) and eluted with 1.2 ml water. L-[^{3}H]Citrulline was quantified by liquid scintillation spectroscopy. The retention of L-[^{3}H]arginine was greater than 98%. Enzymatic activity was determined subtracting the control value, usually less than 1% of the radioactivity added. The activity of nNOS was expressed as picomoles of L-[^{3}H]citrulline produced per milligram of protein per minute.

Purified Rat Brain nNOS Activity Determination. The activity of nNOS present in the commercial source was also measured by the method of Bredt and Snyder (1989). After resuspension of nNOS stock solution in 50 mM HEPES buffer, pH 7.4, aliquots (0.0125U, 0.35 μg of protein) were incubated by 15 min at 37°C in the presence of 15.5 μM $CaCl_2$, 30 μM H_4-biopterin, 10 μM FAD, 1 mg/ml BSA, 0.5 mM inosine, 10 μM L-arginine, 10 ng/ml CaM, 0.75 mM NADPH, and 50 nM L-[^{3}H]arginine, in a total volume of 100 μl. When required, increasing concentrations of L-arginine (0–10 μM), FAD (0.1–10 μM), H_4-biopterin (0.3–30 μM), CaM (0–10 ng/ml), and $CaCl_2$ (0–0.350 mM) were also added to the incubation medium. The reaction was started by the addition of NADPH. The other steps in the procedure were the same as described for nNOS activity determination in rat striatum.

Electrophoresis Studies. Urea-PAGE was done as described previously (Erickson-Vhtanen and De Grado, 1987). To study CaM electrophoretic migration, slab gels (0.75-mm thickness) of 15% polyacrylamide, 4 M urea, 375 mM Tris, pH 8.8, and 1 mM $CaCl_2$ or 2 mM EGTA, in the absence or presence of 1 mM of each compound studied (i.e., aMT, serotonin, or kynurenine derivatives) were run at constant voltage of 100 V. Electrode buffer contained 25 mM Tris, 192 mM glycine, pH 8.3, and 0.1 mM $CaCl_2$ or 2 mM EGTA, in the absence or presence of either 1 mM aMT, 1 mM serotonin, or 1 mM each kynurenine. Samples containing 10 μg of CaM in 100 mM Tris, 4 M urea, pH 7.2, and 0.1 mM $CaCl_2$ in the absence or presence of either 1 mM aMT, 1 mM serotonin, or 1 mM each kynurenine, were incubated at room temperature for 60 min, in a total volume of 50 μl. One half-volume of 50% glycerol with tracer bromphenol blue was added and samples were applied to wells. The gels were fixed in 43% (v/v) methanol/1.6 M acetic acid and stained with 0.1% (w/v) Coomassie brilliant blue R-250. When required, gels were scanned using the QuantiScan System software (Biosoft, Cambridge, UK).

Statistical Analysis. Data are expressed as the mean ± S.E.M. Statistics included two-way analysis of variance and a posthoc test to assess any significant difference between CaM concentrations.

Results

Fig. 1 shows the structure of the six synthetic kynurenines tested. It can be seen that the structure of aAM is the same as that of aM except for the presence of the NH_2 group in position R_1. Similarly, the structure of the bAM is the same as that of bM except for the amino group in position R_1. The structures of aDAM and aBAM are the same as those of aAM,

except that the NH_2 group is blocked by a dimethyl or a benzyl group, respectively.

In previous experiments, we found that aMT and kynurenines aAM and bAM inhibit nNOS activity in a dose-dependent manner (León et al., 1998a,b). The calculated IC_{50} values were aMT, >1 mM; aAM, 40.9 μM; and bAM, >1 mM. The other kynurenines (i.e., aM, bM, aDAM, and aBAM) did not affect nNOS activity at concentrations of up to 1 mM. To investigate the mechanism by which aMT and kynurenines aAM and bAM inhibit nNOS activity, rat striatal homogenates were incubated with each drug (1 mM) in the presence of increasing concentrations of L-arginine (0–10 μM). Striatal nNOS activity was saturable and proportional to substrate concentration (Fig. 2A). The activity of the enzyme, however, was significantly decreased in the presence of 1 mM aMT, aAM, or bAM, as assessed by the Lineweaver-Burk double-reciprocal analysis of the data (Fig. 2B). Although K_m values of control, aMT, aAM, and bAM (Table 1) were similar, the V_{max} values for these compounds were lower than the control. The results suggest that aMT and kynurenines aAM and bAM behave as noncompetitive inhibitors of nNOS activity.

We next tested for the possible existence of any interaction between aMT and the kynurenines aAM and bAM with nNOS cofactors. We used a commercially available purified rat nNOS. In preliminary experiments we found that concentrations of Ca^{2+} between 0 and 17.5 μM in the incubation medium did not affect [^{3}H]citrulline production by purified nNOS. Concentrations of Ca^{2+} above 17.5 μM produced a dose-related inhibition of [^{3}H]citrulline formation with this commercial source of purified nNOS (Fig. 3A). However, inclusion of 1 mM EGTA abolished nNOS activity, highlighting the Ca^{2+} dependence of this NOS isoform (Fig. 3B). The CaM dependence of nNOS activity in our conditions was assessed with the CaM antagonists trifluoperazine and calmidazolium. In the presence of 1 mM trifluoperazine or 100 nM calmidazolium, the activity of nNOS was significantly reduced at CaM concentrations of 0.1 and 10 μg/ml, whereas 1 mM aMT inhibited nNOS at 0.1 but not at 10 μg/ml of CaM (Fig. 4). Figure 4 shows that 100 nM calmidazolium significantly reduced nNOS activity at both concentrations of CaM. In light of these preliminary findings, the effect of aMT and kynurenines aAM and bAM on nNOS activity was investigated in an incubation medium containing 0.0125 U of purified nNOS, 17.5 μM $CaCl_2$, 10 μg/ml CaM, 10 μM FAD, and 30 M H_4-biopterin.

In absence of added CaM, aMT inhibited purified nNOS activity in a dose-dependent manner, the effect being significant at 1 nM ($P < .05$, Fig. 5A). The incorporation of increasing amounts of CaM into the incubation medium resulted in a progressive loss of aMT efficiency to inhibit nNOS (Table 2). At 10 μg/ml of CaM, aMT was unable to inhibit the enzyme activity. Fixing the CaM concentration at 0.1 μg/ml, different concentrations of FAD (0.1–10 μM) (Fig. 5B) or H_4-biopterin (0.3–30 μM) (Fig. 5C) into the incubation medium did not modify the enzyme inhibition by aMT.

Compound	R_1	R_2
aM	-H	$-CH_3$
aAM	$-NH_2$	$-CH_3$
aDAM	$-N(CH_3)_2$	$-CH_3$
aBAM	$-NHCH_2Ph$	$-CH_3$
bM	-H	$-C_3H_7$
bAM	$-NH_2$	$-C_3H_7$

Fig. 1. Molecular structure and location of the substituents in the kynurenine derivatives.

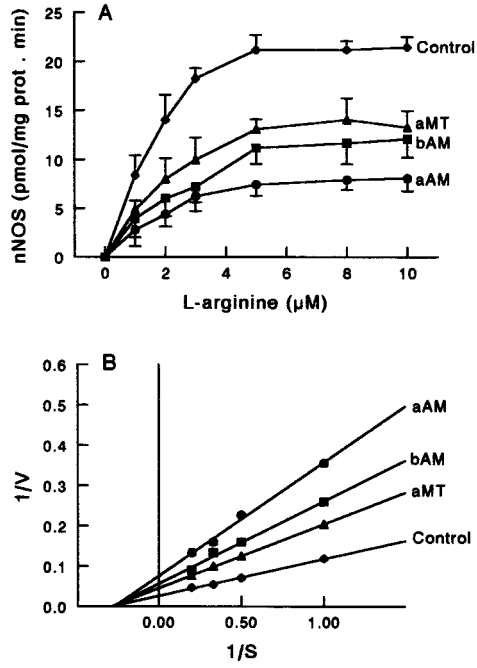

Fig. 2. Experiments with homogenates from rat striatum showing the kinetics of enzyme-substrate reaction in the presence of the indicated drugs. A, effect of aMT (▲), aAM (●), and bAM (■) on rat striatal nNOS activity. Homogenates from rat striatum were incubated for 30 min at 37°C with increasing concentrations of L-arginine and in the absence (◆) or in the presence of 1 mM drug. Each point is the mean ± S.E.M. of three experiments done in triplicate. B, double reciprocal plot of the data showing that the drugs tested modify V_{max} and not K_m values of the enzyme-substrate reaction.

TABLE 1

K_m and V_{max} values for nNOS activity measured in rat striatal homogenates incubated in the presence of a 1 mM concentration of each indicated drug

Kinetic parameters were calculated from Lineweaver-Burk analysis of the data showed in Fig. 2. Each value represents the mean ± S.E.M. of three experiments with four striata, each one performed in triplicate.

Compound	K_m	V_{max}
	μM	*pmol/mg protein/min*
Control	2.5 ± 0.2	30.1 ± 2.1
aMT	2.8 ± 0.3	19.2 ± 1.8*
aAM	3.1 ± 0.3	11.4 ± 2.6**
bAM	3.1 ± 0.4	16.4 ± 2.9**

* $P < .05$ versus control; ** $P < .01$ versus control.

Kynurenines aAM and bAM inhibited nNOS activity in a dose-dependent manner when CaM was absent from the incubation medium, and the effect was significant at 1 nM each compound ($P < .05$, Figs. 6A and 7A, respectively). Increasing the concentration of CaM resulted in a loss of inhibitory potency of aAM and bAM (Table 2) on purified nNOS activity. The behavior of these kynurenines was similar to that of aMT. However, in the presence of 10 μg/ml CaM, aAM (10 μM) inhibited nNOS activity ($P < .05$), an effect absent for bAM and aMT at this concentration of CaM.

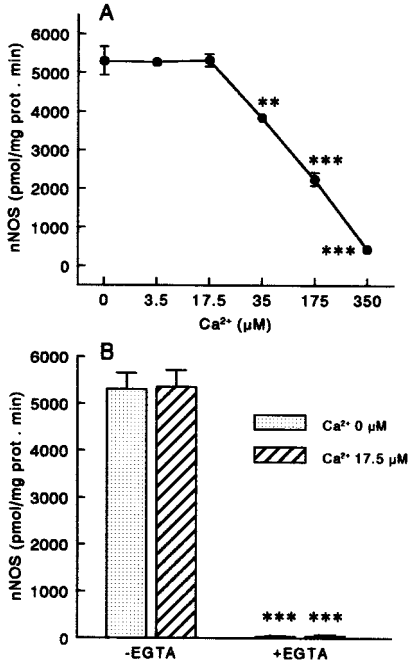

Fig. 3. Experiments with purified rat brain nNOS carried out in presence of 10 μg/ml CaM, 10 μM FAD and 30 μM H_4-bioperin. A, effect of different concentrations of Ca^{2+} on nNOS activity. $^{**}P < .01$ and $^{***}P < .001$ versus $Ca^{2+} = 0$. B, effect of 1 mM EGTA, a specific Ca^{2+} chelator, in the incubation medium. $^{***}P < .001$ versus nNOS activity in absence of EGTA. Each point is the mean ± S.E.M. of three experiments done in triplicate.

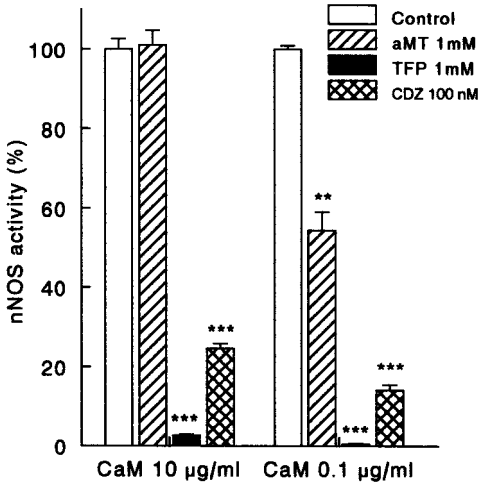

Fig. 4. Effect of aMT (1 mM), trifluoperazine (TFP, 1 mM), and calmidazolium (CDZ 100 nM) on the activity of purified rat brain nNOS incubated in presence of 0.1 or 10 μg/ml CaM, 10 μM FAD, and 30 μM H_4-bioperin. Results show percentage of inhibition of control nNOS activity and are the mean ± S.E.M. of three experiments done in triplicate. $^{**}P < .01$ and $^{***}P < .001$ versus control.

After fixing the concentration of CaM at 0.1 μg/ml, neither changes in FAD (0.1 - 10 μM) nor in H_4-biopterin (0.3 - 30 μM) in the incubation medium affected the inhibitory effect of either aAM and bAM on purified nNOS activity (Fig. 6, B and C, and Fig. 7, B and C, respectively).

To further study any possible interaction of aMT, aAM, and bAM with CaM, urea-PAGE of CaM was made. The

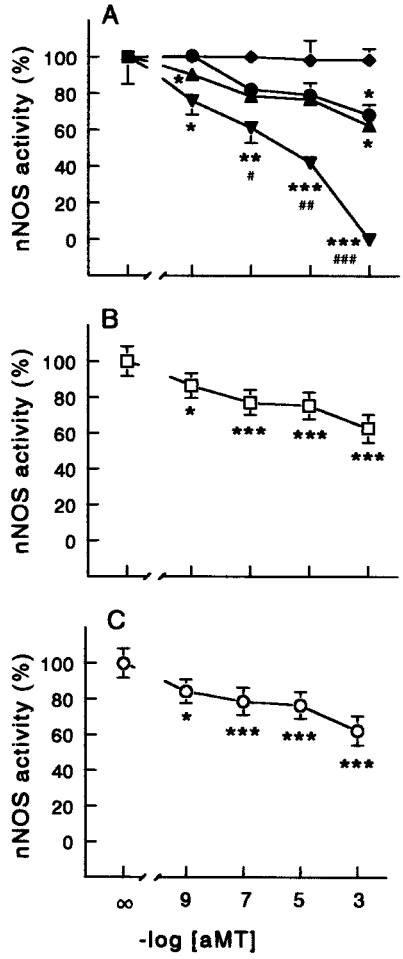

Fig. 5. Effect of aMT (10^{-9} to 10^{-3} M) on nNOS activity in the presence of increasing concentrations of nNOS cofactors. A, CaM (▼, 0 μg/ml; ▲, 0.1 μg/ml; ●, 1 μg/ml; ♦, 10 μg/ml); FAD, 10 μM; and H_4-biopterin, 30 μM. B, FAD (▼, 0.1 μM; ▲, 1 μM; ●, 10 μM); CaM, 0.1 μg/ml; and H_4-biopterin, 30 μM. C, H4-biopterin (▼, 0.3 μM; ▲, 3 μM; ●, 30 μM); CaM, 0.1 μg/ml; and FAD, 10 μM. Results show the percentage of inhibition of control nNOS activity and are the mean ± S.E.M. of three experiments done in triplicate. Comparisons between aMT doses: $^{*}P < .05$ versus ∞; $^{**}P < .01$ versus ∞; $^{***}P < .001$ versus ∞. Comparisons between CaM doses: $^{\#}P < .05$ versus CaM 10 μg/ml; $^{\#\#}P < .01$ versus CaM 0.1, 1 and 10 μg/ml; $^{\#\#\#}P < .001$ versus CaM 0.1, 1 and 10 μg/ml.

TABLE 2

IC_{50} values for nNOS activity inhibition calculated from experiments with purified rat enzyme incubated with aMT, aAM, or bAM at different concentrations of calmodulin

Experiments were performed in the presence of 10 μM FAD, 30 μM H_4-biopterin, and 17.5 μM Ca^{2+}.

Calmodulin	aMT	aAM	bAM
$\mu g/ml$		M	
0	1.32×10^{-6}	0.46×10^{-9}	0.31×10^{-9}
0.1	$>10^{-3}$	2.05×10^{-8}	3.22×10^{-6}
1	$>10^{-3}$	2.92×10^{-8}	4.31×10^{-6}
10		0.70×10^{-3}	

electrophoretic migration mobility of CaM was studied in the absence or presence of both 2 mM EGTA and 1 mM aMT (Fig. 8A), 1 mM aAM (Fig. 8B), and 1 mM bAM (Fig. 8C). The presence of aMT, aAM, or bAM produced a similar CaM mobility into the gel that was faster than the CaM mobility obtained in control gels in which these compounds were absent. In the presence of EGTA, the migration of CaM was slower than the observed in the absence of EGTA. In these conditions (i.e., in the presence of EGTA), aMT, aAM, and bAM were unable to modify the migration pattern of CaM.

To assess whether the interaction of aMT and the kynurenines aAM and bAM with the calcium-CaM (CaCaM) complex is related to their inhibition of nNOS activity, a series of urea-PAGE of CaM were made in the presence of the kynurenines aM and bM. Besides, to test the hypothesis that the differences in the molecular structure (i.e., differences in hydrophobicity) may account for the interaction between these compounds and CaM, a urea-PAGE of CaM with serotonin was also made. The results suggest that none of these compounds affected the migration pattern of CaM either in the presence or absence of EGTA (Fig. 9A, B, and C, respectively). Moreover, blocking the NH_2 group of kynurenine aAM with a dimethyl (aDAM) or a benzyl (aBAM) group made the resultant compounds unable to change the migration pattern of CaM (Fig. 10A and B, respectively).

A dose-dependent study of the interaction of aMT, aAM, and bAM with CaCaM was made. Fig. 11 shows the urea-PAGE gels of these experiments, proving that the kynurenines aAM and bAM bind CaCaM with more affinity than aMT. In fact, at concentrations of 250 μM, the kynurenines aAM and bAM but not aMT were able to affect the migration behavior of CaCaM.

Discussion

The major findings of this study are the demonstration that aMT and some synthetic kynurenines, structurally similar to the natural brain aMT metabolite (Hirata et al., 1974),

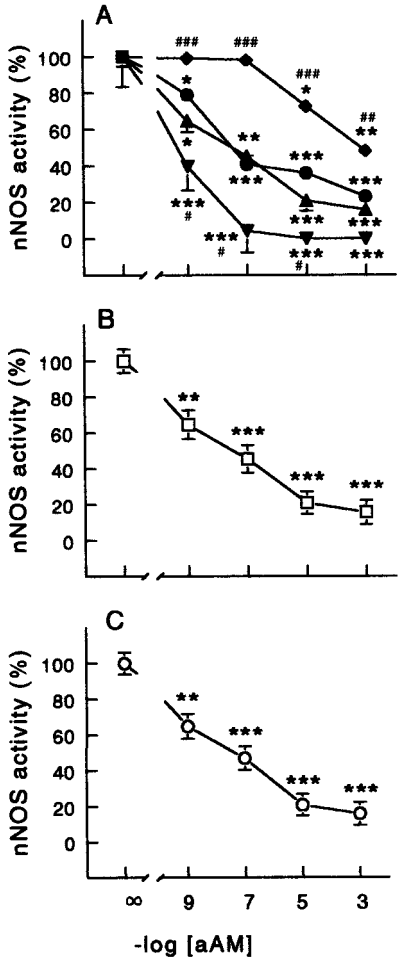

Fig. 6. Effect of aAM (10^{-9} to 10^{-3} M) on nNOS activity in the presence of increasing concentrations of nNOS cofactors. A, CaM (▼, 0 μg/ml; ▲, 0.1 μg/ml; ●, 1 μg/ml; ♦, 10 μg/ml); FAD, 10 μM; and H_4-biopterin, 30 μM. B, FAD (▼, 0.1 μM; ▲, 1 μM; ●, 10 μM); CaM, 0.1 μg/ml; and H_4-biopterin, 30 μM. C, H_4-biopterin (▼, 0.3 μM; ▲, 3 μM; ●, 30 μM); CaM, 0.1 μg/ml; and FAD, 10 μM. Results show the percentage of inhibition of control nNOS activity and are the mean ± S.E.M. of three experiments done in triplicate. Comparisons between aMT doses: $^{*}P < .05$ versus ∞; $^{**}P < .01$ versus ∞; $^{***}P < .001$ versus ∞ 10 μg/ml. Comparisons between CaM doses: $^{\#}P < .05$ versus CaM 1 μg/ml; $^{\#\#}P < .01$ versus CaM 0 μg/ml; $^{\#\#\#}P < .001$ versus CaM 0 μg/ml.

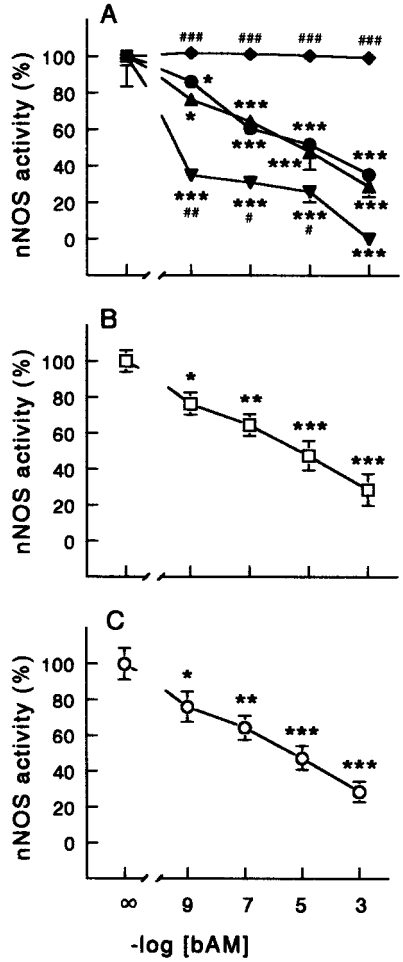

Fig. 7. Effect of bAM (10^{-9} to 10^{-3} M) on nNOS activity in the presence of increasing concentrations of nNOS cofactors. A, CaM (▼, 0 μg/ml; ▲, 0.1 μg/ml; ●, 1 μg/ml; ♦, 10 μg/ml); FAD, 10 μM; and H_4-biopterin, 30 μM. B, FAD (▼, 0.1 μM; ▲, 1 μM; ●, 10 μM); CaM, 0.1 μg/ml; and H_4-biopterin, 30 μM. C, H_4-biopterin (▼, 0.3 μM; ▲, 3 μM; ●, 30 μM); CaM, 0.1 μg/ml; and FAD, 10 μM. Results show the percentage of inhibition of control nNOS activity and are the mean ± S.E.M. of three experiments done in triplicate. Comparisons between aMT doses: $^{*}P < .05$ versus ∞; $^{***}P < .001$ versus ∞ 10 μg/ml. Comparisons between CaM doses: $^{\#}P < .05$ versus CaM 0.1 and 1 μg/ml; $^{\#\#}P < .01$ versus CaM 0.1 and 1 μg/ml; $^{\#\#\#}P < .001$ versus CaM 0 μg/ml.

inhibit nNOS activity through a mechanism involving a complex formation with CaCaM. The results also show interesting structure-related effects of these kynurenines in their ability to bind CaCaM. Although kynurenines carrying an NH_2 group on their molecule (aAM and bAM) bind CaCaM inhibiting nNOS activity, kynurenines lacking the amino group (aM and bM) are unable to bind CaCaM, losing their inhibitory effect on nNOS. That the NH_2 group is decisive for the inhibitory effect on nNOS activity was also demonstrated by blocking it with either a dimethyl or a benzyl group (aDAM and aBAM, respectively); in this case nNOS was not inhibited further. Our results also show that aMT inhibits nNOS activity in a dose-dependent manner. The hormone significantly reduced nNOS activity at 1 nM, corresponding to the concentration of aMT during its nocturnal peak (Reiter, 1991; Bettahi et al., 1996). Interestingly, aAM and bAM showed a 20% reduction in nNOS activity at concentration of 10 pM, reflecting 100 times more activity than aMT itself to inhibit the enzyme (León et al., 1998a).

Kinetic studies showed that the inhibitory effect of aMT and NH_2-kynurenines on nNOS activity measured in rat striatal homogenates could not be prevented by increasing L-arginine concentration, the natural substrate of the enzyme. Similar results were found using a purified rat brain nNOS as a source of the enzyme. The data suggest that these compounds behave as noncompetitive inhibitors of the nNOS. It was surprising that purified nNOS converted L-[^{3}H]arginine to L-[^{3}H]citrulline in the absence of Ca^{2+} or the presence of CaM. These results confirm data elsewhere reported and presumably suggest a significant carry-over of these compounds during enzyme purification (Handy and Moore, 1997). The inclusion of Ca^{2+} up to 17.5 μM did not affect L-[^{3}H]citrulline production; above this concentration, a Ca^{2+}-dependent inhibition of nNOS activity was found. The precise mechanism of this inhibition is poorly understood (Mittal and Jadhav, 1994). Calcium ions and CaM are required for the catalytic conversion of L-arginine to nitric oxide and L-citrulline (Knowles et al., 1989; Mittal and Jadhav, 1994). High Ca^{2+} concentration may account for Ca^{2+} itself or some Ca^{2+}-derivative intermediates interacting with a presumed regulatory (noncatalytic) site of nNOS. In turn, the Ca^{2+}-dependent interaction leads to a diminished generation of oxidizing equivalents at the catalytic site of the enzyme, decreasing nNOS activity. However, the concentration of Ca^{2+} within the cell in vivo is considerably lesser than that required to inhibit nNOS in the in vitro experiments here described. Thus, it is unlikely that Ca^{2+}-induced nNOS inhibition was of physiological significance (Handy and Moore, 1997). Anyway, the activity of nNOS was completely blocked in presence of EGTA in the incubation medium, further supporting a constitutive, Ca^{2+}-dependent nNOS isoenzyme.

The inhibitory role of aMT and NH_2-kynurenines on nNOS activity was independent of FAD or H_4-biopterin concentration in the incubation medium. Increasing concentrations of CaM, however, resulted in a loss of the inhibitory ability of these compounds. That the decrease in nNOS activity was caused by CaCaM inhibition was shown by incubation of nNOS with the CaM antagonists trifluoperazine and calmidazolium. Thus, other experimental approaches were made to further investigate a possible interaction between aMT and NH_2-kynurenines with CaM. Urea-PAGE experiments proved an increase in the mobility of CaM in the presence of

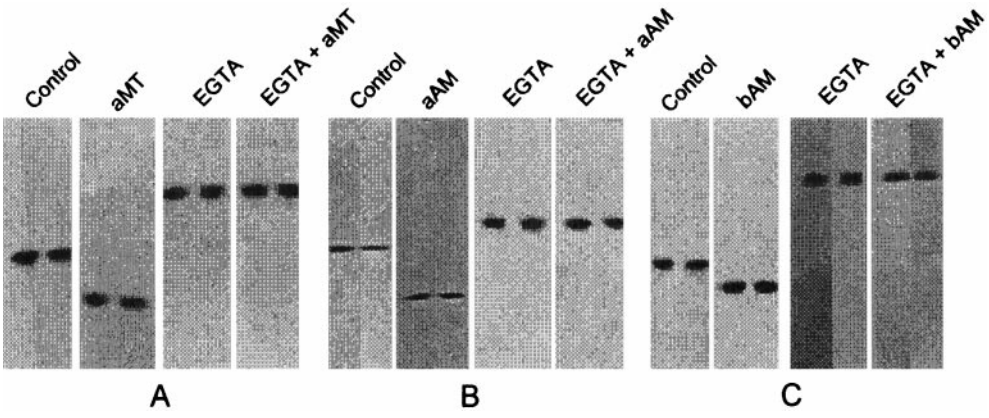

Fig. 8. Effects of aMT (A) and kynurenines aAM (B) or bAM (C) on the electrophoretic mobility of CaM. Urea-PAGE gels were made in the presence of 1 mM Ca^{2+} (control) or 2 mM EGTA and in the absence or presence of 1 mM each compound. See *Results* for explanation.

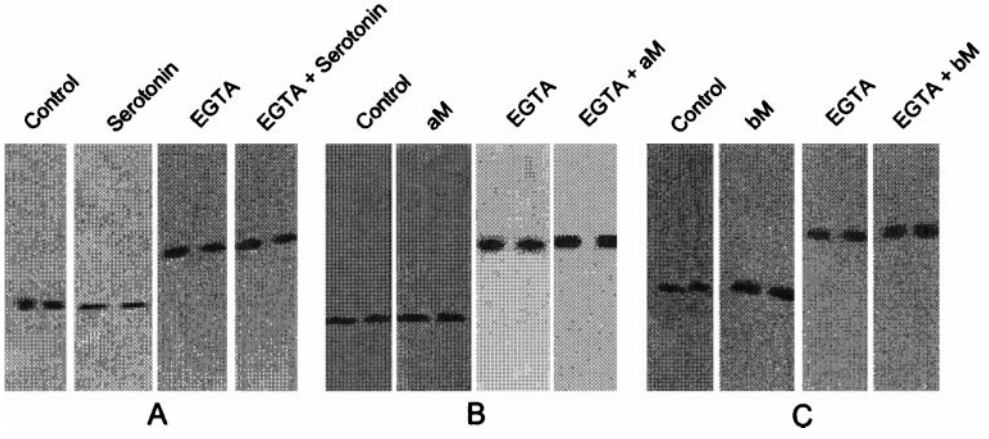

Fig. 9. Effects of serotonin (A) and the kynurenine aM (B) or bM (C) on the electrophoretic mobility of CaM. Urea-PAGE gels were made in the presence of 1 mM Ca^{2+} (control) or 2 mM EGTA and in the absence or presence of 1 mM each compound. See *Results* for explanation.

either aMT, aAM, or bAM. CaM migration pattern in the presence of these drugs was similar for all of them. In the absence of Ca^{2+}, aMT and the kynurenines aAM and bAM were unable to modify the mobility of CaM alone. With the use of NMR, circular dichroism, and fluorescence spectroscopy, it was recently shown that aMT binds CaCaM but neither the CaCaM-NOS complex nor NOS alone (Ouyang and Vogel, 1998). These data together with our results suggest an interaction between aMT and NH_2-kynurenines and the CaCaM complex that may account for the changes in the peptide electrophoretic migration. The results also suggest that Ca^{2+} ions are necessary for the interaction between aMT and NH_2-kynurenines with CaM. Dose-dependent experiments with urea-PAGE demonstrate that the kynurenines aAM and bAM bind CaCaM with higher affinity than aMT, thus supporting the results of kinetic experiments.

In response to high intracellular Ca^{2+} levels, CaM binds Ca^{2+} ions and undergoes a major conformational change after the exposure of two hydrophobic regions on the protein surface (Ouyang and Vogel, 1998). Studies on peptide-CaM interactions show that CaM-binding peptides are normally positively charged, hydrophobic α-helices (O'Neil and DeGrado, 1990). Small, hydrophobic molecules can also bind to CaM and this may account for the interaction between CaCaM and aMT, aAM, and bAM (Ouyang and Vogel, 1998). Besides hydrophobic interactions, the electrostatic interaction between basic residues on the molecule and acidic residues of CaM also contribute to the binding (Crivici and Ikura, 1995). This may be the case for some tryptophan metabolites such as 5-hydroxytryptophan, in which the extra negative charge from its carboxyl group is likely to be repelled by the negative charges from the acidic residues on CaM surrounding the hydrophobic clefts (Ouyang and Vogel, 1998). For serotonin, its hydrophilicity (Benítez-King et al., 1993, 1996) may account for its very weak bind to the C-terminal domain of CaM (Ouyang and Vogel, 1998). Thus, the relative hydrophobic interactions and electrostatic repulsion might be the main reason that serotonin and kynurenines aM and bM did not bind apparently to CaM in our experiments.

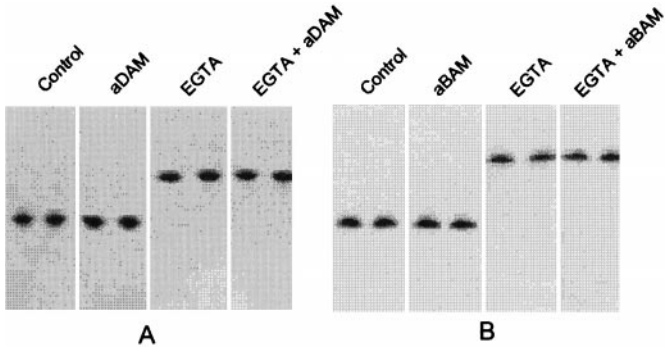

Fig. 10. Effects of the kynurenine aDAM (A) or aBAM (B) on the electrophoretic mobility of CaM. Urea-PAGE gels were made in the presence of 1 mM Ca^{2+} (control) or 2 mM EGTA and in the absence or presence of 1 mM each compound. See *Results* for explanation.

Upon NMDA activation and Ca^{2+} influx into the cell, Ca^{2+} binds to CaM, producing the CaCaM complex responsible for the activation of nNOS (Moncada et al., 1991; Garthwaite and Boulton, 1993). We showed that microiontophoresis of aMT inhibits the excitatory response of the striatal neurons to the sensory-motor cortex stimulation acting via the NMDA receptor in a dose-dependent manner (Castillo-Romero et al., 1995; Escames et al., 1996). The kynurenines aAM and bAM were more potent than aMT in inhibiting this striatal excitatory response; aM displayed potency similar to that of aMT, and bM seemed to act as a functional aMT antagonist (León et al., 1998a,b). Comparison of the electrophysiological data with the results reported here suggest that the break of the pyrrolic ring of the indoleamine during kynurenine synthesis leads to the loss of aMT's ability to bind to CaM. In turn, the addition of an NH_2 group in position R_1 of the kynurenine molecule recovers this ability. Consequently, the synthetic kynurenines here described keep some of the properties of aMT in a structure-related fashion; NH_2-kynurenines keep the ability of aMT to inhibit both NMDA-dependent excitatory response and nNOS activity, whereas kynurenines lacking the NH_2 group or with this group blocked retain the ability to affect NMDA response but do not further affect nNOS activity.

A relationship between aMT, CaM, and cytoskeleton has been demonstrated (Benítez-King et al., 1993; Benítez-King et al., 1996). Glutamate receptors are associated with cytoskeletal proteins, and CaM may inactivate NMDA receptor interacting with the NR1 subunit, an effect involving α-actinin, another cytoskeletal protein (Wyszynski et al., 1997). However, CaM reduces NMDA channel activity but CaM inhibitors were unable to counteract the inactivation of CaM-dependent NMDA activity in whole-cell recordings (Krupp et al., 1999). These data suggest that NMDA inactivation in the intact cell was not a simple phenomenon and the participation of aMT and aMT metabolites in this process should be taken into account. In fact, recent data based on a carefully conducted kinetic study suggest that CaM may act as a membrane/cytosolic aMT receptor (Romero et al., 1998).

One last consideration that should be addressed is the relation between the effects of aMT and kynurenines here described in vitro and their physiological relevance in vivo.

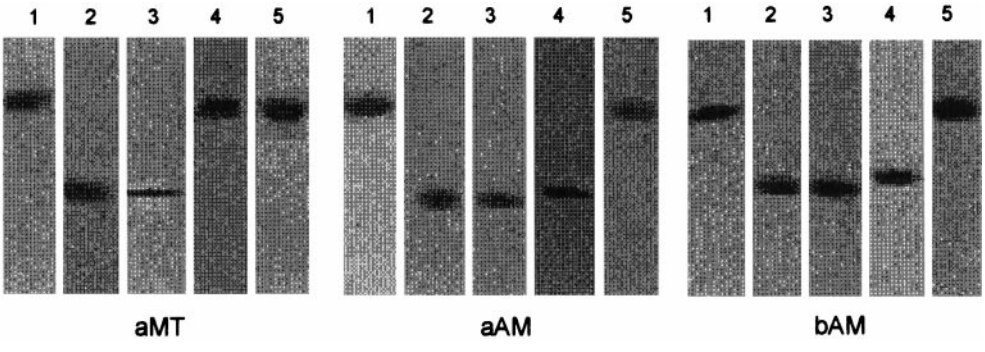

Fig. 11. Dose-response effects of aMT and the kynurenine aAM or bAM on electrophoretic mobility of CaM. Urea-PAGE gels were made in the presence of 1 mM Ca^{2+} (lanes 1) and in the absence or presence of aMT, aAM or bAM at the following doses: 1 mM (lanes 2); 500 μM (lanes 3); 250 μM (lanes 4); 100 μM (lanes 5).

Melatonin, aAM, and bAM significantly inhibit recombinant nNOS activity at 1 nM. These effects shown with recombinant nNOS confirm similar results in homogenates of rat striatum, although in this case, aAM and bAM were effective at concentrations of 10 pM (León et al., 1998a,b). Because 1 nM may be considered a physiological concentration of aMT during the night (Reiter, 1991), the inhibition of nNOS activity by the nocturnal peak of the indoleamine might be a mechanism involved in the depressive effect of aMT on brain excitability. Perhaps this mechanism explains, at least in part, the sedative and hypnotic effects of the indoleamine at night. Anyway, aMT levels into the cell are not clearly stated and aMT may concentrate in such cellular structures as membranes and mitochondria, where it may reach concentrations of 200 nM (Martín et al., 2000). These data lead one to re-examine the "physiological" levels of the hormone. Furthermore, aMT is metabolized to aMK by 2,3-dioxygenase, an enzyme of the cytosolic side of the cellular membrane. It was hypothesized that aMK could have some of the physiological functions assigned to aMT (Kennaway and Hugel, 1991; Acuña-Castroviejo et al., 1994). In experiments in our laboratory, we have found that aMK displays an IC_{50} value of 40 nM for nNOS inhibition in rat striatal homogenates [i.e., more than 25,000 times the IC_{50} for aMT (D. Acuña-Castroviejo, unpublished observations)]. The action's mechanism of aMK is the same as aMT (i.e., the aMT metabolite forms a complex with CaM that hampers the activation of nNOS). Consequently, the actions of aMT in vivo may depend not only on its "physiological" concentrations, but also on the aMK produced endogenously. Because the kynurenines assayed in our study are synthetic, we cannot compare their effects at the doses used with similar compounds produced endogenously. The interest of these synthetic kynurenines was the search for neuroprotective compounds with potential clinical use.

The data discussed highlight the importance of brain tryptophan metabolism in modulating CNS activity. The methoxyindole pathway of tryptophan produces aMT, with inhibitory effects on brain homeostasis, acting on NMDA via CaCaM interaction. The kynurenine pathway produces a series of compounds with both excitatory and inhibitory effects on glutamate receptors (Fukui et al., 1991). In addition, some kynurenines are bioactive metabolites of aMT, and may mediate some actions of aMT (Kennaway and Hugel, 1991; Acuña-Castroviejo et al., 1994; Acuña-Castroviejo et al., 1995). Thus, an imbalance of this tryptophan metabolic pathways may produce widespread changes in CNS excitability (Muñoz-Hoyos et al., 1997). The results also account for the potential use of aMT and kynurenines as neuroprotective drugs in human clinical studies (Molina-Carballo et al., 1997; Crespo et al., 1999) and suggest a pathway for the design and synthesis of other new ones.

Acknowledgments

We thank Drs. David Pozo and Francisco Martínez for help in the preparation of urea-PAGE experiments.

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