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AGN 2979 [3-(3-METHOXYPHENYL)-3-(3-DIMETHYLAMINOPROPYL)-4,4-DIMETHYLPIPERIDINE-2,6-DIONE]

AN INHIBITOR OF THE ACTIVATION OF TRYPTOPHAN HYDROXYLASE

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Abstract—AGN 2979 [3-(3-methoxyphenyl)-3-(3-dimethylaminopropyl)-4,4-dimethylpiperidine-2,6-dione] blocked the increase in tryptophan hydroxylase activity that occurred when slices of brainstem were exposed to a depolarizing medium or to agents that mobilize intracellular pools of calcium, but it had no effect on the activity of enzyme prepared from slices of brainstem incubated in control medium. AGN 2979 also blocked the calcium–calmodulin-dependent activation of tryptophan hydroxylase that was seen when supernatant preparations of the enzyme were exposed to phosphorylating conditions but not the activation induced by calcium-dependent proteases that was triggered by millimolar calcium concentrations. An identical pattern of inhibition has been found with the antipsychotic drugs, haloperidol and fluphenazine [Boadle-Biber, *Biochem. Pharmac.* **31**, 2495 (1982)]. The sensitivity to the same inhibitors of both the activation of tryptophan hydroxylase produced by pretreatment of brainstem slices and that induced by incubation of supernatant preparations of enzyme under phosphorylating conditions suggests involvement of a common mechanism of enzyme activation in response to these different treatments.

The kinetic properties of tryptophan hydroxylase [tryptophan-5-monooxygenase, L-tryptophan, tetrahydropterin:oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4], the rate-limiting enzyme in the synthesis of 5-hydroxytryptamine (5-HT), can be modified by a variety of *in vitro* treatments including incubation under phosphorylating conditions [1–3]. The activation seen under these conditions is fully reversible [4] and involves both calcium and calmodulin [5, 6], probably through the action of a calcium–calmodulin-dependent protein kinase [7]. In addition, another protein that is distinct from calmodulin apparently also has a role in this activation [8].

Of particular interest is the question of whether this calcium–calmodulin-requiring activation mediates the reversible increase in tryptophan hydroxylase activity in supernatant preparations of enzyme obtained following *in vivo* electrical stimulation of 5-HT neurons or depolarization of brainstem slices [9–13] particularly since the depolarization-induced activation of enzyme has also been shown to be calcium dependent in work with brain slices [11–13]. One approach to this question is to examine whether inhibitors of the activation induced by incubation of supernatant enzyme under phosphorylating conditions [6] also block the increase in enzyme activity resulting from depolarization of brain tissue. In recent studies we found that haloperidol and fluphenazine, antipsychotic drugs, which prevent the activation of the enzyme under phosphorylating conditions by virtue of their interaction with calmodulin [14], also block the depolarization-induced enzyme activation [12]. However, one difficulty in the interpretation of experiments on depolarized slices arises from the fact that antipsychotic drugs such as haloperidol or fluphenazine not only bind to calmodulin with affinities in the micromolar range [15], but also may exert a local anesthetic effect at these concentrations [16]. Thus, no distinction can be drawn between an inhibition of enzyme activation due to the block of calcium entry in response to depolarization and that due simply to an interaction with calmodulin. To distinguish between these potential targets of drug action it is necessary to bypass the depolarization-dependent calcium entry step for activation of the enzyme and instead activate the enzyme through exposure of the slices to substances that release intracellular stores of calcium, such as metabolic inhibitors [17] or methylxanthines [18]. Activation of tryptophan hydroxylase in this way is sensitive to antipsychotic drugs [12], but it is unaffected by the local anesthetic, tetracaine, or the calcium channel blocking agent, verapamil [12, 18], both of which block the depolarization-induced enzyme activation [12]. This result suggests that the antipsychotic drugs block the activation of tryptophan hydroxylase produced under these treatment conditions at an intracellular site. It also suggests that the enzyme activation induced in the slices by these treatments may involve the same mechanism as the *in vitro* activation induced when supernatant preparations are incubated under phosphorylating conditions since both are sensitive to these drugs. In the present paper we report results with another drug, AGN 2979 [3-(3-methoxyphenyl)-3,3-

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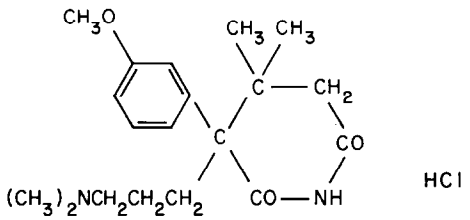

Fig. 1. Structure of AGN 2979.

dimethylaminopropyl)-4,4-dimethylpiperidine-2,6-dione hydrochloride] (Fig. 1), that also blocks the activation of tryptophan hydroxylase resulting from exposure of brainstem slices to depolarizing media, metabolic inhibitors or methylxanthines as well as that induced by incubation of supernatant preparations of the enzyme under phosphorylating conditions. These results provide further evidence for involvement of a common mechanism in the activation of enzyme resulting from these different treatments.

METHODS

Male Sprague–Dawley rats (Flow Laboratories, Rockville, MD) matched for weight in each experiment were killed by decapitation and the brainstems (diencephalon, midbrain and medulla pons) were removed, chilled on ice, weighed and then cut into 250 μm slices with a model MT-2 Dupont Sorvall tissue chopper. Each sliced brainstem preparation was then incubated for 10 min at 25° in 5 ml of control medium bubbled with 100% oxygen. Various additions (drugs, metabolic poisons) or modifications were made to this incubation medium as indicated in Results. Its composition was as follows: 150 mM NaCl; 6 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$; 10 mM glucose; and 10 mM Tris acetate buffer (adjusted to pH 7.4 at 23°). In the depolarizing medium, 40% of the NaCl was replaced with equimolar KCl. Calcium-free control medium contained 100 μM ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA). In drug experiments that were conducted in calcium-free control medium, the slices were first pre-incubated in the calcium-free medium for 5 min to wash out extracellular calcium and were then transferred to fresh calcium-free medium containing the appropriate additions (none in the case of controls). At the end of the incubation, the slices were separated from the medium by centrifugation (12,000 g for 5 min), and the pellet was then homogenized in 0.05 M Tris acetate buffer, pH 7.4 (1:1.5, w/v), with a Kontes Duall all glass homogenizer (Kontes Scientific, Vineland, NJ) and spun at 39,000 g for 30 min at 4° as previously described [11, 17]; the resulting supernatant fraction was used as the source of tryptophan hydroxylase.

In some experiments, drugs were tested for direct effects on the activity of low speed (39,000 g for 30 min) supernatant preparations of tryptophan hydroxylase under both control conditions and conditions known to increase enzyme activity, namely phosphorylating conditions [1] (0.5 mM ATP, 5.0 mM $MgCl_2$, 0.1 mM $CaCl_2$) or high (millimolar) calcium concentrations [11]. In this case, fresh brainstems were used to prepare the enzyme. Dithiothreitol (DTT), final concentration 2 mM, was added to the low speed supernatant fraction, which was then passed over a Sephadex G-25 column (22 × 1.3 cm) equilibrated with 0.05 M Tris acetate, pH 7.4, and 2 mM DTT, in order to remove endogenous tryptophan or other indoles that can raise the blank in the enzyme assay [11, 17]. The assay for tryptophan hydroxylase which has been described in detail elsewhere [11, 17] is based on the procedure of Friedman *et al.* [19] in which 5-hydroxytryptophan (5-HTP), formed in the presence of an aromatic amino acid decarboxylase inhibitor, brocrescine, is measured by its fluoresence in HCl (excitation 295 nm; emission 535 nm). The concentration of L-tryptophan was 200 μM and that of the artificial reduced pterin cofactor, DL-6MPH$_4$, 50 μM, which is subsaturating. D-Tryptophan was added to the blanks. Each brainstem enzyme preparation was assayed in quintuplet and the results were averaged. The assay was checked for linearity with time and protein in each experiment. Results are expressed as pmoles 5-HTP formed per mg protein per min ± the standard error of the mean (S.E.M.). Numbers in parentheses refer to the numbers of individual brainstems enzyme preparations tested. Protein was determined by the method of Lowry *et al.* [20] with bovine serum albumin as the standard.

Of the drugs used in this study, (±) AGN 2979, (+) AGN 2979, (−) AGN 2979 and AGN 3222 [3-(3,5-dimethoxyphenyl)-3-(3-dimethylaminopropyl)-4,4-dimethyl piperidine-2,6-dione hydrochloride] were gifts of Dr. Maurice Gittos, Centre de Recherche, Merrell Dow International, Strasbourg, France; haloperidol was supplied by the McNeil Pharmaceutical Co., Spring House, PA, and fluphenazine by E. R. Squibb & Sons, Inc., Princeton, NJ. Both TMB-8 [8-(*N*,*N*-diethylamino) octyl-3,4,5-trimethoxybenzoate hydrochloride] (from Dr. Richard Feinman, Department of Biochemistry, State University of New York, Brooklyn, NY) and tetracaine (Mann Research Laboratories, New York) were obtained through the kind offices of Dr. Ronald Rubin, Department of Pharmacology, Medical College of Virginia. Guanidine HCl and 3-isobutyl-l-methylxanthine (IBMX) were purchased from the Sigma Chemical Co., St. Louis, MO. All the drugs were dissolved directly in the slice incubation medium except for haloperidol and IBMX which were made up in ethanol and diluted 20-fold in the slice incubation medium to give a final ethanol concentration of 5%. Five percent ethanol was also added to the control slice incubation medium but was without effect on enzyme activity. Calmodulin was purchased from Calbiochem Behring, La Jolla, CA, and was dissolved directly in appropriate amounts in the 0.2 M Tris acetate buffer, pH 7.4, that was used in the enzyme assay.

RESULTS

Figure 2 depicts the action of AGN 2979 on the depolarization-induced increase in tryptophan hydroxylase activity. The drug was added directly to

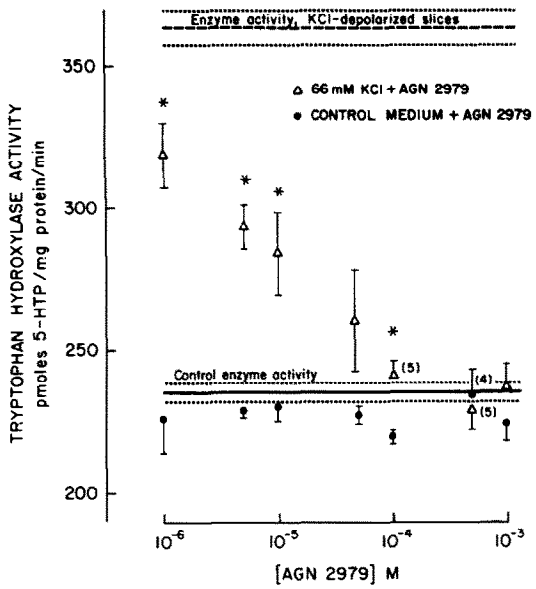

Fig. 2. Effect of increasing concentrations of AGN 2979 on the depolarization-induced increase in tryptophan hydroxylase activity. Slice preparations of rat brainstem were incubated for 10 min at 25° in oxygenated control or 66 mM KCl medium together with various concentrations of AGN 2979. Three brainstem slice preparations were tested under each condition, unless indicated otherwise by numbers in parentheses. Control enzyme activity, 235 ± 3 pmoles 5-HTP/mg protein/min (N = 12). Enzyme activity from depolarized slices, 363 ± 6 pmoles 5-HTP/mg protein/min (N = 12) ($P < 0.001$ by Student's t-test). Significance of the differences between enzyme activity from depolarized and control brainstem slices in the presence of AGN 2979: (*) $P < 0.05$; other values were not significantly different.

the slice incubation medium. It had little effect on the activity of enzyme from slices incubated in control medium, but it did reverse the increase in activity seen in the depolarizing medium in a dose-dependent manner. This reversal was complete when the drug was used at concentrations of 100 μM or greater. The blocking effect, however, was not stereospecific. Thus, both the (+) and (−) isomers of AGN 2979 produced a partial inhibition (39 ± 5 and 43 ± 4% respectively) of the depolarization-induced increase in enzyme activity at 50 μM (N = 3) and a full block at 500 μM. The 3,5-dimethoxy analogue of AGN 2979, AGN 3222, also inhibited the depolarization-induced increase in enzyme activity; at 50 μM the inhibition was 48 ± 6% (N = 3); at 500 μM inhibition was complete.

This inhibitory effect of AGN 2979 could not be reversed by increasing the calcium concentration in the incubation medium to 10 mM (data not shown).

AGN 2979 also blocked the increase in tryptophan hydroxylase activity obtained when slices of brainstem were exposed to a variety of metabolic inhibitors [12, 17] or to high (millimolar) concentrations of methylxanthines [18]. This block was complete at a concentration of 500 μM (Fig. 3). In these experiments, the treatments to the slices were carried out in calcium-free control medium in order to exclude the possibility that extracellular calcium could contribute to the resulting enzyme activation. Under these conditions, the methylxanthine, IBMX, like depolarization [12], was found to produce a reversible increase in enzyme activity (Fig. 4). Thus, when the slices of brainstem were reincubated for 60 min in three changes of calcium-free, IBMX-free control medium, enzyme activity was restored to control levels. Activity could be increased once again by a second exposure to IBMX.

Another drug, TMB-8, that is reported to interfere with the release of calcium from intracellular organelles [21] was tested in these systems for purposes of comparison. It was also found to block the increase in enzyme activity induced by exposure of the brainstem slices to depolarization, to a metabolic inhibitor (guanidine), or to the methylxanthine (IBMX) (Fig. 5).

AGN 2979, when tested on supernatant extracts of tryptophan hydroxylase, antagonized the increase in enzyme activity obtained under phosphorylating conditions but had no effect on control enzyme activity (Fig. 6). This inhibitory effect could be reversed by addition of exogenous calmodulin to the enzyme reaction medium but only in amounts 80 times those needed to reverse a similar degree of

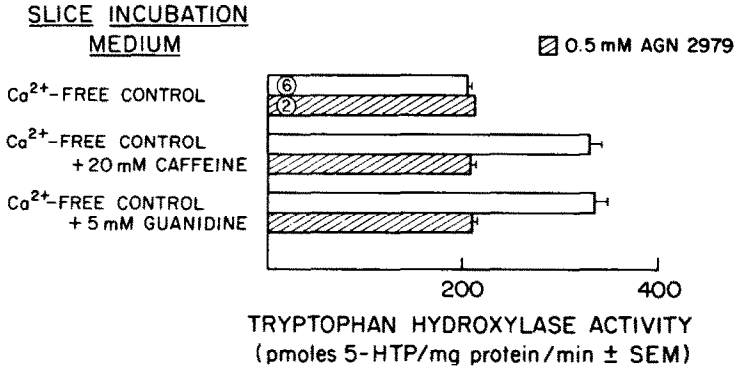

Fig. 3. Reversal by AGN 2979 of the increase in enzyme activity induced by caffeine and guanidine. Three brainstem preparations were tested under each condition unless indicated otherwise by circled numbers. Significance of the increase in enzyme activity induced by caffeine and guanidine compared with untreated control, $P < 0.001$ (Student's t-test).

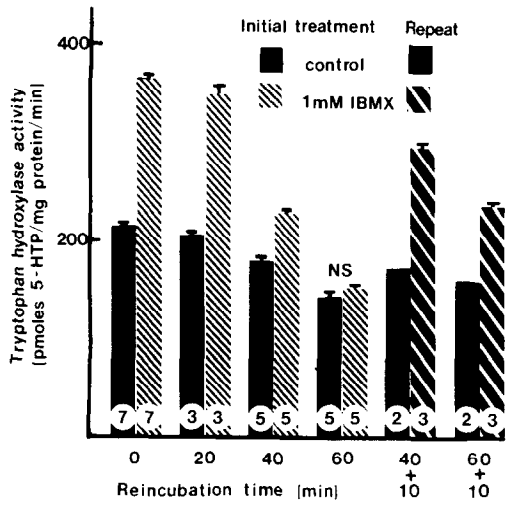

Fig. 4. Reversal of the increase in tryptophan hydroxylase activity induced by incubation of slices of rat brainstem with 1 mM IBMX in calcium-free control medium. Slices of brainstem were exposed to 1 mM IBMX for 10 min at 25° and then were reincubated for different times in calcium-free control medium. The reincubation medium was changed at 5, 10, 20 and 40 min in order to remove IBMX that had diffused out of the slices. Slices that had been reincubated for 40 or 60 min were then reexposed to 1 mM IBMX for 10 min at 25° to test whether enzyme could be reactivated. Significance of the differences between enzyme activity from initially treated and control brainstem slices was determined by student's *t*-test, $P < 0.05$. NS, not significant.

inhibition (50%) produced by haloperidol (Fig. 7). AGN 2979 had no effect on the increase in tryptophan hydroxylase activity that results from the action of a calcium-dependent protease that is activated by high concentrations of calcium [22] (Fig. 6).

In contrast to AGN 2979, TMB-8 did not alter the increase in enzyme activity seen under phosphorylating conditions when it was tested at the same concentration at which it blocked the increase in enzyme activity induced by exposure of slices to high potassium, guanidine or IBMX. The percent increase in enzyme activity induced by incubation of supernatant enzyme preparations under phosphorylating conditions was 77 ± 2 in the absence and 75 ± 3 in the presence of 100 μM TMB-8 (N = 3). TMB-8 had no effect on control enzyme activity.

DISCUSSION

In this paper, we have presented results with AGN 2979 which indicate that, like the antipsychotic drug haloperidol, it not only inhibits the increase in tryptophan hydroxylase activity seen when slices of rat brain stem are exposed to guanidine, caffeine or a depolarizing medium but also blocks the increase in enzyme activity that results when low speed supernatant preparations of the enzyme are incubated under phosphorylating conditions.

Other drugs that have their sites of action in the cell membrane, such as the calcium channel blocker, verapamil, and the local anesthetic, tetracaine, block only the depolarization-induced increase in enzyme activity; they leave unaffected the increase in enzyme activity induced by incubation of brainstem slices with metabolic inhibitors or methylxanthines [12, 17, 18] or by exposure of supernatant enzyme preparations to phosphorylating conditions [12]. Another substance, TMB-8, which has been reported to inhibit release of intracellular calcium stores in muscle [21], prevented the activation of the enzyme that results from the various pretreatments to the brain slices (metabolic inhibitors, methylxanthines), or depolarizing conditions. However, when used at the same concentration that was effective in the slice

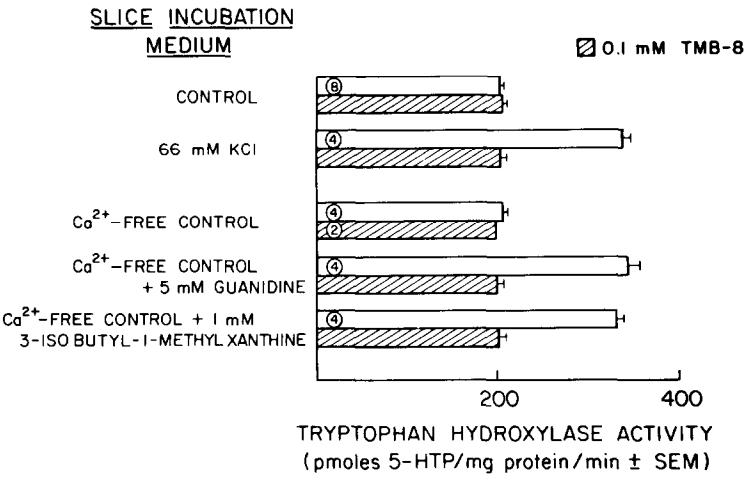

Fig. 5. Effect of TMB-8 on the depolarization-induced increase in tryptophan hydroxylase activity and on the increase in enzyme activity obtained when brainstem slices were incubated in calcium-free control medium in the presence of the metabolic poison, guanidine or the methylxanthine, IBMX. Significance of the increase in enzyme activity in the absence of TMB-8 was at the level of $P < 0.001$ (Student's *t*-test). The number of slice preparations tested under each condition was three unless indicated otherwise by the circled numbers in the figure.

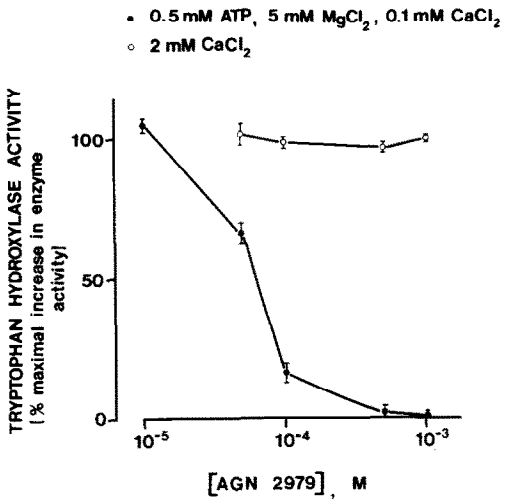

Fig. 6. Effect of AGN 2979 on the increase over control of the tryptophan hydroxylase activity of a low speed supernatant preparation of brainstem incubated in the presence of 0.5 mM ATP, 5.0 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (●---●) or 2 mM $CaCl_2$, (○---○). The increase in enzyme activity in the presence of phosphorylating conditions or high $CaCl_2$ that remained after addition of different concentrations of AGN 2979 (experimental minus control activity in the presence of AGN 2979) was expressed as a percentage of the increase in activity observed in the absence of AGN 2979. Control enzyme activity was unchanged by the concentrations of AGN 2979 used in this experiment. Data points are the mean ± S.E.M. of results from three different enzyme preparations. Enzyme activity in the presence of phosphorylating conditions was 422 ± 18 (3) and control activity 218 ± 3 (3) pmoles 5-HTP per mg protein per min. In the presence of 2 mM $CaCl_2$, enzyme activity was 413 ± 14 (3) and control activity 222 ± 3 pmoles 5-HTP per mg protein per min.

preparations, it did not alter the activation induced by incubation of enzyme supernatant under phosphorylating conditions.

Thus, the shared inhibitory effects of AGN 2979 and haloperidol on the activations of the enzyme resulting from pretreatment of brainstem slices as well as exposure to phosphorylating conditions suggests involvement of some common mediator in all these activation processes. Other evidence that is consistent with a common mechanism of enzyme activation is, first, the shared calcium dependency of the enzyme activations induced by incubation of supernatant extracts under phosphorylating conditions and by exposure of slices to a depolarizing medium or various other treatments [ouabain, sodium-free medium, A23187) [11, 17]. Although the activation induced by metabolic inhibitors or methylxanthines cannot be demonstrated directly to be calcium dependent [17], these treatments have been shown to raise intracellular free calcium levels in other excitable tissue preparations. A second piece of evidence for a common mechanism of enzyme activation is the reversibility of the activation induced by phosphorylating conditions or by depolarization [12] or methylxanthine treatment of brain slices. This reversibility excludes any role for calcium-dependent proteases as mediators of the activation in the brainstem slices [22].

The antipsychotic drugs are presumed to mediate their inhibitory effects on enzyme activation in slices by binding to calmodulin, in a manner similar to the situation for supernatant preparations of enzyme incubated under phosphorylating conditions. In this case, Kuhn *et al.* [6] showed that the supernatant enzyme can no longer be activated under phosphorylating conditions if calmodulin is removed from

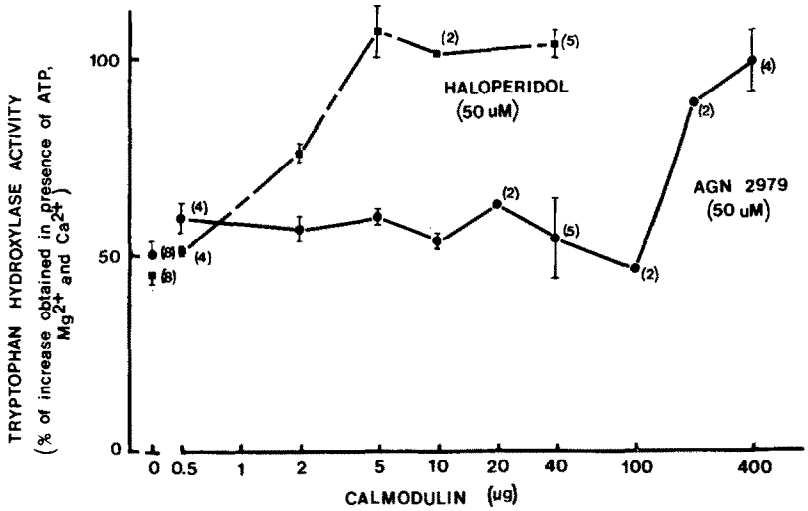

Fig. 7. Effect of exogenous calmodulin on the inhibition by 50 μM AGN 2979 or 50 μM haloperidol of the increase in tryptophan hydroxylase activity produced by incubation of low speed supernatant preparations of brainstem enzyme in the presence of 0.5 mM ATP, 5.0 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The increase in enzyme activity induced by these incubation conditions, that remained after addition of haloperidol or AGN 2979, with or without calmodulin, was expressed as a percentage of the increase observed in the absence of either drug or calmodulin. Results are the mean (±S.E.M. where appropriate) of two or more experiments. Control enzyme activity was 209 ± 3 (4) and enzyme activity obtained under phosphorylating conditions was 423 ± 4 (4) pmoles 5-HTP per mg protein per min.

the enzyme supernatant by a fluphenazine affinity column.

Although AGN 2979 has inhibitory effects on enzyme activation that parallel those of the antipsychotic drugs, the actual target of its inhibitory action in the activation of tryptophan hydroxylase is uncertain. Compared with haloperidol the drug does not appear to interact readily with calmodulin. Thus, many times more exogenous calmodulin is required to reverse a similar degree of inhibition of the enzyme activation induced by phosphorylating conditions in the case of ACN 2979 than with haloperidol. Other potential targets of action for AGN 2979 include the calcium–calmodulin-dependent protein kinase and the activator protein recently isolated by Yamauchi *et al.* [8] which is distinct from calmodulin, but is apparently essential for the calcium–calmodulin-dependent activation of tryptophan hydroxylase seen under phosphorylating conditions. It is worth noting that phenytoin, which has certain features in its heterocyclic ring structure in common with AGN 2979, has been found to inhibit the calmodulin-sensitive protein kinase [23]. Further studies are warranted on AGN 2979 to determine whether it inhibits the calmodulin-sensitive protein kinase. The high levels of calmodulin required to overcome the inhibition of tryptophan hydroxylase activation by AGN 2979 could be explained by tight binding of the drug to the calmodulin binding site of calmodulin-regulated kinase.

Although the precise site of action of this drug remains to be identified, its spectrum of inhibitory effects, taken together with those for the antipsychotic drug, haloperidol, is consistent with the hypothesis that the activation of tryptophan hydroxylase which results from depolarization or a variety of other treatments to brain slices and that obtained in supernatant preparations incubated under phosphorylating conditions share a common mechanism.

Note added in proof: In an open, non-blind trial in depressed patients, AGN 2979 showed antidepressant activity. G. Clerc, J. Noury, M. Gittos, letter to *Am. J. Psychiatry*, 1986, in press.

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