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EFFECT OF THE ANTITUMOR DRUG CARACEMIDE ON THE NEUROCHEMISTRY OF MURINE NEUROBLASTOMA CELLS (CLONE N1E-115)

MICHAEL MCKINNEY,* MICHAEL PFENNING and ELLIOTT RICHELSON
Departments of Pharmacology and Psychiatry, Mayo Foundation, Rochester, MN 55905, U.S.A.

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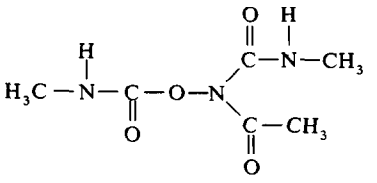
Abstract—Because the antitumor drug caracemide causes neuropsychiatric effects in patients, we investigated its effects on the neurochemistry of cultured neuroblastoma cells (murine clone N1E-115). The drug caused a transient elevation in the level of [^{3}H]cyclic GMP that was not blocked by receptor antagonists or by desensitization of histamine or muscarinic receptors. The EC_{50} for the response to caracemide was 635 μM. Preincubation of cells with caracemide led to the inhibition of muscarinic receptor-mediated [^{3}H]cyclic GMP formation with an IC_{50} of 450 μM. Caracemide inhibited basal guanylate cyclase activity in homogenates noncompetitively with a K_i value of 162 μM. The drug also inhibited sodium nitroprusside-stimulated guanylate cyclase in homogenates. Caracemide did not inhibit basal adenylate cyclase activity in either intact cells or homogenates, but inhibited adenylate cyclase activated by prostaglandin E_1 (PGE_1) or forskolin. The muscarinic receptor-mediated reduction of PGE_1-stimulated [^{3}H]cyclic AMP formation was not affected. The K_i for the inhibition of PGE_1-activated adenylate cyclase in homogenates was 110 μM. Caracemide was a competitive inhibitor of acetylcholinesterase with a K_i value of 8 μM. The drug did not inhibit, but slightly stimulated, monoamine oxidase activity in N1E-115 cells. The results indicate that caracemide can affect several neurochemical systems in neural cells in culture in a way that correlates with its neuropsychiatric effects. The N1E-115 clone thus appears to be useful for evaluating some of the molecular pharmacological effects of drugs interacting with the nervous system.

Caracemide (CARA†) *N*-[(alkylamino)carbonyl]-*N*-[[(alkylamino)carbonyl]oxy]acylamide (I), is a newly-developed antitumor drug presently undergoing clinical trials. It possesses activity against experimentally-induced tumors and leukemia in rodents, inhibits DNA synthesis, and inhibits partially-purified ribonucleotide reductase [1, 2]. Structurally, it is an N-substituted derivative of hydroxylurea.



I

During clinical trials a number of neurological and psychiatric side-effects were noted; several patients developed an irreversible dementia, and there were indications that the effects might be due to an induced cholinergic excess and noradrenergic hyperfunction [3]. It was therefore important to study the neurochemical effects of caracemide in some detail in order to gain insight into possible bases for the effect of this drug on the nervous system.

An appropriate model system to employ is the well-characterized murine neuroblastoma clone N1E-115 [4]. These cultured cells display many characteristics of a neuron, including numerous receptors that when activated can modulate the intracellular level of cyclic nucleotides. The study of this clone has yielded many valuable data that have aided in clarifying the molecular nature of neuronal receptor activation [5, 6].

Our studies of the effects of caracemide on several neurochemical systems in these cultured neuroblastoma cells reveal that this antitumor drug has multiple effects on receptor-linked systems, both stimulatory and inhibitory. The drug first transiently activated, then non-competitively inhibited, guanylate cyclase. Receptor-activated adenylate cyclase was also non-competitively inhibited. Additionally, a moderately potent competitive inhibition of acetylcholinesterase occurred. Caracemide had a mild stimulatory effect on monoamine oxidase activity in N1E-115 cells. Our data show that this drug, if it reaches the brain in significant levels, can be expected to have complex effects on brain neurochemistry. The findings of this study correlate with the neuropsychiatric effects of the drug [3]. Also, this work demonstrates the usefulness of the murine neuroblastoma cell system for evaluating drug effects on the nervous system.

* Address correspondence to: Michael McKinney, Ph.D., Abbott Laboratories, Department 47U, Building AP10, North Chicago, IL 60064.

† Abbreviations: CARA, caracemide; EC_{50}, concentration for half-maximal response; EGTA, ethyleneglycol-bis-(β-aminoethylether)*N*,*N*′-tetraacetic acid; ETYA, eicosatetraynoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; K_i, equilibrium dissociation constant for an inhibitor; NDGA, nordihydroguaiaretic acid; and PGE_1, prostaglandin E_1.

MATERIALS AND METHODS

Cell culture. Murine neuroblastoma cells (clone N1E-115) were subcultured and maintained as described [7]. Dulbecco's modified Eagle's medium was supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY). Cells were grown in 75 cm^2 flasks (Falcon Plastics, Cockeysville, MD) in 20 ml of medium. Cells were fed daily and harvested for assays on days 13–21. Cells were subcultured at a distribution of 1:10 (about 5×10^5 cells/flask).

Assays for cyclic nucleotides. The intracellular levels of [^{3}H]cyclic GMP or [^{3}H]cyclic AMP were assayed by the method of prelabeling intracellular stores of the triphosphate nucleotide substrate with the respective [^{3}H]purine base and isolating the [^{3}H]cyclic nucleotide products with ion-exchange columns, as described [7, 8].

Enzyme assays. Guanylate cyclase was assayed as described [9]. Adenylate cyclase was assayed by a method essentially the same as in Ref. 8, isolating the [^{3}H]cyclic AMP on the same columns as referenced above for the intact cell assay. Acetylcholinesterase activity was measured by the method of Wilson *et al.* [10], using heptanone/tetraphenylboron to isolate the [^{14}C]acetate formed. Monoamine oxidase activity was assayed by the oxidation of [^{14}C]-tryptamine to [^{14}C]indoleacetic acid [11].

Other procedures. Solutions of caracemide (a gift of Dr. F. Adams, M. D. Anderson Hospital, Houston, TX) were made up in the assay buffer immediately before its addition to the cells. In some experiments the drug was preincubated under various conditions in order to test whether active metabolites would form spontaneously [12].

RESULTS

Effect of caracemide on the guanylate cyclase system. Caracemide transiently increased the level of [^{3}H]cyclic GMP in N1E-115 cells (Fig. 1). The peak level was reached in 10 min; the amount of the cyclic nucleotide then returned to basal level after 20 min. The concentration–response curve for the elevation of [^{3}H]cyclic GMP by caracemide, assayed at the time of the peak effect, is shown in Fig. 2. The EC_{50} for the response shown in Fig. 2 was 560 μM. The average of two such experiments was 635 ± 75 μM. After preincubation of caracemide in the assay buffer (a "physiological" phosphate-buffered saline solution, iso-osmolar at 340 ± mOs) at either pH 7.4 or pH 9.0 at 37° for 35 min, the concentration–response curve was not changed substantially (data not shown), indicating that a slowly-produced spontaneous breakdown product of caracemide was not formed or, if it did, it acted similarly to the parent molecule. In general, a maximal concentration of caracemide was about two-thirds as effective as a maximal concentration of a muscarinic receptor agonist in mediating [^{3}H]cyclic GMP formation. This effect of caracemide on the intact cell was blocked by 50 or 100 μM eicosatetraynoic acid (ETYA), 50 μM 15-hydroxyeicosatetraenoic acid (15-HETE), and 100 μM bromphenacylbromide, but not by indomethacin (Tables 1 and 2), a pattern reminiscent of receptor-mediated effects on the guanylate cyclase system ([13]; M. McKinney, manuscript in preparation); however, pyrilamine and atropine, specific histaminergic (H_1) and muscarinic receptor antagonists, respectively, were unable to block the effect of caracemide (Table 1). Desensitizing receptors by preincubating for 30 min with histamine, carbachol, or the two agonists together, also had no effect on the response to caracemide (Table 1). A comparison of the pharmacologic profiles of three stimulating agents, caracemide, sodium nitroprusside, and carbachol, is shown in Table 2. The increase in [^{3}H]cyclic GMP levels elicited by both caracemide and sodium nitroprusside was largely blocked by 3 mM EGTA (Table 2). The muscarinic receptor-mediated response was blocked completely by this concentration of EGTA. Nor-

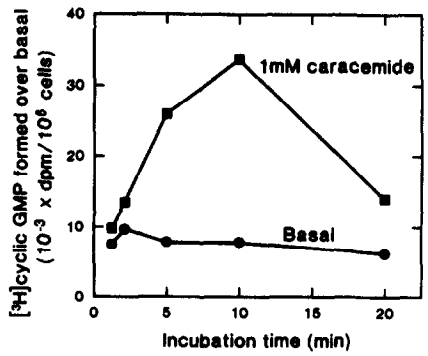


Fig. 1. Time course of the stimulation of [^{3}H]cyclic GMP formation in N1E-115 neuroblastoma cells by caracemide. A fresh solution of caracemide (1 mM final concentration) or buffer ("basal") was added to prelabeled cells at 37°; after incubation for various times the reaction was stopped with trichloroacetic acid, and the [^{3}H]cyclic GMP contents of the wells were determined by ion-exchange chromatography. This particular experiment was performed twice, with each time point assayed in duplicate.

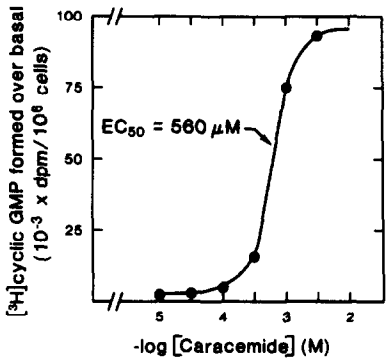


Fig. 2. Concentration–response relation of the stimulation of [^{3}H]cyclic GMP formation in N1E-115 neuroblastoma cells by caracemide. A fresh solution of caracemide was added to prelabeled cells at 37°; the [^{3}H]cyclic GMP formed over basal levels was determined after 10 min. Each point was assayed in duplicate. The EC_{50} for caracemide in this experiment was 560 μM. This experiment was performed twice in this fashion and two other times with different preincubation conditions, as described in the text.

Table 1. Effects of various drugs on increases in [^{3}H]cyclic GMP levels in intact N1E-115 cells induced by caracemide

Inhibition experiment	[^{3}H]Cyclic GMP formed over basal (dpm/10^6 cells)	Percent change
Experiment 1: Receptor agents		
Caracemide (1 mM)	36,600 ± 400	
+Atropine (1 μM)	32,000 ± 1,000	NSD
+Pyrilamine (10 μM)	35,000 ± 3,000	NSD
+Carbachol (1 mM)	33,000 ± 2,000	NSD
+Histamine (1 mM)	34,000 ± 1,000	NSD
+Atropine, pyrilamine	39,000 ± 3,000	NSD
+Carbachol, histamine	35,500 ± 800	NSD
Experiment 2: Arachidonic acid metabolic inhibitors		
Caracemide (1 mM)	20,300 ± 700	
+ETYA (100 μM)	300 ± 700	−99*
+15-HETE (50 μM)	2,400 ± 800	−88*
+Bromphenyacylbromide (100 μM)	20 ± 600	−100*

Inhibitors at the final concentration indicated were incubated at 37° with prelabeled cells for 30 min prior to the addition of caracemide (1 mM final concentration). After a further incubation for 10 min, the reaction was stopped with trichloroacetic acid, and the [^{3}H]cyclic GMP was assayed by ion-exchange chromatography. Values are mean ± S.E. Each assay was performed in triplicate. NSD = not significantly different.

* $P < 0.001$, when compared to the respective control value. Similar experiments were performed three to five times.

Table 2. Comparative pharmacologic profiles for mediation of [^{3}H]cyclic GMP in intact cells by caracemide, sodium nitroprusside, and a muscarinic agonist (carbachol)

Inhibition experiment	[^{3}H]Cyclic GMP formed over basal (dpm/10^6 cells)	Percent change
Caracemide (1 mM)	31,600 ± 800	
+Indomethacin (1 μM)	29,000 ± 2,000	NSD
+ETYA (50 μM)	4,500 ± 2,000	−86*
+NDGA (50 μM)	47,000 ± 4,000	+48†
+DTT (2 mM)	900 ± 960	−97*
+EGTA (3 mM)	2,100 ± 700	−93*
Sodium nitroprusside (250 μM)	216,000 ± 2,000	
+Indomethacin (1 μM)	206,000 ± 5,000	NSD
+ETYA (50 μM)	1,000 ± 2,000	−99*
+NDGA (50 μM)	47,000 ± 3,000	−78*
+DTT (2 mM)	153,000 ± 7,000	−29†
+EGTA (3 mM)	91,000 ± 2,000	−58*
+CARA (1 mM)	69,000 ± 8,000	−68*
Carbachol (1 mM)	52,000 ± 1,000	
+Indomethacin (1 μM)	47,000 ± 1,000	NSD
+ETYA (50 μM)	1,200 ± 800	−98*
+NDGA (50 μM)	8,000 ± 2,000	−84*
+DTT (2 mM)	18,000 ± 2,000	−65*
+EGTA (3 mM)	500 ± 2,000	−99*
+CARA (1 mM)	8,500 ± 600	−84*

Inhibitors at the final concentrations indicated were incubated for 30 min at 37° with prelabeled cells. Caracemide (1 mM final concentration, 10 min), sodium nitroprusside (250 μM final concentration, 5 min), or carbachol (1 mM final concentration, 30 sec) was incubated with the cells; the reactions were stopped with trichloroacetic acid, and the [^{3}H]cyclic GMP was assayed by ion-exchange chromatography. Values are means ± S.E. Each assay shown was performed in triplicate.

* $P < 0.001$ and † $P < 0.02$, when compared with the respective control value. Experiments of this type were performed three times.

dihydroguaiaretic acid (NDGA), which blocks receptor-mediated and sodium nitroprusside-mediated responses, did not inhibit the response to caracemide but actually enhanced it (Table 2). Dithiothreitol (DTT) (2 mM) completely blocked the [^{3}H]cyclic GMP response to caracemide while being less effective against the other two stimulants. The response to caracemide was unaffected by 200 μM physostigmine (data not shown), indicating that acetylcholinesterase was not involved in producing a metabolite of caracemide that caused the [^{3}H]cyclic GMP stimulation.

The effects of NDGA (an antioxidant) and dithiothreitol (an agent that preserves sulfhydryl groups) suggest that caracemide acts to elevate [^{3}H]cyclic GMP levels by oxidizing sulfhydryl groups; the lack of effect with receptor agents suggests that these sulfhydryl groups are not located on receptors. Both the muscarinic response and that due to sodium nitroprusside were inhibited by preincubation with caracemide (Table 2), indicating that the drug does act at a point in the activating mechanism common to all three agents.

The blockade of the muscarinic receptor-mediated elevation of [^{3}H]cyclic GMP, which is effected by the low-affinity agonist–receptor complex in these cells [14, 15], occurred when N1E-115 cells were preincubated with caracemide for periods of time longer than 10 min (Table 2 and Fig. 3). The concentration–response for this effect (Fig. 4), assayed after a 20-min preincubation with caracemide, displayed an IC_{50} of 450 μM, a value similar to the EC_{50} value observed for the initial stimulation. Physostigmine (200 μM) preincubated with the cells to inhibit acetylcholinesterase had no effect on this delayed inhibitory effect of caracemide.

The results with the intact cells suggested that caracemide was acting directly on the effectors involved in activating the system for elevating cyclic GMP or directly on guanylate cyclase itself, bypassing the receptors on the cell surface. We therefore extended the studies to observe the effects of the drug on the guanylate cyclase in crude homogenates. The enzyme in a homogenate of the cells was shown to be transiently activated by caracemide; after 5 min of incubation with the drug, there was a progressive inhibition of the basal activity that reached a nadir by 20 min (Fig. 5). The sodium nitroprusside-activated guanylate cyclase was also inhibited, with a slightly slower time course. A Dixon plot for the inhibition of the basal activity of guanylate cyclase appears in Fig. 6. At concentrations of caracemide up to about 0.4 mM there was no inhibitory effect of the drug; to the contrary, a slight but reproducible activation of the enzyme occurred. Caracemide inhibited guanylate cyclase at concentrations above 0.4 mM. The pattern of the data is compatible with a non-competitive mechanism of inhibition: when the lines

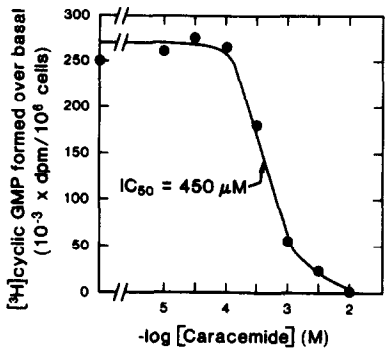


Fig. 4. Concentration-dependence of the inhibition of N1E-115 neuroblastoma muscarinic receptor-mediated [^{3}H]-cyclic GMP formation by caracemide. Various concentrations of caracemide were added to prelabeled cells at 37° for 20 min, in duplicate. Carbachol (1 mM final concentration) was used to activate the muscarinic receptors for 30 sec, at which time the reaction was stopped and [^{3}H]cyclic GMP levels were determined. The IC_{50} for the effect of caracemide in this experiment was 450 μM.

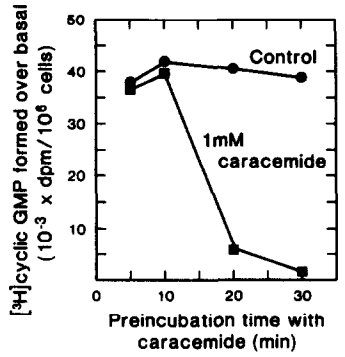


Fig. 3. Time course of the inhibition of N1E-115 neuroblastoma muscarinic receptor-mediated [^{3}H]cyclic GMP formation by caracemide. Caracemide (1 mM final concentration) or buffer ("control") was added to prelabeled cells at 37°. After various times, carbachol (1 mM final concentration) was added. After a further incubation for 30 sec, the [^{3}H]cyclic GMP levels were determined. The points shown were assayed in duplicate.

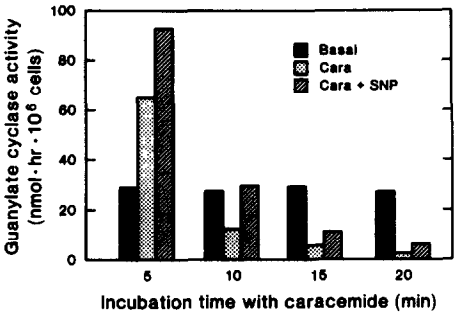


Fig. 5. Time-dependence of the effect of caracemide on basal and sodium nitroprusside-activated guanylate cyclase in a homogenate of N1E-115 neuroblastoma cells. Cells were homogenized in 50 mM Tris (pH 7.6), and caracemide (1 mM final concentration) was preincubated with the homogenate at 37° for the times indicated. Sodium nitroprusside (250 μM final concentration) was added to selected tubes, and then a mixture containing (final concentrations) 5 mM Mg^{2+}, 0.5 mM isobutylmethylxanthine, 220 μM [^{3}H]GTP, and a GTP regeneration system was added in Tris buffer. After a 5-min incubation, the reaction was stopped, and the [^{3}H]cyclic GMP was determined by ion-exchange chromatography. Points were in duplicate.

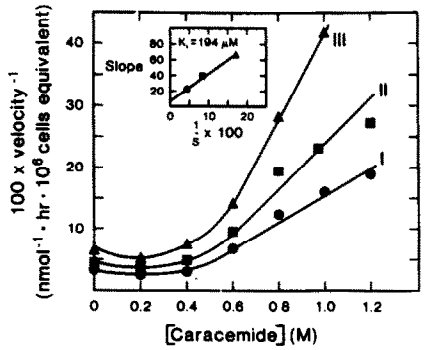


Fig. 6. Dixon plot of the inhibition of N1E-115 neuroblastoma guanylate cyclase by caracemide. Caracemide was incubated with a homogenate of N1E-115 cells at 37° for 20 min prior to the assay for guanylate cyclase activity, performed in triplicate. Assay conditions were the same as in the legend to Fig. 5, except that three different [^{3}H]GTP concentrations were used: I, 220 μM; II, 110 μM; III, 55 μM. This experiment was repeated at least six times.

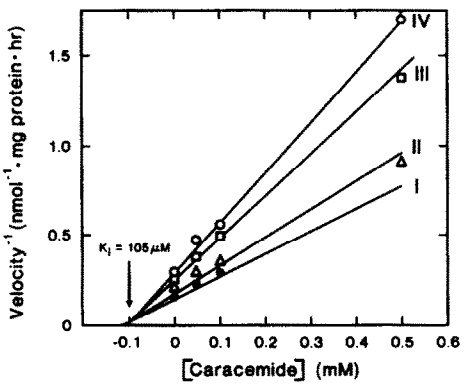


Fig. 7. Dixon plot of the effect of caracemide on PGE_1-mediated [^{3}H]cyclic AMP formation in N1E-115 membranes. Caracemide at various concentrations was incubated in triplicate with membranes at 37° for 15 min prior to the addition of PGE_1 (1 μM final concentration) and the [^{3}H]ATP substrate (four different concentrations: I, 400 μM; II, 200 μM; III, 80 μM; and IV, 40 μM) for 10 min. The experiment was replicated three times. The K_i for caracemide in the experiment shown was 105 μM.

in Fig. 6 are extended, they cross on the abscissa. The inset to Fig. 6, a secondary plot of the data, gave a K_i value of 194 μM for caracemide for the inhibition of guanylate cyclase. The average of five such experiments gave a K_i of 162 ± 39 μM. This is probably an overestimate of the K_i of the apparent metabolite that must be forming in the preparation (which is causing the curvature of the plot), but represents the half-maximal concentration of caracemide required to develop this putative metabolite.

Effect of caracemide on the adenylate cyclase system. The drug did not affect significantly the basal level of [^{3}H]cyclic AMP in intact N1E-115 cells (Table 3). However, 1 mM caracemide inhibited the prostaglandin E_1-mediated increase in the level of this cyclic nucleotide by more than 50% by 10 min. At 30 min of incubation with the cells, 1 mM caracemide inhibited PGE_1-mediated [^{3}H]cyclic AMP increases by more than 80%. At any time point, however, the ability of carbachol to reduce the increases due to the prostaglandin was not affected (Table 3), indicating that the high-affinity muscarinic agonist–receptor complex and its mode of interaction with the prostaglandin/adenylate cyclase system were not inhibited by caracemide. Thus, the drug clearly differentiates between the two effector systems activated by muscarinic receptors in these cells [14, 15].

Caracemide inhibited the PGE_1-activated adenylate cyclase in membranes purified from N1E-115 cells. A Dixon plot for this inhibitory effect of caracemide (Fig. 7) showed a pattern of noncompetitive inhibition. A K_i value of 105 μM was obtained from the data in Fig. 7. The average of three such experiments was 110 ± 15 μM. The data of Table 3 show that caracemide also inhibited increases in [^{3}H]cyclic AMP levels induced by forskolin. We found that caracemide had no effect on adenylate cyclase activity induced by sodium fluoride; this latter agent was effective in activating adenylate cyclase only in broken cell preparations.

Effect of caracemide on acetylcholinesterase. Cara-

Table 3. Effect of caracemide on basal and stimulated adenylate cyclase activity in intact N1E-115 cells

Time of preincubation with caracemide	[^{3}]Cyclic AMP (dpm/10^6 cells)			
	Basal	PGE_1	PGE_1 + Carb	Forskolin
0 (control)	6,400 ± 700	107,000 ± 1,500	53,000 ± 900	212,000 ± 4,500
10 min	5,100 ± 200	22,000 ± 400*	8,800 ± 200†	17,000 ± 600*
20 min	4,300 ± 400	13,000 ± 200*	9,400 ± 500†	14,000 ± 200*
30 min	6,200 ± 200	12,000 ± 500*	8,500 ± 600†	12,000 ± 300*

Caracemide (1 mM final concentration) was preincubated at 37° with prelabeled cells for the times indicated prior to the addition of buffer ("Basal"), PGE_1 (1 μM final concentration), PGE_1 + carbachol (1 mM final concentration), or forskolin (20 μM final concentration). After further incubation for 10 min the reaction was stopped, and the [^{3}H]cyclic AMP was assayed by ion-exchange chromatography. Values are means ± S.E.

* $P < 0.001$ when compared to the respective control level. Similar experiments were performed two times.

† $P < 0.001$ when compared to the level in the presence of PGE_1 alone. The experiment was performed in triplicate.

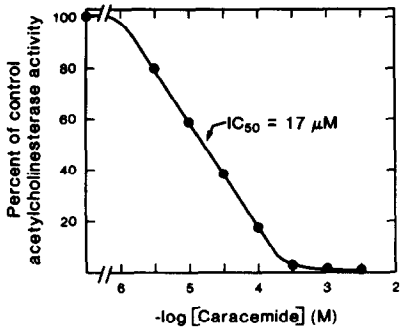


Fig. 8. Concentration-dependence of the inhibition of N1E-115 neuroblastoma acetylcholinesterase by caracemide. Cells were homogenized in 50 mM Tris (pH 7.4) containing 0.2% Triton. The sonicate was incubated with caracemide at 37° for 5 min after which [^{14}C]acetylcholine (0.8 mM final concentration) was added. After 5 min the reaction was stopped with the addition of a solution of tetraphenylboron in 3-heptanone (50 mg/ml). After centrifugation, an aliquot of the aqueous phase containing the [^{14}C]acetate product was counted. Each point was assayed in duplicate. Pseudocholinesterase activity was determined in some tubes after inhibiting true acetylcholinesterase with BW 284C51.

Table 4. Effect of caracemide on monoamine oxidase activity in homogenized N1E-115 cells

Time of preincubation with caracemide	Oxidation of [^{14}C]tryptamine (pmoles/hr-10^6 cells equivalent)
0 (control)	1330 ± 50
5 min	1430 ± 60
10 min	1550 ± 60*
20 min	1480 ± 60†
30 min	1400 ± 10

Cells were homogenized in 5 mM Tris (pH 7.4) containing 0.2% Triton, and caracemide was incubated with the homogenate for various times. [^{14}C]Tryptamine was added in 0.5 M potassium phosphate buffer (pH 7.4), and the incubation was continued for 20 min. The reaction was stopped by the addition of toluene, and an aliquot of the organic phase containing the [^{14}C]product was counted by liquid scintillation. Boiled enzyme blanks were the same as buffer blanks and were about 5% of the control activity shown; the appropriate correction for the blank was applied. Values are means ± S.E. Each assay shown was performed in quadruplicate.

* $P < 0.01$ and † $P < 0.05$, when compared to control activity. This experiment was performed twice.

cemide inhibited acetylcholinesterase in N1E-115 cells in a concentration-dependent fashion (Fig. 8). This inhibition was in full effect as early as 5 min of incubation of the drug with the enzyme. The IC_{50} in Fig. 8 was 17 μM, and the [^{14}C]acetylcholine concentration used (0.8 mM) was about twice the K_i value for this enzyme (445 μM). A Dixon plot for the acute inhibition of acetylcholinesterase by caracemide appears in Fig. 9. The pattern of inhibition in Fig. 9 is competitive, with a K_i value of 9 μM. The average K_i for caracemide in five such experiments was 8 ± 1 μM.

Effect of caracemide on monoamine oxidase. Caracemide did not inhibit acutely the monoamine oxidase of N1E-115 cells when preincubated with the enzyme for as long as 30 min (Table 4). At 10 min there was a slight but significant increase in the activity of the enzyme.

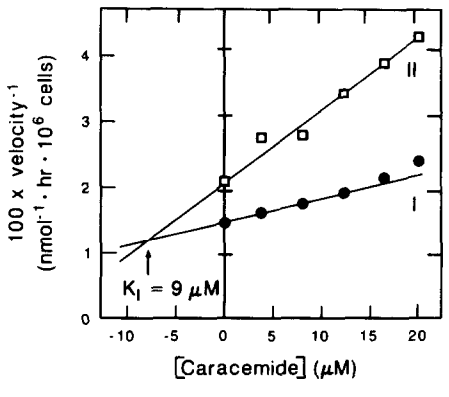


Fig. 9. Dixon plot of the inhibition of N1E-115 neuroblastoma acetylcholinesterase by caracemide. Incubation and assay conditions were as described in the legend to Fig. 8 except that a 12-min incubation and two different concentrations of [^{14}C]acetylcholine were used: I, 2.12 mM and II, 0.27 mM. Each point is the average of triplicate determinations. The K_i for caracemide was 9 μM, expressed as the final caracemide concentration after all additions. Five such Dixon plots for the effect of caracemide on acetylcholinesterase were obtained.

DISCUSSION

Caracemide was shown to have several acute neurochemical effects in N1E-115 cells. The drug transiently elevated cyclic GMP levels in intact cells, apparently by diffusing into the cell and activating intracellular processes that are sensitive to inhibitors of arachidonate metabolism, to calcium depletion, and to dithiothreitol. Receptor-mediated activation of both guanylate cyclase (intact cells) and adenylate cyclase (in either intact cells or homogenates) was inhibited by caracemide. The inhibition of the muscarinic receptor-mediated formation of [^{3}H]cyclic GMP, which is effected by the low-affinity agonist–receptor complex [14, 15], was probably due to the direct effect of a metabolite of caracemide on guanylate cyclase. The effects of the drug on the two cyclic nucleotide systems differed in that basal adenylate cyclase activity was not affected by caracemide. Adenylate cyclase stimulated by either prostaglandin E_1 or forskolin was inhibited by caracemide. The PGE_1-activated enzyme was shown to be inhibited noncompetitively. The ability of the high-affinity muscarinic receptor to inhibit PGE_1-mediated [^{3}H]cyclic AMP increases, however, was not largely affected at any time point tested, even though the response due to PGE_1 itself was inhibited profoundly. Acetylcholinesterase was inhibited rapidly by caracemide; the mechanism observed in this acute phase was competitive in nature. The drug did not inhibit monoamine oxidase significantly with incuations up to 30 min, but was observed to activate this enzyme modestly by 10 min.

The structure of caracemide, which contains several labile bonds, led us to expect that the drug would be subjected to several routes of degradation in solution or by cell metabolism. In serum-free buffer, however, we did not observe any large change in potency or efficacy, even with preincubation of the drug at 37° at pH 9. Our data with guanylate cyclase support the hypothesis that the drug may be converted by a cellular process to some other form before the inhibitory phase of its action on this enzyme begins. After a short preincubation, there was no inhibition of guanylate cyclase below about 0.5 mM caracemide, but there was a mild stimulation of the enzyme. Apparently, the putative inhibitory metabolite did not appear at a significant level in a short incubation until the caracemide concentration approached 0.5 mM. It should be noted, however, that all our evaluations of the drug were performed at times of 30 min or less; thus, only selected acute effects of caracemide are described. Further effects of the drug after chronic incubation may be expected. In clinical practice the drug is infused continuously over a period of several days; under these conditions it might be expected that long-term effects on cellular processes may appear.

The inhibitory action of caracemide or its metabolite(s) on [^{3}H]cyclic GMP responses elicited by the muscarinic receptor was apparently due to an effect of the drug on the guanylate cyclase itself or on the intracellular process by which it was activated, as both the basal enzyme activity (in homogenates) and sodium nitroprusside-mediated activation (in either intact cells or homogenates) of the guanylate cyclase were inhibited by caracemide. We have found that the arachidonate metabolic inhibitors (ETYA, 15-HETE, bromphenacyl bromide) have multiple effects in our system (M. McKinney, manuscript in preparation). The blockade of the effect of caracemide by these agents, shown in Table 1, could therefore have been a reflection of inhibitory action somewhere other than on a lipoxygenase or phospholipase. Interestingly, NDGA, which inhibits receptor-mediated [^{3}H]cyclic GMP responses, augmented the response to caracemide. One possible explanation for this effect is that the antioxidant blocked the degradation of the putative active metabolite formed from caracemide. The responses to carbachol, sodium nitroprusside, and caracemide were all inhibited by dithiothreitol, suggesting the involvement of oxidation processes in guanylate cyclase activation. The response to caracemide was the most sensitive to this agent.

The inhibition of adenylate cyclase was observable only with receptor-mediated or forskolin-mediated activation of the enzyme. Because no effect was seen on the basal enzyme, the result with the receptor agonist would suggest that caracemide does not act on the adenylate cyclase itself, but on the receptor's mechanism of activation of the enzyme. However, the result with the directly activating agent, forskolin, suggests that caracemide has a direct effect on adenylate cyclase that appears only when the enzyme is activated with forskolin. It may be, then, that the receptor-mediated response was also due to a direct effect on adenylate cyclase and not on the guanine nucleotide regulatory subunit, which presumably is involved in receptor-mediated processes. The ability of the high-affinity muscarinic receptor to inhibit PGE_1-mediated elevations in [^{3}H]cyclic AMP was not affected by caracemide; thus, the effects of this drug on this enzyme appear to be only via activating mechanisms. An additional conclusion that can be drawn from these results is that the high-affinity and low-affinity muscarinic agonist–receptor complexes activate distinct effectors in N1E-115 cells [14, 15].

Caracemide appears acutely to bind competitively to the active site of acetylcholinesterase. As the structure of the drug contains the same carbamylating moiety present in physostigmine, an irreversible effect of caracemide on acetylcholinesterase due to its alkylation of the enzyme esteratic site might be expected on chronic treatment with the drug. Either the demonstrated acute competitive action or the possible chronic semi-irreversible inhibition would account for some of the clinical manifestations of cholinergic excess.

The finding that caracemide affects processes within intact neuron-like cells indicates that caracemide or something derived from it diffuses into the neuron. Thus, there is an important implication that the drug could gain nearly immediate access to the nervous system after crossing the blood–brain barrier, diffusing into neurons to act on critical intracellular processes. Because of the seemingly nonselective action of caracemide on the neurochemical systems tested so far, it can probably be expected that the drug will affect numerous other enzymes in the brain. Our hypothesis is that a degradation product, perhaps hydroxylamine, oxidizes sensitive residues (such as sulfhydryl groups) or cofactors of proteins, or disturbs the oxidation–reduction potential of the neuron. This drug, and others like it, then could have global central nervous system effects, in addition to its specific effects on cholinergic systems. Our results with caracemide indicate to us that it is of critical importance that investigators involved in drug testing take into account central nervous system side-effects. One of the most direct ways to do this is to examine the molecular pharmacology of the drug with an *in vitro* system such as the mouse neuroblastoma system.

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