smooth muscle MLCK in the absence of calmodulin, including Ser^{512} [(4); M. E. Payne, M. Elzinga, R. S. Adelstein, *J. Biol. Chem.* **261**, 16346 (1986)]. The tryptic digestion of the phosphorylated enzyme also generated the inactive 64-kD fragment (6), which was not phosphorylated, together with two tryptic phosphopeptides. The largest phosphopeptide, a 23-kD species, purified by Mono Q anion-exchange chromatography, had the amino-terminal sequence Lys^{523}-Ala-Ser(^{32}P)-Gly-Ser-Ser-Pro-Thr^{530}; the same fragment was also generated from the unphosphorylated enzyme. The smaller phosphopeptide was purified by reversed-phase HPLC and found to have the sequence Leu^{510}-Ser-Ser(^{32}P)^{512}-Met-Ala-Met-Ile-Ser-Gly-Met^{519}. The identification of the two sites of phosphorylation, Ser^{512} and Ser^{525}, confirms findings made by others [(4); M. E. Payne *et al.*, see above; Fig. 3B]. Thus the result of the tryptic analysis of the phosphorylated enzyme was consistent with the finding that 64-kD fragment ended at Arg^{505} (Fig. 3). The intervening tryptic peptide Ala^{506}-Ile-Gly-Arg^{509} was not recovered from the tryptic digest of the phosphorylated enzyme because identification of these peptides depended on the presence of ^{32}P.

14. A 60-min incubation of the 64-kD fragment with carboxypeptidase P [ratio of 1:20 (w:w)] yielded only 15% of the activity resulting from tryptic digestion over the same period [ratio of 1:40 (w:w)]. Carboxypeptidase P digestion resulted in the release of Val and Arg as well as Lys from the 64-kD fragment. These measurements were made after subtraction of the carboxypeptidase blank. The presence of Val and Arg was consistent with the assignment of Arg^{505} as the carboxyl terminus of the 64-kD fragment, but the lysine was unaccounted for.
15. The structure of the 61-kD fragment appears more open because it is more susceptible to proteolytic digestion than the 64-kD fragment. Several chymotryptic peptides were unique to the 61-kD fragment and were derived from the internal region; for example, HPLC peak 3, Gly^{228}-Gln-Val-Phe^{231}, and peak 7, Gly^{359}-Leu-Ala-Arg-Arg-Leu^{364}.
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Beta-*N*-Methylamino-L-Alanine Neurotoxicity: Requirement for Bicarbonate as a Cofactor

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Ingestion of the excitotoxic cycad seed amino acid β-*N*-methylamino-L-alanine may be responsible for the neuronal degeneration associated with Guam amyotrophic lateral sclerosis–parkinsonism–dementia in man. However, the basis for the central neurotoxicity of β-*N*-methylamino-L-alanine has been unclear, as it lacks the omega acidic (or equivalent electronegative) moiety characteristic of other excitatory amino acids. β-*N*-methylamino-L-alanine produced neurotoxic and neuroexcitatory effects in murine cortical cell cultures only when physiological concentrations of bicarbonate were available in the extracellular bathing medium. Bicarbonate may interact noncovalently with β-*N*-methylamino-L-alanine to produce, in combination, a molecular configuration that activates glutamate receptors.

THE HIGH INCIDENCE OF AMYOTROPHIC lateral sclerosis (ALS), often with features of parkinsonism and Alzheimer-type dementia (ALS-PD) among the Chamorro people in Guam has prompted intensive epidemiological searches for etiologic clues (*1*). The demonstration that ingestion of β-*N*-methylamino-L-alanine (BMAA), an uncommon amino acid present in the seed of the indigenous food plant *Cycas circinalis*, can induce in primates a neurodegenerative syndrome closely resembling Guam ALS-PD has implicated cycad-BMAA ingestion as the specific cause of the human disease (*2, 3*).

Chemically, BMAA is related to another plant-derived amino acid, β-*N*-oxalylamino-L-alanine (BOAA), which is implicated in the pathogenesis of lathyrism (*4*). BOAA is a dicarboxylic amino acid and, like other such amino acids, can produce neuroexcitatory and neurotoxic effects on central neurons and can induce seizures in rodents (*5*). Although less potent than BOAA, single doses of BMAA can also induce seizures in rodents (*6*). In explants of spinal cord or frontal cortex, BMAA can produce excitotoxic neuronal degeneration that can be attenuated by *N*-methyl-D-aspartate (NMDA) antagonists (*7*). Because BMAA lacks the ω-acidic or electronegative moiety characteristic of other excitatory amino acids (having instead a positively charged β-amino group), this excitotoxic action is curious; Spencer *et al.* hypothesized that it may be mediated indirectly by a metabolite (*3*).

We investigated BMAA neurotoxicity on cortical neurons in cell culture, a system permitting control of the extracellular milieu and a high degree of temporal resolution. Dissociated mouse cortical cultures were prepared (*8*) and studied between 15 and 21 days after plating in media consisting of Eagle's minimal essential medium (MEM–Earle's salts, supplied glutamine-free) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, glutamine (2 m*M*), and glucose (total 21 m*M*). Cells were initially exposed to BMAA (*9*) in a defined Hepes-buffered (*p*H 7.4) physiological salt solution (HSS) with the following composition: 130 m*M* Na^+, 5.4 m*M* K^+, 0.8 m*M* Mg^{2+}, 1.8 m*M* Ca^{2+}, 20 m*M* Hepes, 130.6 m*M* Cl^-, and 15 m*M* glucose. A 1-hour exposure to 3 m*M* BMAA in HSS did not produce any evidence of the neurotoxicity seen with dicarboxylic excitatory amino acids (*8, 10*); that is, acute neuronal swelling, late cell loss, and efflux of lactate dehydrogenase (LDH) to the bathing medium were all absent (Fig. 1A; Fig. 2 shows representative morphological appearance) (five experiments). A similar lack of BMAA neurotoxicity was seen in a tris-buffered exposure solution. These observations contrasted with rapid (within minutes) neuronal vacuolation seen in mouse frontal cortex explants after exposure to 1.6 m*M* BMAA (*3*).

To reconcile our findings with the previous study (*3*), we repeated the experiment and used the Eagle's MEM exposure solution described in (*3*); 1 to 3 m*M* BMAA then produced acute neuronal swelling and substantial late neuronal degeneration (ten experiments). The key difference appeared to be the bicarbonate (HCO_3^-) present in MEM but not in HSS. If 10 m*M* $NaHCO_3$ was added to HSS, then exposure to 3 m*M* BMAA for 1 hour produced a substantial amount of neuronal degeneration by the next day (Fig. 1A). This permissive effect of HCO_3^- on BMAA neurotoxicity was not mimicked by 10 m*M* NaCl (Fig. 1A) and not accounted for by *p*H shifts. The addition of 10 m*M* HCO_3^- to HSS produced only a small alkalinization, to *p*H 7.5 initially and gradually to *p*H 7.7 at the end of 1 hour in room air. If this alkalinization were mimicked by addition of NaOH (both at the beginning and in the middle of the 1-hour exposure), BMAA remained essentially nontoxic (Fig. 1A). BMAA was also nontoxic at a higher *p*H of 8.2 in HSS (Fig. 1A) and

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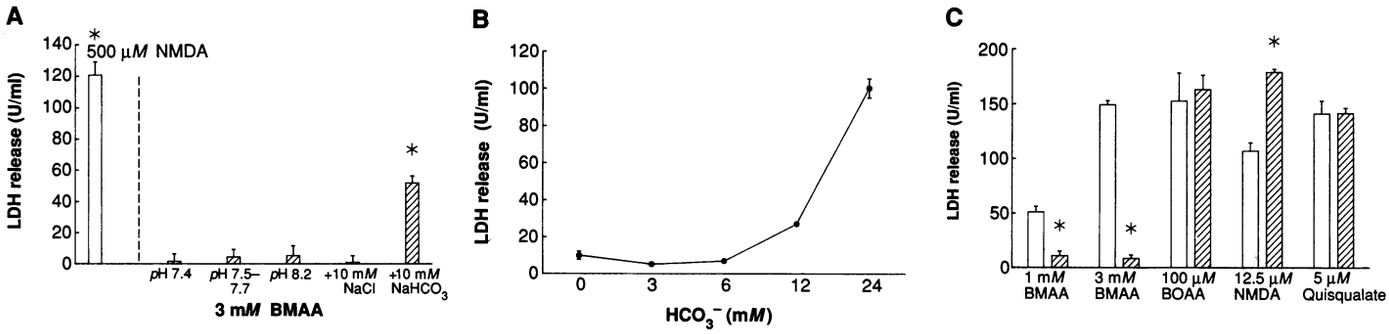

Fig. 1. (**A**) BMAA neurotoxicity requires extracellular bicarbonate. Sister cultures were exposed to 3 m*M* BMAA for 1 hour in HSS in room air under the indicated conditions, then exchanged into Eagle's MEM with augmented glucose (21 m*M*), and returned to the CO_2 incubator (37°C) overnight. The shaded bars depict LDH (mean + SEM, $n = 4$) present in the bathing medium 20 hours after BMAA exposure, following subtraction of the small amount of background LDH present in sister cultures exposed only to sham wash. For comparison, the open bar shows the LDH present in other sister cultures exposed to 500 μ*M* NMDA for 5 min, a toxic exposure sufficient to destroy most of the neurons in the dish. Experience has shown that this specific LDH efflux correlates quantitatively with the number of neurons irreversibly damaged by exposure to an excitatory amino acid (*10*). Description of shaded bars is as follows: *p*H 7.4, control exposure in HSS; *p*H 7.5 to 7.7, HSS titrated with NaOH to *p*H 7.5 initially and to *p*H 7.7 after 30 min; *p*H 8.2, HSS titrated with NaOH to *p*H 8.2; +10 m*M* NaCl, HSS with added 10 m*M* NaCl; and +10 m*M* $NaHCO_3$, HSS with added 10 m*M* $NaHCO_3$. The asterisk indicates significant difference from the *p*H 7.4 condition at $P < 0.05$ by analysis of variance and Student-Newman-Keuls procedure. (**B**) BMAA neurotoxicity is dependent on the bicarbonate concentration. Sister cultures were exposed to 3 m*M* BMAA for 1 hour in HSS, with addition of the indicated concentration of HCO_3^-. LDH (±SEM, $n = 4$) efflux above background was measured in the bathing medium 20 hours later. (**C**) Specificity of bicarbonate dependence. Sister cultures were exposed to the indicated toxic agonist for 1 hour in a standard bicarbonate-buffered salt solution(Earle's BSS) (open bars) with the following composition: 143.5 m*M* Na^+, 5.4 m*M* K^+, 1.8 m*M* Ca^{2+}, 0.8 m*M* Mg^{2+}, 125.3 m*M* Cl^-, 26.2 m*M* HCO_3^-, 0.8 m*M* SO_4^{2-}, and 1.0 m*M* $H_2PO_4^-$. Exposure took place in the CO_2 incubator at 37°C, under conditions ensuring normal *p*H (7.4) of the exposure solution. LDH efflux above background was measured 20 hours later (open bars, mean + SEM, $n = 4$). The shaded bars depict LDH values in cultures treated similarly, but exposed to the indicated toxic agonist in BSS modified by replacing HCO_3^- with 20 m*M* Hepes (HBSS) in a room air (37°C) incubator (also at *p*H 7.4). The asterisk indicates difference from toxicity in BSS at $P < 0.05$ by two-tailed *t* test.

became toxic in the presence of HCO_3^- even in an incubator that contained CO_2, in which *p*H was maintained at 7.4.

The neurotoxicity of BMAA depended on the concentration of added HCO_3^- between 6 and 24 m*M*, with little toxicity in the presence of less than 6 m*M* HCO_3^- (Fig. 1B). With the addition of 24 m*M* HCO_3^- and a 1-hour exposure, 3 m*M* BMAA destroyed 50 to 80% of the cortical neuronal population.

This requirement for HCO_3^- was not found with several other excitatory amino acid neurotoxins. One-hour exposure to 1 or 3 m*M* BMAA, 100 μ*M* BOAA, 12.5 μ*M* NMDA, or 5 μ*M* quisqualate in a standard HCO_3^--containing solution [Earle's balanced salt solution (BSS)] all produced substantial amounts of neuronal degeneration by the following day, accompanied by efflux of LDH to the bathing medium (Fig. 1C). However, substitution of Hepes for HCO_3^- in the exposure solution [Hepes balanced salt solution (HBSS)] abolished only the toxicity of BMAA (Fig. 1C). The toxicity of BOAA and quisqualate was largely unaffected; the toxicity of NMDA was actually increased by this substitution (Fig. 1C).

To investigate the possibility that HCO_3^- unmasked BMAA toxicity by preventing BMAA metabolism or uptake, we incubated cultures in BMAA alone before the addition of HCO_3^-. No morphological changes in neurons were apparent during a 30-min preincubation in 3 m*M* BMAA in HSS. However, within 3 to 5 min of adding

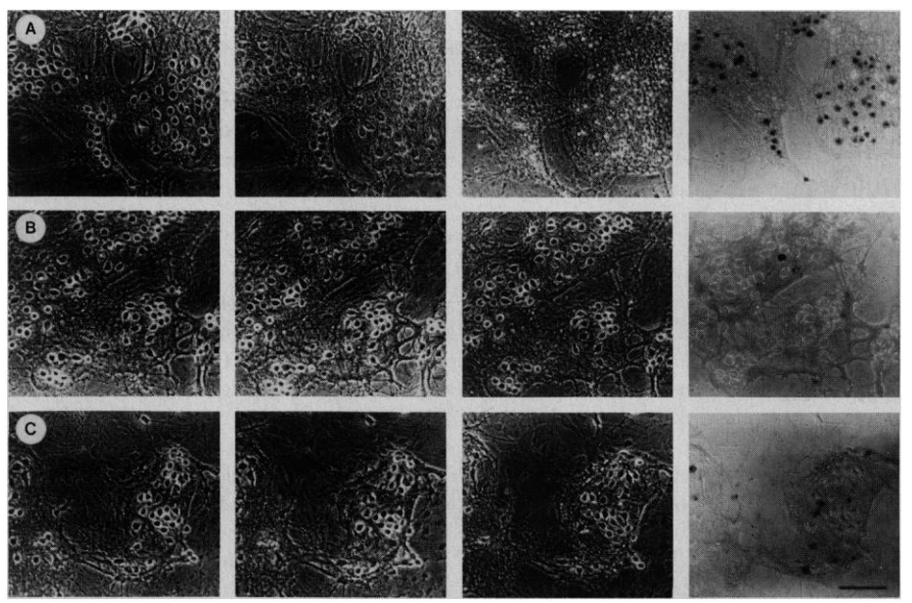

Fig. 2. Effect of bicarbonate on BMAA neurotoxicity. Photomicrographs (×100) from three sister cultures (**A**, **B**, and **C**) are shown before (left column), during (left center), and 20 hours after (right center) onset of a 1.5-hour exposure to 3 m*M* BMAA in HSS at room temperature; rightmost column shows same as right center after incubation in 0.5% Trypan blue for 5 min. After exposure to BMAA in HSS for 30 min, none of the cultures exhibited morphological changes compared with baseline. At that time, a small (<10 μl) aliquot of desired test compounds was added to each culture, and 5 min later the "during" (left center column) micrographs were taken. After 55 more minutes, the exposure solutions were washed out, media (lacking serum) were replaced, and the cultures were returned to the 37°C incubator overnight, until the right center and rightmost micrographs were obtained. The following test compounds were added during BMAA exposure: (A) $NaHCO_3$ to a final concentration of 20 m*M*; (B) NaCl to a final concentration of 20 m*M*, plus enough NaOH to roughly mimic the alkalinizing effect of the HCO_3^- added in (A); and (C) $NaHCO_3$ to 20 m*M* plus APV to 500 μ*M*. Control experiments demonstrated that addition of 20 m*M* HCO_3^- to HSS acutely increased *p*H to about 7.7; over the next hour of exposure in room air, *p*H drifted upward to about 8.0. To control for this alkalinization, in (B) NaOH was added to raise *p*H to 7.7 initially (at the time of NaCl addition), and then another portion of NaOH was added 30 min later to further raise *p*H to 8.0. Photomicrographs were taken with phase-contrast optics, except for the Trypan blue staining, which was taken with bright-field optics. Bar, 50 μm.

$NaHCO_3$, neuronal soma became abruptly swollen, granular, and phase-dark (Fig. 2A); continued joint BMAA-HCO_3^- exposure for 1 hour was followed by the usual widespread neuronal degeneration over the next 20 hours (Fig. 2A). This acute effect of HCO_3^- was not mimicked by addition of 20 m*M* NaCl (and enough base to match the alkalinizing effect of 20 m*M* HCO_3^-) (Fig. 2B), and could be blocked by the NMDA antagonist 2-amino-5-phosphonovalerate (APV) (Fig. 2C).

We also investigated the effect of HCO_3^- on BMAA responses electrophysiologically. Neurons were penetrated with microelectrodes filled with potassium acetate (4*M*) (resistance, 50 to 80 megohms), and current pulses were passed through the recording electrode through a bridge circuit. Pressure ejection of either 3 m*M* BMAA (seven cells) or 20 m*M* $NaHCO_3$ (three cells) in Hepes-buffered recording medium produced little or no change in membrane potential or conductance (Fig. 3A). However, when 3 m*M* BMAA was applied together with 20 m*M* $NaHCO_3$ (in the same micropipette), a rapid membrane response occurred consisting of depolarization and increased membrane conductance. This response could be reversibly attenuated by immediate prior ejection of the glutamate antagonist kynurenate at 2 m*M* (three cells) (Fig. 3B).

These observations document a unique dependence of the neurotoxic effects of BMAA on physiological concentrations of extracellular HCO_3^-. In the presence of HCO_3^-, low millimolar concentrations of BMAA produced acute swelling and substantial late degeneration of cortical neurons in cell culture, an effect consistent with the weak toxicity observed with BMAA in motor cortex explants bathed in a HCO_3^--containing culture medium (7). In both systems, BMAA-HCO_3^- neurotoxicity could be substantially attenuated by selective NMDA antagonists, suggesting important mediation by NMDA receptors. However, in the absence of extracellular HCO_3^-, the neurotoxicity of BMAA was abolished.

The interaction between BMAA and HCO_3^- was highly specific to BMAA, and hence unlikely to represent a direct effect of HCO_3^- on postsynaptic glutamate receptors or on neuronal viability in general. None of the other excitatory amino acid compounds we have studied, including BOAA and NMDA, required the presence of HCO_3^- to produce neuronal injury.

The interaction between BMAA and HCO_3^- cannot be explained by an ability of HCO_3^- to reduce the cellular uptake or metabolism of BMAA. BMAA incubated in cultures without HCO_3^- was not toxic, but was fully available for subsequent rapid activation by addition of HCO_3^-. This interaction was not restricted to the toxic effects of BMAA, but also extended to its neuroexcitatory effects, suggesting that HCO_3^- was required to allow BMAA to open glutamate receptor–linked membrane conductances. The interaction was also fast; HCO_3^- could unmask BMAA neuroexcitation within a fraction of a second when applied directly onto a neuron in cell culture, a result that argues against involvement of indirect metabolites.

The simplest explanation consistent with these observations is that HCO_3^- interacts directly with BMAA to produce a structure suitable for glutamate receptor activation. Structure-activity analysis of NMDA agonists suggests a three-point association between agonist and receptor, with the agonist usually presenting a positively charged amino group, an α-carboxylate group, and an ω-acidic group (*11*). A noncovalent interaction between the positively charged β-amino group of BMAA and HCO_3^- could act both to negate the positive charge of the group and to add a moiety that approximates the ω-acidic group of other glutamate agonists (Fig. 4). Some precedent for this proposal exists; the γ-aminobutyric acid (GABA)–mimetic activity of ethylenediamine also requires the presence of HCO_3^- (*12*). Ethylenediamine is structurally similar to GABA but has a second amino group instead of a carboxylic acid group on one end of the molecule. Bicarbonate could associate with that amino group and simulate the existence of a carboxylic acid group.

The finding that HCO_3^- is required for BMAA neurotoxicity may be important for understanding the pathogenesis of Guam ALS-PD and could provide insights into the pathogenesis of the sporadic forms of ALS, Parkinson's disease, or Alzheimer's disease. In addition, the receptor "cofactor" principle delineated here could have far-reaching implications, perhaps suggesting that other compounds, which by themselves are not structurally recognizable as glutamate agonists, can produce neuronal injury.

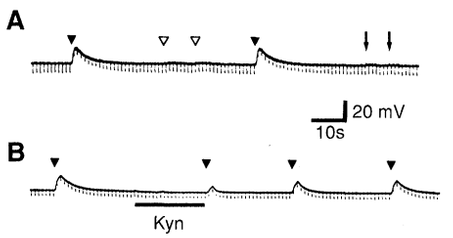

Fig. 3. BMAA neuroexcitation also requires bicarbonate. Cultures were bathed in a recording medium consisting of HSS modified by augmenting Ca^{2+} (3.6 m*M*), removing Mg^{2+}, and adding 1 μ*M* tetrodotoxin; Mg^{2+} was removed to facilitate detection of NMDA receptor-mediated neuroexcitation; tetrodotoxin was added to abolish spontaneous synaptic activity. Recordings were performed at room temperature, and drugs were applied by pressure ejection with recording medium as the carrier. (**A**) The solid triangles indicate the application of 2-s pressure ejection pulses of 3 m*M* BMAA plus 20 m*M* $NaHCO_3$, open triangles indicate 3-s pulses of BMAA plus 20 m*M* extra NaCl, and arrows indicate 3-s pulses of 20 m*M* $NaHCO_3$ alone. Cell resting potential was −60 mV, and injected current pulses were 0.11 nA. (**B**) Pressure ejection of 2 m*M* kynurenate (Kyn) markedly attenuated the subsequent response to 2-s pulses of 3 m*M* BMAA plus 20 m*M* $NaHCO_3$ (solid triangles). Resting potential was −60 mV, and current pulses were 0.09 nA.

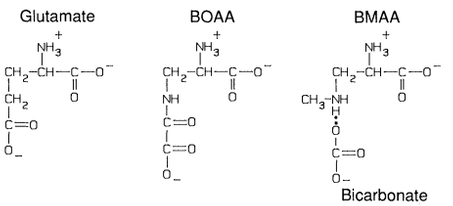

Fig. 4. A noncovalent interaction of BMAA and bicarbonate may simulate a glutamate agonist.

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