ty was not associated with the EGF receptor (*10*).

Neither adenosine triphosphate (ATP) nor bradykinin, both of which stimulate formation of IP_3 in A-431 cells, enhanced the recovery of phospholipase C activity from the phosphotyrosine antibody matrix (*10*). This finding suggests that these agonists induce the formation of IP_3 by a mechanism or mechanisms that do not involve tyrosine phosphorylation. While our experiments have not focused on possible roles for G proteins in mediating EGF-stimulated formation of IP_3, we have been unable to further activate the immunoaffinity-purified phospholipase C by addition of guanosine triphosphate (GTP) or GTP-γ-S. Also, treatment of A-431 cells with toxins or other agents that modify certain G proteins does not affect the EGF-stimulated formation of inositol phosphates in vivo (*10*).

To date, although several proteins, including several enzymes, have been identified in vivo or in vitro as possible exogenous substrates of tyrosine kinases (*5*), none of these is a good candidate as a molecule regulating cell proliferation. In no case has a biochemical function been associated with tyrosine phosphorylation of exogenous substrates. Data obtained in vivo by expression of tyrosine kinase–defective mutants of the EGF receptor indicate that all events distal to receptor occupancy, including the formation of inositol phosphates and the release of stored intracellular Ca^{2+} (*12*), are abrogated directly or indirectly as a consequence of this mutation. Those data support the hypothesis that tyrosine kinase activity is required for EGF-stimulated PIP_2 turnover in vivo, but do not discriminate as to whether autophosphorylation of the receptor or exogenous substrate phosphorylation is critical to the generation of biological responses. Our findings suggest that either phospholipase C or a tightly associated protein is an exogenous substrate for EGF-stimulated tyrosine phosphorylation, and this tyrosine phosphorylation may have functional consequences relating to PIP_2 hydrolysis.

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13. A-431 cells were subcultured in 100-mm Falcon tissue culture plates at a density of 1×10^4 to 2.5×10^4 cell/cm^2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum, 20 m*M* Hepes (*p*H 7.4), and gentamicin (50 mg/ml) and grown for 2 to 3 days to a density of 5×10^4 to 10×10^4 cell/cm^2. Then the cells were washed twice with DMEM and incubated at 37°C in 4 ml of DMEM. Sodium orthovanadate was added to all cultures to a final concentration of 100 μ*M* for 10 minutes, and the cultures were treated without or with EGF (200 ng/ml) for an additional 0 to 60 minutes. (A control experiment demonstrated that treatment with vanadate prior to growth factor addition was not required.) Subsequently, the cultures were placed on ice and washed four times with ice-cold calcium- and magnesium-free phosphate-buffered saline. Soluble cell extracts were prepared by adding 1.4 ml per plate of ice-cold solubilization buffer [20 m*M* Hepes, *p*H 7.2, 1.0% octyl β-D-glucopyranoside, 30 m*M* sodium pyrophosphate, 50 m*M* sodium chloride, 5 m*M* β-glycerophosphate, 1 m*M* EGTA, 1 m*M* phenylmethylsulfonylfluoride, 100 μ*M* sodium orthovanadate, aprotinin (10 μg/ml), and leupeptin (10 μg/ml)]. The soluble cell extracts were centrifuged for 10 minutes at 250,000*g*. The clarified extracts (0.75 to 1.5 mg/ml) were treated with 50 to 100 μl of Sepharose-linked phosphotyrosine antibody (1G2) at a ratio of approximately 1.0 to 1.5 mg extract to 100 μl of packed bead matrix. After adsorption to the matrix for 2 hours at 4°C, the bead matrix was batch-washed four times with ten volumes of ice-cold solubilization buffer. Finally, eluates from the matrix were obtained by addition of 1.5 volumes of elution buffer (solubilization buffer plus 1 m*M* phenylphosphate). After 10 minutes at 4°C the eluates (100 to 200 μl) were recovered by centrifugation into a second tube. The phospholipase C assay was performed by adding 20 μl of the eluates to 30 μl of a reaction solution containing [^{3}H]PIP_2, octylglucoside, calcium chloride plus EGTA, sodium phosphate (*p*H 6.8), and potassium chloride, yielding, in a 50-μl volume, final concentrations of 200 μ*M*, 0.65%, 1 μ*M* free Ca^{2+}, 20 m*M*, and 40 m*M*, respectively.

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Autoregulation of Enzymes by Pseudosubstrate Prototopes: Myosin Light Chain Kinase

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The myosin light chain kinase requires calmodulin for activation. Tryptic cleavage of the enzyme generates an inactive 64-kilodalton (kD) fragment that can be further cleaved to form a constitutively active, calmodulin-independent, 61-kD fragment. Microsequencing and amino acid analysis of purified peptides after proteolysis of the 61- and 64-kD fragments were used to determine the amino-terminal and carboxyl-terminal sequences of the 64-kD fragment. Cleavage within the calmodulin-binding region at Arg^{505} generates the catalytically inactive 64-kD fragment, which is incapable of binding calmodulin. Further digestion removes a carboxyl-terminal fragment, including the pseudosubstrate sequence Ser^{484}-Lys-Asp-Arg-Met-Lys-Lys-Tyr-Met-Ala-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Val-Arg^{505} and results in a calmodulin-independent 61-kD fragment. Both the 61- and 64-kD fragments have the same primary amino-terminal sequences. These results provide direct support for the concept that the pseudosubstrate structure binds the active site and that the role of calmodulin is to modulate this interaction. Pseudosubstrates may be utilized in analogous ways by other allosterically regulated enzymes.

PROTEIN KINASES ARE INVOLVED IN the regulation of protein and enzyme functions necessary for numerous biological processes (*1*). The smooth muscle myosin light chain kinase (MLCK) plays an obligatory regulatory role in the initiation of smooth muscle contraction (*2*). In many instances, protein kinases are inactive and require activation by regulators. In the case of the MLCK, the regulator is calmodulin. Structural studies by Blumenthal *et al.* (*3*) showed that a peptide segment of 26 residues was responsible for binding calmodulin

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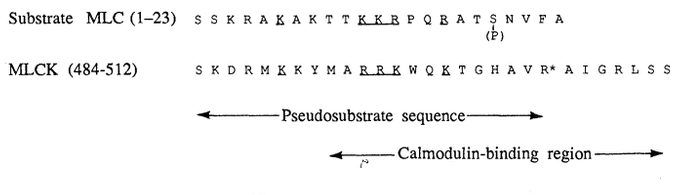

Fig. 1. Amino acid sequence of the MLCK pseudosubstrate and calmodulin-binding regions. The myosin light chain (MLC) phosphorylation site sequence and the MLCK sequence are illustrated with residue numbers in parentheses (*5*). Ser^{19} is the MLC phosphorylation site. The tryptic cleavage site at Arg^{505} for generation of the 64-kD inactive fragment is indicated by an asterisk. The basic residue substrate specificity determinants (*16*) are underlined together with the corresponding residues in the enzyme (*5*). Amino acids are listed using the single letter code; abbreviations are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

to the skeletal muscle MLCK. A structurally related calmodulin-binding region was subsequently found in the smooth muscle MLCK (*4, 5*). Whereas there was no compelling homology between the calmodulin-binding sequence of the skeletal muscle enzyme and other proteins in the database (*3*), the arrangement of basic residues in this region of the enzyme was similar to the pattern of basic residues identified as substrate specificity determinants in the myosin light chains (Fig. 1). This led to the concept that a sequence within the calmodulin-binding region may be responsible for maintaining the enzyme in the inactive form by mimicking a substrate (*5*). The calmodulin-binding region overlaps and extends to the carboxyl-terminal side of the pseudosubstrate sequence (Fig. 1). Synthetic peptides corresponding to the pseudosubstrate calmodulin-binding region are potent substrate antagonists as well as calmodulin antagonists for the enzyme (*5, 6*). Because these functional sequences of the enzyme can be mimicked with synthetic peptides, they are referred to as pseudosubstrate and calmodulin-binding prototopes, respectively (*7*). We have found that other protein kinases, including protein kinase C (*7*) and the calmodulin-dependent protein kinase II (*8*), also contain pseudosubstrate sequences in their primary structures that appear to be responsible for regulating these enzymes. These putative regulatory sequences are located outside the catalytic domains that share homology with all protein kinases (*5, 7, 8*). However, there has been no direct evidence that these pseudosubstrate structures actually function to inhibit the catalytic activity in the enzyme in situ. We used limited proteolysis, protein sequencing, and amino acid analysis to provide direct evidence that the pseudosubstrate sequence in the MLCK is responsible for regulating the enzyme.

Limited tryptic digestion of the MLCK (130 kD) for approximately 1 min generates a 64-kD fragment that has lost the capacity to bind calmodulin and has little or no catalytic activity (*6, 9*). Further digestion of the enzyme yields a 61-kD fragment that is constitutively active. To determine the difference in structure, the 61- and 64-kD fragments were purified by anion-exchange chromatography. Amino-terminal sequence analysis of the 64-kD fragment revealed the sequence Thr-Pro-Pro-Lys-Ala-Ala-Thr-Pro-Pro-Gln-Ile-Thr-Gln-Phe-Pro-Glu (initial yield, 550 pmol, 48% of the 64-kD fragment by amino acid analysis). The amino-terminal Thr of the 64-kD fragment is 41 residues amino terminal to the Trp^1 (*10*) reported for the cDNA-derived sequence (*11*). However, the complete amino-terminal sequence of the smooth muscle MLCK is not yet known. A second overlapping sequence commencing Ala-Ala-Thr-Pro-Pro-Gln (initial yield, 120 pmol, 10% of the 64-kD fragment by amino acid analysis) was also present, indicating a ragged amino terminus. The 61-kD fragment contained four amino-terminal sequences identified by quantitative phenylthiohydantoin amino acid analysis. Of these, Thr-Pro-Pro-Lys-Ala- (initial yield, 90 pmol) and Ala-Ala-Thr-Pro-Pro- (initial yield, 200 pmol) coincided with the amino terminus of the 64-kD fragment. The remaining two sequences, Val^{372}-Leu-Phe-Gly-Thr^{376} (initial yield, 140 pmol) and Ala^{284}-Asn-Ile-Val-Met^{288} (initial yield, 60 pmol), appeared to be derived from low molecular size contaminating fragments resulting from partial internal cleavages during the conversion of the 64-kD fragment to the 61-kD fragment. The results show that both the 61- and 64-kD fragments share the same primary amino-terminal sequences, and the conversion of the 64-kD fragment to the 61-kD fragments results from carboxyl-terminal cleavage.

The carboxyl-terminal sequence of the 64-kD fragment was investigated by comparing the chymotryptic peptides generated from the 64- and the 61-kD fragments and separated by reversed-phase high-performance liquid chromatography (HPLC) (Fig. 2). The chymotryptic peptides that were unique to the 64-kD fragment digest were selected for sequence analysis, and the sequences were compared with the partial primary sequence of the enzyme previously reported (*11*). The most carboxyl terminal peptide identified in the chymotryptic digest of the 64-kD fragment was Gln^{498}-Lys-Thr-Gly-His-Ala-Val-Arg^{505} (Figs. 2 and 3A). Peptides with this structure were isolated from two independent preparations of the 64-kD fragment, and both gave the sequence Gln^{498}-Lys-Thr-Gly-His-Ala-Val. The presence of the carboxyl-terminal Arg was unequivocally identified from the amino acid composition of the peptide (*12*). Independent evidence that $Arg^{505}$was the carboxyl-terminal residue of the 64-kD fragment was also obtained from the tryptic digests of ^{32}P-labeled MLCK (*13*).

Carboxypeptidase P digestion of the 64-kD fragment also generated active enzyme, consistent with the inhibitory region being

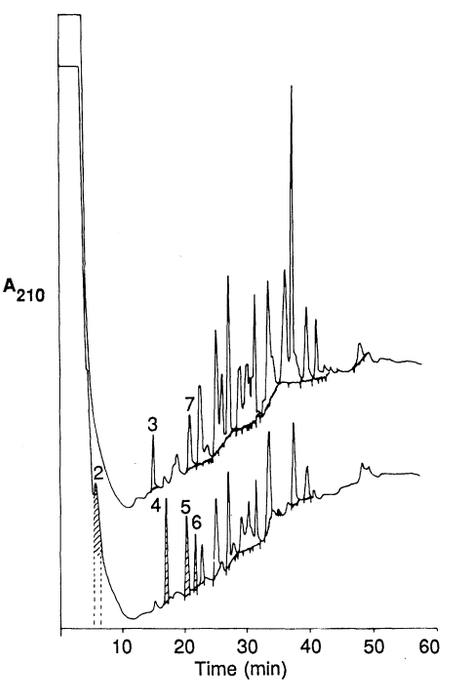

Fig. 2. Chymotryptic peptide maps of MLCK 61- and 64-kD fragments. The 64- and 61-kD tryptic fragments were concurrently digested with chymotrypsin at 25°C [1:50 and 1:32 (w:w), respectively]. After 10 min, the hydrolyses were terminated by the addition of trichloroacetic acid to 5% (w/v). After 15 min at room temperature, the samples were centrifuged in an Eppendorf bench top centrifuge, and the supernatants were neutralized with 1*M* tris base, diluted with an equal volume of 0.1% trifluoroacetic acid, and applied to an Aquapore RP-300 microbore guard column (2.1 mm by 30 mm with 7-μm resin, Brownlee Labs). Peptides were eluted with a linear gradient of 0 to 60% CH_3CN (v/v) in 0.1% CF_3COOH (v/v) over 60 min at a flow rate of 0.5 ml/min, and detected by monitoring the absorbance at 210 nm. The major absorbance peaks (61 kD, upper trace; 64 kD, lower trace) were collected, and those indicated by hatching were sequenced by automated Edman degradation. Peptide 2 was further purified on a Vydac C18 column (4.6 mm by 25 mm, 5-μm resin) before sequencing (*20*).

located close to the carboxyl terminus of the 64-kD fragment, but this activation was much less efficient than with trypsin (*14*).

Analysis of unique chymotryptic peptides associated with the 61-kD fragment provided equivocal information about the carboxyl-terminal region of the constitutively active form of the enzyme. The most distal unique chymotryptic peptide sequence identified was Ile^{405}-Leu-Val-(Ser)-Gly, which would place the most carboxyl-terminal extension of the tryptic cleavage site for the 61-kD fragment at either Lys^{445} or Arg^{459}. The presence of a number of peptides unique to the 61-kD fragment indicated that it was more susceptible to chymotryptic digestion (*15*). An arginine corresponding to Arg^{459} is found at the carboxyl terminus of the catalytic domain of all known protein kinases. Since the 61-kD fragment is active, the protein substrate binding site must be located amino terminal to this arginine in the MLCK.

Studies by Lukas *et al.* (*4*) and our group (*5*) indicate that the MLCK calmodulin-binding region contains the sequence from Ala^{493} to Ser^{512}, and that the tripeptide Gly^{508}-Arg-Leu^{510} is necessary but not sufficient for high-affinity calmodulin binding (Fig. 3B). Thus the tryptic cleavage site at Arg^{505} occurs within the calmodulin-binding site, thereby explaining the loss of calmodulin binding of the 64-kD fragment (*6*). The 64-kD fragment retains the entire pseudosubstrate sequence Ser^{484}-Lys-Asp-Arg-Met-Lys-Lys-Tyr-Met-Ala-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Val^{504} previously postulated (*5*) to act as an inhibitor by binding with the active site (Fig. 1). The substrate-pseudosubstrate homology extends from Ser^{484} to Val^{504}, but it is possible that the sequence most critical for binding with the active site may be smaller, perhaps Arg^{494} - Arg - Lys - Trp - Gln - Lys^{499} (*16*). The failure of trypsin to cleave at the Arg^{494}-Arg-Lys^{496} sequence during the generation of the 64-kD fragment is consistent with the protection of this region, as expected by its binding with the active site. Furthermore, we have shown that during tryptic digestion of the MLCK-calmodulin complex the active 61-kD fragment is generated but there is no accumulation of the inactive 64-kD fragment (*6*), a result consistent with the release of the pseudosubstrate peptide from the active site during calmodulin activation.

The model we developed, that the MLCK is regulated by a pseudosubstrate, is based on the following observations. Synthetic peptides corresponding to the pseudosubstrate sequence act as potent substrate antagonists of both the intact calmodulin-dependent enzyme (*5*) and the 61-kD constitutively active fragment (*6, 17*). The inactive 64-kD fragment contains the pseudosubstrate sequence, and its removal generates the active 61-kD fragment. The 64-kD fragment is missing a segment of the calmodulin-binding region, which explains why it is devoid of calmodulin-binding activity.

The role of pseudosubstrates in the regulation of protein kinases is not restricted to the calmodulin-dependent protein kinases; protein kinase C (*7*), the regulatory subunit of the adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (*18*), and the heat-stable inhibitor of this enzyme (*19*), all contain pseudosubstrate sequences. An important question raised by these findings is whether all protein kinases or other calmodulin-dependent enzymes, such as adenylate cyclase and phosphodiesterase, are regulated in this way. It is not known if the corresponding calmodulin-binding sites in these proteins are associated with sequences that render the enzyme inactive by mimicking substrate binding with the active site. Thus the pseudosubstrate model may be more general and relevant to the regulation of a wide range of enzymes dependent on allosteric regulators. Given the design of enzymes it is perhaps not surprising that one of their most distinguishing features, namely substrate specificity, would be exploited with such masterly economy in their regulation.

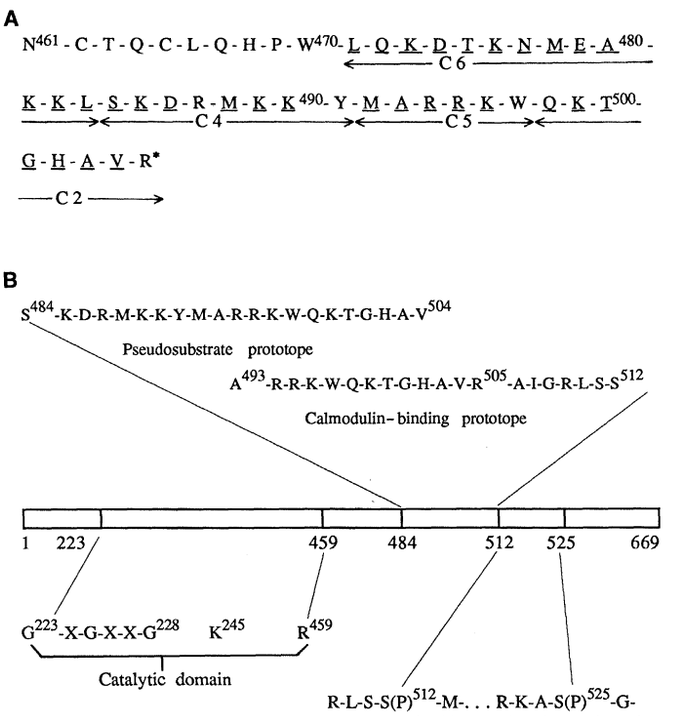

Fig. 3. (**A**) Amino acid sequence of chymotryptic peptides unique to the 64-kD fragment. Chymotryptic peptides are identified within arrows and are prefixed C. The residues identified by quantitative sequential Edman degradation (*20*) are underlined. The yield of peptides C6 and C2 were 33% (383 pmol) and 28% (305 pmol) of the 64-kD digest, respectively. Arg^{505}, indicated by an asterisk, was identified from the amino acid composition of peptide C2 (*12*). Abbreviations are as in Fig. 1. (**B**) Summary of the locations of the catalytic and regulatory features of the MLCK. The sequence 1 to 669 corresponds to the partial cDNA-derived sequence of MLCK (*11*). Residues conserved in the catalytic domain of all protein kinases, including the glycine-rich adenosine triphosphate (ATP)–binding motif and the essential Lys^{245} and Arg^{459}, are shown. The regulatory features, including the pseudosubstrate prototope, the calmodulin-binding prototope, and the phosphorylation sites, are located on the carboxyl-terminal side of the catalytic domain. Abbreviations are as in Fig. 1.

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7. Prototope refers to the functional unit of a protein with properties that can be mimicked with a short synthetic peptide [C. House and B. E. Kemp, *Science* **238**, 1726 (1987)].
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12. The carboxyl-terminal chymotryptic peptide C2 contained the following amino acid ratios: Thr, 0.89; Glu, 1.18; Gly, 1.14; Ala, 1.02; Val, 0.97; Lys, 0.93; Arg, 0.97; and His, 0.92, corresponding to those expected for the peptide Gln^{498}-Arg^{505}. Previously we have shown that the amino acid compositions of the 64- and 61-kD fragments differ in the number of basic residues (*6*). This was confirmed here and is consistent with the removal of the highly basic pseudosubstrate region.
13. Previously the cAMP-dependent protein kinase had been shown to phosphorylate two sites in the

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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