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The Marine Guanidine Alkaloid Crambescidin 816 Induces Calcium Influx and Cytotoxicity in Primary Cultures of Cortical Neurons through Glutamate Receptors

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ABSTRACT: Crambescidin 816 is a guanidine alkaloid produced by the sponge Crambe crambe with known antitumoral activity. While the information describing the effects of this alkaloid in central neurons is scarce, Cramb816 is known to block voltage dependent calcium channels being selective for L-type channels. Moreover, Cramb816 reduced neuronal viability through an unknown mechanism. Here, we aimed to describe the toxic activity of Cramb816 in cortical neurons. Since calcium influx is considered the main mechanism responsible for neuronal cell death, the effects of Cramb816 in the cytosolic calcium concentration of cortical neurons were studied. The alkaloid decreased neuronal viability and induced a dose-dependent increase in cytosolic calcium that was also related to the presence of calcium in the extracellular media. The increase in calcium influx was age dependent, being higher in younger neurons. Moreover, this effect was prevented by glutamate receptor antagonists, which did not fully block the cytotoxic effect of Cramb816 after 24 h of treatment but completely prevented Cramb816 cytotoxicity after 10 min exposure. Therefore, the findings presented herein provide new insights into the cytotoxic effect of Cramb816 in cortical neurons.

KEYWORDS: Guanidine alkaloids, crambescidin 816, cortical neurons, cytosolic Ca2+, cytotoxicity, glutamate receptors

INTRODUCTION

Marine sponges are regarded as a good source of neuroactive natural products.1 The red encrusting marine sponge Crambe crambe, widely distributed in the Western Mediterranean Sea but also in the Macaronesian archipelagos produces several metabolites, including the guanidine alkaloids crambescins and crambescidins.2–4 First, only a few studies assessed the biological activity of these compounds and their pharmaceutical potential, mainly due to the difficulties to obtain large quantities of pure compounds.5 Most of the reports considering the bioactivity of these molecules have revealed their cytotoxic activity in tumoral cells, and thus, these compounds are proposed as potential anticancer drugs. In this sense, some compounds from the crambescin family such as crambescin 800 and crambescidin 816 (Cramb816) are cytotoxic against tumor derived cell lines.6–9

The bioactivity of crambescidins in excitable cells has been less explored, and only a few reports consider the effects of these guanidine alkaloids in central neurons. Initially, an inhibitory activity of crambescidin 816 on voltage gated calcium channels (VGCCs) was reported in 1993. This crambescidin blocked Ca2+ channels with higher potency than the selective blocker of L-type VGCC nifedipine (NIF).2 In this sense, we recently confirmed the inhibitory activity of Cramb816 in primary cultures of cortical neurons demonstrating that Cav1 or L-type calcium channels are the main target for Cramb816 in this cellular model.10 In addition, this compound caused a small blockade of voltage gated sodium channels but did not affect voltage gated potassium channels as crambescins did, which indicated a clear structure–activity difference.10 In another report, Bondu et al. revealed that Cramb816 was cytotoxic to cortical neurons.4 However, so far there is no information regarding the mechanism by which this alkaloid is neurotoxic.

Typically, marine toxins induce cell death by increasing calcium influx into the cell.11,12 Massive calcium influx is regarded as one of the main mechanisms responsible for neuronal cell death.13,14 It has been traditionally accepted that the pharmacological blockade of the calcium overload prevents
neuronal death, and thus VGCCs antagonists are considered good candidates for lowering neuronal damage. In this sense, L-type calcium channel antagonists have been proposed as promising tools for improving age-related working memory deficits. Since antagonists of voltage gated calcium channels have neuroprotective roles, the previously described cytotoxic 

\[ \text{Cramb816} \text{ ranging from 0.01 to 10 } \mu \text{M} \] 

were exposed during 24 h to different concentrations of Cramb816 evaluated in neuronal viability and cytosolic calcium concentration. The results presented here indicate that Cramb816 did not elicit calcium influx at the concentration that blocked L-type calcium channels (1 \( \mu \text{M} \)). However, at this concentration, it was slightly cytotoxic to cortical neurons. At higher concentrations, Cramb816 was neurotoxic and induced calcium influx in a concentration-dependent manner. The pharmacological profile of the calcium influx elicited by Cramb816 was characterized using the N-methyl-D-aspartate receptor (NMDA) selective antagonist d-(-)-2-amino-5-phosphonopentanoate (APV) and the \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor selective antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). The calcium influx elicited by Cramb816 was partially blocked by APV and fully blocked by the simultaneous preincubation of cortical neurons with APV and CNQX, thus indicating that the Cramb816 induced calcium influx was mediated by both NMDA and AMPA glutamate receptor subtypes. In addition, these glutamate receptors antagonists decreased the neuronal death produced by Cramb816. Therefore, the results presented herein provide evidence that Cramb816 is neurotoxic to cortical neurons through a mechanism involving glutamate receptor activation and, in addition, the alkaloid produces an increase in cytoplasmic calcium concentration involving glutamate receptors.

**RESULTS AND DISCUSSION**

Effect of Cramb816 in Neuronal Viability. To study the cytotoxic effect of Cramb816 in cortical neurons, 4–6 DIV cells were exposed during 24 h to different concentrations of Cramb816 ranging from 0.01 to 10 \( \mu \text{M} \). The chemical structure of Cramb816 is shown in Figure 1A. As shown in Figure 1B, Cramb816 lowered cellular viability in a dose-dependent manner. While at concentrations of 0.01 \( \mu \text{M} \) and 0.1 \( \mu \text{M} \) Cramb816 was not toxic, the compound at 1 and 10 \( \mu \text{M} \) lowered cellular viability by 30.5\% \pm 1.0\% \((n = 3); p = 0.002; t = 13.32; df = 4\) and 98.3\% \pm 0.3\% \((n = 3); p < 0.0001; t = 47.12; df = 4\), respectively. The \( ^1 \text{H} \) NMR spectrum of Cramb816 (500 MHz) is shown in Supplementary Figure 1. Additionally, Supplementary Figure 2 shows the HPLC and HRMS data for Cramb816.

These results are in contrast with a previous report where Cramb816 produced almost complete cell death at 1 \( \mu \text{M} \) decreasing cell viability by 86.3\% \pm 6.8\% \((n = 3); p < 0.0001; t = 52.67; df = 6\). A similar result was obtained in those cells co-incubated with 25 mM KCl and 1 \( \mu \text{M} \) Cramb816 where neuronal viability decreased by 86.5\% \pm 4.4\% \((n = 3); p < 0.001; t = 5.614; df = 6\).

High Concentrations of Cramb816 Increase the Cytosolic Calcium Concentration \([\text{Ca}^{2+}]_c\) in Cortical Neurons. Since calcium influx is considered one of the main mechanisms involved in neuronal cell death, the effect of Cramb816 on the cytosolic calcium concentration in cortical neurons was analyzed. Inasmuch as the previous work on crambeScidin cytotoxicity in cortical neurons was performed in cultures of 4–6 DIV, first, young neurons were exposed to Cramb816 concentrations ranging from 1 to 10 \( \mu \text{M} \) in order to evaluate the effect of this compound on \([\text{Ca}^{2+}]_c\). Figure 2 shows the effect of bath application of Cramb816 at 1, 5, and 10 \( \mu \text{M} \) on the cytosolic calcium concentration in cortical neurons. Since depletion of calcium ions from the endoplasmic reticulum (ER) or mitochondria has been suggested as an initial signal for intracellular calcium elevation and neuronal apoptosis, initially the effect of Cramb816 on \([\text{Ca}^{2+}]_c\), was evaluated using \( \text{Ca}^{2+}\)-free media. As observed in Figure 2A, in the absence of \( \text{Ca}^{2+}\) in the extracellular medium, Cramb816 did not affect the \( R_{340/380} \) thus indicating that intracellular \( \text{Ca}^{2+}\) stores were not affected by the guanidine alkaloid. However, when 1 mM \( \text{Ca}^{2+}\) was added to the bath solution, Cramb816 induced \( \text{Ca}^{2+}\) influx in a concentration dependent manner. The alkaloid at concentrations of 5 and 10 \( \mu \text{M} \), but not at the lowest concentration evaluated (1 \( \mu \text{M} \)), induced a rapid calcium influx in cortical neurons. Moreover, the calcium influx induced by 5 \( \mu \text{M} \) Cramb816 started about 100 s after the addition of the compound, while at 10 \( \mu \text{M} \) the effect was immediate. Figure 2B shows the medium \([\text{Ca}^{2+}]_c\) measured 5 min after bath application of 5 and 10 \( \mu \text{M} \) Cramb816 in calcium containing medium. In these conditions the, \( R_{340/380} \) was significantly enhanced by 30.8\% \pm 6.4\% \((n = 3); p = 0.088; t = 4.779; df = 4\) and 53.9\% \pm 9.7\% \((n = 3); p = 0.005; t = 5.518; df = 4\) after addition of 5 or 10 \( \mu \text{M} \) Cramb816, respectively, while at the lower concentration, 1 \( \mu \text{M} \) Cramb816 enhanced \( R_{340/380} \) only by 10.9\% \pm 4.4\%, an effect that was not statistically significant (\( n = 3; p = 0.11 \)).
Finally, we also evaluated the effect of the compound when it was added directly in calcium containing media (Supplementary Figure 4) using young neurons. In this condition, Cramb816 also elicited a concentration-dependent increase in calcium (Supplementary Figure 4A), which was of a similar magnitude to that observed previously. In calcium containing media, the [Ca\(^{2+}\)] in the absence of Cramb816 was not modified by bath application of Cramb816 at 1 μM; however, it increased by 32.2% ± 10.4% (n = 3; p = 0.0367; t = 3.085; df = 4) and 59.4% ± 15.7% (n = 3; p = 0.0195; t = 3.778; df = 4) after addition of Cramb816 at 5 and 10 μM, respectively (Supplementary Figure 4B).

Ca\(^{2+}\) ions may gain access to the neuronal cytoplasm via ion channels or Ca\(^{2+}\) transport systems or through the release of Ca\(^{2+}\) ions from intracellular stores. Since the latter option was discarded because Cramb816 did not affect cortical neurons in the absence of Ca\(^{2+}\) in the extracellular media, the following experiments were performed in media containing 1 mM Ca\(^{2+}\). In the next set of experiments, we aimed to analyze the route underlying the Cramb816-dependent Ca\(^{2+}\) influx in cortical neurons. A concentration of 10 μM Cramb816 was chosen to further elucidate the target for Cramb816 induced calcium influx by using different pharmacological approaches.

**The Voltage-Gated Calcium Channel Blocker Nifedipine Did Not Impede the Cytosolic Calcium Increase Elicited by Cramb816.** In neurons, the entry of calcium is regulated mainly by voltage-gated channels and by receptor operated channels activated by glutamate. Despite the fact that Cramb816 is an antagonist of voltage gated calcium channels, this activity was observed at 1 μM, a concentration that in our cellular model and conditions did not induce calcium influx. To investigate the molecular basis of Cramb816 induced Ca\(^{2+}\) increase, the effect of 10 μM Cramb816 in the presence of the L-type voltage gated calcium channel blocker nifedipine (NIF) was first analyzed. For this purpose, NIF was added 30 min prior to the administration of 10 μM Cramb816. This pharmacological manipulation did not impede the calcium increase elicited by Cramb816 (Figure 3A). As indicated in Figure 3B, the R\(_{340/380}\) was significantly enhanced after the addition of 10 μM Cramb816 by 45.9% ± 12.9% (n = 3; p = 0.03; t = 3.170; df = 4). A similar result was obtained when 10 μM Cramb816 was added after the incubation for 30 min with 10 μM NIF. In this case, the R\(_{340/380}\) was increased by 45.9% ±

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**Figure 2.** Effects of Cramb816 on cytosolic calcium concentration in cortical neurons. (A) In young neurons, Cramb816 did not affect [Ca\(^{2+}\)]\(_i\), in a calcium-free media, but it produced a concentration-dependent rise in [Ca\(^{2+}\)]\(_i\), after addition of calcium to the extracellular media. The arrow indicates solvent/drug and calcium addition to the bathing solution. (B) Pooled results for the increase in [Ca\(^{2+}\)]\(_i\), produced by Cramb816 at the different concentrations after addition of calcium to the bathing media. (C) Effect of 10 μM Cramb816 on [Ca\(^{2+}\)]\(_i\), in young (4–6 DIV) and mature (10–12 DIV) cortical neurons. Arrows indicate drug/DMSO and calcium addition to the bathing solution. (D) Pooled results for the shift in [Ca\(^{2+}\)]\(_i\), produced by 10 μM Cramb816 in the two groups. *p < 0.05; **p < 0.01 versus control; ***p < 0.001 between young and mature neurons. Results were obtained from three different experiments and cultures.

Several reports indicate calcium homeostasis changes during neuronal development.\(^{19}\) In this sense, spontaneous Ca\(^{2+}\) activity during early neocortical development depends mainly on gap junctions and voltage dependent Ca\(^{2+}\) channels, whereas later in development neurotransmitters and synapses exert an influence.\(^{20}\) In the cerebral cortex, synchronized rhythmic activity also develops spontaneously in neuronal networks formed by embryonic neurons in cell culture, and this is accompanied by changes in Ca\(^{2+}\) transients.\(^{21}\) Thus, we investigated whether the calcium influx pattern elicited by Cramb816 was dependent on the time that neurons were maintained in culture. Therefore, in the next set of experiments, the calcium influx elicited by Cramb816 in immature (4–6 DIV) and mature cortical neurons (10–11 DIV) was compared. As observed in Figure 2C, in a Ca\(^{2+}\) free media, 10 μM Cramb816 did not affect the R\(_{340/380}\) in either of the two age groups; however, in the presence of 1 mM Ca\(^{2+}\) in the bath solution, 10 μM Cramb816 induced calcium influx in both groups. As indicated in Figure 2D, the R\(_{340/380}\), measured 5 min after the addition of 1 mM Ca\(^{2+}\) to the bath solution, was enhanced by 48.05% ± 8.1% (n = 3; p = 0.002; t = 5.901; df = 5) in 4–6 DIV cortical neurons and only by 16.9% ± 5.7% (n = 3; p = 0.003; t = 2.929; df = 6) in older neurons. The difference between both groups was statistically significant (p < 0.05), thus indicating that 4–6 DIV neurons were more susceptible than 10–11 DIV neurons to Cramb816.

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**Figure 3.** The cytosolic calcium increase elicited by Cramb816 was not dependent on voltage-gated calcium channels. (A) Incubation of cortical neurons with 10 μM nifedipine (NIF) 30 min before bath application of Cramb816 did not modify the calcium influx elicited by Cramb816 at 10 μM. (B) Pooled results for the shift in [Ca\(^{2+}\)]\(_i\), produced by 10 μM Cramb816 in the absence and presence of the voltage-gated calcium channel blocker nifedipine. *p < 0.05 versus the respective control. Results were obtained from three different experiments and cultures.
Glutamate Receptors Antagonists Impeded the Calcium Influx Induced by Cramb816. In order to study whether NMDARs were involved in the Cramb816-induced calcium influx, NMDARs were blocked by 100 μM APV added to the bath solution 30 min before Cramb816. In this condition, the Cramb816-dependent Ca2+ influx was partially reduced by the presence of APV (Figure 4A). As indicated in Figure 4B, when measured at 5 min after Cramb816 administration, the $R_{\text{340/380}}$ was enhanced by 47.05% ± 5.1% ($n = 3$; $p < 0.05$; $t = 9.248$; $df = 6$). However, when 10 μM Cramb816 was added after the incubation for 30 min with the glutamate receptor antagonists APV and CNQX, the increase in the $R_{\text{340/380}}$ elicited by 10 μM Cramb816 was only 1.7% ± 2.5%. Thus, the complete blockade of glutamate receptors by the combination of APV and CNQX impeded the rise in cytosolic calcium elicited by the guanidine alkaloid. These results indicate that the calcium increase elicited by Cramb816 in cortical neurons involves both AMPA and NMDA receptors.

Glutamate Receptors Antagonists Improved Cramb816-Induced Cytotoxicity in Cortical Neurons. In order to study whether glutamate receptors were also involved in the cytotoxic effects of Cramb816, cortical neurons were co-incubated with the glutamate receptor antagonists APV and CNQX prior to the addition of Cramb816 to the extracellular media. As observed in Figure 5A, when neurons were exposed to Cramb816 for 24 h in a media containing APV and CNQX, the effect of the alkaloid was about 10% or 20% lower than the cytotoxicity in the absence of glutamate receptor antagonists.

Figure 4. The calcium increase evoked by Cramb816 in cortical neurons was mediated by glutamate receptors. (A) Blockade of NMDA receptors by APV partially prevented the calcium increase produced by Cramb816. (B) Pooled results for the shift in $[\text{Ca}^{2+}]_{\text{c}}$ produced by Cramb816. (C) Simultaneous blockade of NMDA and AMPA receptors in the presence of APV and CNQX fully prevented the Cramb816-elicited calcium influx. Basal recordings were obtained in the presence of glutamate receptor blockers. (D) Pooled results for the shift in $[\text{Ca}^{2+}]_{\text{c}}$ produced by Cramb816. Arrow indicates addition of Cramb816 or vehicle (DMSO). **p < 0.01, ###p < 0.005 versus control; ##p < 0.05 and ###p < 0.01 respect to Cramb816.

Figure 5. The cytotoxic effect of Cramb816 in cortical neurons was mediated by glutamate receptors. (A) Exposure (24 h) of cortical neurons to Cramb816 reduced cellular viability in a dose dependent manner (white bars) and this effect was ameliorated by the simultaneous blockade of NMDA and AMPA receptors in the presence of APV and CNQX (gray bars). (B) Exposure (10 min) of cortical neurons to 1 and 10 μM Cramb816 reduced cellular viability only at the highest alkaloid concentration (white bars), and this effect was completely reverted by the simultaneous blockade of NMDA and AMPA receptors in the presence of APV and CNQX. *p < 0.05, ***p < 0.005 versus control; ##p < 0.05 versus Cramb816.
This result indicated that the cytotoxic effect elicited by Cramb816 at 24 h was mitigated but not impeded by the pharmacological blockade of glutamate receptors. As shown in Figure 5A, in the absence of glutamate receptor antagonists Cramb816 elicited a cytotoxic effect at 0.1 μM, while in the presence of APV + CNQX, this alkaloid was not toxic to cortical neurons. The effect of glutamate receptor antagonist was most profound at the higher toxin concentrations. Thus, at 10 μM Cramb816, the mitochondrial function was 1.7% ± 0.2% of control, indicating an almost complete cell death, while when the cells were co-incubated with glutamate receptor antagonists and 10 μM Cramb816 the mitochondrial function was 23.7% ± 0.2% (p < 0.0001; t = 53.49; df = 4).

Since the duration of the experiments assessing the activity of Cramb816 on intracellular calcium concentration was about 5-10 min, the effect of Cramb816 in neuronal viability was studied after exposing cortical neurons to the alkaloid either alone or in the presence of glutamate receptor antagonists during 10 min (Figure 5B). In these conditions, 10 μM Cramb816 reduced cellular viability by 32.37% ± 6.519% (n = 3; p = 0.0077; t = 4.966; df = 4), whereas 1 μM Cramb816 did not significantly affect neuronal viability. In contrast, in those experiments where neurons were preincubated with APV and CNQX, the alkaloid did not significantly modified cellular viability at the highest concentration. A significant difference in the cytotoxicity of Cramb816 in the absence and presence of glutamate receptor antagonists was found (n = 3; p = 0.0145; t = 4.127; df = 4), again indicating that the cytotoxic effect of Cramb816 in cortical neurons involved glutamate receptors.

**Cramb816 Increases the Amplitude of mEPSCs in Cortical Neurons.** Finally, the effect of the toxin on miniature excitatory synaptic events (mEPSCs), which are generally assumed to report synaptic function at the level of a single terminal, representing the postsynaptic response to release of individual vesicles of glutamate, was also evaluated. mEPSCs were pharmacologically isolated as described in the Methods section. These mEPSCs in cortical neurons were glutamatergic as previously described in this cellular model by their complete pharmacological blockade by the combination of APV and CNQX. A representative recording of mEPSC before and after addition of 10 μM Cramb816 is shown in Figure 6A. Amplified recordings from the same neuron, before and after addition of the toxin, are shown in Figure 6B. All together these experiments indicate that bath application of Cramb816 increased the amplitude and frequency of mEPSCs.

Members of the crambescidin family have been reported with antifungal, antibacterial, antiviral, and also antitumoral activities in several preparations. However, less attention has been paid to the activity of these compounds in the central nervous system. In this sense, the major finding for the effects of a member of the crambescidin family in a neuronal model was the selectivity of Cramb816 for L-type voltage-gated calcium channels in cortical neurons. Moreover, it was also described that this compound was cytotoxic in these neurons. The main result of this study was that Cramb816 produced calcium influx in a dose dependent manner, and this effect was blocked by glutamate receptor antagonists. Moreover, at 10 μM, the alkaloid induced neuronal death at 24 h, and this effect was ameliorated but not fully avoided by glutamate receptor antagonists. In addition, exposure of cortical neurons to high concentrations of the compound for 10 min also caused toxicity in cortical neurons, but in this case, the cytotoxic effect was fully blocked by coadministration of NMDA/AMPA receptor antagonists. It is noteworthy that at 1 μM, a concentration known to produce blockade of L-type voltage gated calcium channels, Cramb816 did not acutely produce calcium influx or cellular death but it was slightly cytotoxic to cortical neurons after 24 h treatment.

Previous works had shown that 1 μM Cramb816 did not affect the cellular viability of the tumor cell line OU-31 after a 24 h exposure. However, in other tumor cell lines, this compound reduced cellular viability in a concentration-dependent manner and by percentages ranging between 17% and 39%. Recently, it was also demonstrated that Cramb816 reduced the viability of a wide range of tumor cell lines with IC50 of 0.12 μM after 72 h of treatment. Initial viability assays of cortical neurons treated for 24 h with different concentrations of Cramb816 showed that this compound was highly neurotoxic at a concentration of 1 μM causing almost complete cell death at this concentration, an unexpected fact since at this concentration the alkaloid has been shown to act as a blocker of voltage-gated calcium channels in the same in vitro model. In this work, we reevaluated the neurotoxic effect of Cramb816 in cortical neurons cultured in media containing a physiological concentration of potassium instead of a high potassium concentration, and the results presented herein indicated that exposure of cortical neurons to 1 μM Cramb816 reduced cell viability only by 30% and caused complete cell death at concentrations 10 times higher, an effect similar to that recently described in tumoral cell lines. We suggest that the differences observed between our results and the previous report describing the cytotoxic effect of Cramb816 in cortical neurons can be assigned to the differences between experimental conditions. The higher extracellular potassium concentration used in our previous work was probably exacerbating the toxicity of Cramb816 and could interfere in previous results where Cramb816 produced complete cell death at 1 μM and was cytotoxic at lower concentrations after a 24 h exposure of cortical neurons to the alkaloid. In this sense, we here demonstrated that a medium containing 25 mM KCl is not an adequate media to perform a cytotoxicity assay in cortical neurons.
neurons. In fact, whereas this condition is necessary in primary cultures of rat cerebellar granule neurons, it has been reported to be neurotoxic in cortical neurons and to impede neuronal activity due to the excessive membrane depolarization in cerebral cortex.

On the other hand, one possible mechanism responsible for the neuronal cell death elicited by Cramb816 could be the excessive accumulation of calcium, since intracellular calcium levels are critical to regulate apoptosis. Potentially neurotoxic cytoplasmic calcium concentrations can be produced by the access of Ca ions to the neuronal cytoplasm via ion channels (voltage gated calcium channels), Ca transport systems, release of Ca ions from internal stores, physical damage to mitochondria and the endoplasmic reticulum, or a malfunction of receptors and channels present in their membranes. Here, Cramb816 did not affect internal organelles as evidenced by the lack of effect in a calcium free media. Since other marine compounds have been broadly reported to cause cellular death by inducing calcium influx from the extracellular media, we also explored this possibility. In this case, Cramb816 induced calcium influx from the extracellular media at 5 and 10 μM. However, it did not promote calcium influx at 1 μM, a concentration that was not cytotoxic to cortical neurons when exposed to the compound for 10 min and that is known to produce blockade of L-type calcium channels. This latter result is logical since VGCC antagonists are considered as good candidates for lowering neuronal damage. However, at 24 h, 1 μM Cramb816 was slightly cytotoxic to cortical neurons similar to the effect produced by other calcium channel antagonists such as NIF, which also has been described to be cytotoxic in cerebral corticall cultures after 2 days of treatment. L-type calcium channel antagonists have been proposed as pharmacological tools for improving age-related working memory deficits. However, it might seem controversial that a ten times higher concentration of this L-type channel blocker, in theory neuroprotective, led to massive calcium in...
maintained in the presence of the toxins at 37 °C in a humidified 5% CO2/95% air atmosphere. Saponin was used as cellular death control, and its absorbance was subtracted from the other data. After the exposure time, cells were rinsed and incubated for 60 min with a solution of MTT (500 μg mL−1) dissolved in Locke’s buffer containing in mM 154 NaCl, 5.6 KCl, 1.3 CaCl2, 1 MgCl2, 10 HEPES, and 5.6 glucose (pH 7.4). Addition of drugs was made by aspiration and addition of fresh bathing solution. The cytosolic calcium ratio was obtained from the images collected by fluorescence equipment (Lambda-DG4, Sutter Instrument Company, Novato, CA, USA). The light source was a xenon arc bulb, and the different wavelengths used were chosen with filters. The ratio of intensities of emitted light (505 nm) corresponding to excitation wavelengths of 340 and 380 nm (R380/340) was taken as a measure of [Ca2+]i. The experiments were performed in triplicate. In those experiments requiring L-type calcium channel blockade or glutamate receptor blockade, neurons were preincubated for 30 min with their respective antagonists.14

Electrophysiology. Whole cell patch-clamp recordings, achieved by gentle mechanical suction of the membrane patch, were performed on cortical neurons, between 10 and 11 days in culture, at room temperature (22–25 °C). A computer-controlled current and voltage clamp amplifier (Multiclamp 700B, Molecular Devices) was used. Signals were recorded and analyzed using a Pentium computer equipped with Digidata 1440 data acquisition system and pClamp10 software (Molecular Devices, Sunnyvale, CA). pClamp10 was used to generate current and voltage-clamp commands and to record the resulting data. Signals were filtered at 10 kHz and digitized at 20 μs intervals. Culture medium was exchanged with several washes of cold Locke’s buffer. The glass coverslips were inserted into a thermostated chamber at 37 °C (Life Science Resources), and cells were viewed with a Nikon Diaphot 200 microscope, equipped with epifluorescence optics (Nikon 40X-immersion UV-Fluor objective). Additions of drugs was made by aspiration and addition of fresh bathing solution. The cytosolic calcium ratio was obtained from the images collected by fluorescence equipment (Lambda-DG4, Sutter Instrument Company, Novato, CA, USA). The light source was a xenon arc bulb, and the different wavelengths used were chosen with filters. The ratio of intensities of emitted light (505 nm) corresponding to excitation wavelengths of 340 and 380 nm (R380/340) was taken as a measure of [Ca2+]i.

The experiments were performed in triplicate. In those experiments requiring L-type calcium channel blockade or glutamate receptor blockade, neurons were preincubated for 30 min with their respective antagonists.14

Toxins and Drugs Used. Cramb816 was extracted and isolated from the Mediterranean sponge Crambe crambae. Nifedipine (NIF) and n(-)-2-amino-5-phosphono pentanoic acid (APV) were purchased from Sigma-Aldrich, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was from Tocris. The final concentration of NIF or Cramb816 (solvent DMSO) was less than 0.01%. APV and CNQX were dissolved in H2O.

Statistical Analysis. All data are expressed as means ± SEM of n determinations. Statistical comparison was by Student’s t-test. Values of p<0.05 were considered statistically significant.

1H NMR spectrum of Cramb816, HPLC and HRMS of Cramb816, effect of Cramb816 on neuronal viability in a medium containing 25 mM KCl, and effect of Cramb816 on the cytosolic calcium concentration in a calcium containing media (PDF)

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S.B.L.S. and O.P.T. performed the chemical purification and identification of Cramb816 and revised the manuscript. A.A., C.V., L.M.B., and M.R.V. developed the idea, designed experiments for the study, and wrote the manuscript with the help of A.G.M., A.B.J., and V.M. A.G.M., A.B.J., and V.M. performed the experiments, analyzed the data, and prepared the figures. All authors participated in discussions and proofreading of the manuscript.

Notes
The authors declare no competing financial interest.

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■ ABBREVIATIONS
Cramb816, crambescein 816; NIF, nifedipine; VGCCs, voltage-gated calcium channels; CrambC, crambescin C1; NMDA, N-methyl-D-aspartate receptor; APV, n(-)-2-amino-5-phosphono pentanoate; AMPA, α-amino-3-hydroxy-5-methyl-
4-isoxazolepropionic acid receptor; CNQX, 6-cyano-7-nitroquinazoline-2,3-dione; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); DIV, days in vitro; [Ca\(^{2+}\)]\(_o\), cytosolic calcium concentration in cortical neurons

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