DIFFERENTIAL EFFECTS OF CRAMBESCINS AND CRAMBESCIDIN 816 IN VOLTAGE-GATED SODIUM, POTASSIUM AND CALCIUM CHANNELS IN NEURONS

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DIFFERENTIAL EFFECTS OF CRAMBESCINS AND CRAMBESCIDIN 816 IN VOLTAGE-GATED SODIUM, POTASSIUM AND CALCIUM CHANNELS IN NEURONS

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Key words: guanidine alkaloids, crambescidin 816, crambescins, cortical neurons, voltage-dependent channel.

TABLE OF CONTENTS GRAPHIC:_

Crambescins
K_v Inhibition:
NCrambA2 >
CrambC1 >
CrambA2

\+

Crambescidins
Cramb816:
Ca_v1 inhibition
No K_v inhibition

ABSTRACT:

Crambescins and crambescidins are two families of guanidine alkaloids from the marine sponge *Crambe crambe*. Although scarce information about their biological effect has been reported, it is known that crambescidin 816 (Cramb816) blocks calcium channels in a neuroblastoma X glioma cell line. Taking into account this, and the fact that ion channels are frequent targets for natural toxins, we examined the effect of Cramb816 and three compounds from the crambescin family: Norcrambescin A2 (NcrambA2), Crambescin A2 (CrambA2) and Crambescin C1 (CrambC1), in the main voltage-dependent ion channels in neurons: sodium, potassium and calcium channels.

Electrophysiological recordings of voltage gated sodium, potassium and calcium currents, in the presence of these guanidine alkaloids, were performed in cortical neurons from embryonic mice. Different effects were discovered: Crambescins inhibited K^+ currents with the following potency: NcrambA2 > CrambC1 > CrambA2 while Cramb816 lacked an effect. Only CrambC1 and Cramb816 partially blocked Na^+ total current. On the other hand Cramb816 partially blocked Ca^{2+} currents while NcrambA2 did not. Since the blocking effect of Cramb816 on calcium currents has not been previously reported in detail, we further pharmacologically isolated the two main fractions of HVA Ca^{+2} channels in neurons and investigated the Cramb816 effect on them. Here, we revealed that Cav1 or L-type calcium channels are the main target for Cramb816.

These two families of guanidine alkaloids clearly showed a structure-activity relationship with the crambescins acting on voltage-gated potassium channels while Cramb816 blocks the voltage-gated calcium channel Cav1 with higher potency than nifedipine. The novel evidence that Cramb816 partially blocked Ca_V and Na_V channels in neurons, suggests that this compound might be involved in decreasing the neurotransmitter release and synaptic transmission in the central nervous system. The findings presented here provide the first detailed approach on the different effects of Crambescin and Crambescidin compounds in voltage-gated sodium, potassium and calcium channels in neurons and thus provide a basis for future studies.

INTRODUCTION

Crambescins and crambescidins are guanidine alkaloids first reported in the early 90s. They are produced by the red encrusting marine sponge *Crambe crambe* (Schmidt, 1862) widely distributed in the Western Mediterranean Sea but also in the Macaronesian archipelagos (*1, 2*). There are only limited data on the biological activity of these compounds and its pharmaceutical potential, probably because of the difficulties to obtain large quantities of pure compounds (*3*). Crambescidin alkaloids inhibit HIV-1 cell fusion(*4, 5*) and other protein-protein interactions (*6*).They are also active *in vitro* against Herpes simplex virus type 1 (HSV-1), cytotoxic to L1210 murine leukemia cells and potentially anticancer drugs (*7, 8*). Recently, Bondu *et al* showed that Crambescidin 816 (Cramb816) possess cytotoxic activity against cortical neurons causing a dose-dependent increase on the cytotoxic effect and reaching an almost complete cell death at 1 μM whereas Crambescin C1 (CrambC1) just lowered cellular viability at the same concentration (*9*). To our knowledge, the only reported effect of any of these guanidine alkaloids on ion channels, is related to the activity of Cramb816 on voltage-sensitive calcium channels, which exhibited a higher Ca^{2+} antagonist activity (IC_{50} = 1.5 x 10^{-4} μM) than the selective blocker of L-type Ca^{+2} channels, Nifedipine (NIF) (IC_{50} = 1.2 μM) in neuroblastoma X glioma cell line (NG 108-15) (*1*). It seems to operate through a reversible blockage of Ca^{+2} channels (*1, 3*). Indeed, Cramb816 inhibited acetylcholine-induced contraction of guinea pig ileum at very low concentrations (*1*).

Regulation of calcium, sodium and potassium conductance is of central importance to a great number of neurobiological subjects, such as control of transmitter release(*10-12*) and mechanisms of memory and learning (*13, 14*). In addition, voltage-dependent potassium (K_V) channels play an important role in different aspects of the nervous system electrical responses and are among the most important signaling macromolecules in neuronal cells. In the central nervous system, K_V channels determine the shape, frequency and duration of action potentials (*15*). In addition, blockage of K_V channels is a potential therapy for diverse disorders, including myasthenia gravis, multiple sclerosis, Hungington´s chorea and Alzheimer´s disease. The dominant neuronal delayed K_V channels in mammals are from the voltage-gated channel families K_V1- K_V4 which can be blocked by Tetraethylammonium chloride (TEA) and 4-Aminopyridine (4-AP)(*16*). Voltage dependent sodium channels (Na_V) play an

essential role in neuronal and non-neuronal function, being responsible for the initiation and propagation of action potentials in excitable cells by allowing the influx of sodium ions and are involved in regulation of transmitter release (*17*). High voltage activated (HVA) Ca^{2+} channels are heteromeric transmembrane proteins consisting of α_1, α_2, δ, β and γ subunits, where the α_1 subunit forms the pore of the channel and determines ion selectivity, voltage dependence and toxin sensitivity while the other subunits modulate the functional properties of the α_1 subunit (*18*). By controlling Ca^{2+} entry, these channels regulate several neuronal functions such as spike pattering, neurotransmitter release and gene transcription (*19, 20*). Based on electrophysiological and pharmacological criteria, six types of voltage gated calcium channels (Ca_V) were identified in neurons, however just L-, N-, P, Q- and R-type channels are activated at depolarized membrane potentials thus they are named HVA Ca^{2+} channels (*21-23*). L-type channel antagonists have been proposed to improve age-related working memory deficits (*14*). As preliminary studies on the biological activity of Cramb816 showed that this compound inhibits voltage-dependent calcium channels (*1*), we further investigated its blocking effect in voltage-gated Ca^{2+} channels but also in voltage-gated Na^+ and K^+ channels in neurons. Furthermore, in the present study we aimed at clarifying, for the first time, the effect of three representative compounds of the Crambescin family on voltage-gated Na^+, K^+ and Ca^{2+} channels (Na_V, K_V and Ca_V channels) in primary cultures of cortical neurons using whole-cell voltage clamp recordings.

Here we report that Crambescin C1, Crambescin A2, Norcrambescin A2, partially blocked K_V but not HVA Ca^{2+} channels, whereas Cramb816 (from Crambescidin family) had the opposite effect. Both, Cramb816 and CrambC1, blocked Na_V with a similar potency. These effects suggest a structure-activity dependence in these guanidine alkaloids. We further demonstrate that Cramb816 produces its main antagonist effect on L-type Ca^{2+} channels.

MATERIAL AND METHODS

Primary cultures of cortical neurons. Swiss mice were used to obtain primary cultures of cortical neurons. All protocols were approved by the University of Santiago de Compostela Institutional animal care and use committee. Primary cortical neurons were obtained from embryonic day 16–18 mice fetuses as previously described (*24*). Briefly, cerebral cortices were removed and

dissociated by mild trypsinisation, followed by mechanical trituration in a DNAse solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were suspended in Neurobasal Medium supplemented with 1% B-27 supplement (Invitrogen), 5 mM L-glutamine, and 1% penicillin/streptomycin. Cell suspension was seeded in 12 well plates precoated with poly-D-lysine and the cell culture was kept in a 95% air, 5% CO_2 atmosphere at 37 ºC. Culture medium was replaced every 3-4 days.

Electrophysiology. Whole cell patch-clamp recordings, achieved by gentle mechanical suction of the membrane patch, were performed on cortical neurons, between 3-7 days in culture (unless otherwise noted), at room temperature (22-25 ºC). In order to gain homogeneity within the experiments, only neurons with a bright and smooth appearance were selected for voltage-clamp recordings. 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 a 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. Series resistance was compensated by 80% when possible. After establishing the whole-cell configuration, neurons were allowed to stabilize for at least 5 min before current recording protocols were initiated to ensure adequate equilibration between the internal pipette solution and the cell interior. Recording electrodes were fabricated from borosilicate glass micro capillaries (outer diameter, 1.5mm), and the tip resistance was 5-10 MΩ. Culture medium was exchanged with several washes of recording solution immediately prior to the experiment. The external solution for potassium currents measurements contained (in mM): 120 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-NaOH, 10 glucose, (pH 7.4) while intracellular pipette solutions contained (in mM): 115 $KMeSO_3$, 5 $MgCl_2$, 10 HEPES-KOH, 5 EGTA and 5 K_2ATP (pH 7.2). In addition, in these experiments TTX 1µM was added to the bath solution to block sodium currents. To record sodium currents an external solution containing (in mM): 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-NaOH, 10 glucose, and 10 TEA-Cl (pH 7.4) was used while the intracellular pipette solutions contained (in mM): 110 Csgluconate, 3.7 NaCl, 5 $MgCl_2$, 10 HEPES, 5 EGTA and 5 Na_2 ATP

adjusted to pH 7.2 with CsOH. The external and internal solutions for calcium measurements were designed to eliminate sodium and potassium channel currents. Thus, the bath solution contained (in mM): 110 NaCl, 25 TEA chloride,5 4-AP, 5 $CaCl_2$, 10 HEPES, 1 $MgCl_2$, 5.4 KCl, 25 D-glucose and 1 μM TTX (pH 7.4). The electrode solution contained (in mM): 110 CsCl, 25 TEA chloride, 20 phosphocreatine, 50 units/ml phosphocreatine kinase, 10 EGTA, 10 HEPES, 5 NaCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 0,5 $BaCl_2$, 2 NaATP, 0.1 NaGTP (pH 7.3)(*25*). Gradual rundown of HVA Ca and potassium currents over the recording time was occasionally observed, therefore those with > 1%/min rundown over the course of the experiment were excluded from the analysis. Moreover, most experiments were completed within 20 min. Thus, rundown of the calcium channel current may have played only a minor role in our results.

Toxins and drugs used. CrambC1, CrambA2, NcrambA2 and Cramb816 were extracted and isolated from the Mediterranean Sponge *Crambe crambe (9)*. They were dissolved in DMSO in a 10 mM stock. TEA, 4-AP, ω-AgTx, NIF and TTX were purchased from Sigma-Aldrich. ω-CTx was purchased from Tocris Bioscience. In the presence of NIF, as dihydropyridines are light sensitive compounds, experiments were performed under restricted light conditions, avoiding any major light in the room where the experiments were carried out.

The final concentration of the solvents used for the different compounds or drugs evaluated, DMSO (in most cases) or acetic acid (for TTX), was less than 0.1%, which did not affect none of the currents analyzed (data not shown).

Statistical Analysis. All data are expressed as means ± SEM of *n* determinations. Statistical comparison was by paired Student's t test. P-values < 0.05 were considered statistically significant.

RESULTS

As far as we know, the only reported effect on ion channels of any of the marine compounds from the sponge *Crambe crambe* shows that Cramb816 exhibits a potent Ca^{2+} antagonist activity, more potent than NIF, in neuroblastoma X glioma cell line(*1*). We used the whole cell patch-clamp technique to analyze the effect of CrambC1, NCrambA2, CrambA2 (compounds from Crambescin family) and Cramb816 (from Crambescidin family) on Na_V, K_V and Ca_V channels in mice cortical neurons. The chemical structure of the compounds analyzed in this work is shown in Figure 1.

Crambescins but not Cramb816 partially block K_V channels in mice cortical neurons

We first evaluated the concentration-response effect of these guanidine alkaloids on K_V channels administering consecutive concentrations of 10, 100 and 1000 nM, of each toxin to the same cell at 5 min intervals. Neurons were voltage clamped at a membrane holding potential (V_H) = -60 mV and total potassium currents (I_K) were evoked by 200 ms depolarizing pulses from V_H to +75 mV in 15 mV steps. As shown in Figure 2 (right panel), outward potassium currents were activated around -15 mV. The three Crambescins evaluated in this work blocked the total potassium current in a dose dependent manner whereas Cramb816 lacked this effect. Sensitive currents (the amount of total I_K inhibited by the compounds) were measured by subtracting the remaining current after drug administration from their respective controls. As shown in Figure 2A, when consecutive concentrations of Cramb816 at 10, 100 and 1000 nM were added to the same cell, total potassium current was not inhibited by the compound at any concentration at 75 mV. Consecutive concentrations of NcrambA2 (Figure 2B) at 10, 100 and 1000 nM blocked I_K by 11.6 ± 2.9 **%** (n = 6, p = 0.007) and 20.5 ± 4.5 % (n = 6, p = 0.002) respectively at the two higher concentrations evaluated while its effect was not significant at 10 nM. In a similar way, when consecutive concentrations of CrambA2 (Figure 2C) were added to the cells, it only caused a significant effect at 1000 nM decreasing I_K by 11.6 ± 2.3 **%** (n = 4; p = 0.01). Bath application of CrambC1 (Figure 2D) at 10, 100 and 1000 nM did not show any effect on I_K at the lower concentrations while at the highest concentration evaluated, total I_K decreased by 14.4 ± 5.0 % (n = 6; p = 0.008). In order to exclude a possible rundown of I_K in the presence of consecutive concentrations of the compound, a single concentration of CrambC1 at 1000 nM was directly applied to some cells. In this case, total I_K was blocked by 13.3 ± 2.5 % (n = 5; p = 0.01), therefore indicating that there is no loss of channel function after compound addition. Moreover, in some cells, we measured the current 10 and 15 minutes after application of CrambC1 at 1000 nM, and in this case, total I_K current decreased by 19.9 ± 3.7% (n = 5, p = 0.003) and 31.1 ± 3.7 **%** (n = 4; p < 0.001) respectively, indicating that it acts in a time-dependent way (data not shown).

Cramb816 but not NCrambA2 partially blocks HVA Ca_V channels in primary cortical neurons

To evaluate the effect of the compounds on Ca_V and due to the scarcity of compounds, one representative member of each family was used at 1 μM as at 10 and 100 nM none of them had significant effect on potassium currents. We chose NCrambA2 and Cramb816 since they belong to different structural families and because NCrambA2 showed the most interesting effect in potassium currents among the rest of compounds analyzed from its family. Therefore, here, we report the results of a concentration of 1 μM NCrambA2 and 1 μM Cramb816 on Ca_V channels. In order to obtain a first approximation of their effect, Ca_V currents were elicited by a single step depolarization to -10mV for 600 ms, previously preceded by a preconditioning step of -100 mV and 200 ms of duration (holding potential -60 mV). In Figure 3, representative Ca^{2+} current traces before and after addition of Cramb816 (Fig. 3A) at 1 μM and NCrambA2 at the same concentration (Fig. 3B), are shown. The Cramb816 sensitive current (Fig. 3C), obtained by subtracting Cramb816 current from control current in A, showed a peak of transient current followed by a slow sustained current. In most of the cells, Cramb816 sensitive currents showed this particular shape. In contrast, the NCrambA2 sensitive current was null as shown in Figure 3D. As illustrated in Figure 3E, at 1 μM Cramb816 inhibited Ca^{2+} currents by 22.59 ± 1.6% ($n = 10$; $p < 0.001$) whereas NCrambA2 ($n = 4$) had no significant effect as shown in Figure 3F. Increasing the compounds concentration to 2 μM, NCrambA2 did not show any effect over the control currents while the effect produced by Cramb816 at 2 μM remained similar to that observed at 1 μM (18.4 ± 3.1%; $n = 3$), (data not shown).

In order to gain a deeper insight in the effect of Cramb816 on Ca_V channels, we next examined the I-V relationships in the absence (control) and presence of Cramb816 at 1 μM (Fig. 4). As shown in Figure 4A, currents were elicited by 20 mV depolarizing steps from a holding potential of -80 mV (2-s prepulse) up to +20 mV in 10 mV increments. Inward currents were normally activated at voltages more positive than -50 mV and peaked between -10 and 0 mV. Blockade by Cramb816 did not alter the activation kinetics in the residual

current and this was evident from -10 mV to higher potentials (Fig 4B). A representative trace of Cramb816 effect at 0 mV is shown in Figure 4C and the Cramb816 sensitive current in Figure 4D.

Pharmacological characterization of HVA Ca^{2+} currents and effect of Cramb816 on them

In order to pharmacologically characterize the Ca_V channels affected by Cramb816, we used Ca_V blockers. The main Ca_V families expressed in neurons are L-type or Cav1 channels and Cav2, which include P/Q type (Cav2.1) and N-type (Cav2.2) (*26*). L-type Cav1 channels are blocked by dihydropyridines such as NIF (*27*) while P/Q- and N-type Cav channels are inhibited by the spider toxin ω-AgTx and the cone snail toxin ω-CTx respectively(*26*). All of these Ca^{2+} channels are expressed in the neuronal cortex(*28-31*). Since the different Ca_V channels undergo dynamic changes during neuronal development(*32*), they contribute very differently to the Ca^{2+} influxes over the time. Thus, we performed all the Ca^{2+} electrophysiological recordings in 4-6 DIV neurons. In order to characterize which Ca^{2+} current was blocked by Cramb816, the effect of the compound was analyzed in the presence of the different Ca_V blockers mentioned above. With the protocol described in figure 4A, at -10 mV the selective L-type or Cav1 channels antagonist NIF, at 10 μM, reduced HVA Ca^{2+} currents by 31.5 ± 2.9% of control currents (n = 8; p <0.001), however the remaining current was not significatively affected after addition of Cramb816 (Figure 5A). By contrast, as shown in Figure 5B, the combination of ω-AgTx and ω-CTx, reduced HVA Ca^{2+} currents by 30.1 ± 4.6% (n= 4; p = 0.002) and the further addition of Cramb816 reduced the remaining current by 22.9 ± 5.4% (n = 4, p = 0.003), an effect very similar to the one obtained by the administration of Cramb816 alone. Thus, the simultaneous blockage of Cav2 channels in the presence of both, ω-AgTx and ω-CTx did not affect Cramb816 inhibition of HVA Ca^{2+} currents, which suggests that Cav2 channels are not the main target of Cramb816. These results further indicate that the Ca_V channels blocked by Cramb816 are mainly the NIF-sensitive or Cav1 channels.

Cramb816 does not affect the steady state inactivation of Ca_V channels

The voltage dependence of inactivation of Ca_V channels is based mainly on the voltage dependency of activation, because inactivation depends primarily on the state of the channel, rather than on voltage (*33*). To study the kinetics of HVA Ca^{2+} channel inactivation, Ca^{2+} currents were generated by a 200 ms test pulse (TP) to +10 mV preceded by a 1.5-s conditioning prepulse (CP) from -80 to +10 mV in 10 mV increments (Figure 6A). Figure 6B shows representative current traces in the absence (control) and presence of Cramb816 at 1 µM at CP of 0 and -80 mV. CP to very negative voltages, starting at -80 mV, evoked minimal Ca^{+2} currents and yielded near maximal Ca^{+2} currents on the test pulse. By contrast, a CP to the maximum of Ca^{+2} current activation (about 0 mV) evoked maximal Ca^{+2} entry, and resulted in a minimal current on the test pulse, produced as a result of calcium channel inactivation (CDI)(*33*), which was unaltered by Cramb816 (Figure 6B). Currents elicited from the TP and the CP pulse were normalized to the current associated with their maximal current. The maximal rate of inactivation occurred near the peak of I-V relationship, between 0 and + 10 mV (Figure 6C). Normalized current amplitude against the CP potential was fifted by the Boltzman equation which yielded a V_{50} of -20 mV for steady state inactivation of the Ca^{+2} channels which was not affected in the presence of Cramb816 at 1 µM (Figure 6C).

Effect of Crambescins and Cramb816 on Na_V channels

Voltage dependent sodium currents were elicited in cortical neurons by applying a series of 25 ms depolarizing pulses (voltage steps), in 5 mV increments, from a holding potential of -100 mV(*34*). The effect of the compounds on Na_V was measured by plotting the percent inhibition of the peak Na^+ current amplitude (I_{Na}) at each concentration. As described above for K^+ current, consecutive concentrations of the compounds at 10, 100 and 1000 nM were also added to each cell. As shown in Figure 7A, Cramb816 decreased I_{Na} significatively at 100 nM by 17.4 ± 6.1 **%** (n = 5; p = 0.046) while this decrease did not reach a significant value at the concentration of 1000 nM. On the other hand, CrambC1 at 1000 nM blocked I_{Na} by 20.7 ± 4.8 **%** (n = 5; p = 0.02) whereas NCrambA2 and CrambA2 failed to modify I_{Na} at any of the concentrations evaluated (Fig. 7B, C and D respectively).

DISCUSSION

This study shows the effect of NCrambA2, CrambC1, CrambA2 (Crambescin family compounds) and Cramb816 (from Crambescidin family) in the main ion channels responsible for neuronal excitability and electrical signaling: voltage-gated calcium, sodium and potassium channels (*26*). In 1993, Berlinck *et al* revealed that Cramb816 had a much more potent antagonist action than NIF in blocking Ca^{2+} channels (*1, 2*). Since then, no additionnal work, related with the activity of these compounds on ion channels, has been reported. Considering that ion channels are frequent targets for natural toxins, we further investigated the potential activity of these compounds in Ca_V, K_V and Na_V channels in neurons.

Under voltage clamp conditions, we found that all these guanidine alkaloids affected with similar potency the different ion channels. Cramb816 affected Na_V and Ca_V but not K_V. In contrast, all the representative compounds from the Crambescin family studied here, affected Kv channels. Moreover, CrambC1 also affected Na_V but was not tested in Ca_V as well as CrambA2. Differences in the bioactivity of these two families may be related to their chemical structure. Even if these compounds should share a common biosynthetic pathway, the mono- or bicyclic crambescins are structurally simpler than the corresponding pentacyclic crambescidins. All crambescins exhibit similar bioactivity on K_v channels but not Cramb816 which implies that changes in the cyclisation or the chain lengths do not affect this activity which may be essentially due to the presence of two strongly basic guanidine functions. The absence of the second guanidine group may explain the loss of bioactivity observed for Cramb816. On the contrary, the higher bioactivity of CrambC1 on Na_v than the other crambescins A2 indicates that either a longer chain linked to the terminal guanidine or a non cyclized hydroxypropyl chain is required for an action on Na_v channels. Finally, the bipolar parts of Cramb816 linked by a long alkyl chain seems to be important for an action on Ca_v channels. We also observed that the regulation of the activation threshold of the ion channels studied here, was unaffected by any compound, indicating that they might modulate the conformation of the channels rather than their kinetics.

In our hands, and in full agreement with the report of Berlinck (*1*), Cramb816 blocked the Ca_V channels. The fact that Cramb816 has a more potent effect than NIF as Berlinck proposed, was evidenced since 10 times less

concentrated Cramb816 blocked about 20% of the total Ca^{2+} current which NIF blocked by about 30%. By pharmacologically isolating the main calcium channels in neurons, we further demonstrated that Cramb816 mainly blocks the L-type Ca_V channels or Cav1 family, which play a critical role in somatodendritic calcium influx and are involved in dendritic calcium signaling resulting from back-propagating action potentials, synaptic plasticity, and excitatory activity-dependent modulation of gene transcription in mammalian brain neurons (*35, 36*). Among the four Cav1 family members, only Cav1.2 and Cav1.3 are expressed in mammalian central neurons. The Cav1.2 family is the major constituent of the brain L-type Ca_V channel population, thus, its regulation is critical to calcium entry in response to synaptic activity (*37, 38*). It is also known that L-type Ca_V channels are increased in neurons of aged rats thus L-type channel antagonists might be involved in ameliorating age-related working memory deficits (*14*). Modulation of Nav currents is also, undoubtedly, important *in vivo*. They are responsible for the initiation and propagation of action potentials in excitable cells by allowing the influx of sodium ions (*39, 40*). Cramb816, as well as CrambC1, blocked about 20% of the Nav1 current. Neuromodulation of electrical excitability and synaptic transmission is the basis for many aspects of learning, memory and physiological regulation. Our data suggest that, by decreasing both, Ca_V and Na_V currents, Cramb816 may decrease the transmitter release and thus the propagation of action potentials and synaptic transmission in neurons. The lower release of neurotransmitter would be again in agreement with Berlinck *et al*, who found that Cramb816 inhibited the acetylcholine-induced contraction in guinea pig ileum. In contrast to Cramb816, all the studied representatives from the Crambescin family partially blocked K_V channels, an effect that is known to be neuroprotective and has been proposed as a therapeutic strategy for Alzheimer´s Disease (*41, 42*).

Bondu *et al.* recently demonstrated that Cramb816 has a dose-dependent cytotoxic effect in neurons whereas CrambC1 has much less effect (*9*). Numerous studies link changes in calcium homeostasis in neurons with increased apoptosis (*43-46*), and long-term exposure to Ca_V channel antagonists compromises neuronal survival (*44*). By contrast, attenuating outward K^+ current reduces apoptosis in cortical neurons (*47, 48*). Thus, the differences observed by Bondu *et al.* in cell viability between members from

both families, might be partially explained by their different blocking effects in Ca_V and K_V channels. Therefore, we suggest that the cytotoxic effect that 24 hours exposure of 1 μM Cramb816 produces in these neurons (*9*) may be due, at least in part, to its Ca_V blockade at the same concentration, while the blocking effect of CrambC1 in K_V might explain the higher neuronal survival observed in the same neuronal model.

In summary, this is the first report addressing the effects of some representative members of the Crambescin family and Cramb816 in voltage-gated calcium, sodium and potassium channels in cultured mouse cortical neurons. A detailed study of the contribution of every compound to each current would require more pharmacological and biophysical analyses which we were unable to perform due to the scarcity of compounds. Hence, the sodium and potassium channel type that these guanidine alkaloids affect remains to be verified. However, despite this limitation, we report that Crambescins have different effects compared to Cramb816 in the studied ion channels, probably due to the structural differences of both families. Our experiments indicate that Ca_V1 (L-type) channels are the main target for Cramb816. Indeed, we suggest the possibility that this effect may be involved in the neuronal death produced by the treatment of cortical neurons with Cramb816. It is obvious that these guanidine alkaloids interestingly affect the main neuronal ion channels and thus may have a key role to play in future studies.

ABREVIATIONS

Cramb816, Crambescidin 816; CrambC1, Crambescin C1; CrambA2, Crambescin A2; NcrambA2, Norcrambescin A2; NIF, Nifedipine; K_V channels, voltage-gated potassium channels; TEA, Tetraethylammonium chloride; 4-AP, 4-Aminopyridine; Na_V channels, voltage-gated sodium channels; HVA, High voltage activated; Ca_V channels,voltage-gated calcium channels; ω-AgTx, ω-Agatoxin IVA; TTX, tetrodotoxin; ω-CTx, ω-Conotoxin GVIA; I_K , total potassium current; $I_{Na,}$ total sodium current; CP, Conditioning prepulse; CDI, Calcium dependent inactivation; VDI, voltage dependent inactivation.

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REFERENCES

(1) Berlinck, R. G., Braekman, J. C., Daloze, D., Bruno, I., Riccio, R., Ferri, S., Spampinato, S., and Speroni, E. (1993) Polycyclic guanidine alkaloids from the marine sponge Crambe crambe and Ca++ channel blocker activity of crambescidin 816. *J Nat Prod*, *56*, 1007-1015.

(2) Berlinck, R. G., Braekman, J. C., Daloze, D., Hallenga, K., and Ottinger, R. (1990) Two new guanidine alkaloids from the mediterranean sponge crambe crambe. *Thetrahedron Letters*, *31*, 6531-6534.

(3) Jares-Erijman, E. A., Ingrum, A. A., Sun, F., and Rinehart, K. L. (1993) On the structures of crambescins B and C1. *J Nat Prod*, *56*, 2186-2188.

(4) Bewley, C. A., Ray, S., Cohen, F., Collins, S. K., and Overman, L. E. (2004) Inhibition of HIV-1 envelope-mediated fusion by synthetic batzelladine analogues. *J Nat Prod*, *67*, 1319-1324.

(5) Chang, L., Whittaker, N. F., and Bewley, C. A. (2003) Crambescidin 826 and dehydrocrambine A: new polycyclic guanidine alkaloids from the marine sponge Monanchora sp. that inhibit HIV-1 fusion. *J Nat Prod*, *66*, 1490-1494.

(6) Olszewski, A., Sato, K., Aron, Z. D., Cohen, F., Harris, A., McDougall, B. R., Robinson, W. E., Jr., Overman, L. E., and Weiss, G. A. (2004) Guanidine alkaloid analogs as inhibitors of HIV-1 Nef interactions with p53, actin, and p56lck. *Proc Natl Acad Sci U S A*, *101*, 14079-14084.

(7) Jares-Erijman, E., Sakai, R., and Rinehart, K. L. (1991) Crambescidins: new antiviral and cytotoxic compounds from the sponge Crambe crambe. *J Org Chem*, *56*, 5712-5715.

(8) Laville, R., Thomas, O. P., Berrue, F., Marquez, D., Vacelet, J., and Amade, P. (2009) Bioactive guanidine alkaloids from two Caribbean marine sponges. *J Nat Prod*, *72*, 1589-1594.

(9) Bondu, S., Genta-Jouve, G., Leiros, M., Vale, C., Guigonis, J. M., Botana, L., and Thomas, O. P. (2012) Additional bioactive guanidine alkaloids from the Mediterranean sponge Crambe crambe. *The Royal Society of Chemistry 2*, 2828-2835.

(10) Augustine, G. J., Charlton, M. P., and Smith, S. J. (1987) Calcium action in synaptic transmitter release. *Annu Rev Neurosci*, *10*, 633-693.

(11) Swartz, K. J. (2004) Towards a structural view of gating in potassium channels. *Nat Rev Neurosci*, *5*, 905-916.

(12) Wu, L. G., Westenbroek, R. E., Borst, J. G., Catterall, W. A., and Sakmann, B. (1999) Calcium channel types with distinct presynaptic localization couple differentially to transmitter release in single calyx-type synapses. *J Neurosci*, *19*, 726-736.

(13) Etcheberrigaray, E., Gibson, G. E., and Alkon, D. L. (1994) Molecular mechanisms of memory and the pathophysiology of Alzheimer's disease. *Ann N Y Acad Sci*, *747*, 245-255.

(14) Veng, L. M., Mesches, M. H., and Browning, M. D. (2003) Age-related working memory impairment is correlated with increases in the L-type calcium channel protein alpha1D (Cav1.3) in area CA1 of the hippocampus and both are ameliorated by chronic nimodipine treatment. *Brain Res Mol Brain Res*, *110*, 193-202.

(15) Melishchuk, A., Loboda, A., and Armstrong, C. M. (1998) Loss of shaker K channel conductance in 0 K+ solutions: role of the voltage sensor. *Biophys J*, *75*, 1828-1835.

(16) Gu, C., and Barry, J. (2011) Function and mechanism of axonal targeting of voltage-sensitive potassium channels. *Prog Neurobiol*, *94*, 115-132.

(17) Rahamimoff, R., Butkevich, A., Duridanova, D., Ahdut, R., Harari, E., and Kachalsky, S. G. (1999) Multitude of ion channels in the regulation of transmitter release. *Philos Trans R Soc Lond B Biol Sci*, *354*, 281-288.

(18) Snutch, T. P., and Reiner, P. B. (1992) Ca2+ channels: diversity of form and function. *Curr Opin Neurobiol*, *2*, 247-253.

(19) Lancaster, B., Nicoll, R. A., and Perkel, D. J. (1991) Calcium activates two types of potassium channels in rat hippocampal neurons in culture. *J Neurosci*, *11*, 23-30.

(20) Hernandez-Lopez, S., Bargas, J., Surmeier, D. J., Reyes, A., and Galarraga, E. (1997) D1 receptor activation enhances evoked discharge in neostriatal medium spiny neurons by modulating an L-type Ca2+ conductance. *J Neurosci*, *17*, 3334-3342.

(21) Catterall, W. A. (2000) Structure and regulation of voltage-gated Ca2+ channels. *Annu Rev Cell Dev Biol*, *16*, 521-555.

(22) Llinas, R., Sugimori, M., Lin, J. W., and Cherksey, B. (1989) Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison. *Proc Natl Acad Sci U S A*, *86*, 1689-1693.

(23) Zhang, J. F., Randall, A. D., Ellinor, P. T., Horne, W. A., Sather, W. A., Tanabe, T., Schwarz, T. L., and Tsien, R. W. (1993) Distinctive pharmacology and kinetics of cloned neuronal Ca2+ channels and their possible counterparts in mammalian CNS neurons. *Neuropharmacology*, *32*, 1075-1088.

(24) Vale, C., Alonso, E., Rubiolo, J. A., Vieytes, M. R., LaFerla, F. M., Gimenez-Llort, L., and Botana, L. M. (2010) Profile for amyloid-beta and tau expression in primary cortical cultures from 3xTg-AD mice. *Cell Mol Neurobiol*, *30*, 577-590.

(25) Xiang, K., Earl, D. E., Davis, K. M., Giovannucci, D. R., Greenfield, L. J., Jr., and Tietz, E. I. (2008) Chronic benzodiazepine administration potentiates high voltage-activated calcium currents in hippocampal CA1 neurons. *J Pharmacol Exp Ther*, *327*, 872-883.

(26) Vacher, H., Mohapatra, D. P., and Trimmer, J. S. (2008) Localization and targeting of voltage-dependent ion channels in mammalian central neurons. *Physiol Rev*, *88*, 1407-1447.

(27) Snutch, T. P., Sutton, K. G., and Zamponi, G. W. (2001) Voltage-dependent calcium channels--beyond dihydropyridine antagonists. *Curr Opin Pharmacol*, *1*, 11-16.

(28) Miljanich, G. P., and Ramachandran, J. (1995) Antagonists of neuronal calcium channels: structure, function, and therapeutic implications. *Annu Rev Pharmacol Toxicol*, *35*, 707-734.

(29) Ahlijanian, M. K., Westenbroek, R. E., and Catterall, W. A. (1990) Subunit structure and localization of dihydropyridine-sensitive calcium channels in mammalian brain, spinal cord, and retina. *Neuron*, *4*, 819-832.

(30) Hell, J. W., Westenbroek, R. E., Warner, C., Ahlijanian, M. K., Prystay, W., Gilbert, M. M., Snutch, T. P., and Catterall, W. A. (1993) Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel alpha 1 subunits. *J Cell Biol*, *123*, 949-962.

(31) Westenbroek, R. E., Ahlijanian, M. K., and Catterall, W. A. (1990) Clustering of L-type Ca2+ channels at the base of major dendrites in hippocampal pyramidal neurons. *Nature*, *347*, 281-284.

(32) Pravettoni, E., Bacci, A., Coco, S., Forbicini, P., Matteoli, M., and Verderio, C. (2000) Different localizations and functions of L-type and N-type calcium channels during development of hippocampal neurons. *Dev Biol*, *227*, 581-594.

(33) Budde, T., Meuth, S., and Pape, H. C. (2002) Calcium-dependent inactivation of neuronal calcium channels. *Nat Rev Neurosci*, *3*, 873-883.

(34) Perez, S., Vale, C., Alonso, E., Alfonso, C., Rodriguez, P., Otero, P., Alfonso, A., Vale, P., Hirama, M., Vieytes, M. R., and Botana, L. M. (2011) A comparative study of the effect of ciguatoxins on voltage-dependent Na+ and K+ channels in cerebellar neurons. *Chem Res Toxicol*, *24*, 587-596.

(35) Catterall, W. A., Perez-Reyes, E., Snutch, T. P., and Striessnig, J. (2005) International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. *Pharmacol Rev*, *57*, 411-425.

(36) Johnston, D., Christie, B. R., Frick, A., Gray, R., Hoffman, D. A., Schexnayder, L. K., Watanabe, S., and Yuan, L. L. (2003) Active dendrites, potassium channels and synaptic plasticity. *Philos Trans R Soc Lond B Biol Sci*, *358*, 667-674.

(37) Elliott, E. M., Malouf, A. T., and Catterall, W. A. (1995) Role of calcium channel subtypes in calcium transients in hippocampal CA3 neurons. *J Neurosci*, *15*, 6433-6444.

(38) Magee, J. C., Christofi, G., Miyakawa, H., Christie, B., Lasser-Ross, N., and Johnston, D. (1995) Subthreshold synaptic activation of voltage-gated Ca2+ channels mediates a localized Ca2+ influx into the dendrites of hippocampal pyramidal neurons. *J Neurophysiol*, *74*, 1335-1342.

(39) Cantrell, A. R., and Catterall, W. A. (2001) Neuromodulation of Na+ channels: an unexpected form of cellular plasticity. *Nat Rev Neurosci*, *2*, 397-407.

(40) Nieto, F. R., Cobos, E. J., Tejada, M. A., Sanchez-Fernandez, C., Gonzalez-Cano, R., and Cendan, C. M. (2012) Tetrodotoxin (TTX) as a therapeutic agent for pain. *Mar Drugs*, *10*, 281-305.

(41) Lavretsky, E. P., and Jarvik, L. F. (1992) A group of potassium-channel blockers-acetylcholine releasers: new potentials for Alzheimer disease? A review. *J Clin Psychopharmacol*, *12*, 110-118.

(42) Wesseling, H., Agoston, S., Van Dam, G. B., Pasma, J., DeWit, D. J., and Havinga, H. (1984) Effects of 4-aminopyridine in elderly patients with Alzheimer's disease. *N Engl J Med*, *310*, 988-989.

(43) Takadera, T., Suzuki, R., and Mohri, T. (1990) Protection by ethanol of cortical neurons from N-methyl-D-aspartate-induced neurotoxicity is associated with blocking calcium influx. *Brain Res*, *537*, 109-114.

(44) Koh, J. Y., and Cotman, C. W. (1992) Programmed cell death: its possible contribution to neurotoxicity mediated by calcium channel antagonists. *Brain Res*, *587*, 233-240.

(45) Turner, C. P., Connell, J., Blackstone, K., and Ringler, S. L. (2007) Loss of calcium and increased apoptosis within the same neuron. *Brain Res*, *1128*, 50-60.

(46) Takadera, T., Matsuda, I., and Ohyashiki, T. (1999) Apoptotic cell death and caspase-3 activation induced by N-methyl-D-aspartate receptor antagonists and their prevention by insulin-like growth factor I. *J Neurochem*, *73*, 548-556.

(47) Yu, S. P., Farhangrazi, Z. S., Ying, H. S., Yeh, C. H., and Choi, D. W. (1998) Enhancement of outward potassium current may participate in beta-amyloid peptide-induced cortical neuronal death. *Neurobiol Dis*, *5*, 81-88.

(48) Yu, S. P., Yeh, C. H., Sensi, S. L., Gwag, B. J., Canzoniero, L. M., Farhangrazi, Z. S., Ying, H. S., Tian, M., Dugan, L. L., and Choi, D. W. (1997) Mediation of neuronal apoptosis by enhancement of outward potassium current. *Science*, *278*, 114-117.

FIGURE LEGENDS

Figure 1. **Chemical structures of Crambescin A2, Norcrambescin A2, Crambescin C1 and Crambescidin 816.**

Figure 2. Concentration-dependent effects of Cramb816, NCrambA2, CrambA2 and CrambC1 on potassium currents. Compound-sensitive currents (amount of total potassium current (I_K) inhibited by the compounds) were measured by subtracting the remaining current after drug administration from their respective controls. Pooled results in the left panel of A, B, C and D show the peak of sensitive currents (at +75 mV) for Cramb816, NCrambA2 , CrambA2 and CrambC1 respectively (left panel). The number of cells tested in each condition is indicated in parentheses. I-V relationships of sensitive currents and their respective controls (total I_K) are shown in the right panel. Neurons were voltage clamped at a membrane holding potential (V_H) = -60 mV and I_K was evoked by a 200 ms depolarizing pulse from V_H to +75 mV in 15 mV steps.

Figure 3. Effect of NCrambA2 and Cramb816 at 1 µM in HVA Ca^{+2} currents in cortical neurons. Representative traces of the calcium currents elicited by a single-step depolarization to –10mV from a preconditioning step of -100 mV (holding potential -60 mV), before and after application of Cramb816 (A) and NcrambA (B). C and D are the sensitive currents of Cramb816 and NCrambA2 in A and B obtained by subtracting the current inhibited by Cramb816 and NcrambA from their respective controls. The pooled results of all experiments are shown in the histograms E and F. The number of cells tested is indicated in parentheses. ***$p < 0.005$

Figure 4.Current-voltage relationship of HVA Ca^{+2} currents elicited from cortical neurons in presence and absence of Cramb816 1 µM. A, activation pulse protocol: 200 ms depolarizing voltage steps ranging from -70 to 20 mV in 10 mV increments were preceded by a 2-s prepulse to -80mV. Membrane holding potential (V_H) was -65 mV. B, I-V relationship of HVA Ca^{+2} currents

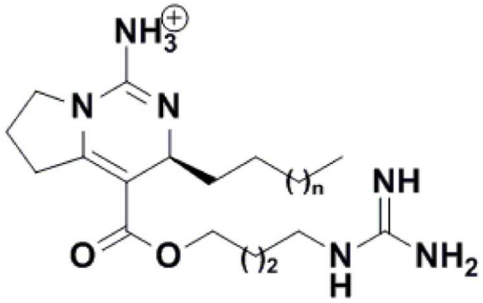
normalized to the Imax in presence and absence of Cramb816. C, representative traces of peak Ca^{+2} currents evoked in the absence (control) and presence of Cramb816 at 0 mV. D, sensitive Cramb816 current obtained by subtracting the current after application of Cramb816 from the control in C.

Figure 5. Inhibition of Cav channels by Cramb816 in the presence of specific HVA Ca^{+2} channel blockers. Left panel, current-voltage relationships of the voltage-dependent calcium currents elicited with the voltage. Protocol shown in Fig 4 in control conditions and after bath application of 10 μM NIF followed by bath application of 1 μM Cramb816 (A). In B the voltage-dependent calcium currents are shown in absence and in the presence of the combination of 300 nM ω-AgTx and 1μM ω-CTx followed by bath application of Cramb816 at 1 μM. The representative traces of the compound-sensitive voltage-dependent calcium currents, at 0 mV before and after drug application are shown in the center panel. As shown in A, in the presence of NIF, Cramb816 is not able to block the remaining current while it blocks the remaining current in the presence of ω-AgTx and ω-CTx. The right panel shows the pooled results of all the experiments performed with protocol in Fig 4, at a holding potential of -10mV. The number of cells tested is indicated in parentheses. **p < 0.01, ***p < 0.005.

Figure 6. Voltage dependence of steady state inactivation of voltage-dependent calcium channels in the absence and presence of Cramb816. A, Inactivation pulse protocol: a 200 ms test-pulse (TP) to 10 mV was preceded by a 1.5-s conditioning prepulse (CP). Voltage steps ranged from -80 to +10 mV in 10 mV increments. V_H was -65 mV. B, representative steady state inactivation calcium current traces in the absence (control) and presence of Cramb816 1 μM at conditioning pulses of 0 and -80 mV (n = 4). C, I-V relationship of steady state inactivation. Current amplitudes were normalized to the maximun current (Imax).

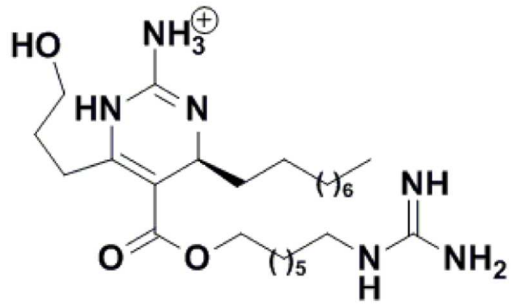
Figure 7. Concentration-dependent effects of Cramb816, CrambC1, NCrambA2 and CrambA2 on sodium currents (I_{Na}). Pooled results for the concentration-dependent effects of Cramb816, CrambC1, NCrambA2 and

CrambA2 on I_{Na} are shown in A, B, C and D respectively. The effect of the compounds was measured by plotting the percent of inhibition of the peak I_{Na} at each concentration. The number of cells tested is indicated in parentheses. *$p < 0.05$.

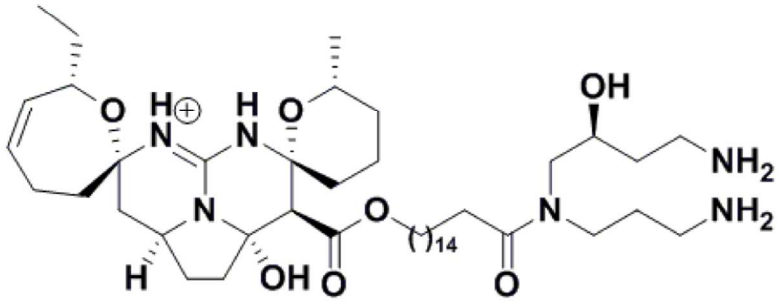


Norcrambescin A2 n = 8

Crambescin A2 n = 9



Crambescin C1



Crambescidin 816

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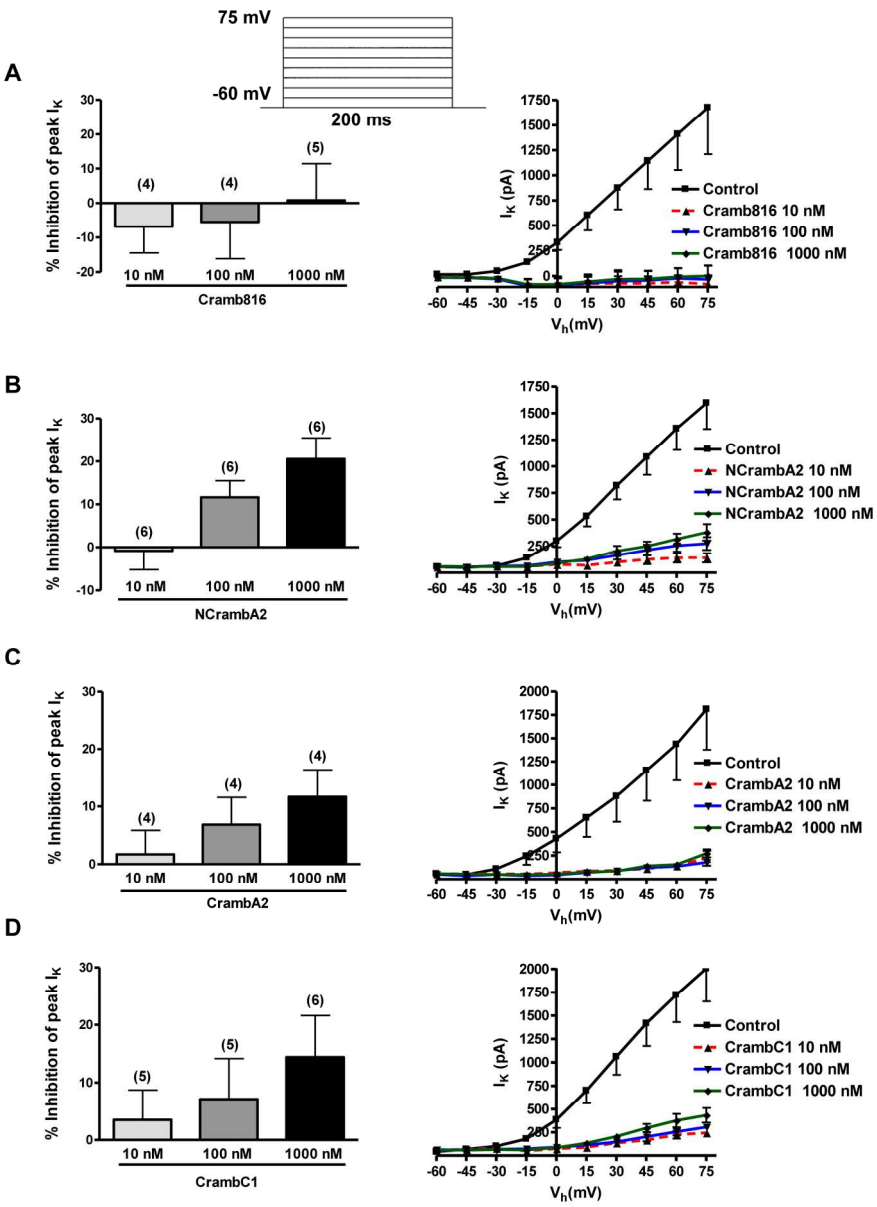

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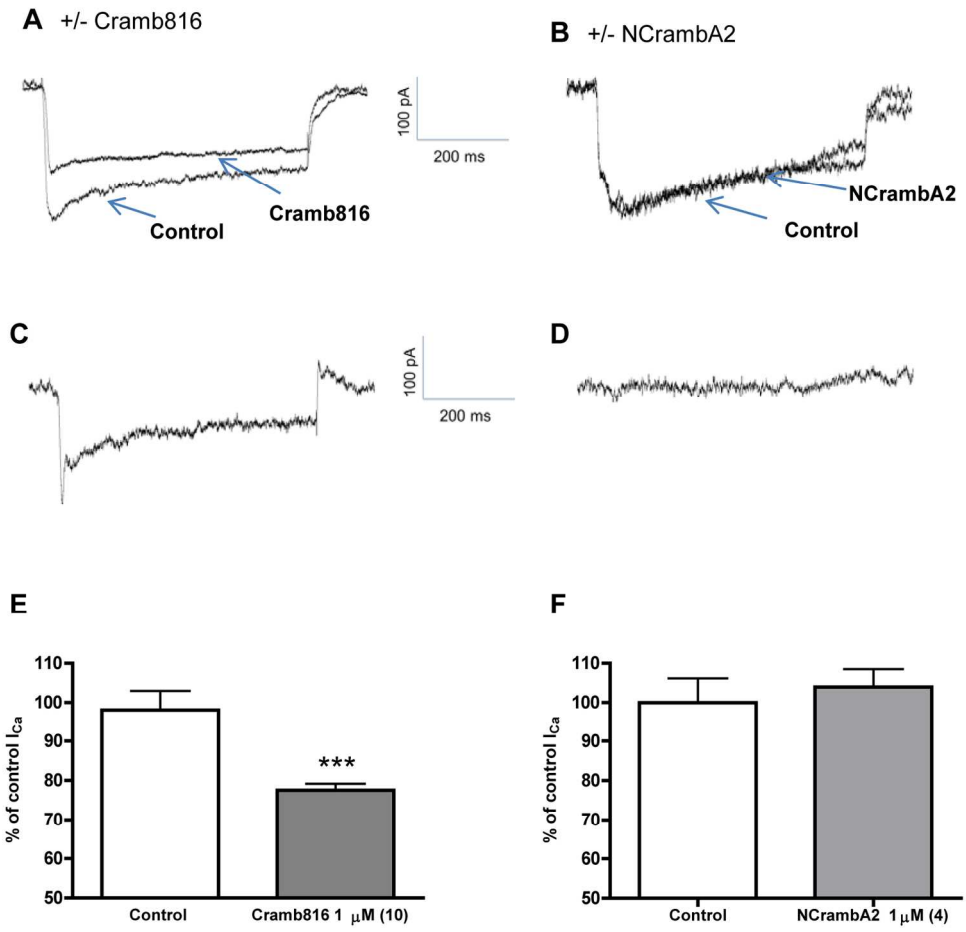

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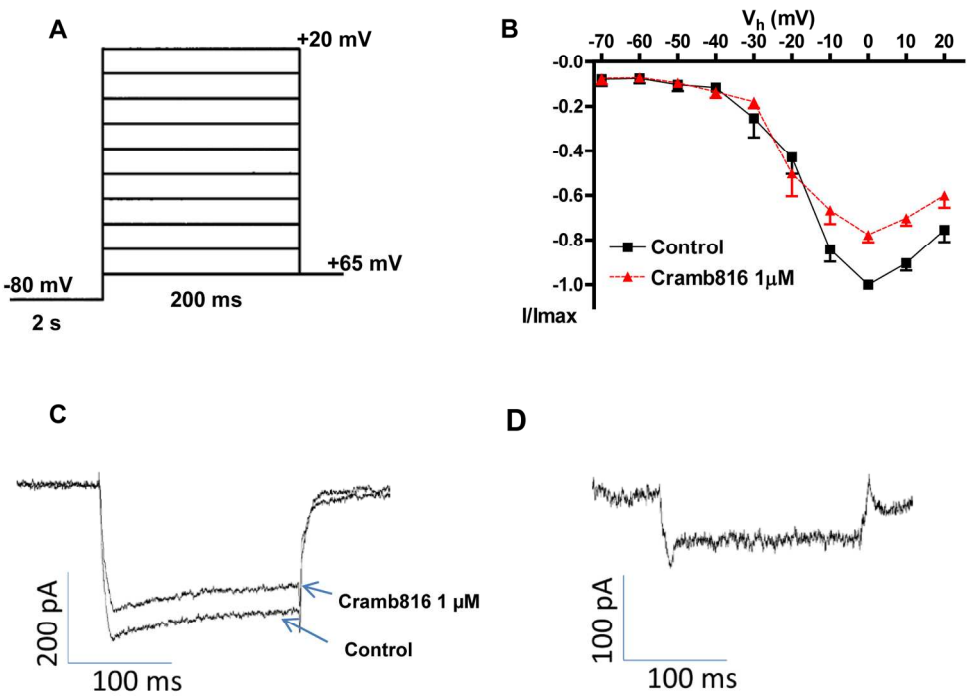

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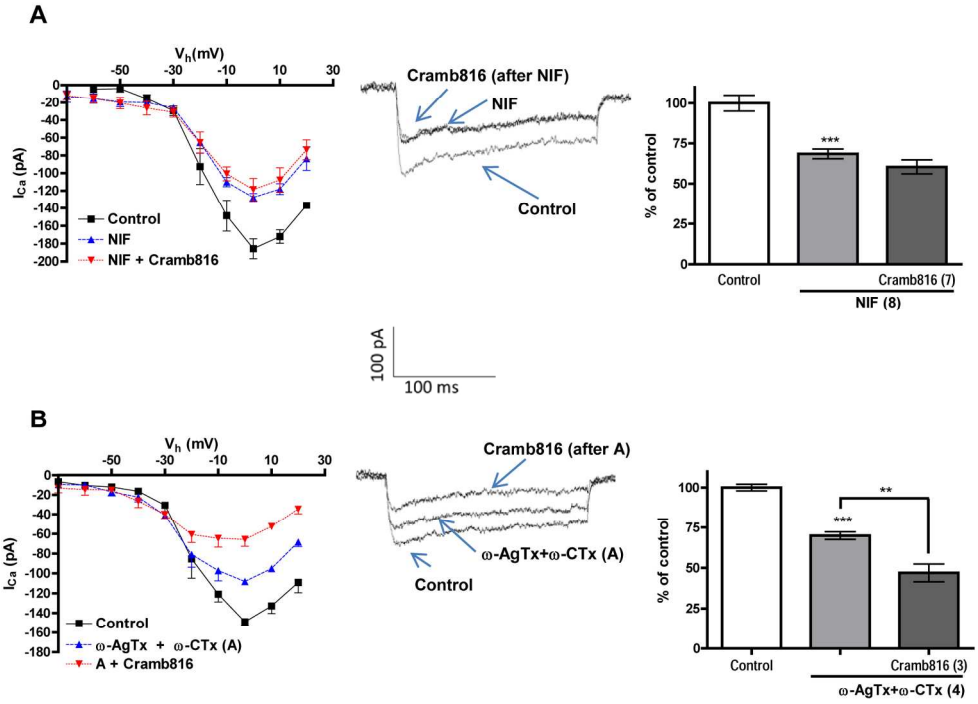

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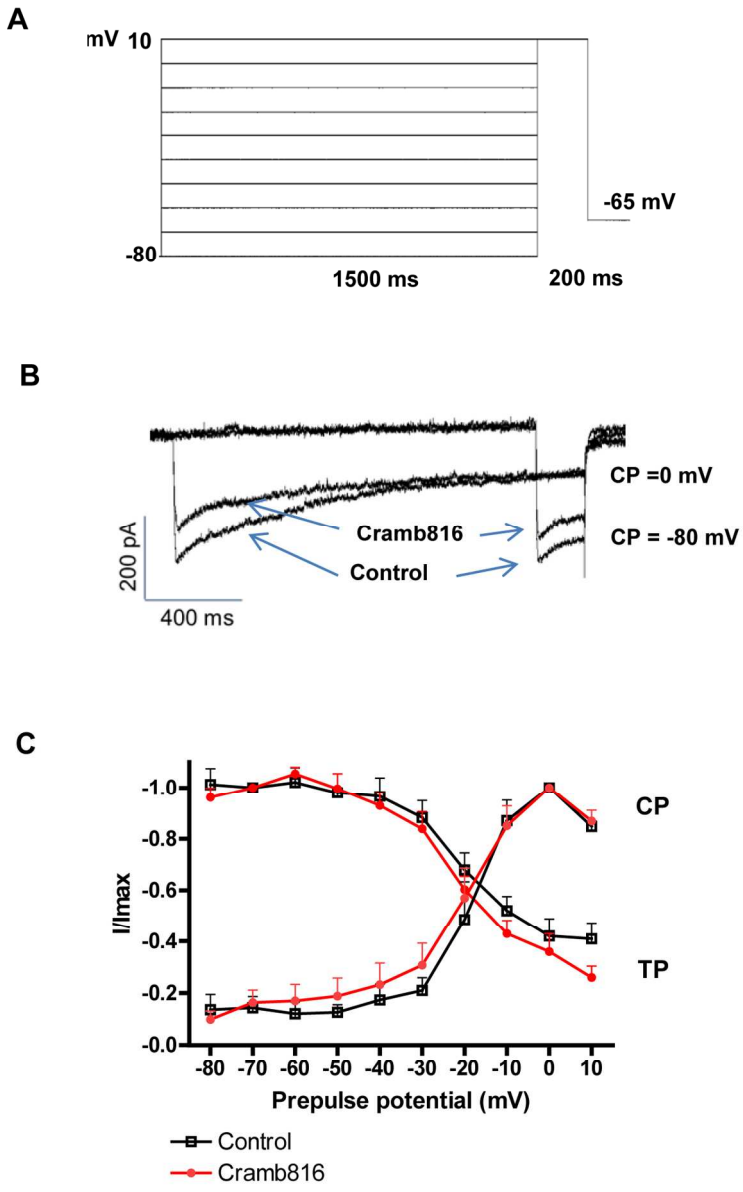

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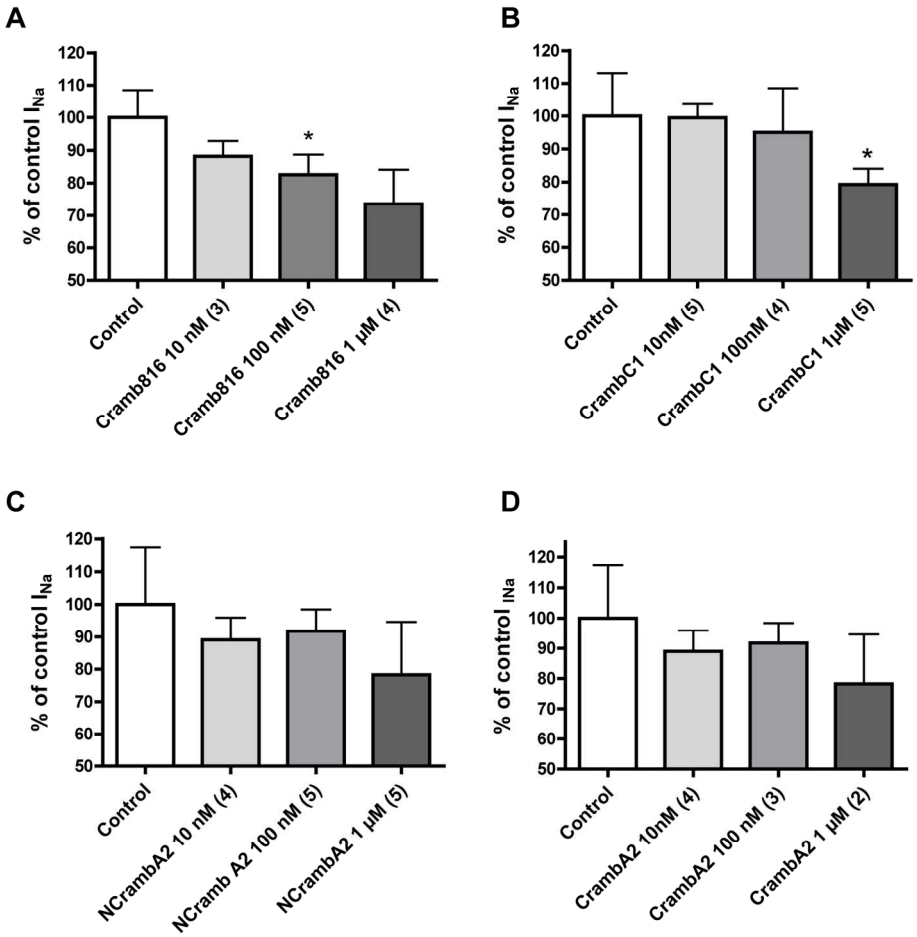

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