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CHARACTERIZATION OF THE Ca^{2+} CURRENT IN ISOLATED TERMINALS OF CRUSTACEAN PEPTIDERGIC NEURONS

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Summary

Ca^{2+} currents (I_{Ca}) were recorded from the neurosecretory terminals of the crab X-organ–sinus gland under voltage-clamp conditions. I_{Ca} was detectable at command potentials above –40 mV, with maximum currents at approximately +20 mV. No differences were observed between current–voltage (*I*/*V*) relationships from holding potentials of –50 or –90 mV, indicating that there were no low-voltage-activated Ca^{2+} channels present in the terminals. The decay of I_{Ca} was best fitted with a single exponential, the extent of inactivation over 50 ms averaging 53 %. The rate of decay of I_{Ca} was reduced by the substitution of Ca^{2+} with Sr^{2+} in the external solution and was eliminated by substitution with Ba^{2+}.

The effect of varying prepulse potential on the amplitude of I_{Ca} at +20 mV was tested. I_{Ca} declined with increasing prepulse depolarization up to +20 mV and then showed partial recovery at more depolarized prepulse potentials. Inactivation curves in solutions containing Sr^{2+} and Ba^{2+} showed much less current-dependent inactivation. Removing Ca^{2+} chelators from the internal solution significantly increased I_{Ca} decay. I_{Ca} was insensitive to nifedipine at a concentration of 1 μmol l^{-1}. Pretreatment of the isolated sinus gland containing the intact terminals with a combination of ω-conotoxin (ω-Ctx) GVIA, ω-Ctx MVIIC and ω-agatoxin IVA had no effect on the levels of K^+-induced peptide release.

Key words: neurosecretion, Ca^{2+} channels, crab, *Cardisoma carnifex*, conotoxins, agatoxin, Crustacea.

Introduction

Ca^{2+} influx through voltage-operated Ca^{2+} channels plays a crucial role in regulating neurosecretion, and yet the small size and inaccessibility of most terminals has impeded the characterization of channels located at the release sites. Notable exceptions include the squid giant synapse (Llinás *et al.* 1981*a*,*b*; Augustine *et al.* 1985*a*,*b*), the rat neurohypophysis (Lemos *et al.* 1994), the chick ciliary ganglion (Stanley and Goping, 1991) and the goldfish retinal bipolar cells (Heidelberger and Matthews, 1992). In particular, little is known about the voltage-dependence and kinetics of activation and inactivation of the Ca^{2+} channels present in nerve terminals.

One preparation amenable to this kind of analysis is the crab neurohemal organ, the X-organ–sinus gland, which contains peptide-secreting neurons with exceptionally large terminals. This system has been used extensively to study aspects of excitation–secretion coupling (for a review, see Stuenkel and Cooke, 1988). The Ca^{2+} current (I_{Ca}) in isolated X-organ somata and growth cones has been well characterized (Lemos *et al.* 1986; Meyers *et al.* 1992; Meyers, 1993; Richmond *et al.* 1995), but little is known of I_{Ca} at the peptide release sites in the terminals of these neurons, although regenerative, tetrodotoxin (TTX)-resistant depolarizations indicative of Ca^{2+} spikes have been recorded from terminals using intracellular electrodes (Cooke, 1985; Nagano and Cooke, 1987). Recently a terminal preparation was developed from the sinus gland, producing isolated nerve endings routinely measuring 5–10 μm in diameter which can be on-cell patch-clamped and whole-terminal voltage-clamped (Lemos *et al.* 1986; Stuenkel *et al.* 1990). Consistent with the calcium hypothesis of neurosecretion, one component of the inward current in these terminals has been shown to involve Ca^{2+} channels on the basis of Cd^{2+} sensitivity, resistance to TTX and evidence of run-down (Lemos *et al.* 1986). Given the relative scarcity of preparations in which terminals can be voltage-clamped and the interest in the biophysical and pharmacological properties of terminal Ca^{2+} channels, we have investigated I_{Ca} in sinus gland terminals.

Materials and methods

Dissection

The sinus gland of the crab *Cardisoma carnifex* Herbst was separated from the adjoining eyestalk tissue in normal crab

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saline (NCS), consisting of (in mmol l^{-1}): NaCl, 440; KCl, 11.3; $CaCl_2$, 13.3; $MgCl_2$, 26; Na_2SO_4, 23; Hepes, 10; pH adjusted to 7.4 with NaOH, and mechanically dissociated by trituration in defined medium (DM, as described in Cooke *et al.* 1989). The resulting isolated terminals were plated in Primaria culture dishes (Becton Dickinson) and left to adhere to the dish for a minimum of 1 h prior to experimentation.

The DM was exchanged with filtered extracellular solution immediately prior to experimentation. Any further changes in extracellular milieu were achieved by pressure-ejection of solutions onto individual terminals.

Electrophysiology

Voltage-clamp recordings were obtained in the whole-terminal patch-clamp configuration with the use of an EPC9 amplifier. Data acquisition and storage were performed by HEKA software (Instrutech) run on a Macintosh Centris 650. Analysis and graphics were achieved with Igor Pro software (Wavemetrics). Capacitance-compensated current signals were leak-subtracted with four scaled pulses obtained at hyperpolarizing command potentials (filtered with a four-pole Bessel filter, corner frequency 2.9 kHz). Pipettes used to obtain tight-seal whole-terminal recordings were pulled from Kimax thin-walled glass capillaries (1.5–1.8 mm o.d.) on a vertical puller (David Kopf Instruments, TW 150F-4). Pipettes were coated with dental wax to reduce capacitance and fire-polished with a microforge (Narishige, model MF-83). Typically pipettes filled with the intracellular solution and immersed in the bath had resistances ranging from 1.5 to 6 MΩ. All experiments were conducted at room temperature (24–26 °C).

I_{Ca} in terminals was isolated using an extracellular solution that contained (in mmol l^{-1}): *N*-methyl-D-glucamine methane sulphonate (NMG-$MeSO_3$), 240; $MgCl_2$, 24; $CaCl_2$, 52; Hepes, 10; CsCl, 100; tetraethylammonium bromide (TEABr), 20; 4-aminopyridine 3; TTX, 0.0005; pH 7.4. The intracellular solution, applied through the pipette contained (in mmol l^{-1}): NMG-$MeSO_3$, 200; CsCl, 100; NaCl, 10; MgATP, 5; *bis*-(*o*-aminophenoxy)-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (Cs^+ salt), 20; Hepes, 50; TEABr, 10; pH 7.4. The concentration of chelator was varied in some experiments as indicated in the Results. In Ba^{2+} substitution experiments, NMG-$MeSO_3$ and CsCl were replaced with NaCl in the extracellular solution to prevent precipitation of Ba^{2+} salts. The tonicity of all solutions was adjusted with sucrose to 1100 mosmol l^{-1}.

A nifedipine (Sigma) stock solution dissolved in 95 % ethanol, stored in the dark at 4 °C, was diluted to a final concentration of 1 μmol l^{-1} in external solution, protected from light during use and applied through pressure-ejection.

Secretion assay

Since successful whole-terminal recordings were acquired infrequently, it proved impractical to test limited supplies of conotoxins and agatoxin on isolated terminals. Therefore, we examined the potential effects of these toxins on terminal I_{Ca} indirectly, by monitoring the Ca^{2+}-dependent release of crustacean hyperglycemic hormone (CHH) from an isolated nerve tract–sinus gland preparation (as previously described; Keller *et al.* 1994).

Release of CHH was chosen as it and its co-localized gene products represent 90 % of the peptide content of the sinus gland and account for the bulk of peptide released in response to high-K^+ stimulation (Stuenkel and Cooke, 1988). CHH is present in the majority of the X-organ neurons and terminals of the sinus gland (Dircksen *et al.* 1988). A sensitive enzyme-linked immunosorbent assay (ELISA) has been developed to detect levels of CHH release from individual sinus glands. CHH release (Keller *et al.* 1994) and the secretion of other peptides (Stuenkel and Cooke, 1988) from isolated X-organ–sinus gland preparations have been shown to be abolished by removal of external Ca^{2+} or blocking with Mn^{2+} or Cd^{2+}, indicating that Ca^{2+} influx through terminal voltage-operated Ca^{2+} channels (VOCCs) is necessary for CHH secretion.

The nerve tract was retained and pinned to the base of a small perfusion chamber to hold the sinus gland in place during release experiments. Constant perfusion of the micro-chamber was achieved with a peristaltic pump. Reduced-Ca^{2+} (5 mmol l^{-1}) and reduced-Mg^{2+} salines (2.6 mmol l^{-1}) were used in these experiments to facilitate toxin binding. Release was stimulated by perfusing a high-K^+ saline (50 mmol l^{-1}) onto the preparation. Fractions of the perfusate were collected every 2 min and subsequently analyzed for CHH content using an ELISA (Keller *et al.* 1994).

50 μl of 5 mmol l^{-1} Ca^{2+}, 2.6 mmol l^{-1} Mg^{2+} saline containing ω-conotoxin (ω-Ctx) GVIA (10 μmol l^{-1}), ω-agatoxin (ω-Aga) IVA (500 nmol l^{-1}) and ω-Ctx MVIIC (5 μmol l^{-1}) was applied directly to the 25 μl micro-chamber after the perfusion had been halted and was left on for 2 min. The perfusion system was then restarted and the sinus gland was stimulated with K^+. The same protocol was used in control experiments with the omission of the toxins.

Results

The acutely isolated terminals from X-organ neurons were examined under conditions designed to isolate Ca^{2+} currents and minimize outward current contamination. Breaking into the terminals following seal formation often caused the terminal to rupture, severely limiting the number of successful whole-terminal voltage-clamp recordings. The quantity of releasable peptide contained in successfully voltage-clamped terminals was not determined.

Activation of terminal Ca^{2+} currents

From a holding potential (V_{hold}) of −50 mV, 10 mV incrementing depolarizing steps beyond −40 mV produced inward currents in the terminals which peaked at approximately +20 mV, which is typical of high-voltage-activated (HVA) I_{Ca} (Fig. 1A). An equation incorporating a Boltzmann and a linear term was used to fit the normalized current–voltage (*I*/*V*) data from six terminals and gave a value for the $V_{1/2}$ of activation of +3.7 mV, a valence (*z*) of 3.5*e* and

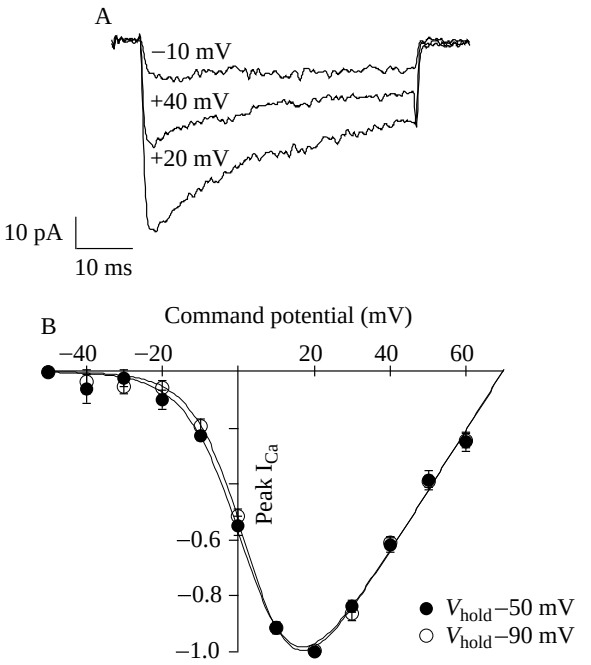

Fig. 1. Voltage-dependent characteristics of Ca^{2+} current (I_{Ca}) activation in isolated terminals. (A) Examples of terminal I_{Ca} traces to the potentials indicated from a holding potential (V_{hold}) of −50 mV. (B) Averaged, normalized peak I_{Ca} amplitude (N=6, mean ± S.E.M.) plotted against command potential from a V_{hold} of −50 mV (●) and after 200 ms prepulses to −90 mV (○). Test potentials (50 ms) ranged from −50 mV to +60 mV with 10 mV increments. Both *I*/*V* relationships were fitted with the equation $I=(V-V_{rev})[g_{max}/\{1+\exp-[z(V-V_{1/2})/25.7]\}]$, where V is the command potential, V_{rev} is the reversal potential, g_{max} is the maximum conductance, z is the valence and $V_{1/2}$ is the potential at which half the channels are activated.

a reversal potential (E_{Ca}) of +69.7 mV. A hyperpolarizing prepulse of 200 ms to −90 mV failed to reveal more current (Fig. 1B), and the characteristics of the terminal Ca^{2+} *I*/*V* relationship ($V_{1/2}$=+4.2 mV, z=3.8e, E_{Ca}=62.1 mV) showed no significant difference from those at a V_{hold} of −50 mV, implying that the terminals do not have a transient, low-voltage-activated I_{Ca}. The *I*/*V* relationships from acutely isolated terminals are indistinguishable from those previously obtained from X-organ somata (Richmond *et al.* 1995), which also lack a low-threshold-activated Ca^{2+} current.

The average peak amplitude for the terminal I_{Ca} in 52 mmol l^{-1} Ca^{2+} was 43.2±4.2 pA with an average membrane capacitance of 4.8±0.7 pF, giving a current density of 10.5±4.9 μA cm^{-2} (mean ± S.E.M., N=9, assuming a capacitance of 1 μF cm^{-2} for the cell membranes). Interestingly, the current density of acutely dissociated X-organ somata in 52 mmol l^{-1} Ca^{2+} has previously been shown to be 28.8±6.3 μA cm^{-2} (N=6), more than twice that of the terminals (Richmond and Penner, 1994).

Inactivation

The terminal I_{Ca} showed considerable time-dependent inactivation immediately following the peak. The decay was best fitted in most traces by a single exponential function. Fig. 2A plots the time constant (τ) of inactivation as a function of the command potential. The τ of inactivation was fastest at +20 mV (18.6±1.22 ms, N=6), the potential producing maximal Ca^{2+} influx, suggesting that inactivation is current-dependent. To test the Ca^{2+}-dependence of inactivation, a double-pulse protocol was used, in which the effect of varying a 200 ms prepulse potential from −80 mV to +60 mV on the test current amplitude at +20 mV was investigated. The average test-pulse I_{Ca} amplitude (normalized to the peak amplitude with a prepulse of −80 mV) was plotted against prepulse potential

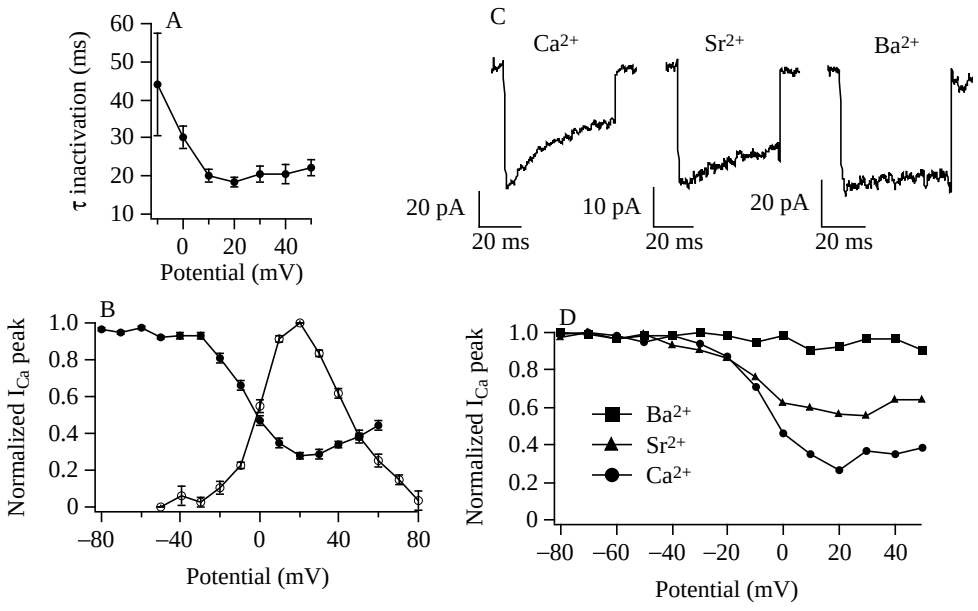

Fig. 2. Evidence for Ca^{2+}-dependent inactivation of terminal Ca^{2+} current (I_{Ca}). (A) A single-exponential was used to fit I_{Ca} decay (V_{hold} −50 mV, 50 ms command pulses) to generate the relationship between the time constant, τ, of inactivation and the command potential. Values are means ± S.E.M., N=6. (B) The average inactivation curve (solid line and ●) was obtained by applying 200 ms prepulses ranging from −80 mV to +60 mV, followed by a test pulse to +20 mV. An inverted *I*/*V* relationship from the prepulse peak amplitudes is superimposed on the graph (dotted lines and ○), N=6. (C) Comparison of the kinetics of inactivation of currents carried by 52 mmol l^{-1} Ba^{2+}, Sr^{2+} and Ca^{2+}. (D) Comparison of inactivation curves obtained, as in B, for Ba^{2+}, Sr^{2+} and Ca^{2+} currents.

(Fig. 2B). Maximal inactivation coincided with peak Ca^{2+} entry, with partial recovery at more positive potentials, indicative of Ca^{2+}-dependent inactivation. This was further investigated by substituting 52 mmol l^{-1} Ba^{2+} or Sr^{2+} for Ca^{2+} as the charge carrier. Ba^{2+} largely removed inactivation, and Sr^{2+} caused a marked reduction in the extent of inactivation compared with Ca^{2+} (Fig. 2C). A comparison of the double-pulse inactivation curves from three terminals illustrates the relative current-dependent inactivation produced by the different divalent cations (Fig. 2D).

We also examined the effects on inactivation of altering the level of Ca^{2+} chelator in the internal pipette solution. We compared the percentage of current remaining at the end of a 50 ms step depolarization to +20 mV in terminals loaded with 0 chelator, 5 mmol l^{-1} EGTA, 5 mmol l^{-1} BAPTA or 20 mmol l^{-1} BAPTA. There was no significant difference between the extent of inactivation in terminals loaded with 5 mmol l^{-1} EGTA, 5 mmol l^{-1} BAPTA or 20 mmol l^{-1} BAPTA; therefore, these results were grouped and compared with the inactivation in 0 chelator. As exemplified in Fig. 3A,B, inactivation was significantly higher in terminals lacking intracellular chelator compared with terminals with either BAPTA or EGTA present (P=0.0002, Student's t-test). A possible explanation for the similarity of action of BAPTA and EGTA is that, at such high concentrations of each (5 mmol l^{-1}), the faster binding kinetics and higher affinity of BAPTA over EGTA are no longer discernible. In support of this speculation, 20 mmol l^{-1} BAPTA was no more effective in reducing inactivation than 5 mmol l^{-1} BAPTA. In Fig. 3B, the average percentage of inactivation at the end of a 50 ms pulse in the presence or absence of chelator is summarized.

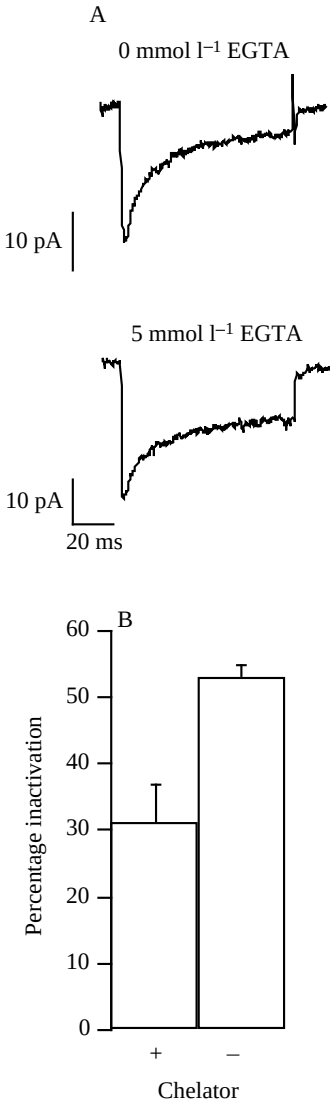

Fig. 3. (A) Representative traces of Ca^{2+} current (I_{Ca}) obtained with 0 or 5 mmol l^{-1} EGTA in the pipette solution. (B) Graph showing the extent of I_{Ca} inactivation expressed as a percentage (mean + S.E.M.) of the peak current amplitude remaining at the end of a 50 ms depolarizing pulse to +20 mV in the presence (+, N=14) or absence (–, N=4) of chelator (EGTA or BAPTA). The values are significantly different (P=0.0002).

Pharmacology

Whole-terminal voltage-clamp recordings

The effects of the L-type Ca^{2+} channel blocker nifedipine were tested on I_{Ca} elicited by a command potential to +20 mV. Nifedipine at a concentration of 1 μmol l^{-1} had no effect on the kinetics (Fig. 4A) or the peak amplitude of the terminal I_{Ca} (Fig. 4B). To ensure that the negative result with nifedipine

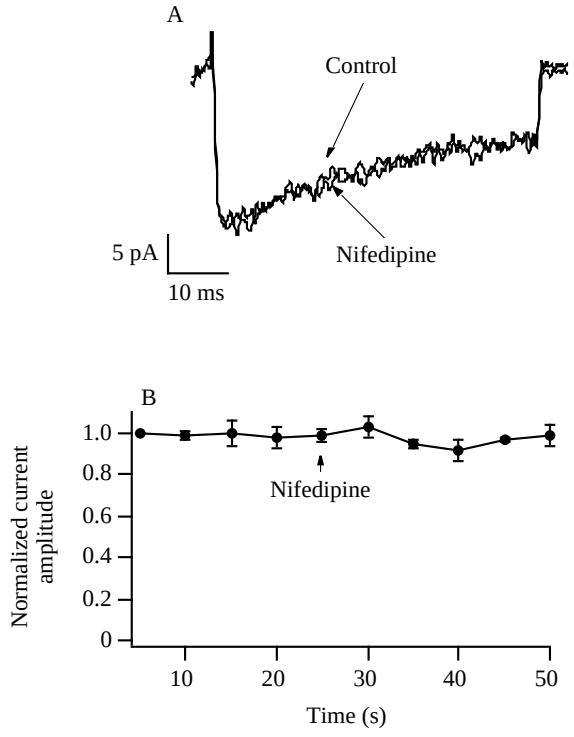

Fig. 4. Terminal Ca^{2+} current (I_{Ca}) is nifedipine-insensitive. (A) Representative I_{Ca} before and during pressure-ejection of 1 μmol l^{-1} nifedipine. (B) Averaged data showing the normalized peak current amplitude before and during the application of nifedipine (mean ± S.E.M., N=3).

was not due to the voltage-dependence of blocking effects, a standing holding potential of −10 mV was implemented prior to ramp commands from −100 to +100 mV. Under these circumstances, the current amplitude over the entire voltage range of the ramp command was unaffected by nifedipine.

These results indicate that the terminal Ca^{2+} channels are not of the dihydropyridine-sensitive L-type.

Secretion

Owing to the very low success rate in obtaining whole-cell recordings from terminals, we used an indirect approach to test for the presence of N- and P/Q-type Ca^{2+} channels. The isolated sinus gland was used to measure Ca^{2+}-dependent release of CHH from terminals. By monitoring the K^+-stimulated release of CHH in control and toxin-treated preparations, we were able to study, indirectly, whether any block of Ca^{2+} channels occurred in CHH-containing terminals. ω-Ctx GVIA (10 μmol l^{-1}) was used to block N-type channels in combination with ω-Aga IVA (500 nmol l^{-1}) and ω-Ctx MVIIC (5 μmol l^{-1}) to block P/Q-type channels. In both control and toxin-treated terminals, CHH release was stimulated twice with 50 mmol l^{-1} K^+ and the second release amplitude was normalized to the first. The toxin combination was added to the chamber containing the terminals for 2 min in a saline containing low concentrations of divalent cations prior to the second K^+ stimulation. Averages of three control and three toxin-treated preparations revealed no significant difference in the extent of CHH release (Fig. 5). These data suggest that the Ca^{2+} channels involved in CHH release from the terminals are insensitive to a combination of mammalian N- and P/Q-type channel blockers. In one experiment, it was also shown that both the L-type channel agonist BayK and the blocker nifedipine (1 μmol l^{-1}) had no effect on CHH secretion compared with controls.

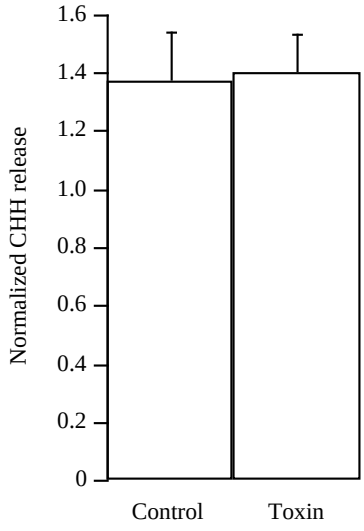

Fig. 5. Crustacean hyperglycemic hormone (CHH) secretion under control conditions and after pretreatment with 10 μmol l^{-1} ω-conotoxin GVIA, 500 nmol l^{-1} ω-agatoxin IVA and 5 μmol l^{-1} ω-conotoxin MVIIC. CHH release resulting from the second 50 mmol l^{-1} KCl stimulation was normalized to that in response to the first and averaged for three control and three toxin experiments (mean + S.E.M.). Note that in both control and toxin-treated preparations the secretion in response to the second KCl stimulation was greater than that in response to the first. The values are not significantly different.

Discussion

We have examined I_{Ca} in the isolated peptidergic terminals of the crab neurohemal organ, the X-organ–sinus gland. As demonstrated by the *I*/*V* relationship, these terminals have high-voltage-activated (HVA) Ca^{2+} channels, with no evidence of low-threshold I_{Ca}. Both the features of the *I*/*V* relationship and the dominant Ca^{2+}-dependent inactivation of the terminal Ca^{2+} channels closely resemble those of the X-organ somata from which the terminals originate (Meyers *et al.* 1992; Richmond *et al.* 1995).

The heterogeneity of HVA Ca^{2+} channels has been demonstrated in an increasingly large number of neurons. In many instances, the voltage-dependent properties of these channels are sufficiently similar that the whole-cell current of mixed populations of channel types appears as a single V-shaped *I*/*V* relationship, as seen in the sinus gland terminals. Pharmacological tools have been useful in some preparations to reveal the presence of heterogeneous channel populations. In some terminal preparations, mixed populations of HVA channels have been found; for example, the neurohypophyseal terminals contain L-type, N-type and P-type channels (Lemos *et al.* 1994). The I_{Ca} in chick ciliary ganglia is insensitive to dihydropyridines and is predominantly, although incompletely, blocked by ω-Ctx GVIA (Yawo and Momiyama, 1993). In retinal bipolar cells, however, the somata and terminals both appear to contain only L-type Ca^{2+} channels (Heidelberger and Matthews, 1992). In the present study, no effects of specific Ca^{2+} channel blockers and toxins were observed. Similar results were obtained previously in the X-organ somata (Richmond *et al.* 1995). The lack of any pharmacological distinction between terminal and somata I_{Ca} in the X-organ–sinus gland raises the possibility that these neurons have a homogeneous population of Ca^{2+} channels. However, this possibility remains tentative until specific blockers for these channels become available. Furthermore, while toxins have been tested directly on the somata of X-organ neurons, the paucity of successful terminal recordings necessitated an indirect assessment of terminal VOCC pharmacology through CHH secretion. If mixed populations of VOCCs exist in these terminals and each subtype is able to support CHH release fully, then inhibition of one channel type would not be expected to alter the level of CHH release. However, since we have shown that the terminals do not respond to the L-type antagonist nifedipine and that in the simultaneous presence of N- and P/Q-type blockers release is unaffected, at least one VOCC subtype supporting release of CHH must be pharmacologically distinct from these channel types.

Few studies have addressed the pharmacology of crustacean Ca^{2+} channels. Similarly negative results for the effects of

nifedipine were found on crab pyloric neurons (Golowasch and Marder, 1992) and the lobster neuromuscular junction (Grossman *et al.* 1991), although the crayfish giant axon reportedly has a nifedipine-sensitive I_{Ca} (Nishio *et al.* 1993). Indirect evidence, based on a reduction in synaptic transmission, indicates that Ca^{2+} channels sensitive to ω-Ctx GVIA and ω-Aga IVA are present at presynaptic loci of the lobster abdominal neuromuscular junction (Grossman *et al.* 1991) and the crayfish leg opener muscle (Araque *et al.* 1994), respectively. It appears, therefore, that crustacean neuromuscular terminals have Ca^{2+} channels pharmacologically similar to the N-type channels found at several vertebrate synapses (Dayanithi *et al.* 1988; Hirning *et al.* 1988) and the P-type channels found in other invertebrate and mammalian synapses (Llinás *et al.* 1989, 1992; Uchitel *et al.* 1992). Since no effect of a combined N- and P/Q-type block was observed on CHH release, the pharmacological profiles of the X-organ, CHH-secreting terminals may differ from those found at crustacean neuromuscular synapses.

It has been shown previously using intracellular electrodes that Ca^{2+}-dependent depolarization is restricted to the sinus gland terminals and somata and is not detectable in the adjoining axons of X-organ neurons (Nagano and Cooke, 1987), as has been found in other systems (Stockbridge and Ross, 1984). Interestingly, the isolated X-organ somata have more than double the channel density of the terminals (see Richmond *et al.* 1995) in contrast to the goldfish retinal bipolar cells where the reverse pattern is observed (Heidelberger and Mathews, 1992). Whereas blockade of the terminal Ca^{2+} channels inhibits CHH release (Keller *et al.* 1994), supporting their role in secretion, the functional significance of a high Ca^{2+} channel density in the somata is less well understood (Richmond *et al.* 1995). It is also unclear whether the Ca^{2+} channels of the terminals are equally distributed on the terminal membrane or are clustered at release sites, giving a locally higher current density within microdomains, as has been found at the squid giant synapse (Llinás *et al.* 1992).

The sinus gland terminals exhibit several characteristics which indicate that the predominant mode of Ca^{2+} channel inactivation is Ca^{2+}- rather than voltage-dependent. The U-shaped relationship between prepulse potential and test-pulse current amplitude is a characteristic of Ca^{2+}-dependent inactivation. The Ca^{2+} influx during the prepulse produces inactivation and hence a reduction in test-pulse amplitude which is greatest at prepulse potentials producing the largest I_{Ca} (approximately +20 mV in these terminals). Similarly, the rate of inactivation exhibits a voltage-dependence, peaking at +20 mV. Substituting Sr^{2+} or Ba^{2+} for Ca^{2+} as the charge carrier progressively attenuated the extent of inactivation in the terminals, further indicating that inactivation is primarily regulated by a Ca^{2+}-dependent mechanism in which Sr^{2+} but not Ba^{2+} can partially imitate Ca^{2+}. Finally, a small but significant (P=0.0002) increase in the extent of inactivation was observed when the terminals were internally dialyzed without the Ca^{2+} chelators BAPTA or EGTA.

The presence of Ca^{2+}-dependent inactivation is expected to have important functional consequences in terms of regulating the secretory output of the terminals, in that it would act as a negative feedback loop closing Ca^{2+} channels that have been extensively stimulated. The CHH-containing neurons exhibit sustained release, which is somewhat at odds with the idea that their terminals may exhibit Ca^{2+}-dependent inactivation (Keller *et al.* 1994). One explanation for this discrepancy may be that although Ca^{2+}-dependent inactivation was present in all terminals studied under voltage-clamp none of these terminals was of the CHH phenotype. Interestingly, it is known that the release of the much less prevalent (3 % total peptide content) red pigment concentrating hormone rapidly inactivates in the presence of a maintained secretory stimulus (Stuenkel and Cooke, 1988).

Alternatively, we have previously shown that, in identified CHH-containing X-organ somata, hyperpolarization partially reverses Ca^{2+}-dependent inactivation (Richmond *et al.* 1995). Therefore, hyperpolarization during the repolarizing phase of the action potentials known to occur in the terminals during secretion could alleviate inactivation. In addition, the physiological Ca^{2+} concentration of the crab hemolymph of 13 mmol l^{-1} would be expected to produce less Ca^{2+}-dependent inactivation than that observed in the present experiments in which [Ca^{2+}] was elevated fourfold to enhance current amplitude. This, combined with the unknown capacity of the intact terminal to sequester Ca^{2+}, may allow sufficiently prolonged I_{Ca} activation to account for the sustained pattern of CHH release observed.

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