Calpain inhibitors alter the excitable membrane properties of cultured Aplysia neurons

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Running title: calpain inhibitors and membrane excitability
Abstract
The calpain superfamily of calcium-dependent papain-like cysteine proteases are highly conserved proteases that function to post-translationally modify substrates by partial proteolysis. Calpains are known to proteolyse over 100 substrates that lack strong sequence homology. Consequently, the calpain superfamily has been implicated in playing a central role in diverse physiological and pathological processes. Investigation of the physiological functions of calpains, on the one hand, and the need to develop pharmacological reagents to inhibit calpain-mediated pathological processes, on the other hand, led to the development of numerous calpain inhibitors.

Using cultured Aplysia neurons and voltage clamp analysis, we report here that the calpain inhibitors calpeptin, MG132, and the calpain inhibitor XII inhibit voltage-gated potassium conductance and moderately reduce the sodium conductance. These consequently lead to spike broadening, and increased calcium influx. Such alterations of the excitable membrane properties may alter the normal patterns of neuronal and muscle electrical activities, and thus should be taken into account when evaluating the effects of calpain inhibitors as protective/therapeutic drugs, and as research tools.
Introduction

The calpain superfamily of calcium-dependent papain-like cysteine proteases (Guroff 1964) are highly conserved, and function to post-translationally modify substrates by partial proteolysis rather than by complete proteolytic digestion (Carragher and Frame 2002; Evans and Turner 2007). Calpains are known to proteolyse over 100 substrates that lack strong sequence homology in various tissues. Consequently, the calpain superfamily has been implicated in playing a central role in diverse physiological and pathological processes (Bevers and Neumar 2008; Evans and Turner 2007; Goll et al. 2003; Wu and Lynch 2006). For example, calpains play physiological roles in cell motility (Perrin and Huttenlocher 2002), apoptosis (Squier et al. 1994), cell differentiation (Simonson et al. 1985), and synaptic transmission (for a review, see Wu and Lynch 2006). On the other hand, calpains are also thought to play roles in the pathogenesis of Huntington's, Parkinson's and Alzheimer's diseases, cataract formation, diabetes, ischemic and traumatic brain injury, the pathogenesis of stroke, myocardial infarction, and muscular dystrophy (Bevers and Neumar 2008; Higuchi et al. 2005; Lescop et al. 2005; Ray et al. 2003; Saatman et al. 1996). Understanding the physiological roles of calpains, on the one hand, and the potential use of calpain inhibitors in preventing pathological processes, on the other, led to the development of a large number of calpain inhibitors (Bevers and Neumar 2008; Ray 2006; Ray et al. 2003; Saez et al. 2006).

Interpretation of the experimental results obtained by using these inhibitors and the translation of these results to clinical applications are complicated by the fact that calpains cleave multiple substrates, and that many calpain inhibitors also inhibit other cysteine proteases, serine proteases, and the proteasome (Barrett et al. 1982; Wang and Yuen 1994).
In a series of earlier studies, we found that a transient elevation of \([\text{Ca}^{2+}]_i\), in cultured Aplysia neurons, after axotomy, activates cytoplasmic proteases. Since the proteolytic activity was induced by a transient elevation of \([\text{Ca}^{2+}]_i\), blocked by the membrane-permeable calpain inhibitor, calpeptin (Tsujinaka et al. 1988), and cleaved the classical calpain substrate, spectrin, we functionally classified this proteolytic activity as generated by calpain (Gitler and Spira 1998, 2002; Khoutorsky and Spira 2005; Sahly et al. 2006). While studying the role of calpain in synaptic facilitation of cultured Aplysia sensory motor synapses, we noticed that inhibition of calpain initially increases evoked neurotransmitter release (Khoutorsky and Spira 2005). The initial increase in neurotransmitter release was associated with broadening of the presynaptic action potentials. Therefore, in the present study we undertook to examine the effects of calpain inhibitors on the excitable membrane properties.

We found that bath application of the calpain inhibitors calpeptin, calpain inhibitor XII, and MG132 leads to spike broadening, and increased calcium influx due to a significant reduction of the inactivating and non-inactivating voltage-gated potassium conductances. Consistent with recent reports (Suzuki et al. 2002; Wu et al. 2005; Yuen et al. 2007), our observations suggest that constitutive calpain activity regulates the properties of integral membrane proteins. The above observations should be taken into account when considering the physiological roles of calpains as "house-keeping" proteases, and when evaluating the use of calpain inhibitors as neuroprotective drugs.

**Materials and Methods**

**Cell cultures**

Left upper quadrant (LUQ) neurons from the abdominal ganglia of *Aplysia californica* were isolated and maintained in culture, as described by (Schacher and Proshansky 1983). Briefly, animals were anesthetized by injection of isotonic MgCl₂ solution. The
ganglia were isolated and incubated for 1.5-3 hours in 1% protease (type IX, Sigma) at 34°C. Next, the ganglia were desheathed, and the cell bodies of their neurons with their axons were pulled out with sharp micropipettes and placed on poly-L-lysine-coated (Sigma) glass bottomed culture dishes. The culture medium consisted of 10% filtered hemolymph from *Aplysia faciata* collected along the Mediterranean coast, and L-15 (Gibco-BRL) supplemented for marine species. Twenty-four hours after plating, the dishes were transferred to an 18°C incubator. The experiments were performed 1-2 days after plating, at room temperature (between 20 and 24 °C) after replacing the culture medium with artificial sea water (ASW).

Electrophysiology

For current-clamp experiments, LUQ neurons were impaled by a sharp 5-10 MΩ glass microelectrode filled with 2M KCl. The microelectrode served for both current injection and voltage recording (Axoclamp-2A; Axon Instruments). The membrane potential was kept at -50 mV by passing direct current. Single spikes were generated by 5-10 msec-long depolarizing current pulses. At the end of the experiments, the stimulation intensity was gradually reduced until no spike was elicited, and the subthreshold stimulus artifact was subtracted from the signal.

Voltage-gated ionic currents were analyzed using conventional two-electrode voltage-clamp. For these experiments, the main axon of the neuron was trimmed off approximately 20 min prior to the experiment with the sharp tip of a micropipette under visual control, as previously described (Benbassat and Spira 1993; Hasson et al. 1993; Hasson et al. 1995). In most cases this procedure enabled better space clamping. The cell body of a cultured neuron was impaled by two microelectrodes: one for current
injection, and the other for voltage recordings. Current records were corrected by subtracting the linear components of leak and capacitative currents.

Solutions
Control experiments were carried out in artificial sea water (ASW) composed of NaCl 460 mM, KCl 10mM, CaCl$_2$ 11 mM, MgCl$_2$ 55 mM, and HEPES [N-(2-hydroxyethyl)piperazine-N’-2ethanesulfonic acid, Sigma] 10 mM, adjusted to pH 7.6.

To isolate the sodium current, we used a solution composed of NaCl 410 mM, CsCl 10mM, MgCl$_2$ 65 mM, HEPES 10 mM, TEA (tetrathlammonium chloride) 50 mM, 3,4-DAP ([3,4-diaminopyridine) 0.1 mM, Co 1 mM. The pH was adjusted to 7.6.

To isolate potassium currents, we blocked the sodium and calcium currents by a solution composed of NaCl 460 mM, KCl 10mM, MgCl$_2$ 65 mM, HEPES 10 mM, Co 1 mM, and 200 μM tetrodotoxin (TTX, Alomone labs, Jerusalem, Israel). The pH was adjusted to 7.6.

To inhibit potassium current, we used the potassium channel blocking solution (PCBS) composed of NaCl 410 mM, CsCl 10 mM, 11 mM CaCl$_2$, 55 mM MgCl$_2$, TEA (tetrathlammonium chloride) 50 mM, 3,4-DAP ([3,4-diaminopyridine) 0.1 mM, and HEPES 10 mM, adjusted to pH 7.6.

Calcium current was isolated using a solution composed of TEA 460 mM, MgCl$_2$ 1 mM, CsCl 10 mM, CaCl$_2$ 65 mM, and HEPES 10 mM. The pH was adjusted to 7.6.

Drugs
Calpeptin, MG132, and calpain inhibitor XII (Calbiochem) were prepared as 50 mM stock solutions in DMSO (Sigma) and diluted to the final concentration just before the experiments. *Clasto*-lactacystin β-lactone (Calbiochem) was prepared as 10 mM stock
solutions in DMSO. In control experiments, bath application of a vehicle solution composed of 0.2% DMSO in artificial sea water (ASW) had no effect on the action potential shape.

Calcium imaging

The system used for confocal imaging consisted of an Olympus microscope IX70 and a Bio-Rad (Hercules, CA) Radiance 2000/AGR-3 confocal imaging system. The objective used was an Olympus planApo 60x 1.4 NA oil objective. The images were collected and processed by using LaserSharp and LaserPix BioRad software, respectively. For calcium imaging, fluo-4 10mM (pentapotassium salt, Invitrogen) in 2M KCl was loaded into the neurons by pressure injection. Imaging was performed after the dye had equilibrated throughout the main axon (approximately 30 min). The dye was excited by a 488-nm laser line, and the emitted lights were collected at 500–560 nm.

Imaging of proteolytic activity

Neurons were continuously incubated in ASW containing 10 μM bis(CBZ-Alanyl-Alanine amine)-Rhodamine 110 (bCAA-R110, Molecular Probes) (Leytus et al. 1983) and were confocally imaged to determine the relative levels of proteolytic activity as previously described (Gitler and Spira 1998). The effect of MG132 and calpain inhibitor XII on the axotomy-induced increase in the bCAA-R110 fluorescent signal was tested (Gitler and Spira 1998). Whereas MG132 (100 μM, n=7) and calpain inhibitor XII (100 μM, n=8) prevented the elevation of the bCAA-R110 signal, Clasto-lactacystin β-lactone (10 μM, n=5) had no significant effect. In control experiments,
bath application of a vehicle solution composed of 0.2% DMSO in artificial sea water (ASW) had no effect on calcium-activated cleavage of the proteolytic substrate.

Statistics

All the data are presented as mean ± SEM. For all statistical analyses of significance, the paired Student's $t$-test was used.

**Results**

**Current clamp analysis**

Bath application of the calpain inhibitor - calpeptin (100 μM) led, within 5 minutes, to an increase in spike duration, and a slight attenuation of its amplitude. In most of the experiments (71%, n=30/42 experiments), applying calpeptin resulted in the appearance of a second low-amplitude delayed spike (Fig. 1A-C). In the remaining experiments (29%, n=12/42), calpeptin resulted in decreased after-hyperpolarization as well as spike broadening without the appearance of the delayed spike (E). When a train of action potentials was generated by a 1-sec-long intracellular depolarizing pulse, the time interval between the first spike and the delayed one was reduced, until the second action potential fused with the falling phase of the first action potential (Fig 1 C). Following a wash, the shape of the action potential recovered within 5-10 min (Fig. 1 D and E wash).

The above alterations could be generated by a number of mechanisms: (a) Reduction in voltage-gated potassium conductances could lead to decreased after-hyperpolarization, increased spike duration, and the appearance of a second spike by delayed development of sodium or calcium conductances that are normally suppressed by the prolonged potassium conductances. (b) The broadening of the action potential could also be
related to a decreased rate of sodium inactivation. (c) The reduction in the action potential amplitude could be related to reduced sodium conductance. And finally, (d) the appearance of a second spike could be generated by "back reflection" of an action potential initiated at remote sites.

We began to investigate the above possibilities by testing the hypothesis that the second action potential, generated by a single stimulation after applying calpeptin, is a "back reflection" of a spike initiated at an electrically remote location. To that end, we decreased the electrical length of the neuron by cutting away a large part of the axon, leaving the cell body and a short segment of the main axon intact. After a rest period of approximately 30 min, the neuron recovered from the transection (Benbassat and Spira 1993; Spira et al. 1993) and became isopotential (Hasson et al. 1995). Since under these conditions a second spike was still generated in response to a single stimulus in the presence of calpeptin, we concluded that it represents alterations in voltage-gated currents (n=7).

To differentiate between the possibilities that the second low-amplitude spike (that in some experiments fuses with the first spike and appears as a prolonged "shoulder" on the falling phase of the first spike) is generated by voltage-gated calcium channels or by latent sodium conductance, we applied 200 μM TTX to neurons that fired broadened action potentials in the presence of calpeptin (Fig. 2, n=5). This led to blockade of the first peak (which is generated by inward sodium current) but not the prolonged shoulder, suggesting that the "shoulder" is generated either by voltage-gated calcium channels or by TTX-insensitive sodium channels (Yoshida 1994). Since the shoulder was blocked by a solution in which CaCl₂ was replaced by MgCl₂, and 1 mM Cobalt (Fig. 2D), we concluded that the "shoulder" as well as the second action potential are generated by calcium currents. Consistent with this conclusion, we found that spikes
generated in the presence of calpeptin result in a larger increase in the free intracellular calcium levels ([Ca^{2+}]_i) than in controls. For these experiments, neurons were loaded with fluo-4 and the changes in the [Ca^{2+}]_i were measured by confocal microscopy in the line scan mode, as previously described by our laboratory (Malkinson and Spira 2006). We found that the [Ca^{2+}]_i is elevated to a higher value in the presence of calpeptin than in the control (Fig. 3, n=4). The increase in the [Ca^{2+}]_i was observed only after the broadening of the spike. This temporal correlation suggests that the elevation in [Ca^{2+}]_i represents increased calcium influx due to spike broadening. However, we cannot rule out the possibility that a fraction of the elevated [Ca^{2+}]_i represents calcium release from intracellular stores.

To gain insights into the question whether the above-described observations are related to inhibition of calpain or to the direct action of calpeptin on voltage-gated ion channels, we examined the effects of additional calpain inhibitors on membrane excitability and compared them to those of calpeptin. These included MG132, a calpain and proteasome inhibitor (Figueiredo-Pereira et al. 1994; Lee and Goldberg 1998), calpain inhibitor XII (Li et al. 1996), and the specific proteasome inhibitor clasto-lactacystin β-lactone (Dick et al. 1996; Fenteany et al. 1995). We found that MG132 (100 μM, n=6 and 200 μM n=5) and calpain inhibitor XII (100 μM, n=5 and 200 μM n=4) decreased the hyperpolarizing after-potential, reduced the peak amplitude of the action potentials, generated a delayed spike in a fraction of the experiments (200μM MG132, 3 out of 5, and calpain inhibitor XII 2 out of 4, not shown), and broadened the action potentials (Fig. 4B and C, left-hand side) in a similar manner to calpeptin. As in the case of calpeptin, the effects of MG132 and calpain inhibitor XII reversed after wash. In contrast, a 30-min incubation of the neurons in the proteasome
inhibitor *clasto*-lactacystin β-lactone (10 μM, n=6) had no significant effect on the action potential waveform (not shown).

**Voltage clamp analysis**

To gain better insights into the mechanisms by which the calpain inhibitors alter the action potential shape, we analyzed the underlying voltage-gated ionic currents using the standard two-electrode voltage-clamp method (Hasson et al. 1995; McIntosh et al. 1995). In all experiments, the neurons were depolarized from a holding potential of -50 mV to various potentials.

Recording the overall macroscopic currents revealed that 100 μM calpeptin decreases both the inward and outward currents within 5 min of application (Fig. 4A right-hand side, n=7). Removing the calpeptin by washing resulted in partial recovery of the currents.

The overall effects of MG132 (n=6) and calpain inhibitor XII (n=5) on the inward and outward currents were similar to those of calpeptin (compare Figs. 4A right-hand side and Figs. 4B and C right-hand side). *Clasto*-lactacystin β-lactone (10 μM) had no effect on inward and outward voltage-gated currents (not shown, n=4).

To examine the effect of calpeptin on IK⁺, INa⁺, and ICa²⁺, we systematically blocked two ion currents while measuring the third one.

To study the effects of calpeptin on potassium currents (Fig. 5), we blocked INa⁺ and ICa²⁺ (see Methods). Under these conditions, application of calpeptin resulted in a significant decrease in IK⁺ (5A, compare control and calpeptin n=5). Whereas the mean early inactivating IK⁺, which lasts approximately 20 msec, from the onset of the voltage clamp step, decreased by 79%, when the membrane potential was stepped to +25 mV
(p<0.05 open arrow in Fig. 5A and B), the delayed non-inactivating IK⁺ decreased by 87\% (p<0.01 dark arrow in Fig. 5A and C).

To study the blocking kinetics of the early – inactivating, and late - noninactivating IK⁺ by the calpain inhibitors, we repeatedly applied voltage-clamp steps (from a holding potential of - 50 to +20 mV at a rate of 0.05 Hz) to the neuron. After 15 control voltage-clamp steps, we applied the inhibitor and examined its effects on the early and late IK⁺. The normalized peak amplitudes of the early and late IK⁺ are shown in Fig. 6 as a function of time. We found that the noninactivating IK⁺ is reduced with faster kinetics than the early inactivating IK⁺ by the three calpain inhibitors. This observation is sufficient to account for the appearance of the delayed spike, which with time, when the early IK⁺ is reduced, fuses with the first spike (Fig.1).

The reduction in IK⁺ is dose dependent. Complete blockage of IK⁺ by calpeptin was observed with 400 μM calpeptin (n=3).

We next examined the effects of calpeptin on INa⁺ (Fig. 7). To that end, ICa²⁺ and IK⁺ were blocked by replacing all potassium ions by cesium and all calcium ions by magnesium (see Methods). In addition, the solution contained 50 mM TEA, 0.1 mM 3,4 diaminopyridine, and 1 mM cobalt. Under these conditions, in addition to the classical early inward INa⁺, we also measured a residual non-inactivating outward current (Fig. 7Ab).

Following calpeptin application, a single stimulation generated a broadened sodium spike with reduced amplitude in the current clamp mode (Fig. 7Ac). In voltage-clamp mode, application of calpeptin resulted in a 36\% reduction in peak INa⁺ (p<0.05 n=4, Fig. 7Ad and B), and a partial decrease in the residual outward current. The partial blockage of the sodium current was not associated with alterations in its steady-state inactivation curves (Fig. 7C, n=4).
To examine whether calpeptin alters the inward calcium current (Fig. 8), we blocked INa⁺ and IK⁺ (see Methods). Under these conditions, in the current clamp mode, a short stimulus generates a long-lasting calcium action potential. Application of calpeptin shortened the calcium spike (compare 8A control and calpeptin left, n=4). Voltage clamp experiments revealed that calpeptin slightly reduces the peak ICa²⁺ (11% p<0.05 n=4, Fig. 8A right and B).

To investigate the rate by which calpeptin blocks the inward and outward currents, we repeatedly measured the overall macroscopic currents using constant voltage steps ranging from -50 to +20 mV at 0.1 Hz (Fig. 9, n=5). Bath application of calpeptin reduced the early inactivating outward current to a steady-state level (SS) within about 400 sec and the noninactivating potassium current within 90 sec. The inward currents were reduced to a SS level within about 150 sec (Fig. 9C). Recovery of the outward and inward currents occurred within 150 and 300 sec of washing, respectively.

**Discussion**

The main findings of the present study are that the calpain inhibitor calpeptin, the calpain inhibitor XII, and MG132, but not the proteasome inhibitor clasto-lactacystin β-lactone, alter within minutes of application the excitable membrane properties of cultured Aplysia neurons.

Voltage clamp analysis revealed that applying calpeptin results in (a) a significant reduction in the early inactivating and the non-inactivating potassium currents, (b) blockade of a residual non-inactivating outward current that is not blocked by the potassium and calcium currents blocking solution used in this study, (c) a moderate (36%) reduction in the sodium current, and (c) a small 11% decrease in the calcium current. The reduction in these currents is not associated with significant alterations in
the sodium current-voltage relationships, and its steady-state inactivation properties. The kinetics of the blockade of potassium currents by the calpain inhibitors is sufficient to account for the appearance of the delayed spike that fuses with the first spike (Fig.1). Under control conditions, the voltage-dependent potassium conductances are sufficient to prevent the development of a second spike, or spike broadening. Following the application of the calpain inhibitors, the noninactivating potassium conductance is reduced, allowing for the development of a second calcium-dependent spike. Initially, the early inactivating potassium conductance (that lasts for about 20 msec) repolarizes the membrane potential, terminates the first spike, and prevents the calcium conductance from contributing to the falling phase of the spike. Nevertheless, the calcium conductance is sufficient to generate a second spike when the early inactivating potassium conductance is terminated. With time, when the early inactivating potassium conductance is also reduced by the calpain inhibitors, the second spike fuses with the falling phase of the first spike, generating the "shoulder". The fact that in a fraction of the experiments a second spike was not observed is attributed to variability in the rates and levels to which the early inactivating potassium conductances were reduced.

**The possible mechanisms by which calpain inhibitors alter the ionic conductances**

Theoretically, the reduction in the voltage-gated potassium and sodium conductances by the calpain inhibitors could be attributed to two principal mechanisms: (a) alterations in the voltage-gated potassium and sodium currents by inhibition of calpain-mediated proteolysis, and (b) via binding and blockage of the ion channels by the inhibitors.

When considering the first hypothesis, it should be noted that inhibition of $I_{K^+}$ and $I_{Na^+}$ by the calpain inhibitors takes place following incubation of resting, non-stimulated neurons, in which the $[Ca^{2+}]_i$, at the bulk of the axoplasm, is about 100 nM.
(Ziv and Spira 1993). Despite the very low $[\text{Ca}^{2+}]_i$, constitutive cleavage of a fluorogenic proteolytic substrate, bCAA-R110, was imaged (Gitler and Spira 1998). The constitutive calpain activity may represent compartmentalized submembrane domains in which the $[\text{Ca}^{2+}]_i$ is high in the vicinity of calcium channels (Berridge 2006). Thus, theoretically, the alterations in the excitable membrane properties could be attributed to inhibition of constitutive calpain activity.

The onsets of $\text{IK}^+$ and $\text{INa}^+$ inhibition by calpeptin were detected by voltage clamp measurements within less than a minute of calpeptin application (Fig. 9). The rapid onset of the inhibition may indicate that the calpain inhibitors directly block the channels. Nevertheless, on-line imaging of the proteolysis of an exogenous proteolytic calpain substrate - bCAA-R110 in axotomyzed cultured Aplysia neurons revealed that the onset of calpain inhibition by bath application of calpeptin is detected within less than a minute of application (Gitler and Spira 2002). Thus, based on the onset time for inhibition, we cannot favor one hypothesis over the other. The rapid recovery of the channels functions after calpeptin washout also corresponds to the rapid recovery of proteolytic activity after calpeptin removal, as corroborated by images of bCAA-R110 in transected axons (Gitler and Spira 2002). Thus, this parameter also cannot be used to elucidate the above possibilities.

Another parameter that we considered is the correlation between the effects of the inhibitors on the ionic conductances and their effects on the proteolysis of the fluorogenic proteolytic substrate bCAA-R110. Whereas calpeptin, calpain inhibitor XII, and MG132 inhibit $\text{IK}^+$ and $\text{INa}^+$ and inhibit the proteolysis of bCAA-R110 (see Methods and Gitler and Spira 1998), the proteasome inhibitor clasto-lactacystin $\beta$-lactone did not induce spike broadening and did not block the cleavage of bCAA-R110.
These observations support the hypothesis that inhibition of calpain modulates the channels' conductances.

**Generalization of the present findings**

Inhibition of proteolytic activity, as indicated by the use of the fluorogenic substrate bCAA-R110 in cultured Aplysia neurons, is almost total at a concentration of 100 μM calpeptin (Gitler and Spira 1998; 2002). This concentration also blocks the supply of vesicles to the readily releasable store in sensory-motor synapses of cultured Aplysia neurons (Khoutorsky and Spira 2005). Here we found that 100μM calpeptin blocks approximately 80% of the potassium conductance (Fig. 5) and 36% of the sodium conductance (Fig. 7).

A comparable analysis of the effects of calpain inhibitors on excitable membrane properties in vertebrate neurons is not available. It is therefore not known whether similar alterations in the properties of ion channels are induced by calpain inhibitors and if so, at what concentrations. Since the potential use of calpain inhibitors as protecting drugs is extensively considered, it would be beneficial to examine its effects on vertebrate neurons and cardiac muscles.
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**Figure Legends**

**Fig. 1. Bath application of calpeptin alters the firing pattern of the cultured neurons.**

A single spike (left-hand side) or a train of action potentials (right-hand side) elicited by short -5 msec, or long -1sec, intracellular depolarizing pulses. The single spikes and the train of action potentials were generated by the same neuron using the same stimulation intensity. (A) Control in ASW. (B) Five minutes in DMSO 0.2% ASW. (C) Five minutes in 100 μM calpeptin. Note that the same stimulation used in (B) generated in the presence of calpeptin a broadened action potential followed by a delayed spike of lower amplitude. The delayed spike merges with the first spike in the course of the stimulation (right hand side). (D) After a 10-min wash, the spike shape of the neuron shown in (C) recovered. (E) In a fraction of the experiments, applying calpeptin resulted in spike broadening without intermediate stages in which a second delayed spike was recorded (E).

**Fig. 2. The broadening of the spike by calpeptin is calcium dependent.**

A single spike was elicited in ASW by a 10-msec intracellular depolarizing pulse (A). (B) Five minutes after applying calpeptin, the same stimulus elicited a broadened spike. (C) Bath application of 200 μM TTX blocked the first peak of the spike but not the long-lasting shoulder. (D) Replacing the normal ASW by a calcium channel blocking solution completely blocked the spikes. (E) Washing with ASW for 10 minutes restored the normal appearance of the action potential (n=5).
Fig. 3. A single action potential generates larger intra-axonal calcium concentration transients in the presence of calpeptin than in normal ASW.

A neuron was loaded with the calcium indicator fluo-4. After the dye equilibrated throughout the axon, a spike was elicited by an intracellular microelectrode to fire a single action potential (A, left-hand side), while the fluo-4 fluorescence was monitored by line scanning (B). Then 100 μM calpeptin was applied and 5 min later, a spike was elicited again (A, right-hand side), while the axon was line-scanned (B, right-hand side). The line scans were analyzed off line. The changes in the free calcium concentration are represented as ΔF/F₀ (C). Note that the transient calcium concentration is elevated to a higher level in the presence of calpeptin (p<0.05 n=4).

Fig. 4. Calpeptin reduces outward and inward currents.

Using the standard current clamp and two-electrode voltage-clamp techniques, we examined the effect of calpeptin (A), MG132 (B), and calpain inhibitor XII (C) on spike shape (left), and inward and outward currents (right). In current-clamp mode (left), the spike was generated by a 5-msec depolarizing pulse. In the voltage-clamp mode, the neuron was depolarized from a holding potential of -50 mV to +70 mV in 10 mV steps for 100 msec. Current traces, generated by seven voltage steps from a holding potential of – 50 mv to 10 up to +60 mV are depicted. After the currents in ASW (Aa) were recorded, 100 μM calpeptin was added to the experimental dish and the currents were measured 5 minutes later (n=7). Note the significant decrease of outward current and the slight decrease in inward current (Ab). After a wash, the currents recovered (Ac). The same protocol was used for MG132 100 μM (B, n=6) and calpain inhibitor XII 100 μM (C, n=5). Note that the effects of MG132 and calpain inhibitor XII on
inward and outward currents are very similar to those of calpeptin. The effect of both MG132 and calpain inhibitor XII were reversible upon washing.

**Fig. 5. Potassium currents are significantly decreased by calpeptin.**

The potassium currents were measured in a solution that blocks the sodium and calcium currents (see Methods). Potassium currents were evoked by 100-msec-long depolarizing steps from a holding potential of -50 mV to +50 mV in increments of 5 mV, under control conditions and 5 minutes after applying calpeptin (n=5). Shown are the potassium currents generated by voltage steps from the holding potential to -10, 0, 10, 20, 30, 40, and 50mV (A). The potassium currents were substantially decreased by calpeptin. The early inactivating potassium current (open arrow) and the delayed non-inactivating potassium current (dark arrow) were altered differently by calpeptin. The average (n=5) current-voltage relation curves for the early inactivating potassium current (B) and the delayed potassium current (C) reveal that the delayed potassium current is reduced more extensively than the early inactivating potassium current.

**Fig. 6. The depression rates of the early inactivating and the late noninactivation potassium currents.**

Potassium currents were generated by stepping the membrane voltage from a holding potential of -50mV to +20 mV at 0.05Hz. After 15 control stimuli, 100 μM calpeptin (A), 200 μM MG132 (C), or 200 μM calpain inhibitor XII (E) were applied to the bathing solution. The peak of the early inactivating (open circles) and the late noninactivating potassium conductances were measured and plotted as a function of the number of the applied voltage step. The rate of reduction of the late $I_{K^+}$ proceeds faster than the early $I_{K^+}$. (B, D, F) The ratio of the early and late peaks of potassium currents
as a function of the voltage clamp step (B) calpeptin, (D) MG132, and (C) calpain inhibitor XII.

**Fig. 7. Calpeptin decreases the sodium current without changing its current-voltage relationship and steady-state inactivation.**

To investigate the effect of calpeptin on the sodium currents, we blocked the calcium and potassium currents (see Methods). After 10 minutes of incubation in the blocking solution, the sodium action potential was recorded (Aa). Then the sodium current was measured by depolarizing the membrane potential from a holding potential of -50 mV to +70 mV in 5 mV steps for 50 msec. The sodium currents generated by seven consecutive voltage steps are depicted (Ab). Next, calpeptin (100 μM) was added, and 5 minutes later, the sodium action potential (Ac) and currents (Ad) were recorded. (B) The average, normalized current-voltage relation in the control and calpeptin (n=4). Calpeptin decreases the sodium currents by 36% without significantly changing its current-voltage relations. (C) The effect of calpeptin on the steady-state inactivation of sodium currents (n=3). The sodium current inactivation curve was generated by holding the membrane potential at various indicated potentials for 2 sec, and stepping up the voltage clamp to +20 mV for 20 msec. The relative conductance value for each holding potential was calculated.

**Fig. 8. Calpeptin slightly decreases calcium currents.**

To examine the effect of calpeptin on calcium currents, we blocked the sodium and potassium currents (see Methods). Calcium spikes were recorded in current clamp mode (Aa). In calpeptin (100 μM), the spike is significantly shorter and slightly smaller than under control conditions (Ab). Calcium currents were evoked by 400-msec
depolarizing steps from a holding potential of -50 mV to +50 mV (in 10 mV steps), in control ASW (Ac) and 5 minutes after applying calpeptin (Ad, n=4). Shown are examples of calcium current traces, generated by seven voltage steps from the holding potential, to -10 up to +50 mV in 10 mV increments in control and calpeptin. The normalized current-voltage relations curve (B, average of n=4) reveals a slight decrease in calcium currents in calpeptin without a significant alteration in current-voltage relations.

**Fig. 9. The rates of inward and outward current depression following calpeptin application.**

To examine the rate at which calpeptin reduces the outward and inward currents, we repeatedly measured the inward and outward currents at 0.1 Hz by 100 msec depolarizing steps from a holding potential of -50 mV to +20 mV following a bath application of calpeptin (n=5). (A) Current traces of seven voltage steps from a holding potential of -50 mV to -10 mV up to +50 at 10 mV increments. Samples of the current and voltage are shown in (B) ASW (left), in calpeptin 100 μM (middle), and after a wash (right). The amplitude of the outward (sampled at the time point indicated by the black arrow in A) and the peak inward currents (C) are shown as a function of time before and after applying calpeptin to the bathing solution, and after it was washed out. The time that calpeptin was applied and washed out are indicated by arrows.
control

A

calpeptin

B

calpeptin, TTX

C

calpeptin, TTX, 0 Ca²⁺, Co

D

washing

E

100 msec 10 mV
A

ASW

calpeptin

B

C

30% ΔF/F₀

30% ΔF/F₀
\textbf{A}

\begin{itemize}
  \item \textit{a} ASW
  \item \textit{b} calpeptin
  \item \textit{c} wash
\end{itemize}

\textbf{B}

\begin{itemize}
  \item \textit{a} ASW
  \item \textit{b} MG132
  \item \textit{c} wash
\end{itemize}

\textbf{C}

\begin{itemize}
  \item \textit{a} ASW
  \item \textit{b} calpain inhibitor XII
  \item \textit{c} wash
\end{itemize}
A

control

calpeptin

early IK+

delayed IK+

B

early IK+

Normalized current

Control
Calpeptin

C

delayed IK+

Normalized current

Control
Calpeptin