Calcium-activated proteases are critical for refilling depleted vesicle stores in cultured sensory-motor synapses of *Aplysia*

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We report that the calpain inhibitors, calpeptin, MG132, and ALLN (Figueiredo-Pereira et al. 1994; Lee and Goldberg 1998), but not the proteasome inhibitors, lactacystin and *clasto*-lactacystin β-lactone, inhibit 5HT-induced facilitation of depressed synapses. Likewise, the 5HT-induced enhancement of spontaneous miniature excitatory postsynaptic potentials (mEPSPs) frequency of depressed synapses is significantly reduced by calpeptin. In contrast, neither the facilitation of nondepressed synapses nor the enhancement of their mEPSPs frequency is affected by the inhibitor. The data suggest that action potentials-induced calcium influx activate calpains. These, in turn, play a role in the refilling processes of the depleted, releasable vesicle store.

**Results**

Calpain inhibitors accelerate the rate of synaptic depression and inhibit 5HT-induced facilitation of highly depressed synapses

We began by examining the effects of calpeptin on the rate of synaptic depression and 5HT-induced facilitation. To that end, the excitatory post synaptic potentials (EPSPs) were depressed by 40 consecutive extracellular stimuli delivered at 0.05 Hz to the presynaptic sensory neuron in the absence and presence of 100 µM calpeptin.

In control experiments, carried out in the presence of the carrier solution, 0.2% DMSO ASW, following synaptic depression, 5HT application led to the recovery of the EPSP’s amplitude to 91 ± 9.1% SEM (n = 6) of the initial level (Fig. 1A). In the presence of 100 µM calpeptin, the rate of synaptic depression was partially opposed by a Ca2+-mediated facilitatory process, which may be described in terms of vesicles mobilization to a readily releasable pool. We report that the calpain inhibitors, calpeptin, MG132, and ALLN (Figueiredo-Pereira et al. 1994; Lee and Goldberg 1998), but not the proteasome inhibitors, lactacystin and *clasto*-lactacystin β-lactone, inhibit 5HT-induced facilitation of depressed synapses. Likewise, the 5HT-induced enhancement of spontaneous miniature excitatory postsynaptic potentials (mEPSPs) frequency of depressed synapses is significantly reduced by calpeptin. In contrast, neither the facilitation of nondepressed synapses nor the enhancement of its mEPSPs frequency is affected by the inhibitor.

Our results are consistent with the hypothesis that calcium influx during homosynaptic depression activates calpain. The active calpain in turn assists in the replenishing processes of the depleted vesicle stores, possibly by facilitating the mobilization of vesicles from a nonreleasable compartment to a readily releasable store. When calpain activation is inhibited, and the releasable vesicle store is depleted, 5HT application fails to induce synaptic dishabitation, as vesicle mobilization is impaired. The effect of calpain activation by calcium influx on vesicle mobilization is transient and lasts for minutes, since the structural integrity of the cleaved protein recovers after mild proteolysis.

Chemical synapses formed by cultured *Aplysia* mechanosensory and motoneurons have proven to be an extremely useful model system to study mechanisms underlying different forms of short and long-term synaptic plasticity (for review, see Kandel 2001). These synapses undergo depression in response to repetitive stimuli delivered at low frequencies. The depressed synapse undergoes rapid facilitation in response to a single bath application of 10 µM 5HT (Hochner et al. 1986). Based on pharmacological experiments, it was suggested that the 5HT-induced facilitation of depressed synapse results from PKC activation (Braha et al. 1990; Ghirardi et al. 1992; Byrne and Kandel 1996; Manseau et al. 2001), which, in turn, induces the mobilization of neurotransmitter-containing vesicles from a nonreleasable pool to the depleted pool of readily releasable vesicles (Gingrich and Byrne 1985; Bailey and Chen 1988; Klein 1995; Byrne and Kandel 1996; Zhao and Klein 2002, 2004). Alternative explanations have also been considered; namely, that PKC activates voltage-gated calcium channels strategically located close to the release site, or that PKC act directly on the exocytotic machinery (Byrne and Kandel 1996; Manseau et al. 2001; Zhao and Klein 2002).

Theoretical considerations led Gingrich and Byrne (1985) to suggest that the process of sensory-motor synaptic depression is partially opposed by a Ca2+-mediated facilitatory process, which may be described in terms of vesicles mobilization to a ready releasable status. This view was further supported by Eliot et al. (1994) and Bao et al. (1997) who argued that post-tetanic potentiation (PTP) in the synapse is expected to be mediated by a calcium-dependent enzymatic reaction. Nevertheless, the mechanisms that link alterations in the free intracellular calcium concentration ([Ca2+]i) and the refilling processes of the depleted releasable vesicle store were not investigated.

Here, we examine the hypothesis that the calcium-activated protease—calpain (Sato and Kawashima 2001; Goll et al. 2003) is the linking molecular element between the elevation of the [Ca2+]i, and vesicle refilling.
significantly accelerated and the degree of synaptic depression was significantly higher. Thus, while in control experiments, 40 stimuli depressed the EPSP amplitude from a mean value of 16.9 ± 4.7 mV to 18 ± 4.5% SEM of the initial level (n = 6) in the presence of calpeptin, the rate of depression was significantly faster and resulted in synaptic depression from a mean value of

Figure 1. The inhibition of calcium-dependent proteinases accelerates the rate of homosynaptic depression and inhibits SHT-induced facilitation of depressed synapses. To examine the roles of proteinases in the cascade leading to homosynaptic depression and facilitation of highly depressed synapse by SHT, we compared the rates of synaptic depression and S-HT-induced facilitation in the presence and absence of protease inhibitors; (A) 100 µM calpeptin, (B) 100 µM MG132, (C) 100 µM ALLN, and (D) intracellularly injected lactacystin into the sensory neuron. Examples of EPSPs recorded during synaptic depression induced by 40 stimuli at 0.05 Hz and SHT-induced facilitation (arrows) in control experiments are shown in aI, bI, cI, and dI. The traces shown in aI, bI, cI, and dI depict the first EPSP, the 40th EPSP, and the second EPSP after SHT application (42 stim). The effects of protease inhibitors are depicted in aII, bII, cII, and dII and the mean values are shown in aIII, bIII, cIII, and dIII (n = 6).

For experiments A, B, and C, the preparations were bathed in the protease inhibitor containing ASW for 15 min before the onset of the experiment. The paired controls were incubated for 15 min in 0.2% DMSO ASW (the vehicle solution of the inhibitors). For the experiment in D, lactacystin was pressure injected into the presynaptic neuron (reaching an estimated concentration of 25 µM) 1 h prior to the experiment. Note that in the presence of (A) calpeptin and (B) MG132, the rate of synaptic depression is significantly accelerated with respect to the control (F_{39,390} = 1.971, P < 0.01 repeated measures one-way ANOVA for calpeptin and F_{39,312} = 2.063, P < 0.001 for MG132) and that the S-HT-induced facilitation is significantly reduced (F_{4,40} = 8.348, P < 0.001 repeated measures one-way ANOVA for calpeptin and F_{4,32} = 5.909, P < 0.01 for MG132). The 100 µM ALLN (C) does not accelerate the rate of synaptic depression, but inhibits SHT-induced synaptic facilitation. In contrast, the proteasome inhibitor lactacystin neither affected the rate of synaptic depression nor the facilitation induced by SHT.

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Calpain has no significant effect on 5HT-induced facilitation of nondepressed synapses

Finally, we studied the effect of calpeptin on 5HT-induced facilitation of nondepressed synapses. The experiment was done as follows: the EPSP amplitude evoked by a single test stimulus to the sensory neuron was recorded. This was followed by calpeptin application. Fifteen minutes later, a second stimulus was delivered in the presence of calpeptin (Fig. 2B). In this series of experiments, the EPSP’s amplitude measured after 15 min in 100µM calpeptin was higher by 14.5 ± 8.9% (SEM; n = 6) than the control EPSP’s. 5HT was then applied, and 3 min later, a third stimulus was delivered. This led to an increase in EPSP’s amplitude by 118 ± 28% (n = 6) over the initial control value. The control experiments were conducted using the same protocol, except for application of 0.2% DMSO ASW rather than calpeptin-containing solution. In these controls, a slight decrease of 6.3 ± 3.4% (n = 6) in the PSP amplitude was recorded. 5HT application doubled the EPSP amplitude (by 100 ± 18.6%) of its initial level, n = 6 (Fig. 2B). Thus, calpeptin has no significant effect on 5HT-induced facilitation of nondepressed synapses (P > 0.05; t-test). These results are consistent with earlier reports demonstrating that calpain inhibitors have no significant effects on 5HT-induced facilitation of nondepressed *Aplysia* sensory motor synapses (Chain et al. 1999).

Calpain activity with respect to the PKC cascade of synaptic dishabituation

In a series of studies, it was suggested that 5HT-induced facilitation of depressed synapses involves the activation of PKC (Brahao et al. 1990; Ghirardi et al. 1992; Byrne and Kandel 1996; Manseau et al. 2001). It was argued that the activation of PKC, in turn, leads to the mobilization of neurotransmitter-containing vesicles from a nonreleasable pool to a readily releasable store (Gingrich and Byrne 1985; Hochner et al. 1986; Bailey and Chen 1988; Braha et al. 1990; Dale and Kandel 1990). The experimental results described above are phenomenologically similar to those described for the PKC inhibitor, H7, by Braha et al. (1990) and Ghirardi et al. (1992). Namely, H7 does not block 5HT-induced facilitation of rested synapses, yet it blocks the facilitation of depressed synapses. These earlier studies also demonstrated that direct activation of PKC by phorbol dibutyrate-PDBu is sufficient to induce facilitation of depressed synapses (Brahao et al. 1990; Ghirardi et al. 1992).

Thus, we began to explore the role of calpain action with respect to PKC within the cascade leading to 5HT-induced synaptic facilitation of depressed synapses. It activated calpain operates by cleavage of substrates that impede vesicle translocation from a reserve pool to a readily releasable pool, then the facilitation of depressed synapses by PDBu application would be inhibited in the presence of calpeptin (Fig. 5, below, and see Discussion). It should be noted that, in this case, the hypothetical vesicle translocation-impeding substrates could operate downstream (Fig. 5, #5a) or upstream (Fig. 5, #5b) with respect to PKC (Fig. 5, #10). Furthermore, it is expected that if the putative calpain substrates impede vesicle translocation, but do not block...
The role of calpain in facilitation of depressed synapses

The effect of calpeptin on spontaneous neurotransmitter release

As stated earlier, SHT-induced facilitation following synaptic depression is thought to depend on the mobilization of synaptic vesicles from a nonreleasable store into a readily releasable store (Gingrich and Byrne 1985; Hochner et al. 1986; Bailey and Chen 1988; Dale and Kandel 1990; Zhao and Klein 2002). The availability of a releasable pool of vesicles is reflected by the mEPSP frequency (Dale and Kandel 1990) (but, see Eliot et al. 1994). To gain some insight into these processes, we depressed the synaptic potential, and then applied 20 nM PDBu in the presence or absence of calpeptin (Fig. 3). In control experiments, PDBu induced significant synaptic facilitation from 22 to ±3% (3.7 ± 0.5 mV) to 90 ± 3.9% of the initial level (n = 5, Fig. 3, P < 0.001; paired t-test). In the presence of 100 µM calpeptin, PDBu induced a much smaller facilitation, from 9.2 ± 1.8% after depression (1.5 ± 0.29 mV) to 22 ± 6% of the initial level (P > 0.05; paired t-test, n = 4). In view of the insignificant facilitatory effects of PDBu on depressed synapses in the presence of calpeptin, it is reasonable to assume that calpain and PKC exert their effect on the same cascade of events as described in Figure 5, below.

Figure 2. Effect of calpeptin on SHT-induced synaptic facilitation of moderately depressed and nondepressed synapses. Examples of SHT-induced facilitation of moderately depressed synapses in controls (0.2% DMSO ASW) (AII) and preparations bathed in 100 µM calpeptin (AII). The figure shows representative traces of EPSP in the course of the experiments. The number shown under the EPSPs in A and AII corresponds to the order of the EPSP’s generation. To moderately depress the synaptic potentials to about 45% of the initial level, four stimuli at 0.05 Hz were given to the sensory neuron in control experiments. Since the rate of depression is faster in calpeptin, only three stimuli at 0.05 Hz were given to depress the synapses to about 45% of the initial level in calpeptin. The arrow (time 0) indicates SHT application. In the control experiments, SHT induces facilitation of the depressed EPSPs. In the presence of calpeptin, SHT induces partial recovery, which then rapidly declines. The kinetics of synaptic depression and SHT-induced facilitation in control (AII, #) and in the presence of calpeptin (AII, ⊗) are shown. Significant difference between control and calpeptin experiments becomes apparent from the fourth stimulus after SHT application, (P < 0.05; t-test). (B) In resting synapses, calpeptin has no effect on SHT-induced facilitation. BII and BIII are examples of SHT-evoked facilitation of rested synapses in the controls and in the neurons incubated in 100 µM calpeptin for 15 min, respectively. (BII) EPSP before (ASW), 15 min after calpeptin application (calpeptin) and 3 min following SHT application (SHT + calpeptin). In control experiments, DMSO 0.2% was added instead of calpeptin (BIII). (BIII) Summary of the short-term facilitation of rested synapses in the presence and absence of calpeptin. Each bar represents the mean percent change ± SEM of the EPSP amplitude with respect to the first EPSP. Note that facilitation produced by SHT in rested synapses is not affected by calpeptin (P > 0.05; t-test).

Thus, we next examined the effects of calpeptin on SHT-induced enhancement of spontaneous release.

In control experiments, performed on nondepressed synapses, the mean mEPSP frequency was 0.091 ± 0.03 Hz. Bath application of 0.2% DMSO (the vehicle solution for calpeptin) did not induce any significant effects on mEPSP frequency (n = 4) (Fig. 4A). Application of SHT led to a significant 5.57 ± 0.31-fold increase in mEPSP frequency (P < 0.01, paired t-test) (Fig. 4A). Application of 100 µM calpeptin alone induced a 2.4 ± 0.37-fold insignificant increase in mEPSPs frequency (P > 0.05, paired t-test, n = 6) (Fig. 4A). This increase in mEPSP frequency is most likely related to a small (~100 nM) increase in the free intraneuronal calcium concentration as revealed by fura-2 imaging (Ziv and Spira 1995; data not shown). In the presence of calpeptin bath, application of SHT induced a significant 5.9-fold increase in mEPSP frequency with respect to the frequency measured with calpeptin alone (n = 6, P < 0.05; paired t-test) (Fig. 4A, III). These results demonstrate that calpeptin does not inhibit the effects of SHT on mEPSP frequency in rested synapses.

An insignificant decrease in the mean mEPSP amplitude from 1.025 to ±0.24 mV in control to 0.92 ± 0.26 mV was noted after calpeptin application (n = 3). This clearly indicates that calpeptin has no significant postsynaptic actions.

We next turned to examine whether calpeptin alters the effects of SHT on mEPSP frequency in highly depressed synapses. In these experiments, we first sampled the mEPSP frequency for 5 min. Then, the synapse was depressed by 40 stimuli delivered at
produced an insignificant (t-test). In the presence of calpeptin, 5HT application produced a 0.4-fold enhancement of mEPSP frequency (n = 6, P < 0.05; paired t-test). In the presence of calpeptin, SHT application produced an insignificant (P > 0.05; paired t-test) 1.83 ± 0.4-fold enhancement of mEPSP frequency (n = 6) (Fig. 4B). These results demonstrated that calpains are involved in the SHT-induced increase of spontaneous release in depressed synapses, but not in rested synapses.

Discussion

The main observation of the present study is that the calpain inhibitors, calpeptin, MG132, and ALLN, largely inhibit the SHT-induced facilitation of depressed sensory-motor synapses. These observations are consistent with the interpretation that the activation of calcium-dependent protease is essential for synaptic vesicle mobilization from a nonreleasable pool into a readily releasable pool (Fig. 5).

The refilling of the releasable pool of neurotransmitter-containing vesicles in the sensory motor synapse appears to be a rate-limiting process. A single bath application of SHT leads to significant facilitation of the synaptic potential of both depressed and rested synapse (Hochner et al. 1986). A series of experiments lead to the conclusion that the SHT-induced facilitation of depressed synapses results from the activation of a calcium-independent PKC (Braithwaite et al. 1990; Ghirardi et al. 1992; Sossin et al. 1993; Byrne and Kandel 1996; Manseau et al. 2001), which, in turn, induces the mobilization of neurotransmitter-containing vesicles from the nonreleasable pool to the depleted pool of readily releasable vesicles (Gingrich and Byrne 1985; Bailey and Chen 1988; Klein 1995; Byrne and Kandel 1996; Zhao and Klein 2002, 2004) (Fig. 5, #9 and #10). It is important to note that the contribution of PKC to synaptic facilitation was always detected only after the depression of the synaptic potentials by repetitive stimuli. Thus, in the experimental paradigm used to examine the role of PKC, elevation of [Ca^{2+}], always precedes the activation of PKC.

The results presented here suggest that the activation of calpain by the incoming calcium underlies the refilling process. Since calpain inhibitors do not significantly alter evoked release of rested synapses, we conclude that the process of neurotransmitter release itself, including the exocytotic machinery, is not affected by calpain blockade. Calpains appear to regulate two processes, i.e., the rate of homosynaptic depression (Figs. 1A, B and 2A) and the facilitation of depressed synapses by SHT (Figs. 1, 2, 4). Both processes can be interpreted as indications that activated calpains regulate vesicle mobilization from nonreleasable to releasable pools (Fig. 5, #5a and #5b).

Figure 3. Effect of PDBu on SHT-evoked facilitation of depressed synapses. Facilitation of the depressed synapses by 20 nM PDBu (A) in control cells (0.2% DMSO) and (B) in cells incubated for 15 min in 100 μM calpeptin EPSPs before depression, after depression, and 1 min following 20 nM PDBu application (B) in the presence and (A) absence of calpeptin are shown. (C) Summary of the synaptic depression and PDBu (20 nM) induced facilitation in the presence (n = 4) and absence (n = 5) of calpeptin expressed as the mean percent change ± SEM in the amplitude of the EPSP compared with the EPSP before depression. PDBu-induced facilitation is greatly inhibited by calpeptin (P > 0.05; paired t-test).

Specificity of the protease inhibitors

Most of the arguments for the role of calpains in the sensory motor synapse rest on the specificity of the calpain inhibitors used. We have shown that calpeptin, a membrane-permeable cystein protease inhibitor (Tsujinaka et al. 1988) inhibits calcium-dependent proteolytic processes in cultured Aplysia neurons (Gitler and Spira 1998, 2002; Spira et al. 2003). In a preliminary study, MG132 and ALLN were also shown to inhibit the calcium-dependent protease in Aplysia neurons (Spira et al. 2001). While MG132 and ALLN are known to function as calpain and proteasome inhibitors (Figueiredo-Pereira et al. 1994; Lee and Goldberg 1998), calpeptin is only a weak proteasome inhibitor (Figueiredo-Pereira et al. 1994). Since we found that the proteasome inhibitors lactacystin and clasto-lactacystin β-lactone have no short-term effects on SHT-induced facilitation of depressed synapses, we suggest that the described inhibition of SHT-induced facilitation of depressed synapses is indeed related to inhibition of calpains.

Interestingly, the concept that synaptic depression is opposed by a Ca^{2+}-mediated process that mobilizes vesicles was suggested 20-yr ago by Gingrich and Byrne (1985), and see also Klein et al. (1980). This view was further supported by Elliot et al. (1994) and Bao et al. (1997), who argued that post-potentiation in the sensory motor synapse is expected to be mediated by a calcium-activated molecule that exerts its effects long after the [Ca^{2+}], is down-regulated (see also Fisher et al. 1997). These observations are consistent with the proposed model that action potential-induced Ca^{2+} influx activates calpains, which, in turn, facilitate the mobilization of vesicles for minutes (see below).

Activation of calpain

Calpains are the only known proteolytic enzymes that depend on direct calcium binding (Perrin and Huttenlocher 2002; Goll et al. 2003). Following activation, calpains undergo autocatalytic processing, yielding a calcium-independent isoform (Goll et al. 2003). Thus, calpains can (1) integrate the number of spikes over time, as calpain activation depends on the [Ca^{2+}]; (2) once the calcium concentration reaches a level sufficient for calpain activation, the calpain undergoes an autoproteolytic process leading to the formation of a calcium-independent isoform, and thus, may serve as molecular memory.

During a train of action, potentials leading to synaptic depression, the [Ca^{2+}], is elevated in the sensory neuron neurites (Blumenfeld et al. 1990). We hypothesize that this, in turn, activates an Aplysia form of μ-calpain that facilitates vesicle mobilization from a nonreleasable to readily releasable domains (Fig. 5, #5a and #5b). When calpain activation is inhibited (Fig. 5, #8), the refilling rate of the depleted pool is reduced, leading to an enhanced depression rate of the evoked EPSP (Figs. 1 and 5, #5a),...
and the inhibition of SHT-induced facilitation of evoked and spontaneous neurotransmitter release (Figs. 1,4,5, #5b).

**Mechanisms by which calpain regulate the facilitation of depressed synapses**

Several mechanisms have been proposed to account for the observation that SHT induces facilitation of depressed synapses. These include mobilization of vesicles (Dale and Kandel 1990; Angers et al. 2002; Zhao and Klein 2002), increased calcium influx at the release site, or priming of vesicles that are already docked (Zhao and Klein 2002). While the molecular mechanisms underlying the facilitation are still not totally understood, most studies reveal that the activation of PKC by DAG is the key event leading to facilitation. Consistently, PKC inhibition by pharmacological reagents, such as H7, blocks the SHT-induced facilitation of depressed synapses (Brahal et al. 1990; Sacktor and Schwartz 1990; Ghirardi et al. 1992) and PDBu application is sufficient to induce the facilitation (Brahal et al. 1990).

In that respect, it is important to note that Munc-13 is an additional phorbol ester receptor (Betz et al. 1998). It was shown that in some experiments in which facilitation of neurotransmitter release by phorbol esters was attributed to PKC activation, the facilitation was in fact related to the effect of phorbol esters on Munc13 (Rhee et al. 2002; Silinsky and Searl 2003). Nevertheless, we are not aware of studies showing that Munc13 or related proteins serve as calpain substrate.

The calpain substrate, spectrin (Bennett 1990; Bennett and Gilligan 1993) is considered as a submembrane skeletal component that may impede the refilling processes of depleted synapses. In neurons, spectrin is often preferentially localized to synapses (Bloch and Morrow 1989; Daniels 1990; Goodman et al. 1995; Kordeli 2000). Spectrin interacts with many of the synaptic components, and thus, could affect vesicle mobility. Indeed, it was suggested that spectrin cleavage might expose release sites to incoming vesicles (Gitler and Spira 1998; Sikorski et al. 2000; Zimmer et al. 2000). Interestingly, it was suggested by the laboratory of G. Lynch (Lynch and Baudry 1987) that calpain is involved in long-term potentiation of hippocampal neurons in slices. Inhibition of calpain reduced the incidence and magnitude of long-term potentiation. It was suggested that calpain pro teaseolyses postsynaptic spectrin (Lynch and Baudry 1987). These observations were not followed up, and the mechanism of calpain action was not determined. Another potential site for calpain could be the anchorage of vesicles to the actin skeleton by synapsins (Landis et al. 1988; Goodman et al. 1995; Kordeli 2000). Spectrin interacts with many of the synaptic components, and thus, could affect vesicle mobility. Indeed, it was suggested that spectrin cleavage might expose release sites to incoming vesicles (Gitler and Spira 1998; Sikorski et al. 2000; Zimmer et al. 2000). Interestingly, it was suggested by the laboratory of G. Lynch (Lynch and Baudry 1987) that calpain is involved in long-term potentiation of hippocampal neurons in slices. Inhibition of calpain reduced the incidence and magnitude of long-term potentiation. It was suggested that calpain pro teaseolyses postsynaptic spectrin (Lynch and Baudry 1987). These observations were not followed up, and the mechanism of calpain action was not determined. Another potential site for calpain could be the anchorage of vesicles to the actin skeleton by synapsins (Landis et al. 1988; Goodman et al. 1995; Sikorski et al. 2000). Thus, both synapsin (Fig. 5, #5a) and spectrin (Fig. 5, #5b) could serve as impeding-substrates.

Other substrates including calcineurin, Ca$^{2+}$/calmodulin-protein kinase, Ca$^{2+}$ ATPase, and phospholipase C should be considered as a potential calpain substrate that might influence the mobilization of vesicles (for review, see Chan and Mattson 1999).

In conclusion, the data presented here are interpreted to
suggest the following model: (1) action potentials delivered to the presynaptic sensory neurons elevate the [Ca\(^{2+}\)](Fig. 5, #1–#3) and lead to neurotransmitter release (Fig. 5, #7). Repetitive stimulation of the sensory neuron leads to homosynaptic depression by depletion of the available neurotransmitter pool; (2) in parallel, the increased [Ca\(^{2+}\)] activates calpains (Fig. 5, #4), and in parallel, invokes neurotransmitter release (#7). The activated calpain cleaves substrates (#5a; #5b) that impede vesicle translocation from the reserve pool to a readily releasable pool of neurotransmitter (#6). The nature of the calpain substrate is not known. Nevertheless, based on the experimental results, the impeding substrates could be located upstream (#5a) or downstream (#5b) with respect to the vesicle-mobilization functions of PKC. When calpain activation is inhibited (#8), the impeding substrates are not cleaved, and as a consequence, the translocation of vesicles is slowed down, leading to an increased rate of homosynaptic depression. Furthermore, following homosynaptic depression, calpain cleaves substrates (#5a; #5b) that impede the mobilization of vesicles from a nonreleasable into a readily releasable store of vesicles (Fig. 5, #5a or #5b). We propose that while PKC acts by removal of barriers that impede the translocation of the vesicles. Thus, when calpain is inhibited the rate of homosynaptic depression is accelerated and SHT-induced synaptic dishabitation is inhibited (Fig. 1). The proposed model also accounts for the observations that in the presence of calpeptin, the partially depolarized synapse undergoes only a transient SHT-induced facilitation (Fig. 3). Thus, under these conditions, application of SHT results in action potential broadening, which leads to increased calcium influx and increased neurotransmitter release from the readily releasable store. As a consequence, the available store is depleted. Since calpain is not activated in the presence of calpeptin, the mobilization of vesicles to the releasable pool is inhibited and the EPSP amplitude is further depressed. The same mechanism could account for the observation that in the presence of calpeptin, SHT fails to increase the spontaneous miniature frequency following homosynaptic depression (Fig. 4).

We propose tentatively that the substrate for calpain action could be the un tethering of vesicles from the cytoskeleton (Fig. 5, #5a, upstream to PKC) or proteolysis of spectrin that impede vesicles from reaching the release sites (Fig. 5, #5a, downstream to PKC). It is reasonable to assume that the formation of a calcium-independent isoform of calpain by an autocatalytic process may serve as short-term molecular memory that promotes vesicle mobilization and refilling of depleted stores. It should be noted, however, that calpain activity is limited by the presence of endogenous inhibitors such as calpastatin. Thus, the duration of calpain action is limited by endogenous inhibition and dilution, and opposed by spontaneous recovery and delivery of its substrates.

Materials and Methods

Cell cultures

Sensory neurons from the pleural ganglia of adult animals (60–80 g) were cocultured with either an L7, or an LFS motor neuron from the abdominal ganglia of juvenile (2–5 g), or adult animal, respectively, as described by Schacher and Proshansky (1983). Briefly, animals were anesthetized by injection of isotonic MgCl\(_2\) solution. The ganglia were isolated and incubated for 1.5–3 h in 1% protease (type IX, Sigma) at 34°C. The ganglia were then dehydrated, and the cell bodies of their neurons with their long axons were pulled out with sharp micropipettes and placed on poly-L-lysine-coated (Sigma) glass-bottom 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, dishes were transferred to an 18°C incubator. Experiments were performed 3–5 d after plating.

Electrophysiology

All recordings were performed at room temperature in artificial sea water (ASW) composed of NaCl 460 mM, KCl 10 mM, CaCl\(_2\) 10 mM, MgCl\(_2\) 55 mM, and HEPES [N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid, Sigma] 11 mM, adjusted to pH 7.6.

Sensory neurons were extracellularly stimulated. EPSPs were intracellularly recorded from the motor neurons with 7–9 MΩ sharp microelectrodes filled with 2 M KCl. EPSPs were recorded while holding the motor neurons transmembrane potential at approximately −70 mV. Intracellular stimulation was done by sharp microelectrodes (12–15 MΩ) filled with 2 M KCl.

Spontaneous miniature potentials were recorded in culture formed by a single sensory neuron in contact with a single LFS motor neuron. Throughout the experiments, the LFS motor neurons were hyperpolarized to −80 mV in current-clamp mode with microelectrodes filled with 2 M KCl (15–20 MΩ). Spontaneous miniature potentials were identified visually.

Drugs

Calpeptin, MG132, and ALLN (Calbiochem) were prepared as a 50-mM stock solution in DMSO (Sigma) and diluted to the final concentration in ASW just before the experiments. Clasto-lactacystin β-lactone (Calbiochem) and PDBu (Sigma) were prepared as 10-nM stock solutions in DMSO. Stock solution of S-Hydroxycitrpta mine (Sigma) was prepared fresh on the same day of the experiment.

Loading of lactacystin (Calbiochem) into the sensory neurons was done by pressure injection of a 1-M KCl solution containing 5 mM lactacystin and 5 mM fura-2 (Molecular Probes). The intracellular concentrations of fura-2 were estimated by measuring the fura-2 fluorescent intensity within the main neurite of the sensory neuron by excitation at 360 nm. Using a fluorescent calibration curve of fura-2 intensity/fura-2 concentration, the final concentration of lactacystin was estimated (see Ziv and Spira 1995).

Statistical analysis

The effects of various treatments are presented as the percent change of EPSP’s amplitude after treatment with respect to the initial EPSP’s amplitude pretreatment. All of the data are presented as mean ± SEM. Statistical analysis was performed with the software package SPSS 10.0 (Genius Systems, Israel) for Windows using repeated measures one-way ANOVA in Figure 1, in-
dependent samples t-test, and the paired samples t-test in Figures 1C and 2, and 3 and 4, respectively.

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