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Protein Kinase C Acts as a Molecular Detector of Firing Patterns to Mediate Sensory Gating in *Aplysia*

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Abstract

Habituation of a behavioral response to a repetitive stimulus enables animals to ignore irrelevant stimuli and focus on behaviorally important events. In *Aplysia*, habituation is mediated by rapid depression of sensory synapses, which could leave an animal unresponsive to important repetitive stimuli, making it vulnerable to injury. We identified a form of plasticity that prevents synaptic depression depending on the precise stimulus strength. Burst-dependent protection from depression is initiated by trains of 2–4 action potentials, and is distinct from previously described forms of synaptic enhancement. The blockade of depression is mediated by presynaptic Ca^{2+} influx and protein kinase C (PKC), and requires localization of PKC via a PDZ domain interaction with *Aplysia* PICK1. During protection from depression, PKC acts as a highly sensitive detector of the precise pattern of sensory neuron firing. Behaviorally, burst-dependent protection reduces habituation, enabling animals to maintain responsiveness to stimuli that are functionally important.

It is critical for both animals and humans to distinguish between stimuli and events that require attention and those that are irrelevant and should be ignored. Even simple animals must continually discriminate between stimuli that merit a response and those that have no behavioral relevance. Habituation to repeatedly occurring stimuli represents one of the simplest forms of this discrimination. An extensively studied example of habituation is the decrement of the defensive gill and siphon withdrawal reflexes in the marine mollusc *Aplysia*. The synapses between the mechanosensory neurons (sensory neurons) that innervate the siphon and the motor neurons that control the siphon and gill undergo rapid and profound homosynaptic depression (HSD) with repeated activation over a wide range of

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X.-Y.J. and T.W.A. discovered the BDP phenomenon. Q.W., K.S.M. and T.W.A. designed the experiments. Q.W., X.-Y.J., A.M.N. and S.-G.L. did the experiments analyzing the mechanism of BDP. K.S.M. did the behavioral experiments. T.W.A., K.S.M. and Q.W. wrote the paper.

intervals (from 1 sec to >100 sec) [1–3]. HSD contributes to the habituation of the defensive withdrawal reflexes elicited by repeated weak tactile stimuli to the siphon skin [4–6]. This synaptic depression persists for minutes or tens of minutes after only 5 to 15 stimuli [7]. Although it may be behaviorally adaptive to ignore recurring, innocuous stimuli, it is also critical to remain attentive to biologically significant stimuli, even if they occur repetitively. These same sensory neurons are believed to function as nociceptors [8]. If rapid and persistent HSD occurs at sensory neuron-motor neuron connections in the behaving animal, how can these synapses still transmit important mechanosensory information? What prevents these marine snails from becoming chronically unresponsive to tactile stimuli, including noxious stimuli? Heterosynaptic facilitatory input activated by stronger stimuli can strengthen previously depressed synapses [9]; however, once synapses from sensory neurons innervating a site on the skin have been depressed, more intense stimuli to this same site would be relatively ineffective in activating heterosynaptic facilitatory pathways, leaving the animal vulnerable. Such an interruption of afferent input could also impair learning. We recently identified a mechanism for regulating synaptic strength in which brief bursts of action potentials in siphon sensory neurons prevent the development of HSD. Whereas the first action potential during a burst initiates silencing of release sites, the subsequent action potentials activate PKC, which interrupts the silencing process.

RESULTS

Bursts of presynaptic spikes prevent synaptic depression

Whereas individual action potentials result in rapid homosynaptic depression (HSD) of siphon sensory neuron-to-siphon motor neuron synaptic connections, we observed that when sensory neurons fired bursts of 4 spikes at 20 Hz, HSD was largely or entirely prevented. After 12 to 15 bursts, the first EPSP within each burst typically remained between 80% and 130% of the initial amplitude; bursts of only 2 spikes per trial also produced significant protection from HSD (Fig. 1a,b). We call this maintenance of synaptic strength produced by bursts of several action potentials in a presynaptic sensory neuron “burst-dependent protection” from synaptic depression (BDP). Effective protection is evident from the outset; by the second trial, the synaptic connections activated by bursts of either 2 or 4 spikes were significantly stronger than the sensory neuron-motor neuron synapses activated with single spikes [$p = 0.020$ and $p = 0.024$, for 2 and 4 spikes per burst, respectively, compared with single spikes; all pairwise comparisons done with Sidak adjustment for multiple comparisons (see Methods)]. All synaptic experiments were conducted in high-divalent saline to eliminate polysynaptic PSPs from interneurons; however, bursts also protect against depression in normal saline (Supplementary Fig. 1a).

We examined the effect of brief bursts of spikes on sensory neuron-motor neuron synapses that had previously been depressed. When the stimulation pattern was switched from single spikes to 4 spikes per trial, synaptic strength recovered partially, increasing 89 ± 16% within 4 trials (Fig. 1c,d). Bursts of two spikes resulted in a more modest increase in the depressed EPSP. Bursts were significantly less effective in producing recovery at recently depressed synapses than in protecting naïve synapses from depression ($p < 0.001$, Fig. 1e). Out of 11 experiments in which the stimulus pattern was switched from 1 spike per trial to 4 spikes per

trial, we observed only two cases in which the recovered EPSP amplitude exceed 75% of the initial amplitude during the series of 15 trials with bursts (Fig. 1d). Therefore we believe that bursts of spikes actually prevent the stabilization or consolidation of HSD, rather than reversing depression that has developed after the first action potential in the burst.

BDP is distinct from known forms of synaptic plasticity

Is BDP a distinct form of synaptic plasticity or simply a previously known form of activity-dependent synaptic plasticity in a different context? These same sensory neuron-motor neuron connections display posttetanic potentiation (PTP), at both non-depressed and depressed synapses [2, 10]. Initiation of PTP required a train of more than 6 or 8 action potentials (Supplementary Fig. 2a), whereas BDP was effectively initiated by bursts of as few as 2 spikes (Fig. 1b). The time courses of BDP and PTP were also very different. When the EPSP amplitudes for BDP and HSD protocols were examined 20 min after the end of the 15 trial protocols, there was no decline in protection from depression (Fig. 2a,b). In contrast, as is typical at other synapses [11], PTP at sensory neuron-motor neuron synapses declines substantially within minutes (Supplementary Fig. 2b) [2, 10]. Even after a single trial with bursts, BDP is stable, as seen by comparing the second trials during a 15 s and a 60 s ITI (Figs. 1b and 2b). In addition, as discussed below, the protein kinases involved in BDP and PTP are different.

There are almost certainly interactions between BDP and PTP at these synapses. After PTP wanes, the sensory neuron-motor neuron synapse exhibits little or no depression (Supplementary Fig. Fig. 2b and [2]); this suggests that the depression that would be expected to have been induced during the high frequency activity has actually been prevented from developing. We predict that the BDP mechanism is powerfully activated by the prolonged burst that initiates PTP (this prediction is consistent with experiments with kinase inhibitors, discussed below). Indeed, BDP should be recruited during any form of high frequency activity in sensory neurons, such as the bursts of action potentials used to initiate activity-dependent heterosynaptic facilitation in which paired activity enhances the response to facilitatory transmitter [12, 13].

Although both BDP and paired-pulse facilitation are both induced by a pair of spikes at 20 Hz (Fig. 1), these two forms of activity dependent synaptic plasticity are distinct in at least two fundamental respects. First, paired-pulse facilitation persists only hundreds of milliseconds. Second, at these sensory neuron-motor neuron synapses, paired-pulse facilitation is unusual in that it is labile and decreases dramatically after only a single paired-pulse trial [14] (e.g. PSPs indicated by arrow and arrowhead in Fig. 1a, c,). In contrast, BDP remains robust across at least 15 trials and the effects are stable for at least 20 minutes (Fig. 2). Interestingly, paired-pulse facilitation at these synapses is substantially independent of HSD [14], whereas BDP prevents or reverses HSD. (For example, compare the first 2 EPSPs in the following: for 2 spikes per trial in Fig. 1a, trial #1 at a naïve synapse and trial #12 during the BDP protocol, and in Fig. 1c, trial #16, the first burst at a depressed synapse.)

Intense activity in sensory neurons initiates heterosynaptic facilitation of sensory neuron-motor neuron connections by activating interneurons that release facilitatory transmitter, such as 5HT and SCP [15]. Do these bursts of 2–4 action potentials in individual sensory

neurons during BDP produce synaptic enhancement by triggering release of facilitatory transmitter? To determine whether facilitatory transmitter was released by the bursts of 4 spikes, we monitored the modulatory change in the sensory neurons that is the most sensitive response to the facilitatory transmitters 5HT and SCP: increased excitability [16], which results from a decrease in the S-K^+ currents [17, 18]. The change in sensory neuron excitability is also the most sensitive modulatory response to peripheral nerve stimulation (Supplementary Fig. 3). For these measurements of excitability, during each trial, the presynaptic sensory neuron was activated with a 200 msec depolarizing pulse at sufficient intensity to elicit 2 to 5 spikes (see Methods). We observed no increase in sensory neuron excitability during a series of 200 msec stimuli (mean no. of spikes = 3.1 ± 0.60 spikes as compared with 3.1 ± 0.56 spikes in the pretest, n=5). Therefore, we conclude that there was no activation of heterosynaptic facilitatory mechanisms. Nevertheless, the synaptic connections from these same sensory neurons were effectively protected from depression (EPSP #15 ÷ EPSP #1 = 0.86 ± 0.07). Consistent with BDP involving exclusively homosynaptic mechanisms, we have also observed BDP at synapses between VC cluster sensory neurons and LFS motor neurons in culture (Supplementary Fig. 1b). Thus, BDP does not involve either PTP or heterosynaptic facilitation by modulatory neurotransmitters.

Role of Ca^{2+} influx in initiation of BDP

To ask whether BDP is initiated by Ca^{2+} influx, we reduced Ca^{2+} influx by removing 60% of the extracellular Ca^{2+}; low Ca^{2+} saline decreased release approximately 4.5-fold. BDP was eliminated in 40% normal Ca^{2+} saline (Fig. 3a,b). In contrast, HSD was unaffected by the reduction in Ca^{2+} influx (Fig. 3b); this is consistent with HSD at these synapses involving a release-independent mechanism that switches off release sites, rather than a depletion of the readily releasable pool of vesicles [7, 19]. To further explore whether BDP is initiated by an increase in presynaptic Ca^{2+}, we injected presynaptic sensory neurons with EGTA. EGTA is a relatively slow Ca^{2+} chelator that eliminates slower effects of Ca^{2+}, but is less effective in reducing rapid Ca^{2+}-triggered exocytosis [14, 20, 21]. In sensory neurons injected with EGTA, protection from synaptic depression was largely eliminated (Fig. 3c). Postsynaptic increases in Ca^{2+} have been implicated in two forms of synaptic enhancement at these same sensory neuron-motor neuron synapses: PTP and Hebbian LTP [21–23]. BDP was unaffected by postsynaptic injection of either EGTA or the faster chelator BAPTA (Fig. 3c).

BDP requires protein kinase C

We explored the possibility that during BDP, the protection against HSD by presynaptic Ca^{2+} influx might be mediated by a Ca^{2+}-activated protein kinase: CaM kinase II or the conventional PKC isoform, Apl-I. We injected peptides corresponding to the autoinhibitory domains of mammalian CaMKII or PKCα, which are specific inhibitors of each of the respetive classes of protein kinases. Presynaptic injection of PKC(19-31) almost completely eliminated BDP (Fig. 4a,b,c). Injection of the alanine-substituted autoinhibitory domain of CaMKII [CaMKII(281-302)], at a 20-fold higher concentration, had no effect (Fig. 4a,c). It is possible that with PKC(19-31), the gradual decrease in EPSP amplitude during the bursts occurred because inhibiting PKC enhanced synaptic depression; however, blocking PKC had no effect on HSD elicited with single spikes (Fig. 4b). To further evaluate differences

between BDP and PTP at these synapses, we tested PTP with these kinase inhibitors. Presynaptic injection of CaMKII(281-302) completely prevented PTP. The elimination of PTP by CaMKII(281-302) confirms that this peptide reached sensory neuron terminals at effective concentrations. Therefore we conclude that CaMKII does not contribute to BDP. In contrast to BDP, injection of PKC(19-31) into the presynaptic sensory neuron only marginally reduced PTP ($p = 0.051$, Fig. 4d). This partial reduction is consistent with the concept that a component of the synaptic enhancement observed after the tetanus actually reflects elimination of HSD through activation of the BDP mechanism. Results obtained with two other kinase inhibitors, H7 and KN-62, were also consistent with the conclusion that BDP is mediated by PKC and not CaMKII (Supplementary Fig. 4). *Aplysia* neurons express one conventional, one novel and one atypical form of PKC [24, 25]. PKC(19-31) is a selective inhibitor of conventional and novel PKCs, and is relatively ineffective in inhibiting atypical PKCs [26, 27]. It seemed possible that Ca^{2+} could either stimulate conventional PKC Apl-I directly, or it could stimulate the novel PKC Apl-II indirectly by activating phospholipase C (PLC). The PLC inhibitor U73122, which blocks 5HT-induced facilitation of depressed sensory neuron-motor neuron synapses, had no effect on BDP (Supplementary Fig. 5). Thus, BDP does not involve activity-dependent liberation of diacyl glycerol; rather Ca^{2+} influx must act directly to stimulate PKC Apl-I.

Localization of PKC during BDP mediated by interaction with PICK1

The observation that short bursts of action potentials protect these sensory neuron synapses from undergoing depression is surprising because with only 2 to 4 spikes, the increase in Ca^{2+} in presynaptic varicosities is very modest. During a train of action potentials, global Ca^{2+} in sensory neuron varicosities rises only ~12 nM per spike [28], which is substantially below the concentration required to activate Ca^{2+}-dependent isoforms of PKC [29]. This discrepancy suggests that the PKC responsible for initiating BDP must be localized within a microdomain at the active zone near Ca^{2+} channels, where peak Ca^{2+} levels are substantially higher. The C terminus of Ca^{2+}-activated PKCα in mammals contains a PDZ domain recognition motif that mediates the interaction between PKCα and the scaffold protein PICK1 [30]. The Ca^{2+}-activated PKC in *Aplysia*, Apl-I, ends in VTV [31, 32], which resembles a PDZ recognition motif. A highly conserved PICK1 ortholog with a PDZ domain is expressed in *Aplysia* CNS (Supplementary Fig. 6). Apl-PICK1 binds PKC Apl-I, but not the Ca^{2+}-independent PKC, Apl-II (Fig. 5). To test the importance of PDZ interactions for localizing PKC Apl-I during BDP, we injected presynaptically a peptide corresponding to the C terminus of PKC Apl-I, which acts as a dominant negative, displacing PKC Apl-I bound to PICK1 (Supplementary Fig. 7). This Apl-I C terminus peptide completely eliminated BDP (Fig. 6a,b). In contrast, a peptide corresponding to the C terminus of Apl-II had no effect on BDP. To test specifically the involvement of PICK1, as opposed to other PDZ domain proteins that might interact with the PKC Apl-I PDZ domain recognition motif, we injected Fab fragments of affinity purified antibody against the PDZ domain of PICK1 (Supplementary Fig. 8). The anti-Apl-PICK1 antibody effectively blocked BDP, whereas a control antibody had no effect (Fig. 6a,c). These results are consistent with the hypothesis that interactions of PKC Apl-I with the PDZ domain of Apl-PICK1 ediate localization of this kinase near Ca^{2+} channels in the active zone.

BDP helps maintain responsiveness to repetitive stimuli

We next asked whether BDP plays an actual role behaviorally in maintaining the animal's responsiveness to stimuli that are sufficiently intense to activate brief bursts of spikes in siphon sensory neurons. To test whether BDP contributes to maintaining the response to local tactile stimuli during repeated presentations, it was necessary to stimulate sensory neurons to fire in a defined pattern. We used focal capillary electrodes covering approximately a 2 mmdiameter spot of siphon skin in a reduced preparation and measured the siphon withdrawal response (Supplementary Fig. 9) [6]. To distinguish the contribution of BDP from that of "classical" heterosynaptic facilitation at these sensory neuron-motor neuron synapses [9], it is necessary to ensure that these bursts of afferent activity do not initiate facilitation by releasing facilitatory transmitter, primarily 5HT. To prevent any modulation by 5HT of the sensory neuron-motor neuron synapses or other sites in the defensive siphon withdrawal circuit, the abdominal ganglion was superfused with methiothepin and spiperone, which block the AC-coupled and the PLC-coupled 5HT receptors, respectively [33]. We confirmed that 5HT receptors were effectively blocked by testing for increases in sensory neuron excitability after trains of 6 shocks to the siphon nerve, with sufficient current intensity to activate all of the siphon sensory neuron axons. No increase in sensory neuron excitability was observed (Fig. 7a), indicating that 5HT receptors were blocked and that these brief trains of stimuli did not trigger release of physiologically effective levels of the other facilitatory transmitters, all of which affect sensory neuron excitability [17, 34]. Therefore, any synaptic enhancement produced by the brief bursts of sensory neuron action potentials initiated by the siphon skin stimuli must involve exclusively homosynaptic mechanisms.

In these behavioral experiments, the habituation of the siphon withdrawal response activated by single 1.5 msec shocks was compared with the decrement of the response activated by bursts of 4 shocks per trial. With one skin stimulus per trial, the withdrawal response rapidly habituated to less than a third of the initial amplitude. In contrast, with bursts of skin stimuli, the response decremented more gradually. At the end of 15 trials, the response with bursts of 4 stimuli was more than three-fold larger than the habituated response with single stimuli (Fig. 7). Because siphon sensory neuron-motor neuron connections should be maintained at >80% of initial strength due to BDP (Figs. 1b, 2b, 3b,c and 4b), we suggest that most of the habituation in the siphon withdrawal response observed with bursts of 4 stimuli (Fig. 7) is due to changes at other loci in the neural circuit for the reflex [35]. Indeed, the results of these behavioral experiments provide an independent estimate of the contribution of changes at the monosynaptic sensory neuron-motor neuron connection to habituation of the reflex. A comparison of the habituation induced by one stimulus per trial with the habituation induced by bursts of four stimuli per trial suggests that approximately half of the habituation of the siphon withdrawal reflex is due to HSD at this synapse (Fig. 7c). The remaining habituation may be due to plasticity in interneurons that mediate the polysynaptic component of the reflex or to depression at synapses from yet unidentified, low-threshold siphon sensory neurons [5]. (It is not known whether these synapses from low-threshold sensory neurons undergo BDP, as it is not possible to control their firing pattern with peripheral electrical stimuli, without also activating the LE siphon sensory neurons.)

DISCUSSION

We observed in this study that very brief bursts of spikes in presynaptic sensory neurons act to block the dramatic depression that typically occurs at sensory neuron-motor neuron synapses with a series of single action potentials. HSD is effectively prevented by bursts of as few as 2 to 4 spikes. Does BDP actually protect against HSD, or does BDP reactivate transmission at sensory neuron synapses immediately after they have become depressed? At synapses that were already depressed, bursts of spikes acted rapidly, within a few trials, to partially restore synaptic transmission. However, bursts are substantially more effective at preventing HSD than in reversing HSD once it has been established (Fig. 1e). We therefore propose that when non-depressed sensory neuron synapses fire in bursts, the initiation of HSD is interrupted, rather than the bursts reversing HSD that has just developed. Thus, induction of stable HSD must require a few hundred milliseconds because 50 to 200 msec after the first spike, the later spikes in a burst are able to interrupt the shutting off of synaptic sites.

The protection from depression by bursts of several spikes is surprising because depletion of the readily releasable pool of vesicles was previously considered to be the main mechanism underlying HSD at *Aplysia* sensory neuron-motor neuron synapses [36–38], as at many other synapses [11]. During BDP, sensory neurons fire 1 to 3 additional spikes, which evoke additional vesicle release, particularly in the earlier trials (e.g. Figs. 1a and c and Fig. 4b), yet HSD is reduced or eliminated. BDP is clearly incompatible with a vesicle depletion model for HSD. Several independent lines of recent evidence also indicated that vesicle depletion is not the primary mechanism of depression at the sensory neuron-motor neuron synapse [7, 19]. In the present study, when release was reduced in low Ca^{2+} saline, the induction of HSD with single action potentials was unchanged (Fig. 3b). Furthermore, presynaptic EGTA, which decreased release 50%, converted the protection observed with bursts of spikes to HSD with normal kinetics (Fig. 3c). This lack of reduction in HSD when transmitter release is decreased is consistent with an alternative mechanism proposed for HSD: activity-dependent, release-independent, silencing of active zones [7, 19]. Despite the normal initiation of HSD by single action potentials both when Ca^{2+} influx is either decreased (discussed above) or increased [7], the initiation of HSD does involve Ca^{2+}; when Ca^{2+} influx is eliminated completely, sensory neuron activity does not result in HSD (Liu, Negroiu and Abrams, in preparation).

Initiation of BDP involves both Ca^{2+} influx and PKC in the presynaptic sensory neurons. Reducing Ca^{2+} influx, chelating presynaptic Ca^{2+} or inhibiting PKC presynaptically all block BDP. In *Aplysia*, there is a Ca^{2+}-dependent conventional PKC isoform, Apl-I, and a Ca^{2+}-independent novel isoform, Apl-II [31, 32], both of which are inhibited by PKC(19-31). The development of BDP was not affected by the presence of a PLC inhibitor, indicating that PKC Apl-II, which requires DAG for stimulation, does not contribute to BDP. We therefore propose that Ca^{2+} influx during the second, third and fourth spikes activates PKC Apl-I, and that this Ca^{2+}-dependent isoform is responsible for BDP. How is PKC activated by only two to four action potentials? Based on published fura-2 imaging during spike trains [28], we estimate that in sensory neuron varicosities, Ca^{2+} rises approximately 12 nM per spike from a basal level of ~75 nM, which is consistent with measurements on crayfish

neuromuscular boutons [39, 40]. Following 2 to 4 action potentials, the predicted global free Ca^{2+} within a varicosity should be <150 nM. Half-maximal lipid binding of the C2 domains of conventional mammalian PKC isoforms b voccurs at Ca^{2+} concentrations ranging from 0.7 to 5 μM [29]. Modest translocation of mammalian PKC-GFP can be detected only when Ca^{2+} levels exceed 200 nM [41]. In *Aplysia* sensory neurons, translocation of CFP-tagged PKC Apl-I is not observed with moderate activity (as much as 100 spikes at 10 Hz), but rather requires high concentrations of Ca^{2+} (or pairing activity with 5HT) [42]. For PKC Apl-I to be activated by Ca^{2+} transients during bursts of just 2–4 action potentials, this kinase population must be localized within a microdomain near Ca^{2+} channels at the active zone, where peak Ca^{2+} concentrations reach levels substantially higher than the average concentration in the varicosity. Such spatially restricted populations would not be readily resolved in conventional imaging experiments [42]. In mammals, the PDZ domain-containing scaffold protein, PICK1 (protein interacting with C kinase) was identified because it interacts with PKCα [30]. We found that in *Aplysia*, the PICK1 ortholog similarly binds to PKC Apl-I, but not to PKC Apl-II. We explored the possibility this interaction is responsible for localizing PKC at the active zone adjacent to sites of Ca^{2+} influx. Injecting sensory neurons with a dominant negative peptide corresponding to the Apl-I C terminus, which displaces Apl-I bound to Apl-PICK1, powerfully inhibited induction of BDP. An antibody against the PDZ domain of Apl-PICK1 similarly blocked BDP. These results suggest that interactions of PKC Apl-I with PICK1 may mediate localization of PKC near Ca^{2+} channels at exocytosis sites. We cannot distinguish from our results whether this PDZ domain interaction is responsible for localization of PKC Apl-I near the sites of Ca^{2+} influx or for targeting PKC to a substrate that mediates BDP, or both. The concept that PICK1 mediates the localization of PKC Apl-I in the vicinity of Ca^{2+} channels and is responsible for the unparalleled sensitivity of this kinase to Ca^{2+} influx is illustrated in the cartoon in Supplementary Fig. 10.

How could PKC function as a sensitive discriminator of the pattern of activity in sensory neurons, initiating BDP when sensory neurons fire bursts of only 2 to 4 spikes, yet not with single action potentials? In the presence of the K^+ channel blocker TEA, which causes a >5-fold increase in the duration of the action potential and a substantial increase in Ca^{2+} influx, single action potentials still trigger HSD [43]. Analysis of C2 domains of conventional mammalian PKC isoforms indicates that binding of the full complement of Ca^{2+} ions occurs only when the C2 domain interacts with phospholipid [29]. Figure 8 illustrates a model in which Ca^{2+} initiation of PKC translocation and the formation of an additional phospholipid-dependent Ca^{2+} binding site provide a temporal filter by which PKC could discriminate the pattern of firing in sensory neurons. In this model, Ca^{2+} influx during the first spike initiates translocation of PKC to the membrane (Fig. 8a). However, because this translocation requires time, when PKC interacts with the membrane the Ca^{2+} concentration has already dropped below the level required to occupy the phospholipid-dependent binding site. A second action potential is then required to cause additional Ca^{2+} influx, which activates the membrane-associated PKC or stabilizes its association with the membrane; the active PKC then prevents the synapse from switching to a silent state (Fig. 8b). Because Ca^{2+} influx initiates HSD even with reduced extracellular Ca^{2+} levels or with intracellular EGTA, we predict that the Ca^{2+} sensor that initiates HSD has high affinity and is located very proximal

to the Ca^{2+} channel. Thus, Ca^{2+} has two opposing consequences. Ca^{2+} influx during a single spike initiates HSD, but also triggers translocation of PKC. Ca^{2+} influx during subsequent spikes in a burst activates or stabilizes the translocated PKC, interrupting the development of HSD. Recent experiments demonstrated that bursts at frequencies as low as 5 Hz are quite effective in protecting against depression (Wan et al. in preparation). Therefore, we hypothesize that PKC must remain in the membrane for at least 200 msec after the first spike, and that the silencing of release sites by the HSD mechanism requires more than 200 msec to be consolidated. In summary, the translocation of PKC and the appearance of the phospholipid-dependent Ca^{2+} binding site could represent a sensitive molecular detector for the pattern of presynaptic spike activity.

At highly depressed sensory neuron-motor neuron synapses, 5HT acts via the Ca^{2+}-independent PKC isoform Apl-II to restore synaptic transmission to the same level or above the level prior to depression [44]. Interestingly, when 5HT is present during continued low frequency stimulation of sensory neurons in an HSD protocol, the development of HSD is interrupted only transiently, for a few minutes [33, 44]. Thus, 5HT and Apl-II are more effective than Bursts and Apl-I in reversing HSD (Fig. 1), but less effective in blocking the development of HSD. This difference could reflect different substrates of the two PKC isoforms at the active zone. Alternatively, the lack of efficacy of continuous exposure to 5HT in preventing the development of HSD could result from desensitization of the 5HT-PKC pathway [33].

We hypothesized that BDP plays a critical behavioral role, preventing animals from becoming unaware of stimuli in their environment that are potentially important. If highly localized stimuli rapidly caused HSD at sensory neuron-motor neuron connections, as we would predict from earlier electrophysiological experiments, animals would quickly become insensitive to potentially important stimuli. Once sensory neuron synapses become depressed with initial activation, even if a local stimulus became stronger and could potentially be damaging, these sensory neurons would be unable to transmit information to the CNS. It is believed that a strong local stimulus would activate facilitatory interneurons and enhance sensory neuron synapses; however, this heterosynaptic facilitatory mechanism might not be effectively recruited if sensory synapses were already substantially depressed. BDP should provide a homosynaptic mechanism to maintain or partially restore transmitter release when local tactile stimuli have at least moderate intensity. We tested behavioral habituation of the siphon withdrawal reflex in a preparation where we could precisely control the pattern of sensory neuron firing and where heterosynaptic modulatory input was either blocked by 5HT receptor antagonists or was not effectively recruited, in the case of non-serotonergic inputs. In the absence of heterosynaptic modulation, bursts of 4 spikes in sensory neurons produced an approximately two-fold reduction in habituation. These results suggest that BDP plays an important role in the behaving animal, contributing to maintaining responses to stimuli when they are sufficiently intense to have behavioral significance. With sufficiently strong tactile stimuli, the defensive withdrawal response undergoes little or no habituation [45]. Presumably, with strong stimuli, heterosynaptic modulation acts, together with BDP, to maintain the amplitude of the withdrawal response.

This switch between HSD and BDP, which depends upon the pattern of afferent activity, acts to gate sensory input. The animal remains responsive to moderate intensity stimuli, while ignoring weak stimuli. Although HSD at the sensory neuron-motor neuron synapse in *Aplysia* has traditionally been considered in the context of cellular mechanisms of learning, sensory gating typically functions to select those stimuli to which an animal responds or ignores. It is difficult to distinguish between sensory gating that changes depending upon stimulus intensity and simple non-associative learning. Nevertheless, we believe that the switch between HSD and BDP at the sensory neuron-motor neuron synapse displays features more typical of sensory gating than of learning. Habituation typically occurs with repeated stimulus presentations over many trials, and dishabituation typically involves additional pathways and heterosynaptic modulatory mechanisms. The “decision” as to whether a sensory neuron synapse will undergo HSD or BDP occurs rapidly, with very modest activity in a single sensory neuron and in the absence of heterosynaptic modulatory input. After only a single trial, synaptic strength is significantly reduced when a presynaptic sensory neuron is activated with single spikes as compared with bursts of only two spikes (Fig. 1b). Not only does induction of HSD occur rapidly with minimal presynaptic activity, but HSD is at least partially reversible if the pattern of firing changes to bursts; even bursts of two spikes produced significant reversal of HSD. The BDP-HSD switch is functionally similar to attention in that it determines whether an animal will respond to a particular stimulus. Sensory gating that involves regulation of the gain of afferent inputs is an important mechanism in spatial attention in both birds and primates [46, 47]. Attention in higher vertebrates has been categorized as being either “bottom-up,” where stimuli draw attention, or “top-down,” where attention is voluntarily directed [48, 49]. The BDP-HSD switch is functionally similar to bottom-up mechanisms of attention that are driven by stimulus salience.

Regardless of whether this bidirectional switch of release sites between silent and active states itself represents sensory gating or a simple mechanism of learning, the BDP-HSD switch is clearly important in the context of learning. During conditioning, these same sensory neuron-motor neuron synapses undergo associative facilitation involving Hebbian plasticity [22, 50]. Hebbian mechanisms during learning cannot be effectively recruited if afferent synapses are weakened and postsynaptic neurons are not sufficiently depolarized. Thus, BDP may contribute to learning by ensuring that afferent inputs remain effective even when the stimulus that activates these inputs has been presented repeatedly. From this perspective, BDP could be a cellular mechanism enabling an animal to attend during learning to a stimulus that occurs repeatedly. These mechanosensory neurons have relatively high thresholds, and appear to function as nociceptors [8]; the same BDP mechanism could also play a role in gating the afferent input from these sensory neurons in response to painful stimuli.

METHODS

Electrophysiology

Aplysia californica, weighing 70 to 150 g (obtained from Alacrity or Marinus, Inc.) were anesthetized by injection with isotonic $MgCl_2$. Abdominal ganglia were removed and the

ventral surface of the left hemiganglion was desheathed in a 1:1 mixture of $MgCl_2$ and artificial sea water. Ganglia were superfused at room temperature with high divalent saline (6 × normal Ca^{2+}, 1.6 × normal Mg^{2+}) [14] to reduce spontaneous synaptic activity: 328 mM NaCl, 10 mM KCl, 66 mM $CaCl_2$, 88 mM $MgCl_2$, 10 mM Na-HEPES, pH 7.6, supplemented with nutrients [7 mM glucose, MEM essential and non-essential amino acids (0.2 × normal concentration, Invitrogen), and MEM vitamin solution (0.7 × normal concentration, Invitrogen)]. This high-divalent saline does not alter synaptic strength [14].

Siphon sensory neurons and LFS motor neurons were penetrated with single 12–20 MΩ microelectrodes filled with 2 M K-acetate and 0.4 M KCl. During penetration, 0.5 nA hyperpolarizing current was injected to prevent sensory neuron firing. Injection of EGTA, BAPTA, H7 or peptides was by passive diffusion from the beveled recording pipettes; injected compounds were diluted into 20 mM K-HEPES and 400 mM KCl, pH 7.3, and electrodes filled with this solution were beveled to 7 to 10 MΩ. Control neurons were penetrated with beveled pipettes containing the same buffered saline. sensory neuron action potentials were elicited by injection of 2 msec depolarizing current pulses. The membrane potential of postsynaptic motor neurons was maintained at 60 mV below the resting potential to prevent action potentials. After a synaptic connection was identified, the synapse was rested for a minimum of 15 min before beginning an experimental protocol. During bursts and tetanic stimulation, the intensity of the depolarizing current injected was increased 20 to 40% above threshold to ensure that an action potential was elicited during each depolarizing pulse. For BDP protocols, when sensory neurons were activated with bursts of 2–4 stimuli, only the amplitude of the first EPSP in a burst was measured. The amplitudes of the subsequent EPSPs in a burst change with repeated bursts in a manner independent of synaptic depression as described previously [14] (see Fig. 1 and Supplementary Note 1). For BDP experiments, the intertrial interval was 15 sec, except in Figure 2, where it was 60 sec.

In experiments examining the effect of intracellular injection of compounds, stimulation protocols were begun after allowing ≥30 minutes for diffusion to presynaptic or postsynaptic regions of the sensory neurons or motor neurons: a minimum of 30 minutes for BAPTA and EGTA, 40 minutes for H7 (Biomol) and 45 min for peptides. KN-62 (Biomol) was dissolved in DMSO and diluted to a final DMSO concentration of 0.1%; this concentration of DMSO had no detectable effect on synaptic connections. Ganglia were incubated 30 min in saline with 20 μM KN-62 to allow penetration of the compound into neuropilar processes.

Measurement of excitability during BDP protocol

To test for excitability changes in sensory neurons that might occur if heterosynaptic modulatory input were activated by the bursts of sensory neuron activity, we stimulated a sensory neuron with a 200 msec depolarizing current pulse, at an intensity that produced 2–5 action potentials in a pretest. After identifying the effective current intensity, we used this 200 ms pulse to activate sensory neurons in each of 15 trials at a 15 sec ITI. During the protocol, the amplitude of the EPSP in a postsynaptic motor neuron was measured as well as the firing pattern during the depolarizing pulse. To test efficacy of drugs in blocking 5HT

receptors, excitability in sensory neurons was tested by injecting 500 msec depolarizing current pulses at 2 times threshold.

Peptides

The auto-inhibitory domain peptide of PKCα [PKC(19-31)] and the autoinhibitory domain of CaMKII [CaMKII(Ala286, 281-302) in which alanine was substituted for threonine 286], were purchased from Bachem. A peptide corresponding to the 10 residues at the C terminus of PKC Apl-I (YVNPEFVVTV) was purchased from SynPep (Dublin, CA); a peptide corresponding to the C terminus sequence of PKC Apl-II (LEMEASGQAH) was purchased from Bio-Synthesis (Lewisville, TX). All peptides were aliquoted and stored dry at −20 °C. Peptides were injected in intracellular saline: 400 mM KCl and 20 mM K-HEPES, pH 7.3. The concentrations in the pipette were 1 mM and 20 mM for PKC(19-31) and CaMKII(281-302), respectively. The concentration in the pipette of both PKC C-terminus peptides was 2 mM. Approximate estimates of peptide concentrations in neuropilar processes of injected neurons are ~1% of pipette concentrations based on fluorescein-labeled peptides.

Anti-Apl-PICK1 antibody

Rabbit antibodies were generated against a peptide corresponding to residues 13–32 of Apl-PICK1 (DKLGMTVTSGHVVLKKDTQN) purchased from Bio-Synthesis (Lewisville, TX). Antibodies were affinity purified on an antigen column, and Fab fragments were generated by overnight treatment with immobilized papain (Thermo Scientific). Undigested antibody and Fc fragments were removed with a Protein A column. The resulting Fab fragments were injected in intracellular saline (described above). Control antibody was similarly prepared Fab fragments from affinity purified antibody against AC Apl-B [51].

Behavior

Behavioral experiments on the siphon withdrawal reflex were conducted following the protocol of Antonov et al. [6]. The siphon was dissected from the animal together with the abdominal ganglion; care was taken not to stretch the siphon nerve. The siphon was partially cut along the longitudinal axis from the distal end, so that one side could be immobilized for stimulation while the other side remained free to move. The immobilized side was attached at the edge with minuten pins to the Sylgard bottom of a Plexiglas chamber. The distal end of the unpinned side of the siphon was attached via suture thread to a strain gauge (Harvard Apparatus model #724486) (Supplementary Fig. 10). At the end of dissection, filtered artificial sea water (ASW) was injected into the siphon sinus from the proximal end to flush out the $MgCl_2$ used for anesthesia. The chamber was perfused with aerated, filtered ASW. The abdominal ganglion was isolated from the rest of the chamber with a ring and the gap through which the nerve passed was sealed with petroleum jelly. The ganglion compartment was superfused separately with normal saline [460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, 10 mM Na-HEPES, pH 7.6, supplemented with nutrients (7 mM glucose, MEM essential and non-essential amino acids (0.2 × normal concentration, Invitrogen), and MEM vitamin solution (0.7 × normal concentration, Invitrogen)] with the 5HT receptor antagonists methiothepin (150 μM) and spiperone (100 μM) [33]. Spiperone was prepared as a

1000x stock in DMSO and stored in aliquots at −20 °C; methiothepin was dissolved fresh for each experiment in saline.

A pair of capillary electrodes with tips that covered approximately a 2 mm diameter spot of skin were used to directly activate sensory neuron terminals in the siphon skin with stimulus pulses 1.5 msec duration from a stimulus isolation unit (WPI #A385). In some experiments, the left ventral surface of the abdominal ganglion was desheathed to permit intracellular recording from the siphon sensory neurons during the delivery of stimuli to the skin. When we monitored action potentials in the somata of siphon sensory neurons, action potentials typically followed the skin stimuli one-for-one; occasionally, a sensory neuron initiated one or two additional spikes during the series of 4 stimuli (extra spikes were not observed with single stimuli). Thus, the number of spikes during a burst was 4–6 (most commonly 4); therefore, to verify that heterosynaptic facilitation was not activated during this protocol, we delivered a series of 6 stimuli.

Data analysis

Data were acquired digitally with DT 2821 A–D interface (Data Translation, Marlboro, MA) and analyzed using Spike software (Hilal Associates). Analysis of variance (ANOVA) was performed using SPSS. Differences between groups in the time course of synaptic depression or of the decrease in the paired-pulse ratio were evaluated by a repeated measure's ANOVA, testing trial x treatment interactions. EPSP data were first normalized to the amplitude of the initial EPSP and then transformed by arcsine-square root transformation prior to ANOVA. Log transforms of EPSPs for PSP data were used for experiments involving PTP or facilitation, rather than HSD. Transformed normalized EPSP data during depression protocols (n = 39) were tested for normality using the Kolmogorov Smirnov test; EPSP amplitudes for all time points after transformation were distributed normally. After ANOVA, pairwise posthoc comparisons were done Sidak adjustment for multiple comparisons. Recovery of depressed synapses with after changing the stimulus protocol from single stimuli to trains of 4 stimuli was analyzed with paired two-tailed *t* tests (comparing EPSPs on trials 19–21 with EPSPs on trials 13–15). Differences with a $p < 0.05$ were considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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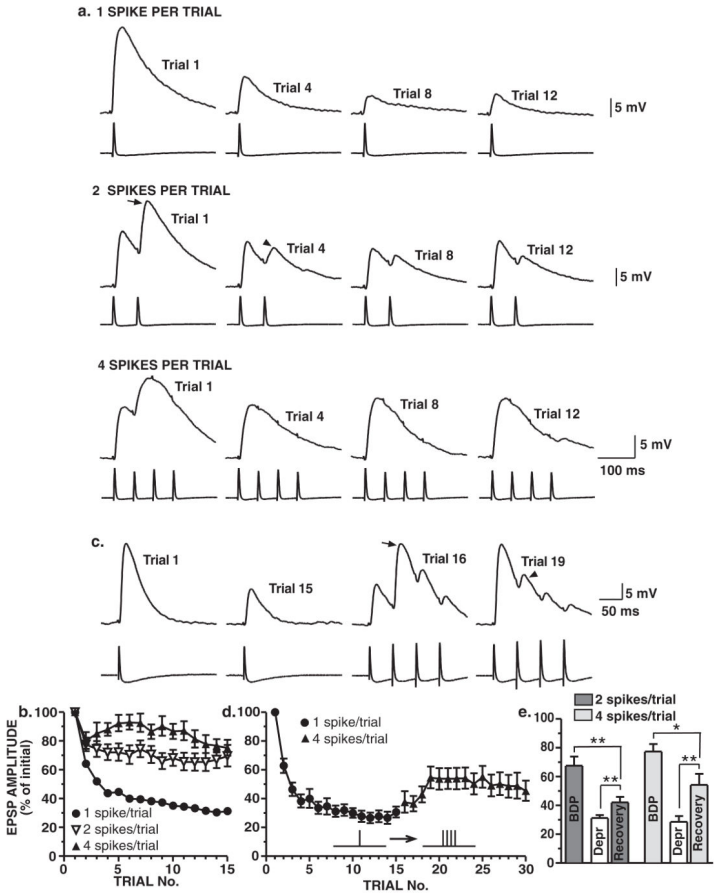

Figure 1.

Bursts of 2 to 4 spikes in presynaptic sensory neurons protect against development of HSD and partially reverse previously developed HSD. **a.** Sensory neuron (SN)-motor neuron (MN) EPSPs when sensory neurons were activated with single stimuli per trial or two to four stimuli per trial at 20 Hz. Intertrial interval (ITI) was 15 sec. In quantifying BDP, only the amplitude of the first EPSP during each burst is measured, which can be compared with the EPSP when sensory neurons are activated by single stimuli. In these experiments, the amplitude of the second EPSP within a burst is largely independent of the amount of depression of the first EPSP [14]. [Arrow and arrowhead highlight the transient paired-pulse facilitation during the burst, which disappears with repeated testing (as discussed in Results.] Amplitude calibration bars apply to postsynaptic records. **b**. Combined data for experiments when sensory neurons fired 1, 2 or 4 spikes per trial (n = 39, 26 and 18, respectively). Both 2 spikes per trial and 4 spikes per trial resulted in significant reduction in HSD. [(see Supplementary Table 1 for all repeated measures ANOVA results); both 2 spikes per trial and 4 spikes per trial differed significantly from 1 spike per trial, $p < 0.001$ for both comparisons, but not from each other, $p = 0.113$. With one spike per trial, trial #1 differed significantly from all subsequent trials, $p < 0.001$, indicating that significant HSD was induced by trial #2.] All data plotted are mean ± SEM.

c, d. Reversal of HSD by bursts of spikes. sensory neurons were stimulated to fire a single spike per trial for 15 trials; the stimulation pattern was then changed to 4 spikes per trial on

trial #16. **c.** Example of reversal of HSD by bursts. In this synapse, the reversal of HSD was unusually effective. Arrows, as in panel a. **d.** Group data for experiments on reversal of HSD by bursts of spikes (n = 11). **e.** Bursts are less effective in restoring transmission at depressed synapses than in protecting sensory neuron synapses from undergoing HSD. Depressed ("Depr") is the mean EPSP amplitude on trials #13–15 (with single spikes). "Recovery" is the mean EPSP amplitude on trials #19–21 (with bursts). (Data for 4 spikes per trial are from panel b.) Both bursts of 2 and 4 spikes produced significant reversal of synaptic depression ($p < 0.001$ for both, n = 18 and 11 respectively). BDP is the mean EPSP amplitude on trials #13–15 from experiments in Fig. 1b. Bursts are significantly less effective in restoring transmission at depressed synapses than in protecting sensory neuron synapses from undergoing HSD ($F_{1,70} = 15.3$, $p < 0.001$; with both 2 spikes and 4 spikes, reversal of depression was less effective than protection of naïve synapses, $p = 0.002$ and 0.017, respectively).

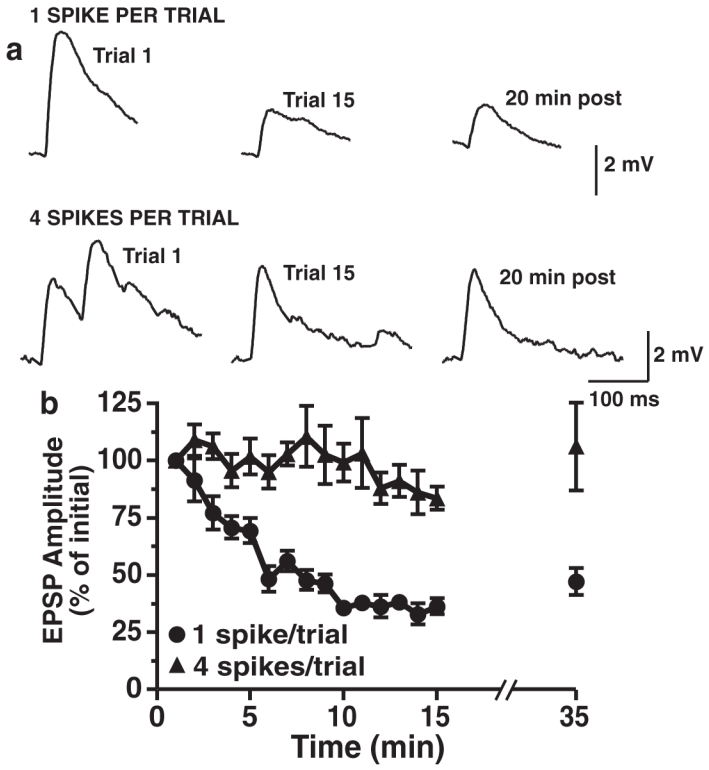

Figure 2.

Effect of BDP on sensory neuron synapses is stable, distinguishing BDP from PTP. **a.** Examples of EPSPs from sensory neuron-motor neuron synapses activated with either one spike per trial or 4 spikes per trial at a 1 min ITI. EPSPs are shown for trials #1 and #15, and for a posttest 20 min after trial #15. **b.** BDP persists after induction for tens of minutes. Group data from experiments in **a** (n = 6). Amplitudes of EPSPs with BDP protocol were significantly greater than with HSD protocol on trials #13–15 ($p < 0.001$) and at 20 min posttest ($p = 0.017$). For HSD or BDP, there was no significant difference between the EPSP amplitudes at the posttest and during trials #13–15 ($p = 1.0$ for both); at the 1 min posttest, mean EPSP = 98.0 ± 10.0% of initial. (Thus, there was neither recovery from HSD nor waning of BDP after 20 min.)

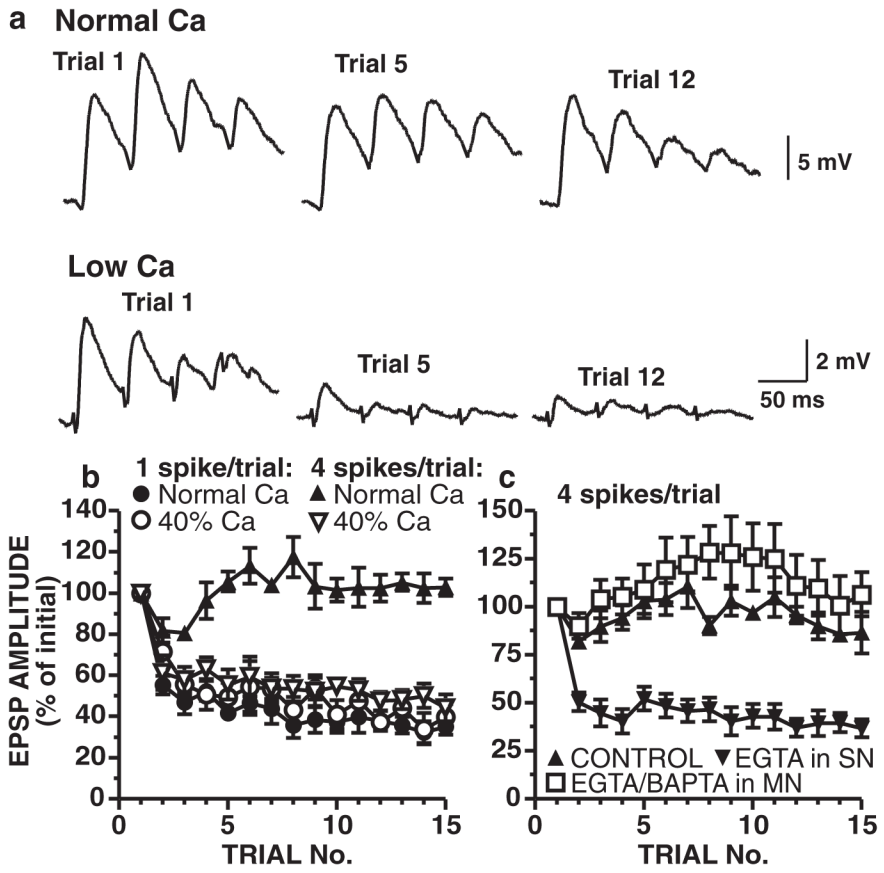

Figure 3.

Initiation of BDP involves presynaptic Ca^{2+} influx. **a**. Examples of EPSPs when sensory neurons fired bursts of 4 spikes in normal high-divalent saline or high-divalent saline with a 60% reduction in Ca^{2+}. **b.** BDP was eliminated in low Ca^{2+} saline. Group data for BDP experiments as in a. BDP in low Ca^{2+} was significantly different than in normal Ca^{2+} ($p < 0.001$), but was not significantly different from normal HSD ($p = 0.691$, pairwise comparisons). In contrast, the induction of HSD with 1 spike per trial was unaffected by this reduction in extracellular Ca^{2+} ($p = 0.234$), which resulted in a 4.5-fold decrease in initial EPSP amplitude. **c.** Presynaptic injection of EGTA eliminated BDP elicited with 4 spikes per trial, whereas postsynaptic EGTA or BAPTA did not affect BDP. When sensory neurons were injected with EGTA (50 mM in pipette, $n = 7$), synapses showed significantly less protection from depression than controls ($n = 4$, $p = 0.009$ for pairwise comparison) or than when motor neurons were injected with EGTA (100 mM in pipette, $n = 3$, $p = 0.011$) or BAPTA (200 mM in pipette, $n = 4$, $p < 0.001$). Postsynaptic injection of either EGTA or BAPTA did not affect induction of BDP, as compared with vehicle-injected controls ($p = 1.00$ and 0.72, respectively). [There was no significant difference during the BDP protocol between synapses with postsynaptic EGTA or postsynaptic BAPTA ($p = 0.907$); therefore these two sets of results have been combined and plotted as a single set of data.]

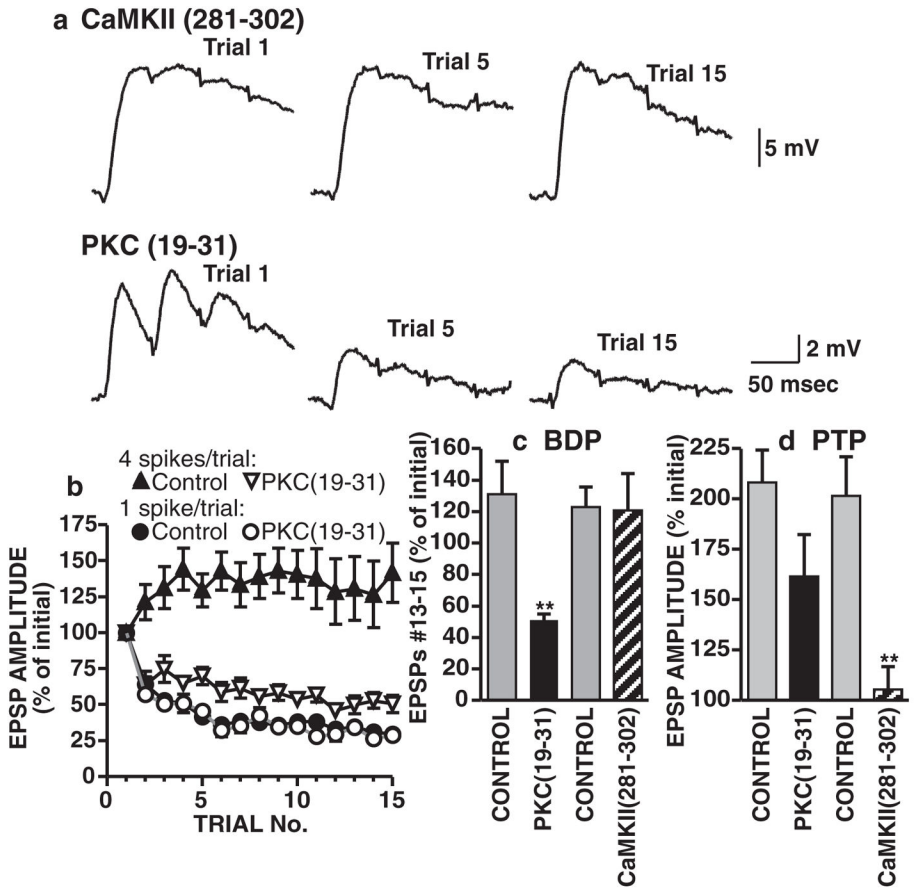

Figure 4.

Initiation of BDP involves PKC, but not CaMKII. **a.** Examples of synapses, activated with 4 spikes per trial, after presynaptic injection of autoinhibitory domain peptides from either CaMKII or PKC. The concentration in the pipette was 1 mM for PKC(19-31) and 20 mM for CaMKII(281-302). **b.** PKC(19-31) in sensory neurons blocks BDP, but does not affect HSD. (n = 8 and 12, for BDP and HSD, respectively). BDP was significantly reduced by presynaptic PKC(19-31) ($p < 0.001$), whereas there was not a significant effect on depression with single spikes ($p = 0.69$). [For PKC(19-31), the small residual difference between 1 spike and 4 spikes per trial was still significant ($p = 0.001$).] PKC(19-31) did not significantly affect the amplitude of EPSP #1 compared with pre-injection amplitude. **c.** BDP was not affected by CaMKII(281-302) (n = 9, $p = 0.97$). Average of EPSP amplitudes on trials 13–15 is expressed as a percent of initial EPSP amplitude. For comparison, EPSPs #13–15 from BDP experiments in **b** are plotted; BDP was effectively inhibited by PKC(19-31) (n = 8, $p = 0.005$). **d.** PTP was blocked by presynaptic injection of CaMKII(281-302) (n = 5, $p = 0.003$). PKC(19-31) resulted in a marginally significant, partial decrease in PTP (n = 8, $p = 0.051$). A small contribution of PKC could reflect activation of the BDP mechanism by the train of spikes that was used to induce PTP. PTP was measured 1 min after stimulation of sensory neurons at 20 Hz for 2 sec.

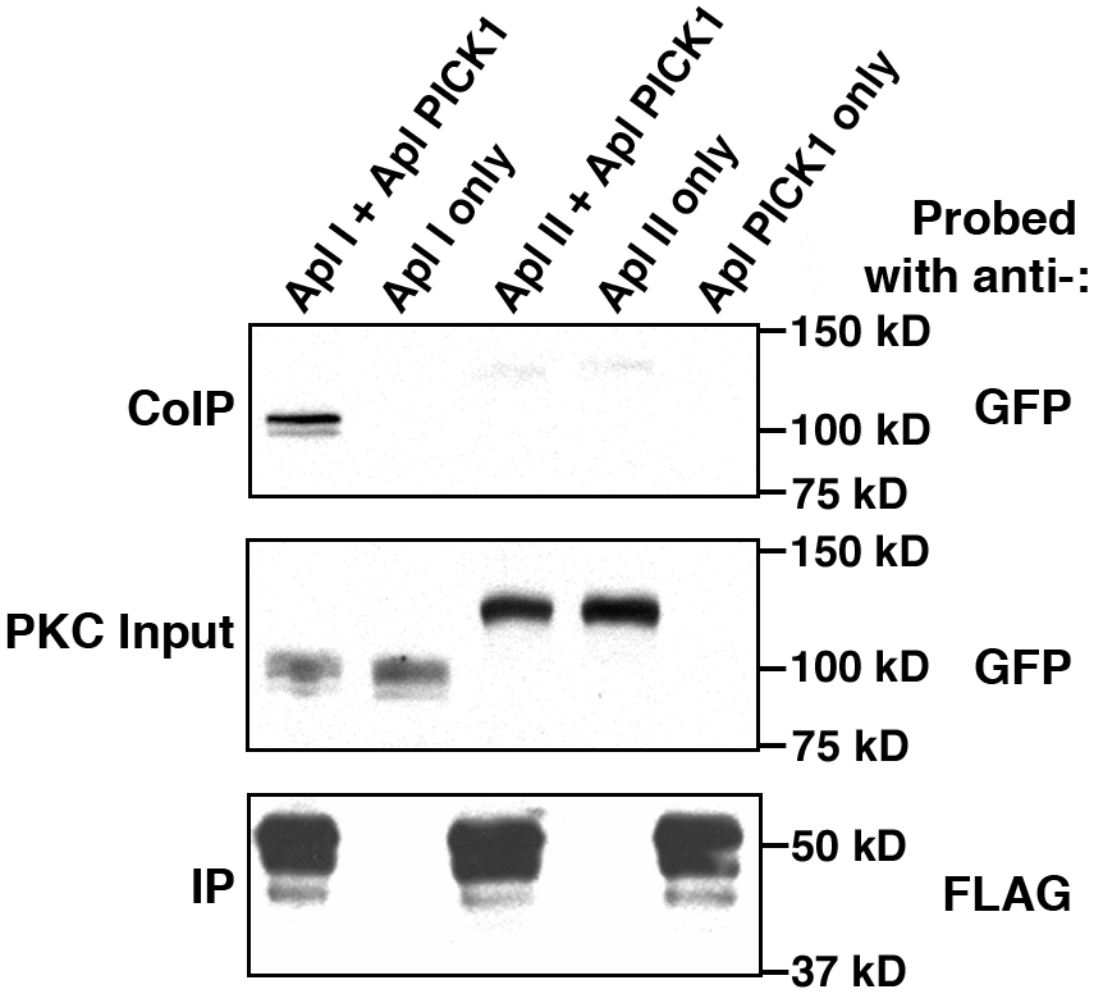

Figure 5.

The Ca2+-activated PKC Apl-I interacts with the PDZ domain protein *Aplysia* PICK1. To test whether PKC Apl-I binds to Apl-PICK1, CFP-tagged PKC Apl-I was co-expressed with FLAG-tagged Apl-PICK1 in HEK 293 cells. Alternatively, GFP-tagged PKC Apl-II was co-expressed with FLAG-tagged Apl-PICK1. Agarose beads coupled to anti-FLAG antibody were used to immunoprecipitate FLAG-Apl-PICK1. PKC Apl-I co-immunoprecipitated with anti-FLAG beads, but only in the presence of FLAG-Apl-PICK1. (Very faint bands for PKC Apl-II are visible; unlike Apl-I, this binding does not require FLAG-Apl-PICK1.) Immunoblots were probed with anti-GFP or anti-FLAG antibodies; the anti-GFP antibody also recognizes CFP. (Blots shown have been cropped to show the relevant bands. Full-length blots/gels are presented in Supplementary Figure 11.)

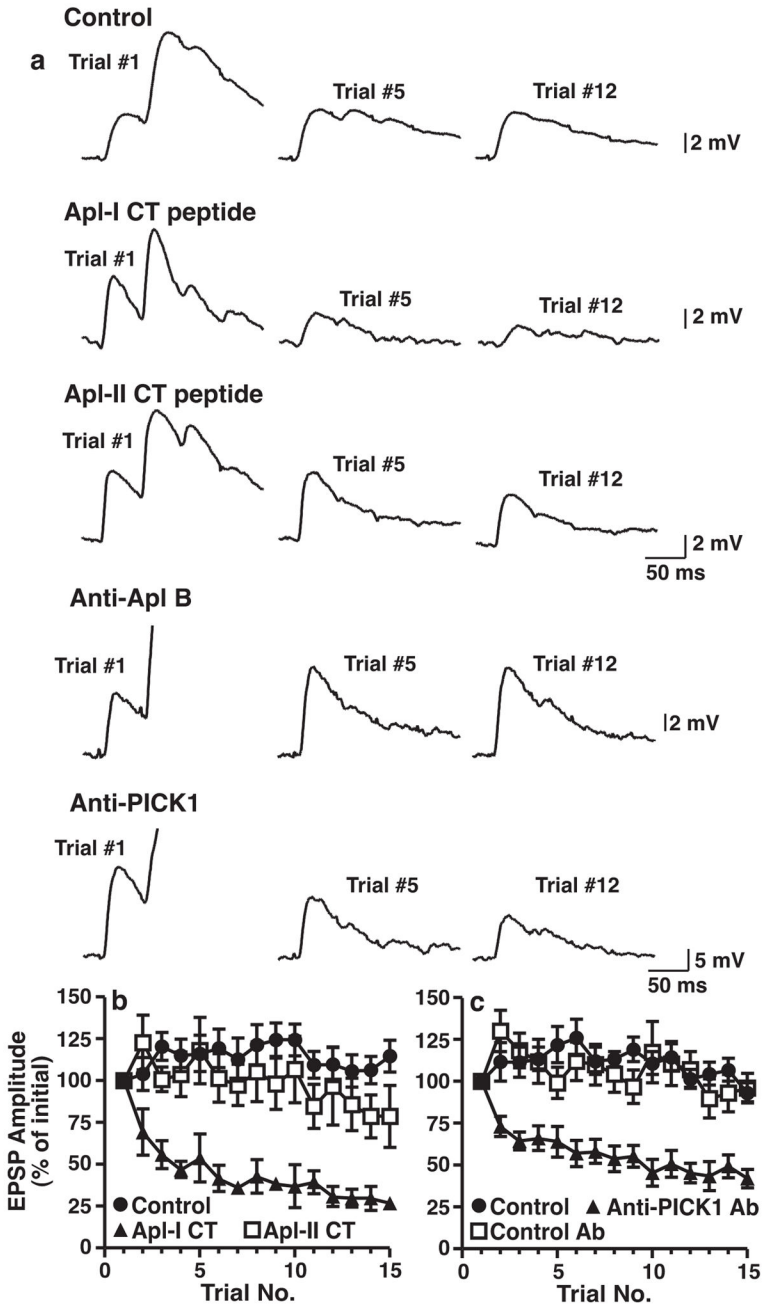

Figure 6.

BDP depends upon PDZ domain interactions. Presynaptic sensory neurons were injected with either a 10 AA peptide corresponding to the C terminus of PKC Apl-I, which ends in a PDZ binding motif, a 10 AA peptide corresponding to the C terminus of PKC Apl-II, or an antibody against the PDZ domain of *Aplysia* PICK1. **a.** Example of synapses activated with 4 spikes per trial from sensory neurons injected with either vehicle (Control), Apl-I C terminus peptide, Apl-II C terminus peptide (3 mM peptide in pipette), affinity purified antibody against the PDZ domain of *Aplysia* PICK1 or control antibody (affinity purified

antibody against *Aplysia* adenylyl cyclase AC-*Apl*B, which is not expressed in sensory neurons (see Methods). Note, BDP was eliminated by both the Apl-I C terminus peptide and by the anti-Apl-PICK1 antibody. **b.** Group data for sensory neurons injected with Apl-I C terminus peptide (n = 8), Apl-II C terminus peptide (n = 7) or vehicle (n = 15). Apl-I C terminus peptide significantly reduced BDP ($p = 0.001$, pairwise comparison). BDP was unaffected by Apl-II peptide [$p = 0.474$, pairwise comparison; the effect of the two peptides was significantly different, $p = 0.006$]. c. Group data for sensory neurons injected with anti-Aplysia PICK1 antibody (n = 7), control (anti-AC-*Apl*B) antibody (n = 8), or vehicle (n = 12). The anti-Apl-PICK1 antibody significantly reduced BDP compared with either vehicle or the control antibody ($p < 0.001$ and $p = 0.001$, respectively, pairwise comparisons), whereas the control antibody had no effect ($p = 0.96$).

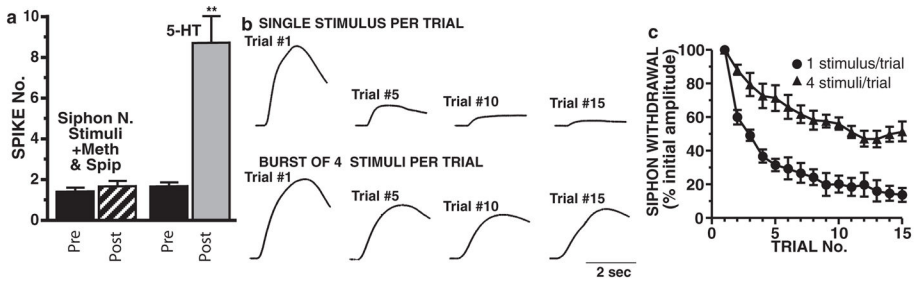

Figure 7.

Bursts of spikes in siphon sensory neurons reduce habituation of siphon withdrawal response via a homosynaptic mechanism. Two 5HT receptor antagonists, 150 μM methiothepin and 100 μM spiperone, were used to block effects of any 5HT released by sensory neuron activity. **a.** To confirm blockade, the siphon nerve was stimulated at intensity that activated all LE sensory neuron neurons with 6 shocks at 20 Hz; no increase in sensory neuron excitability occurred ($p = 0.197$). 5HT (5 μM), in the absence of receptor antagonists, substantially increased sensory neuron excitability ($p = 0.001$) in same ganglia. **b.** Examples of siphon withdrawal response elicited by one shock per trial or 4 shocks per trial at 20 Hz, delivered focally to 2 mm diameter area of siphon skin (ITI = 1 min). To deliver methiothepin and spiperone, abdominal ganglion was superfused independently from the siphon (Supplementary Fig. 8). **c.** Group data from experiments in b. With bursts of 4 skin shocks per trial, the siphon withdrawal response decremented more gradually than with 1 shock per trial ($F_{14,126} = 4.66$; $p < 0.001$). After 15 trials with bursts of 4 stimuli, the habituated response amplitude was 3.5 times greater than with single stimuli. There was no difference in average peak amplitude of the response on trial #1 for single stimuli and bursts of 4 stimuli (776 ± 164 vs. 784 ± 64, respectively, arbitrary units), suggesting the brief bursts of sensory neuron spikes did not dramatically enhance the synaptic input to interneurons and motor neurons compared with single sensory neuron spikes.