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Presynaptic mechanism of action induced by 5-HT in nerve terminals: Possible involvement of ryanodine and IP_3 sensitive Ca^{2+} stores

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Abstract

Although modulation of transmitter release by serotonin (5-HT) at crayfish neuromuscular junctions has been known since 1965, the mechanisms of action have not been established in this classical synaptic preparation. We show that injections of adenophostin-A (an IP_3 analog) in the nerve terminals greatly enhances synaptic transmission. Exposure to ryanodine (Ry) produces a biphasic response: at low concentration it is excitatory and high concentration it is inhibitory. Likewise, a low concentration (1 μM) of caffeine enhances synaptic transmission, whereas a high concentration (10 mM) has little effect on transmission. The varied responses and sensitivity to Ry and caffeine suggest a Ca^{2+}-induced Ca^{2+}-release mechanism and/or the presence of an IP_3-receptor within the terminal. Thus, it is likely 5-HT's response is due to activation of intracellular pathways, which subsequently release Ca^{2+} from internal stores.
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1. Introduction

Transmitter release at chemical synapses is well known to depend on the intracellular concentration of Ca^{2+}, which can be increased by influx of extracellular Ca^{2+} through voltage-gated calcium channels and/or by release of Ca^{2+} from internal stores. Major sources of intracellular sources of Ca^{2+} include smooth endoplasmic reticulum (ER), mitochondria, Golgi apparatus, synaptic vesicles, and calcium bound to molecules such as calmodulin (Brailoiu et al., 2002). Although ER has not traditionally been ascribed a role in neurotransmitter release, attention to its involvement in neuronal function is increasing (reviewed by Petersen and Cancela, 1999; Mattson et al., 2000).

Release of Ca^{2+} from ER can be affected by intracellular mediators such as inositol 1,4,5-trisphosphate (IP3) (Berridge, 1997, 2005; Berridge et al., 2000). Release of Ca^{2+} is also possible by activation of ryanodine receptors, cyclic adenosine diphosphate-ribose (cADPR), Ca^{2+}, NAADP, ryanodine, caffeine, or Ca^{2+} itself (the so-called Ca^{2+}-induced Ca^{2+}-release or CICR) (Bouchard et al., 2003; Brailoiu et al., 2005; Lee, 2000, 2001; Mattson et al., 2000; Petersen and Cancela, 1999). Ryanodine receptors within presynaptic nerve terminals have been established to alter evoked transmitter release (Galante and Marty, 2003) and the size of the quantal unit at neuromuscular junctions (Liu et al., 2005).

One way to learn more about the ER function is to perturb it directly or indirectly with the use of neuromodulators. The effects of the neuromodulator, serotonin (5-HT), are commonly investigated in many neural systems, particularly in arthropods, because of the ease of the systems. For example, the opener muscle of the crayfish walking leg is innervated by a single excitatory motor neuron which is influenced by exogenous exposure to 5-HT. Understanding the mechanism of action of 5-HT and the possibility of interaction with the ER in single identified neurons at crustacean neuromuscular junctions (NMJs) may well prove useful for other animal models (Cooper et al., 2001, 2003; Southard et al., 2000; Sparks and Cooper, 2004; Tabor and Cooper, 2002).

Serotonin's ability to enhance transmitter release at the crayfish NMJ was reported to be due to an increase in intracellular calcium levels resulting from an activation of an IP_3 cascade (Dixon and Atwood, 1989b). With direct measures of free-calcium ions by calcium-sensitive indicators, 5-HT did

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promote an increase in $[Ca^{2+}]_i$ within the axon but not in the terminals (Delaney et al., 1991). Thus, alternative possibilities were put forth for an IP_3 cascade working to enhance transmission (Cooper et al., 2001, 2003; He et al., 1999; Southard et al., 2000; Strawn et al., 2000.) A likely scenario is that 5-HT triggers IP3-induced phosphorylation for calcium-induced cellular responses and altered vesicular dynamics within the nerve terminal (Wang and Zucker, 1998; Sparks and Cooper, 2004).

The absence of observed increase in $[Ca^{2+}]_i$ within the terminals is at odds with the well-characterized serotonin-ergic sensory-motor circuit in *Aplysia*, which has been shown to increase intracellular calcium levels in the postsynaptic cells as well as to effect changes in ionic conductance and lead to spike broadening in rested synapses (Byrne and Kandel, 1996). Also, calcium entry is enhanced at the release sites in *Aplysia* presynaptic neurons in which facilitation is induced by 5-HT (Eilot et al., 1993). In *Aplysia* sensory neurons, 5-HT has been shown to activate both PKA and PKC, which in part explains the temporal differences of the effects of a slow K^+ channel and an increase in Ca^{2+} influx (Braha et al., 1993). In the *Aplysia* network, there are differences in which second-messenger systems are utilized, depending on the amount of exposure to 5-HT. The varied responses to 5-HT in the invertebrates suggest a possible family of 5-HT receptor subtypes, as in vertebrates. Recent pharmacological evidence indicates 5-HT_1 and 5-HT_2 receptors to be present in the crayfish (Cooper et al., 2001; Sparks et al., 2003; Tabor and Cooper, 2002; Yeh et al., 1996).

Second-messengers also show a role in synaptic transmission based on the degree of electrical activity in the absence of neuromodulators. For example, in crayfish motor neurons, short-term facilitation is dependent on Ca^{2+}, whereas long-term facilitation is dependent on the induction of cAMP (Dixon and Atwood, 1989a). Since 5-HT is thought to activate an IP_3 cascade in the crayfish motor nerve terminals (Dixon and Atwood, 1989a,b), it was of interest to see if an analog of IP_3, such as adenophostin-A placed directly in the nerve terminal, could mimic the effects of 5-HT. Adenophostin-A has a high receptor affinity and is resistant to inactivation, unlike IP_3 (Broad et al., 1999). We used agents such as caffeine, which activates calcium release from ryanodine-sensitive calcium stores and inhibits the IP_3 receptors on the ER, to investigate whether these receptors are activated by 5-HT when pretreated with caffeine or when caffeine is in the presence of 5-HT. We also wanted to know the direct effects, independently for 5-HT and caffeine, on synaptic transmission. Since blocking or activating the ER in neurons has been shown to alter Ca^{2+}-induced oscillations (Cseresnyes et al., 1999), it is reasonable to expect varied responses, depending on the concentration used of caffeine and ryanodine (Ry).

2. Methods

2.1. Preparation

Experiments were performed on the opener muscle of the first walking leg of *Procambarus clarkii* (4 to 6 cm in length), obtained from Atchafalaya Biological Supply Co., Raceland, LA, USA. Autotomized legs were dissected (Dudel and Kuffler, 1961) and pinned out in a Sylgard-lined dish for viewing with a Nikon Optiphot-2 upright fluorescent microscope and 40× (0.55 NA) Nikon water immersion objective. Preparations were maintained at 14 °C in a modified Van Harreveld's solution consisting of (mM): NaCl 205; KCl 5.3; $CaCl_2$ 13.5; $MgCl_2$ 2.45; and HEPES 0.5 adjusted to pH 7.4 (Cooper et al., 1995).

2.2. Electrophysiology

Conventional microelectrode (3 M KCl) techniques were used to carry out intracellular recordings from the opener muscle. The excitor nerve was stimulated with supramaximal pulses using a suction electrode and Grass S-88 stimulator to elicit trains of excitatory postsynaptic potentials (EPSPs). Recorded signals were fed into an AxoClamp 2A preamplifier, a Power Mac 9500 computer via a MacLab/4 s interface (10 kHz digitization frequency). All events were scaled to known test pulses applied through the electrode and directly measured on an oscilloscope. The corrected scale was then adjusted with MacLab Scope software (version 3.5.4).

2.3. Delivery of agents

Pharmacologic agents were applied by the rapid exchange in the bath of drug-containing saline with normal saline. Standards of 10 or 100 mM of these compounds were made in saline. Aliquots were freshly made from a frozen stock before each experiment. A 100 μM stock solution of adenophostin-A was made by dissolving the compound in 115 mM KCl. Immediately prior to injection, the protein-containing solution was mixed with a 5% Texas Red (TR) dextran solution (70 kDa, Molecular Probes, Eugene, OR, USA) to a final concentration of 0.05% TR dextran. This combination was then loaded into the microelectrode by capillary action. The microelectrode was inserted into the excitor axon of the opener muscle, close to the axon bifurcation. The fluorescent 70 kDa TR dextran entered the nerve terminals, loading each varicosity with 30 min. The molecular mass of adenophostin-A is 669.3 g. Compared to the 70 kDa TR dextran, adenophostin-A should have reached the varicosities before the TR dextran. This same type of axon injection of compounds was used earlier for proteins (He et al., 1999). Impalement of the excitatory or the inhibitory axon was determined by the presence of an evoked action potential, since the excitor axon is selectively stimulated in the meropodite.

Adenophostin-A was obtained from Calbiochem, and TR dextran (MW 70 kDa) was obtained from Molecular Probes (Eugene, OR). 5-HT, caffeine, and ryanodine were all obtained from Sigma (St. Louis, MO).

2.4. Analysis of data

A stimulation paradigm of 10 pulses at 40 Hz, repeated every 5 to 10 s, was used to induce short-term facilitation

(STF). Amplitudes of the 5th and 10th EPSPs in the train were used to provide an index of STF. Preceding the event to the peak response, amplitudes were measured from the trough. Since the EPSP amplitudes vary among preparations, a percent change was calculated, using the difference among the average of the events during the baseline recording prior to exposure and the average of the events around the maximum response during exposure to various compounds. The difference was divided by the baseline value, as shown in the following equation: (|Baseline − maximum response|)/Baseline] × 100 = % Difference. (Crider and Cooper, 1999, 2000). Numerical data were expressed as either mean ± SEM or mean percent difference from "saline" control. Students' *t*-test was used for parametric data, whereas the Wilcoxon rank sum test was used for non-parametric tests.

3. Results

3.1. Effect of 5-HT

The amplitudes of the 5th and 10th EPSPs were used as a measure of the effect of different agents (Fig. 1A). The saline bath was exchanged with one containing 5-HT (100 nM) increased EPSP amplitudes throughout the train, as indicated by measures of the amplitude in the 5th and 10th EPSPs (Fig. 1B). The mean of 20 measurements induced from trains every 10 s is shown. The response to 5-HT occurs within seconds and usually reaches a maximum effect in several minutes. This is an expected result since Dudel (1965) and Southard et al. (2000) reported an enhancement with single stimuli in the presence of 5-HT.

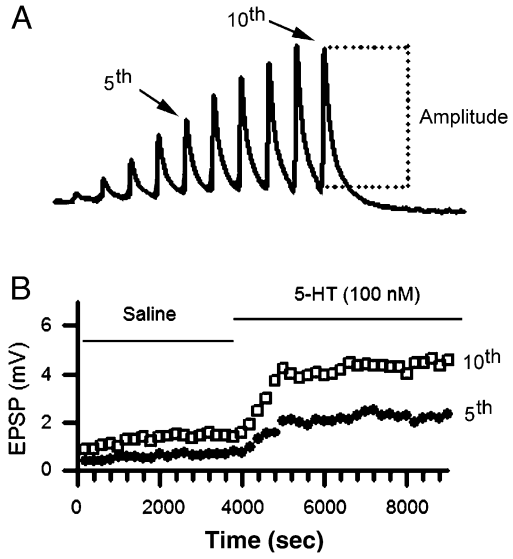

Fig. 1. Illustration of the synaptic response and method of quantifying synaptic response. (A) In normal saline, tetanic stimulation of the excitatory nerve (10 pulses at 40 Hz) causes synaptic facilitation, as indicted by the progressive increase in EPSP amplitudes. Preceding the event to the peak response (dotted line), amplitudes were measured from the trough. The 5th and 10th EPSPs in each train were used to provide a numerical index of facilitation (see Methods). (B) The application of 5-HT (100 nM) was used to produce an increase in synaptic responses, as indicated by the increase in the 5th and 10th EPSPs after 4000 s. The values shown represent a mean amplitude for twenty consecutive trials.

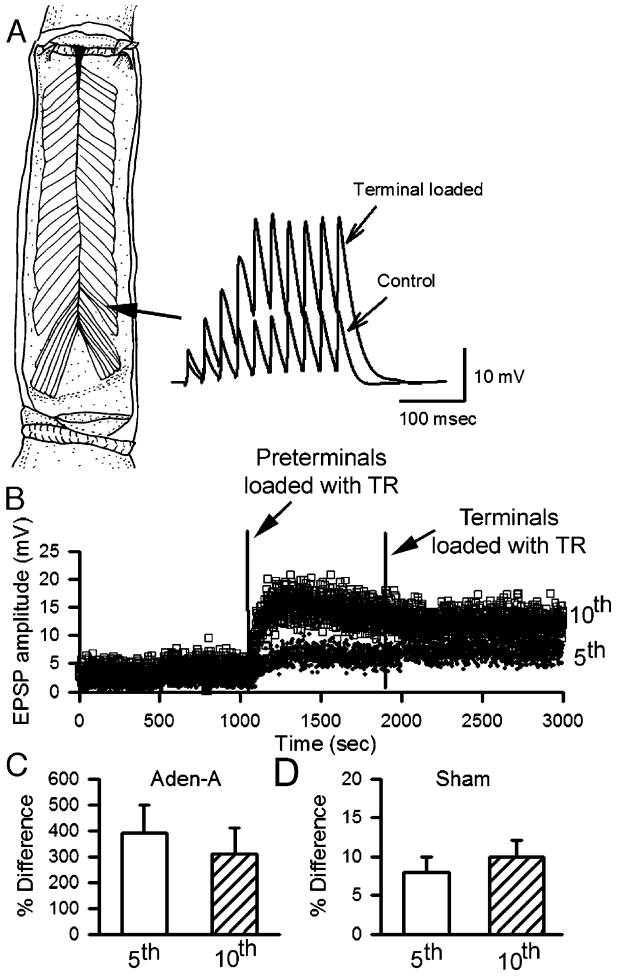

Fig. 2. Effect of intraterminal administration of adenophostin-A (100 μM) on synaptic facilitation. (A) Schematic diagram shows the dissected opener muscle within the cuticle and the location from which EPSPs are recorded. The inserts show recordings of EPSP trains before ("Control") and after ("Terminal loaded") injection of adenophostin-A. Note the increase in EPSP amplitudes. (B) The increase in the EPSPs due to adenophostin-A was usually seen before the co-injected Texas Red (TR) dextran (70 kDa) reached the terminal, presumably due to a faster diffusion by the lower molecular weight adenophostin-A. (C) Injection of adenophostin-A (Aden-A) always resulted in an increase in the EPSP amplitude ($n = 5$, $p < 0.05$, Wilcoxon rank sum). However, each preparation varied in the degree of enhancement and the time to peak response, since the degree of loading the terminals could not be rigorously controlled. The peak responses were used to determine the percent differences shown. (D) Sham injections of the carrier solution without adenophostin-A were performed in three preparations. These control experiments showed that there is a slight enhancement (<10%) due to the carrier solution.

3.2. Effect of adenophostin-A

The long-lasting activator of the IP_3 receptor, adenophostin-A, was injected directly into the preterminal of the excitatory motor axon. The injection solution also contained 70 kDa TR dextran to assess the amount and rate of loading of the terminals. The axon and terminals were periodically inspected during the injection process, while the axon was being stimulated. EPSPs were recorded in the central muscle fibers

close to the proximal end of the opener nearest to the loaded terminals (Fig. 2A). When the terminal was loaded with adenophostin-A, all EPSP amplitudes in the train were enhanced and maintained at an elevated level (Fig. 2B). The net response in the percent difference for all the preparations ($n=5$) is shown for both the 5th and 10th EPSPs (Fig. 2C).

Because of variation in the rate of loading, the peak response, rather than the time after injection, was used to determine the percent change. In three experiments, sham injections of the carrier solution without adenophostin-A produced a 10% increase in the EPSP amplitude. This indicated that the carrier solution itself had a facilitatory effect, although the magnitude of the effect was much less than that of adenophostin-A (Fig. 2D).

3.3. Effect of caffeine

Since caffeine is known to block IP_3 receptor mediated responses, we examined the effect of caffeine on the response to 5-HT. Recordings were carried out in control solution followed by the application of 5-HT (100 nM). Caffeine (10 mM) was then added in the continuous presence of 5-HT (100 nM). However, the addition of caffeine dampened the increase in EPSPs (Fig. 3A). This was observed in 7 out of 7 preparations ($p<0.05$ Wilcoxon rank sum test). The mean in the percent difference compiled within each preparation is shown in Fig. 3B. The period of time taken for determining a change is a factor, since the amplitudes changed over time with exposure to caffeine. Thus, an early and a late period of exposure time were used for assessment (Fig. 3B).

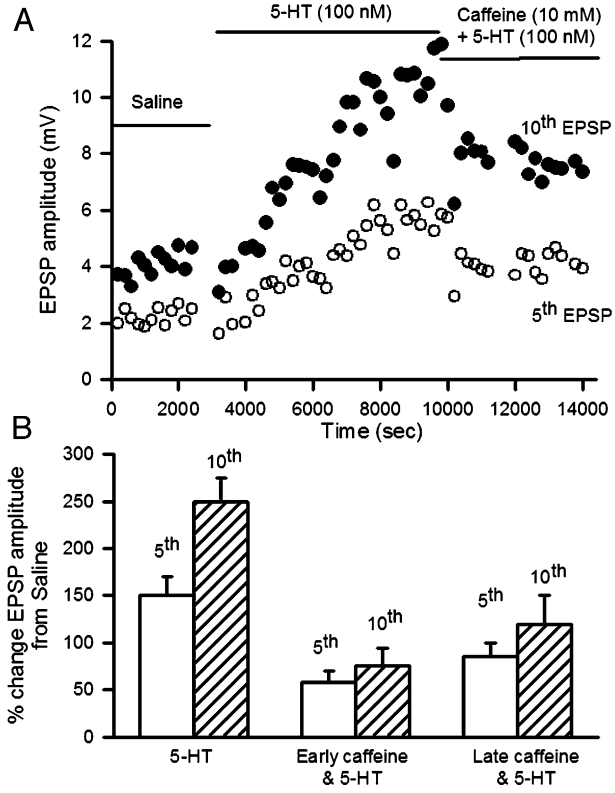

Fig. 3. (A) Initial enhancement of synaptic transmission by 5-HT is depressed by subsequent application of 5-HT and caffeine, as seen in the representative experimental response shown. (B) 5-HT (100 nM) had an excitatory effect in each preparation examined ($n=7$, $p<0.05$ Wilcoxon rank sum). Since there is individual variation in the initial amplitudes among each preparation, a mean of the percent differences is shown with the standard error of the mean (SEM). Additional application of 5-HT (100 nM) and caffeine (10 mM) resulted in a reduction in the EPSP amplitudes ($n=7$, $p<0.05$, Wilcoxon rank sum). The mean values obtained in each period were as follows: early caffeine and 5-HT exposure is from 10,000 to 11,000 s and the late caffeine and 5-HT is from 13,000 to 14,000 s.

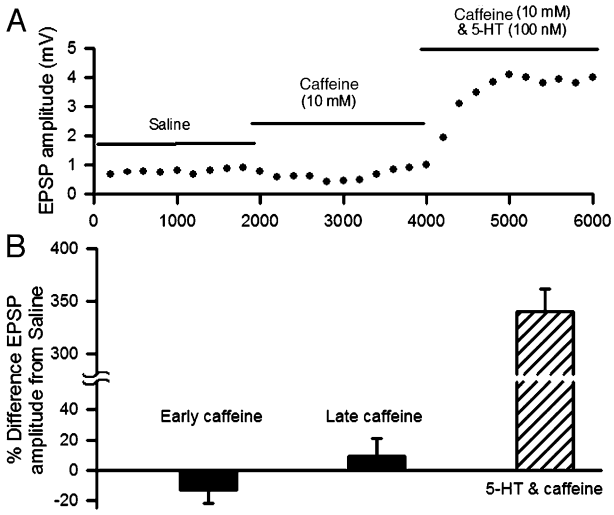

Fig. 4. (A) High concentration of caffeine (10 mM) results in a slight reduction of the EPSP amplitudes from basal conditions in saline. This reduction is most common initially, upon addition, but is followed by a leveling off after several minutes. When caffeine (10 mM) and 5-HT (100 nM) are combined and exposed to the NMJ, an enhancement in the EPSP amplitudes will occur. Only the 10th EPSP amplitudes are shown. (B) The percent difference is shown for both the early and late responses of caffeine as well as for the cocktail of caffeine and 5-HT ($n=5$, $p<0.05$, Wilcoxon rank sum). The mean values obtained in each period were as follows: early caffeine exposure is from 2000 to 2500 s and the late caffeine is from 3500 to 4000 s.

Exposure to caffeine (10 mM) alone initially produced a slight reduction in EPSP amplitude, which usually recovered and sometimes led to a slight enhancement over baseline values (Fig. 4A). Subsequent additions of caffeine (10 mM) and 5-HT (100 nM) together produced an increase in EPSP amplitudes (Fig. 4A) that was the same or even slightly greater than that produced by 5-HT (100 nM) alone (Fig. 3B).

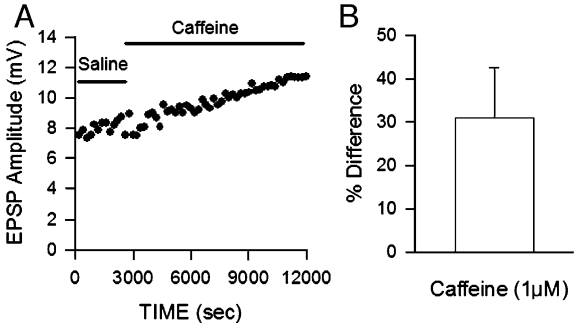

Fig. 5. (A) Low concentration of caffeine (1 μM) produced a slight enhancement of the EPSP amplitudes, as shown in the representative preparation. (B) The responses varied among preparations from a slight enhancement to substantial changes, as indicated by the SEM. The amplitude of the 10th EPSP within the trains was used for analysis.

To illustrate the early and late effects of caffeine, an average response within the first 5 min and the last 5 min of a 17-min exposure were taken (shown as percent difference compared to basal response in saline) (Fig. 4B). In this case, only the 10th EPSP amplitudes are shown for clarity. This trend was observed in 5 out of 5 preparations ($p<0.05$ Wilcoxon rank sum test).

A lower concentration of caffeine (1 μM) produced a slight enhancement of the EPSP amplitudes, as shown in the representative preparation ($p<0.05$ Wilcoxon rank sum test, Fig. 5A). One out of 7 preparations showed a slight reduction in the EPSP amplitude. The average percent change in the response ($n=7$) is shown in Fig. 5B.

3.4. Effect of ryanodine (Ry)

We tested the effect of 1 and 10 μM Ry (no effect was seen with 100 nM). Ry at 10 μM occasionally produced muscle contraction that was independent of nerve stimulation, and higher concentrations (50 to 100 μM) usually produced waves of muscle contraction. 1 μM Ry produced a small enhancement of all EPSP amplitudes within the train. Addition of 10 μM Ry generally resulted in a continuous enhancement followed by a depression of the EPSP amplitudes (Fig. 6A). In 2 out of 5 cases the onset of the depression was rapid (<1 min) without a significant increase in the EPSP amplitudes upon exposure to 10 μM Ry. In 5 out of 5 trails, 1 μM Ry produced an enhancement of EPSP amplitude (Fig. 6B, $p<0.05$ Wilcoxon rank sum test).

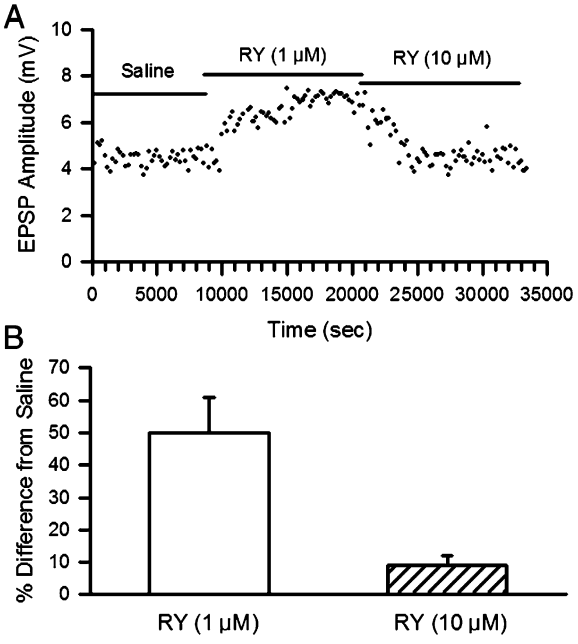

Fig. 6. (A) Ryanodine produced an enhancement of the EPSP amplitudes at low concentrations (1 μM). However, subsequent application of a higher dose (10 μM) produced a depression of the enhanced response. (B) There was considerable variation among the preparations in the initial EPSP amplitudes and the magnitude of the alterations to both low and high concentrations of ryanodine. However the trends were the same among the preparations ($n=5$, $p<0.05$, Wilcoxon rank sum). The mean value for the percent differences (±SEM) within each preparation is indicated.

4. Discussion

The primary observation in this study is that synaptic transmission is enhanced following intraterminal injection of adenophostin-A, a stable analog of IP_3. Considering the effects on synaptic transmission by adenophostin-A, caffeine and Ry, the presence of an IP_3-sensitive system that is able to enhance transmitter release at the crayfish neuromuscular junction is feasible. Such a system would be consistent with those in frog motor nerve terminals, in which IP_3 delivered into the terminal with liposomes is able to increase quantal transmitter release (Brailoiu and Miyamoto, 2000). Although the existence of SER in crayfish nerve terminals has not yet been demonstrated, recent studies using electron microscope spectroscopic imaging have shown, with high sensitivity and spatial resolution, the presence of Ca^{2+}-loaded smooth ER, as well as mitochondria, SER and synaptic vesicles in frog motor nerve terminals (Pezzati et al., 2001).

Llinas et al. (1994) reported that IP_3 injection in squid giant synapse had no effect. In contrast, Dixon and Atwood (1989b) and Yang et al. (2001) did report IP_3 enhancement of transmitter release in the invertebrates they examined. At the frog NMJ, using liposomes for intracellular delivery, IP_3 increases both MEPP frequency and amplitude (Brailoiu and Miyamoto, 2000). One possible explanation for these contradictory results is that IP_3 is short lived. Thus, by injecting adenophostin-A, we found an enhancement in transmitter release which can only be accounted for by presynaptic actions. In neurons, from a functional point of view, internal Ca^{2+} stores have traditionally been described by sensitivity to IP_3, caffeine/ryanodine and NAADP/bafilomycin (Lee, 2001; Brailoiu et al., 2003, 2005). Both IP_3- and Ry-stores are likely to be located in the SER. Caffeine, like cADPR, at high (5 mM) concentrations releases Ca^{2+} from ryanodine-sensitive calcium stores, and at low concentrations, potentiates the effect of cADPR (Galione et al., 1991; Lee et al., 1995).

Caffeine (1 μM) produced a slight increase in EPSP amplitudes, but at higher concentration (10 mM) the EPSP amplitudes did not increase as much. In the bullfrog sympathetic neuron (Hua et al., 1994) and in NG108-15 cells (Empson and Galione, 1997) Ry inhibits the cells' response. It has also been postulated that a large release of internal Ca^{2+} via Ry receptor activation could lead to inactivation of voltage dependent calcium channels and thus lead to a reduction in synaptic transmission (Schwartz et al., 1999). In contrast, Ry stimulates sea urchin eggs (Lee et al., 1995), as we have also observed for Ry at a low concentration for the crayfish NMJ. The results with caffeine support the idea that Ry receptors are present in crayfish motor nerve terminals. However, we cannot exclude the involvement of other mechanisms such as phosphodiesterase inhibition.

Application of Ry (1 μM) enhanced EPSP amplitude relatively quickly, as also observed for 5-HT. At the highest concentration we examined (10 μM) Ry lost its ability to substantially enhance transmitter release. Like that of high concentrations of caffeine, this effect is probably due to an inhibition of Ca^{2+}-induced Ca^{2+}-release mechanism (CICR).

The fact that both caffeine and Ry modulate transmitter release at crayfish neuromuscular junctions suggests a possible involvement of Ry operated calcium stores. These stores are physiological targets for cADP ribose and Ca^{2+}-induced Ca^{2+}-release processes (Galione et al., 1991; Lee, 2001). In neurons, such processes involve the Ry receptor subtype 3 (Guse, 2000).

In concordance with Dixon and Atwood (1989b), this observation suggests that inositol system may be involved in crayfish presynaptic neuromuscular junction response to 5-HT, possibly involving IP_3 sensitive Ca^{2+} stores. Caffeine, although best known as an activator of ryanodine-sensitive calcium pool, also inhibits the opening of IP_3 receptors (Petersen and Cancela, 1999), probably by a direct effect on the IP_3 receptor or a closely associated protein. It was expected that caffeine would inhibit the enhancement induced by 5-HT. However, this did not occur at high concentration (10 mM). If there is a convergence of mechanisms, then the actions of caffeine would have produced a synergistic effect. This possibility is hinted at since the average enhancement was greater when the compounds were combined than when exposure was to individual compounds. However, the percent increase in the EPSPs when caffeine and 5-HT were combined was not significantly different due to the large variation among preparations. The results suggest there are caffeine-sensitive Ca^{2+} stores in the crayfish motor nerve terminals. The 5-HT mediated IP_3 response may indeed work on the same Ca^{2+} stores, or there might be separate Ca^{2+} stores as proposed for ascidian eggs (Lee, 2000). In a preliminary report, the drug theophylline as well as caffeine was examined on other NMJs of the crayfish (i.e., superficial flexor muscles) which showed alterations in synaptic responses as those reported herein (Gupta and Velez, 2005). It would be of interest to examine other arthropods, particularly *Drosophila melanogaster* NMJs, for similarities in responses and regulation of synaptic transmission.

Dixon and Atwood (1989b) postulated that the mechanism of 5-HT effects at the crayfish neuromuscular junction might be due to an increase in intracellular calcium levels because of an activated IP_3 cascade. This hypothesis was tested by Delaney et al. (1991) with the calcium-sensitive indicator Fura 2 directly loaded into the axons. The results with the calcium imaging suggested that 5-HT did not significantly increase the resting calcium concentration $[Ca^{2+}]_i$ in presynaptic terminals during or after application. In addition, they injected the calcium buffer EGTA to reduce the buildup of $[Ca^{2+}]_i$ during frequency-dependent facilitation, but there was still an enhancement of the EPSPs in the presence of 5-HT. This suggested to the authors that 5-HT does not release calcium from intracellular stores. However, the spatial and temporal resolution of monitoring Ca^{2+} fluxes close to the internal face of the synapses may not have been adequate for monitoring small spatial alterations, which could have affected synaptic efficacy substantially. In addition, EGTA with its relatively low Kd is not as effective as BAPTA in buffering rapid fluxes of calcium influx. Even with the use of calcium imagining, the agents used in this study could have triggered transient and localized $[Ca^{2+}]_i$ release which may have escaped Fura-2 detection.

In conclusion, the results of the present study indicate that apart from the cAMP/PKA pathway, the 5-HT response is also modulated by internal Ca^{2+} stores from two distinct pools: a IP_3- and a ryanodine/cADPR-sensitive source.

Acknowledgments

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References

Berridge, M.J., 1997. Elementary and global aspects of calcium signalling. J. Physiol. 499, 291–306.

Berridge, M.J., 2005. Unlocking the secrets of cell signaling. Annu. Rev. Physiol. 67, 1–21.

Berridge, M.J., Lipp, P., Bootman, M.D., 2000. The versatility and universality of calcium signalling. Nat. Rev., Mol. Cell Biol. 1, 11–21.

Bouchard, R., Pattarini, R., Geiger, J.D., 2003. Presence and functional significance of presynaptic ryanodine receptors. Prog. Neurobiol. 69, 391–418.

Braha, O., Edmonds, B., Sacktor, T., Kandel, E.R., Klein, M., 1993. The contributions of protein kinase A and protein kinase C to the actions of 5-HT on the L-type Ca^{2+} current of sensory neurons in *Aplysia*. J. Neurosci. 13, 1839–1851.

Brailoiu, E., Miyamoto, M.D., 2000. Inositol trisphosphate and cyclic adenosine diphosphate-ribose increase quantal transmitter release at frog motor nerve terminals: possible involvement of smooth endoplasmic reticulum. Neuroscience 95, 927–931.

Brailoiu, E., Miyamoto, M.D., Dun, N.J., 2002. Calmodulin increases transmitter release by mobilizing quanta at the frog motor nerve terminal. Br. J. Pharmacol. 137, 719–727.

Brailoiu, E., Patel, S., Dun, N.J., 2003. Modulation of spontaneous transmitter release from the frog neuromuscular junction by interacting intracellular Ca(2+) stores: critical role for nicotinic acid-adenine dinucleotide phosphate (NAADP). Biochem. J. 373, 313–318.

Brailoiu, E., Hoard, J.L., Filipeanu, C.M., Brailoiu, G.C., Dun, S.L., Patel, S., Dun, N.J., 2005. Nicotinic acid adenine dinucleotide phosphate potentiates neurite outgrowth. J. Biol. Chem. 280, 5646–5650.

Broad, L.M., Armstrong, D.L., Putney Jr., J.W., 1999. Role of the inositol 1,4,5-trisphosphate receptor in Ca(2+) feedback inhibition of calcium release-activated calcium current (I(crac)). J. Biol. Chem. 274, 32881–32888.

Byrne, J.H., Kandel, E.R., 1996. Presynaptic facilitation revisited: state and time dependence. J. Neurosci. 16, 425–435.

Cooper, R.L., Marin, L., Atwood, H.L., 1995. Synaptic differentiation of a single motor neuron: conjoint definition of transmitter release, presynaptic calcium signals, and ultrastructure. J. Neurosci. 15, 4209–4222.

Cooper, R.L., Chase, R.J., Tabor, J., 2001. Altered responsiveness to 5-HT at the crayfish neuromuscular junction due to chronic p-CPA and m-CPP treatment. Brain Res. 916, 143–151.

Cooper, R.L., Dönmezer, A., Shearer, J., 2003. Intrinsic differences in sensitivity to 5-HT between high- and low-output terminals innervating the same target. Neurosci. Res. 45, 163–172.

Crider, M.E., Cooper, R.L., 1999. The importance of the stimulation paradigm in determining facilitation and effects of neuromodulation. Brain Res. 842, 324–331.

Crider, M.E., Cooper, R.L., 2000. Differentially facilitation of high- and low-output nerve terminals from a single motor neuron. J. Appl. Physiol. 88, 987–996.

Cseresnyes, Z., Bustamante, A.I., Schneider, M.F., 1999. Caffeine-induced [Ca^{2+}] oscillations in neurones of frog sympathetic ganglia. J. Physiol. 514, 83–99.

Delaney, K., Tank, D.W., Zucker, R.S., 1991. Presynaptic calcium and serotonin-mediated enhancement of transmitter release at crayfish neuromuscular junction. J. Neurosci. 11, 2631–2643.

Dixon, D., Atwood, H.L., 1989a. Adenylate cyclase system is essential for long-term facilitation at the crayfish neuromuscular junction. J. Neurosci. 9, 4246–4252.

Dixon, D., Atwood, H.L., 1989b. Conjoint action of phosphoinositol and adenylate cyclase systems in serotonin-induced facilitation at the crayfish neuromuscular junction. J. Neurophysiol. 62, 1251–1259.

Dudel, J., 1965. Facilitatory effects of 5-Hydroxy-trytamine on the crayfish neuromuscular junction. Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol. 249, 515–528.

Dudel, J., Kuffler, S.W., 1961. The quantal nature of transmission and spontaneous miniature potentials at the crayfish neuromuscular junction. J. Physiol. 155, 514–529.

Eilot, L.S., Kandel, E.R., Siegelbaum, S.A., Blumenfeld, H., 1993. Imaging terminals of *Aplysia* sensory neurons demonstrates role of enhanced Ca^{2+} influx in presynaptic facilitation. Nature 361, 634–637.

Empson, R.M., Galione, A., 1997. Cyclic ADP-ribose enhances coupling between voltage-gated Ca^{2+} entry and intracellular Ca^{2+} release. J. Biol. Chem. 272, 20967–20970.

Galante, M., Marty, A., 2003. Presynaptic ryanodine-sensitive calcium stores contribute to evoked neurotransmitter release at the basket cell-Purkinje cell synapse. J. Neurosci. 23, 11229–11234.

Galione, A., Lee, H.C., Busa, W.B., 1991. Ca($^{2+}$)-induced Ca^{2+} release in sea urchin egg homogenates: modulation by cyclic ADP-ribose. Science 253, 1143–1146.

Gupta, A.C., Velez, S.J., 2005. Effect of theophylline on synaptic transmission at crayfish neuromuscular junctions. Soc. for Neurosci. Meeting Program No. 730.15.

Guse, A.H., 2000. Cyclic ADP-ribose. J. Mol. Med. 78, 26–35.

He, P., Southard, R.C., Whiteheart, S.W., Cooper, R.L., 1999. Role of alpha-SNAP in promoting efficient neurotransmission at the crayfish neuromuscular junction. J. Neurophysiol. 82, 3406–3416.

Hua, S.Y., Tokimasa, T., Takasawa, S., Furuya, Y., Nohmi, M., Okamoto, H., Kuba, K., 1994. Cyclic ADP-ribose modulates Ca^{2+} release channels for activation by physiological Ca^{2+} entry in bullfrog sympathetic neurons. Neuron 12, 1073–1079.

Lee, H.C., 2000. Multiple Calcium Stores: Separate but Interacting. Science's STKE (Science's Signal Transduction Knowledge Environment). www.stke.org/cgi/content/full/OC_sigtrans;2000/40/pe1.

Lee, H.C., 2001. Physiological functions of cyclic ADP-ribose and NAADP as calcium messengers. Annu. Rev. Pharmacol. Toxicol. 41, 317–345.

Lee, H.C., Aarhus, R., Graeff, R.M., 1995. Sensitization of calcium-induced calcium release by cyclic ADP-ribose and calmodulin. J. Biol. Chem. 270, 9060–9066.

Liu, Q., Chen, B., Yankova, M., Morest, D.K., Maryon, E., Hand, A.R., Nonet, M.L., Wang, Z.W., 2005. Presynaptic ryanodine receptors are required for normal quantal size at the *Caenorhabditis elegans* neuromuscular junction. J. Neurosci. 25, 6745–6754.

Llinas, R., Sugimori, M., Lang, E.J., Morita, M., Fukuda, M., Niinobe, M., Mikoshiba, K., 1994. The inositol high-polyphosphate series blocks synaptic transmission by preventing vesicular fusion: a squid giant synapse study. Proc. Natl. Acad. Sci. U. S. A. 91, 12990–12993.

Mattson, M.P., LaFerla, F.M., Chan, S.L., Leissring, M.A., Shepel, P.N., Geiger, J.D., 2000. Calcium signaling in the ER: its role in neuronal plasticity and neurodegenerative disorders. Trends Neurosci. 23, 222–229.

Petersen, O.H., Cancela, J.M., 1999. New Ca^{2+}-releasing messengers: are they important in the nervous system? Trends Neurosci. 22, 488–495.

Pezzati, R., Meldolesi, J., Grohovaz, F., 2001. Ultra rapid calcium events in electrically stimulated frog nerve terminals. Biochem. Biophys. Res. Commun. 285, 724–727.

Schwartz, A.D., Whitacre, C.L., Wilson, D.F., 1999. Do ryanodine receptors regulate transmitter release at the neuromuscular junction of the rat? Neurosci. Lett. 274, 163–166.

Southard, R.C., Haggard, J., Crider, M.E., Whiteheart, S.W., Cooper, R.L., 2000. Influence of serotonin on the kinetics of vesicular release. Brain Res. 871, 16–28.

Sparks, G., Cooper, R.L., 2004. 5-HT offsets homeostasis of synaptic transmission during short-term facilitation. J. Appl. Physiol. 96, 1681–1690.

Sparks, G.M., Brailoiu, E., Brailoiu, C., Dun, N.J., Tabor, J., Cooper, R.L., 2003. Effects of m-CPP in altering neuronal function: blocking depolarization in invertebrate motor and sensory neurons but exciting rat dorsal root neurons. Brain Res. 969, 14–26.

Strawn, J.R., Neckameyer, W.S., Cooper, R.L., 2000. The effects of 5-HT on sensory, central and motor neurons driving abdominal superficial flexor muscles in the crayfish. Comp. Biochem. Physiol., B 127, 533–550.

Tabor, J., Cooper, R.L., 2002. Physiologically identified 5-HT2-like receptors at the crayfish neuromuscular junction. Brain Res. 932, 91–98.

Wang, C., Zucker, R.S., 1998. Regulation of synaptic vesicle recycling by calcium and serotonin. Neuron 21, 155–167.

Yang, F., He, X., Feng, L., Mizuno, K., Liu, X.W., Russell, J., Xiong, W.C., Lu, B., 2001. PI-3 kinase and IP3 are both necessary and sufficient to mediate NT3-induced synaptic potentiation. Nat. Neurosci. 4, 19–28.

Yeh, S.-R., Fricke, R.A., Edwards, D.H., 1996. The effect of social experience on serotonergic modulation of the escape circuit of crayfish. Science 271, 366–369.