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FACILITATORY AND INHIBITORY TRANSMITTERS MODULATE SPONTANEOUS TRANSMITTER RELEASE AT CULTURED *APLYSIA* SENSORIMOTOR SYNAPSES

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SUMMARY

1. The monoamine transmitter 5-hydroxytryptamine (5-HT) and the peptide Phe-Met-Arg-Phe-amide (FMRFa), which appear to contribute to presynaptic facilitation and inhibition of the sensorimotor synapse in the abdominal ganglion of *Aplysia*, can modulate the frequency of spontaneous transmitter release at synapses formed between dissociated *Aplysia* sensory neurones and motoneurones *in vitro*.

2. 5-HT caused a decrease in the mean time interval between consecutive miniature EPSPs (mEPSPs), while FMRFa, applied either by itself or together with 5-HT, caused an increase in the mean time interval between consecutive mEPSPs.

3. Depolarization of the presynaptic neurone caused a decrease in the mean time interval between consecutive mEPSPs. This modulation required external Ca^{2+}.

4. 5-HT and FMRFa were able to modulate spontaneous release when applied in saline solutions lacking Ca^{2+} and containing Ca^{2+}-chelating agents, suggesting that the modulation of spontaneous release by 5-HT and FMRFa did not require a Ca^{2+} influx. Similarly, spontaneous release could still be modulated by 5-HT and FMRFa in saline solutions containing 1 mM-Cd^{2+}, which blocked both the voltage-gated Ca^{2+} channels and the evoked transmitter release.

5. To prevent a rise in intracellular Ca^{2+}, we buffered the concentration of Ca^{2+} in the presynaptic terminals by injecting into the sensory neurone the Ca^{2+} chelator 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA). The injection of BAPTA blocked evoked transmitter release, suggesting that it acted as an effective buffer of Ca^{2+} in the terminals. However, spontaneous release could still be observed and was still modulated by 5-HT and FMRFa. This suggests that the modulation of spontaneous release does not require an elevation of intracellular Ca^{2+}.

6. We propose that 5-HT and FMRFa can modulate the rate of spontaneous release directly by mechanisms that do not require changes in the intracellular concentration of Ca^{2+}. These mechanisms might contribute an additional component to the presynaptic inhibition and facilitation of evoked transmitter release.

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INTRODUCTION

Modulation of ionic currents has long been shown to play a role in altering the efficacy of transmitter release from neurones (Klein & Kandel, 1980; Kretz, Shapiro, Connor & Kandel, 1984). For example at sensorimotor synapses of *Aplysia* facilitatory transmitters such as 5-HT can cause the reduction of a specific K^+ current, the serotonin-sensitive K^+ current (S-current; Klein, Camardo & Kandel, 1982) and result in broadening of the action potential. This occurs in the growth cones of cultured cells (Belardetti, Schacher, Kandel & Siegelbaum, 1986) and is thought to occur in the terminals and allow more Ca^{2+} to enter during each action potential and hence more transmitter release (Castellucci & Kandel, 1976; Klein & Kandel, 1980). Conversely, inhibitory transmitters in *Aplysia*, such as the peptide Phe-Met-Arg-Phe-amide (FMRFa), can cause both an increase in the S-current (Belardetti, Kandel & Siegelbaum, 1987) and a reduction of a Ca^{2+} current involved in transmitter release (Brezina, Eckert & Erxleben, 1987; Edmonds, Kandel & Klein, 1988). The modulation of these two currents is thought to reduce the Ca^{2+} influx during the action potential and contribute to the presynaptic inhibition.

However, other mechanisms, independent of the modulation of the S-current, contribute to facilitation of sensorimotor synapses in *Aplysia*. For example there is a second process by which more transmitter can be released from *Aplysia* sensory neurones which does not involve changes in the width of the action potential (Hochner, Klein, Schacher & Kandel, 1986*a*). This second process may involve the release of more transmitter, either by changing the efficiency of the release process itself, or by increasing the availability of transmitter for release (Gingrich & Byrne, 1985; Hochner *et al.* 1986*a*).

The aim of this paper is threefold. First, we try to provide more direct evidence for a second process in presynaptic facilitation by examining whether transmitter release or the availability of transmitter for release can be modified by mechanisms independent of the modulation of ionic currents. Second, we attempt to elucidate the possible role of Ca^{2+} (either a Ca^{2+} influx or release of Ca^{2+} from internal stores) in the modulation of the transmitter release process or availability of transmitter. Finally, we try to examine whether direct modulation of the release process or the availability of transmitter contributes to other forms of plasticity at this synapse, in particular presynaptic inhibition.

To address these issues, we have studied the modulation of spontaneous release at cultured *Aplysia* sensorimotor synapses. By forming cultures consisting of only one sensory neurone and one motoneurone, we have been able to study, unambiguously, the incidence of spontaneous miniature EPSPs originating from a single presynaptic neurone (Dale *et al.* 1988). Although many factors influence the rate of spontaneous release, including the basal concentration of Ca^{2+} in the presynaptic terminals (Zucker & Haydon, 1988), an alteration of either the efficiency of the release mechanism or the availability of transmitter for release might also be expected to alter the rate of spontaneous release. We have therefore used spontaneous release to examine new aspects of the actions of modulatory transmitters.

The use of spontaneous release provides several advantages to studying processes contributing to presynaptic facilitation: (1) It allows modulation of release to be

studied under conditions in which membrane currents essential for evoked release need play no role (Martin, 1966). (2) Facilitatory mechanisms can be studied in the absence of homosynaptic depression produced by repeated stimulation. This is particularly advantageous at the sensorimotor synapses in *Aplysia*, which decrement rapidly when an action potential is fired (Castellucci, Pinsker, Kupfermann & Kandel, 1970). (3) The role of Ca^{2+} in mediating the facilitation of spontaneous release can be elucidated since, unlike evoked release, spontaneous release can occur in the absence of external Ca^{2+} (Fatt & Katz, 1952; Katz & Miledi, 1967; Rahamimoff, Lev-Tov & Meiri, 1980).

In this paper we provide evidence that both 5-HT and FMRFa can modulate spontaneous release by mechanisms which are independent of the modulation of ionic currents and do not require either a Ca^{2+} influx or a change in the internal Ca^{2+} concentrations. We argue that these mechanisms not only modulate spontaneous release but also contribute to the modulation of evoked transmitter release.

METHODS

Cell culture

Pleural sensory neurones and L7 gill motoneurones were isolated from the pleural ganglia of adult *Aplysia* (75–150 g) and the abdominal ganglia of juvenile *Aplysia* (1–3 g), respectively, using techniques pioneered by Schacher and colleagues (Schacher & Proshansky, 1983; Schacher, 1985). After dissociation, L7 motoneurones and sensory neurones were plated close together on dishes (Falcon, 50 mm) coated with polylysine (Sigma) and grown in a medium consisting of *Aplysia* haemolymph mixed with L15 culture medium (Flow Laboratories, McLean, VA, USA), with salts added to adjust the medium to an appropriate composition (400 mM-NaCl, 11 mM-$CaCl_2$, 10 mM-KCl, 27 mM-$MgSO_4$, 27 mM-$MgCl_2$, and 2 mM-$NaHCO_3$). Each dish contained a single L7 motoneurone in close physical contact with a single sensory neurone. As described in earlier studies (Schacher, 1985; Rayport & Schacher, 1986) the cells extended neurites and made synaptic contact. The presumed site of synapse formation is between the short newly extended neurites of the sensory neurone and the initial segment of the motoneurone axon (Masurovsky, Glanzman & Schacher, 1988). Experiments were performed between 3 and 6 days after the initial dissociation and plating of the cells. Occasionally LFS, small motoneurones from adult abdominal ganglia, were used instead of the L7 motoneurone. These cultures responded to 5-HT and FMRF in the same manner as those made with L7.

Recording techniques

Conventional recording techniques were used to make intracellular recordings from the sensory and motoneurones. Results were stored for later analysis on a 7-channel FM tape-recorder (Racal 7 DS). The voltage trace of the motoneurone was recorded at high and low gain. The low-gain record was filtered at 3 kHz (−3 dB) and DC coupled, while the high-gain recording was filtered at 300 Hz (−3 dB) and was AC coupled. To reduce the noise of the electrode, recordings from the L7 motoneurone were made with bevelled electrodes filled with a mixture of 1 M-potassium acetate and 1 M-KCl; the sensory neurones and LFS motoneurones were penetrated with unbevelled 2·5 M-KCl electrodes. A conventional bridge balance circuit was used to inject current through the recording electrode. Pharmacological agents were applied by a microperfusion system that allowed rapid changing of the medium surrounding the cells. Recordings were made in a saline that contained L15 medium diluted by an equal volume of artificial sea water. The pH was adjusted to 7·8 and buffered with 5 mM-HEPES.

Measurements

To analyse the effects of transmitters on spontaneous release, the time interval between consecutive mEPSPs was measured. A computer was used to digitize and display the data.

Manually controlled cursors were used to mark the beginning of the mEPSPs. The amplitude, rise time, half-fall time and time interval between consecutive mEPSPs were then determined automatically by the computer. At the high gains necessary to observe mEPSPs, noise and slow oscillations in membrane potential could be observed (especially in low-Ca^{2+} saline solutions). Nevertheless, mEPSPs could clearly be distinguished visually by the following three criteria: they

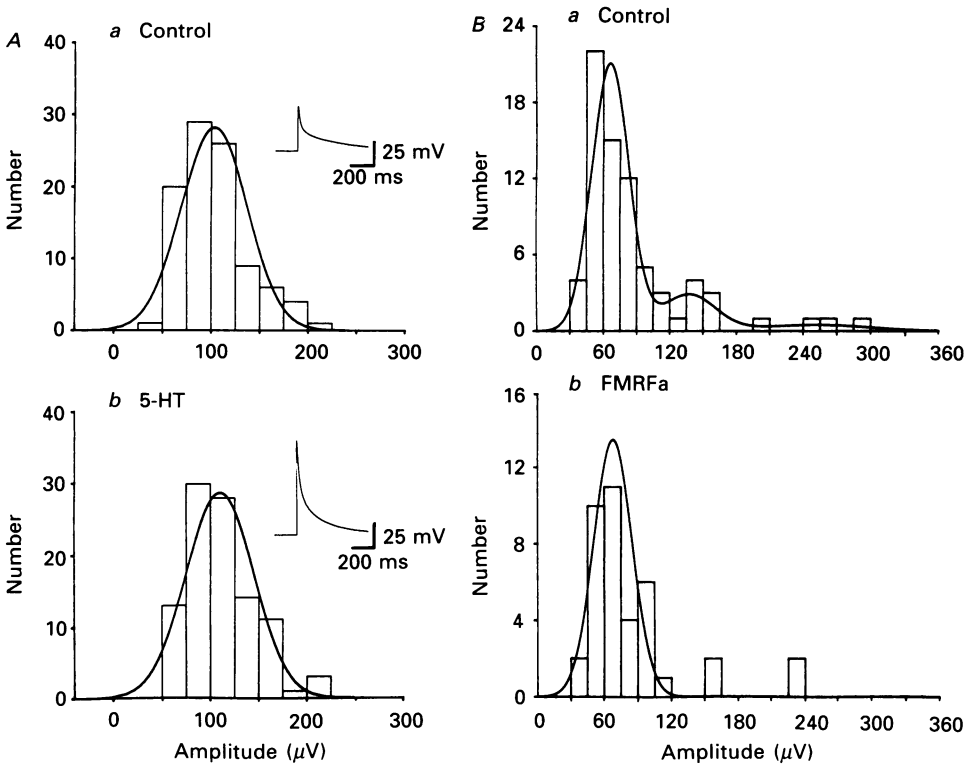

Fig. 1. Effect of 10 μM-5-HT and 100 nM-FMRFa on the amplitude of the spontaneous mEPSPs. Amplitude histograms are shown before (*A a*) and after (*A b*) the application of 5-HT. The smooth line is the Gaussian curve specified by the mean and standard deviations of the underlying distributions. The insets shown the evoked synaptic potential and demonstrate the facilitation of the EPSP caused by 5-HT. In *B* the amplitude histograms before (*Ba*) and after (*Bb*) the application of FMRFa to a different pair of cells are shown. The smooth lines are the best-fitting Gaussian distribution determined by using the simplex algorithm and the method of maximum likelihood. Before FMRFa three Gaussians were fitted (mean $\pm$ S.D.): $65 \pm 16{\cdot}4$, $135 \pm 23{\cdot}8$ and $246 \pm 48{\cdot}3$ μV. After application of FMRFa a single Gaussian was fitted (mean $\pm$ S.D.): $67 \pm 16{\cdot}9$ μV. The experiment in *B* was performed in a 0 Ca^{2+} saline.

had to have (1) a rise time between 3 and 20 ms, (2) a half-fall time of at least 5 ms, and (3) an amplitude greater than 40 μV. As reported in a previous paper (Dale, Schacher & Kandel, 1988), application of these criteria gave mEPSPs with amplitudes which were normally distributed about a mean in the range 60–120 μV, with a standard deviation ranging from 15 to 25 μV. The spontaneous mEPSPs which were very similar in time course to the evoked EPSP (of Dale *et al.* 1988) remained consistent in amplitude and shape throughout each experiment, and had mean rise times in the range 6–13 ms and mean half-fall times ranging from 10 to 16 ms. While both 5-HT and FMRFa altered the input resistance and resting potential of the motoneurone slightly, neither 5-HT (mean difference 1·5%, S.E.M. 2·01%, 10 experiments, $t = 0{\cdot}75$, $P > 0{\cdot}4$) nor FMRFa (mean difference $-2{\cdot}7$%, S.E.M. 2·20, 8 experiments, $t = 1{\cdot}23$, $P > 0{\cdot}2$) changed the mean amplitude of the unitary spontaneous miniature potentials significantly (Fig. 1).

For each experiment, the mean time interval between consecutive mEPSPs under control conditions was computed and normalized to 100%. The mean time intervals between consecutive mEPSPs under the various experimental conditions were then calculated and expressed as a percentage of the control. Enough data were analysed to obtain a minimum of fifteen time intervals. In practice, this meant analysing at least 1 min of data. To assess the effect of 5-HT and/or Phe-Met-Arg-Phe-amide (FMRFa) on spontaneous release, the portions of data analysed for each treatment were chosen immediately before the application of 5-HT or FMRFa, immediately after their maximal action and immediately after complete wash-out. Since both 5-HT and FMRFa affect the membrane potential and input resistance of the sensory neurone, the onset of the actions of these transmitters and the extent of their wash-out were assessed by continuously measuring the membrane potential and input resistance of the sensory neurone. The results reported in the text are the overall averages of the normalized mean time intervals obtained by performing the same experiment on several different cultures. Statistical comparisons were performed by using the pair-sample t test on the normalized data. For each experiment the mean time interval under the experimental condition was subtracted from 100 (the normalized control). The mean of the differences obtained in a series of experiments was then compared to zero with the number of degrees of freedom being equal to the number of experiments minus one. The values of t and associated probabilities are reported in the text.

'Zero' Ca^{2+} saline solutions

In some experiments, it was necessary to examine the role of external Ca^{2+} in mediating the modulation of spontaneous release. Two types of saline were used. The first was made without adding Ca^{2+}. Measurements of the free Ca^{2+} in the deionized water, together with the amounts of Ca^{2+} contaminating the $MgCl_2$, suggested that the free Ca^{2+} in these saline solutions was about 20 μM (K. Karl, personal communication). The second saline included 1 mM-1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA; Tsien, 1980). This was calculated to reduce the free Ca^{2+} to 13 nM if the total contaminating Ca^{2+} was 20 μM, and 72 nM if the total contaminating Ca^{2+} was 100 μM. Thus, even assuming the worst conditions, the inclusion of BAPTA reduced the free concentration of Ca^{2+} in the external medium to below the basal levels found inside the cell (Spira, Blumenfeld, Schacher & Siegelbaum, 1987).

Intracellular injection of BAPTA

For the intracellular injection of BAPTA, microelectrodes were filled with BAPTA dissolved in 2 M-potassium acetate. The concentrations of BAPTA in the micropipette ranged from 100 to 400 mM. In most experiments, Ca^{2+} was added to the microelectrode solution to give a free Ca^{2+} concentration of around 100 nM. The electrodes were bevelled to lower their resistance and facilitate the ionophoretic injection of BAPTA. In two experiments, the presynaptic cell was perfused from a patch pipette containing 10 nM-BAPTA dissolved in an intracellular perfusion medium containing 6·82 mM-$MgCl_2$, 10 mM-NaCl, 496 mM-KCl, 20 mM-HEPES, 5 mM-ATP, 10 mM-glutathione, 100 mM-glucose, 2·84 mM-$CaCl_2$, 0·1 mM-GTP and 0·1 mM-leupeptin. Leupeptin, a protease inhibitor, was included to avoid possible proteolytic degradation of Ca^{2+} channels reported by Chad & Eckert (1986). Some 20 min after intracellular perfusion, evoked release was blocked but spontaneous release was maintained. The experiments with whole-cell perfusion gave similar results to those performed with ionophoresis of BAPTA from microelectrodes.

RESULTS

Application for 1–2 min of 1–10 μM-5-HT, a transmitter that is thought to mediate facilitation at the sensorimotor synapse of *Aplysia* (Hochner, Klein, Schacher & Kandel, 1986*b*), caused a large increase in spontaneous release (Fig. 2) with a time course similar to that of the facilitation of the evoked EPSP (Fig. 2*B*). Under control conditions in eleven experiments, the mean time interval between consecutive mEPSPs was 3·07 s (S.E.M. 0·44). During the application of 1 and 10 μM-5-HT, the mean time interval between consecutive mEPSPs was reduced to 0·78 s (S.E.M. 0·16, 7 experiments) and 0·52 s (S.E.M. 0·18, 4 experiments), respectively. Thus during the

application of 1 and 10 μM-5-HT, the mean time interval between consecutive mEPSPs was reduced to 28·8% (S.E.M. 5·11%, $t = 11{\cdot}87$, $P < 0{\cdot}001$) and 17·7% (S.E.M. 2·88%, $t = 28{\cdot}75$, $P < 0{\cdot}001$), respectively.

In three further experiments, the dose–response relation between the con-

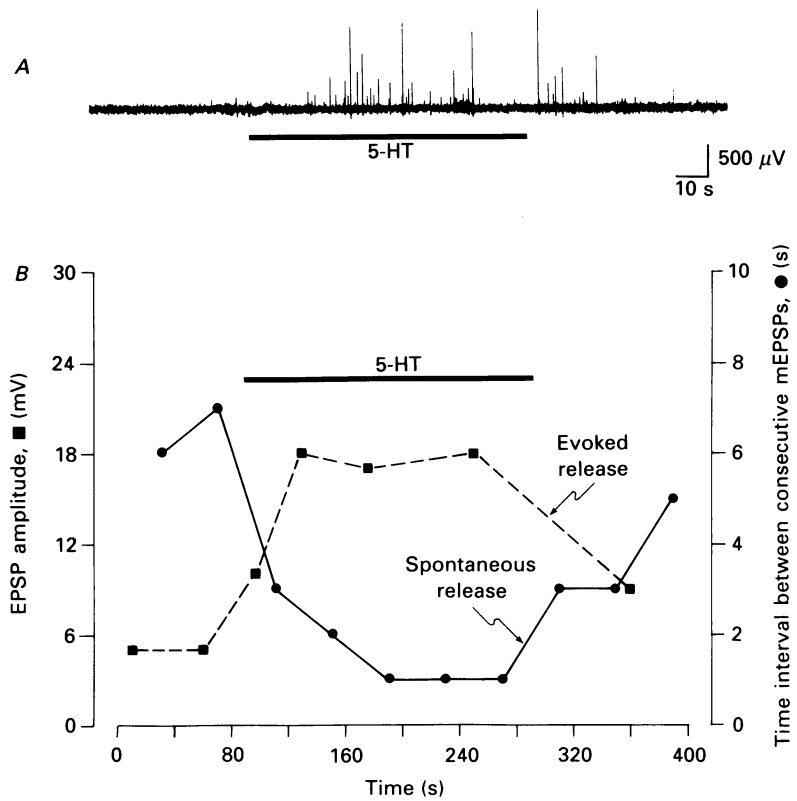

Fig. 2. Bath application of 5-HT causes an enhancement of spontaneous release. *A*, a slow record showing the increases in spontaneous release caused by 10 μM-5-HT. The mEPSPs are seen as upward deflections. In this and all subsequent figures, the high-gain voltage record of the motoneurone is AC coupled and filtered at 300 Hz (−3 dB). *B*, a comparison in a different culture of the time course of the reduction in the mean time interval between consecutive mEPSPs during the application of 10 μM-5-HT (●) and the onset of synaptic facilitation (■). In order to follow the change in spontaneous release, the mean time interval between consecutive mEPSPs was calculated from consecutive 40 s segments of data throughout the experiment. The motor cell in this culture was an LFS cell.

centration of bath-applied 5-HT and the reduction in the mean time interval between consecutive mEPSPs and facilitation of the evoked EPSP at the same synapse was determined (Fig. 3). In all three cases, the evoked and spontaneous transmitter release were maximally enhanced by 10 μM-5-HT (Fig. 3), with a higher dose of 5-HT giving a submaximal enhancement of both the evoked EPSP and spontaneous release.

The tetrapeptide FMRFa is thought to mediate presynaptic inhibition at sensorimotor synapses in *Aplysia* (Abrams, Castellucci, Camardo, Kandel & Lloyd,

1984; Montarolo, Kandel & Schacher, 1987; Mackey, Glanzman, Small, Dyke, Kandel & Hawkins, 1987). We therefore examined the effects of FMRFa on spontaneous release. In eight experiments, FMRFa at 100 nM so greatly depressed spontaneous release that only a few mEPSPs could be observed during the period of its application (1–2 min). FMRFa also caused a small depolarization (< 5 mV) of the motor cell. When spontaneous release was enhanced by prior application of 5-HT, and FMRFa was then applied in conjunction with the 5-HT, it could overcome the action of 5-HT and cause a great reduction in spontaneous release (Fig. 4).

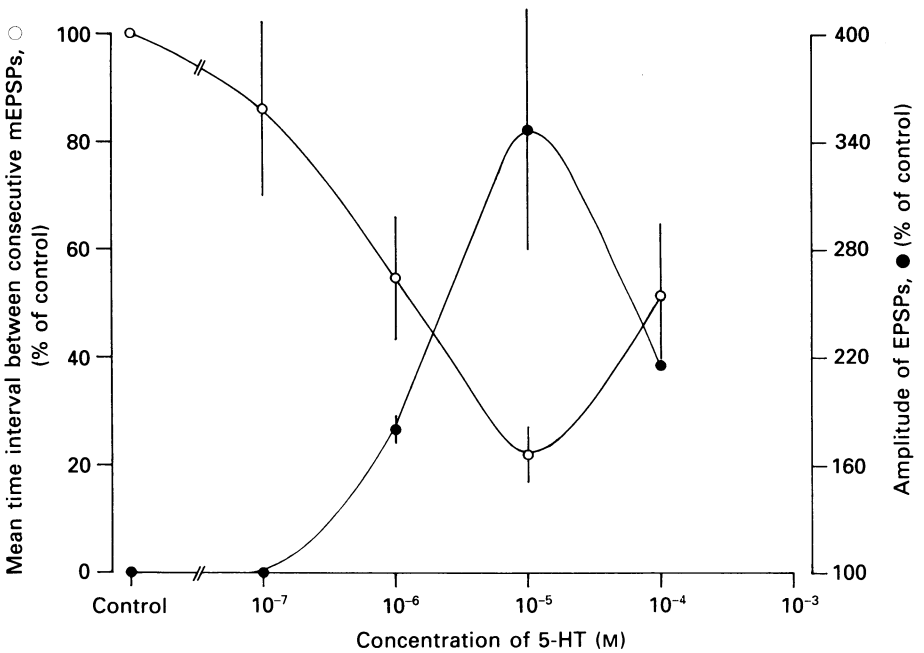

Fig. 3. The dose–response relation between the concentration of 5-HT, the degree of heterosynaptic facilitation (●), and the decrease in the mean time interval between consecutive mEPSPs (○) of the mean.

Since 5-HT depolarizes and FMRFa hyperpolarizes the sensory neurones, the modulation of spontaneous release by these transmitters could be ascribed to alterations of the resting potential, either directly or indirectly through consequent changes of the influx of Ca^{2+} through voltage-gated Ca^{2+} channels.

Effect of membrane potential on spontaneous release

Depolarization of the soma of the presynaptic neurone decreased the mean time interval between consecutive mEPSPs (Fig. 5*A*), suggesting that the membrane potential of the terminal could be altered by injecting current into the soma. However, hyperpolarization by 1–2 nA, sufficient current to hyperpolarize the soma by approximately 20 mV, had no effect on the mean time interval between consecutive mEPSPs. The effect of depolarization required external Ca^{2+}: when saline solutions containing no added Ca^{2+} were applied, depolarization no longer

caused a decrease in the mean time interval between consecutive mEPSPs (Fig. 5*A* and *B*). This suggests that the membrane potential of the presynaptic neurone is unable to alter the rates of spontaneous release directly, but that loading of the terminals with Ca^{2+} might increase spontaneous release.

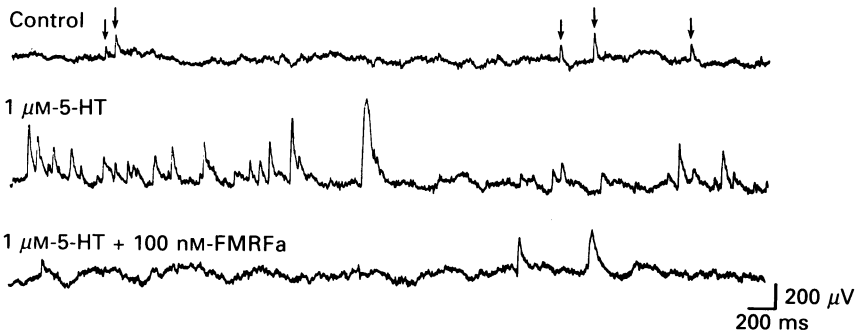

Fig. 4. Application of 1 μM-5-HT increases the frequency of mEPSPs. When 100 nM-FMRFa is applied in conjunction with the 5-HT, the incidence of mEPSPs is greatly reduced. The arrows above the top trace indicate mEPSPs.

To test whether 5-HT might enhance spontaneous release by depolarizing the presynaptic terminals, the sensory neurone was hyperpolarized by 1–2 nA during the application of 5-HT. This amount of current hyperpolarized the neurones by some 20–30 mV below their resting potential. The mean time interval between consecutive mEPSPs was compared just before and during the hyperpolarization. There was no significant difference between the mean time intervals between consecutive mEPSPs before and during the hyperpolarization (mean difference −0·086 s, S.E.M. 0·055 s, 5 experiments, $P > 0·1$). Since 5-HT increases the membrane resistance, injection of current into the soma might be expected to have a greater effect on the membrane potential of the terminals than under control conditions. The lack of effect of hyperpolarization during the actions of 5-HT therefore suggests that the enhancement of spontaneous release by 5-HT was not due to depolarization of the presynaptic neurone.

Modulation of spontaneous release in saline solutions lacking added Ca^{2+}

To test whether the modulation of spontaneous release by 5-HT and FMRFa might be due to alterations in a Ca^{2+} influx, experiments were performed in saline solutions lacking added Ca^{2+}. Spontaneous release persisted in the absence of added Ca^{2+}. Under these conditions, bath application of 10 μM-5-HT still caused a decrease in the mean time interval between consecutive mEPSPs to 31·5% of the control value (17·7% in Ca^{2+}) (S.E.M. 4·94%, 7 experiments, $t = 13·87$, $P < 0·001$, Fig. 6; see also Table 1). When applied in conjunction with the 5-HT, 100 nM-FMRFa significantly increased the mean time interval between consecutive mEPSPs to 75·8% of the control value (S.E.M. 10·96%, 7 experiments, $t = 5·19$, $P < 0·01$, Fig. 6). A large component of the modulation of spontaneous release by 5-HT and FMRFa can clearly occur in the absence of external Ca^{2+}.

Since saline solutions lacking added Ca^{2+} can still contain significant amounts of Ca^{2+} (as a contaminant from $MgCl_2$ or the water; see Methods), four further experiments were performed in which the Ca^{2+} chelator BAPTA was added at 1 mM to the saline. The application of 10 μM-5-HT reduced the mean time interval between

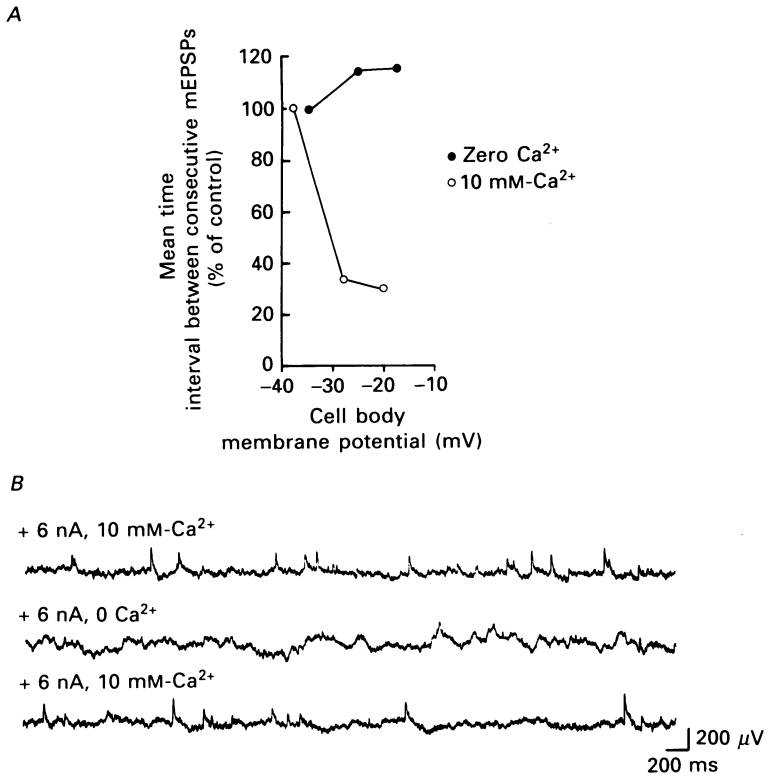

Fig. 5. The effect of cell body membrane potential on spontaneous release. *A*, depolarization of the sensory neurone soma decreases the mean time interval between consecutive mEPSPs when the bathing medium contains 10 mM-Ca^{2+}. If the neurone is bathed in a saline containing no added Ca^{2+}, depolarization is no longer effective. *B*, in a different culture, the sensory neurone soma was depolarized by +6 nA, in a saline containing 10 mM-Ca^{2+}, to elevate the frequency of spontaneous release. Using a microperfusion system, the bathing medium was quickly replaced by one containing no added Ca^{2+}; the frequency of spontaneous release was greatly diminished. When 10 mM-Ca^{2+} was reintroduced to the bathing medium, the frequency of spontaneous release became elevated once more. In the 0 Ca^{2+} salines, slow fluctuations in membrane potential are seen (middle trace of *B*). Spontaneous mEPSPs superimposed on these slow fluctuations are clearly distinguishable by virtue of their fast rise time (see Methods).

consecutive mEPSPs to 15·2% of the control (S.E.M. 3·57%, 4 experiments, $t = 23·8$, $P < 0·001$; see also Table 1).

The effect of FMRFa applied alone in saline containing no added Ca^{2+} and 1 mM-BAPTA was examined. At a concentration of 100 nM (Fig. 7), FMRFa caused a small but significant increase (4 experiments, $t = 3·18$, $P < 0·05$) in the mean time interval between consecutive mEPSPs. At a concentration of 1 μM ($n = 4$), FMRFa caused a

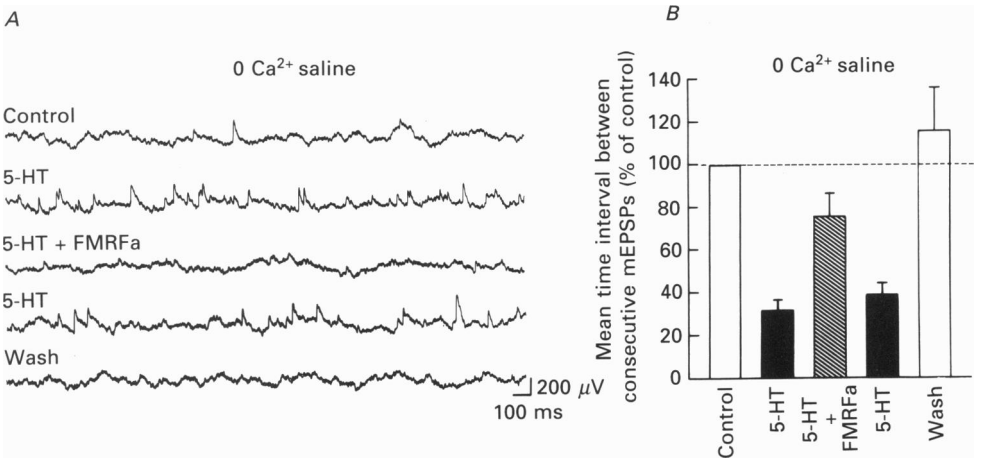

Fig. 6. Modulation of spontaneous release in saline solutions containing no added Ca^{2+}. *A*, 10 μM-5-HT elevates spontaneous release in a saline containing no added Ca^{2+}; when 100 nM-FMRFa is added in conjunction with the 5-HT, spontaneous release is depressed; this effect of FMRFa reverses when the FMRFa is washed out, leaving the 5-HT. The effect of the 5-HT reverses when the 5-HT is washed off. *B*, a histogram summarizing seven similar experiments showing the effect of 5-HT and FMRFa on the mean time interval between consecutive mEPSPs in a saline containing no added Ca^{2+}. Error bars are one standard error of the mean.

TABLE 1. Comparison of the effects of 10 μM-5-HT and 100 nM-FMRFa on the absolute mean time interval between consecutive mEPSPs in the presence and absence of external Ca^{2+}, Cd^{2+} and BAPTA

Experimental conditions	Mean time interval between mEPSPs (s) Control	5-HT	5-HT + FMRFa
ASW with 10 mM-Ca^{2+}	3·07 ± 0·44	0·52 ± 0·18	—
ASW with no added Ca^{2+}	2·82 ± 0·39	0·94 ± 0·22	2·15 ± 0·45
ASW with 1 mM-BAPTA, no added Ca^{2+}	1·99 ± 0·22	0·29 ± 0·07	—
ASW with 1 mM-Cd^{2+}	1·95 ± 0·18	0·57 ± 0·09	1·49 ± 0·10
BAPTA injection	6·97 ± 0·79	2·82 ± 0·65	4·15 ± 0·49

ASW = artificial sea water.

roughly twofold increase in the mean time interval between consecutive mEPSPs ($t = 15{\cdot}69$, $P < 0{\cdot}01$, Fig. 7). FMRFa by itself can therefore modulate spontaneous release in the absence of external Ca^{2+}.

Modulation of spontaneous release in saline solutions containing 1 mM-Cd^{2+}

To investigate further whether the modulation of spontaneous release required a Ca^{2+} influx, experiments were performed in the presence of 1 mM-Cd^{2+} (with normal

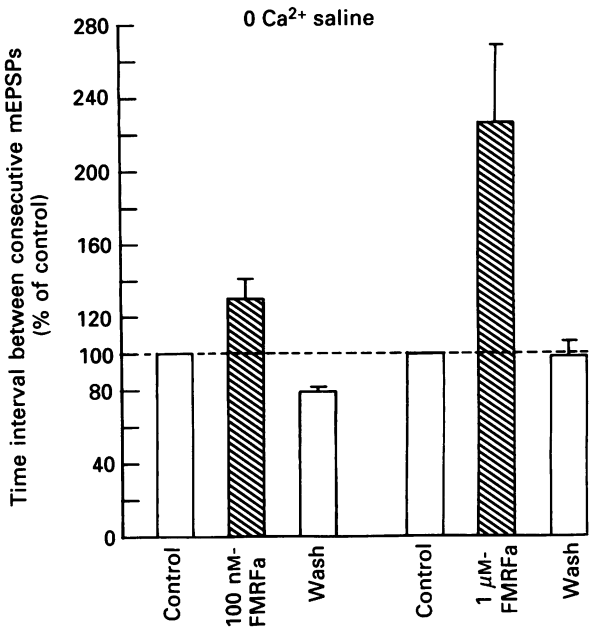

Fig. 7. The effect of 100 nM and 1 μM-FMRFa alone on the mean time interval between consecutive mEPSPs in saline solutions containing no added Ca^{2+} and 1 mM-BAPTA. Error bars are one standard error of the mean.

external Ca^{2+}). At this concentration, Cd^{2+} blocks all voltage-gated Ca^{2+} currents, and completely abolished evoked transmitter release. Both 5-HT and FMRFa, in combination with 5-HT, were able to modulate spontaneous transmitter release (Figs 8 and 9) by amounts very similar to those seen in the 0 Ca^{2+} saline solutions (compare Figs 6, 8 and 9, see also Table 1). 5-HT (10 μM) caused a significant decrease in the mean time interval between mEPSPs to 29% of the control ($t = 20{\cdot}76$, $P < 0{\cdot}001$) and 100 nM-FMRFa significantly increased the time interval between mEPSPs to 79·8% of the control ($t = 28{\cdot}28$, $P < 0{\cdot}01$). This confirms that the modulation of spontaneous release does not depend on a Ca^{2+} influx. Unlike the 0 Ca^{2+} saline solutions, Cd^{2+} caused a slow and progressive decrease in the mean time interval between consecutive mEPSPs. A similar effect has been reported at the vertebrate neuromuscular junction and it may be due to a blockade of the Ca^{2+}–Na^{+} exchanger by Cd^{2+} which could gradually elevate the levels of Ca^{2+} in the terminals (Trosper & Philipson, 1983).

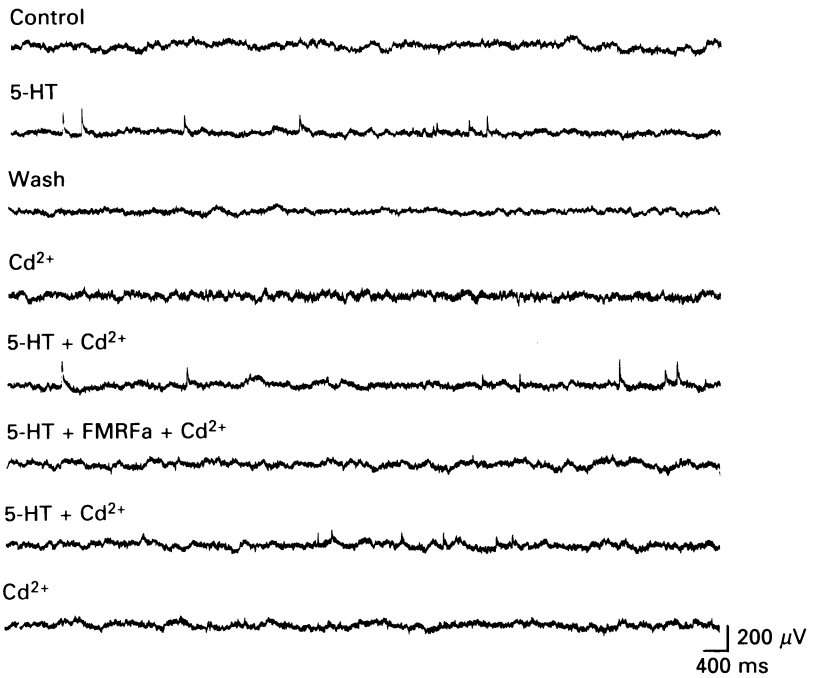

Fig. 8. The effects of 5-HT and FMRFa in saline solutions containing 1 mM-Cd^{2+}. The effect of 10 μM-5-HT on spontaneous release in a control saline was determined; a saline containing 1 mM-Cd^{2+} was introduced. Under these conditions, 5-HT still increased spontaneous release, and FMRFa applied in conjunction with the 5-HT reduced it.

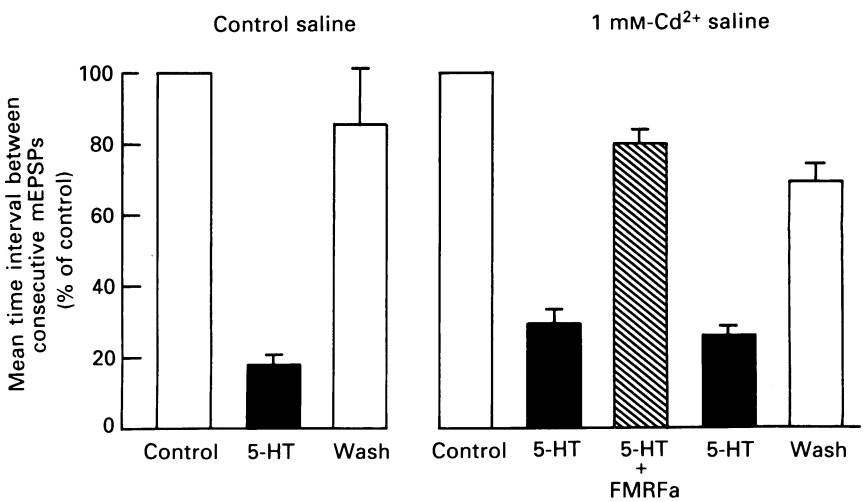

Fig. 9. A summary histogram showing the effect of 10 μM-5-HT under control conditions and in the presence of 1 mM-Cd^{2+} ($n = 5$), and the effect of FMRFa when applied together with the 5-HT in the presence of Cd^{2+} ($n = 3$). Error bars are one standard error of the mean.

Modulation of spontaneous release in cells injected with BAPTA

To test whether release of Ca^{2+} from internal stores could be involved in the modulation of spontaneous release by 5-HT and FMRFa, sensory neurones were injected with the Ca^{2+} chelator BAPTA. Two criteria were used to determine whether the BAPTA was acting as an effective buffer at the terminals of the sensory neurones. The first and more rigorous condition was whether, with the normal concentration of Ca^{2+} in the medium, evoked transmitter release was blocked after injection of BAPTA. This condition was verified both before and during the application of 5-HT (Fig. 10*A*). The second test was whether the injection of BAPTA could block the increase in spontaneous release caused by depolarization of the sensory neurone with normal Ca^{2+} in the extracellular saline. This second condition was always fulfilled if BAPTA had blocked evoked transmitter release. The effect of the BAPTA was to cause a progressive decline in the amplitude of the evoked EPSP from its full size to the level of single quanta. The evoked EPSP was tested at intervals of 5 min with trains for four stimuli. Successful blockade was considered to be achieved only when all four stimuli failed to evoke even a single quantum. In all the experiments reported here, after some 20–40 min of injection, the first of these conditions was met, suggesting that the BAPTA was present in the presynaptic terminals and able to chelate the fast Ca^{2+} transient during the action potential sufficiently well to prevent evoked transmitter release even during the actions of 5-HT (cf. Storm, 1987).

The injection of BAPTA also caused a gradual and progressive increase in the mean time interval between consecutive mEPSPs. For control cells, the mean time interval between consecutive mEPSPs was 3·07 s (S.E.M. 0·44 s, 11 experiments). In the BAPTA-treated cells, when enough BAPTA had been injected over a period of 20–40 min to block evoked release, the mean time interval between consecutive mEPSPs was increased to 6·97 s (S.E.M. 0·79 s, 10 experiments). This is further evidence that the BAPTA had reached the presynaptic terminals and was presumably able to reduce the basal levels of Ca^{2+}, and hence the amount of spontaneous release.

Bath application of 10 μM-5-HT to the cells injected with BAPTA was still able to cause an enhancement of spontaneous release, reducing the mean time interval between consecutive mEPSPs to 38·7 % of the control (S.E.M. 5·55 %, 10 experiments, $t = 11{\cdot}05$, $P < 0{\cdot}01$, Fig. 10*B*; see also Table 1). When 100 nM-FMRFa was applied in conjunction with the 5-HT, it partially reversed the effect of 5-HT, significantly increasing the mean time interval between consecutive mEPSPs to 63·8 % of the control (S.E.M. 7·17 %, 6 experiments, $t = 4{\cdot}95$, $P < 0{\cdot}01$ Fig. 10*B*; see also Table 1). The magnitude of these changes is similar to those seen with the 0 Ca^{2+} and Cd^{2+} saline solutions, and suggests, therefore, that a large component of the modulation of spontaneous release by 5-HT and FMRFa does not require changes in the internal Ca^{2+} concentration.

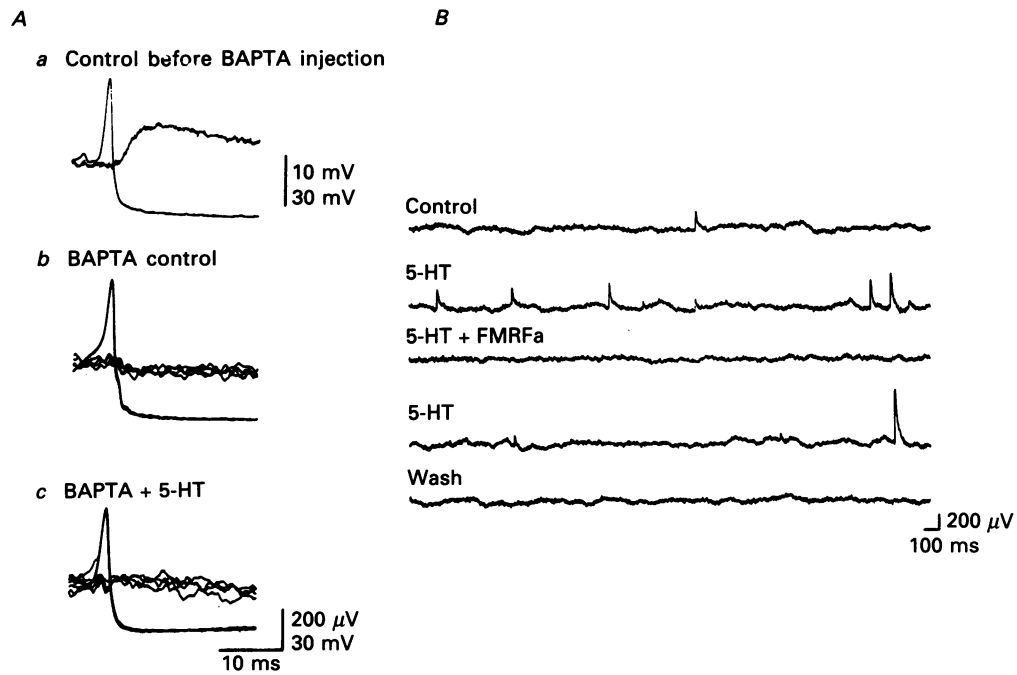

Fig. 10. The effect of injection of BAPTA into the presynaptic neurone. *A a*, before the injection of BAPTA into the sensory neurone, the sensory neurone could evoke an EPSP in the motoneurone of about 10 mV. *A b*, after the injection of BAPTA, action potentials in the sensory neurone could no longer evoke an EPSP (four superimposed traces). *A c*, the BAPTA injection blocked synaptic transmission even during the application of 5-HT (four superimposed traces). Note the difference in the gain of the motoneurone recording between *A a* and *A c* and *d*. *B*, in the same cells as in *A*, 10 μM-5-HT could cause an increase in spontaneous release after the BAPTA injection had blocked evoked release. FMRFa (100 nM) applied in conjunction with the 5-HT caused a decrease in the amount of spontaneous release from which there was partial recovery when the FMRFa was washed out.

DISCUSSION

The dependence of the modulation of spontaneous release on Ca^{2+}

Both 5-HT and FMRFamide produce substantial modulation – increases and decreases respectively – in the spontaneous release of transmitter from the presynaptic terminals of individual sensory neurones in culture. A large component of this modulation remained when the external Ca^{2+} was removed or Cd^{2+} was added to abolish voltage-gated Ca^{2+} currents. This suggests that the modulation of spontaneous release was not due to a Ca^{2+} influx. Similar results have been obtained in other preparations. For example, at the frog neuromuscular junction, adrenocorticotrophic hormone (ACTH) enhances spontaneous release. This effect can be seen in saline solutions containing no added Ca^{2+} (Johnston, Kravitz, Meiri & Rahamimoff, 1983). In the CA1 region of the hippocampus, both evoked synaptic

transmission and the frequency of spontaneous release are enhanced by activation of protein kinase C by application of phorbol esters (Malenka, Ayoub & Nicoll, 1987). Here too the modulation of spontaneous release by the phorbol esters can be seen in the absence of added Ca^{2+} and with elevated Mg^{2+} concentrations. In apparent contrast, while elevation of spontaneous release during post-tetanic potentiation can occur at the frog neuromuscular junction in the absence of extracellular Ca^{2+}, it is much reduced when compared to the elevation recorded in normal Ca^{2+} saline solutions (Lev-Tov & Rahamimoff, 1980). Similarly post-tetanic potentiation in *Aplysia* is reduced by injection of EGTA (Kretz *et al.* 1984).

Our experiments with the intracellular injection of BAPTA were designed to test whether alterations in the concentrations of intracellular Ca^{2+} might mediate the effects of 5-HT and FMRFa on spontaneous release. Our criteria for the effective action of BAPTA in the terminals were: (1) a blockade of evoked release; (2) the blockade of the depolarization-induced enhancement of spontaneous release; and, (3) the gradual and progressive increase in the mean time interval between consecutive mEPSPs which occurred during the injection of BAPTA. We cannot fully discount the possibility that the BAPTA was unable to buffer some localized changes in the internal Ca^{2+} levels in the terminals, which could underlie the actions of 5-HT and FMRFa. However, a localized increase in Ca^{2+} seems unlikely to account for the modulation of spontaneous release for two reasons: (1) the BAPTA was able to block evoked release even in the presence of 5-HT which would be expected to increase both evoked release and the invasion of the terminals by the action potential; (2) if there were a localized increase in Ca^{2+} it might be expected to be greatly attenuated by the BAPTA and, hence, the resulting modulation of release would also be attenuated. This was not seen; a substantial amount of the modulation remained.

That 5-HT and FMRFa were still able to modulate spontaneous release after the injection of BAPTA, suggests that a major component of the actions of 5-HT and FMRFa are not mediated by a change in the basal levels of Ca^{2+} caused by release of Ca^{2+} from internal stores. This conclusion is further supported by observations in the sensory neurones with the Ca^{2+} indicator dye Fura-2, which show that 5-HT does not alter the resting level of Ca^{2+} (Spira *et al.* 1987). We propose, therefore, that these transmitters can modulate spontaneous release either by altering directly the availability of transmitter for release, or the efficiency of the release process (e.g. the docking or fusion of vesicles) by a mechanism that is independent of transmembrane ionic fluxes or changes in Ca^{2+}. This finding suggests further that the second messenger, which might mediate this process, cannot require changes in the basal concentrations of Ca^{2+}.

An alternative interpretation of the observation that spontaneous release remained at a reduced rate after injection of BAPTA into the sensory neurone is that this spontaneous release might represent a constitutive Ca^{2+}-independent form of release. If this were the case, modulation of this constitutive spontaneous release process might not be representative of the more normal Ca^{2+}-dependent release. Although we cannot discount this possibility, we think it unlikely. In all the experiments with BAPTA injection, the synapse went through a phase, some 20–40 min after injection, when evoked release was blocked and spontaneous release remained (albeit at a reduced rate) to a phase where spontaneous release seemed to have disappeared

completely. This suggests that prolonged injection of BAPTA into the presynaptic cell was able to reduce the Ca^{2+} levels gradually to a point where spontaneous release could no longer occur. This in turn would suggest that there is not a constitutive release process that is independent of Ca^{2+}.

Possible mechanisms for modulating spontaneous release

Our results suggest that presynaptic facilitation and inhibition utilize an additional component, one that involves either a change in the release process or the availability of transmitter. The mechanism by which this component operates is not known. We shall here outline two main alternatives.

One possibility, proposed by Gingrich & Byrne (1985) and Hochner *et al.* (1986*a*), is that there could be a storage pool and a releasable pool of transmitter, and that 5-HT and FMRFa could cause the movement of transmitter between the two pools. This hypothesis has recently received support. Klein, Braha, Dale, Kandel, Hansen & Abrams (1989) have found that both the modulation of spontaneous release and a component of heterosynaptic facilitation may be mediated by an additional protein kinase, perhaps protein kinase C, acting either in series or in parallel with the cyclic AMP-dependent protein kinase (see also Higashida, 1988). This suggests that the modulation of spontaneous release and a component of heterosynaptic facilitation are two manifestations of the same underlying mechanism. This component of facilitation is particularly important for increasing transmitter release at synapses that have previously been depressed by repeated stimulation (Hochner *et al.* 1986*a*; Klein *et al.* 1989). The modulation of spontaneous release could involve a replenishment of the depleted pool of releasable transmitter. A synapsin-like molecule (Llinás, McGuiness, Leonard, Sugimori & Greengard, 1985) could be involved in such a process by, for example, controlling translocation of vesicles or their attachment to the cytoskeleton (De Camilli & Greengard, 1986). 5-HT and FMRFa could alter the availability of transmitter for release by altering the translocation of vesicles or the proportion that might be anchored to the cytoskeleton. However, on the basis of our data in *Aplysia*, it seems unlikely that Synapsin I itself is involved, since Synapsin I is thought to require phosphorylation by Ca^{2+}/calmodulin kinase before it will release vesicles from their cytoskeletal attachment. Moreover, spontaneous release and its modulation by 5-HT are unaffected by brief applications (1–5 min) of 5–15 μM-cytochalasin B or 1 mM-colchicine, both of which might be expected to alter the cytoskeleton (N. Dale, personal observations).

A second alternative is that 5-HT and FMRFa could affect the release process directly. For example, perhaps only a proportion of the morphological release sites in the synapse are active and release transmitter. The effects of 5-HT and FMRFa would be to increase or decrease, respectively, the proportion of active release sites, possibly by phosphorylating proteins in the active zones.

Relationship between spontaneous and evoked transmitter release

We suggest that the mechanisms which modulate spontaneous release also contribute to the modulation of evoked release during presynaptic facilitation and inhibition, and that the study of spontaneous release is a useful additional approach to examining the mechanisms of synaptic plasticity. Several observations support

this suggestion. (1) Spontaneous mEPSPs have the same amplitude, rise time, and half-fall time as the unitary evoked EPSPs elicited under conditions of low release (Dale *et al.* 1988). This suggests that the same quantal release event underlies both evoked and spontaneous release, and that the transmitter pools for spontaneous and evoked release might be the same. (2) The modulation of spontaneous release and the enhancement of evoked transmitter release share a very similar time course and dose–response relation to the application of 5-HT, suggesting that they are but two manifestations of a related underlying process. (3) The modulation of spontaneous release and a component of heterosynaptic facilitation may share the same second messenger pathway: possibly activation of protein kinase C (Klein *et al.* 1989).

We therefore argue that our conclusions can be extended to the facilitation of evoked release itself, and propose that the component of heterosynaptic facilitation, which is thought to be independent of K^+ and Ca^{2+} currents (see Hochner *et al.* 1986*a*), could involve a direct modulation of the release process or the availability of transmitter for release.

Presynaptic facilitation and inhibition

Although our results suggest that a major component of the modulation of spontaneous release and, by extension, a component of heterosynaptic facilitation, does not require a change in the intracellular Ca^{2+}, there is evidence that Ca^{2+} plays an important role in heterosynaptic facilitation. First, the reduction of the K^+ S-current produced by cyclic AMP that accompanies facilitation allows a broadening of the action potential, a consequent increase in Ca^{2+} entry during the action potential (Hochner *et al.* 1986*b*), and hence more evoked transmitter release. Second, there is a change in the Ca^{2+} handling in response to depolarization and action potentials (Boyle, Klein, Smith & Kandel, 1984; Spira *et al.* 1987). The increase in intracellular Ca^{2+} that results from these two mechanisms is important for evoked transmitter release. This additional Ca^{2+} could play a synergistic role, together with the Ca^{2+}-independent mechanisms, and further enhance the release process or availability of transmitter. Indeed, spontaneous release can be enhanced by depolarization of the presynaptic neurone, both under control conditions (Fig. 5) and during the application of 5-HT (data not shown), suggesting that a Ca^{2+} influx during the actions of 5-HT will provide an additional enhancement of facilitatory mechanisms.

The peptide FMRFa mediates presynaptic inhibition in *Aplysia*. Two actions which could contribute to presynaptic inhibition have already been described (cf. Kretz, Shapiro, Bailey, Chen & Kandel, 1986*a*). The first involves an increase in the probability in the opening of the serotonin-sensitive K^+ channel (Belardetti *et al.*, 1987). A second effect involves a reduction of a voltage-sensitive Ca^{2+} current (Brezina *et al.* 1987; Edmonds, Kandel & Klein, 1988). Our results suggest a third physiological effect of FMRFa, which appears to be independent of both K^+ and Ca^{2+} currents: a direct modulation of spontaneous release either by altering transmitter availability or the release process itself.

Thus, for both facilitation and presynaptic inhibition, it appears that several changes act in concert at several different targets to modulate the synapse. (1) Modulation of K^+ channels which alters the width of the action potential, thereby indirectly altering the Ca^{2+} influx during the action potential (Hochner *et al.* 1986*a*, *b*;

Klein & Kandel, 1980). (2) Modulation of the Ca^{2+} influx (cf. Kretz, Shapiro & Kandel, 1986*b*; Kretz *et al.* 1986*a*) or handling of intracellular Ca^{2+} (Boyle *et al.* 1984), both of which could alter the availability of transmitter for release. (3) A direct modulation of either transmitter availability or the release mechanism through a Ca^{2+}-independent process.

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