Partial Uncoupling of Neurotransmitter Release From $[\text{Ca}^{2+}]_i$ by Membrane Hyperpolarization

R. RAVIN, H. PARNAS, M. E. SPIRA, AND I. PARNAS

The Otto Loewi Minerva Center for Cellular and Molecular Neurobiology, Department of Neurobiology, The Hebrew University, Jerusalem 91904, Israel

Ravin, R., H. Parnas, M. E. Spira, and I. Parnas. Partial uncoupling of neurotransmitter release from $[\text{Ca}^{2+}]_i$ by membrane hyperpolarization. J. Neurophysiol. 81: 3044–3053, 1999. The dependence of evoked and asynchronous release on intracellular calcium ($[\text{Ca}^{2+}]_i$) and presynaptic membrane potential was examined in single-release boutons of the crayfish opener neuromuscular junction. When a single bouton was depolarized by a train of pulses, $[\text{Ca}^{2+}]_i$, increased to different levels according to the frequency of stimulation. Concomitant measurements of evoked release and asynchronous release, from the same bouton, showed that both increased in a sigmoidal manner as a function of $[\text{Ca}^{2+}]_i$. When each of the depolarizing pulses was immediately followed by a hyperpolarizing pulse, $[\text{Ca}^{2+}]_i$, was elevated to a lesser degree than in the control experiments, and the rate of asynchronous release and the quantal content were reduced; most importantly, evoked quantal release terminated sooner. The diminution of neurotransmitter release by the hyperpolarizing postpulse (HPP) could not be entirely accounted for by the reduction in $[\text{Ca}^{2+}]_i$. The experimental results are consistent with the hypothesis that the HPP reduces the sensitivity of the release machinery to $[\text{Ca}^{2+}]_i$, thereby not only reducing the quantal content but also terminating the quantal release process sooner.

INTRODUCTION

The $\text{Ca}^{2+}$-voltage hypothesis (CVH) (Parnas and Parnas 1994; Parnas et al. 1990) asserts that in fast synapses two voltage-dependent factors are required to evoke release of neurotransmitter, the opening of $\text{Ca}^{2+}$ channels and the ensuing increase in intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) and the voltage-dependent transformation of a putative inactive release apparatus, $T$, to its active state, $S$. According to the CVH, the quantal content depends on both $[\text{Ca}^{2+}]_i$ and $S$, whereas the time course of release depends on the $T \Rightarrow S$ transformation (Lustig et al. 1989; Parnas and Parnas 1994; Parnas et al. 1986a,b). Other investigators support the notion that it is the kinetics of entry and removal of $\text{Ca}^{2+}$ that determine the time course of release. In particular, transient local elevation of $\text{Ca}^{2+}$ to hundreds of micromolar concentration initiates release, and the subsequent collapse of such $\text{Ca}$ domain terminates release. This hypothesis, termed the $\text{Ca}^{2+}$ microdomain hypothesis for neurotransmitter release, is described in several reviews (Smith and Augustin 1988; Zucker 1996).

Numerous findings support the conclusion that it is not the influx of $\text{Ca}^{2+}$ that triggers release, and it is not the removal of $\text{Ca}^{2+}$ from the microdomain of the transmitter release site that terminates release. Experimental manipulations known to affect $\text{Ca}^{2+}$ influx or its removal did not affect the time course of release (Andrew and Barrett 1980; Arechiga et al. 1990; Datyner and Gage 1980; Hochner et al. 1991; Parnas et al. 1984, 1989; Van der Kloot and Molgo 1994). In other experiments, conditions were such that no significant influx of $\text{Ca}^{2+}$ was possible. In one case, the external solution included EGTA with no added $\text{Ca}^{2+}$ (Silinsky et al. 1995). In another case, $\text{Ca}^{2+}$ was omitted from the circulation solution (Mosier and Zengel 1994), or $\text{Ca}^{2+}$ entry was blocked by increasing $[\text{Mg}^{2+}]_o$ (Hochner et al. 1989). In these experiments, $[\text{Ca}^{2+}]_i$, was increased by means other than entry via voltage-gated $\text{Ca}^{2+}$ channels. Evoked release was obtained only when the presynaptic terminal was depolarized, although there was little or no influx of $\text{Ca}^{2+}$. Recently, Mochida et al. (1998) demonstrated that depolarization directly enhances neurotransmitter release and that the $\text{Ca}^{2+}$ channel itself is a voltage sensor connected via the synprint protein to the exocytic machinery.

The third line of experiments tested whether the quantal content and time course of release are affected by the rate of presynaptic repolarization after a test depolarizing pulse. The CVH predicts that if $S$ transforms faster to the $T$ state, the quantal content will be smaller, and termination of release will occur sooner. This was tested experimentally by Dudel (1984), Parnas et al. (1986b), and Arechiga et al. (1990). These authors compared synaptic delay histograms obtained by a control depolarizing test pulse with histograms obtained by the same stimulus followed by a brief hyperpolarizing postpulse (HPP). Indeed, when the test pulse was followed by an HPP, quantal content was reduced, and the duration of evoked release was shortened. These results indicated that the HPP caused a faster $S \rightarrow T$ transformation. However, as suggested by Zucker (1987), the reduction in quantal content can well be explained if the HPP merely causes a faster closure of $\text{Ca}^{2+}$ channels (Klockner et al. 1989; Matteson and Armstrong 1986; Swandulla and Armstrong 1988).

Because Ravin et al. (1997) have since developed a technique to monitor $[\text{Ca}^{2+}]_i$ and quantal release from a single-release bouton simultaneously, we reinvestigated the effect of a HPP on $[\text{Ca}^{2+}]_i$ on evoked and asynchronous release. We found that in addition to accelerating closure of $\text{Ca}^{2+}$ channels, HPP directly affects the release apparatus, probably by facilitating the $S \rightarrow T$ transformation.

METHODS

We used the opener neuromuscular preparation of the first walking leg of the crayfish Procambarus clarkii. Crayfish (3–4 cm long) were...
Ca\textsuperscript{2+} imaging

[Ca\textsuperscript{2+}], was measured after fura-2 injection into one of the secondary branches of the excitatory axon and with ratiometric imaging techniques (Grynkievicius et al. 1985; Ziv and Spira 1993). The final intraxonal fura-2 concentration was 50–100 \(\mu\)M (Ravin et al. 1997). For digital video imaging (\(\times 40\) upright microscope, Nikon, Optiphoto 2) we used an intensified charge-coupled device video camera (model 2400–77, Hamamatsu, Hamamatsu City, Japan). The images taken before, during, and after the stimulation period were stored on 1/4-inch videotape. Thus, although release was measured after each impulse of the train, Ca\textsuperscript{2+} imaging reflected the overall Ca\textsuperscript{2+} concentration during the stimulation period. For further technical details, the reader should consult Ravin et al. (1997) where detailed procedures are provided.

**Stimulation and recording**

We used the macropatch technique (Dudel 1981), which enables depolarization of a single-release bouton and simultaneous recording of single quanta events with the same electrode (Ravin et al. 1997).

**RESULTS**

**Basic experiment**

Figure 1 shows results of a “typical” experiment. Figure 1A shows Ca\textsuperscript{2+} images of a single-release bouton injected with fura-2. In Fig. A, first row, the bouton was stimulated with a train of depolarizing pulses (\(-0.7 \mu\text{A}, 0.6 \text{ ms}\) at 100 Hz for 8 s (control). The test depolarizing pulse appears as a negative current pulse on the right. In the second row, a similar train was delivered (7 min later), but now each test depolarizing pulse was followed immediately (0 delay) by an HPP (+0.6 \(\mu\)A, 0.5 ms) (see stimulation protocol on right). In the third row, the train of the test pulse was administered again (7 min later) as a second control. Clearly, the HPP reduced Ca\textsuperscript{2+} accumulation.

Figure 1B shows the temporal change in intracellular Ca\textsuperscript{2+} concentration for the stimulation regimes described. The HPP reduced the rate of [Ca\textsuperscript{2+}]\textsubscript{i} accumulation and its plateau level. Under conditions of the first control, [Ca\textsuperscript{2+}]\textsubscript{r} reached a plateau level of 4.4 \(\mu\)M, and with the HPP the plateau level of [Ca\textsuperscript{2+}]\textsubscript{r} fell to 2.37 \(\mu\)M (56\% of control) and rose again to 3.9 \(\mu\)M during the second control.

During the plateau levels of [Ca\textsuperscript{2+}], quantal release (presented as delay histograms) after individual pulses was measured. The results are shown in Fig. 1C. Quantal content of the first control was 1.2. Quantal content declined to 0.4 with the HPP and recovered in the second control to 1.1. The shape of the delay histograms (time course of release) (Katz and Miledi 1965a,b) was actually the same for the first and second controls. The delay histogram corresponding to the HPP condition clearly shows that release stopped sooner than in the two controls. The median for the two controls was 2.4 ms, and with the HPP it fell to 2.1 ms (similar to the findings of Arechiga et al. 1990). In the delay histogram with the HPP, the minimal delay seems to be longer. This is an artifact resulting from the longer duration of saturation of the amplifier when both the depolarizing and hyperpolarizing pulses were administered in comparison with the period of saturation under control (compare stimulation artifacts in Fig. 1D, left and right columns). During the stimulation artifacts, no recording of quanta is possible. This introduces a small error at the beginning of the histogram but not at the decline of the histogram, the relevant phase for termination of release.

Figure 1D shows samples of quantal events recorded while [Ca\textsuperscript{2+}]\textsubscript{r} remained at the plateau level. In the left column, the traces show responses obtained for the test pulse. In the right column, the test pulse was followed by HPP (ascending stimulus artifact).

Is the reduction in Ca\textsuperscript{2+} accumulation due to reduced Ca\textsuperscript{2+} entry or to accelerated removal?

Hyperpolarizing pulses might reduce Ca\textsuperscript{2+} accumulation by facilitating closure of Ca\textsuperscript{2+} channels and thereby reducing Ca\textsuperscript{2+} influx. Alternatively, HPP may predominantly accelerate Ca\textsuperscript{2+} removal by facilitating the Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange at the more-negative membrane potentials (Blaustein 1988; Carafoli 1987). In the first case, HPP is expected to reduce Ca\textsuperscript{2+} accumulation only if administered immediately or very soon after the depolarizing pulse. In the second case, reduction in Ca\textsuperscript{2+} accumulation is expected to be independent of the interval between depolarization and the HPP.

Figure 2 shows that at 11°C, when the HPP was administered 2 ms or even 1 ms after the end of the depolarizing test pulse, it did not affect Ca\textsuperscript{2+} accumulation (Fig. 2, B and C). In contrast, when the postpulse was administered with zero delay after the test pulse, Ca\textsuperscript{2+} accumulation was reduced by 36\%, from 3.23 to 2.06 \(\mu\)M (Fig. 2A). In seven such experiments, HPP with zero delay reduced Ca\textsuperscript{2+} accumulation by 24.7 ± 9.7\% (SD). HPP after a 1- or 2-ms delay had no effect on Ca\textsuperscript{2+} accumulation (0.85 ± 6.4\% and 1 ± 4\%, respectively, \(n = 7\)). The difference between Ca\textsuperscript{2+} accumulation in the control and accumulation where an HPP was administered at zero delay is very significant (\(P = 0.0005\)). These results are consistent with the assumption that HPP accelerates closure of Ca\textsuperscript{2+} channels, consequently reducing Ca\textsuperscript{2+} influx.

Figure 2, D–F, shows the corresponding synaptic delay histograms for the Ca\textsuperscript{2+} profiles given in Fig. 2, A–C. In Fig. 2D, when an HPP was given with zero delay, the quantal content declined from a control value of 0.74 to 0.19 (a decline of 75\%). The median of the control histogram was 2.4 ms, and with the HPP it became 2.15 (a shift of 11\%). In Fig. 2, E and F, because of the stimulus artifact and saturation of the amplifier, the histograms are incomplete. Yet it is clear that the decay of the histograms is the same with and without HPP. Thus termination of release was not accelerated when the HPP was given with a delay of 1 or 2 ms. Similar results were obtained in seven additional experiments.
FIG. 1. Effects of hyperpolarizing postpulses (HPPs) on intracellular calcium $[Ca^{2+}]_i$ and on the time course of release. A: fura-2 ratio images of a single-release bouton. Note the macropatch electrode over the bouton. First row, control: bouton stimulated with a train of depolarizing pulses ($-0.7 \mu A, 0.6 \text{ ms}$) at $100 \text{ Hz}$ for $8 \text{ s}$ (right insert). Second row: similar train delivered, but now each depolarizing pulse was followed by an HPP ($+0.6 \mu A, 0.5 \text{ ms}$, right insert). Third row: control train administered again. Time between trains was $7 \text{ min}$. Scale bar: $12 \mu m$. B: temporal changes in $[Ca^{2+}]_i$, —: first control; $\cdots \cdots$: with HPP; $\cdots \cdots$: second control. $[Ca^{2+}]_i$ at the plateau was $4.4, 2.37, \text{ and } 3.9 \mu M$, respectively. C: delay histograms established during the plateau of $[Ca^{2+}]_i$, —: first control, $\cdots \cdots$: with HPP; $\cdots \cdots$: second control. Quantal contents were $1.19, 0.39, \text{ and } 1.1$ with the corresponding medians of $2.43, 2.1, \text{ and } 2.4 \text{ ms}$, respectively. D: samples of recordings. Left column: responses recorded during first control test pulse. Right column: responses recorded for test pulses followed by HPP. Note the change in stimulus artifact.
2.58, and 3.25 m

In 10 experiments stimulation, the average change in the median for 35 histograms was 1.4 M. Here with a fourfold change in 

We examined whether the average level of 

The time course of release was found to be insensitive to experimental manipulations that affect 

Time course of release is not affected by experimental manipulations that affect 

The time course of release was found to be insensitive to treatments known to affect Ca$^{2+}$ entry or removal (Andrew and Barrett 1980; Datyner and Gage 1980; Hochner et al. 1991). We examined whether the average level of $[Ca^{2+}]_i$ that was found to significantly affect the quantal content (Ravin et al. 1999) affects also the time course of release. As shown in Fig. 3, the normalized delay histograms were the same for different levels of $[Ca^{2+}]_i$, whether $[Ca^{2+}]_i$ was increased by stimulation at different frequencies (Fig. 3D) or by application of ionomycin (Fig. 3B). When $[Ca^{2+}]_i$ was increased by ionomycin (Fig. 3, A and B), the medians of the delay histograms were 2.02, 1.97, 2.02, 1.97, and 2.02 ms for $[Ca^{2+}]_i$ levels of 0.5, 0.87, 1, 1.3, and 1.45 M, respectively. Thus, with a threefold change in $[Ca^{2+}]_i$, the median of the histograms did not change. Similarly, when $[Ca^{2+}]_i$ was increased by stimulation at different frequencies (a different bouton, a different animal Fig. 3, C and D), the medians of the delay histograms were 2.45, 2.42, 2.45, 2.42, and 2.52 ms for $[Ca^{2+}]_i$ levels of 0.68, 1.43, 1.84, 2.58, and 3.25 M. Here with a fourfold change in $[Ca^{2+}]_i$, the medians were almost the same. Altogether, in 14 boutons (different preparations) where $[Ca^{2+}]_i$ was raised by repetitive stimulation, the average change in the median for 35 histograms was $3.3 \pm 5.3\%$ for $[Ca^{2+}]_i$, ranging between a few hundred nanomolars and a few micromolars. In 10 experiments where $[Ca^{2+}]_i$, was raised by ionomycin, the average change in the median for 93 histograms was $1.4 \pm 4.7\%$ for $[Ca^{2+}]_i$.

Is reduced Ca$^{2+}$ entry responsible for the faster termination of release?

Because we found that the HPP caused a reduction in Ca$^{2+}$ entry, it is possible that this reduction is responsible for shortening the delay histograms. One way to test this question is to reduce Ca$^{2+}$ entry by means other than a faster closure of the voltage-dependent Ca$^{2+}$ channels and study the effect on the synaptic delay histogram. In the experiment depicted in Fig. 4, we compared the consequences of reduction in $[Ca^{2+}]_i$, under two conditions, HPP and increased extracellular Mg$^{2+}$ concentration ([Mg$^{2+}]_o$). For both treatments, on the same release bouton, the reduction in $[Ca^{2+}]_i$ was similar (compare Fig. 4, A and D). In Fig. 4A (with HPP), Ca$^{2+}$ accumulation declined from a level of 4.3 to 3.0 M with recovery to a level of 4.1 M (a reduction of 29%). The quantal content was reduced from 1.45 to 0.58 (a reduction of 60%) with recovery to 1.26. The corresponding delay histograms show once again that with HPP release terminated sooner (Fig. 4B). The median was 2.35 ms for both controls, and it declined to 2.05 for the postpulse (a shift of 13%). Normalization (Fig. 4C) shows that with the postpulse the peak of the histogram shifted to the left, and release clearly terminated sooner. Figure 4, D–F, shows the effects of increasing [Mg$^{2+}]_o$ to 8.5 M. In the control, $[Ca^{2+}]_i$, was 4.1 M (this is the second control value from Fig.

**FIG. 2.** Effect of time interval between the depolarizing pulse and HPP on $[Ca^{2+}]_i$ and on the synaptic delay histogram. $[Ca^{2+}]_i$, was increased by stimulating bouton with a test pulse (−0.7 μA, 0.6 ms) at 100 Hz for 7 s. HPP was +0.6 μA, 0.5 ms. First control (−) and second control (−−−) were taken at the beginning and end of each experiment. $[Ca^{2+}]_i$ levels during the plateau phase of the controls were 3.23 and 3.18 M, respectively. A: HPP (−−−) was administered with 0 delay. B: HPP delivered with 1-ms delay. C: HPP given with 2-ms delay. For 3 HPP conditions, Ca$^{2+}$ concentrations during the plateau levels were 2.06, 3.4, and 3.16 M, respectively. D–F: corresponding delay histograms for the $[Ca^{2+}]_i$ profiles given in A–C. Note in E and F incomplete histograms because of the saturation of the amplifier when the HPP was given.
reduces $[\text{Ca}^{2+}]_i$, $[\text{Ca}^{2+}]_o$, and $[\text{Mg}^{2+}]_o$; the quantal content declined from 1.26 to 0.9 (a reduction of 28%). With increased $[\text{Mg}^{2+}]_o$, $[\text{Ca}^{2+}]_i$ dropped to 2.36 $\mu$M at 60 Hz (a reduction of 27%) and to 0.71 $\mu$M at 20 Hz (a reduction of 34%). The corresponding quantal content was 0.36 (a reduction of $\sim$78%). $[\text{Ca}^{2+}]_i$ was elevated by an HPP at 100 Hz $[\text{Ca}^{2+}]_i$ reached a plateau level of 4.7 $\mu$M. The corresponding quantal content was 1.64. With the HPP, $[\text{Ca}^{2+}]_i$ dropped to 2.36 $\mu$M ($\sim$50% reduction), and quantal content was reduced to 0.36 (a reduction of $\sim$78%). $[\text{Ca}^{2+}]_i$ in the second control reached a level of 4.6 $\mu$M, and the quantal content recovered to 1.45 (Fig. 5B). For the lower frequencies of 60 and 20 Hz, $[\text{Ca}^{2+}]_i$ reached plateau levels of 2.49 $\mu$M (Fig. 5C) and 1.08 $\mu$M (Fig. 5E), respectively. After an HPP, $[\text{Ca}^{2+}]_i$ declined to 1.83 $\mu$M at 60 Hz (a reduction of 27%) and to 0.71 $\mu$M at 20 Hz (a reduction of 34%). The corresponding quantal content was 0.74 in the control at 60 Hz, and it fell to 0.16 after an HPP (a reduction of 78%) (Fig. 5D). For 20 Hz, the quantal content was 0.17 in the control and declined to 0.04 after HPP (a reduction of 77%) (Fig. 5F).

These results show that the effect of the HPP on quantal content was fairly constant at the various frequencies, irrespective of the plateau level of $[\text{Ca}^{2+}]_i$. Furthermore, the effect of the postpulse on quantal content was also independent of the magnitude of reduction in $[\text{Ca}^{2+}]_i$ caused by the HPP. Similar results were obtained in five experiments. These results therefore support the possibility that the HPP, in addition to reduc-
ing Ca\textsuperscript{2+} influx, also causes the release machinery to be less sensitive to the level of [Ca\textsuperscript{2+}].

At this stage, however, an alternative explanation cannot be excluded. Such an explanation is as follows. The HPP reduces Ca\textsuperscript{2+} influx. As a result of this reduction, the local (below release sites) Ca\textsuperscript{2+} concentration is reduced to the same extent at all frequencies, and hence the percentage of reduction in quantal content is always the same. This possibility is not very likely, as Ravin et al. (1999) showed that evoked release is very sensitive to the average level of [Ca\textsuperscript{2+}]. Nevertheless, in the following section we describe results of experiments designed specifically to distinguish between these two possibilities.

**Effect of HPP on asynchronous release**

It is well accepted that when asynchronous release occurs the level of Ca\textsuperscript{2+} concentration below release sites is the same as the average [Ca\textsuperscript{2+}] in the terminal (Aharon et al. 1994; Miledi 1973; Rahamimoff et al. 1978; Ravin et al. 1997). Therefore if the only effect of HPP is to reduce Ca\textsuperscript{2+} influx, and hence [Ca\textsuperscript{2+}], it is expected that the decline in the rate of asynchronous release will correlate with the decline in [Ca\textsuperscript{2+}] caused by the HPP. On the other hand, if the HPP also causes the release machinery to be less sensitive to [Ca\textsuperscript{2+}], then, as in the case of evoked release, the rate of asynchronous release should be much less sensitive to changes in [Ca\textsuperscript{2+}]. Figure 6 shows pooled results (*) relating the rate of asynchronous release given as number of quanta/s to [Ca\textsuperscript{2+}], raised by repetitive stimulation. Asynchronous release was measured as described by Ravin et al. (1997), 5 ms after each depolarizing pulse. Asynchronous release was also measured when each of the depolarizing pulses was followed by an HPP. Figure 6A clearly shows that with HPP the rate of asynchronous release is almost independent of [Ca\textsuperscript{2+}]. The points with HPP fall well below the control points (*). For example, for [Ca\textsuperscript{2+}], ranging between 1.6 and 2.1 μM, the average rate of asynchronous release (average of the values in this range of [Ca\textsuperscript{2+}]) was 22.12 ± 15.6 quanta/s (n = 36); with postpulses the average was only 8.2 ± 6.6 quanta/s (n = 14). The difference is extremely significant (P = 0.0001). For [Ca\textsuperscript{2+}] levels in the range of 2.8–4 μM, the average rate of asynchronous release declined from a value of 70.16 ± 58 quanta/s (n = 24) in the control to 18.13 ± 7.5 quanta/s (n = 4) with HPP (P = 0.0001).

On the other hand, in 13 experiments when [Ca\textsuperscript{2+}] was reduced by increasing [Mg\textsuperscript{2+}] (Fig. 6B), the reduced rate of asynchronous release related fully to [Ca\textsuperscript{2+}]. For [Ca\textsuperscript{2+}], ranging between 1.6 and 2.1 μM, the average rate of asynchronous release was similar in the presence of elevated [Mg\textsuperscript{2+}] and in the control (23.34 ± 14.4 quanta/s (n = 5) and 22.12 ± 15.6 quanta/s, respectively, an insignificant difference, P = 0.58). For [Ca\textsuperscript{2+}] levels ranging between

---

**FIG. 4. Effect of HPP and [Mg\textsuperscript{2+}] on [Ca\textsuperscript{2+}] accumulation and on evoked release.** A: changes in [Ca\textsuperscript{2+}] during stimulation with a train of depolarizing pulses (0.7 μA, 0.6 ms) at 100 Hz for 7 s with and without HPP. —: first controls; - - - : second controls. The plateau levels of [Ca\textsuperscript{2+}] of the first and second control were 4.3 and 4.1 μM, respectively. HPP of +0.6 μA, 0.5 ms with a 0 delay (--) reduced plateau level of [Ca\textsuperscript{2+}] to 3.0 μM. B: delay histograms obtained during the first and second controls and with HPP. Medians of the first and second controls were 2.35 ms. Corresponding quantal contents were 1.45 and 1.26. HPP with 0 delay (--) reduced the quanta content to 0.58 and shifted the median to 2.05 ms. C: normalization of the delay histograms shown in B to their peak. With HPP the peak shifted to the left and release terminated sooner. Symbols as in A. D: same bouton as in A. Changes in [Ca\textsuperscript{2+}], for the same pulse trains (as in A) at 2 levels of [Mg\textsuperscript{2+}]: 2.5 mM at the 2 controls (— and - - - ) and 8.5 mM. Corresponding [Ca\textsuperscript{2+}], plateau levels for the controls were 4.1 and 3.9 μM. Increasing [Mg\textsuperscript{2+}] to 8.5 mM (--) reduced the [Ca\textsuperscript{2+}], plateau level to 2.9 μM. E: corresponding delay histograms for controls and elevated [Mg\textsuperscript{2+}]. The quantal content declined from 1.26 to 0.9 without a change in the medians, which remained 2.35 ms. F: normalization to the peak shows overlapping of the delay histograms.
2.8 and 4 μM, the average rate of asynchronous release in the presence of elevated [Mg$^{2+}$]$_i$ was 65.12 ± 26.6 quanta/s (n = 4), an insignificant change from the control of 70.16 ± 58 quanta/s.

These results strengthen the conclusion that the HPP exerts two effects. It clearly reduces Ca$^{2+}$ influx, but in addition to that effect it also affects the release apparatus associated with asynchronous release, rendering it less sensitive to [Ca$^{2+}$]$_i$.

To further support this conclusion and to test whether the apparatus associated with evoked release is also affected by the HPP, we compared the relationship between the percent of reduction in [Ca$^{2+}$]$_i$, (achieved either by HPP or elevated [Mg$^{2+}$]$_i$) and the percent of reduction in either asynchronous release or evoked release.

Percent reduction in release is given as $(L_1 - L_2)/L_1$, and the percent reduction in Ca$^{2+}$ is given as $(C_1 - C_2)/C_1$. Here, $L_1$ is the control release, and $L_2$ is the release after an HPP or after increasing [Mg$^{2+}$]$_i$. $C_1$ stands for the control [Ca$^{2+}$]$_i$, and $C_2$ is the Ca$^{2+}$ concentration after the treatments. This way of presenting the data was selected as it generates a straight line (for moderate changes in [Ca$^{2+}$]$_i$, mathematical derivation not shown). The data to consider must be in the range where $C_2$ is not very small in comparison with $C_1$ (hence the same applies also to $L_2$ in comparison with $L_1$) such that the ratio of $(L_1 - L_2)/L_1$ or $(C_1 - C_2)/C_1$ is sufficiently smaller than 1. When $C_2$ or $L_2$ is too small, these ratios approach 1, and the existence or lack of correlation between changes in release and changes in [Ca$^{2+}$]$_i$ cannot be resolved. For this reason we limited our analysis to the results where the change in [Ca$^{2+}$]$_i$ did not exceed 25%. The results presented in Fig. 7 show that, when [Ca$^{2+}$]$_i$ was reduced by increasing [Mg$^{2+}$]$_i$, the lines relating percent change in evoked release (Fig. 7A) or asynchronous release (Fig. 7B) show slopes of 3 and 2.5, respectively. The coefficients of determination ($r^2$) were 0.6 and 0.5, respectively, showing a high correlation between the change in percent release as a function of the change in [Ca$^{2+}$]$_i$. Furthermore, the intercept of the two lines with the x-axis is near the zero point. In other words, without a change in Ca$^{2+}$ entry there is no change in the level of evoked release or asynchronous release. On the other hand, with HPP, the slopes relating the percent changes in evoked (Fig. 7C) or asynchronous release (Fig. 7D) as a function of the percent change in [Ca$^{2+}$]$_i$ where 0.5 and 0.4, respectively, and the coefficients of determination were only 0.06 and 0.03, respectively. Thus with the HPP the percent change of release practically does not depend on the changes in [Ca$^{2+}$]$_i$. Also, the intercept of these
two lines with the y-axis is at a level of ~55%. This result shows that not as with Mg$^{2+}$ the HPP has an appreciable effect on release even when there is no reduction in Ca$^{2+}$ entry.

**DISCUSSION**

The effect of HPPs on evoked release (quantal content and time course of release) was first described by Dudel (1984) in the frog neuromuscular junction and by Parnas et al. (1986b) in the crayfish. These authors investigated the effects of HPP on the time course of release because according to the CVH (see INTRODUCTION) an HPP is expected to accelerate termination of release caused by a faster transition of the active release machinery, $S$, into its inactive form, $T$. Indeed, although all other experimental treatments to date, except one (Schweizer et al. 1998), affected only the quantal content, the HPP affected both quantal content and the time course of release, reducing quantal content and accelerating termination of release. These results were interpreted to support the CVH. At the time of these earlier studies, the method for measuring intracellular Ca$^{2+}$ concentration from single release boutons depolarized by a macropatch electrode (Ravin et al. 1997) was not available. For this reason, an indirect method (test pulse facilitation)
(Parnas et al. 1986a) was used to estimate whether an HPP reduced Ca\(^{2+}\) influx (Klockner et al. 1989; Mattheson and Armstrong 1986; Swandulla and Armstrong 1988) or accelerated the transition \(S \rightarrow T\) or did both. Accepting that facilitation depends on residual Ca\(^{2+}\) (Katz and Miledi 1968), which in turn depends on Ca\(^{2+}\) entry and removal, it was assumed that, if the HPP reduces Ca\(^{2+}\) entry, both the magnitude and duration of facilitation will be reduced. Because this was not found, it was concluded that the HPP does not reduce Ca\(^{2+}\) entry but rather facilitates the \(S \rightarrow T\) transition. This interpretation was criticized by Zucker (1987), who argued that the method of test pulse facilitation is not sensitive enough to detect small changes in Ca\(^{2+}\) entry. Our current results corroborate Zucker’s criticism. Indeed we found that as shown by others (Klockner et al. 1989; Mattheson and Armstrong 1986; Swandulla and Armstrong 1988), an HPP facilitates closure of Ca\(^{2+}\) channels, thus causing a reduction in Ca\(^{2+}\) influx.

However, our results show that HPP exerts, in addition to the previous effect, an effect on the release machinery itself. These results are consistent with the earlier interpretation of Parnas et al. (1986a) that the HPP increases the rate constant associated with the \(S \rightarrow T\) transition. As a result, \(S \rightarrow T\) transition is faster, and a larger fraction of \(S\) is transformed to the \(T\) state. Consequently, the quantal content is expected to decline, not only because of the reduction in Ca\(^{2+}\) influx but also because of the faster reduction in \(S\). The duration of quantal release is expected to be shorter because of the faster transition \(S \rightarrow T\). Finally, for a given concentration of \([\text{Ca}^{2+}]_i\), the rate of asynchronous release, immediately after evoked release, is expected to be lower because of the lower level of \(S\) at the resting potential. With time, \(S\) will eventually reach the steady-state level typical for resting potential. However, in the absence of depolarization to cause \(T \rightarrow S\) transition, recovery of \(S\) will take quite a long time.

The following experimental results support the conclusion that the postpulse affected the release machinery. 1) HPP reduced quantal content and accelerated termination of quantal release, whereas elevated \([\text{Mg}^{2+}]_o\) only reduced the quantal content and did not affect the time course of quantal release. 2) With elevated \([\text{Mg}^{2+}]_o\), a strong correlation was found between the percent reduction in quantal content and percent reduction in \([\text{Ca}^{2+}]_i\), whereas with HPP this correlation was poor. The poor correlation observed in the latter case is anticipated by analysis of the “complex model” of the CVH (Lustig et al. 1989). Accordingly, a faster \(S \rightarrow T\) transition causes the release machinery to exhibit a lower affinity to Ca\(^{2+}\) at the time when evoked release takes place (see Eq. 19 in Lustig et al. 1989). 3) Asynchronous release retained its control dependence on \([\text{Ca}^{2+}]_i\), when \([\text{Ca}^{2+}]_i\) was reduced by elevated \([\text{Mg}^{2+}]_o\). In contrast, asynchronous release became less sensitive to \([\text{Ca}^{2+}]_i\) after administration of an HPP.

In conclusion, with the aid of the technique developed by Ravin et al. (1997) for measuring \([\text{Ca}^{2+}]_i\), and release directly from single-release boutons, we were able to discern the two effects that HPP exerts and fully distinguish one from the other.

M. E. Spira is Jacomo De Viall Professor for Neurobiology and I. Parnas is Greenfield Professor for Neurobiology. This work was supported by Grant I-392–216.01/94 from the German-Israel Foundation to M. E. Spira.

Address for reprint requests: I. Parnas, Dept. of Neurobiology, Life Sciences, Hebrew University, Jerusalem 91904, Israel.

Received 30 November 1998; accepted in final form 1 March 1999.

REFERENCES


