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Release of LHRH Is Linearly Related to the Time Integral of Presynaptic Ca^{2+} Elevation above a Threshold Level in Bullfrog Sympathetic Ganglia

Yan-yi Peng* and Robert S. Zucker
Department of Molecular and Cell Biology
University of California at Berkeley
Berkeley, California 94720

Summary

To study the Ca^{2+} dependency of luteinizing hormone-releasing hormone (LHRH) release in the bullfrog sympathetic ganglia, a method was developed to fill the preganglionic nerve terminal boutons with membrane-impermeant fura-2. We found that as stimulation frequency increased from 0.5 to 40 Hz, the peak $[Ca^{2+}]_i$ ($[Ca^{2+}]_p$) and the rate of rise in $[Ca^{2+}]_i$ increased, the decay of $[Ca^{2+}]_i$ transients followed up to three exponentials, and release of LHRH was linearly related to $\int([Ca^{2+}]_i - [Ca^{2+}]_t)dt$. The threshold level of $[Ca^{2+}]_i$ for LHRH release for a given set of boutons on a C cell, $[Ca^{2+}]_t$, was estimated by the $[Ca^{2+}]_p$ evoked by 0.5 Hz stimulation that does not induce LHRH release.

Introduction

Neuropeptides are coexpressed with classical transmitters in many central and peripheral neural circuits. Because peptides act as neural modulators in many systems, it is important to understand the mechanisms and dynamics of peptide release.

In bullfrog sympathetic ganglia, the preganglionic C terminals release both acetylcholine (ACh) and luteinizing hormone-releasing hormone (LHRH). A previous study (Peng and Horn, 1991) has shown that for a given number of stimuli, the amount and rate of LHRH being released increase as the stimulation frequency increases from 2 to 20 Hz. Also, for electrical stimulation delivered as various bursting patterns, the amount and the rate of release are dictated by the stimulation frequency within a burst. Furthermore, this frequency dependence of peptide release can be changed by altering extracellular Ca^{2+} concentration. This implies that the frequency dependence of LHRH release might be a manifestation of its dependence on presynaptic Ca^{2+} concentration. In this study, a method was devised to fill the presynaptic terminal boutons of fine, lightly myelinated axons with the fluorescent Ca^{2+} indicator fura-2. This enabled us to study directly the Ca^{2+} transients evoked by various stimulation conditions used in the study of the frequency dependence of LHRH release (Peng and Horn, 1991). We found that there is a threshold level of peak $[Ca^{2+}]_i$ ($[Ca^{2+}]_p$) for LHRH release to occur. Furthermore, the time integral of presynaptic Ca^{2+} elevation above this threshold is linearly related to the postsynaptic charge evoked by release of LHRH.

* Present address: Department of Pharmacological and Physiological Sciences, The University of Chicago, Chicago, Illinois 60637.

Results

The Filling of Synaptic Terminals and Boutons with Fura-2 and the Ca^{2+} Signals Monitored

The fura-2-filled boutons have diameters of 1–5 μm. As illustrated in Figure 1, the preganglionic fiber came in contact with the axon of the ganglionic cell, coiled around the latter several turns at the proximal region (Figure 1E), forming connections with the ganglion cell en passent, and then wrapped around the soma and formed more en passent boutons (Figures 1A–1D). The size of the filled boutons and the organization of the presynaptic axon and terminal boutons with respect to the ganglionic neurons are similar to that observed using horseradish peroxidase (Jan and Jan, 1982) and by scanning electron microscopy (Baluk, 1986). Furthermore, the fluorescence at the center of a bouton was more intense than at the periphery, as would be expected if boutons were spheroid.

Typically, in sympathetic paravertebral ganglion 9 (G9), at least 30 surface cells possessed filled boutons. For a given neuron, up to 40 individual boutons were observed (Figures 1A–1D). Different boutons on a given cell appeared to have similar resting $[Ca^{2+}]_i$ ($[Ca^{2+}]_r$). Fura-2 concentration in boutons was 100–800 μM. In the presence of the dye, these boutons still transmitted because a single stimulus to the presynaptic fiber evoked both $[Ca^{2+}]_i$ elevation in synaptic boutons detectable by a photomultiplier tube as a decrease of the intensity of fluorescence excited at 385 nm (Figure 2A) and an orthodromic action potential or an excitatory postsynaptic potential in the postsynaptic cell. When dye concentration was high, orthodromic transmission tended to fail, but the $[Ca^{2+}]_i$ elevation at the boutons due to a single shock was always detectable.

Because a C neuron is typically innervated by one or two preganglionic axons (Dodd and Horn, 1983b), data collected from a set of fura-2-filled boutons on a C cell might have belonged to one or two axons. If Ca^{2+} influx occurred at all the boutons under study, the elevation in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) thus monitored would be the average $\Delta[Ca^{2+}]_i$ across a bouton along the axis that was orthogonal to the plane of focus. Otherwise, the measured $\Delta[Ca^{2+}]_i$ would be less than the average $\Delta[Ca^{2+}]_i$ across a bouton along this axis. Since filled boutons were typically spheroid, then the monitored $\Delta[Ca^{2+}]_i$ should also be approximately the average $\Delta[Ca^{2+}]_i$ across a bouton in dimensions that are parallel to the plane of focus.

Assuming LHRH release is via fusion of the dense core vesicles with the plasma membrane (details in Discussion), there are two reasons for believing that

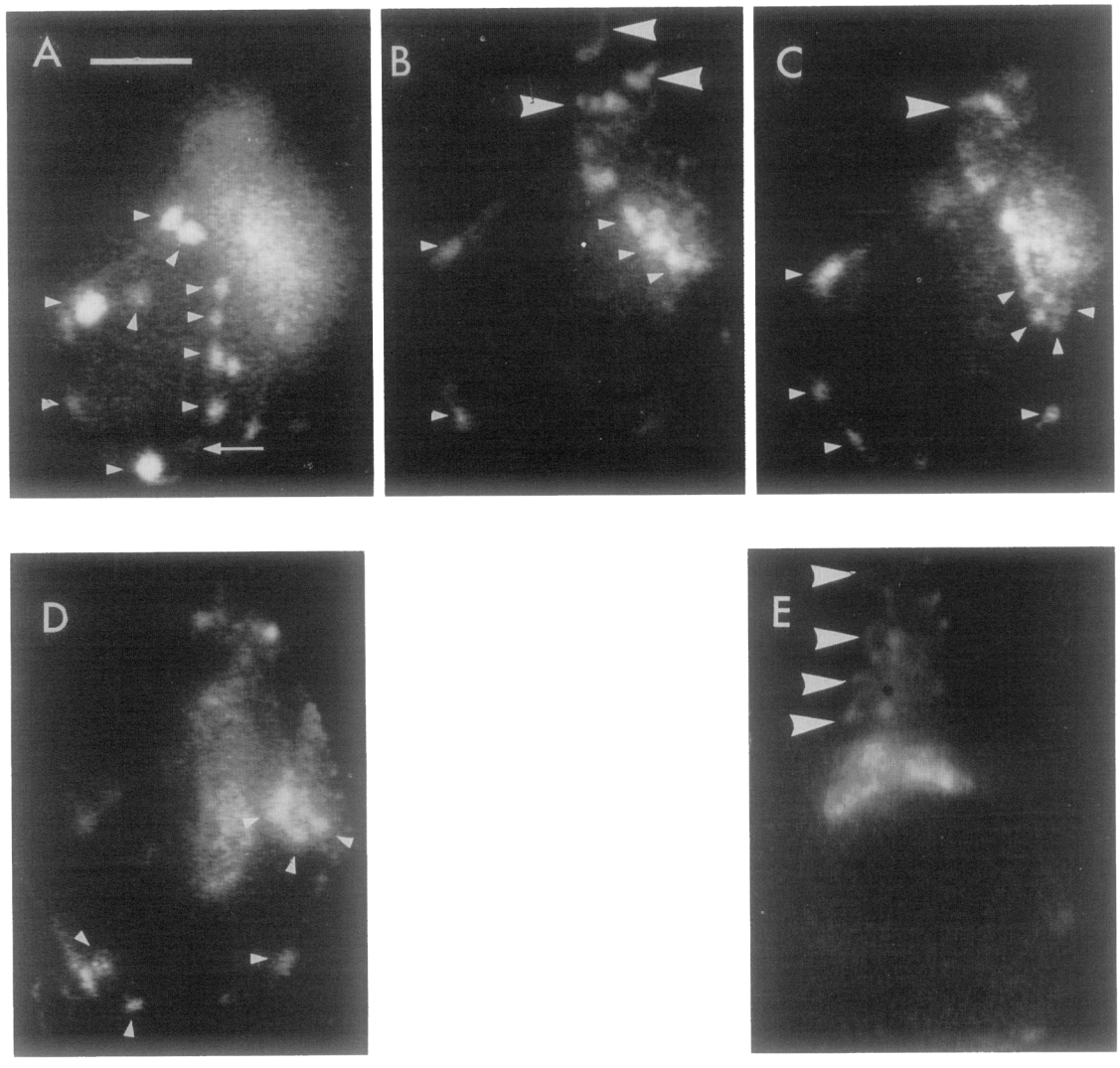

Figure 1. Fura-2-Filled Synaptic Terminals and Boutons

([A]–[D]) Fura-2-filled structures in a C cell taken at four different focal planes from the top to the bottom surfaces of the cell. (E) Fura-2 labeling of a presynaptic axon wrapped around a ganglionic C cell and dense labeling at the axon hillock region of the cell. Small arrow heads indicate synaptic boutons, whereas large arrowheads and arrows indicate the presynaptic axon and its terminals, respectively. The calibration bar is 15 μm.

the measured $[Ca^{2+}]_i$ transients would be relevant to LHRH release. The first is a morphological reason. Typically, within a bouton, the dense core vesicles tend to be away from the plasma membrane and there is no specialized release zone for them (Taxi, 1967; Uchizono, 1964; Belhumeur and Tremblay, 1986). The second reason is based on several physiological observations: repetitive stimulations are required to evoke LHRH release; typical stimulation trains last for many seconds (at least 1 s at 20 Hz is required for LHRH release); $[Ca^{2+}]_i$ increased gradually as the stimulation train continued (see below). Using a model similar to that of Sala and Hernandez-Cruz (1990) and a cytosolic Ca^{2+} buffer that can bind 99% of entering Ca^{2+} (see Discussion), it would take only 10 ms for Ca^{2+} to reach 80% of complete equilibration throughout the largest boutons. This calculation shows that the time required for Ca^{2+} to equilibrate throughout the boutons is much shorter than the briefest stimulation time required for the observed postsynaptic LHRH responses. Moreover, the reaction of fura-2 binding to Ca^{2+} is orders of magnitude faster than the time required for LHRH release: the time constant for Ca^{2+} binding to fura-2 molecules can be calculated using the equation $1/\tau = K_{on}[Ca^{2+}] + K_{off}$, where on rate $K_{on} = 6.02 \times 10^8$ $M^{-1}s^{-1}$ and off rate $K_{off} = 96.7\ s^{-1}$ at 20°C, 0.14 M ionic strength (pH 7.4) (Kao and Tsien, 1988). For Ca^{2+} concentrations of 100 nM and 1 μM, τ is 6.4 ms and 1.4 ms, respectively. Therefore, fura-2 should be able to monitor the spatial and temporal changes in $[Ca^{2+}]_i$ that are relevant to the release of LHRH from these boutons.

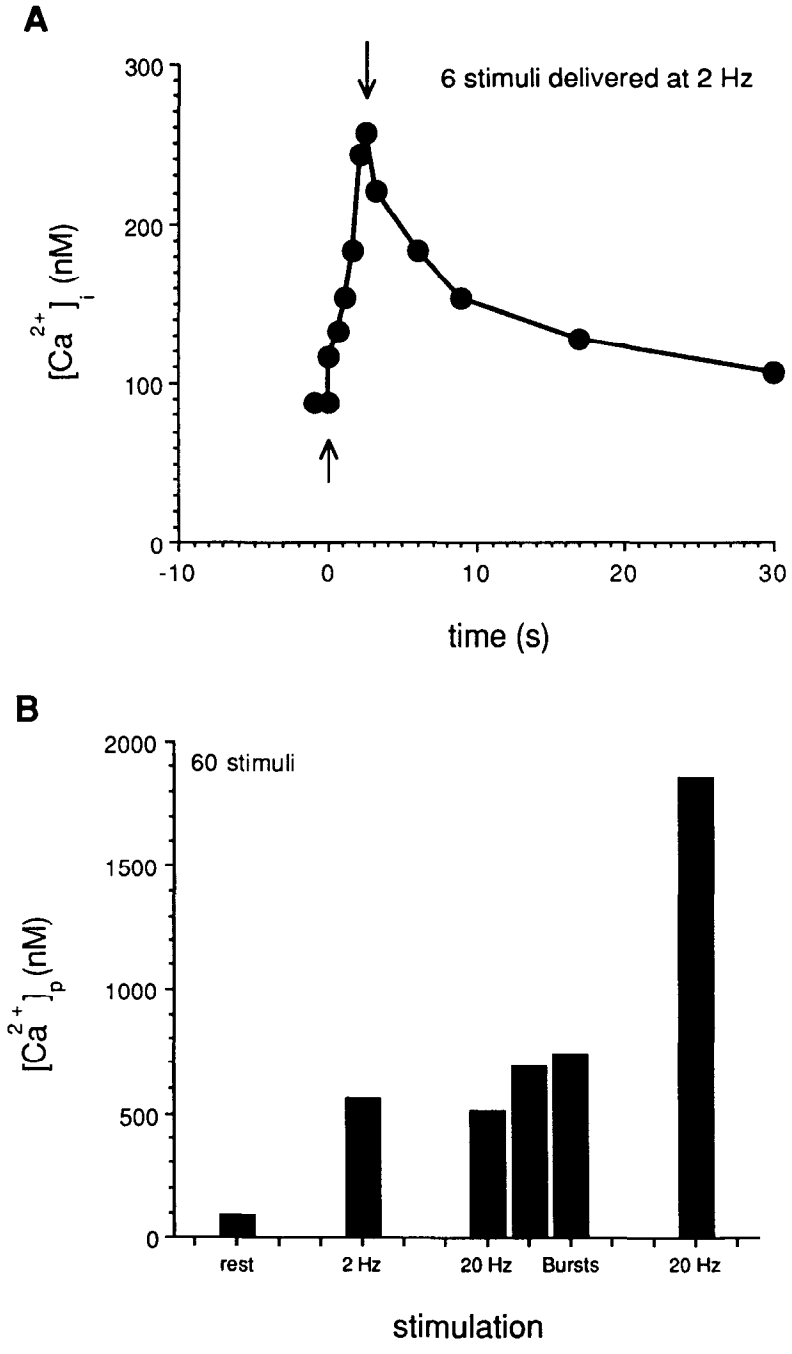

Figure 2. Changes in Average $[Ca^{2+}]_i$ across a Bouton

Recorded from the boutons shown in Figure 1A. (A) Changes in $[Ca^{2+}]_i$ in response to six stimuli delivered at 2 Hz. (B) $[Ca^{2+}]_p$ in response to 60 stimuli delivered at 2, 20B, and 20 Hz. 20B refers to bursting stimulation of 20 shocks at 20 Hz every 10 s.

When presynaptic terminal boutons were filled with fura-2, LHRH secretion was typically depressed when compared with the release study done on preparations without filling the terminals with the dye (Peng and Horn, 1991). In some cases when [fura-2] was high, no response was seen until stimulation frequency exceeded 10 Hz. We believe this was due to a Ca^{2+}-buffering action of fura-2. Because of this observation, the LHRH release data collected using the same set of stimulation patterns but without the release being distorted by the presence of fura-2 (Peng and Horn, 1991) were used. To make comparisons between different sets of boutons on different neurons, for a given set of boutons on a C cell, measurements of presynaptic Ca^{2+} were normalized to those evoked by 20 Hz stimulation. Data for the postsynaptic charge evoked by LHRH release had been similarly normalized (Peng and Horn, 1991). The grouped normalized data for the presynaptic Ca^{2+}-related measurements were then compared with grouped normalized data for the postsynaptic current or the total postsynaptic charge evoked by LHRH release.

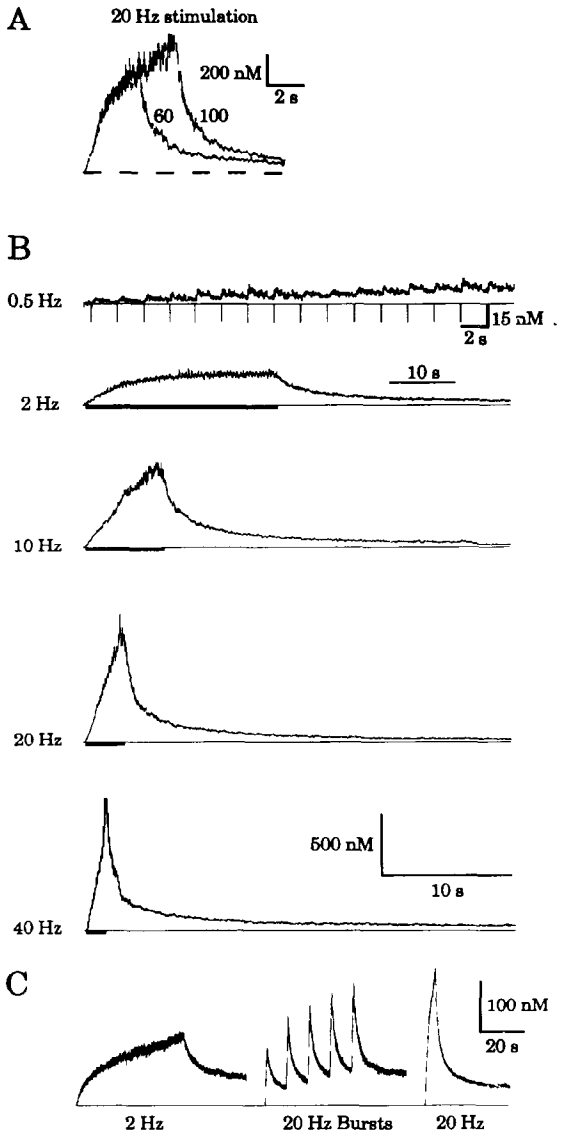

Figure 3. Peak $[Ca^{2+}]_i$ Is Proportional to Number of Stimuli and Stimulation Frequency

(A) Superimposed $[Ca^{2+}]_i$ traces in response to 60 and 100 stimuli delivered at 20 Hz recorded from a set of boutons synapsing onto a C cell. Peak $[Ca^{2+}]_i$ increases as the number of stimuli increases.

(B) Changes in $[Ca^{2+}]_i$ were recorded from a set of boutons synapsing onto another C cell in response to 60 stimuli delivered to the preganglionic nerve at 0.5, 2, 10, 20, and 40 Hz. Peak $[Ca^{2+}]_i$ increases as the stimulation frequency increases.

(C) $[Ca^{2+}]_i$ traces were recorded from a set of boutons synapsing onto a third C cell. 20B pattern of stimulation causes higher $[Ca^{2+}]_p$ than that by 2 Hz stimulation but less than that evoked by 20 Hz stimulation.

Peak Ca^{2+} Concentration and LHRH Release

For a given stimulation frequency, peak Ca^{2+} concentration ($[Ca^{2+}]_p$) increased as the number of stimuli in the stimulation train increased (Figure 3). This is also illustrated in the rising phases of the traces in Figure 4A; i.e., for a given stimulation frequency, $[Ca^{2+}]_i$ continued to rise as more stimuli were delivered. For a given number of stimuli, $[Ca^{2+}]_p$ increased as the stimulation frequency increased from 0.5 to 40 Hz (Figure 3B). Bursting at 20B (see Experimental Procedures) produced $[Ca^{2+}]_p$ higher than that produced by continuous 2 Hz stimulation, but less than that evoked by 20 Hz continuous stimulation (Figure 3C). Also, as illustrated in Figures 3B and 4A, the rate of rise of $[Ca^{2+}]_i$ increased as the stimulation frequency increased from 0.5 Hz to 40 Hz.

When the frequency-dependent behavior of $[Ca^{2+}]_i$ (Figure 5A) was compared with that for LHRH release studied by monitoring its postsynaptic effect (see Figure 2; Peng and Horn, 1991), they were found to be different in three ways. First, 0.5 Hz stimulation evoked elevation of $[Ca^{2+}]_i$ while this stimulation frequency failed to induce LHRH release monitored in most cells. This implies that there might be a threshold level of $[Ca^{2+}]_i$ for release of LHRH to occur. Operationally, it can be estimated from $[Ca^{2+}]_p$ evoked by 0.5 Hz stimulation. Second, 40 Hz stimulation produced higher $[Ca^{2+}]_p$ (see Figures 3B and 5A) though it has been shown to produce less release than 20 Hz stimulation. Third, the rate of rise in $[Ca^{2+}]_i$ due to nerve stimulation at 40 Hz was higher than that evoked by 20 Hz stimulation (see Figure 4A) though the rate of rise of the postsynaptic current evoked by 40 Hz stimulation was the same as that evoked by 20 Hz.

The Total Time Integral of Ca^{2+} Elevation and LHRH Release

In trying to understand the discrepancy between $[Ca^{2+}]_p$ and postsynaptic current evoked by 20 and 40 Hz stimulation, we noticed that though 40 Hz stimulation induced higher $[Ca^{2+}]_p$, both the rising and the falling phases of the Ca^{2+} transients were briefer than those caused by 20 Hz stimulation (see Figures 3B and 4B). It is conceivable that the higher $[Ca^{2+}]_p$ in the boutons produced by 40 Hz stimulation might have activated an additional intracellular Ca^{2+} removal mechanism(s), such that 40 Hz stimulation induced an elevation of $[Ca^{2+}]_i$ that decayed more rapidly than that evoked by 20 Hz stimulation. To investigate this possibility, the falling phase of $[Ca^{2+}]_i$ transients were plotted logarithmically against time. Typically, at low stimulation frequencies, e.g., 0.5 and 2 Hz, the semilog plot of the falling phase can be fitted with a single straight line. At higher stimulation frequencies, i.e., 20 and 40 Hz, the falling phase has three different discernible components. For any given set of boutons on a C neuron, these slopes overlap as illustrated by Figure 4B. Thus, 40 Hz stimulation did not appear to activate Ca^{2+} removal mechanisms not present at 20 Hz stimulation. This was confirmed by studying the falling phase of Ca^{2+} transients evoked with a prolonged 20 Hz train such that it reached a $[Ca^{2+}]_p$ equal to or higher than that induced by 40 Hz stimulation with the original train length. Even though there was no additional Ca^{2+} removal mechanism involved at 40 Hz stimulation other than those involved in 20 Hz

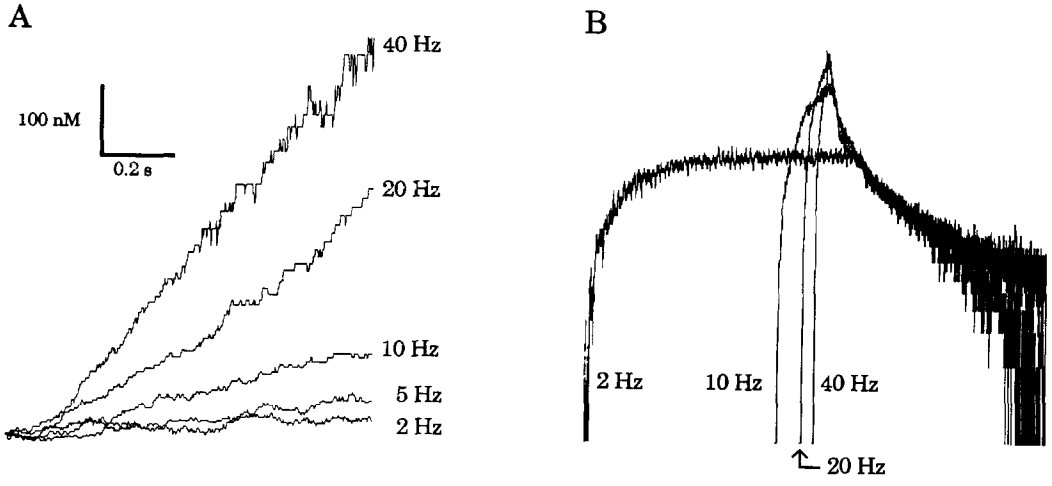

Figure 4. The Frequency Dependence of the Rate of Rise and Decay of $[Ca^{2+}]_i$

(A) Superimposed $[Ca^{2+}]_i$ traces in response to stimulation delivered at 2, 5, 10, 20, and 40 Hz. The traces were recorded from a set of boutons synapsing onto a C cell. For each trace, only the baseline and the first second following the first stimulus are shown. The rate of rise in $[Ca^{2+}]_i$ increases as the stimulation frequency increases.

(B) The decaying phases of $[Ca^{2+}]_i$ traces in response to 60 stimuli at 2, 10, 20, and 40 Hz. The traces were recorded from the same set of boutons as in Figure 3B. The traces were superimposed using the final phase of the falling phase. The stimulation frequency for each trace is labeled beside the trace.

stimulation, the removal process that had the fastest rate appeared to be responsible for removing a larger proportion of the $\Delta[Ca^{2+}]_i$ in the case of 40 Hz stimulation than in the case of 20 Hz stimulation.

In comparing the frequency dependence of $[Ca^{2+}]_p$ with postsynaptic responses, we expected a relationship between $[Ca^{2+}]_i$ and the rate of LHRH release. However, the postsynaptic current outlasts the stimulus for so long (Jan and Jan, 1982; Peng and Horn, 1991) that it is probably not simply related to the rate of release, but also to the amount of LHRH released. Although we do not have a good measure of the rate of LHRH release, the total postsynaptic charge (or the time integral of the postsynaptic current, $\int I_{LHRH}dt$) is probably proportional to the total amount of LHRH released and therefore might be related to the time integral of changes in Ca^{2+} above the resting level. It might be objected that Peng and Horn (1991) did not find a linear relationship between the duration of pressure application of LHRH and total postsynaptic charge, but this method of applying LHRH is subject to variable dilution of LHRH in the tip of the puffer pipette and other nonlinearities in the behavior of small pipettes subjected to prolonged pressure pulses. We note in this regard the linear relationship between LHRH concentration and postsynaptic current (Jones et al., 1984) as evidence that the postsynaptic receptor is a linear detector of this substance. Therefore, the normalized time integrals of the change in $[Ca^{2+}]_i$ ($\int([Ca^{2+}]_i - [Ca^{2+}]_r)dt$) evoked by different frequencies from different sets of boutons were grouped and plotted in Figure 5B.

We found that the value evoked by 40 Hz is similar to that evoked by 20 Hz. But more importantly, $\int([Ca^{2+}]_i - [Ca^{2+}]_r)dt$ is inversely related to stimulation frequencies from 0.5 to 20 Hz. The fact that 0.5 Hz, the frequency known not to evoke release of LHRH, evoked a $\int([Ca^{2+}]_i - [Ca^{2+}]_r)dt$ that is almost four times that evoked by 20 Hz, the optimal frequency for inducing LHRH release, shows that release of LHRH is not simply related to $\int([Ca^{2+}]_i - [Ca^{2+}]_r)dt$.

$\int[Ca^{2+}]_i dt$ above a Threshold and LHRH Release Depend Similarly upon Stimulation Frequency

Since neither $[Ca^{2+}]_p$ nor $\int[Ca^{2+}]_i - [Ca^{2+}]_r)dt$ resemble the frequency dependence of either the peak or the time integral of LHRH response and since the previous study of release (Peng and Horn, 1991) showed that 0.5 Hz stimulation typically does not evoke LHRH release, we defined $[Ca^{2+}]_p$ evoked by 0.5 Hz stimulation as an operational threshold ($[Ca^{2+}]_t$). However, because in the release study 0.5 Hz did not evoke release but 2 Hz always did, a $[Ca^{2+}]_t$ based on $[Ca^{2+}]_p$ for 0.5 Hz stimulation would be a lower bound of the real threshold. The relationship between normalized $\int([Ca^{2+}]_i - [Ca^{2+}]_t)dt$ and stimulation frequency is shown in Figure 5C. This frequency dependence is similar to that for the total charge of LHRH responses. For these sets of boutons, the $[Ca^{2+}]_t$ for LHRH release was 186 ± 41 nM (mean ± SEM, n = 6) where $[Ca^{2+}]_r$ was 83 ± 26 nM.

The Postsynaptic Charge Evoked by LHRH Release Is Linearly Related to the Time Integral of $[Ca^{2+}]_i$ Elevation above a Threshold

As the Ca^{2+}-related variable, $\int([Ca^{2+}]_i - [Ca^{2+}]_t)dt$, and the LHRH release-related variable, $\int I_{LHRH}dt$ or evoked postsynaptic charge, had similar dependence on stimulation frequency, these two variables in their normalized form were plotted against each other. As illustrated in Figure 5D, they are linearly related (with a

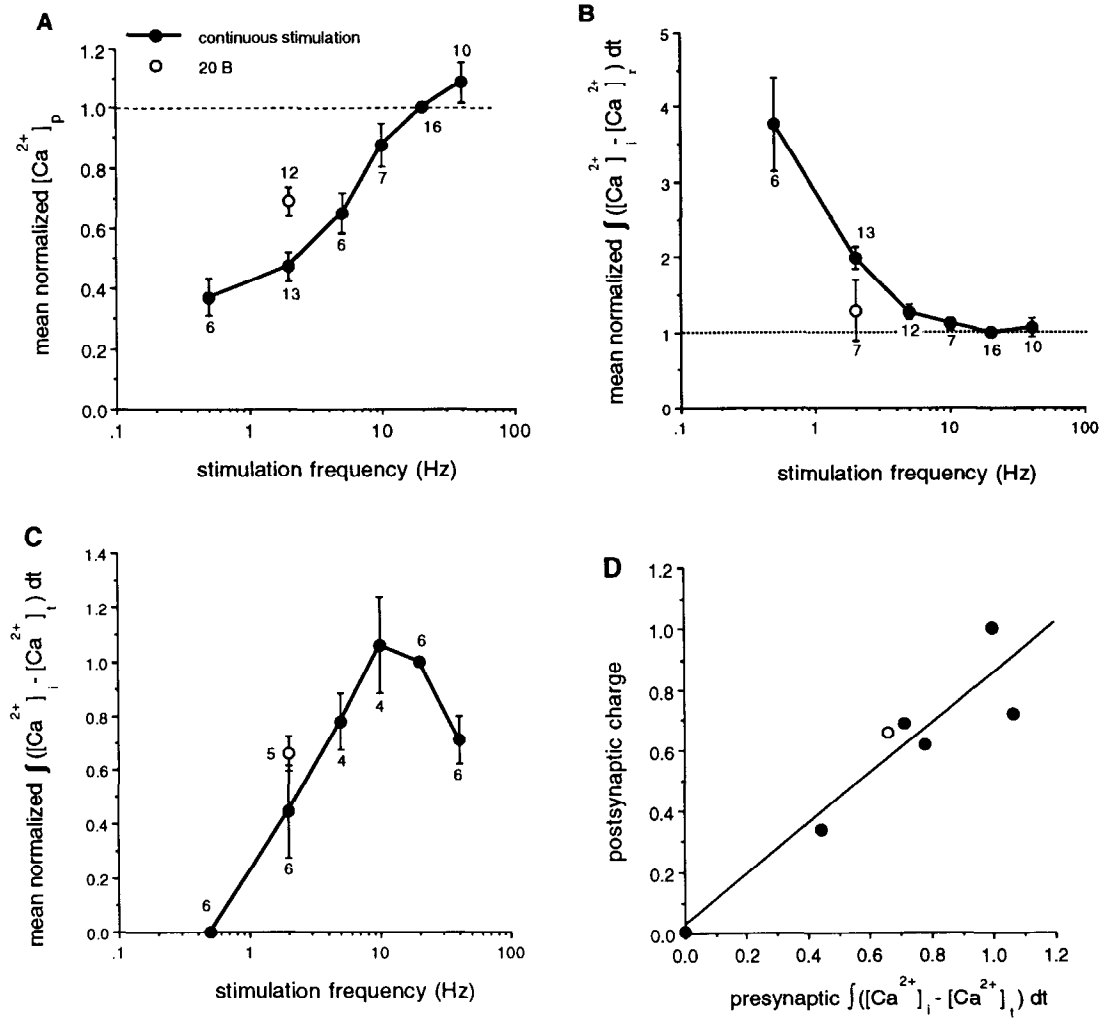

Figure 5. Plots of Various Functions of $[Ca^{2+}]_i$ versus Stimulation Frequency

(A) $[Ca^{2+}]_p$ increases with stimulation frequency. (B) The time integral of $[Ca^{2+}]_i$ above $[Ca^{2+}]_r$ decreases with stimulation frequency. (C) The time integral of $[Ca^{2+}]_i$ exceeding the threshold level $[Ca^{2+}]_t$ resembles the frequency dependence of LHRH release (Peng and Horn, 1991). In ([A]-[C]) data from each set of boutons were normalized to the response to 20 Hz stimulation. Sample size is given next to each data point. The error bars represent 1 SEM. (D) Postsynaptic charge is plotted against the time integral of $[Ca^{2+}]_i$ exceeding $[Ca^{2+}]_t$, with both variables normalized to those in response to 20 Hz stimulation. Response data from Peng and Horn (1991).

slope of 0.83, $r^2 = 0.88$, $p < 0.01$). This linear relationship strengthens the assumption of a threshold in $[Ca^{2+}]_i$ for the release of LHRH.

Discussion

Possible Cellular Mechanism Whereby Fura-2 Descends the Axon into Terminal Boutons

One intriguing characteristic of the filling was that though fura-2 went down the axons at a rate (~2 mm/hr) twice the rate of fura-2 diffusion in other cells (Pusch and Neher, 1988; Strautman et al., 1990) and it would take only milliseconds for fura-2 molecules to diffuse across a bouton, hours were required between the arrival of dye in G9 and its appearance in boutons. Furthermore, a cooling period seemed crucial for a large number of cells to have many of their presynaptic terminal boutons filled. One possibility is that after reaching G9, fura-2 molecules did diffuse across boutons without additional hindrance; however, the dye molecules were pumped out of the boutons at least as fast as they diffused in at room temperature. Perhaps cooling stopped this pumping while only slowing down diffusion moderately. As experiments done on a given set of fura-2-filled boutons typically lasted for 1–2 hr at room temperature, during which there was no significant loss of dye from the boutons, this could have happened only if the cooling permanently damaged the pumping mechanism. Another possibility is

that fura-2 molecules were bound to cytosol, a cytoskeleton component, or both and that dye entered boutons only after more proximal binding sites were saturated. If cooling slowed binding of fura-2 to these sites, it might allow the dye to enter the boutons. In neurons, unlike muscle (Baylor and Hollingworth, 1988), there is no report of fura-2-binding sites in cytosol. If such preterminal binding sites exist, it would be interesting to identify them since they might serve as a barrier for charged molecules to enter synaptic terminals and boutons.

The Assumed Vesicular Nature of Release of LHRH

In interpreting the present results, we assumed a vesicular release of LHRH from the dense core vesicles, although there is no physiological evidence for this. However, in adrenal chromaffin cells, the release of catecholamine in discrete quantities has been demonstrated to correspond to fusion of vesicles with the plasma membrane (Chow et al., 1992). In these cells the catecholamines have been shown to be encapsulated in dense core vesicles (Fenwick et al., 1978). Morphologically, during stimulation, Ω-shaped figures showing the fusion of dense core vesicles with plasma membrane have been reported for neurohypophysial neurons (Pow and Morris, 1989) and in nerve terminals of the mammalian sympathetic nervous systems (Fillenz, 1971; Thureson-Klein et al., 1979; Thureson-Klein and Stjärne, 1981). Unlike the Ω figures for release of the contents of clear synaptic vesicles where they occurred at active zones (Heuser et al., 1974, 1979; Heuser and Reese, 1981), the release of neuropeptide has been reported not to occur at any morphologically specialized zone (Fillenz, 1971; Buma and Nieuwenhuys, 1987, 1988; Pow and Morris, 1989).

The critical physiological test for vesicular release (namely, quantal analysis) is not feasible for study of the release of LHRH. Single quantal events are not detectable, presumably because of the large and variable distance between LHRH release and postsynaptic receptors. Statistical analysis of responses composed of events that should be highly variable is also inappropriate, especially when neither the mean nor variance of the quantal event can be estimated. Moreover, the recording of many prolonged responses in small cells to repeated stimulation is impractical.

Inferences about Ca^{2+} Buffering in Synaptic Boutons

An action potential could elevate $[Ca^{2+}]_i$ about 30 nM, as measured for the boutons in Figure 1A. In a 4 μm diameter bouton, this corresponds to about 600 free Ca^{2+} ions. Each preganglionic bouton has space for only one active zone for attachment of clear vesicles containing ACh. If 60 Ca^{2+} channels open in an active zone during an action potential, as has been calculated for the squid giant synapse (Fogelson and Zucker, 1985), with a single channel current of 0.6 pA and 1 ms open time, then about 100,000 Ca^{2+} ions will enter the bouton. Since fura-2 detects 0.6% of these, it is likely that over 99% of entering Ca^{2+} is rapidly bound to cytoplasmic buffers.

The Meaning of Threshold in $[Ca^{2+}]_i$ for Release and the Linear Relationship between the Time Integral of $[Ca^{2+}]_i$ above the Threshold and Postsynaptic Charge

The apparent existence of a threshold of $[Ca^{2+}]_i$ for the release of LHRH and the fact that higher stimulation frequencies cause both $\Delta[Ca^{2+}]_i$ to exceed the threshold within a shorter time and a shorter delay in the postsynaptic LHRH response (Y.-y. P., unpublished data) suggest that there is a Ca^{2+}-dependent process that precedes release of LHRH. A possible candidate for this could be the detachment of the dense core vesicles from a cytoskeleton component that holds them in place, the transport of these vesicles toward the plasma membrane, or both. The linear relationship between $\int([Ca^{2+}]_i - [Ca^{2+}]_t)dt$ and $\int I_{LHRH}dt$ suggests that the rate-limiting step for exocytosis of dense core vesicles requires the binding of only one Ca^{2+} at a critical site. It appears likely that the release of LHRH depends on Ca^{2+} acting in two different processes sequentially, but it is not yet clear whether the mobilization of vesicles to the membrane or their subsequent exocytosis is rate limiting.

This situation is in sharp contrast with the rapid release of docked vesicles with a fourth power dependence on Ca^{2+} that characterizes release of ACh at the frog neuromuscular junction (Dodge and Rahamimoff, 1967). Peptide release from rat pituitary melanotrophs (Thomas et al., 1990) and glutamate release from squid giant synapse (Augustine and Charlton, 1986; Stanley, 1986) also depend nonlinearly on $[Ca^{2+}]_i$. In these other synapses, it is not known whether the cooperativity of Ca^{2+} in transmitter release occurs at one or more than one site, although recent simulations of synaptic facilitation suggest more than one binding site (Yamada and Zucker, 1992).

The frequency dependence of $[Ca^{2+}]_i$ at pituitary terminals in rat hypophysis was reported to be generally similar to the frequency dependence of vassopressin release (Jackson et al., 1991). Although differences were attributed to a presumed nonlinear dependence of release on $[Ca^{2+}]_i$, they could also be due to the existence of a threshold for Ca^{2+} activation of release in that system.

An apparent $[Ca^{2+}]_i$ threshold of about 700 nM has been reported in a different study on hormone secretion from isolated nerve terminals from the posterior pituitary (Lindau et al., 1992). Lee et al. (1992) have recently described a Ca^{2+}-activated nonspecific cation channel isolated from dense core vesicles of neurohypophysis terminals that may be involved in neurosecretion. It is interesting to note that this channel also displays an apparent submicromolar threshold for

Ca^{2+} activation and that the open probability declines above a few micromolars $[Ca^{2+}]$. The latter property might be partly responsible for the reduction in LHRH release above 20 Hz.

Differential $[Ca^{2+}]$ Sensitivity of LHRH and ACh Release

Maximal LHRH release occurred at 20 Hz when $[Ca^{2+}]_i$ reached only about 1 μM in preganglionic terminals. At the squid giant synapse, glutamate release is apparently triggered by local $[Ca^{2+}]_i$ at hundreds of micromolars (Adler et al., 1991; Llinàs et al., 1992; Yamada and Zucker, 1992). If ACh release from these boutons is similar to glutamate release, it would appear to be much less sensitive to $[Ca^{2+}]_i$ than LHRH release. Indirect evidence for this view comes from our observation that 100–800 μM of fura-2 often prevented LHRH release at lower frequencies and at higher frequencies reduced release to less than in preparations without fura-2 (Peng and Horn, 1991). On the contrary, the same boutons usually released ACh with a single shock. Therefore, the same Ca^{2+}-buffering capacity diminished LHRH release to a large extent while leaving ACh release almost intact. In other words, the binding sites for ACh release are likely to have much lower affinity for Ca^{2+} than those for LHRH release.

However, much less electrical stimulation is required to evoke ACh release than LHRH release. This discrepancy may reflect different spatial relations between Ca^{2+} entry sites and Ca^{2+}-binding sites for ACh and LHRH release. A similar difference in Ca^{2+} sensitivity and localization of Ca^{2+}-binding sites between peptidergic and amino acid secretion was suggested recently in studies of synaptosomes excited by potassium depolarization and Ca^{2+} ionophores (Verhage et al., 1991).

Experimental Procedures

The Method for Filling the Presynaptic Terminal Boutons with Membrane-Impermeant Fura-2

Preparations of paravertebral ganglia 8–10 were isolated from 12–18 cm bullfrogs (Rana catesbiana) of both sexes. The sympathetic chain was cut ~4 mm rostral to G9. The cut end of the nerve was then placed on a Sylgard platform with a small well at one end of the platform. The segment of the nerve that was on the platform was isolated from the rest of the preparation and the bulk of the saline solution of the dish by silicone grease (Dow-Corning high vacuum grease). A grain of fura-2, pentapotassium salt, or 0.2 μl of 40 mM fura-2 solution was put in the well. The preparation was put into a moist chamber for 2–5 hr at room temperature. Then the platform was taken out, and the preparation was rinsed with saline and placed in a refrigerator for 2 hr or overnight. Sometimes ~0.1% L-α-lysophosphatidylcholine was added to the fura-2 solution. This did not appear to affect the filling of the axon terminals and the boutons by fura-2. Fura-2 molecules went down the axons at ~2 mm/hr. Fura-2 appeared to be transported to the terminal boutons via a fast axonal anterograde transport system because it could be reversibly stopped by cooling to 4°C. Intriguingly, after the dye passed through G9, it took a few hours before any axon terminals and boutons were seen to be labeled. Cooling to ~7°C appeared to facilitate this filling of terminals and particularly the boutons (see Discussion). These terminals and synaptic boutons have been similarly filled with dimethylaminomethyl-nitrophen and fluo 3.

Electrical Recording and Stimulation

After observing fura-2-filled boutons and the presynaptic axon wrapping around the axon hillock region of a neuron, the cell was impaled with a microelectrode. Electrical stimulation orthodromically to the sympathetic chain and antidromically to the sciatic nerve was applied via suction electrodes. C cells were identified by their long latencies for antidromic action potentials (Dodd and Horn, 1983a).

Once a cell has been identified as a C neuron, then the filled boutons synapsing on it must contain ACh and LHRH (Jan and Jan, 1982). We focused the field of observation of the photomultiplier tube on a subset of these boutons. For a given set of boutons, a total number of stimuli (60, 100, or 200) was chosen such that the intensity of fluorescence excited at 385 nm would continue to decrease during the entire train. There are two reasons for this procedure: to prevent evoking an elevation in $[Ca^{2+}]_i$ too high to be measured reliably by fura-2 and to match the number of stimuli used to study LHRH release by monitoring excitatory postsynaptic currents (Peng and Horn, 1991). Once the total number of stimuli was determined for a set of boutons on a given cell, trains of stimuli of this number were delivered at frequencies of 0.5, 2, 5, 10, 20, and 40 Hz. One pattern of bursting consisting of 20 shocks at 20 Hz every 10 s was also used. This bursting pattern is abbreviated as 20B. These are the stimulation patterns studied for the frequency dependence of release of LHRH by monitoring postsynaptic current evoked by release of LHRH in this system (Peng and Horn, 1991). Trains of stimuli were separated by 5–10 min.

Recording of Fura-2 Signals and Conversion to $[Ca^{2+}]_i$

The morphological study of the filling technique was made by a fura-2 imaging system utilizing a SIT camera. Pseudocolor images of the filled axons, their terminals, and boutons were formed by ratiometric measurements of fura-2 fluorescence excited at 350 and 385 nm. For the study of the dynamic responses of presynaptic $[Ca^{2+}]_i$ due to electrical stimulations, to limit the temporal resolution of $[Ca^{2+}]_i$ transients only by the kinetics of Ca^{2+} binding to fura-2, the intensity of fluorescence excited at 385 nm alone from a set of boutons on a C cell was monitored with a photomultiplier tube. A ratiometric measurement was made before each stimulation train. Then, the boutons were excited at 385 nm for 2–4 s before the stimulation train started. For all the sets of boutons studied, the fluorescent intensity was stable for this duration. Because of this stability, we stored only 100 points of the baseline before the first stimulus in the computer for calculation of F_0 in equation 1 below. The reason for choosing this short segment of baseline for the computer records was to maximize the number of data points for recording the signal. The computer data buffer allows 32,000 points for each trace. Experimental data was digitized at 0.5, 1, or 10 KHz and stored in an Intel 80386-based computer using commercial software EGAA (RC Electronics Incorporated). The intensity records were converted into $[Ca^{2+}]_i$ afterward using the following equation (Monck et al., 1988, Equation 1):

$$[Ca^{2+}]_i = K_d \frac{(F/F_0)A-(S_f/S_b)B}{B-(F/F_0)A}, \quad (1)$$

where

$$A = 1 + (S_f/S_b)\,(K_d/[Ca^{2+}]_r) \quad (2)$$

and

$$B = 1 + K_d/[Ca^{2+}]_r. \quad (3)$$

The resting $[Ca^{2+}]_i$, i.e., $[Ca^{2+}]_r$ in Equations 2 and 3, was calculated according to Equation 5 of Grynkiewicz et al. (1985) using the ratio measurements taken just before the stimulation train.

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