Synchronous and Asynchronous Exocytosis Induced by Subthreshold High K$^+$ at Cs$^+$-Loaded Terminals of Rat Hippocampal Neurons

FANG-MIN LU AND KENJI KUBA
Department of Physiology, School of Medicine, Nagoya University, Nagoya 466-8550, Japan

Received 23 April 2001; accepted in final form 14 November 2001

Lu, Fang-Min and Kenji Kuba. Synchronous and asynchronous exocytosis induced by subthreshold high K$^+$ at Cs$^+$-loaded terminals of rat hippocampal neurons. J Neurophysiol 87: 1222–1233, 2002; 10.1152/jn.00323.2001. Transmitter release at Cs$^+$-loaded autaptic terminals was selectively activated by the subthreshold concentration of external K$^+$, and Ca$^{2+}$ channel types and transmitter pools involved in synchronous and asynchronous exocytosis were studied. When a neuron was depolarized to +30 mV by applying a current through a pipette containing Cs$^+$ for >30 s, a rapid external K$^+$ jump to 3.75–10 mM, otherwise ineffective, produced an outward current (K10 response). K10 responses were initially graded (type-1) and then became a spike and plateau-shape with (type-2) or without a latency (type-3). On repolarization to ~60 mV, a high K$^+$ jump induced inward currents (called also K10 response) similar to those at +30 mV, whose shape changed from that of type-3, then type-2 and finally type-1 over 30 min. During a period favorable for inducing a type-3 response, a current similar to this response was generated by a voltage pulse (+80 or 90 mV, 20 or 30 ms) to the cell soma. Currents similar to K10 responses were rarely induced by a high K$^+$ jump without a conditioning depolarization except for some cells, but consistently produced when 3 mM Cs$^+$ and 50 μM 4-aminopyridine were externally applied for tens of minutes. Picrotoxin, 6-cyano-7-nitroquinoxaline-2,3-dione with 3-[(RS)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid or Cd$^{2+}$ in, or Ca$^{2+}$ removal from, a high-K$^+$ solution blocked all the K10 responses, while a plateau remaining after a high K$^+$ jump was not blocked by Ca$^{2+}$ removal immediately after the K$^+$ jump. Thus Cs$^+$ loading and decreased K$^+$ concentration in autaptic terminals by a conditioning depolarizing current selectively sensitizes the terminals to a subthreshold high K$^+$ jump for depolarization to activate synchronous or asynchronous transmitter release. Nicardipine (5–10 μM) blocked type-1 and -2 responses but not type-3 responses, while α-conotoxin (10 μM) blocked all the types of K10 response in the presence of nicardipine. Increasing the interval of high K$^+$ jumps biphasically increased the magnitude of K10 response, preferentially in the postjumpl fraction reflecting purely the asynchronous activation of exocytotic machinery, and decreased the reduction of miniature postynaptic current frequency after a K10 response. These results suggest the roles of N(P/Q)-type Ca$^{2+}$ channels in synchronous exocytosis at the terminals, L-type Ca$^{2+}$ channels in initiating a Ca$^{2+}$ action potential at the parent axon and both types in asynchronous exocytosis and also suggest the different releasable pools of transmitter for two modes of exocytosis in cultured hippocampal neurons.

INTRODUCTION

Ca$^{2+}$ entry through voltage-gated Ca$^{2+}$ channels in response to a nerve impulse activates synchronous transmitter exocytosis, contributing to synaptic transmission (Dunlap et al. 1995; Katz 1969). In the absence of nerve activity, spontaneous activation of asynchronous release also occurs (Katz 1969), although it seems to elicit physiological actions only during and/or after high nerve activity (Kobian et al. 2000; Lu and Trussell 2000).

There are several distinctions between synchronous and asynchronous release of transmitter in terms of the mechanisms. First, the high concentration of Ca$^{2+}$ close to the orifice of Ca$^{2+}$ channel is required for the activation of impulse-evoked, synchronous transmitter release via a low-affinity Ca$^{2+}$ receptor coupled with the exocytotic machinery (Bollmann et al. 2000; Heidelberger et al. 1994; Llinás et al. 1992; Schneggenburger and Neher 2000; Schweitzer et al. 1995; see Stanley 1997; Zucker 1996), while asynchronous exocytosis appears to be mediated by a high-affinity Ca$^{2+}$ receptor presumably activated at the basal level of cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$])$_{i}$ or by its global rise (see Lu and Trussell 2000; Wu et al. 1999). The latter idea is supported by the enhancement of transmitter release during the sustained residual rise in [Ca$^{2+}$])$_{i}$ after repetitive nerve activity (Delaney and Tank 1994) and no effect of the gene-knock-out or mutation of a Ca$^{2+}$-binding protein, synaptotagmin, a Ca$^{2+}$ sensor for impulse-evoked exocytosis (DiAntonio and Schwarz 1994; Fernández-Chacón et al. 2001; Littleton et al. 1993). Second, types of Ca$^{2+}$ channels involved in two modes of exocytosis appear to differ. In rat cerebellar nuclear neurons, P/Q-type Ca$^{2+}$ channels were suggested for synchronous exocytosis (Takahashi and Momiyama 1993), while L-type as well as P/Q-type Ca$^{2+}$ channels were suggested for high-K$^+$-induced asynchronous release (Momiyama and Takahashi 1994). Synchronous transmitter release was blocked by inhibitors of high-voltage-activated Ca$^{2+}$ channels in spinal cord neurons, while asynchronous release was blocked by inhibitors of low-voltage-activated Ca$^{2+}$ channels (Bao et al. 1998). This aspect of distinction between synchronous and asynchronous release, however, has still not been established in other terminals. Third, it is possible that the sites of exocytosis and population of synaptic vesicles differ for synchronous and asynchronous transmitter release. Strong evidence for this possibility was reported for the motor nerve terminals of Drosophila (Koenig and Ikeda 1999). Furthermore, it was reported that repetitive nerve activity increased the failure of impulse-evoked transmitter release but enhanced the rate of asynchronous release in cochlear nucleus neurons (Lu and Trussell 2000). Thus it is possible that synaptic vesicle pools for two modes of transmitt-
ter release are not identical and/or their sites of exocytosis differ in central neurons.

To further characterize distinction between synchronous and asynchronous transmitter release, both modes of transmitter exocytosis must simultaneously be recorded under the same condition. Ca\(^{2+}\)-dependent synchronous exocytosis is usually elicited by a nerve impulse and recorded as excitatory or inhibitory postsynaptic potentials or currents, whereas Ca\(^{2+}\)-dependent asynchronous release is recorded as miniature postsynaptic potentials or currents under the enhancement of their occurrence by a high-K\(^+\) solution. The application of high-K\(^+\) solution to central neurons, however, has a drawback in that it activates presynaptic terminals of heterogeneous inputs. To overcome this problem, a single bouton must be activated by the local application of high-K\(^+\) solution (Liu and Tsien 1995). This method may be impractical for routine experiments and require many experiments to obtain averaged responses over many terminals. In this study, we have developed a method to selectively activate high-voltage-activated Ca\(^{2+}\) channels at the autaptic terminals by a subthreshold concentration of the external K\(^+\) (3.75–10 mM). This is achieved by loading Cs\(^+\) to inhibit K\(^+\) channels and reduce intracellular K\(^+\) concentration in autaptic terminals. With this method, the sensitivity of presynaptic terminals to [K\(^+\)]\textsubscript{P} can be regulated by the duration of Cs\(^+\) injection.

Using this novel technique, we have studied types of Ca\(^{2+}\) channel and populations of transmitter pool involved in the synchronous and asynchronous exocytosis of neurotransmitter at the autaptic terminals of cultured glutamatergic or GABAergic hippocampal neurons. The analyses revealed that N(P/Q)-type Ca\(^{2+}\) channels are involved in both the synchronized and asynchronous exocytosis of transmitter from autaptic terminals of cultured hippocampal neurons, while L-type Ca\(^{2+}\) channels play roles in asynchronous exocytosis and the induction of a Ca\(^{2+}\) action potential at the parent axon, which leads to the synchronous transmitter release. The results further suggested that synchronous and asynchronous release of transmitter occur from the different pools of synaptic vesicles.

**METHO\d\d**

Hippocampal neurons were cultured from E20-day-old Wistar rat embryos as described previously (Lu and Kuba 2001). Briefly, pregnant rats were anesthetized with ether and killed by decapitation. Fetal hippocampi were dissected, cut into pieces, and maintained in Ca\(^{2+}\)-, Mg\(^{2+}\)-free Dulbecco’s phosphate-buffered saline (Sigma, St. Louis, MO) containing 0.1% trypsin (Gibco, Detroit, MI) for 5 min at 37°C. Hippocampal neurons were mechanically dissociated by trituration and plated onto poly-L-lysine (Sigma)-treated glass cover slips and Hippocampal neurons were mechanically dissociated by trituration. Media (MO) containing 0.1% trypsin (Gibco, Detroit, MI) for 5 min at 37°C. High-K\(^+\) solutions (3.75–10 mM) were locally applied through one channel of multibarreled polyethylene tubes (300 μm ID) placed at ~0.8–1 μm away from the cell soma. The flow to the recording neuron was rapidly changed by a shift of laminar flow from one channel to another with a servo motor (Rapid solution exchanger, RSC-100, Biologic, Claixs, France). The rate of change in a solution superfusing a neuron was <100 ms as seen in the rate of a change in leak current by 10 mM K\(^+\) solution (Fig. 2D). Some of solutions containing drugs and/or consisting of ionic compositions different from the standard 10 mM K\(^+\) solution or BSS solution were also applied by the rapid solution changer. The change of solutions other than high K\(^+\) solutions unless specified was made by altering the flow of solution to the bath. In some experiments, a solution of laminar flow contained antagonists of glutamate receptors, 6-cyano-7-nitrolonoxaline-2,3-dione (CNQX: 10 μM) and 3-((RS)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP: 20 μM), or an antagonist of GABA receptors, picrotoxin, or Cd\(^{2+}\) (0.5 mM). In some other experiments, Ca\(^{2+}\) was removed from a solution of laminar flow. When ω-conotoxin MVIIIC was applied, perfusion was stopped. Then, one-third of the bathing solution was replaced with a solution containing ω-conotoxin MVIIIC (30 μM) so that the final toxin concentration was 10 μM.

- 4-Aminopyridine (4-AP), picrotoxin, and nicardipine hydrochloride were purchased from Sigma Chemical. CNQX and CPP were from Tocris Cookson (Bristol, UK). TTX and ω-conotoxin MVIIIC were from Alomone Labs (Jerusalem, Israel).

Data are expressed as means ± SE. The number of cells studied in a type of, or whole, experiments is shown as \textit{N}, while the number of cells that showed similar results is expressed as \textit{n}.

**RESULTS**

Selective activation of autaptic terminals by a moderately high-K\(^+\) solution after conditioning depolarization

Hippocampal neurons, which had either pyramidal, spindle, or stellar shape, frequently formed autapses (Bekkers and Stevens 1991) in dissociated culture. Neurons forming autapses (\textit{N} = 157) were used in all the experiments in the present study. The existence of autapses were identified by applying a depolarizing command pulse (from –60 to 0 to +20 mV for 2–4 ms) under the whole cell clamp condition with a patch pipette filled with a solution containing Cs\(^+\) and recording autaptic excitatory or inhibitory postsynaptic currents (EPSCs or IPSCs, respectively: Fig. 1B). TTX was not added to the bathing solution only in this type of experiments, but given throughout all other experiments. A rapid jump of [K\(^+\)]\textsubscript{P} from 2.5 to 10 mM caused only a small inward current at the holding potential (\textit{V}_H) of ~60 mV (Fig. 1Ca). Shifting \textit{V}_H from –60 to +30 mV produced a transient outward current occasionally with an increase in the frequency of miniature postsynaptic currents (MPSCs: Figs. 1A and see Fig. 7B). The holding outward current decayed to a steady level, which was maintained as long as the \textit{V}_H was held constant (Figs. 1A and 7B).

Under this condition, a rapid [K\(^+\)]\textsubscript{P} jump to 10 mM produced an outward current. The currents induced by high [K\(^+\)]\textsubscript{P} (K10 response) grew in amplitude and changed their shape during the course of holding the membrane potential at the depolar-
induced an inward current, which consisted of steep spike and plateau or slow decay components, similar to the type-3 of K10 response at +30 mV but in opposite direction (Fig. 1D, e–i; see also Figs. 5B, g, j, and o, and 7C). The spike component rose within 10 ms (Fig. 2Db). The high-[K⁺]o-induced inward current slowly decreased in amplitude with the appearance of a slowly growing component preceding to the spike component over a period of a few to tens of minutes (Fig. 1E, l–n; see also Fig. 8B, left), being similar to the type-2 of K10 response at +30 mV. There were sometimes the mixture of these patterns of the high-[K⁺]o-induced responses like type-2 and -3 K10 responses (Fig. 1D, j and k). Finally, the spike component disappeared leaving only a slow, graded component (Fig. 1E, o–r; see also Figs. 5B, d–f, h, i, k–n, p, and q, and 8B, right), resembling the type-1 of K10 response at +30 mV. Thus the patterns of high-[K⁺]o-induced currents induced after repolarization were heterogeneous, depending on the course of the growth of K10 response during and after the conditioning depolarization, those in the absence of the depolarization, and the rates of rises of a K10 response at +30 mV and the interval of high-[K⁺]o-induced leak current. A: the time course of the growth of K10 response during the conditioning depolarization. The areas of K10 responses recorded from 10 cells were plotted in relative scale against time after the beginning of the conditioning depolarization. B: the time course of the decay of K10 response after the conditioning depolarization. The areas of K10 responses recorded from 5 cells were plotted in relative scale to that of the maximum against time after the beginning of the conditioning depolarization. C: the time course of the growth of K10 response without the conditioning depolarization. The areas of K10 responses recorded from 4 cells were plotted in relative scale against time after the formation of whole cell patch. D: the high-[K⁺]o-induced leak current and the rates of rise of a K10 response. a: the rate of rise of the leak current induced by a high-[K⁺]o jump. A response of quickly rising phase in 2 steps is the summation of two miniature postsynaptic currents. Note the scale of the ordinate is much smaller than that in b. b: the rate of rise of a K10 response.
ization to $-60$ mV are exactly similar to those of K10 response generated at $+30$ mV, although their direction and the order of their appearance were opposite. Accordingly, they are also called as type-3, -2, and -1 of K10 response, according to their similarity in shape. The rate of decay of $10 \text{ mM}[K^+]_o$-induced currents is shown as that of the area of the response (Fig. 2B). The half decay time of the area of the inward current varied from a few to tens of minutes. The effect of the conditioning depolarization to prime the mechanism of K10 response was faithfully repeatable in most cells studied (see Fig. 5; $n = 25$).

In some cells ($n = 6$), a $10 \text{ mM}[K^+]_o$ jump induced inward currents similar to K10 responses at $-60$ mV without the conditioning depolarization (Fig. 2C).

The minimum concentration of $[K^+]_o$ to cause a significant high-$[K^+]_o$-induced outward current at the maintained $V_{h}$ of $+30$ mV was 3.75 to 5 mM, and the amplitude increased with an increase in $[K^+]_o$ (Fig. 3A: $n = 7$). Furthermore, a spontaneous outward or inward current similar to a K10 response with the shape of spike and plateau occasionally occurred during and after the conditioning depolarization (Fig. 1, Ag and Dg). These characteristics of K10 responses suggest that they are caused by an increase in the sensitivity of the cell membrane to a high $[K^+]_o$ in some regions of a patch-clamped neuron as a result of the conditioning depolarization. Such regions are the autaptic terminals and adjacent, parent axon as shown in the following text.

**Transmitter release induced by Ca$^{2+}$ entry at autaptic terminals underlies a K10 response**

The application of either picrotoxin (100 $\mu$M) or a combination of CNQX (10 $\mu$M) and CPP (20 $\mu$M) completely blocked K10 responses (Fig. 3, B and C). Forty cells that had stellar or spindle form showed K10 responses sensitive to picrotoxin and/or insensitive to CNQX and CPP, while 18 cells produced K10 responses that were blocked by CNQX and CPP and/or not affected by picrotoxin. The results suggest that K10 responses are caused by the release of GABA or glutamate from autaptic terminals and that most neurons showing K10 responses are GABAAergic neurons. It may be noted that the K10 responses induced by the release of GABA as well as those by the release of glutamate are inward at $-60$ mV, while they are outward at $+30$ mV, because the major intracellular anion is Cl$^-$. The involvement of neurotransmitter release can also be supported by the dependence of the area of K10 responses on the preceding interval, indicating the depletion of transmitter pool by their generation (see the later section: Fig. 9).

Removing Ca$^{2+}$ from (Fig. 4A), or adding Cd$^{2+}$ (0.2–0.5 mM; Fig. 4C) or Co$^{2+}$ (1–5 mM; not shown) to, a high-[K$^+$]$_o$ solution blocked all the types of K10 responses ($n = 3$ for Ca$^{2+}$ removal, $n = 8$ for Cd$^{2+}$ addition and $n = 6$ for Co$^{2+}$ addition), indicating the essential role of Ca$^{2+}$ entry in K10
responses. The removal of external Ca$^{2+}$ immediately after the end of a high [K$^{+}$]$_{o}$, however, did not affect asynchronous responses after the end of high-K$^{+}$ solution (Fig. 4B). Similar results were seen in three cells. This obviously contrasts with the abolition of the plateau phase of a Ca$^{2+}$-dependent action potential (recorded from the cell soma) by the similar removal of external Ca$^{2+}$ (Fig. 4D; n = 4) in the cell having no autapse. Thus the transmitter exocytosis in the absence of external Ca$^{2+}$ immediately after high [K$^{+}$]$_{o}$ indicates the involvement of the residual Ca$^{2+}$ remaining after Ca$^{2+}$ entry and/or Ca$^{2+}$ release from internal Ca$^{2+}$ stores (see DISCUSSION).

**Mechanism of priming of K10 responses**

How does the conditioning depolarization set up the condition for transmitter release in response to a moderately high [K$^{+}$]$_{o}$ that is otherwise not effective? One possible mechanism would be that the conditioning depolarization primes the mechanism of Ca$^{2+}$-induced Ca$^{2+}$ release mechanism (CICR) via ryanodine receptors (see Kuba 1994) by loading Ca$^{2+}$ into Ca$^{2+}$ stores via large Ca$^{2+}$ entry (Garaschuk et al. 1997) and the Ca$^{2+}$ entry produced by 10 mM [K$^{+}$]$_{o}$ activates CICR. If Ca$^{2+}$ loading was to occur, the removal of external Ca$^{2+}$ during the conditioning depolarization should block the priming effect of the conditioning depolarization for K10 responses. K10 responses, however, were elicited even after the conditioning depolarization applied in a Ca$^{2+}$-free, high-Mg$^{2+}$ solution (Fig. 5: n = 2) or a solution containing Cd$^{2+}$ (0.5 mM: not shown: n = 1). Thus the loading of Ca$^{2+}$ stores by Ca$^{2+}$ entry during the conditioning depolarization is unlikely to occur, although the involvement of CICR is not completely ruled out (see DISCUSSION).

Another possible mechanism for the priming of K10 responses would be that the conditioning, depolarizing current given at the cell soma would cause iontophoresis of Cs$^{+}$ into the dendrites and the axon including autaptic terminals and efflux of K$^{+}$ there. The resultant blockade of K$^{+}$ channels by Cs$^{+}$ as well as the reduction in K$^{+}$ concentration would further depolarize the membrane of the processes. Under this condition, raising [K$^{+}$]$_{o}$ from 2.5 to 10 mM could depolarize the membrane of the processes to a level enough to activate voltage-dependent Ca$^{2+}$ channels and activate the release of neurotransmitters at the terminals (see DISCUSSION). On the other hand, stopping the conditioning outward current would resume the K$^{+}$ concentration in the processes and result in the passive extrusion of Cs$^{+}$ at the cell membrane. This was the case as shown in the following text.

The external application of Cs$^{+}$ (3 mM) together with another K$^{+}$ channel blocker, 4-AP (50 μM), gradually produced a condition for the generation of K10 responses in the cells whole cell clamped with a pipette containing a solution, whose major salt was K-aspartate (Fig. 6). A rapid jump of 10 mM [K$^{+}$]$_{o}$ under the initial period of this condition produced only a change in leak current. In 17–21 min, a 10-mM [K$^{+}$]$_{o}$ jump produced an increase in the frequency of miniature postsynaptic currents. In 37–41 min, the spike and plateau-shaped currents similar to those induced under the patch clamp with a CsCl-filled pipette occurred in response to 10 mM [K$^{+}$]$_{o}$. These 10-mM [K$^{+}$]$_{o}$-induced currents in the presence of Cs$^{+}$ and 4-AP were seen in all five cells studied irrespective of having autapses and blocked partially (n = 2) or completely (n = 2) by the application of either picrotoxin (100 μM) or CNQX (10 μM) with CPP (20 μM) or completely by the co-application of all of them (n = 1). Thus external Cs$^{+}$ and 4-AP mimicked the effect of intracellular Cs$^{+}$ loading through a patch pipette and a depolarizing current.

The experiments shown in the preceding text indicate that the entry of Cs$^{+}$ preferentially into the dendrites and the axon including the terminals (for the greater volume/surface ratio) would have caused the accumulation of Cs$^{+}$ there as seen in the previous study (Lu and Kuba 2001). Consequently, Cs$^{+}$ loading into the processes and a decrease in the intracellular...
K^+ concentration during a conditioning depolarization are the priming mechanisms for the generation of K10 responses in the cells patch-clamped with a pipette filled with CsCl. Under this condition, 10 mM [K+]o that is normally ineffective produced a depolarization enough to activate voltage-gated Ca^{2+} channels. In support of this, a strong depolarizing pulse (20–30 ms) to +80 or 90 mV produced a current similar to a K10 response at both −60 mV (n = 6; Fig. 7) only for the response at −60 mV. The size of the pulse-evoked current depended on the preceding interval (Fig. 7Db) as seen in that of K10 response (see Fig. 9).

Types of Ca^{2+} channel involved in synchronous and asynchronous exocytosis

K10 responses obviously consist of the components caused by synchronous and asynchronous components of transmitter release. The spike component of K10 response could be caused by synchronous transmitter release due to the simultaneous generation of Ca^{2+}-dependent action potentials at presynaptic terminals that are activated at each presynaptic terminal by 10-mM [K+]o-induced depolarization or by the conduction of a Ca^{2+} action potential evoked at the parent axon. On the other hand, the graded and plateau mode of K10 responses appears to be caused by the asynchronous activation of the exocytotic machinery by rises in [Ca^{2+}]i, produced by asynchronous activation of Ca^{2+} channels and/or by the residual rise in [Ca^{2+}]i after the activation.

To examine what types of voltage-gated Ca^{2+} channel are involved in these modes of K10 responses, we have observed effects of Ca^{2+} channel antagonists on K10 responses. Nicardipine (10 μM) applied to both the locally superfusing and bathing solutions almost completely blocked the spike component of type-2 K10 response consisting of slow, graded, and then spike components in four cells (Fig. 8B). Similar blockade of the spike component was also seen at a lower concentration of nicardipine (5 μM) in two cells. On the other hand, type-3 K10 responses consisting of spike and subsequent plateau phases without latency were only slightly reduced by nicardipine in four cells (Fig. 8A). These results indicate that nicardipine blocks the regenerative activation of a Ca^{2+} action potential presumably at the parent axon but not Ca^{2+} action potentials at the terminals (see DISCUSSION). Another action of nicardipine was to decrease the slow, graded, asynchronized component of K10 response (Fig. 8A). This effect was seen in all the cells studied and shown as reductions in the total area of
K10 response (55.6 ± 5.5% of the control, N = 11 at 10 μM nicardipine, and 63.2 and 67.6% at 5 μM). This suggests that nicardipine blocks to some extent the asynchronous activation of exocytosis presumably caused by the global rise in [Ca^{2+}], in presynaptic terminals (see Discussion).

ω-conotoxin (10 μM), a blocker of N- and P/Q-type Ca^{2+} channels, added to the bathing solution blocked both the spike and asynchronized components of K10 response in all the types under the effect of nicardipine (10 μM; N = 6: Fig. 8, C and D: the total area, 21.7 ± 5.8%, n = 4). It may be noted that all the spike components of K10 response in the presence of nicardipine were those without latency. This indicates that Ca^{2+} entry caused by the opening of N- and P/Q-type Ca^{2+} channels at the autaptic terminals, but not at the parent axon, swiftly activates the synchronized transmitter exocytosis (see Discussion). Furthermore, asynchronized activation of N- and P/Q-type Ca^{2+} channels at the autaptic terminals would cause the asynchronized activation of exocytosis via a mechanism closely coupled to Ca^{2+} channel opening or a global rise in [Ca^{2+}] (see Discussion).

Transmitter pools involved in synchronous and asynchronous exocytosis

The foregoing results suggest that Ca^{2+} influx through different types of Ca^{2+} channels are involved in stimulating synchronous and asynchronous transmitter release. This would imply that the site of the asynchronous exocytosis is different from that for synchronous exocytosis. Then it may be possible that the two modes of exocytosis occur from different pools of synaptic vesicles. This was examined in the following text.

We first observed how the transmitter pool for K10 responses changes with the variation of the interval of stimulation. The area of K10 response depended on the interval of the application of high K^{+} jumps: the longer the preceding interval, the greater and longer the K10 response (Fig. 9A). This can be explained by the depletion of transmitter pool in autaptic terminals by the preceding activation of transmitter release. The interval-dependence of K10 response obviously occurred in two phases over a few tens of seconds and a few minutes (Fig. 9B), indicating the involvement of two different processes for the replenishment of transmitter pool. Furthermore the time course of the recovery of the component of K10 response remaining after a K^{+} jump (K-off fraction) apparently differed from that during the jump (K-on fraction: Fig. 9B; see the definition of separation into 2 fractions in Fig. 11). The rate of recovery of K-off fraction was twofold greater than that of K-off fraction (2.1 ± 0.46, n = 5: measured as the ratio of the rate of recovery of K-on fraction to that of K-off fraction at 2–28% of the time interval for full recovery). The recovery of the amplitude of a depolarizing pulse-induced exocytosis roughly corresponding to the K-on fraction of K10 response was also faster than the residual asynchronous exocytosis seen after a pulse, which is equivalent to the K-off fraction (Fig. 7Db).

We next examined how asynchronous transmitter release in the absence of stimulation is affected by the generation of a K10 response. If part of, if not all, the asynchronous component of K10 response results from the transmitter pool involved in asynchronous release that occur in the absence of nerve activity, the frequency of MPSCs would decrease after a K10 response. This was indeed the case (Fig. 10A). The maximum reduction in MPSC frequency occurred immediately after a high [K^{+}] jump or after the subsidence of the residual facilitatory effect of high [K^{+}], lasting for a few seconds. There was an inverse relationship between the area of K10 response and the change in the frequency of MPSCs after a K10 response (Fig. 10B). MPSCs frequency recovered in two phases over a few tens of seconds and 1 min, which are similar to those of the recovery of K10 response (Fig. 10C).

Finally, we examined whether the synchronous and asynchronous modes of exocytosis occur from different pools of synaptic vesicles by comparing changes in the fractions of K10 response during and after a K^{+} jump with variation of the total size. The K-on fraction is obviously caused by both synchronous and asynchronous exocytosis in response to the synchronous and asynchronous activation of Ca^{2+} channels and the resultant rise in [Ca^{2+}]. On the other hand, the K-off fraction must predominantly be caused by the residual rise in [Ca^{2+}], and independent of Ca^{2+} entry as evidenced by no effects of the removal of external Ca^{2+} (Fig. 4B). This component there-
fore purely consists of asynchronous release. If the synchronous and asynchronous exocytosis use the common pool of synaptic vesicles, both the K-on and K-off fractions should remain constant irrespective of changes in the size of pool (see Fig. 12), namely the total size of K10 response. This was not the case, as already indicated by the different interval dependence of the two fractions (Fig. 9). The total area of a K10 response relative to that of the maximum one and its fractions during and after a high \([K^+]_o\) jump were plotted against the preceding interval. The total area; and , the fractions of K10 response during and after a high \([K^+]_o\) jump, respectively. See the records in Fig. 11 for the separation of the fractions of K10 response.

Fig. 9. Interval dependence of K10 responses. A: K10 responses recorded at different intervals. The neuron was voltage-clamped at \(V_{m} = +30\) mV. , the time for the application of 10 mM \([K^+]_o\), (1 s in duration). The times shown above the traces (a–d) indicate the intervals between the applications of 10 mM \([K^+]_o\). Note the gradual decrease in the size of K10 responses when induced at short intervals. B: the interval dependence of the area of K responses and its fractions during and after a high \([K^+]_o\) jump. The total area of a K10 response relative to that of the maximum one and its fractions during and after a high \([K^+]_o\) jump were plotted against the preceding interval. , the total area; , and , the fractions of K10 response during and after a high \([K^+]_o\) jump, respectively. See the records in Fig. 11 for the separation of the fractions of K10 response.

Fig. 10. Decreases in miniature postsynaptic current (MPSC) frequency after the generation of a K10 response. A: the records of MPSCs before and after a K10 response. a: a marked decrease in MPSC frequency after the largest K10 response. The records are continuous from the top to the bottom. b: a moderate decrease in MPSC frequency after the K10 response of a moderate size. The records are continuous from the top to the bottom. B: the relationships between the relative change in MPSC frequency after a high \([K^+]_o\) jump and the size of K10 response. Changes in MPSC frequency relative to that immediately before a K10 response were plotted against the total area of the corresponding K10 response in relative size to the maximum. The data obtained from 8 cells are shown by different groups of symbol. C: the time courses of the recovery of MPSC frequency after a K10 response. Changes in MPSC frequency before and those immediately, or in the period after the maximum reduction, after a K10 response were plotted against time in relative value to the average before the response. Each data point is the average of MPSC frequencies (32 s) obtained from 3 cells.
Ca2+ entry through voltage-gated Ca2+ channels depends at least on two factors. First, the effective-ness of high [K+]o to depolarize the membrane of the processes depends on extent of Cs+ loading into and the reduction of K+ in the processes, both of which would depend on where and how voltage-gated Ca2+ channels are activated by a high [K+]o jump for 0.2 and 0.5 s, respectively. Some of the data points in the graph correspond to the records in the right-hand side as indicated by a corresponding letter.

FIG. 11. Changes in the K-on and K-off fractions of the area of K10 response with changes in the preceding interval. A: changes in the K-on and K-off fractions of the area of K10 responses recorded at +30 mV. High K+ jumps were made at the interval of 20–240 s during a period indicated by a horizontal bar below each record of K10 response. The K-on and K-off fractions are indicated by dotted line in the records of right-hand side and plotted against the total area of K10 response in relative size to the maximum. Filled triangles and circles are the K-off fractions of the K10 responses induced by a high [K+]o jump for 0.2 and 0.5 s, respectively. Open triangles and circles are the K-on fractions of the K10 responses induced by a high [K+]o jump for 0.2 and 0.5 s, respectively. Some of the data points in the graph correspond to the records in the right-hand side as indicated by a corresponding letter. B: changes in the K-on and K-off fractions of the area of K10 responses recorded at –60 mV. K10 responses were recorded at the constant interval of 20 s except for the record a, which was made by the first high [K+]o jump at 10 s after repolarization to –60 mV from +30 mV. High K+ jumps were made during a period indicated by a horizontal bar above each record of K10 response. Filled and open circles are the K-on and K-off fractions, respectively, of the K10 responses induced by a high [K+]o jump for 0.5 s. Some of the data points in the graph correspond to the records in the right-hand side as indicated by a corresponding letter.

DISCUSSION

The present study demonstrates the activation of transmitter release at autaptic terminals by the rapid jump of [K+]o from 2.5 mM to a moderately high level (3.75–10 mM) during and after the depolarization by a long-lasting current to the cell soma. This transmitter release (K10 response) was found to be caused by Ca2+ entry through voltage-gated Ca2+ channels activated by moderately high-[K+]o-induced depolarization, however, did not result from Ca2+ entry through K+-activated Ca2+ channels activated by the direct action of external K+ (Déak et al. 1998) for the following reasons. First, they were blocked by blockers of voltage-gated Ca2+ channels and induced only during or after the passage of long-lasting depolarizing current. Second, a current similar to a K10 response was induced by a strong depolarizing voltage pulse to the cell soma during and after the conditioning depolarization. The priming effect of the conditioning depolarization for K10 responses was not due to the loading of Ca2+ stores for the activation of CICR (see Garaschuk et al. 1997) because of no effect of the removal of external Ca2+ or the addition of Cd2+ during the depolarization. It was ascribed to the loading of Cs+ and a reduction of K+ concentration in the presynaptic terminals as well as the parent axon. The detailed mechanism will be discussed in the following text.

Mechanism of priming of K10 response

The inward current applied to the cell soma causes the electrophoresis of Cs+, a major cation in a patch pipette, into the axon and dendrites, increasing the Cs+ concentration there. The outward current at the process membranes produced by the inward holding current would predominantly be carried out by efflux of K+ and therefore should decrease the K+ concentration in the processes. Blockade of K+ channels by loading of Cs+ and the decreased K+ in these processes depolarize the membrane of the processes and autaptic terminals, although the former reduces to some extent the effect of the latter. Under this condition, a moderate rise in [K+]o, which is otherwise ineffective, further depolarizes the cell membrane of the processes to a level sufficient to activate voltage-gated Ca2+ channels. Because the activation of voltage-gated Ca2+ channels occurs at a level, say –10 to –30 mV (Fox et al. 1987; Miller 1987; Mintz et al. 1992; Usowicz et al. 1992), [K+]o must be decreased to a level as low as 15–33 mM for 10 mM [K+]o to achieve this level of depolarization. In rare cases (see Fig. 2D), the effects of loading of Cs+ and the reduction of [K+]o to enhance the sensitivity of the processes to 10 mM [K+]o occur without a conditioning depolarization, presumably because the distance between the presynaptic terminals and the cell soma was short.

Modes of activation of synchronous and asynchronous exocytosis and Ca2+ channel types

A rapid jump of [K+]o produces the three modes of transmitter release; the graded mode (type-1), the spike and plateau mode with a latency (type-2), and the spike and plateau mode without a latency (type-3). Which mode of release occurs would depend on where and how voltage-gated Ca2+ channels are activated and how a Ca2+-dependent action potential is generated in the terminals and/or parent axon. The activation of Ca2+ channels depends at least on two factors. First, the effectiveness of high [K+]o to depolarize the membrane of the processes depends on extent of Cs+ loading into and the decrease of [K+]o in the processes, both of which would decrease with an increase in distance from the cell soma. The second factor is the efficiency of the regenerative activation of voltage-gated Ca2+ channels by high-[K+]o-induced depolarization at the processes. This increases with an increase in distance from the cell soma for the reduction of the effectiveness of space-clamping. Consequently, a region of a process somewhere between the autaptic terminals and the cell soma would most effectively be depolarized by a moderately high.
and therefore voltage-gated Ca\(^{2+}\) channels there are most effectively activated.

If these conditions for the activation of Ca\(^{2+}\) channels are fully met at each terminal, Ca\(^{2+}\) action potentials are simultaneously activated there, producing a spike-shaped K10 response without a latency (type-3). In fact, the spike of type-3 response was blocked by \(\omega\)CGTX, a blocker of N(P/Q)-type Ca\(^{2+}\) channels directly involved in impulse-evoked transmitter release (Dunlap et al. 1995). On the other hand, if the conditions for Ca\(^{2+}\) channel activation are met at the parent axon membrane, a Ca\(^{2+}\) action potential initiated there must be conducted to each terminal and would cause a spike-shaped K10 response with a notable latency (type-2). In support of this, the type-2 K10 response was blocked by nicardipine, a blocker of L-type Ca\(^{2+}\) channel not directly involved in impulse-evoked transmitter release (Dunlap et al. 1995) and elicited earlier than type-3 responses on conditioning depolarization as expected from the growth of Cs\(^{+}\) loading and decreased [K\(^+\)], through the axon toward the terminals. The latency for a spike may be explained by the growth of a regenerative Ca\(^{2+}\) action potential and its conduction to the terminals.

Asynchronous transmitter release would occur at two different sites. One would be the exocytotic site for impulse-induced exocytosis involving a high [Ca\(^{2+}\)]c closely coupled to the opening of N(P/Q)-type Ca\(^{2+}\) channel. Asynchronous release from this site would occur in part by the asynchronous activation of Ca\(^{2+}\) channel when the depolarization of the terminal membrane by 10 mM [K\(^+\)]o is not strong enough for the regenerative activation. This can be evidenced by the blockade of type-1 response by \(\omega\)CGTX under the effect of nicardipine. In strict sense, however, this is not the “real” asynchronous exocytosis generally known. Another site must be the exocytotic sites activated via high-affinity Ca\(^{2+}\) sensors by the resting and globally increased [Ca\(^{2+}\)]c (Hua et al. 1998; Wu et al. 1999). This was indeed supported by no effect of the removal of external Ca\(^{2+}\) on the K-off fraction of K10 response and the proportional decrease in MPSC frequency after a jump. This “real” mode of asynchronous exocytosis is reduced by simply eliminating Ca\(^{2+}\) entry, as seen in the blockade of type 3 response by nicardipine as well as \(\omega\)CGTX. The possible involvement of CICR from intracellular Ca\(^{2+}\) stores (Llano et al. 2000; Narita et al. 1998, 2000) is not ruled out by the present findings but appears to be indicated by preliminary observations (unpublished).

Transmitter pools involved in synchronous and asynchronous exocytosis

The K-off fraction of K10 response purely consisting of the real asynchronous exocytosis was reduced with a decrease in the interval of K\(^{+}\) jumps. This suggests the different pools of synaptic vesicles for synchronous and asynchronous exocytosis (Fig. 12B) because the constant fractions of two components of release are expected for the common pool of vesicles (Fig. 12A). Other possible mechanisms for the dependence of K-off fraction on the total size of K10 response could be ruled out as follows. First, the efficiency of 10 mM [K\(^+\)]o to activate Ca\(^{2+}\) channels might change during the course of repetitive induction of K10 responses. This is apparently not the case for K10 responses generated at +30 mV because of their constant size and also for those induced at −60 mV under the stable condition for a relatively long period.
pal neurons are separate and supplied from the common reserve pool (Fig. 12B).

Physiological and technical significance

The present study demonstrates distinction between synchronous and asynchronous exocytosis in terms of $\mathrm{Ca}^{2+}$ channel types, transmitter pools, and the mode of $[\mathrm{Ca}^{2+}]_\text{i}$ dependence in cultured hippocampal neurons. Under the physiological conditions, asynchronous release is only slightly enhanced by a nerve impulse that activates synchronous exocytosis and contributes to synaptic transmission. When the global rise in $[\mathrm{Ca}^{2+}]_\text{i}$ is sustained, however, the rate of asynchronous release is greatly enhanced. This indeed occurred in K10 responses. Preferential activation of asynchronous GABA release by high-frequency stimulation of synaptic inputs under the physiological stimulation was recently observed in cochlear nucleus neurons of chick embryos (Lu and Stevens 1991) but can apply the method to autaptic neurons. This novel method is useful in two respects. First, one does not need to prepare singly isolated autaptic neurons in island culture (Bekkers and Stevens 1991) but can apply the method to autaptic neurons in simple primary culture. Second, the method allows one to control the amount of depolarization of the presynaptic terminals and parent axon by controlling the extent of loading of Cs$^+$ or to provide variable conditions during the course of the loading (during the conditioning depolarization) and unloading (after repolarization) of Cs$^+$. On the other hand, this method can be a warning for those using patch pipette solutions, whose major cation is Cs$^+$. Such solutions have been widely used to record the N-methyl-d-aspartate receptor component of excitatory postsynaptic current at a positive voltage for the ease of holding such a voltage. Under this condition, if there are autaptic terminals in the neurons studied, the action of transmitter released asynchronously from depolarized autaptic terminals would add up to the current response by the transmitter released from the foreign terminals.

Present address of F.-M. Lu: Center for Neurobiology and Behavior, Columbia University, 1051 Riverside Dr., New York, NY 10032.

REFERENCES


