Empty Synaptic Vesicles Recycle and Undergo Exocytosis at Vesamicol-Treated Motor Nerve Terminals

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Parsons, Rodney L., Michelle A. Calupca, Laura A. Merriam, and Chris Prior. Empty synaptic vesicles recycle and undergo exocytosis at vesamicol-treated motor nerve terminals. J. Neurophysiol. 81: 2696–2700, 1999. We investigated whether recycled cholinergic synaptic vesicles, which were not refilled with ACh, would join other synaptic vesicles in the readily releasable store near active zones, dock, and continue to undergo exocytosis during prolonged stimulation. Snake nerve–muscle preparations were treated with 5 mM vesamicol to inhibit the vesicular ACh transporter and then were exposed to an elevated potassium solution, 35 mM potassium propionate (35 KP), to release all preformed quanta of ACh. At vesamicol-treated endplates, miniature endplate current (MEPC) frequency increased initially from 0.4 to >300 s⁻¹ in 35 KP but then declined to <1 s⁻¹ by 90 min. The decrease in frequency was not accompanied by a decrease in MEPC amplitude. Nerve terminals accumulated the activity-dependent dye FM1–43 when exposed to the dye for the final 6 min of a 120-min exposure to 35 KP. Thus synaptic membrane endocytosis continued at a high rate, although MEPCs occurred infrequently. After a 120-min exposure in 35 KP, nerve terminals accumulated FM1–43 and then destained, confirming that exocytosis also still occurred at a high rate. These results demonstrate that recycled cholinergic synaptic vesicles that were not refilled with ACh continued to dock and undergo exocytosis after membrane retrieval. Thus transport of ACh into recycled cholinergic vesicles is not a requirement for repeated cycles of exocytosis and retrieval of synaptic vesicle membrane during prolonged stimulation of motor nerve terminals.

INTRODUCTION

Neuromuscular transmission occurs when ACh, which is released from motor nerve terminals through exocytosis of synaptic vesicles, crosses the synaptic cleft and activates postsynaptic nicotinic receptors. Each synaptic vesicle normally contains and releases a discrete amount of ACh, called a quantum. The postsynaptic current caused by one quantum of ACh is the miniature endplate current (MEPC). Normally, sufficient ACh is released so that the postsynaptic response, the endplate potential, remains suprathreshold even with continued repetitive stimulation, thereby ensuring efficient neuromuscular transmission. To sustain neuromuscular transmission, appropriate numbers of synaptic vesicles must be docked and primed for release at the active zones at the inner side of the presynaptic terminal (reviewed in van der Kloot and Molgo 1994). Two pools of synaptic vesicles are proposed, the readily releasable pool positioned near the active zones and a larger, more distant main store (reviewed in Parsons et al. 1993; van der Kloot and Molgo 1994). Two mechanisms maintain the needed supply of synaptic vesicles in the readily releasable pool. First, synaptic vesicles are mobilized from the main store into the releasable pool during continued stimulation. Second, after exocytosis, synaptic membrane must be retrieved through endocytosis quite rapidly. The retrieved vesicular membrane must be transformed into vesicles, refilled with ACh, and then returned to the readily releasable pool near active zones. Recent studies at the neuromuscular junction and at synapses between cultured hippocampal neurons suggest that exocytosis and endocytotic retrieval of synaptic vesicle membrane are closely coupled (Betz et al. 1992b; Murphy and Stevens 1998). Thus the rate of synaptic vesicle recycling is determined by the rate of exocytosis (Betz et al. 1992b; Wu and Betz 1996). In addition, Betz and colleagues (Betz and Bewick 1992, 1993; Betz et al. 1992a) have shown that recycled, refilled vesicles mix randomly and remain within the vesicular pool so that the recycled, refilled synaptic vesicles are indistinguishable from their nonrecycled neighbors.

It might be expected that some mechanism would exist to ensure that vesicles are refilled, at least partially, with transmitter before entering the readily releasable pool. Previous electrophysiological measurements indicate that partially filled vesicles undergo exocytosis (Elmqvist and Quastel 1965; Searl et al. 1990, 1991). However, it is not established whether exocytosis continues or stops once the recycled vesicles no longer contain any transmitter. Thus it is unknown whether refilling of synaptic vesicles is a prerequisite for recycling synaptic vesicles to merge with the readily releasable pool of vesicles and continue to undergo exocytosis (reviewed in van der Kloot and Molgo 1994).

Recently, Cousin and Nicholls (1997) presented evidence that recycled, unfilled glutamatergic vesicles re-enter the available pool of vesicles and undergo exocytosis in cerebellar granule cells. However, key characteristics of synaptic transmission differ between central neuronal and peripheral neuromuscular synapses, such as vesicle pool size, probability of synaptic vesicle release, mechanism of recycling, and need for sustained suprathreshold transmission. Thus it is reasonable to propose that mechanisms regulating the replenishment of recycled vesicles might differ between central nerve terminals and motor nerve terminals.

Consequently, we asked the basic question do empty, recycled cholinergic synaptic vesicles at continually stimulated...
motor nerve terminals re-enter the releasable vesicle pool near active zones, dock, and undergo exocytosis? To test this question, we inhibited the ACh transporter with vesamicol and then stimulated the nerve terminal until all preformed stores of quanta were depleted. Once preformed transmitter stores were depleted and postsynaptic currents were eliminated, we then tested whether endocytosis and exocytosis continued to occur at the motor nerve terminal by using the activity-dependent dye FM1–43.

METHODS

Experiments were performed on twitch muscle fiber neuromuscular junctions in costocutaneous muscles of garter snakes (Thamnophis) at room temperature (21–23°C). Nerve–muscle preparations were initially bathed in a control physiological solution containing (in mM) 159 NaCl, 2.5 KCl, 1.0 CaCl₂, 4.2 MgCl₂, and 5.0 HEPES, pH 7.3 (Connor et al. 1984, 1997). Depolarization-stimulated transmitter release was induced by exposure to an elevated potassium solution (35 mM potassium propionate, 35 KP) (Connor et al. 1997). CsCl (5 mM) was included in the 35 KP solution to facilitate voltage clamping of depolarized muscle fibers to hyperpolarized potentials (Connor et al. 1984, 1997). Stimulation by K⁺ depolarization rather than nerve stimulation was chosen because with repetitive neural stimulation muscle movement made it impossible to maintain stable intracellular stimulation was chosen because with repetitive neural stimulation muscle movement made it impossible to maintain stable intracellular

FIG. 1. Miniature endplate currents (MEPCs) recorded from different endplates exposed to 35 mM potassium propionate (35 KP) and voltage clamped to −150 mV. Traces 1–3: MEPCs recorded from vesamicol-treated endplates after different durations in 35 KP. 1: 2 min, MEPC frequency only estimated, >30 s⁻¹; 2: 35 min, MEPC frequency 12 s⁻¹; 3: 105 min, MEPC frequency 0.6 s⁻¹. Trace 4: MEPCs recorded from an untreated preparation after 127 min in the 35 KP solution, MEPC frequency only estimated, >300 s⁻¹.

confirm the success of the loading procedure. While still in 35 KP, nerve terminals were then examined over time to determine whether the FM1–43 staining diminished. FM1–43 commonly stained the myelinated preterminal axon (Fig. 3). This nonspecific staining served as a control for the staining conditions and imaging. FM1–43 accumulation and destaining of nerve terminals was examined with a Zeiss fluorescence photomicroscope equipped with filter sets appropriate for FM1–43 (green emission filter, 520–560 nm) or rhodamine (red emission filter, >590 nm). A ×40 water immersion lens was used to locate individual nerve terminals.

RESULTS

MEPC frequency declines at vesamicol-treated endplates exposed to 35 KP

MEPCs were recorded at control snake twitch fiber endplates and at endplates that had been treated with vesamicol (5 µM), a drug that potently inhibits the cholinergic vesicular transporter (IC₅₀ of ~40 nM) (Anderson et al. 1983; Marshall 1970). Transmitter release was stimulated by depolarization in an elevated potassium solution (35 KP). In both vesamicol-treated and control preparations, MEPC frequency increased from ~0.4 s⁻¹ to >300 s⁻¹ on exposure to 35 KP (Fig. 1, trace 1). At control endplates, MEPC frequency remained elevated during a 120-min exposure to 35 KP (Fig. 1, trace 4) (see also Connor et al. 1997). In contrast, at vesamicol-treated endplates, MEPC frequency declined progressively in 35 KP so that by 90 min MEPCs were either not recorded or were recorded at a very low frequency (Fig. 1, trace 3).

The decrease in MEPC frequency was not accompanied by a decrease in MEPC amplitude at vesamicol-treated endplates (Fig. 2, A and B). In addition, although mean MEPC amplitude
varied among individual endplates, the MEPC amplitude distribution histograms remained unimodal with no evidence of a population of smaller-amplitude MEPCs (Fig. 2C). Thus no ACh was released from partially filled vesicles that contributed to MEPC generation.

We then used the MEPC frequency-versus-time relationship to approximate the number of preformed quanta released at vesamicol-treated endplates. The time-dependent decline in MEPC frequency (Fig. 2A) was fitted to a single exponential described by

$$\text{MEPC frequency } f(t) = \text{MEPC freq}_{0} e^{-\frac{t}{\tau}}$$

The area under the exponential was integrated to estimate the total number of quanta released. The estimation of preformed quanta released was 490,000, a value comparable with that reported previously for preformed quantal stores in vertebrate motor nerve terminals (van der Kloot and Molgo 1994). Thus we concluded that the decline in MEPC frequency at vesamicol-treated endplates resulted from the depletion of preformed quanta of ACh, a process that took ~100 min in 35 KP.

**FM1–43 accumulates in vesamicol-treated nerve terminals when the MEPC frequency is low**

We next compared the accumulation of FM1–43 into control and vesamicol-treated nerve terminals maintained in 35 KP for 120 min. All nerve terminals in both control and vesamicol-treated preparations incorporated FM1–43 during the final 6 min of a 120-min exposure to 35 KP (10–15 PNA-identified endplates/muscle; 6 control muscle preparations from 4 different snakes and 9 vesamicol-pretreated muscle preparations from the same 4 snakes). Representative examples in Fig. 3, C and D, illustrate that the FM1–43 fluorescence is comparable for nerve terminals innervating control and vesamicol-treated endplates. Also we confirmed that nerve terminals that were not stimulated (i.e., kept in control physiological solution for 120 min) only exhibited background FM1–43 staining (Fig. 3B). The lack of FM1–43 accumulation in these preparations was consistent with the low resting MEPC frequency (~0.4 s⁻¹) recorded at endplates in control physiological solution (Connor et al. 1997).
**FM1–43 destains with continued exposure to 35 KP**

FM1–43 staining of vesamicol-treated nerve terminals indicated that endocytosis of synaptic vesicle membrane was continuing at a high rate after 120 min in 35 KP, although MEPC frequency was very low. FM1–43 destaining experiments were completed to directly demonstrate that exocytosis also continued at vesamicol-treated terminals after 120 min in 35 KP. For the destaining experiments, two groups of vesamicol-treated nerve–muscle preparations were exposed to FM1–43 for the final 6 min of a 120-min exposure to 35 KP. In one group of muscles, bath solution containing FM1–43 was exchanged by dye-free 35 KP, and the muscles were viewed immediately to verify FM1–43 accumulation. These muscles were then maintained in control physiological solution with PNA for 10 min, and then maintained in control physiological solution for 65 min. Terminal was stained by PNA (C) and exhibited FM1–43 fluorescence (D). Calibration bar = 40 μm.

**DISCUSSION**

At a concentration of 5 μM, vesamicol should have effectively inhibited the vesicular ACh transporter so that all of the quanta of ACh released by K+ stimulation must have come from preformed stores. The observation that the MEPC amplitude histograms were unimodal confirmed that no partially filled vesicles contributed to MEPC generation (Searl et al. 1990). In addition, the MEPC average amplitude did not decrease in vesamicol-treated preparations during the 120-min exposure to 35 KP. Thus it appears that preloaded stores of ACh remained within the vesicles under the conditions of these experiments. We concluded therefore that the decline in MEPC frequency was the result of a depletion of the preformed quantal stores. We estimated that 490,000 preformed quanta were released during the 120 min in 35 KP, a value consistent with other published values for ACh stores (reviewed in van der Kloot and Molgo 1994). However, our value is somewhat larger than that reported previously (290,000) for snake twitch motor nerve terminals (Searl et al. 1990). This difference may reflect differences in experimental approach. We stimulated release by elevating K+, whereas Searl et al. (1990) estimated numbers of quanta released when nerve stimulation was used to exhaust vesamicol-treated (5 μM) motor nerve terminals.

Our results show that vesamicol-treated snake twitch nerve terminals accumulated FM1–43 after 120 min in 35 KP, a time when the MEPC frequency was <1 s⁻¹. This frequency of MEPCs is well below the frequency normally required (25–50 MEPCs s⁻¹) to enable twitch nerve terminals to accumulate enough FM1–43 (with the 6-min exposure) to exhibit noticeable fluorescence with our optical system (Connor et al. 1997). Thus, although the frequency of synaptic currents was very low, endocytosis of synaptic vesicle membrane must have continued at a high rate.

Betz and coworkers (Betz and Bewick 1992, 1993; Betz et al. 1992a,b) demonstrated that FM1–43 accumulates in synaptic vesicles that have undergone recycling via endocytosis. It was also concluded that the extent of endocytosis is dependent on the amount of preceding exocytosis. Thus the rate of endocytosis appears to be closely coupled to the rate of exocytosis (Betz et al. 1992b; Wu and Betz 1996). The accumulation of FM1–43 at vesamicol-treated terminals after 120 min in 35 KP provided strong, albeit indirect, evidence that endocytosis continued at a high rate, although very few MEPCs were recorded. Destaining experiments provided direct evidence that exocytosis was continuing at a high rate. The nerve terminals were destained with continued exposure to 35 KP. In contrast, FM1–43 fluorescence remained in terminals that were washed in control physiological solution and not continually stimulated by exposure to 35 KP. We have not quantitated the time course of FM1–43 destaining in the current studies. However, the extent of destaining we observed after 15 min is very similar to that reported previously by Lindgren et al. (1997) in studies of FM1–43 staining and destaining induced by exposure to elevated potassium at lizard nerve terminals in the absence of vesamicol.

The ability to destain at a time when recorded MEPC frequency was consistently <1 s⁻¹ indicated that exocytosis continued but the vesicles did not contain ACh. We conclude that at vesamicol-treated terminals exocytosis con-
ued at an elevated rate throughout the 120-min exposure to 35 KP, just as that which occurred with preparations not exposed to vesamicol but kept in 35 KP. However, in vesamicol-treated preparations, more and more of the docked vesicles were unfilled, recycled vesicles. Consequently, as the percentage of the empty, docked vesicles undergoing exocytosis increased, the number of recorded postsynaptic currents progressively declined. We conclude therefore that recycled cholinergic synaptic vesicles that were not refilled with ACh continued to undergo exocytosis and endocytosis during prolonged K+ stimulation. Thus, like glutamatergic terminals in cerebellar granule cells (Cousin and Nicholls 1997), transport of transmitter, in this case ACh, into recycled cholinergic vesicles is not a requirement for repeated cycles of exocytosis and retrieval of synaptic vesicle membrane during prolonged stimulation of motor nerve terminals.

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