Neurotransmission in central neuronal synapses is supported by the recycling of synaptic vesicles via endocytosis at different time scales during and after transmitter release. Here, we examine the kinetics and molecular determinants of different modes of synaptic vesicle recycling at a peripheral neuronal synapse formed between superior cervical ganglion neurons in culture, via acute disruption of endocytosis with Dynasore, an inhibitor of dynamin activation, or a dynamin peptide (P4) that perturbs linkage of dynamin to clathrin coats through amphiphysin. When paired action potentials are generated to produce excitatory postsynaptic potential responses, the second response was reduced after application of Dynasore but not P4. In addition, graded reduction in synaptic transmission during a train of action potentials was accelerated by Dynasore but enhanced by P4. After full depletion of releasable vesicles, P4 delayed the recovery of synaptic transmission while Dynasore limited recovery to 10%. In control neurons, synaptic transmission is stable for more than 1 h under low frequency presynaptic stimulation (0.2 Hz), but was reduced gradually by P4 and rapidly but incompletely blocked by Dynasore at a much lower stimulation frequency. These results suggest two essential modes of dynamin-mediated synaptic vesicle recycling, one activity-dependent and the other activity-independent. Our findings extend the current understanding of synaptic vesicle recycling to sympathetic nerve terminals and provide evidence for a physiological and molecular heterogeneity in endocytosis, a key cellular process for efficient replenishment of the vesicle pool, and thus for synaptic plasticity.

The cycling of synaptic vesicles (SVs) through repetitive episodes of exocytosis and endocytosis is fundamental to synaptic transmission (1). The classic endocytic cycle consists of vesicle exocytosis from a readily releasable pool (RRP) (2) followed by retrieval via a clathrin-mediated endocytic pathway (3) that passes through a reserve pool (RP) (4) en route to the RRP (5, 6). The classic pathway, well studied at the frog neuromuscular junction (3), also functions in brain central synapses (7, 8). In addition, other nontraditional modes of recycling such as “kiss-and-run” (9) and fast recycling, which bypasses the RP (10), have been described (11–14). Endocytic pathways have also been categorized in terms of their kinetics, as fast or slow (1, 15). Together, it seems likely that various forms of SV recycling pathways function under different conditions of synaptic activity and in a cell type-specific manner (15, 16).

Although the precise mechanisms of synaptic reformattion remain a matter of debate (3–7), the GTPase dynamin has a key role for this process (17–19). Dynamin oligomerization in endocytic pits mediates neck constriction and scission (20). As a component of the clathrin coat, amphiphysin interacts with dynamin and links clathrin-coats to dynamin (21). Dynamin binds to the Src homology 3 (SH3) domain of amphiphysin I via the PSRPNR sequence in the dynamin polyproline domain near its C terminus (22, 23). A myristoylated peptide derived from this sequence called P4 (QVPSR-PNRAP) is able to competitively block dynamin binding to amphiphysin I and II in vitro (24) and inhibits SV endocytosis, thus resulting in the depression of transmitter release (25). Dynasore, a specific cell-permeable dynamin inhibitor (26), completely blocks SV endocytosis, suggesting an essential role for dynamin in all forms of compensatory SV endocytosis, including “kiss-and-run” events (27). However, in the calyx of Held, a dynamin-independent endocytosis was detected in the presence of Dynasore (28).

The mechanism of SV recycling in sympathetic neurons remains an open question. To investigate this issue, we studied the cholinergic synapse formed between rat SCG neurons in culture (29). In this model system, it is possible to introduce reagents directly into presynaptic terminals by microinjection, and their effect on acetylcholine release evoked by action potentials can be monitored by recording EPSPs from neighboring neurons (29, 30). By perturbing dynamin function with either P4 peptide or Dynasore, we examine both activity-dependent and -independent modes of endocytosis, as well as dynamin-dependent and -independent pathways for refilling of the RRP in sympathetic neurons.
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EXPERIMENTAL PROCEDURES

Cultured SCG neurons were prepared as described previously (29, 30). For immunocytochemistry, SCG neurons in culture (8 weeks) were fixed and stained as described previously (30) with polyclonal anti-dynamin 1 and anti- amphiphysin II antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal anti-synaptophysin antibody (Sigma-Aldrich) (supplemental Fig. S1). For electrophysiology, SCG neurons 6–8 weeks in culture were studied. EPSP recording and injection of peptides were performed as described previously (29, 30). To measure the replenishment of the RRP with readily releasable SVs, either a paired-pulse protocol or 5–30 Hz stimulation for 2 s was applied. For each neuron pair, three recordings were performed every 2 min for each interval of stimuli, and the EPSP peak amplitudes were averaged to account for variations in transmitter release following repetitive action potentials. For the depletion of synaptic vesicles, action potentials were applied at 5 Hz for 4 min, and replenishment of readily releasable SVs was monitored by tracking the recovery of baseline EPSP via recording every 1 s. The peak amplitudes of EPSP were averaged, and the resultant values were smoothed by an eight-point moving average algorithm. To measure the change in readily releasable SVs during prolonged repetitive activity, EPSPs were recorded at either 0.2 or 0.05 Hz. The peak amplitudes were averaged and plotted against recording time with $t = 0$ corresponding to the pre-synaptic injection of P4 (QVPSRPNRAP), a scrambled control peptide (QPPASNPRV), or bath application of Dynasore. 1 mM peptide in the injection pipette was applied as this is the concentration producing a maximum reduction of EPSP amplitude, whereas 5 mM peptide showed no further reduction. For Dynasore bath application, 100 μl of 1 mM Dynasore dissolved in 5% DMSO was drop-applied to a 1.25-ml bath. A final concentration of 80 μM (0.4% DMSO) Dynasore was used to achieve maximum inhibition of endocytosis, because this concentration completely blocked all forms of endocytosis in hippocampal neurons (28). As a control, 5% DMSO was drop-applied, producing a bath concentration of 0.4%. To reach the final concentration it takes a few minutes after bath superfusion was stopped (31).

Before recording EPSPs at 20 min after the Dynasore application, cultured SCG neurons showed no spontaneous synaptic activity (32), suggesting that reduction of SVs in the RRP with the treatment is unlikely. Error bars shown in the text and figures represent mean ± S.E. A two-tailed Student t test was applied as indicated.

RESULTS

Readily Releasable SVs after Evoked Transmitter Release—To examine the readily releasable SVs after single action potential-evoked transmitter release, a paired-pulse protocol was applied under acute disruption of endocytosis by P4 or Dynasore (Fig. 1). Synthetic responses induced by two consecutive action potentials showed a depression of the second response (paired-pulse depression) with inter-stimulus interval (ISI) of 20–100 ms. In contrast, at longer ISIs (200–2000 ms) the amplitudes of the second response were similar to the first (Fig. 1A). Paired-pulse responses were subsequently recorded 20 min after either P4 (Fig. 1A) or Dynasore application (Fig. 1B). The amplitude and the ratio of the EPSPs did not change with P4 (Fig. 1A). In contrast, with Dynasore the amplitude of the second EPSP decreased more than the first EPSP (with ISI 50 ms: 13.2 ± 0.5 mV1st and 6.6 ± 1.0 mV2nd after Dynasore, mean ± S.E., n = 5; p < 0.01, paired Student t test) (Fig. 1B, panel b). Thus the paired-response ratio decreased with Dynasore.

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sore (with ISI = 50 ms, from 0.62 ± 0.03 to 0.45 ± 0.06; with ISI = 120 ms, from 1.1 ± 0.03 to 0.70 ± 0.08; p < 0.05, paired t test) (Fig. 1B, panel c). These results suggest that dynamin dysfunction prevented replenishment of readily releasable SVs with an ISI of <120 ms, although the clathrin-mediated pathway might not function in replenishment of readily releasable SVs with an ISI of <2 s. The time for the dynamin-mediated replenishment after a single action potential is much shorter than previous reports for fast endocytosis in hippocampal synapses in imaging studies (τ = 0.4–6 s) (15). Dynasore is a specific inhibitor for endocytosis (24, 28) and unlikely to affect SV fusion. Although the mechanism of paired-pulse depression in central synapses is thought to be associated with a decrease in release probability (33), SVs are able to fully release from the RRP within tens of milliseconds in synapses of cultured SCG neurons when depression is removed (34). The results indicate that rapid replenishment of the RRP via dynamin-mediated SV recycling occurs after an evoked transmitter release.

Readily Releasable SVs during and after Repetitive Transmitter Release—To examine readily releasable SVs during and after repetitive action potential firing, 2-s trains of 5–30 Hz action potentials were elicited every 2 min in five synaptic couples. The EPSP amplitude decreased during the train (Fig. 2, A–D, upper graphs), and the rate of the decay was dependent on the frequency of action potential generation (Fig. 2I). The train-evoked decrease in EPSP amplitude returned to the initial value 2 min after cessation of each train. Normalized first EPSP amplitudes recorded at 10, 20, and 30 Hz to those at 5 Hz were around 1 (Fig. 2, A–D and L). At 2 min after a series of recording, P4 or Dynasore was applied. EPSP recordings with trains of action potentials were resumed at 20 min after reagent application. In the presence of P4 or Dynasore, the EPSP amplitude did not return to the initial value during a 22-min cessation of firing. Normalized first EPSP amplitudes recorded at 5 Hz were 0.45 ± 0.06 for P4 and 0.2 ± 0.03 for Dynasore (Fig. 2, A, C, J, and L). Dynasore decreased EPSP amplitude rapidly with each successive train of action potentials, especially ≥10 Hz (Fig. 2, E–H and K). P4 enhanced the EPSP amplitude decrease at 20 and 30 Hz (G–I). As a control, the scrambled P4 peptide and DMSO control showed no change in EPSP amplitude (Fig. 2, B and D). The inhibition of the recovery with Dynasore was stronger than that with P4 (Fig. 2J). These results suggest that activity-dependent endocytosis could be activated by a 2-s train of action potentials at >10 Hz, in addition to dynamin-mediated endocytosis seen after a single action potential (Fig. 1B). It should be noted that, in addition to recycling from the plasma membrane, Dynasore will inhibit vesicle budding from sorting endosomes that supply de novo synaptic vesicles to the RRP (35). Interestingly, the train-evoked decrease in EPSP amplitude in the presence of Dynasore returned to the initial value 2 min after cessation of each train. Normalized first EPSP amplitudes recorded at 10, 20, and 30 Hz were ~0.2 (Fig. 2, C, J, and L), suggesting a possible dynamin-independent pathway for replenishment of readily releasable SVs through the transport route from the RP to the RRP during the 2-min cessation of each train. We note that an incomplete effect of Dynasore or an effect of dynamin 3 at this synapse cannot be excluded with these data. However, inhibition of available dynamin 1 and 2 and most if not all components of endocytosis would be consistent with prior work employing similar concentrations of Dynasore (27, 28).

Replenishment of Readily Releasable SVs after Depletion of Readily Releasable SVs—To test whether both dynamin-dependent and -independent pathways can replenish readily releasable SVs, SVs in the presynaptic terminals were depleted with 4-min trains of 5-Hz action potentials, and the recovery of EPSP amplitude was measured every 1 s. At the end of the train, the EPSP amplitude was within baseline noise levels (Fig. 3, A and B), and subsequently recovered at two distinct rates: fast and slow (see arrows in Fig. 3A, panel a). A control scrambled P4 peptide or DMSO did not show any reduction in the recovery rate (Fig. 3, A (panel b) and B (panel b)). In contrast, both P4 and Dynasore inhibited fast recovery (Fig. 3, A (panel a), B (panel a), and C). At 20 s after the train, the EPSP amplitude was significantly smaller than that before reagent applications (Fig. 3C, panel b). These results indicate that releasable SVs are depleted, while SVs in the RP may persist at the end of a 4-min train of action potentials. Furthermore, SVs in the RP may refill the RRP through the dynamin-dependent and clathrin-mediated pathway. In addition, readily releasable SVs may also be replenished through a dynamin-independent pathway in the presence of Dynasore. At 5 min after the train, the EPSP amplitude recovered to 41.4 ± 4.5% with P4, but remained at 12.3 ± 2.1% with Dynasore (Fig. 3C, panel b). The slow recovery could be described by a linear relationship (Fig. 3C, panel a). The slopes before and after P4 injection were 8.1 ± 1.7%/min and 6.7 ± 1.6%/min (p = 0.63, unpaired t test), whereas the slope with Dynasore was 0 ± 0.4%/min (Fig. 3C, panel a). These results demonstrate that the slow recovery rate was not significantly affected by P4 but blocked completely by Dynasore, suggesting the replenishment of readily releasable SVs through dynamin-dependent recycling (18) or de novo sorting via an endosomal pool (35). Together, the results suggest that the fast replenishment of the readily releasable SVs may involve SV transport from the RP via dynamin-mediated and non-dynamin-mediated pathway, whereas the slow replenishment of the readily releasable SVs may be achieved solely through dynamin-mediated endocytosis.

Readily Releasable SVs during Low Frequency Repetitive Transmitter Release—To examine the role of the dynamin-mediated pathway in replenishment of readily releasable SVs during low frequency repetitive transmitter release, changes in the amplitude of EPSPs evoked by presynaptic action potentials at 0.2 or 0.05 Hz were measured (Fig. 4). P4 gradually reduced the EPSP amplitude at 0.2 Hz (Fig. 4A, panels a and c), but not at 0.05 Hz (Fig. 4A, panels b and c). At 40 min after P4 injection, reduction of EPSP amplitude was −47.9 ± 9.4% at 0.2 Hz (n = 7), and −11.7 ± 6.4% at 0.05 Hz (n = 4). This value was similar to the control value with the scrambled P4 peptide (−8.9 ± 4.9% at 0.2 Hz; n = 6) (Fig. 4A, panel d). In contrast, Dynasore reduced the EPSP amplitude at 0.2 and 0.05 Hz (Fig. 4B). The reduction rate was more rapid than that of P4 with 0.2 Hz stimuli (Fig. 4A (panel a) versus B (panel a)). The decay time constant of the EPSP amplitude in the presence of Dynasore was 4.8 ± 0.12 min at 0.2 Hz, and 13.2 ± 0.17 min at 0.05 Hz (p < 0.01, unpaired t test) (Fig. 4B, panel d), whereas it was 28 ± 1.0
min at 0.2 Hz in the presence of P4. These results suggest that dynamin also mediates replenishment of readily releasable SVs during low frequency firing. Surprisingly, the EPSP amplitude was very small at 60 min after Dynasore application, but not completely blocked. The amplitudes were 6.8 ± 1.6% (at 0.2 Hz) and 6.8 ± 0.9% (at 0.05 Hz) of the initial value before Dynasore application, suggesting that non-dynamin-mediated processes may function in replenishing readily releasable SVs, although further experiments will be necessary to address this issue in future.

DISCUSSION
In this study, we demonstrate that sympathetic neurons maintain synaptic transmission via the recycling of SVs through dynamin-mediated pathways during and after action potential activity. In addition, we provide evidence for a non-dynamin-
mediated endocytic pathway, assuming that P4 and Dynasore blockade of dynamin-mediated recycling is complete. Refilling of the RRP via dynamin-mediated endocytic pathways was dependent on both rate and number of action potential firing (Figs. 2–4), in accord with activity-dependent recycling of synaptic vesicles observed at other synapses. In contrast, another mode of the RRP refilling through a dynamin-mediated endocytic pathway was activated independently of action potential firing rate and number (Figs. 1–4), consistent with an activity-independent pathway. The third pathway, not affected by dynamin dysfunction, was also activated at all rates or numbers of action potential firing tested (Figs. 1–4), and was thus activity-independent; it is estimated that 10% of SVs in readily releasable SVs were replenished via this pathway to maintain efficient synaptic vesicle recycling with long lasting repetitive firing of the SCG neuron. Compared with neurons in the central nervous system, sympathetic nerve fibers show relatively low firing activity in the 0.5- to 7.5-Hz range in vivo (36). Thus, evidence for activity-dependent refilling of the RRP observed in this study may reflect physiological synaptic transmission in autonomic neurons in vivo.

The kinetics of endocytosis is variable at different presynaptic terminals (15, 16). In hippocampal neurons, imaging studies have shown a wide range of time constants for endocytosis from \( \tau = 0.1 - 6 \) s for fast components (13, 15) and \( \tau = 4 - 90 \) s for slow clathrin-mediated endocytosis (15). In Drosophila neuromuscular synapses, two pathways of vesicle recycling (37) and two or three SVs pools, the RRP and the RP (38) or "immediately releasable pool" (39), were documented. During short period or low frequency presynaptic activity, SVs in the RRP, including the immediately releasable pool but not those in the RP, participate in transmitter release, whereas vesicles in the RP are required during intense neuronal activity (>10 Hz). A dynamin mutant, shibire, exhibits rapid synaptic fatigue within 0.02 s of repetitive stimulation, a phenotype that cannot be explained by vesicle depletion, suggesting that dynamin is required for rapid replenishment of the RRP with synaptic vesicles (40). In the present study, reduction of the second of two consecutive EPSPs with an ISI of 0.05 s by Dynasore (Fig. 1B) suggests that in sympathetic neurons, dynamin is also required for rapid replenishment of readily releasable SVs, in addition to its role in slow clathrin-mediated endocytosis, which contributes to SVs recycling via the RP. Our data suggest approximate time constants for endocytosis in cultured sympathetic neurons. The dynamin-mediated pathway is able to mobilize SVs to the RRP in <0.05 s after an action potential (Fig. 1B), and the dynamin- and clathrin-mediated pathway is able to transport SVs to the RRP in under 20 s (Fig. 4A), if P4, as proposed, could perturb clathrin-coats formation. Measuring the time constant of readily releasable SV replenishment via the non-dynamin-mediated pathway is technically difficult because of the small synaptic responses after dynamin inhibition.

Using primary cortical cultures from dynamin 1 knockout mice, Ferguson et al. (18) recently demonstrated that neuron-
specific dynamin 1 is required for rapid SV recycling during high frequency (>10 Hz) stimulation, but not after cessation of the stimulus train. Dynamin 3 appears to share a similar role to dynamin 1. In contrast, ubiquitously expressed Dynamin 2 may play a role in slow activity-independent constitutive replenishment of clathrin-coated vesicles. Here we report that presynaptic terminals of a sympathetic neuron have two dynamin-mediated SV replenishment pathways, which differ in activity dependence. The relationship of these two recycling modes to specific dynamin isoforms will require further investigation.

The differential effects of P4 peptide and Dynasore may be accounted for different steps in which they are likely to participate in the endocytic pathway. P4 disrupts the interaction of dynamin with amphiphysin (24), which may interfere with formation of the clathrin-coat (21) or fission complex. On the other hand, Dynasore may act at a downstream step involving dynamin activity and subsequently provide constitutive inactivation of dynamin across the endocytic cycle. It is also possible that, by targeting different parts of the dynamin endocytic complex, P4 peptides and Dynasore may act on different time scales and efficacies and thus account for their differential effects. Clearly the two reagents provide unique insight into distinct roles of dynamin in endocytosis with respect to synaptic vesicle pool recovery during different patterns of action potential stimulation.

We should caution that one cannot exclude incomplete inhibition of dynamin function by injected P4 peptides, which may be unable to fully target preassembled dynamin endocytic complexes due to steric hindrance and lower concentrations at nerve terminals. Because it was technically unfeasible to collect enough injected neurons for in vitro biochemical co-immunoprecipitation study, we are unable to provide experimental evidence to verify the assumption that injected P4 peptide could completely block the dynamin-amphiphysin interaction in our SCG neurons. Although the amphiphysin I/II knockout mice exhibit some defects in synaptic vesicle recycling (41), its essential role in clathrin-mediated endocytosis remains further investigation. It is possible that P4 might target not only to amphiphysin but also to other SH3 domain-containing proteins (24), thus playing a less specific role in blocking endocytosis than that by Dynasore. However, previous work in the SCG neuron system has indicated that injected peptides at even lower concentrations can disassemble other preformed synaptic protein complexes (42). Furthermore, P4 peptides have the efficacy to block clathrin-dependent endocytosis at intracellular concentrations (25) that are comparable to those used in the current study, although it was injected into different neuronal types and the effect was measured by different readouts.

Likewise, one must interpret the Dynasore inhibition data with caution. Dynasore is a fast-acting cell-permeable small molecule that inhibits the GTPase activity of dynamin 1, dynamin 2, and Drp1, the mitochondrial dynamin (43). Thus, it remains possible that in SCG neurons Dynasore may inhibit endocytosis dependent on dynamins 1 and 2 but not dynamin 3. Incomplete inhibition can also not be excluded without an
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independent measure of endocytosis inhibition. To address these issues, investigation of the role of dynamin isoforms in synaptic vesicle endocytic pathways in SCG neurons is under further study. However, it should be noted that robust inhibition of dynamin 1 and 2 and most if not all kinetic components of endocytosis is consistent with prior work employing similar concentrations of Dynasore (27, 28).

The results in the present study suggest a dynamin-independent pathway for SV recycling in a sympathetic neuron synapse. Recent studies at other synapses support this conclusion. SV endocytosis at the large presynaptic terminal of the calyx of Held is partially independent of dynamin (29). Endocytosis activated during intense stimulation persists in the calyx synapse dialyzing with Dynasore and PQVPSRPNRAp (called pP11, one amino acid longer than P4) (29). In peripheral dorsal root ganglion neurons, a calcium- and dynamin-independent form of rapid endocytosis, which is controlled by protein kinase A-dependent phosphorylation, has been described (44). It will be of interest to further examine the molecular and physiological basis of vesicle fission via dynamin-independent endocytosis and its relationship to kiss-and-run and other proposed fast, non-classic modes of synaptic vesicle recycling.

The synaptic short-term depression observed during repetitive neuronal firing may be attributed to multiple mechanisms, including a decrease in vesicle fusion probability, inactivation of voltage-gated Ca$_{2+}$ channels (34), or use-dependent inhibition of the vesicle release machinery (33). In hippocampal or neocortical neurons, rapidly recycled SVs in the RRP are capable of rapid reuse (45) and slow the rate of synaptic depression (46). Our study shows that synaptic transmission in cultured SCG neurons also decreases rapidly in response to repetitive action potential firing (Fig. 2). The decrease was strongly accelerated in the presence of Dynasore, suggesting that a rapid reduction in the number of vesicles available for fast release may contribute to synaptic depression. These results may suggest that dynamin-mediated pathways are critical to maintain baseline levels of neurotransmission. Together, our results along with other results in the literature indicate that distinct endocytic pathways may be engaged under distinct patterns of synaptic activity history, short- and long-term neuromodulation and cell type. For example, the release probability of presynaptic terminals (11), Ca$_{2+}$ and protein kinase activity may regulate the relative engagement of endocytic pathways as a function of firing history and modulation (5, 13, 16). In addition, dynamic engagement of multiple modes of recycling may be useful in homeostatic maintenance of a working amplitude and gain of synaptic transmission under different stimulation patterns. In summary, the present data characterize the fundamental modes of SV recycling and refilling of the RRP in SCG neurons during and after single or sustained firing of action potentials, supporting the involvement of activity-dependent and -independent pathways with distinct molecular requirements for synaptic vesicle endocytosis.

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