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Perturbation of synaptic vesicle delivery during neurotransmitter release triggered independently of calcium influx

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Although much evidence suggests that calcium (Ca^{2+}) usually triggers synaptic vesicle exocytosis and neurotransmitter release, the role of Ca^{2+} in vesicle endocytosis and in the delivery of fusion-competent vesicles (i.e. mobilisation and/or priming) in nerve terminals remains unclear. To address this issue, we have studied synaptic vesicle dynamics in cultured rat neurones under conditions where neurotransmitter release is triggered independently of Ca^{2+} using the secretagogue Ruthenium Red (RR). Using a prolonged stimulation protocol, we find that RR causes a rapid increase in neurotransmitter release followed by a gradually decrementing response. In contrast, when release is triggered by moderate membrane depolarisation caused by saline containing 18 mM K^+, release is sustained. These observations suggest that when release is triggered independently of a rise in Ca^{2+}, endocytosis or vesicle mobilisation/priming are perturbed. Using FM2-10, a fluorescent indicator of synaptic vesicle cycling, we find that neurotransmitter release triggered by RR is accompanied by both uptake and release of this dye, thereby suggesting that vesicle endocytosis is not blocked. To evaluate whether synaptic vesicle mobilisation/priming is perturbed in the absence of a rise in Ca^{2+}, we compared the kinetics of FM2-10 loss during prolonged stimulation. While 18 mM K^+ induced gradual and continuous dye loss, RR only induced substantial dye loss during the first minute of stimulation. In the presence of low concentrations of the Ca^{2+} ionophore ionomycin, release caused by RR was prolonged. Taken together, these results provide evidence suggesting that, although a rise in intraterminal Ca^{2+} is not required for endocytosis, it is essential for the continuous delivery of fusion-competent vesicles and to maintain neurotransmitter release during prolonged stimulation.

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Neurotransmitter release occurs through synaptic vesicle exocytosis (Couteaux, 1974; Heuser *et al.* 1974; Sudhof, 1995). The sequence of events leading to exocytosis is triggered by Ca^{2+} influx through voltage-dependent channels that are tightly associated with the secretory machinery (Smith *et al.* 1985; Kim & Catterall, 1997; Bollmann *et al.* 2000). Although the Ca^{2+} dependence of exocytosis is well known, the role of this ion in the rest of the synaptic vesicle life-cycle is less well established. Work performed in non-neuronal secretory cells has suggested that Ca^{2+} plays an active role in triggering endocytosis (Artalejo *et al.* 1995, 1996; Beutner *et al.* 2001). Endocytosis in neurones has been suggested to be inhibited by Ca^{2+} under some circumstances (von Gersdorff & Matthews, 1994; Ryan *et al.* 1996; Rouze & Schwartz, 1998; Neves & Lagnado, 1999; Cousin & Robinson, 2000; Richards *et al.* 2000), while others have provided evidence in favour of a stimulatory role (Ceccarelli & Hurlbut, 1980; Sankaranarayanan & Ryan, 2001) or a requirement only for early stages of endocytosis (Gad *et al.* 1998).

A role for Ca^{2+} at other steps of the recycling pathway has also been proposed. The importance of Ca^{2+} in the delivery of fusion-competent vesicles and granules (i.e. mobilisation/priming) is well established in non-neuronal cells (Neher & Zucker, 1993; von Ruden & Neher, 1993; Parsons *et al.* 1995; Vitale *et al.* 1995; Niwa *et al.* 1998; Smith *et al.* 1998; Gromada *et al.* 1999; Trifaro, 1999; Xu *et al.* 1999). In neurones, the picture is less clear but a number of observations argue for some role of intraterminal Ca^{2+} in accelerating the transit of synaptic vesicles from the 'reserve pool' to what is commonly referred to as the 'readily releasable pool' (RRP; Greengard *et al.* 1993; Koenig *et al.* 1993; Stevens & Wesseling, 1998; von Gersdorff *et al.* 1998; Wang & Kaczmarek, 1998; Leenders *et al.* 1999; Weis *et al.* 1999). For example, at the Calyx of Held synapse, a fast phase of recovery from depletion of the RRP evoked by high-frequency firing is blocked the Ca^{2+} chelator EGTA and enhanced by facilitating Ca^{2+} entry using the K^+ channel blocker TEA (Wang & Kaczmarek, 1998). Such recovery is also blocked

by a calmodulin inhibitor (Sakaba & Neher, 2001). Similarly, at hippocampal synapses in culture, high-frequency firing accelerates recovery of the RRP following an initial depletion evoked by hypertonic stimulation (Stevens & Wesseling, 1998).

One limitation of much previous work on the role of Ca^{2+} in endocytosis and vesicle mobilisation/priming in neurones has been the difficulty of studying these processes under conditions where exocytosis occurs but Ca^{2+} influx does not. Such a situation is provided by secretagogues such as Ruthenium Red (RR; Trudeau *et al.* 1996*a*; Sciancalepore *et al.* 1998; Bouron & Reuter, 1999; Koyama *et al.* 1999; Hutcheon *et al.* 2000) or hypertonic saline (Hubbard & Kwanbunbumpen, 1968; Rosenmund & Stevens, 1996). Although the specific molecular mechanism of neither of these two stimuli is yet determined, both have been reported to trigger quantal release independently of Ca^{2+} elevations (Rosenmund & Stevens, 1996; Trudeau *et al.* 1996*a*). It has been shown that externally applied RR triggers quantal neurotransmitter release without causing a rise in intracellular Ca^{2+} by acting on external binding sites on synaptic terminals (Trudeau *et al.* 1996*a*; Sciancalepore *et al.* 1998), presumably by stimulating exocytosis of vesicles from the RRP. Considering several advantageous properties which will be discussed hereafter, we have decided to use RR as a stimulus for the present set of experiments.

In the present study we have therefore investigated synaptic vesicle endocytosis and mobilisation/priming in cultured neurones under conditions where neurotransmitter release is triggered in a Ca^{2+}-independent manner by RR. Our results suggest that in the absence of Ca^{2+} influx, endocytosis still occurs, but the delivery of fusion-competent vesicles is blocked, leading to an impaired ability of nerve terminals to maintain a sustained level of neurotransmitter release.

METHODS

Cell culture

Postnatal (1- to 3-day-old) Sprague-Dawley rat pups were cryo-anaesthetised on ice by slowly lowering their body temperature to approximately 4 °C and then decapitated. The brain was quickly removed from the skull and the mesencephalon isolated from a coronal slice. The detailed animal handling protocol used was approved by the University of Montreal institutional animal ethics committee. Primary standard cultures of mesencephalic neurones were prepared as previously described (Bourque & Trudeau, 2000; Trudeau, 2000; Congar *et al.* 2002) and maintained in medium containing 5% fetal calf serum (Gibco, Burlington, Ontario, Canada) and Mito+ serum extender (VWR CanLab, Montreal, Québec, Canada). Neurones were used after 10–20 days in culture.

Electrophysiology

Whole-cell patch-clamp recordings were performed with a PC-501 amplifier (Warner Instruments Inc., Hamden, CT, USA). The normal external saline contained (mM): NaCl 140, $MgCl_2$ 2, $CaCl_2$ 2, KCl 5, Hepes 10, sucrose 6 and glucose 10, pH 7.35. The saline solutions containing 18 mM (18K) and 40 mM potassium (40K) were prepared by replacing equimolar amounts of NaCl with KCl. The saline solution used for recording miniature excitatory synaptic currents (mEPSCs) also contained 0.5 μM TTX to block sodium channels and 1 μM SR-95531 to block $GABA_A$ receptors. For recording mEPSCs, the patch pipettes contained (mM): Cs gluconate 117.5, NaCl 10, $MgCl_2$ 4, EGTA 5, Mg-ATP 2, Tris-GTP 0.2 and Hepes 15, pH 7.35 (adjusted with CsOH). All recordings were performed at room temperature (20–22 °C). Currents were low-pass filtered at 2 kHz and digitised at 5 or 10 kHz. The data were acquired using pClamp software (v. 8.0) from Axon Instruments (Foster City, CA, USA). Miniature synaptic currents were analysed using MiniAnalysis (Synaptosoft Inc., Leonia, NJ, USA). In experiments in which hypertonic solution was used, the solution (sucrose, 150 mosmol kg^{-1} above normal osmolality) was applied through the bath perfusion for 3 min. In the experiments with ionomycin, the drug was bath applied at a concentration of 1–50 nM for 3 min preceding the application of RR. Group data presented in the text are expressed as means ± S.E.M. Unless otherwise indicated, data were analysed using ANOVAs or non-parametric tests, as appropriate. A probability value of $P < 0.05$ was used as an indication of significant differences. All chemicals were from Sigma (St Louis, MO, USA), except FM2-10 and fura-2 (Molecular Probes, Eugene, OR, USA).

Fluorescence imaging

Imaging of FM2-10 was performed using standard epifluorescence imaging through a $GenIII^+$-intensified progressive line scan CCD camera (Stanford Photonics, Palo Alto, CA, USA) and a computer-controlled two-channel fast excitation wavelength switcher (DX-1000, Stanford Photonics). Images were acquired every 5 s and analysed using Axon Imaging Workbench software 2.2 and 4.0 (Axon Instruments). In FM2-10 imaging experiments, release was calculated from background-subtracted images. Terminals were identified by first exposing the neurones to 200 μM FM2-10 in the presence of a depolarising saline solution containing 40 mM K^+ (40K) for 60 s. After a washout period of 10 min, terminals were again stimulated with 40K saline to release the FM2-10 that was sequestered in vesicles and determine sites of activity-dependent release. The recycling pool was estimated by subtracting for each release site the amount of fluorescence remaining after the 40K release period from that before. For the Ca^{2+} imaging experiments shown in Fig. 2, individual neurones were first loaded with fura-2 pentapotassium salt (2 mM intrapipette concentration) through the patch pipette for 10 min. The internal pipette solution for these experiments contained (mM): KCl 150 and Hepes 10, pH 7.35 (adjusted with KOH). Following removal of the patch pipette, a recovery period of 30 min in the presence of 0.5 μM TTX was allowed before beginning image acquisition. Fura-2 images were acquired every 5 s at the 380 nm excitation wavelength immediately before FM2-10 images (490 nm excitation) using a two-channel galvanometric excitation source. The measurement of fura-2 signal at sites which displayed activity-dependent uptake and release of FM2-10 permitted the measurement of relative variations in intracellular Ca^{2+} at sites which were presumed synaptic boutons. In the Ca^{2+} imaging experiments described in Fig. 6*B*, cells were loaded with fura-2 AM for 30–40 min. Standard image ratio pairs at 340 and 380 nm were acquired every 5 s and ratio values were converted to Ca^{2+} concentrations using Grynkiewicz's equation (Grynkiewicz *et al.* 1985) and an *in situ* calibration protocol to determine R_{max} and R_{min} values.

RESULTS

Use of Ruthenium Red to trigger calcium-independent quantal transmitter release

As previously described (Trudeau *et al.* 1996*a*), extracellular application of RR (40 μM) caused a robust elevation of the frequency of mEPSCs recorded under the whole-cell mode from cultured mesencephalic neurones (Fig. 1*A*). On average, basal mEPSC frequency rose from 7.0 ± 0.8 to 21.2 ± 2.8 Hz (n = 10) at the peak of the effect. The ability of RR to increase mEPSC frequency was expressed as a difference score (Δ) obtained by subtracting the number of mEPSCs within a 30 s baseline period from that during the stimulation, using 30 s time bins. Although we have previously shown that during short (30 s) applications of RR the frequency of mEPSCs rapidly stabilizes to a plateau within a few seconds (Trudeau *et al.* 1996*a*), we found in the present set of experiments that during a prolonged application of RR (10 min), the frequency of mEPSCs abruptly rises to a maximum within 30 s and then rapidly decreases to a low level within 3–4 min (n = 10; Fig. 1*B*). On average, RR-evoked release peaked at 390.0 ± 72.8 mEPSCs above baseline and rapidly decreased to 21.1 ± 7.1 % of this peak value after 3 min (n = 10).

Considering that RR is presumed to stimulate release from the RRP of vesicles and that intracellular Ca^{2+} is hypothesised to be necessary to mobilise new vesicles from the reserve pool, one potential explanation for the decline in RR-evoked release over time is that the RRP becomes depleted because it is not replenished. In order to provide a first test of this hypothesis, we stimulated synaptic terminals using mild membrane depolarisation caused by saline containing 18 mM K^+ (18K). We hypothesized that because such a stimulus evokes Ca^{2+} influx, it should cause vesicle mobilisation and thus should not lead to a decrementing response such as that evoked by RR. As expected, 18K saline caused an enhancement of mEPSC frequency (Fig. 1*A*). The enhancement peaked at a Δ value of 660.9 ± 88.6 mEPSCs above baseline (n = 9). Moreover, as predicted, the time course of this response was very different from that observed with RR (n = 9) (Fig. 1*B*). The enhancement caused by 18K saline was relatively stable over time, remaining at 96.8 ± 10.4 % of the peak value after 3 min (n = 9). Overall, the total number of mEPSCs (Δs) evoked by 18K saline during the first 5 min of treatment was approximately 3.3-fold larger than the number induced by RR.

In these experiments, the amplitude of mEPSCs was not significantly changed by RR, as previously reported (Trudeau *et al.* 1996*a*; Sciancalepore *et al.* 1998). Over a 10 min period, only a small, gradual and nonsignificant

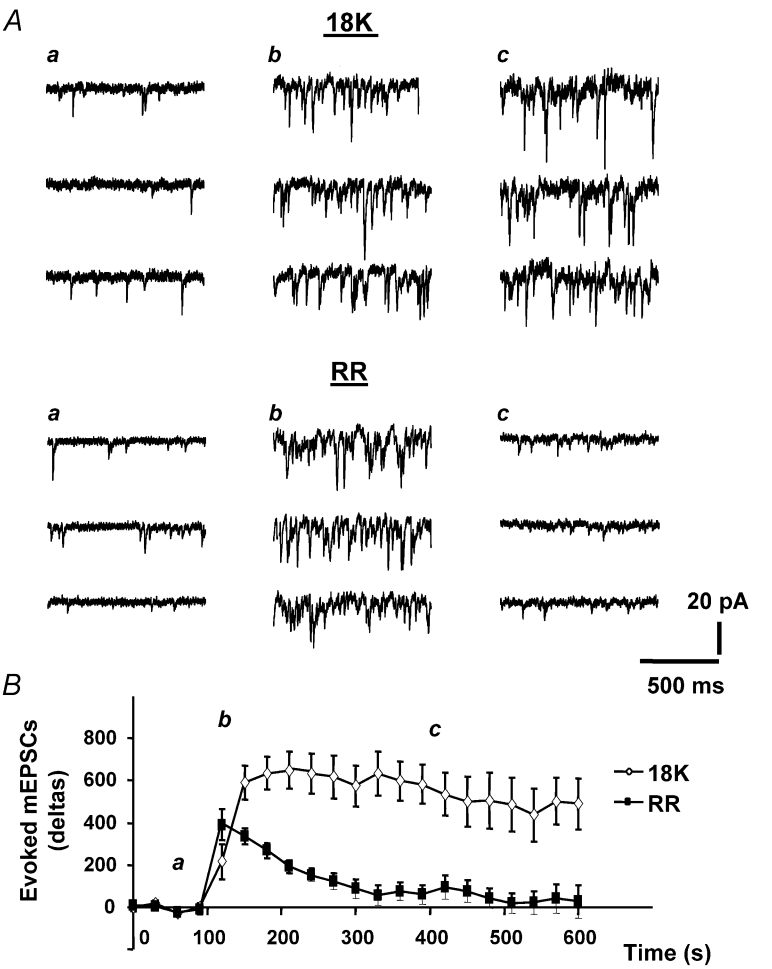

Figure 1. Differential efficacy of RR and depolarisation to evoke mEPSCs during prolonged stimulation

A, neurones were voltage clamped at −60 mV and mEPSCs recorded in the presence of SR-95531 (1 μM) to block $GABA_A$ receptors and 0.5 μM TTX (*a*). Perfusion of saline containing 18 mM K^+ (18K) induced an increase in the frequency of mEPSCs which was comparable to that induced by perfusing 40 μM RR when measured after 1 min of application (*b*). After 5 min of application (*c*), the increase in mEPSC frequency remained substantial with 18K saline but declined to a low rate in the presence of RR. *B*, summary graph comparing the ability of 18K saline and RR to increase the frequency of mEPSCs. After a baseline period of 2 min, 18K saline (18K) or RR (40 μM) were perfused continuously for a period of 10 min. The occurrence of mEPSCs is expressed in 30 s bins as a difference score (Δ) obtained by subtracting the number of mEPSCs within a 30 s baseline period from that during the stimulation. Note that, unlike RR, 18K saline produced a relatively stable increase in mEPSC frequency.

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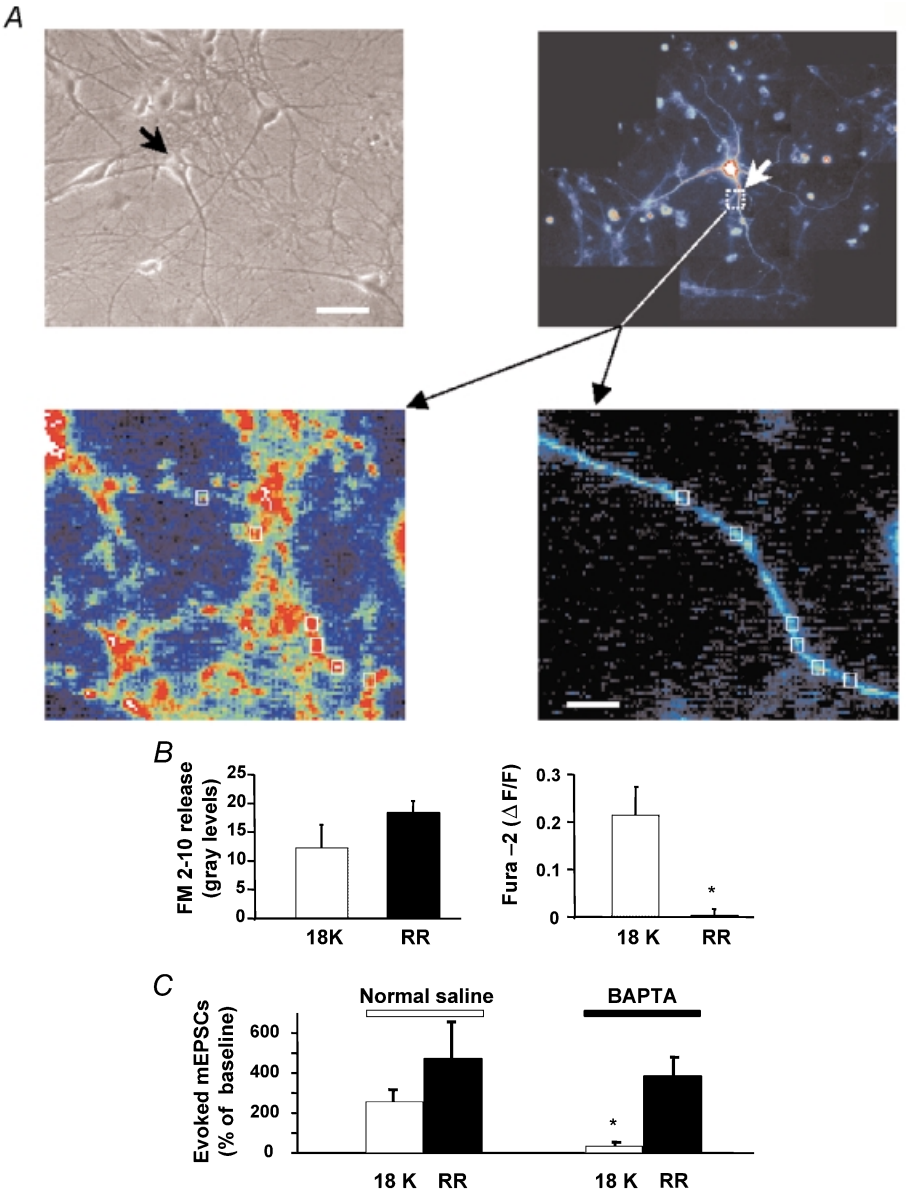

Figure 2. RR triggers calcium-independent quantal transmitter release

A, phase contrast image of a field (upper left image) showing multiple neuronal processes and a small number of neurones(scale bar 40 μm; scale bar also applies to the upper right image). A single neurone (arrow) was filled with fura-2 through a patch pipette (upper right image). The lower images represent a magnification of the section of the upper right image indicated by the dotted white box. Active synaptic boutons were identified by activity-dependent loading with the fluorescent indicator of synaptic vesicle recycling FM2-10 (lower left image) using a 1 min exposure to 40K saline and FM2-10 (200 μM). Fluorescence was measured at synaptic boutons (white boxes, lower images) localised along the fura-2-loaded axon (lower right image; scale bar 4 μm; scale bar also applies to the lower left image). *B*, summary histograms comparing the ability of 18K and RR to trigger FM2-10 release (left graph) and fura-2 fluorescence variations (right graph) in individual FM2-10-loaded synaptic boutons. Variation in exocytosis and intracellular Ca^{2+} were simultaneously monitored by alternately exciting fura-2 and FM2-10. The left histogram represents the average FM2-10 release (grey levels) evoked by 18K or RR, calculated by subtracting the signal intensity after stimulation from before, for each individual bouton. The right histogram represents the average non-ratiometric fura-2 signal change ($\Delta F/F$) upon 18K or RR stimulation. Note that although both 18K and RR evoked FM2-10 release, only 18K caused a rise in intracellular Ca^{2+} in FM2-10-labelled boutons. * $P < 0.05$ (in comparison to the 18K stimulation). *C*, summary histogram comparing the ability of 18K saline and RR to increase the frequency of mEPSCs in control conditions (normal saline) and after loading with BAPTA-AM (BAPTA). The occurrence of mEPSCs at the peak of the stimulatory effect is expressed as a percentage of the baseline period of individual experiments. Note that, unlike 18K, the ability of RR to evoke mEPSCs was completely unaffected by BAPTA-loading of neurones. * $P < 0.05$ (in comparison to the normal saline group).

rundown of mEPSC amplitude was noted (not shown; one-way ANOVA, $F_{(14)} = 0.98$, $P = 0.48$).

Intracellular calcium measurements at synaptic boutons

To confirm that the differences in release time course between RR and 18K are related to their differential ability to raise intraterminal Ca^{2+}, we filled single neurones with fura-2 (pentapotassium salt) through the patch pipette to measure intracellular Ca^{2+} levels. Neurones were filled for 10 min, followed by a 30 min rest period. To estimate variations in intracellular Ca^{2+} levels at synaptic boutons, we performed measurements from sites along the axon that were labelled with the fluorescent indicator of synaptic vesicle recycling FM2-10. This and related fluorescent styrylpyridinium dyes have been shown to be accumulated by recycling synaptic vesicles in an activity-dependent manner (Betz & Bewick, 1992; Ryan *et al.* 1993; Henkel *et al.* 1996*a*; Klingauf *et al.* 1998).

Fura-2-loaded neurones were depolarised for 60 s by saline containing 40 mM K^+ (40K) in the presence of 200 μM FM2-10 to effectively label the recycling pool in synaptic boutons. This was followed by a 10 min wash to remove extracellular FM2-10. This labelling protocol permitted the identification of active terminals at sites along the fura-2-loaded axon of the labelled neurones (Fig. 2*A*). The axon was clearly distinguishable from dendrites because of its length and uniform calibre. Variation in intracellular Ca^{2+} and exocytosis were simultaneously monitored by alternately exciting fura-2 and FM2-10 (see Methods). We found that synaptic boutons identified in this way demonstrated FM2-10 release when exposed to RR or to 18K for 3 min (Fig. 2*B*). Although both 18K and RR evoked FM2-10 release (see also Fig. 4*D*), only 18K caused a detectable rise in intracellular Ca^{2+} at FM2-10-labelled boutons (Fig. 2*B*).

Although our fura-2 measurements allow us to exclude the possibility that RR causes a rise in bulk intracellular Ca^{2+}, they cannot exclude the possibility that small highly localized rises could occur near release sites. To evaluate this, we loaded neurones with BAPTA-AM, a Ca^{2+} chelator with high affinity and rapid binding kinetics. After loading, a neurone was patch clamped to record mEPSC frequency. We found that in control neurones not loaded with BAPTA, both 18K and RR caused a rise in mEPSC frequency, as expected (Fig. 2*C*). However, while 18K-evoked enhancement of mEPSC frequency was almost completely blocked in BAPTA-loaded neurones ($P < 0.05$), RR-evoked release was completely unaffected (Fig. 2*C*; $P > 0.05$).

Implication of the readily releasable pool

An interpretation of the decrementing time course of RR-evoked release as a partial depletion of the RRP depends on the fact that RR only causes release from this

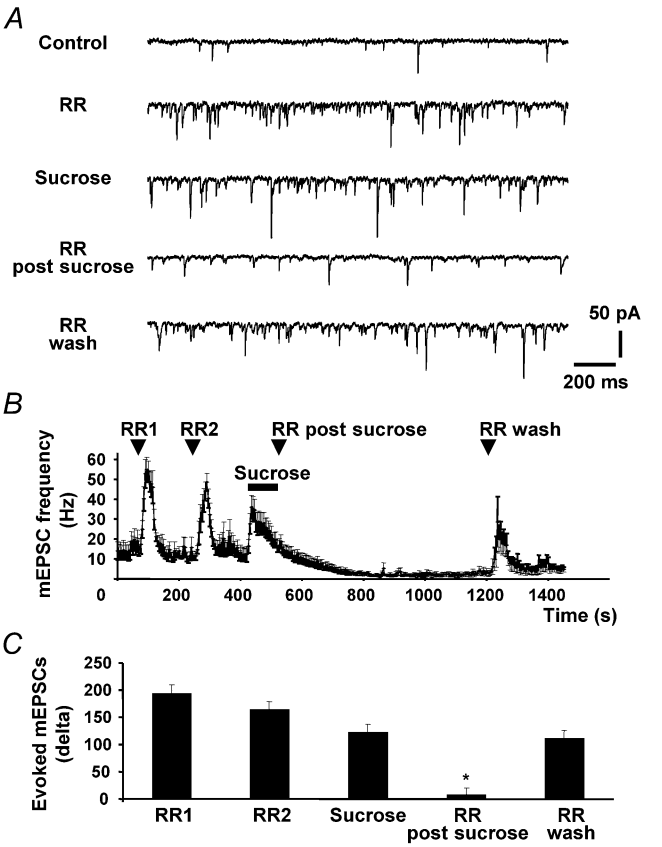

Figure 3. RR-evoked exocytosis is occluded by stimulation of synaptic terminals with hypertonic saline

A, neurones were voltage clamped at −60 mV and mEPSCs recorded in the presence of SR-95531 (1 μM) to block $GABA_A$ receptors and 0.5 μM TTX (control). A brief bath application of RR (40 μM, 30 s) evoked a reversible and reproducible increase in the frequency of mEPSCs (RR). Upon return of the mEPSC frequency to near-basal level following the second application of RR, hypertonic saline (Sucrose, 150 mosmol kg^{-1} above normal osmolality) was bath applied for 3 min, and a subsequent application of RR (RR post sucrose) was made at the end of the sucrose application. Note that the RR-evoked increase in mEPSC frequency was occluded by the sucrose application. When possible, a fourth application of RR (RR wash) was made following a 10 min wash. This evoked a reversible increase in the frequency of mEPSCs, comparable to the control response (RR). Note that following washout of hyperosmotic saline, the basal frequency of mEPSCs has a tendency to decrease below initial levels. *B*, average time course of 5 experiments such as illustrated in *A*, comparing the ability of brief bath applications of RR to increase the frequency of mEPSCs before (RR1, RR2) and after (RR post sucrose) bath application of hypertonic saline (sucrose). The frequency of mEPSCs (Hz) is represented in 5 s bins. *C*, summary histogram illustrating the average effect of RR in control conditions (RR1, RR2), following bath application of hypertonic saline (RR post sucrose) and after a washout period (RR wash). For each stimulation, the ability of RR to increase mEPSC frequency was expressed as a difference score (Δ) obtained by subtracting the number of mEPSCs within a 15 s baseline period from that during the stimulation, at the peak of the effect. Note that stimulation with hypertonic saline occluded the effect of RR. * $P < 0.05$ (in comparison to RR1).

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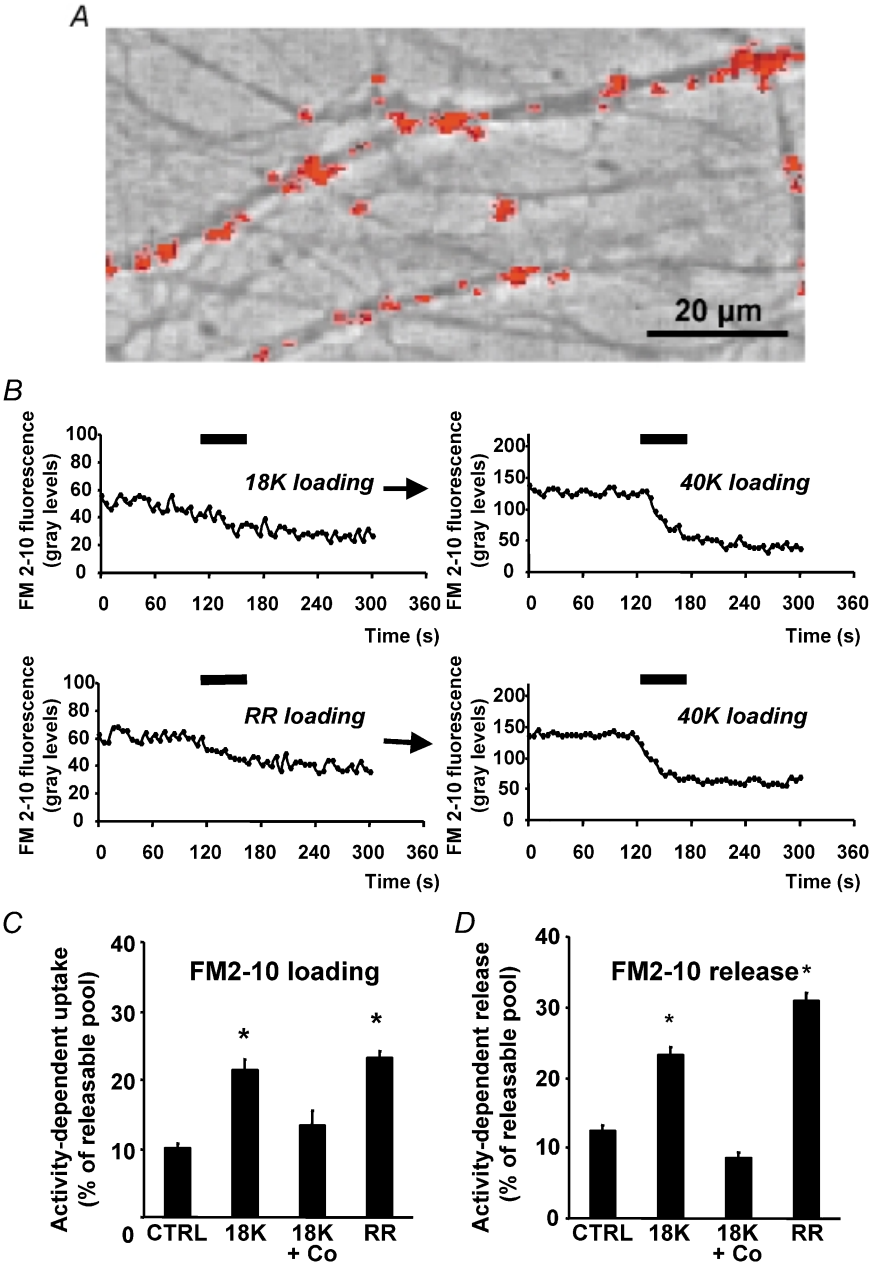

Figure 4. RR stimulates both uptake and release of FM2-10

A, overlay image showing sites of FM2-10 uptake (red spots) superimposed on the phase contrast image of a field containing multiple neuronal processes. Note that all sites of activity-dependent FM2-10 uptake were localised to areas which contained visually identifiable nerve processes. *B*, graphs representing the background-subtracted fluorescence intensity (in grey levels) of individual FM2-10-loaded boutons. The left graphs show the response to a 1 min exposure to 40K saline (filled bar) of boutons loaded by a 3 min exposure to RR (40 μM) or 18K together with 200 μM FM2-10. The right graphs show the response of the same boutons after a second round of FM2-10 loading using 40K saline and FM2-10. Stimulation with 40K saline caused more substantial release of FM2-10. *C*, summary histogram illustrating the quantification of FM2-10 loading experiments. The bars represent the average uptake of FM2-10 after different loading protocols (control saline, 18K saline, 18K saline plus 1 mM cobalt, RR). The uptake was calculated by subtracting the signal intensity after 40K stimulation from before, for each individual bouton. This uptake was expressed as a percentage of the releasable pool, which was estimated following a subsequent loading–unloading cycle using 40K. Stimulation of cells with 18K saline for 3 min in the presence of FM2-10 caused detectable FM2-10 uptake (18K). This effect was blocked by the Ca^{2+} channel blocker cobalt (1 mM; 18K + Co). Stimulation of nerve terminals with RR in the presence of FM2-10 (RR) caused an activity-dependent uptake of FM2-10 beyond that produced by exposing cells to FM2-10 alone for 3 min (CTRL). * $P < 0.05$ (in comparison to the CTRL group). *D*, summary histogram illustrating the quantification of FM2-10 release experiments. The bars represent the average release of FM2-10 from preloaded boutons

pool and not from the reserve pool. Although this is something difficult to demonstrate conclusively, a functional definition of the RRP has been proposed based on the observation that hypertonic saline depletes the pool of vesicles normally released by low-frequency action potential firing (Rosenmund & Stevens, 1996). Considering that RR blocks voltage-dependent Ca^{2+} channels, it was not possible here to determine whether RR-evoked release depletes the pool of vesicles released by action potentials. However, since hypertonic saline depletes the RRP, we predicted that following exposure of terminals to hypertonic saline, RR should fail to cause additional release of neurotransmitter.

In these experiments, a neurone was patch clamped to record mEPSCs in the presence of TTX. For each stimulus, the ability of RR and/or hypertonic solution to increase mEPSC frequency was expressed as a difference score (Δ) obtained by subtracting the number of mEPSCs within a 15 s baseline period from that during the stimulation, at the peak of the effect. The number of mEPSCs detected under basal conditions was estimated as an average of the number of mEPSCs within two 15 s periods taken before and after the stimulation, to account for any gradual rundown in the frequency of mEPSCs. After a 1 min period of control recording, a first 30 s application of RR (40 μM) was performed, which evoked a robust and fully reversible elevation of the frequency of mEPSCs (Fig. 3*A*). The net effect of each additional stimulation was expressed as a percentage of the Δ of the first application of RR. On average, the response to a second stimulation with RR, performed after 3 min, reached 86.0 ± 2.2 % of the first response (n = 5; Fig. 3*B* and *C*), confirming the absence of significant synaptic fatigue under such conditions, as previously reported (Trudeau *et al.* 1996*a*; Sciancalepore *et al.* 1998). Hypertonic saline (150 mosmol kg^{-1} above normal osmolality) was then perfused for 3 min and evoked a reversible increase in the frequency of mEPSCs to 58.0 ± 5.3 % of the first response to RR (n = 5; Fig. 3*A*, *B* and *C*). As previously reported, hypertonic saline-evoked release was short lasting. Upon return of the mEPSC frequency to near-basal level, RR (40 μM) was applied for a third time for 30 s. A complete block of RR-evoked release was observed (2.6 ± 3.6 % of the first response to RR, $P < 0.01$, n = 5; Fig. 3*A*, *B* and *C*). In three out of the five neurones, a fourth application of RR was performed following a 10 min wash. This induced an increase in mEPSC frequency, reaching 57.8 ± 6.7 % of the first response to RR. These results confirm that RR causes release from the RRP of synaptic vesicles.

FM2-10 imaging of synaptic vesicle endocytosis

Considering the possibility that some aspect of synaptic vesicle endocytosis may be Ca^{2+} dependent (Sankaranarayanan & Ryan, 2001), one possible reason for the gradual decrement of RR-evoked release is that in the absence of Ca^{2+} influx, synaptic vesicles can undergo exocytosis in response to RR, but endocytosis is prevented, leading to exhaustion of the RRP of vesicles. An alternative hypothesis is that endocytosis still occurs locally but the delivery of new fusion-competent vesicles to the active zone (mobilisation/priming) is blocked. To test the first hypothesis, we combined the use of RR with the imaging of FM2-10 uptake to monitor synaptic vesicle endocytosis.

To determine whether RR leads to staining of synaptic boutons with FM2-10, cultures were exposed to FM2-10 (200 μM) for 3 min in the presence of RR (40 μM), 18K saline or normal saline. This was followed by a 10 min wash, after which 40K saline was applied for 1 min to depolarise the neurones strongly and release the dye sequestered in synaptic vesicles in an activity-dependent manner. The preparation was then reloaded with FM2-10 using 40K saline as a stimulus (followed by a final 40K stimulation) in order to localise most synaptic boutons in the field of view (Fig. 4*A*). Tetrodotoxin (TTX; 0.5 μM) was present throughout the experiments to limit dye loss caused by spontaneous action potentials. The results were analysed by randomly selecting small punctate structures (presumed synaptic boutons) that showed clearly detectable activity-dependent uptake and release of FM2-10 using the 40K loading–unloading protocol. The ability of 18K saline or RR to induce activity-dependent uptake of FM2-10 in the first part of the protocol was then examined in retrospect in these same structures by measuring the loss of fluorescence caused by 40K saline after loading with RR or 18K saline. The labelling was expressed as a fraction of the recycling pool estimated by the maximum amount of fluorescence loss evoked during the 40K loading–unloading protocol.

We found that 18K saline reliably induced uptake of FM2-10 in synaptic boutons (Fig. 4*B*). The amount of uptake corresponded to 21.5 ± 1.4 % of the recycling pool estimated using the 40K saline (n = 231 boutons in

using different stimulation protocols (control saline, 18K saline, 18K saline plus 1 mM cobalt, RR). The release was calculated by subtracting the signal intensity after the stimulation from before, for each individual bouton. This release was expressed as a percentage of the releasable pool, which was estimated following a subsequent loading–unloading cycle using 40K. After initial loading of FM2-10 using 40K saline, stimulation of cells with 18K saline caused detectable activity-dependent release of FM2-10 (18K). This effect was blocked by the Ca^{2+} channel blocker cobalt (1 mM; 18K + Co). Stimulation of nerve terminals with RR (RR) also caused detectable activity-dependent release of FM2-10, beyond that produced by exposing terminals to normal saline alone for 3 min (CTRL). * $P < 0.05$ (in comparison to the CTRL group).

11 coverslips). As expected, the effect of 18K saline was dependent on Ca^{2+} influx, since its ability to cause FM2-10 uptake was blocked by 1 mM cobalt (Fig. 4*C*) ($n = 105$ boutons in 5 coverslips), a broad-spectrum blocker of voltage-dependent Ca^{2+} channels. Similar to 18K saline, RR also reliably caused detectable FM2-10 uptake at a level above that caused by normal saline alone (10.0 ± 0.7 % of the estimated recycling pool, $n = 92$ boutons in 5 coverslips). The uptake evoked by RR corresponded to 23.3 ± 0.9 % of the estimated recycling pool ($n = 51$ boutons in 3 coverslips; Fig. 4*B* and *C*). Group means were compared using a one-way ANOVA ($F_{(2)} = 16.9$, $P < 0.01$). Both 18K and RR caused significantly more uptake of FM2-10 than the control treatment (Tukey test, $P < 0.01$).

A second set of experiments was performed to obtain a similar quantification of FM2-10 release (instead of uptake) evoked by 18K and RR. In these experiments, synaptic boutons were first loaded with FM2-10 by depolarisation with 40K saline in the presence of 200 μM FM2-10. After a 10 min wash, FM2-10 release was evoked by stimulating neurones with 18K saline or 40 μM RR in the presence of 0.5 μM TTX for a period of 3 min. As expected from the results shown in Fig. 2*B*, both 18K and RR caused a clear release of FM2-10 (Fig. 4*D*). The release caused by 18K amounted to 23.2 ± 1.1 % of the estimated recycling pool ($n = 309$ boutons in 16 coverslips) and was blocked by 1 mM cobalt ($n = 105$ boutons in 5 coverslips) and thus dependent on the activation of voltage-dependent Ca^{2+} channels. The release evoked by RR amounted to 30.9 ± 1.2 % of the estimated recycling pool ($n = 218$ boutons in 11 coverslips). In control, non-stimulated boutons, a decrease in signal corresponding to 12.5 ± 0.7 % of the estimated recycling pool was observed ($n = 200$ boutons in 10 coverslips) and probably corresponded to a mixture of spontaneous release and photo-bleaching. Group means were compared using a one-way ANOVA ($F_{(2)} = 78.9$, $P < 0.01$). Both the 18K and RR caused significantly more release of FM2-10 than the control treatment (Tukey test, $P < 0.01$). Taken together, these results show that RR causes both uptake and release of FM2-10 from synaptic vesicles, thus arguing against an obvious impairment of endocytosis.

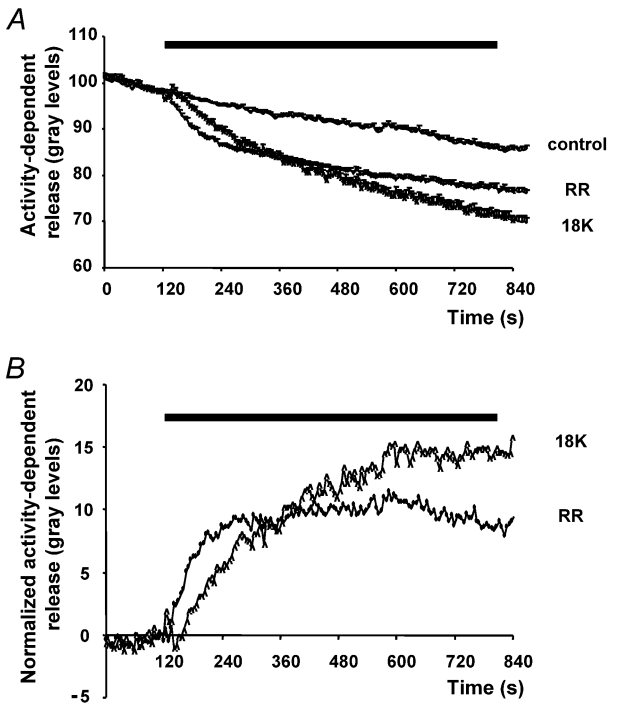

Figure 5. Time course of FM2-10 release

A, summary graph illustrating the average time course of FM2-10 release (grey levels) evoked by a 10 min stimulation with 18K saline (18K) or RR (40 μM; RR). In all experiments, nerve terminals were loaded with FM2-10 using a 1 min stimulation with 40K saline in the presence of 200 μM FM2-10. After a 2 min baseline period, continuous stimulation with 18K saline produced a gradual loss of FM2-10 signal from presumed synaptic boutons. A 10 min stimulation with 40 μM RR caused a short-lasting decrease in signal which reached a plateau after 1–2 min. The time course of spontaneous dye loss in the absence of stimulation is represented by the upper trace (control). Individual data points represent means ± S.E.M. *B*, activity-dependent release of FM2-10 evoked by a 10 min stimulation with 18K saline (18K) or RR (40 μM; RR), normalized by subtracting the corresponding spontaneous release (control in *A*). This graph illustrates that FM2-10 release evoked by RR reached a plateau after approximately 2 min of stimulation. In comparison, 18K evoked continuous dye loss.

Perturbation of the delivery of fusion-competent synaptic vesicles

The gradual decrease in RR-evoked mEPSC frequency shown in Fig. 1 could have resulted from an impairment in synaptic vesicle mobilisation and/or priming due to the absence of Ca^{2+} influx. As an additional test of this possibility, we performed a series of FM2-10 imaging experiments to determine the time course of FM2-10 release at individual synaptic boutons during prolonged stimulation. We predicted that if synaptic vesicle mobilisation and/or priming is slowed down or blocked, then FM2-10 release at preloaded boutons should rapidly cease during stimulation with RR, while during stimulation with 18K it should proceed continuously and gradually. Terminals were first loaded with FM2-10 (200 μM) using a 60 s exposure to 40K saline. After a 10 min wash, neurones were continuously exposed to RR (40 μM) or 18K saline for 10 min. A control set of experiments was performed without any stimulation to monitor the rate of spontaneous release and photo-bleaching ($n = 125$ boutons in 6 coverslips; Fig. 5*A*). We found that, unlike 18K saline, which caused a continuous and gradual release of FM2-10 ($n = 162$ boutons in 8 coverslips; Fig. 5*A*), RR caused detectable release only during the first 2–3 min of the 10 min stimulation ($n = 289$ boutons in 14 coverslips; Fig. 5*A*). After that time, the rate of fluorescence loss closely followed that observed in control, nonstimulated boutons. The time course of FM2-10 release was better visualised by normalising the

RR-evoked and 18K-evoked FM2-10 release to the rate of dye loss in the control group (Fig. 5*B*). This plot clearly illustrates that, unlike 18K saline, RR only caused significant amounts of FM2-10 release for the first 2 min at most, followed by a near-complete arrest of dye loss (Fig. 5*B*).

Prolongation of RR-evoked release by ionomycin

If the decrement in the occurrence of RR-evoked mEPSCs over time is due to a block or a slowing of synaptic vesicle mobilisation and/or priming due to the lack of intra-terminal Ca^{2+} elevation, then one would predict that, under conditions which cause a rise in intracellular Ca^{2+}, the release-activating action of RR should be enhanced and prolonged. To test this hypothesis, we used the Ca^{2+} ionophore ionomycin. Although often used at micromolar concentrations to directly evoke neurotransmitter release in various preparations (Capogna *et al.* 1996; Cousin & Robinson, 2000; Congar *et al.* 2002), we found that in the nanomolar range, ionomycin caused modest increases in intracellular Ca^{2+} and significantly prolonged the time course of RR-evoked release visualised by both mEPSCs recording and FM2-10 imaging.

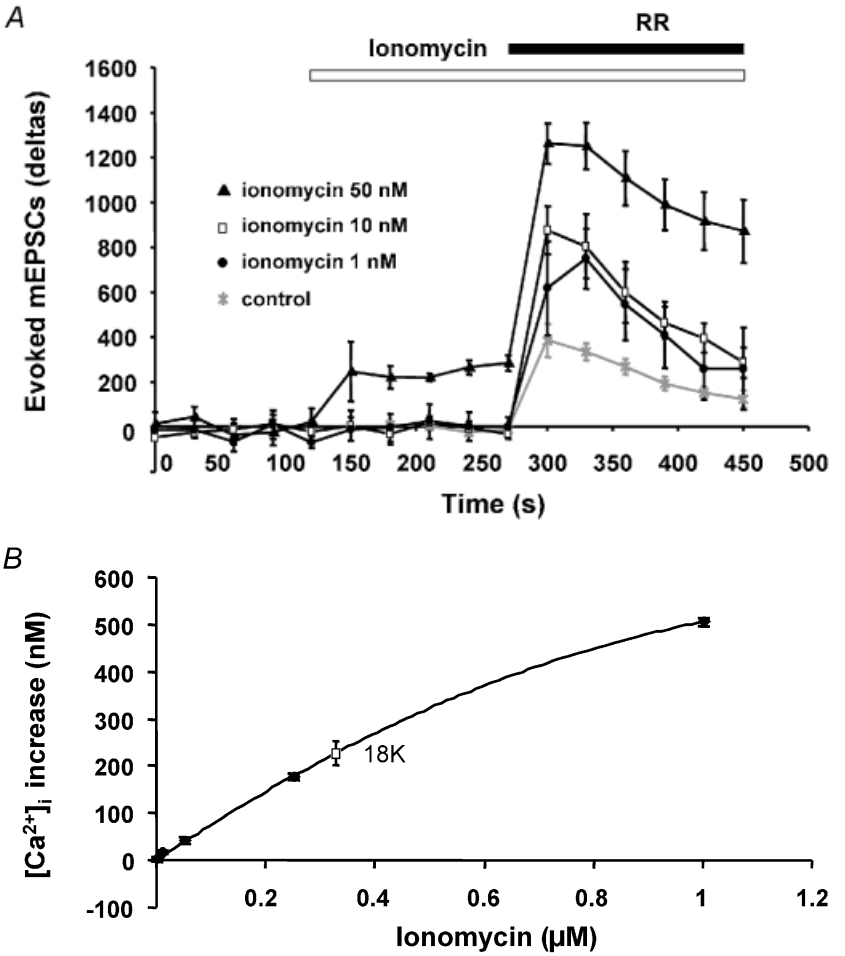

Figure 6. The Ca^{2+} ionophore ionomycin prolongs the time course of RR-evoked increase in mEPSC frequency

A, summary graph comparing the ability of different concentrations of ionomycin to increase and prolong the enhancement in mEPSC frequency induced by RR. Neurones were voltage clamped at −60 mV and mEPSCs recorded in the presence of SR-95531 (1 μM) to block $GABA_A$ receptors and 0.5 μM TTX. After a baseline period of 2 min, ionomycin was perfused at concentrations of 1, 10 or 50 nM. After 3 min of ionomycin, RR (40 μM) was applied for 3 min in the continued presence of ionomycin. The occurrence of mEPSCs is expressed in 30 s bins as a difference score (Δ) obtained by subtracting the number of mEPSCs within a 30 s baseline period from that during the stimulation. Note that RR produced a significantly larger and longer lasting increase in mEPSC frequency in the presence of 50 nM ionomycin. *B*, summary graph illustrating the quantification of the intracellular Ca^{2+} concentration elevations induced by increasing concentrations of ionomycin. Neurones were loaded with fura-2 AM and exposed to various concentrations of ionomycin. Ratiometric measurements were made from cell bodies and major dendritic processes of fura-2-loaded neurones. Note that although no detectable increase was observed at 1 nM, ionomycin caused a near-linear increase in intracellular Ca^{2+} concentration at 10, 50, 250 and 1000 nM. For comparison, the increase in $[Ca^{2+}]_i$ evoked by 18K saline is presented (□; 229 ± 31 nM).

In a first set of experiments, mEPSCs were recorded from neurones in the presence of TTX (0.5 μM). For each condition, the ability of RR to increase mEPSC frequency was expressed as a difference score (Δ) obtained by subtracting the number of mEPSCs within a 30 s baseline period from that during the stimulation, using 30 s time bins. Ionomycin was perfused at concentrations of 1, 10 and 50 nM, 3 min before application of RR. Although it had no significant effect on the basal frequency of mEPSCs at 1 and 10 nM, ionomycin caused by itself a significant rise in mEPSC frequency in a subset of cells when used at 50 nM. This represented a threshold concentration because at 100 nM or above, ionomycin induced large enhancements of mEPSC frequency which ultimately compromised the detection of the effect of RR (not shown). Further experiments were therefore performed with 50 nM ionomycin and data were only analysed from experiments in which ionomycin induced more than a 50 % increase in mEPSC frequency. The enhancement of mEPSC frequency induced by ionomycin (50 nM) stabilised to a plateau level of 265.9 ± 49 % of baseline by 3 min ($n = 7$; Fig. 6*A*). After 3 min in ionomycin, RR (40 μM) was perfused for 3 min in the continued presence of the Ca^{2+} ionophore. While all concentrations of ionomycin gradually increased the RR-evoked enhancement in mEPSC frequency, the duration of this enhancement

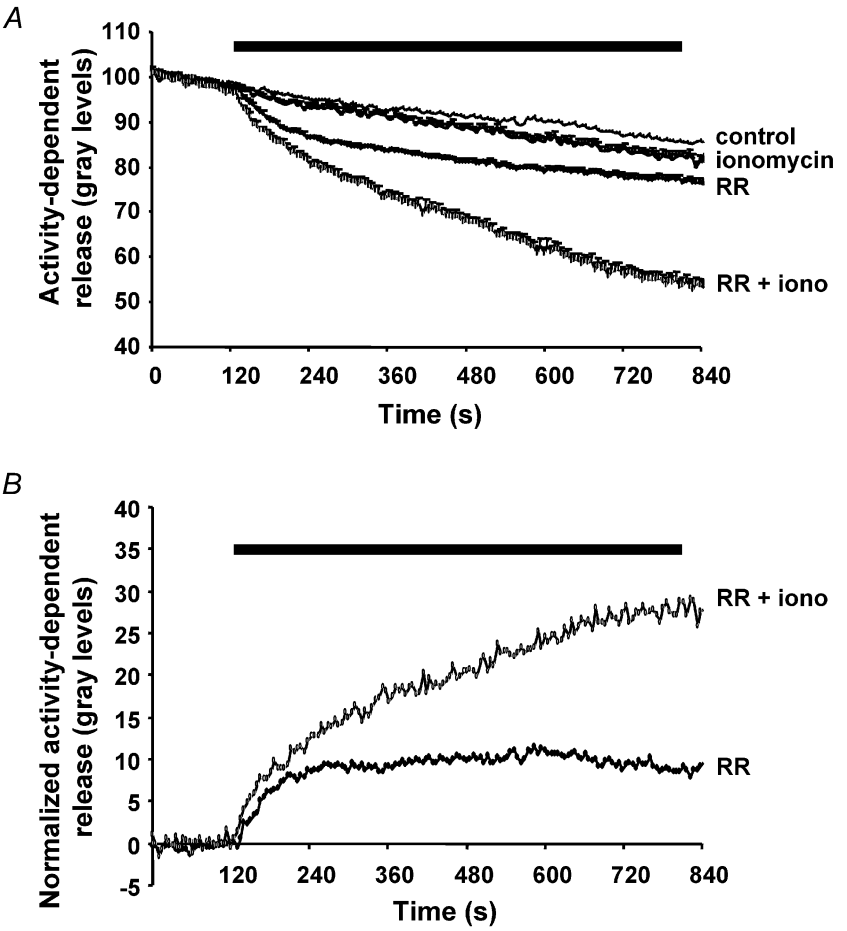

Figure 7. The Ca^{2+} ionophore ionomycin significantly prolongs the time course of RR-evoked FM2-10 release

A, summary graph illustrating the average time course of FM2-10 release (grey levels) evoked by a 10 min stimulation with RR (40 μM; RR), ionomycin (50 nM) or RR + ionomycin (RR + iono). In all experiments, nerve terminals were loaded with FM2-10 using a 1 min stimulation with 40K saline in the presence of 200 μM FM2-10. After a 2 min baseline period, a 10 min stimulation with 40 μM RR caused a short-lasting decrease in signal which reached a plateau after 1–2 min. While ionomycin alone caused only a modest and gradual release of FM2-10, stimulation with RR + ionomycin produced a more substantial and gradual loss of FM2-10 signal. The time course of spontaneous dye loss in the absence of stimulation is represented by the top trace (control). Individual data points represent means ± S.E.M. *B*, activity-dependent release evoked by a 10 min stimulation with RR (40 μM; RR) or RR + ionomycin (50 nM; RR + iono), normalised by subtracting the corresponding spontaneous release (control and ionomycin in *A*) for each condition. This graph illustrates that FM2-10 release evoked by RR alone reached a plateau after approximately 2 min of stimulation. In contrast, in the presence of ionomycin (RR + iono), RR evoked more substantial and near-continuous dye loss.

was prolonged only by 50 nM ionomycin when compared to the control condition (i.e. in the absence of ionomycin). The enhancement of RR-evoked release peaked at 748.9 ± 101.9 mEPSCs at 1 nM ionomycin ($n = 5$, $P < 0.01$), 876.0 ± 106.9 mEPSCs at 10 nM ionomycin ($n = 5$, $P < 0.01$) and 1063.1 ± 89.3 mEPSCs at 50 nM ionomycin ($n = 7$, $P < 0.01$), respectively, all significantly larger than under control conditions (Fig. 6*A*). However, after 3 min of application of RR in the presence of 1 and 10 nM ionomycin, the mEPSC frequency had returned to a level not significantly different from the control condition in the absence of ionomycin (at 23.4 ± 9.8 % ($n = 5$, $P > 0.05$) and 31.2 ± 6.2 % ($n = 5$, $P > 0.05$) of the peak value, respectively). In contrast, in the presence of 50 nM ionomycin, the RR-evoked response remained at 58.1 ± 10.1 % of the peak ($n = 7$, $P < 0.05$; Fig. 6*A*).

To gain insight into the range of intracellular Ca^{2+} concentration increases evoked by ionomycin under these conditions, neurones were loaded with fura-2 AM and exposed to various concentrations of ionomycin. Ratiometric measurements were made from cell bodies and major dendritic processes of fura-2-loaded neurones. At 1 nM, no detectable increase was observed. However, at 10, 50, 250 and 1000 nM, ionomycin caused a near-linear increase in intracellular Ca^{2+} (Fig. 6*B*). At 10 nM the average increase in Ca^{2+} was 17 ± 3 nM above resting level ($n = 9$), while at 50 nM it was 42 ± 8 nM ($n = 13$). Although these measurements are likely to provide an underestimate considering the lower surface-to-volume ratio of terminals relative to cell bodies, they suggest that increases in intracellular Ca^{2+} of more than 40 nM are likely to be required to facilitate synaptic vesicle mobilisation or priming. For comparison, we also determined the rise in intracellular Ca^{2+} caused by 18K saline, which causes non-decrementing increases in mEPSC frequency (Fig. 1) and continuous mobilisation of vesicles (Fig. 5*A* and *B*). We found that on average, 18K saline caused an increase in intracellular Ca^{2+} of 229 ± 31 nM ($n = 16$ neurones).

Complementary FM2-10 release experiments were performed with ionomycin to confirm the electrophysiological findings. We predicted that if ionomycin-evoked Ca^{2+} elevations enhance vesicle mobilisation, the time course of FM2-10 release evoked by RR (40 μM) should be prolonged in the presence of ionomycin. Terminals were first loaded with FM2-10 (200 μM) using a 60 s exposure to 40K saline. After a 10 min wash, neurones were exposed to ionomycin (50 nM) for 3 min, then continuously exposed to RR (40 μM) plus ionomycin (50 nM) for 10 min. A control set of experiments was performed without any RR to monitor the rate of spontaneous release and photo-bleaching in the continuous presence of ionomycin ($n = 63$ boutons in 3 coverslips). We found that in the presence of ionomycin, RR caused continuous and gradual release of FM2-10 ($n = 126$ boutons in 6 coverslips; Fig. 7*A*). The normalized time course of FM2-10 release (Fig. 7*B*) illustrates that in the presence of ionomycin, RR indeed caused continuous and gradual release of FM2-10, much like that induced by 18K saline (Fig. 5*A* and *B*).

DISCUSSION

We have found that during prolonged stimulation of synaptic terminals with the Ca^{2+}-independent secretagogue RR, the frequency of mEPSCs rises abruptly and then rapidly falls off over time, reaching a plateau level of approximately 20 % of the peak effect after 3–4 min (Fig. 1). This time course is quite different from that observed with a Ca^{2+}-dependent stimulus such as 18K saline (Fig. 1) and in the presence of the Ca^{2+} ionophore ionomycin (Figs 6 and 7), which caused sustained exocytosis. These observations suggest that some aspect of the synaptic vesicle life-cycle is compromised when exocytosis is triggered in the absence of a rise in intraterminal Ca^{2+}.

Two major hypotheses can be proposed. The first is that synaptic vesicle endocytosis is either blocked or less efficient leading to a gradual depletion of synaptic vesicles in the RRP at active zones. The second hypothesis is that endocytosis still occurs following RR-evoked exocytosis, but the availability of fusion-competent synaptic vesicles is decreased. This could result from an impairment of vesicle mobilisation or priming, perhaps leading to an accumulation of fusion-incompetent vesicles at release sites. Our finding that RR causes as much FM2-10 uptake in synaptic terminals as 18K saline (Fig. 4) argues against the first hypothesis. Although a small reduction in the efficacy of endocytosis cannot be formally excluded, our findings show that the exocytotic events triggered by RR are followed by some form of endocytosis, leading to the subsequent availability of recycling vesicles. We provide two arguments in favour of the second hypothesis. First, RR caused only short-lasting dye release from FM2-10-loaded nerve terminals (Fig. 5). In comparison, a Ca^{2+}-dependent stimulus (18K saline) caused prolonged FM2-10 release (Fig. 5). Second, we found that a modest enhancement of intracellular Ca^{2+} with ionomycin caused a prolongation of the time course of RR-evoked release quantified by mEPSCs recordings (Fig. 6) or by FM2-10 release (Fig. 7). The simplest interpretation of these sets of data is that RR evokes release from the RRP of vesicles, but fails to mobilise vesicles from the reserve pool because of the inability of this stimulus to cause a rise in intraterminal Ca^{2+}. Alternatively, the priming of vesicles may be compromised, leading to an occupation of release sites by fusion-incompetent vesicles. These observations suggest that in neurones, an elevation of intraterminal Ca^{2+} concentration above resting level is required to maintain a supply of fusion-competent vesicles.

Mechanism of action of RR

In order to be able to probe the synaptic vesicle recycling pathway in the absence of Ca^{2+} influx, we took advantage of the ability of RR to induce quantal neurotransmitter release. This stimulus is known to trigger secretion reversibly without causing detectable Ca^{2+} influx by interacting with extracellular sites at the presynaptic terminal (Trudeau *et al.* 1996*a*,*b*, 1998; Sciancalepore *et al.* 1998). In addition, RR also directly blocks Ca^{2+} channels (Hamilton & Lundy, 1995; Trudeau *et al.* 1996*a*; Cibulsky & Sather, 1999). The miniature events triggered by RR display kinetics that are indistinguishable from spontaneous mEPSCs and are of the same amplitude (Raastad *et al.* 1990; Trudeau *et al.* 1996*a*; Sciancalepore *et al.* 1998). Although the exact molecular mechanism of action of RR is still undetermined, all available evidence suggests that RR acts on the same pool of predocked vesicles that is released by low-frequency action potentials. As for spike-evoked release, RR-evoked release is dependent on the integrity of the SNARE (SNAP receptor) complex of synaptic proteins; it is blocked by cleaving synaptobrevin/vesicle-associated membrane protein (VAMP) using tetanus toxin (Trudeau *et al.* 1996*a*). It is also blocked by cleaving syntaxin using Botulinum toxin C (L.-E. Trudeau, unpublished results). However, RR-evoked release is insensitive to cleavage of SNAP-25 by Botulinum toxin A (Trudeau *et al.* 1998), a protein thought to be involved in regulating the Ca^{2+} sensitivity of exocytosis (Capogna *et al.* 1997; Xu *et al.* 1998). Recent work has also shown that RR-evoked release is subject to modulation by the activation of presynaptic receptors and second messenger pathways in a way similar to action potential-evoked release (Trudeau *et al.* 1996*a*,*b*, 1998; Bouron & Reuter, 1999; Koyama *et al.* 1999; Hoffman & Lupica, 2001).

In the present study we have extended these previous observations concerning the mechanism of action of RR in three ways. First, we have shown that RR-evoked exocytosis is occluded by previous stimulation of synaptic terminals with hypertonic saline (Fig. 3). Considering that hypertonic stimulation has previously been described as selectively inducing release from the RRP, our observations confirm the previous suspicion that RR also acts on the RRP, a fraction of the total synaptic vesicle pool that is thought to be in a state close to exocytosis, awaiting Ca^{2+} influx (Rosenmund & Stevens, 1996).

A second new observation of the present study is that RR fails to cause any detectable rise in intracellular Ca^{2+} in nerve terminals (Fig. 2). This finding complements our previous observation that RR also fails to cause a rise in intracellular Ca^{2+} in cell bodies and major processes of neurones (Trudeau *et al.* 1996*a*). In addition, we show that, unlike release evoked by depolarisation, RR-evoked release is insensitive to the Ca^{2+} chelator BAPTA, thereby excluding the implication of highly localised and brief rises in intraterminal Ca^{2+} that could have escaped detection by fluorescence imaging.

Third, our finding that RR causes both uptake and release of FM2-10 from identified synaptic boutons suggests that, whatever the exact molecular mechanism of RR-evoked release, vesicular release triggered in this way does not prevent the endocytosis and reuse of stimulated vesicles.

Taken together, our results suggest that combining RR with FM2-10 labelling may provide a unique method for studying the modulation of late phases of the secretory pathway in neurones using optical techniques. Using RR as a stimulus, we found that approximately 20 % of the recycling pool was labelled. This may represent an estimate of the relative size of the RRP in central nerve terminals. This number is in close agreement with the findings of Pyle *et al.* (2000) and Richards *et al.* (2000), who suggested figures in the range of 20–25 % using different approaches. However, it is important to note that in our experiments, the size of the recycling pool was estimated by measuring the maximum amount of fluorescence loss evoked during a 40K loading–unloading protocol. This may slightly underestimate the total recycling pool since depolarisation using higher concentrations of potassium, such as 90 mM, can release up to 35 % more signal in some experiments (P. Congar & L.-E. Trudeau, unpublished results). However, such a systematic underestimation of the total recycling pool, which should result in a slight overestimation of the RRP, should not have affected our quantification of the relative efficacy of the different releasing stimuli evaluated in the present work.

Role of calcium in endocytosis

Our observations suggest that in neurones, a rise in intraterminal Ca^{2+} above resting levels is not absolutely necessary to trigger endocytosis following neurotransmitter release. This finding is compatible with a number of recent reports providing indirect support for the hypothesis that endocytosis may be at least partly Ca^{2+} independent in neurones (von Gersdorff & Matthews, 1994; Ryan *et al.* 1996; Gad *et al.* 1998; Rouze & Schwartz, 1998; Neves & Lagnado, 1999; Cousin & Robinson, 2000). The present findings differ from such previous work by providing experimental conditions that allow one to examine endocytosis under conditions where Ca^{2+} influx is not used to trigger exocytosis, thereby eliminating a major complicating variable. It is important to note that our conclusion is not incompatible with the recent suggestion that, at some concentrations, intracellular Ca^{2+} could actually increase the rate of endocytosis (Sankaranarayanan & Ryan, 2001). Our findings simply show that, even in the absence of such a rise in Ca^{2+}, endocytosis can still occur at basal levels of intraterminal Ca^{2+}.

Figure 1 shows that RR increases the frequency of mEPSCs for 3–4 min followed by a plateau at a low frequency. In

comparison, RR stimulates FM2-10 release for at most 2 min (Fig. 5). The reason for this small but apparent discrepancy is unclear. However, considering that the short duration of FM2-10 release is likely to be due to a block of vesicle mobilisation under conditions where Ca^{2+} influx does not occur, one possible explanation is that after releasing their neurotransmitter content, vesicles in the RRP can be recycled locally at or near the active zone and can serve for more than one round of exocytosis. This could occur through two main mechanisms. The first would be through a classical but local exocytosis and endocytosis cycle. The second would be through a form of 'kiss and run' mechanism whereby neurotransmitter is released through a fusion pore, followed by pore closure, refilling and priming of the vesicle for further use. One type of such rapid recycling has been called 'reuse' by Tsien and collaborators (Klingauf *et al.* 1998; Pyle *et al.* 2000). The existence of a dual proximal–distal recycling pathway in nerve terminals has also been suggested by Koenig & Ikeda (1996, 1999) from ultrastructural work at neuromuscular junctions of the *Drosophila* endocytosis mutant *Shibire*. Our results do not allow us to distinguish between 'kiss and run', reuse and local classical endocytosis. However, they suggest that local recycling, whatever its mechanism, is likely to be insufficient to maintain release rates at a high level during prolonged neurotransmitter release (Fig. 1). It is conceivable that in the absence of mobilisation or priming of new vesicles from the reserve pool, vesicles that are recycled locally at the active zone may gradually become fusion incompetent. Mobilisation and classical endocytosis may be crucial to maintain high release rates over long time periods.

Role of calcium in the delivery of fusion-competent vesicles

Our conclusion of a Ca^{2+}-dependent mechanism of synaptic vesicle mobilisation/priming is in agreement with previous models of vesicle mobilisation (Greengard *et al.* 1993; Weis *et al.* 1999) and with electrophysiological investigations relying on high-frequency action potential trains and large intracellular Ca^{2+} elevations to stimulate mobilisation (Stevens & Wesseling, 1998, 1999; Wang & Kaczmarek, 1998; Sakaba & Neher, 2001). Previous ultrastructural investigations of synaptic terminal have also suggested the hypothesis of an involvement of Ca^{2+} in mobilisation (Koenig *et al.* 1993; Leenders *et al.* 1999). In the present work, we conclude that a rise in intracellular Ca^{2+} above 40 nM is required to facilitate mobilisation (Fig. 6*B*), since 50 nM ionomycin prolonged the duration of RR-evoked release and caused a rise in intracellular Ca^{2+} just above 40 mM (Fig. 6*B*). Depolarisation with 18K saline also caused mobilisation and was associated with an increase in intracellular Ca^{2+} of 230 nM. The specific molecular mechanism by which Ca^{2+} influx promotes vesicle mobilisation or priming is still unclear. It has been proposed that interactions between vesicles and a restraining matrix or cytoskeletal elements might be an important variable (Rosahl *et al.* 1995; Henkel *et al.* 1996*b*; Kraszewski *et al.* 1996; Cole *et al.* 2000). Phosphoproteins such as synapsin appear to be implicated in regulating such interactions (Pieribone *et al.* 1995; Hilfiker *et al.* 1998). Recent work has also suggested that small GTP-binding proteins (Takahashi *et al.* 2000), myosin light-chain kinase (MLCK; Ryan, 1999), protein kinase A (PKA; Kuromi & Kidokoro, 2000) and protein kinase C (Stevens & Sullivan, 1998; Waters & Smith, 2000) regulate the rate of vesicle mobilisation. The impact of Ca^{2+} in these GTP-, MLCK-, PKA-, PKC- and synapsin-dependent processes has not been fully established. However, it has been suggested that Ca^{2+} could free vesicles from interactions with cytoskeletal elements through the stimulation of Ca^{2+}–calmodulin-dependent kinase II and the phosphorylation of synapsin-I (Llinás *et al.* 1991; Doussau & Augustine, 2000). The kinase MLCK is also known to be regulated by Ca^{2+}–calmodulin (Edelman *et al.* 1992). Finally, calmodulin has recently been suggested to play a crucial role in the recruitment of a subset of synaptic vesicles at the Calyx of Held synapse (Sakaba & Neher, 2001).

In summary, we have provided evidence for Ca^{2+}-independent synaptic vesicle endocytosis and for a perturbation of the delivery of fusion-competent synaptic vesicles under conditions where neurotransmitter release is triggered in the absence of an elevation of intraterminal Ca^{2+}. Additional work will be required to identify the specific signal transduction pathway involved in this process.

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