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SYNAPTIC MECHANISMS

Adenosine drives recycled vesicles to a slow-release pool at the mouse neuromuscular junction

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Abstract

The effects of adenosine on neurotransmission have been widely studied by monitoring transmitter release. However, the effects of adenosine on vesicle recycling are still unknown. We used fluorescence microscopy of FM2-10-labeled synaptic vesicles in combination with intracellular recordings to examine whether adenosine regulates vesicle recycling during high-frequency stimulation at mouse neuromuscular junctions. The A_1 adenosine receptor antagonist (8-cyclopentyl-1,3-dipropylxanthine) increased the quantal content released during the first endplate potential, suggesting that vesicle exocytosis can be restricted by endogenous adenosine, which accordingly decreases the size of the recycling vesicle pool. Staining protocols designed to label specific vesicle pools that differ in their kinetics of release showed that all vesicles retrieved in the presence of 8-cyclopentyl-1,3-dipropylxanthine were recycled towards the fast-release pool, favoring its loading with FM2-10 and suggesting that endogenous adenosine promotes vesicle recycling towards the slow-release pool. In accordance with this effect, exogenous applied adenosine prevented the replenishment of the fast-release vesicle pool and, thus, hindered its loading with the dye. We had found that, during high-frequency stimulation, Ca^{2+} influx through L-type channels directs newly formed vesicles to a fast-release pool (Perissinotti *et al.*, 2008). We demonstrated that adenosine did not prevent the effect of the L-type blocker on transmitter release. Therefore, activation of the A_1 receptor promotes vesicle recycling towards the slow-release pool without a direct effect on the L-type channel. Further studies are necessary to elucidate the molecular mechanisms involved in the regulation of vesicle recycling by adenosine.

Introduction

Neuronal communication depends on the release of neurotransmitter from a presynaptic terminal and the subsequent activation of postsynaptic receptors. Neurotransmitters are stored in presynaptic vesicles that are organized into distinct pools with different kinetics of release (Rizzoli & Betz, 2005). There is general agreement that vesicles are arranged into three main pools: a rapidly releasable pool (RRP), reserve pool (RP) and resting pool. The RRP constitutes those vesicles closest to the plasma membrane, docked at the active zone and ready for immediate release (Schikorski & Stevens, 2001). The RP acts as a source of vesicles and serves to refill the empty release sites at the RRP. Vesicles in the resting pool are refractory to release in response to electrical activity and their function is still unknown (Südhof, 2000).

The efficacy of synaptic transmission depends on the ability to recycle spent vesicles for reuse and to sustain vesicle supply from the RP. Two pathways for vesicle recycling have been described. One involves clathrin-mediated endocytosis (Heuser & Reese, 1973), whereas, in the other, subsets of vesicles are recycled at, or close to, individual active zones without entering the RP (Ceccarelli *et al.*, 1973). At the mouse neuromuscular junction (NMJ), we have previously found that there are two functionally different vesicle pools according to FM dye loading/unloading patterns: (i) a fast-destaining vesicle pool that is rapidly recycled during high-frequency stimulation and that is modulated by Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels and (ii) a slow-destaining vesicle pool that is recycled during prolonged stimulation and keeps on refilling after the end of stimulation (Perissinotti *et al.*, 2008). Whether these pools are affected by a neuromodulator such as adenosine has not been analyzed.

Neuromodulators at the NMJ are well known to have an effect on presynaptic receptors by regulating transmitter release. Adenosine triphosphate is co-stored with acetylcholine in cholinergic vesicles (Dowdall *et al.*, 1974; Nagy *et al.*, 1976) and is co-released with acetylcholine to the synaptic cleft where it is degraded to adenosine via ectonucleotidases (Ribeiro & Sebastiao, 1987; Meriney & Grinnell, 1991; Redman & Silinsky, 1994). Studies using selective adenosine agonists and antagonists have demonstrated that the presynaptic inhibitory effects of adenosine are mediated mainly by A_1 G-protein-coupled receptors (Nagano *et al.*, 1992; Hirsh & Silinsky, 2002).

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At mammalian NMJs, the A_1 adenosine receptor activation is associated with a reduction in nerve terminal calcium currents (Hamilton & Smith, 1991; Silinsky, 2004). Furthermore, studies with botulinum toxins suggest that the proteins that are members of the core complex of the secretory apparatus, the synaptosome-associated protein receptors (SNAREs) are key elements in mediating the effects of adenosine at mouse NMJs (Kalandakanond & Coffield, 2001; Silinsky, 2005, 2008). The SNARE syntaxin (a protein that is intimately linked to Ca^{2+} channels) and synaptosome-associated protein of 25 kDa have been proposed to be the minimal SNARE requirements for the presynaptic modulation of calcium currents by adenosine (Silinsky, 2008). However, Veggetti *et al.* (2008) found that adenosine decreases the hypertonicity-induced transmitter release in mouse NMJ. As this process is not mediated by Ca^{2+} influx, this result suggests that there is a Ca^{2+} -independent effect of adenosine on the cascade of exocytosis. In addition, genetic deletion of Rab3A, a vesicular protein involved in the recruitment of vesicles to docking sites, alters presynaptic adenosine modulation (Hirsh *et al.*, 2002; Silinsky, 2004, 2008). In summary, adenosine action on the SNARE–Ca^{2+} channel complex affects both calcium currents and vesicle fusion readiness.

Synaptic vesicle exocytosis and endocytosis are tightly coupled processes that sustain neurotransmission in presynaptic terminals, and both are regulated by Ca^{2+} (Ceccarelli & Hurlbut, 1980; Neher & Zucker, 1993; von Gersdorff & Matthews, 1994; Wang & Zucker, 1998; Perissinotti *et al.*, 2008). Molecular evidence suggests that proteins critical for exocytosis, such as synaptotagmin, the SNARE synaptobrevin and the Rab family of small G-proteins, are also essential for endocytosis (Novick & Brennwald, 1993; Simons & Zerial, 1993; Pfeffer, 1994; Poskanzer *et al.*, 2003; Deák *et al.*, 2004; Nicholson-Tomishima & Ryan, 2004). As both exocytosis and endocytosis occur almost simultaneously, changes in endocytocis will also produce an apparent modification in exocytosis.

Although the effects of adenosine on neurotransmission have been widely studied by monitoring transmitter release, the action on vesicle recycling is still unknown. The present work was designed to analyze whether adenosine regulates vesicle recycling at mouse NMJs. Fluorescence microscopy of FM2-10-labeled synaptic vesicles in combination with intracellular recordings was used to examine the potential modulatory effect on specific vesicle pools by adenosine. We found that adenosine affects vesicle retrieval, favoring vesicle recycling towards the slow-release pool.

Materials and methods

Experiments were carried out on the left 'levator auris longus' muscle of male Swiss mice weighing from 25 to 30 g. Animals were supplied by the animal house of the School of Pharmacy and Biochemistry of the University of Buenos Aires. Animals were cared for in accordance with national guidelines for the humane treatment of laboratory animals, similar to those of the US National Institutes of Health. Animals were anesthetized with an overdose of 2% tribromoethanol (0.15 mL/10 g body weight) injected in the peritoneal cavity and exsanguinated immediately. The muscle with its nerve supply was excised and dissected on a Sylgard®-coated Petri dish containing physiological saline solution of the following composition (in mM): 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 12 $NaHCO_3$, 1 Na_2HPO_4 and 11 glucose; continuously bubbled with 95% O_2/5% CO_2; pH 7.4. Bovine serum albumin (0.1 mg/mL) was added to the saline solution to reduce unspecific fluorescence background. The preparation was then transferred to a 1 mL recording chamber. Experiments were performed at room temperature (20–23°C).

Electrophysiology

Evoked endplate potentials (EPPs) and spontaneous endplate potentials (S-EPPs) were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (10–15 MΩ resistance). For recordings, μ-conotoxin GIIIB (10 μM) was added to the bath solution to prevent muscle fiber contraction. Recordings were rejected if the 10–90% EPP rise time exceeded 1 ms. The resting membrane potential of muscle fibers (V_m) ranged from −60 to −70 mV. The nerve was stimulated via two platinum electrodes coupled to a pulse generator (Grass S88). Signals were amplified with Axoclamp 2B (Axon Instruments, USA) and digitized with Digidata 1200 (Axon Instruments). The pClamp 8.2 software (Axon Instruments) was used for analysis.

The quantal content (QC) was determined by a direct method and corrected for non-lineal summation (McLachlan & Martin, 1981), using the formula

$$\mathrm{QC} = \frac{E}{m(1 - 0.8E/V_m)} \quad (1)$$

where E is the amplitude of the EPP and m is the mean amplitude of S-EPPs. Both amplitudes were normalized to −75 mV.

The QC rundown during an evoked train was fitted with a monoexponential function

$$\mathrm{QC}(t) = y_0 + A\exp\left(\frac{-t}{\tau}\right) \quad (2)$$

where QC(t) is the QC during the stimulation time t, y_0 is the QC at the asymptotic value at $t = \infty$ and A is the QC released with the time constant τ during the train. The QC(t) was normalized to the QC released during the first EPP.

Activity-dependent staining and destaining: fluorescence quantification

Synaptic vesicles were labeled during nerve stimulation in the presence of FM2-10 (40 μM) (Molecular Probes, Eugene, OR, USA) and α-bungarotoxin (4–8 μM) to prevent muscle fiber contraction. Under these conditions, the dye was taken up into the vesicles during activity-induced vesicle turnover. The nerve was stimulated via two platinum electrodes coupled to a Grass S88 stimulator associated with a stimulus isolation unit. Extracellular dye was washed using a gravity-fed perfusion.

Fluorescent images were acquired using an upright microscope (BX50WI, Olympus) equipped with epifluorescence optics. We used a 40 × /0.80 NA water immersion objective lens (LUMPlan FI, Olympus) for observation. The excitation light came from a USH-I 02DH 100 W mercury arc lamp, through 6% neutral density transmission filters to prevent excessive bleaching and phototoxicity, and through excitation filters (460–490 nm), a dichroic mirror (505 nm) and emission filters (515 nm). Photobleaching, measured by repetitive image acquisition of FM2-10 loaded at the NMJ, was < 3%. Images were acquired with a cooled Quantix CCD camera (Photometrics Inc., Tucson, AZ, USA) connected to a computer running Axon Imaging software (Axon Instruments). The exposure time was adjusted between 250 and 400 ms (binning was adjusted and gain was set to 3). Image analysis was performed using Image J software (Wayne Rasband, National Institute of Health, USA). Regions in focus of each NMJ were manually outlined and the average intensity of the pixels inside this area was calculated. The background fluorescence estimated from an outlined region surrounding the NMJ was subtracted from this average fluorescence.

FM dye loading protocols

Nerve terminals were loaded using different stimulation protocols.

1. Long loading protocol (used to fully load vesicle recycling pool). Nerve terminals were incubated for 5 min with FM2-10 prior to the loading by 20 Hz nerve stimulation for 10 min. After stimulation, the dye remained for 5 min to allow the loading of vesicles recycled from delayed endocytosis. Extracellular dye was then washed out for at least 20 min (flow rate ~2.5 mL/min). This protocol ensured the full loading of the recycling vesicle pool.
2. Short loading protocol (used to preferentially load the fast-release vesicle pool). Nerve terminals were incubated for 5 min with FM2-10 prior to the loading by 50 Hz nerve stimulation for 5 s. The dye was then quickly washed out for at least 5 min (flow rate ~10 mL/min).
3. Delayed loading protocol (used to preferentially load the slow-release vesicle pool). The dye was added for 5 min after the end of stimulation (15 s at 50 Hz). The dye was then washed out for at least 20 min (flow rate ~2.5 mL/min).

FM dye destaining experiments

After a 30 min resting period, vesicle unloading was performed at 50 Hz stimulation frequency.

Analysis of FM fluorescence signals

To compare different preparations, fluorescence was quantified as the percentage of maximum load.

Absolute fluorescence was converted to percentage of fluorescence with the following equation

$$\%F(t) = \frac{F(t) - F^{N-V}}{F^{MAX} - F^{N-V}} \times 100\% \quad (3)$$

where $F(t)$ is the absolute fluorescence at time t, F^{MAX} is the absolute fluorescence after maximum loading and F^{N-V} is the non-vesicular fluorescence background (i.e. fluorescence remaining after treatment with 90 mM K^+ for 5 min).

Destaining kinetics were fitted using monoexponential and biexponential functions. The monoexponential function is given by

$$\%F(t) = \%A \exp\left(-\frac{t}{\tau}\right) + \%F^R \quad (4)$$

This function models a first-order release, with a single time constant, τ for all vesicles. In Eqn (4), $\%A$ is the size of the vesicle pool that is available for exocytosis at any given frequency and $\%F^R$ is the percentage of fluorescence that remained after stimulation (but could be liberated under high [K^+] conditions).

Biexponential fitting functions were also used

$$\%F(t) = \%A_1 \exp\left(-\frac{t}{\tau_1}\right) + \%A_2 \exp\left(-\frac{t}{\tau_2}\right) + \%F^R \quad (5)$$

These functions model first-order release processes when two different time constants (τ_1 and τ_2) are involved. These time constants are associated with different vesicle pools of different sizes ($\%A_1$ and $\%A_2$).

The Fisher test (F-test) was used to determine the statistical improvement in regressions (Sigma Plot 9). This test compares two equations of the same family (in our case monoexponential and biexponential functions) to determine if the higher order provides any statistical improvement to the quality of the fit. The F-test uses the residuals from two regressions to compute the sums of squares of the residuals and computes an approximate P-value for the significance level. If $P < 0.05$, the higher order equation can be expected to provide a statistically better fit.

In those experiments where different drugs were present during the loading but absent during the unloading, differences in destaining kinetics were attributed to the effect of the drug on the preferential loading of the fast-release or slow-release vesicle pool. We used exponential functions to analyse these experiments. Because the unloading was performed in the absence of drugs, time constants for the fast-release and slow-release vesicle pools were very similar for all of the experiments (no change in the destaining kinetics of each individual pool), but the relative contribution of each of these pools to the total fluorescence (i.e. the amount of dye present in each pool at $t = 0$) changed with the loading treatment. However, when the drug was absent during the loading but present during the unloading (Fig. 2), an effect on the time constants of release cannot be discarded. Therefore, in such cases, we preferred to use a simple analysis based on the time required to reach 50% of the initial fluorescence value ($t_{50\%}$) and the remaining fluorescence after the stimulation ($\%F^R$).

Toxin and chemicals

Tribromoethanol, bovine serum albumin, α-bungarotoxin, adenosine, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), *N*6-cyclopentyl-adenosine (CPA) and all salts of analytical grade were purchased from Sigma (St Louis, MO, USA), μ-conotoxin GIIIB was purchased from Alomone Labs (Jerusalem, Israel) and nitrendipine was purchased from RBI (Natick, MA, USA). DPCPX, CPA and nitrendipine were dissolved in dimethylsulfoxide; the final dimethylsulfoxide concentration was 0.05% (v/v). Dimethylsulfoxide did not affect the parameters under study (data not shown). Sucrose was purchased from Merck (Darmstadt, Germany).

Data presentation and statistics

Average data are expressed and plotted as mean ± SEM. Statistical significance was determined using Student's t-test ($P < 0.05$) and ANOVA ($P < 0.05$). The exponential order of fitting curves was determined using the F-test. Residual values (observed–fitted differences), calculated with either monoexponential or biexponential fittings, were used to show the accuracy of the fitting.

Results

Adenosine decreases the neurotransmitter release

When a motor nerve terminal is stimulated repeatedly at a high rate, vesicle release drops dramatically and eventually reaches a low steady-state level. Such a use-dependent synaptic depression reflects the depletion of the pool of release-competent vesicles and the steady-state level of release corresponds to the rate at which vesicles are replenished into this pool by recycling or recruitment from a RP. As synaptic depression reflects the vesicle refilling efficiency, we analyzed the effects of adenosine on synaptic depression.

The presynaptic inhibitory effects of adenosine are mediated mainly by A_1 G-protein-coupled receptors at mammalian NMJs (Nagano *et al.*, 1992; Hirsh & Silinsky, 2002). In order to study the inhibitory effects of adenosine on transmitter release depression during high-frequency stimulation, the QC was estimated (Eqn 1) at the NMJ during a 50 Hz train in the absence or presence of adenosine (200 μM)

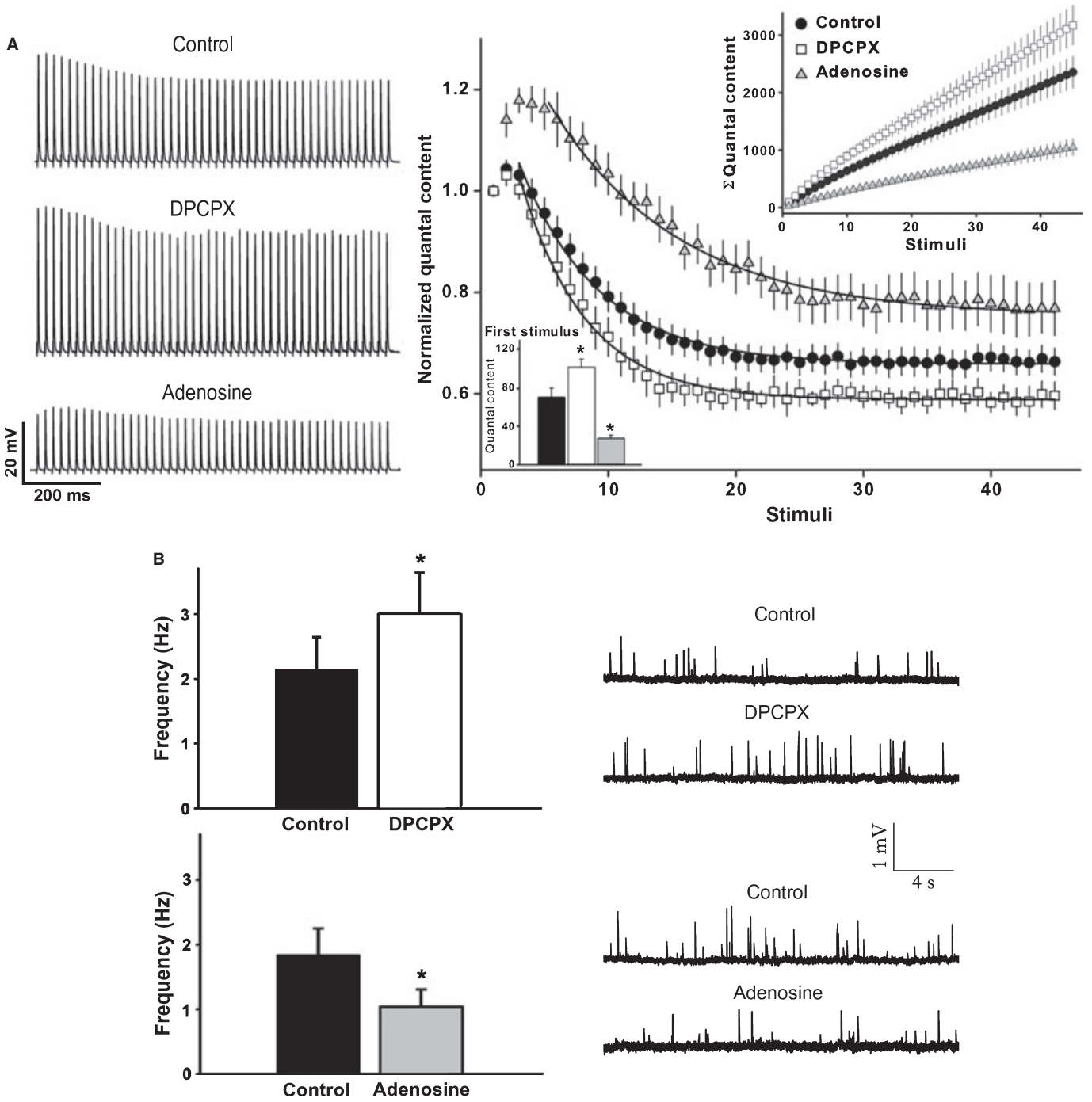

FIG. 1. Adenosine decreases neurotransmitter release. (A) Normalized QC rundown during 50 Hz stimulation in the absence (n = 10) and presence of DPCPX (0.1 μM, n = 4) or adenosine (200 μM, n = 6). Values were fitted with a monoexponential decay function (Eqn 2, Materials and methods): y_0 = 0.65 ± 0.02%, A = 0.35 ± 0.02%, τ = 130 ± 9 ms (control), y_0 = 0.59 ± 0.05%, A = 0.41 ± 0.05%, τ = 114 ± 8 ms (DPCPX), y_0 = 0.75 ± 0.05%*, A = 0.25 ± 0.05%*, τ = 197 ± 14 ms* (adenosine). *P < 0.05, Student's t-test. Bottom inset: QC release during the first stimulus. *P < 0.05, Student's t-test. Top inset: QC release during 45 pulses summed successively to obtain a cumulative transmitter output before and after DPCPX or adenosine treatment. Electrophysiological recordings are shown on the left. (B) Spontaneous release recorded in the same fiber before and after DPCPX (0.1 μM, n = 5) or adenosine (200 μM, n = 5) treatment. Mean frequencies of S-EPPs normalized to control values were plotted. Electrophysiological recordings are shown on the right. *P < 0.05, Student's paired t-test.

or DPCPX, the selective A_1 adenosine receptor antagonist (0.1 μM). The train duration (1 s) was long enough to reach significant synaptic depression. The temporal course of QC depression was fitted with a monoexponential function (Eqn 2). We found that exogenously applied adenosine significantly increased the characteristic time of synaptic depression (τ; $t_{14} = -2.99$, $P = 0.01$) and decreased the percentage of depression (y_0; $t_{14} = -3.18$, $P = 0.01$) in comparison to control experiments (control: τ = 130 ± 9 ms, y_0 = 0.65 ± 0.02%; adenosine: τ = 197 ± 14 ms, y_0 = 0.75 ± 0.05%) (Fig. 1A). As adenosine significantly decreased the QC of the first evoked potential ($t_{14} = 3.68$, $P = 0.002$; bottom inset, Fig. 1A), more facilitation of the second evoked potential was observed as expected (Fig. 1A).

Likewise, the cumulative transmitter output during the train decreased by 53% in the presence of adenosine (top inset, Fig. 1A). There was no significant difference between the kinetic parameters obtained from the time-course of depression (τ: $t_{12} = 1.1$, $P = 0.3$; y_0: $t_{12} = 1.4$, $P = 0.2$) in control and DPCPX treatments (control: $\tau = 130 \pm 9$ ms, $y_0 = 0.65 \pm 0.02\%$; DPCPX: $\tau = 114 \pm 8$ ms, $y_0 = 0.59 \pm 0.05\%$) (Fig. 1A). However, the QC of the first evoked potential ($t_{12} = -2.56$, $P = 0.03$; bottom inset, Fig. 1A), as well as the cumulative transmitter output during the train ($t_{12} = -2.8$, $P = 0.03$; top inset, Fig. 1A), increased significantly in the presence of DPCPX as compared with control experiments. The cumulative transmitter output during the train increased by 24% compared with control.

Furthermore, the frequency of S-EPPs was recorded in the same muscle fiber before and after 20 min of adenosine or DPCPX application. Exogenously applied ($t_4 = 2.35$, $P = 0.04$) and endogenous ($t_4 = -3.14$, $P = 0.02$) adenosine significantly reduced the frequency of S-EPPs (Fig. 1B), indicating an inhibitory effect on spontaneous transmitter release. No differences in the S-EPP amplitude were observed between treatments (data not shown), discarding changes in postsynaptic sensitivity to acetylcholine.

In summary, exogenously applied adenosine, by decreasing the QC release and increasing the characteristic time of synaptic depression, has an inhibitory effect on exocytosis as reported in other preparations (Ribeiro & Walker, 1975; Silinsky, 1984; Bennett *et al.*, 1991). The reduced percentage of depression (y_0) in the presence of exogenous adenosine shows the efficiency of the replenishment process when vesicle release is diminished. However, DPCPX seemed not to affect the kinetic parameters of the time-course of depression but increased the QC release during the first EPP, suggesting that vesicle exocytosis can be restricted by A_1 receptor activation by endogenous adenosine. Furthermore, the fact that DPCPX increased the QC of the first EPP suggests the permanent presence of endogenous adenosine at the synaptic cleft.

Adenosine modulates vesicle recycling

In order to study the effects of adenosine on vesicle mobilization from the recycling vesicle pool, nerve terminals were maximally loaded with FM2-10 using a staining protocol that loads all recycled vesicles (long loading protocol). Unloading was then elicited in the absence and presence of adenosine (200 μM) or DPCPX (0.1 μM) (Fig. 2). No significant difference was seen in the required time to reach 50% of the initial fluorescence value ($t_{50\%}$) between control and DPCPX treatments ($t_{13} = -1.2$, $P = 0.25$; Table 1). However, exogenously applied adenosine significantly increased the $t_{50\%}$ ($t_{11} = -2.22$, $P = 0.04$; Table 1), showing an inhibitory effect on vesicle mobilization from the recycling pool. However, the percentage of fluorescence that remained after the end of stimulation ($\%F^R$) significantly decreased in the presence of DPCPX ($t_{13} = 2.21$, $P = 0.04$), suggesting that activation of A_1 receptor by endogenous adenosine decreased the recycling vesicle pool size. These results agree with the inhibitory effect of adenosine on QC released.

Activation of A_1 receptors by endogenous adenosine recycles vesicle towards the slow-release vesicle pool

Staining protocols were previously designed to label specific vesicle pools that differ in the kinetics of release, i.e. the fast-release and slow-release pools (Perissinotti *et al.*, 2008). In order to determine whether the presynaptic effects of adenosine might alter the replenishment of these pools, we analyzed the FM2-10 destaining kinetics after preferential loading of the fast-vesicle or slow-vesicle pool in the presence and absence of adenosine. In these experiments, destaining was performed in the absence of drugs to determine the destination of vesicles that were recycled during loading.

Figure 3A shows the destaining kinetics recorded after preferentially loading the fast-release vesicle pool using the short FM2-10

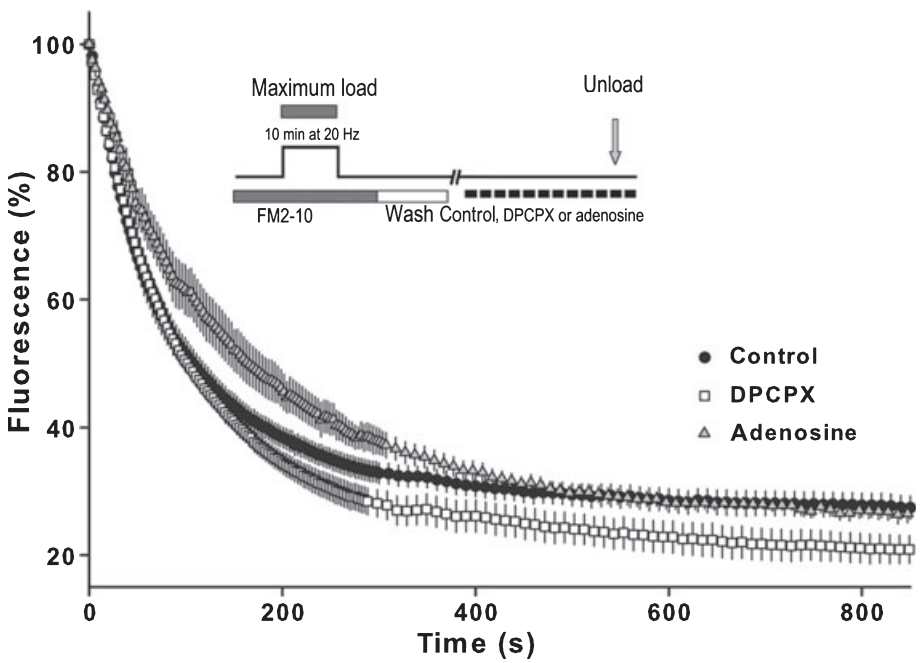

FIG. 2. Adenosine decreases the time-course of FM2-10 destaining and the size of the recycling pool. Time-course of destaining recorded for the loading and unloading protocol shown at the top. NMJs were maximally labeled with FM2-10 by nerve stimulation (10 min at 20 Hz), the dye was incubated for 5 min before and after stimulation and then washed. After 30 min rest, the time-course of destaining was examined during stimulation in the absence ($n = 8$) and presence of adenosine (200 μM, $n = 5$) or DPCPX (A_1 antagonist) (0.1 μM, $n = 7$). Fluorescence values were plotted as the percentage of remaining fluorescence.

TABLE 1. Effects of adenosine on destaining parameters of nerve terminals loaded with the long loading protocol

Parameter	Control	Adenosine	DPCPX
$t_{50\%}$ (s)	59.2 ± 8.2 (8)	98.5 ± 18.5 (5)*	72.9 ± 7.7 (7)
F^R (%)	27.5 ± 2.5 (8)	26.2 ± 1.3 (5)	19.9 ± 2.5 (7)*

Average data are expressed as mean ± SEM (n). $t_{50\%}$, time required to reach 50% of the initial fluorescence value; $\%F^R$, percentage of fluorescence that remained after stimulation. *$P < 0.05$, Student's t-test.

loading protocol in the presence and absence of adenosine (200 μM), the selective A_1 adenosine receptor agonist (CPA, 0.5 μM) or DPCPX (0.1 μM). These destaining curves were fitted with exponential functions and the best-fitting parameters are shown in Table 2. The time-course of dye release was slower in nerve terminals loaded in the presence of adenosine or CPA. For these loading treatments, as well as for the control experiments, we found that a monoexponential function was unable to provide a good fit to the destaining kinetics and therefore a biexponential function was used (Fig. 3B). The fitting parameters showed that the weight of the slow time constant

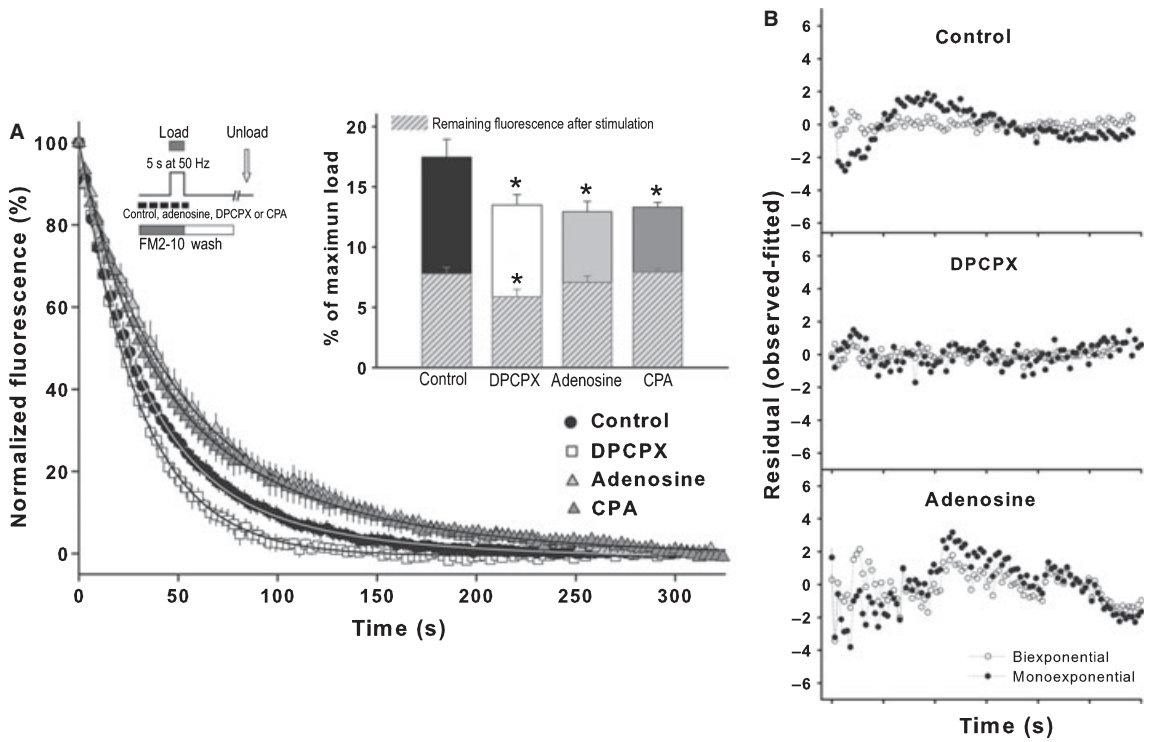

FIG. 3. Adenosine modulates the fast-release vesicle pool. All vesicles are recycled towards this pool in the absence of endogenous adenosine. (A) Time-course of destaining recorded for the loading and unloading protocol shown at the top. NMJs were loaded using a stimulation protocol that preferentially loads the fast-release vesicle pool (short loading protocol). Nerve terminals were incubated for 5 min with FM2-10 and then stimulated for 5 s at 50 Hz in the absence (n = 8) and presence of DPCPX (0.1 μM) (n = 5), adenosine (200 μM) (n = 6) or CPA (0.5 μM) (n = 7). The dye was quickly washed just after stimulation. After a 30 min rest period, the unloading was examined during 50 Hz in the absence of drug. Destaining curves were corrected to the percentage of fluorescence that remained after stimulation ($\%F^R$) and scaled to their initial values. Values were fitted with an exponential decay function (see Eqns 4 and 5 in Materials and methods; fitted parameters in Table 2). Inset: Mean percentages of maximum load during the treatments (Eqn 3, Materials and methods). The percentages of fluorescence that remained after the unloading ($\%F^R$) are shown in striped light gray columns. *$P < 0.05$, Student's t-test. (B) Residual values (observed–fitted difference), calculated with monoexponential or biexponential fittings, were used to evaluate the goodness of fit.

TABLE 2. Exponential destaining parameters of nerve terminals loaded with the short and the delayed loading protocols in the absence or presence of adenosine, DPCPX and CPA

Loading protocol	A_1 (%)	τ_1 (s)	A_2 (%)	τ_2 (min)	F-test F-value	P-value
Short loading (control)	81 ± 1 (8)	33.3 ± 3 (8)	19 ± 1 (8)	1.6 ± 0.4 (8)	386	< 0.05
Short loading (DPCPX)	100 (5)	31 ± 3 (5)			0.02	0.99
Short loading (adenosine)	33 ± 7 (7)	38 ± 12 (6)	67 ± 8 (6)	1.1 ± 0.1 (6)	48.9	< 0.05
Short loading (CPA)	37 ± 6 (8)	30 ± 6 (8)	63 ± 6 (8)	1 ± 0.2 (8)	209	< 0.05
Delayed loading (control)	34 ± 3 (8)	31 ± 2 (8)	66 ± 5 (8)	1.4 ± 0.3 (8)	53	< 0.05
Delayed loading (adenosine)	30.4 ± 1 (5)	33.1 ± 1 (5)	69.6 ± 5 (5)	1.1 ± 0.1 (5)	3.5	< 0.05

The loading was performed in the absence and presence of DPCPX, adenosine or CPA in the first protocol; and in the absence and presence of adenosine in the second protocol. The unloading was performed at 50 Hz stimulation in the absence of both drugs. Average data are expressed as mean ± SEM (n). A_1 and A_2, sizes of the vesicle pools associated with the time constants τ_1 and τ_2, respectively. Exponential order of fitting curves was determined using the F-test (see Materials and methods).

component (%A_2, Table 2) was larger in the presence of adenosine and CPA. We also estimated the amounts of FM2-10 loaded during these short loading procedures, expressed as percentages of maximum loading (inset, Fig. 3A). The amount of FM2-10 loaded significantly decreased in the presence of adenosine ($t_{12} = 2.81$, $P = 0.02$) or CPA ($t_{13} = 2.99$, $P = 0.02$) in comparison to the control condition, in accordance with the QC reduction observed in the presence of adenosine (Fig. 1A). In contrast, dye release occurred faster in nerve terminals loaded in the presence of DPCPX. For this loading condition, we were able to fit the time-course of destaining with a monoexponential function (Fig. 3B), obtaining a fast time constant similar to those observed in control and after treatments with adenosine and CPA. These results suggest that only the fast-release vesicle pool is replenished when the effect of endogenous adenosine is blocked. Despite the fact that DPCPX increased the QC release (Fig. 1A), DPCPX also decreased the amount of FM2-10 loaded ($t_{11} = 2.23$, $P = 0.047$) (inset, Fig. 3A). This was an expected outcome as recycling involves only vesicles from the fast-release pool, and those that are loaded can also be unloaded during the 5 s loading period, and thus reach a steady loading state. However, DPCPX increased the amount of dye that could be released in the following round of stimulation (inset, Fig. 3A; see the remaining fluorescence values). Our results suggest that vesicles that were recycled in the absence of inhibitory effects of endogenous adenosine have a higher reuse probability, i.e. they are more readily available to be released.

Figure 4 shows the destaining kinetics recorded after preferentially loading the slow-release vesicle pool using a delayed FM2-10 loading protocol in the presence and absence of adenosine (200 μM). It also shows the destaining kinetics recorded after preferentially loading the fast-release vesicle pool in the presence of adenosine. No differences in the destaining kinetics and the amount of dye loaded ($t_8 = 1.58$, $P = 0.15$; inset, Fig. 4) were observed between treatments. Thus, loading with the short stimulation protocol in the presence of adenosine showed the same unloading behavior as loading with the delayed protocol. These results indicate that adenosine promotes the recycling of vesicles towards the slow-release vesicle pool favoring its loading with FM2-10. In accordance with this effect, adenosine prevented the replenishment of the fast-release vesicle pool and, thus, hindered its loading with FM2-10 (Figs 3 and 4).

Activation of A_1 receptors by endogenous adenosine decreases the proportion of vesicles that is recycled toward the rapidly releasable pool

Exposure to a hypertonic solution is known to be a reliable method of causing fusion of the RRP in a Ca^{2+} -independent manner (Stevens & Tsujimoto, 1995; Rosenmund & Stevens, 1996; Pyle *et al.*, 2000). Taking advantage of the fact that only the fast-release vesicle pool is recycled and refilled in the presence of DPCPX, we decided to analyze whether this fast-recycling pool corresponded to the RRP and confirm the effects of DPCPX on vesicle replenishment towards the RRP.

In order to determine if the RRP is an integral part of this fast-recycling pool, nerve terminals were loaded with the long loading and short loading protocols. The latter was performed in the absence or presence of DPCPX. After loading, nerve terminals were exposed to a sucrose-containing solution (500 mOsm, 30 s) to release the RRP (Fig. 5). The fraction of fluorescence released by the hypertonic shock increased by 85% in the nerve terminals loaded with the short loading procedure in comparison to those loaded with the long loading procedure ($t_8 = -8.89$, $P < 0.001$; Fig. 5C). These results show that, during the short loading protocol, a higher proportion of labeled

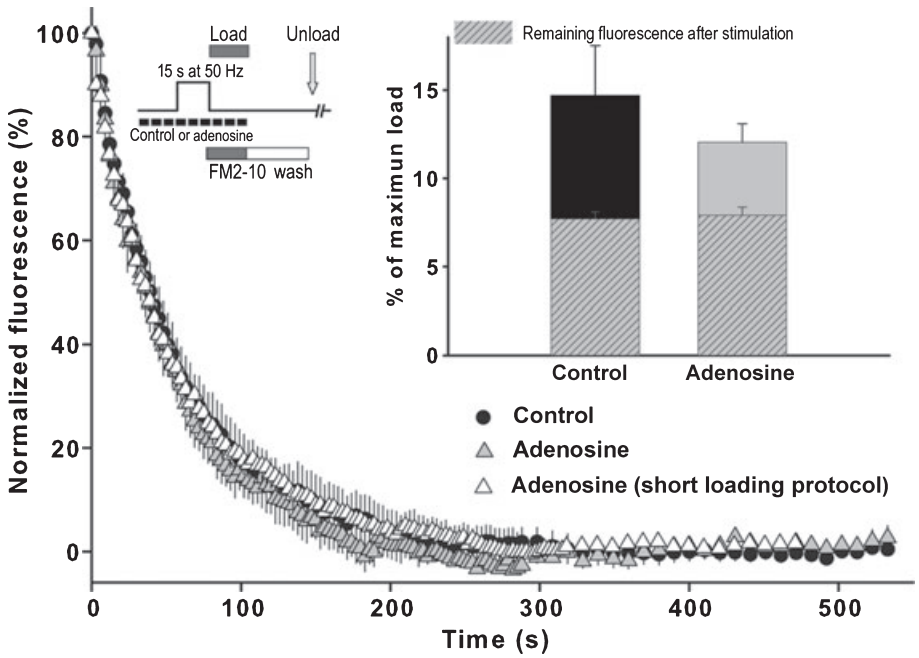

FIG. 4. Adenosine drives recycled vesicles towards the slow-release vesicle pool. The time-course of destaining recorded for the loading and unloading protocol is shown at the top. NMJs were loaded using a stimulation protocol that preferentially loads the slow-release vesicle pool (delayed loading protocol). Nerve terminals were stimulated for 15 s at 50 Hz in the presence ($n = 5$) or absence ($n = 5$) of adenosine (200 μM). The dye was added after the end of stimulation during 5 min. After a 30 min rest period, unloading was examined during 50 Hz in the absence of drug. Destaining curves were corrected to the percentage of fluorescence that remained after stimulation (%F^R) and scaled to their initial values. Inset: Mean percentages of maximum load during the treatments (Eqn 3, Materials and methods). The percentages of fluorescence that remained after unloading (%F^R) are shown in striped light gray columns. The curve for the short loading protocol corresponds to the curve for adenosine treatment in Fig. 3A.

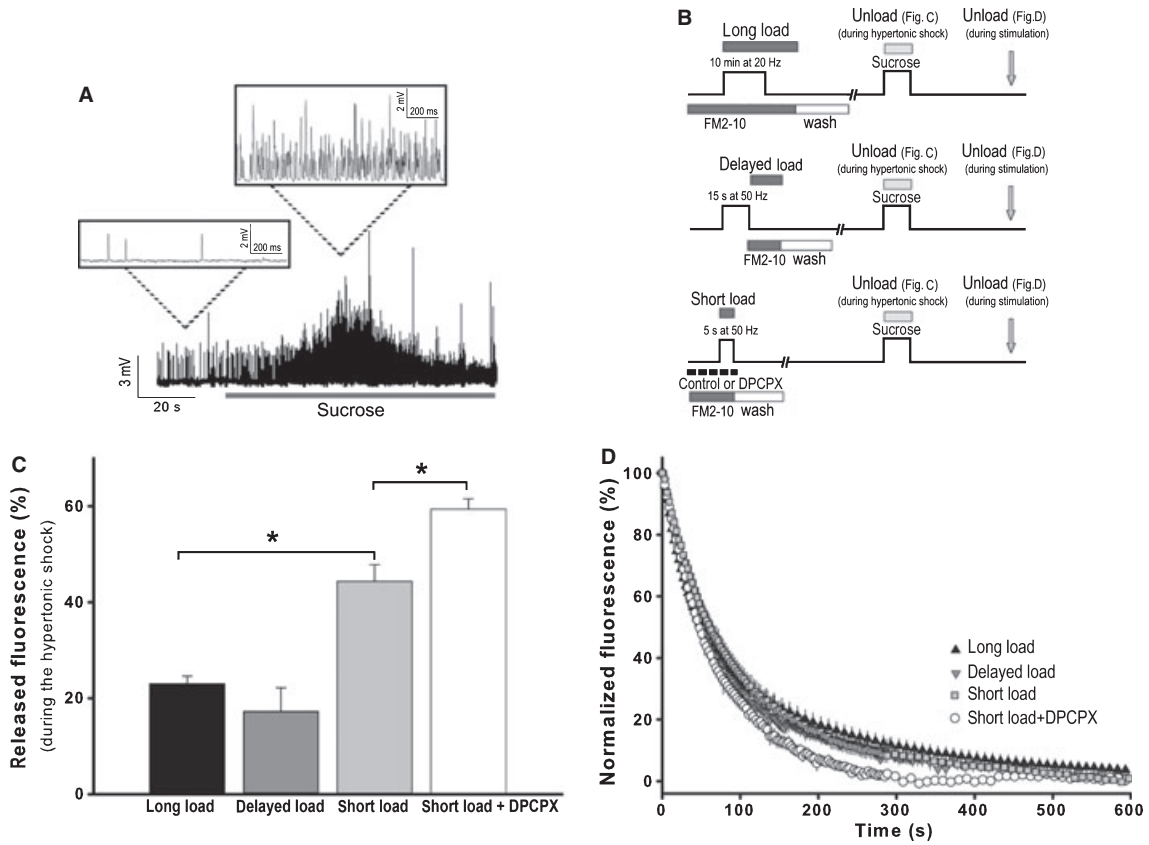

FIG. 5. The RRP is mainly but not exclusively an integral part of the fast-release vesicle pool. Vesicles are recycled towards this pool in the absence of endogenous adenosine. (A) Postsynaptic response to hypertonic sucrose application (bar). This treatment selectively releases the RRP. (B) Schematic diagrams of loading and unloading protocols. NMJs were labeled with the long (n = 5), delayed (n = 3) and short loading protocols. The latter protocol was performed in the absence (n = 5) and presence of DPCPX (0.1 μM) (n = 4). (C) After loading, nerve terminals were exposed to a sucrose-containing solution (500 mOsm, 30 s) to release the RRP. Mean percentages of released fluorescence during the hypertonic treatments were plotted. *$P < 0.05$, Student's t-test. (D) After sucrose addition, unloading was examined during 50 Hz stimulation. Destaining curves were corrected for the percentage of fluorescence that remained after stimulation (%F^R) and scaled to their initial values.

vesicles are placed within the RRP as a consequence of the preferential refilling of the fast-release vesicle pool. Likewise, the fraction of fluorescence released by the hypertonic shock increased by 34% in nerve terminals loaded with the short loading protocol in the presence of DPCPX in comparison to controls loaded with the same protocol ($t_7 = -2.48$, $P = 0.04$; Fig. 5C). In addition, after sucrose treatment, the remaining fluorescence was released by high-frequency stimulation (Fig. 5D). Even after sucrose treatment, the remaining fluorescence of nerve terminals labeled in the presence of DPCPX during the short loading protocol decayed with faster kinetics.

Hypertonic shocks were also applied at NMJs loaded with the delayed protocol. The hypertonic shock was able to release 17% of the dye, suggesting that vesicles from the slow-recycling pool could also be released in a Ca^{2+} -independent manner.

These results reinforce the idea that all vesicles recycled in the absence of inhibitory effects of endogenous adenosine refill the fast-release vesicle pool, which in turn replenishes the RRP, and imply that the RRP defined by sucrose application is mainly, but not exclusively, an integral part of the fast-recycling pool.

Adenosine action does not involve a direct effect on the L-type channel

In a previous work, we found that the blockage of L-type channels decreases the endocytosis of synaptic vesicles and directs newly formed ones to a slow-release vesicle pool during high-frequency stimulation (Perissinotti *et al.*, 2008). As adenosine and the blockage of L-type channels have a similar effect on vesicle recycling, this effect could be explained by the inhibition of L-type channels by adenosine. We therefore decided to test this hypothesis.

In the absence of L-type calcium influx, QC release and endocytosis were diminished by approximately 15 and 40%, respectively, and synaptic vesicles were retrieved towards a slow-release vesicle pool (Perissinotti *et al.*, 2008). In order to test the effects of adenosine on L-type channels, we calculated the QC release elicited by 50 Hz trains before and after nitrendipine (L-type calcium blocker, 10 μM) application during the following treatments: control, adenosine (200 μM) and DPCPX (0.1 μM). The train duration (1 s) was long enough to observe the effects of nitrendipine on QC release. We observed that there were no significant differences in the percentage of QC reduction after nitrendipine application between treatments ($F_{2,9} = 0.54$, $P = 0.6$; Fig. 6), suggesting that adenosine does not inhibit the L-type channel.

Discussion

The present study provides insights into the modulation of vesicle recycling by adenosine at mouse NMJs. Our findings show that, apart from the presynaptic effect of adenosine on the exocytosis process, adenosine also has a role in vesicle retrieval; adenosine decreases

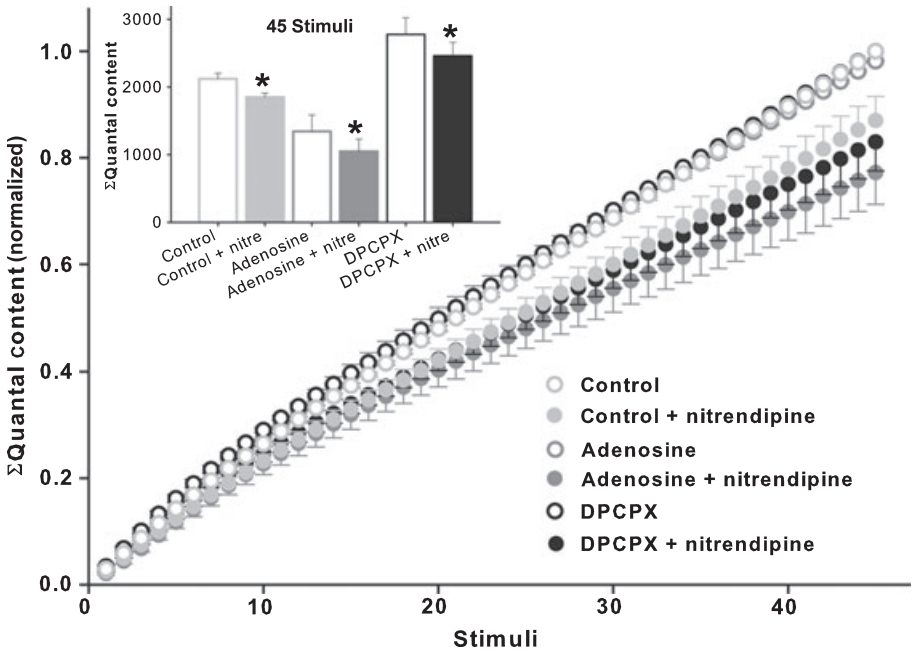

FIG. 6. Adenosine does not inhibit the L-type channel. QC release during 45 pulses at 50 Hz was recorded in the same fiber before and after nitrendipine application (10 μM) in control (n = 4), adenosine (200 μM, n = 5) and DPCPX (0.1 μM, n = 3) treatments. The QC was summed successively and normalized to the total QC obtained at the end of the train before nitrendipine application. Inset: mean QC for 45 stimuli at 50 Hz before and after nitrendipine treatments. *$P < 0.05$, Student's paired t-test (control: $t_3 = 4.05$, $P = 0.01$; adenosine: $t_4 = 2.64$, $P = 0.03$; DPCPX: $t_2 = 2.15$, $P = 0.049$).

transmitter release and directs recycled vesicles towards a slow-release vesicle pool.

A complete explanation of the modulation of vesicle recycling by adenosine should consider both its effect on the exocytosis process and the coupling of this process with endocytosis. In other words, endogenous adenosine may determine activity-dependent differences in vesicle recycling, which arise from the tight coupling between exocytosis and endocytosis that is needed to sustain neurotransmission. Under conditions where the rate of vesicle fusion is high, this coupling step is likely to be rate-limiting and therefore a sensitive point for regulation.

Our electrophysiology studies have shown that adenosine inhibits the impulse-evoked and spontaneous transmitter release at mouse NMJs as already reported for mouse (Nagano *et al.*, 1992; De Lorenzo *et al.*, 2004; Silinsky, 2004), frog (Ribeiro & Walker, 1975; Silinsky, 1984; Bennett *et al.*, 1991; Meriney & Grinnell, 1991; Redman & Silinsky, 1994) and rat (Ribeiro & Walker, 1975; Hamilton & Smith, 1991) NMJs. Indeed, exogenous adenosine decreases the QC at the first evoked potential and increases the characteristic time of synaptic depression, showing its inhibitory effect on the exocytosis process. Studies using selective adenosine agonists and antagonists have demonstrated that presynaptic inhibitory effects of adenosine are mediated mainly by A_1 G-protein-coupled receptors (Nagano *et al.*, 1992; Hirsh & Silinsky, 2002). In our experiments, despite the fact that the A_1 receptor antagonist (DPCPX) did not affect the kinetic parameters of synaptic depression, an increment of QC release for the first stimuli and the cumulative transmitter output during the train that induced synaptic depression was observed. These results suggest that endogenous adenosine, acting on A_1 receptors, can restrict vesicular exocytosis. Furthermore, the fact that the A_1 receptor antagonist increased the QC release of the first EPP suggests that the basal concentration of endogenous adenosine at the synaptic cleft results from adenosine triphosphate released from muscle fibers (Smith, 1991), Schwann cells (Liu *et al.*, 2005) and/or spontaneous fusion of synaptic vesicles.

It has been demonstrated that endogenous adenosine is a modulator of synaptic depression during high-frequency nerve stimulation (Meriney & Grinnell, 1991). As synaptic depression has been associated with a reduction in release probability, as well as in the availability of synaptic vesicles for release (Betz, 1970; Christensen & Martin, 1970), we studied the adenosine modulation of vesicle recycling. Imaging experiments using FM2-10 showed that endogenous adenosine decreases the size of the recycling vesicle pool in accordance with its inhibitory effect on the QC released. We performed FM2-10 experiments using previously described protocols that preferentially label the fast-release or slow-release vesicle pool (Perissinotti *et al.*, 2008). Such loading protocols were utilized in the absence or presence of exogenously applied adenosine or the A_1 adenosine receptor antagonist (DPCPX) to study the effect of the endogenous adenosine. The results showed that all vesicles retrieved when the effect of endogenous adenosine is blocked are recycled towards the fast-release pool, suggesting that endogenous adenosine favors vesicle recycling towards the slow-release pool. Furthermore, this was also the case in the presence of exogenous adenosine. In summary, adenosine inhibits vesicle recycling towards the fast-release vesicle pool and thus favors vesicle recycling towards the slow-release vesicle pool.

In addition, imaging experiments using hypertonic solutions suggest that the RRP is mainly, but not exclusively, an integral part of the fast-release vesicle pool in accordance with reported findings in hippocampal neurons (Moulder & Mennerick, 2005; Chung *et al.*, 2010). Vesicles retrieved towards the slow-release pool also populate the RRP, contributing to a heterogeneous RRP vesicle population. In these experiments, the RRP is supposed to be released by the application of a high concentration of sucrose, although it is not possible to determine if all vesicles placed in the RRP are in fact released during this treatment. Nevertheless, our results are in line with the findings in frog NMJ, where the RRP is an integral part of a fast-recycling pool

that is also replenished (although slowly) with vesicles from the RP (Richards *et al.*, 2003; Rizzoli & Betz, 2005).

However, we observed that a higher proportion of retrieved vesicles were placed within the RRP as a consequence of the preferential refilling of the fast-release vesicle pool in the presence of the A_1 receptor antagonist. This result suggests that the RRP is mainly supplied with recycled vesicles of the fast-release pool. After a hypertonic treatment, synaptic vesicles sorting in specific vesicle pools did not change, suggesting that endogenous adenosine determines synaptic vesicle recycling towards pathways that differ in their availability of release.

It has been established that vesicles endocytosed after high-frequency stimulation are mainly incorporated into the RP in *Drosophila* (Kuromi & Kidokoro, 2002), frog (Richards *et al.*, 2000) and mouse (Perissinotti *et al.*, 2008) NMJs. Therefore, it can be proposed that endogenous adenosine inhibits vesicle recycling towards the fast-release vesicle pool and retrieves recycled vesicles towards the RP. Whether the fate of the endocytic vesicle is linked to a rapid (kiss and run) or slow (clathrin) type of endocytosis remains to be determined.

The presence of adenosine A_{2A} receptors has been reported at the mouse NMJ (Baxter *et al.*, 2005). Our experiments show that exogenous adenosine decreased QC release by 60%, indicating an inhibitory effect of adenosine on neurotransmission. However, we observed a similar effect of exogenously applied adenosine and the A_1 receptor agonist on vesicle recycling, suggesting a predominant action of adenosine on A_1 receptors.

In a previous work, we found that the blockage of L-type channels decreases the endocytosis of synaptic vesicles and directs newly formed vesicles towards a slow-release vesicle pool during high-frequency stimulation (Perissinotti *et al.*, 2008). Not only the L-type channel blocker but also adenosine drove vesicle recycling towards the slow-release pool. Therefore, an attractive hypothesis was that adenosine might inhibit the L-type channel. In the presence of adenosine or DPCPX, the blockage of the L-type channel decreased exocytosis by 15% during high-frequency stimulation as reported in control conditions (Perissinotti *et al.*, 2008). This result suggests that the activation of the A_1 receptor does not inhibit the L-type channel. Furthermore, it suggests that calcium influx through L-type channels and the effects of adenosine might regulate vesicle recycling in opposite ways acting on a common pathway that directs vesicles towards the fast-release or slow-release pool. However, it has been reported that adenosine inhibits P/Q-type calcium currents at mouse NMJs (Hamilton & Smith, 1991; Silinsky, 2004). Therefore, adenosine and nitrendipine inhibit calcium influx acting on P/Q-type and L-type channels, respectively. Thus, it is possible that a reduction of calcium influx, regardless of which calcium channel was inhibited, may explain the effects observed on vesicle recycling. In accordance with this assumption, it has been established that calcium affects vesicle recycling in a number of systems (Ceccarelli & Hurlbut, 1980; Neher & Zucker, 1993; von Gersdorff & Matthews, 1994; Wang & Zucker, 1998; Perissinotti *et al.*, 2008). A proposed model based on our experimental evidence is shown in Fig. 7.

Recent studies on mouse motor nerve terminals have demonstrated that the inhibitory effect of adenosine is mediated via the SNARE–Ca^{2+} channel complex affecting both P/Q-type calcium currents and vesicle fusion readiness (Silinsky, 2005, 2008; Veggetti *et al.*, 2008). In addition, vesicle membranes have been shown to possess all or some of the presently identified synaptic proteins, including synaptophysin, synaptobrevin, synaptotagmin, SV2, protein 29 and Rab3A. Interestingly, it has been found that the genetic deletion of Rab3A increases the affinity of G$\beta\gamma$ for the SNARE–Ca^{2+} channel complex, altering presynaptic adenosine modulation (Hirsh *et al.*, 2002; Silinsky, 2004, 2008).

However, P/Q-type channels play a role in synaptic vesicle exocytosis through direct interactions with sintaxin, synaptosome-associated protein of 25 kDa and synaptotagmin at the so-called synaptic protein interaction (synprint) site at the α_1-subunit (Sheng *et al.*, 1996; Catterall, 2000). The fact that L-type channels lack the synprint region could explain their position farther from the release

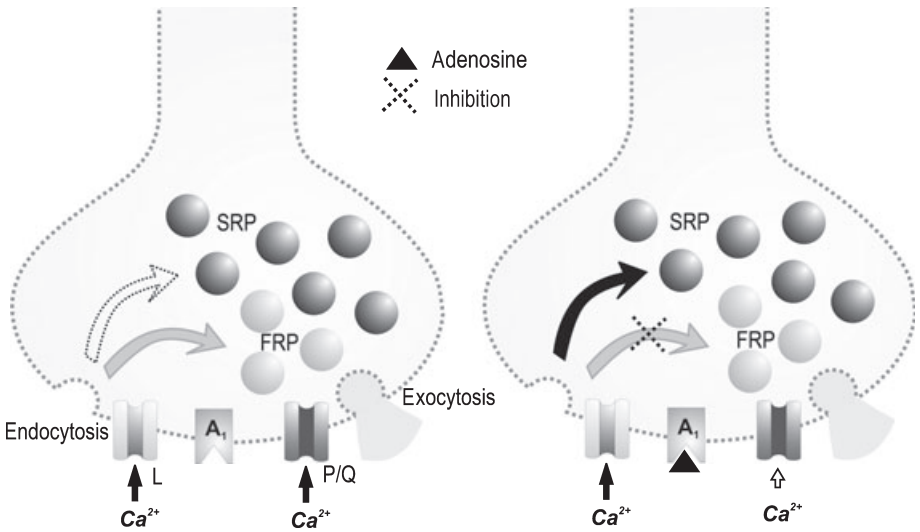

FIG. 7. Proposed model for the recycling of adenosine-driven vesicles towards the slow-release vesicle pool. Scheme of an NMJ showing vesicle pools that differ in their releasability: the fast-release vesicle pool (FRP) (gray) and the slow-release vesicle pool (SRP) (black). At mature mouse NMJ, neurotransmitter release is mediated primarily by P/Q-type (Uchitel *et al.*, 1992; Protti & Uchitel, 1993) and L-type channels calcium influx directs the newly formed vesicles to a fast-release vesicle pool (Perissinotti *et al.*, 2008). During high-frequency stimulation, in the absence of endogenous adenosine, vesicles are recycled towards the FRP (gray arrow) and the recycling towards the SRP is diminished (dotted white arrow). Adenosine prevents the replenishment of the FRP, favoring vesicle recycling towards the SRP (black arrow). In this work we showed that adenosine action does not involve a direct effect on the L-type channel, whereas it has been demonstrated that it reduces P/Q-type calcium currents (small white arrow) (Hamilton & Smith, 1991; Silinsky, 2004).

sites (Polo-Parada *et al.*, 2001; Urbano *et al.*, 2001; Flink & Atchison, 2003) and the lack of effects of adenosine on these channels as was reported in this work.

Considering the effects of adenosine on calcium currents, both synaptic vesicle exocytosis and endocytosis processes are regulated by Ca^{2+}. Voltage-gated Ca^{2+} channels provide pathways for calcium entry during neurotransmission (Uchitel *et al.*, 1992; Takahashi & Momiyama, 1993). Moreover, it has recently been demonstrated that the synprint site of the P/Q-type voltage-gated Ca^{2+} channel is involved in synaptic vesicle endocytosis, rather than exocytosis, via a Ca^{2+} -dependent interaction with an adaptor protein (AP-2) in a clathrin-mediated mechanism (Watanabe *et al.*, 2010). In addition, L-type and P/Q-type channels interact with another protein involved in clathrin-mediated endocytosis (endophilin) and this interaction is also calcium dependent (Chen *et al.*, 2003). Both of these proteins are involved in the fast clathrin-mediated recycling pathway (Morgan *et al.*, 2002; Galli & Haucke, 2004; Voglmaier *et al.*, 2006) and therefore calcium influx, through L-type and P/Q-type channels, regulating these protein interactions, could modulate vesicle recycling.

Although further studies are necessary to elucidate the molecular mechanisms involved in the regulation of vesicle recycling by adenosine, our experiments demonstrate that, at the mouse NMJs, the fate of retrieved synaptic vesicles is modified by the activation of adenosine A_1 receptors, which in turn reduce the size of the fast-release pool of synaptic vesicles at high-frequency stimulation.

Under physiological conditions, high-frequency stimulation starts to operate with a high QC of transmitter release due to the usage of the fast-releasable pool. Due to the fact that the maximal inhibitory effect that adenosine exerts is considerably less than complete inhibition, adenosine generated during the initial period of activity reduces the amount of Ca^{2+} influx and directs vesicles towards the slow-releasable pool. This process assures a lower but sustained level of transmitter release, thus ensuring the rapidity and fidelity of synaptic comunication.

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Abbreviations

CPA, *N*6-cyclopentyl-adenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EPP, evoked endplate potential; NMJ, neuromuscular junction; QC, quantal content; RP, reserve pool; RRP, rapidly releasable pool; S-EPP, spontaneous endplate potential; SNAREs, synaptosome-associated protein receptors.

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