nature neuroscience

Synaptic vesicles recycling spontaneously and during activity belong to the same vesicle pool

Teja W Groemer & Jurgen Klingauf

Recently, it has been claimed that vesicles recycling spontaneously and during activity belong to different pools. Here we simultaneously measured, using spectrally separable styryl dyes, the release kinetics of vesicles recycled spontaneously or upon stimulation and the effects of the v-ATPase blocker folimycin on the frequency of miniature postsynaptic currents in rat hippocampal neurons. Our results provide evidence as to the identities of the vesicle pools recycling at rest and during stimulation.

It is commonly thought that evoked transmission is supported by the same quanta as miniature activity[1]: that is, it is merely the result of a stimulation-enhanced probability of fusion for the very same synaptic vesicles that are also fusing spontaneously. This notion has not been proven directly, but the similar regulation of fusion probability by internal and external calcium[2–4] provides strong indirect evidence to support it. By contrast, it has been proposed recently that vesicles driving spontaneous neurotransmission belong to a separate pool that is only reluctantly released on stimulation[5]. This hypothesis is based on the observation that FM styryl dyes taken up during resting are released much more slowly than those taken up during activity. If correct, this could markedly change scientists' views on the relationship between spontaneous and evoked transmission, as a spontaneously recycling pool might possess its own characteristic subset of proteins or at least bear them in a different state. However, it completely contradicts an earlier study[3] in which spontaneously FM-labeled synaptic boutons were found to have slightly enhanced dye release rates on stimulation compared with those labeled by action potentials (APs). Furthermore, the same study showed that the frequency of miniature synaptic activity is a reliable index for the evoked release efficacy.

Obviously, both modes of fusion can be differentially affected by mutations or genetic ablations of molecules that are important for vesicle fusion. For example, knockout of synaptotagmin 1, the putative calcium sensor, or of the SNARE complex–binding complexin results in greatly decreased evoked release rates but equal or even enhanced spontaneous release rates[6,7]. But such differential effects do not, contrary to what has been recently postulated[5,8], require the existence of an isolated vesicle population. The dual effects of

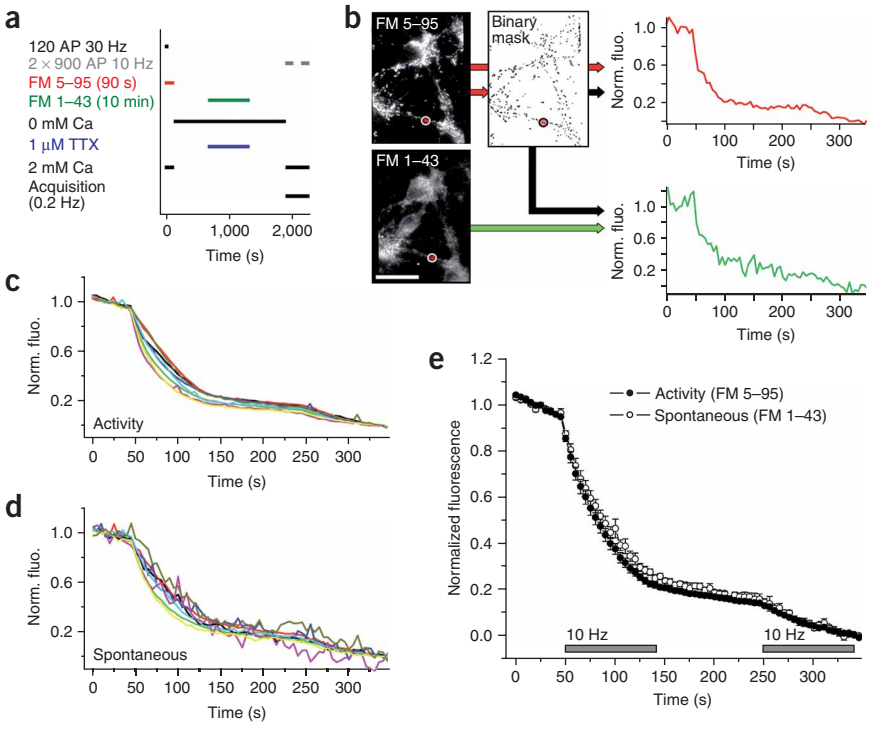

Figure 1 Simultaneous measurement of release kinetics of synaptic vesicles stained by styryl dyes both spontaneously and during stimulation. (**a**) Experimental procedure: boutons of rat hippocampal neurons were first labeled with FM 5–95 by 120 APs and then, after washout, with FM 1–43 by spontaneous uptake during a 10-min period. Both vesicle pools, those labeled spontaneously and by APs, were destained simultaneously at $t = 50$ s and $t = 250$ s with 900 APs at 10 Hz. (**b**) Image analysis: a binary mask of bouton-sized spots where AP-evoked dye loss occurred was created from the FM 5–95 image stack and deployed to select the very same spots blindly in the FM 1–43 image stack for spontaneous labeling. Scale bar, 25 μm. (**c**,**d**) Normalized average fluorescence destaining profiles of individual experiments for FM 5–95 (**c**, AP loading) and FM 1–43 (**d**, spontaneous loading). (**e**) Average fluorescence destaining profiles (closed circles, AP load; open circles, spontaneous load) from all experiments depicted in **c** and **d** (± s.e.m., $N = 8$, comprising $n = 916$ boutons).

Department of Membrane Biophysics, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Goettingen, Germany. Correspondence should be addressed to J.K. (J.Klingauf@mpi-bpc.mpg.de).

Received 8 August 2006; accepted 13 December 2006; published online 14 January 2007; doi:10.1038/nn1831

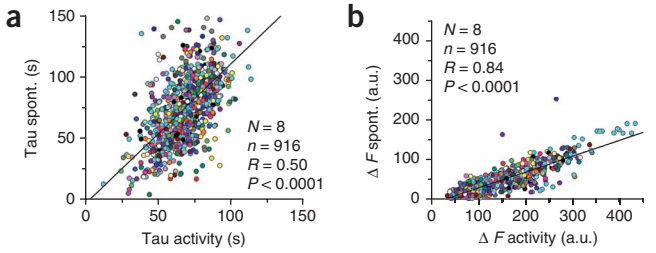

Figure 2 Release kinetics and fluorescence amplitudes are highly correlated for spontaneous and activity-dependent recycling at individual boutons, but vary highly between boutons. (**a**) Correlogram of time constants (Tau) of exponential fits for single bouton destaining profiles ($n = 916$) of all experiments ($N = 8$, color coded). (**b**) Correlogram of fluorescence amplitudes (ΔF) of single boutons from all experiments (color coded). a.u., arbitrary units.

synaptotagmin 1 and complexin could be explained if they served both as fusion triggers or enhancers during stimulation and as fusion clamps at rest[9].

On the other hand, spontaneous transmission does not merely seem to represent background noise in the nervous system, but is necessary for the stabilization of synaptic function via the inhibition of dendritic protein synthesis, maintaining dendritic spines and postsynaptic receptor clustering[10,11]. Therefore, delineating the origin and regulation of miniature synaptic signaling is of great interest.

Here we simultaneously measured the release rates of styryl dyes taken up spontaneously and during stimulation, using a recently developed[12] dual-color approach with two spectrally different FM dyes. Release kinetics for both vesicle populations can be monitored simultaneously at single boutons, thereby avoiding artifacts that may arise from sequential stainings and destainings, such as endosomal dye accumulation or bouton drift, which would both result in a decrease of the signal-to-noise ratio (**Supplementary Discussion**). We stained rat hippocampal neurons with the red dye FM 5–95 by 120 APs at 30 Hz and then, after washout, allowed them to take up the green FM 1–43 for 10 min under resting conditions (1 μM TTX, nominally 0 mM Ca^{2+}, see **Supplementary Methods**). We then evoked release with two trains of 900 APs at 10 Hz (**Fig. 1a**). Emitted photons were spectrally split and projected on either half of the charge-coupled device chip, yielding simultaneous images for both dyes (**Fig. 1b**, images). The image stack for FM 5–95 (AP loading) was used to automatically detect spots[13] of synaptic bouton size where AP-evoked dye loss had occurred and to build a binary mask (**Fig. 1b**, mask). This mask was then applied to both image stacks (for FM 5–95 and FM 1–43) to extract the fluorescence destaining profiles of single boutons (**Fig. 1b**, traces). We found that the fractional release rates for individual experiments (**Fig. 1c**,**d**) and for both labeling conditions were virtually identical (**Fig. 1e**). Their exponential fit time constants of destaining were not significantly different ($\tau_{120AP} = 69.5 \pm 3.3$ s; $\tau_{spont} = 78 \pm 4.8$ s; mean ± s.e.m. $P > 0.1$) and had a high correlation, even at the level of individual boutons ($R = 0.50$; $P < 0.0001$, **Fig. 2a**), where the poor signal-to-noise ratio of profiles for spontaneous staining may easily have led to a breakdown of correlation—results that highlight the power of simultaneous dual-color measurements. In fact, one can easily show (**Supplementary Discussion** and **Fig. 1**) that a poor signal-to-noise ratio, together with the erroneous normalization of fluorescence signals of spontaneously labeled boutons, results in slowly and monotonically declining profiles, similar to those that led to the proposal that a separate pool exists[5]. Our findings of high correlations for both fractional release rates and fluorescence amplitudes ($R = 0.84$; $P < 0.0001$) (**Fig. 2b**) for both staining paradigms at the single-bouton level strongly suggests the contrary, however.

The v-ATPase blocker folimycin is a convenient tool for differentiating between putative pools of vesicles[5] that recycle spontaneously and those that recycle in an activity-dependent manner. If there was indeed a small, separate pool of vesicles that spontaneously fuse and recycle in a few tens of seconds, then this pool should be effectively depleted of neurotransmitter by folimycin application in the absence of action potentials. In fact, folimycin treatment significantly reduces mPSC frequency in 10 min[5]. We applied folimycin 10 min before and during whole-cell recording. Miniature events were detected using a semiautomated scaled template algorithm[14]. We found that folimycin affected neither the frequency nor the amplitude of mPSCs, even within 30 min at room temperature (approximately 23–25 °C; **Supplementary Discussion**). To test whether miniature events are depletable by activity, contrary to the expectation if a separate pool of spontaneously recycling vesicle existed, we stimulated with a 47 mM K^+ solution twice for 60 s during folimycin treatment and before whole-cell recording. We observed a significant tenfold reduction in mPSC frequency, but not amplitude (**Fig. 3**). Our results agree with an earlier observation in cultured autaptic hippocampal neurons that mEPSC frequency and evoked responses are equally affected by v-ATPase blockers[15].

Taken together, our FM dye experiments, as well as the whole-cell recordings showing cross-depletion of the spontaneous pool by activity, provide evidence for the theory that activity-dependent and spontaneous vesicle turnover draw upon the same pool of vesicles.

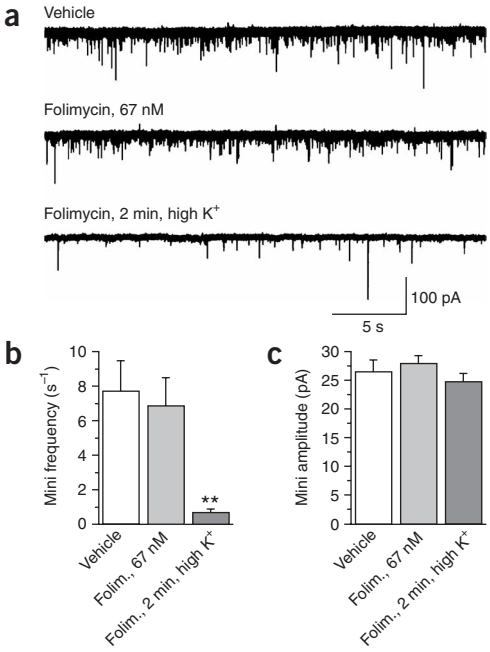

Figure 3 Folimycin decreases the frequency of spontaneous release events in an activity-dependent manner. (**a**) Sample traces from whole-cell recordings of rat hippocampal neurons during the application of vehicle (0.2% DMSO; $n = 10$), 67 nM folimycin ($n = 11$) or 67 nM of folimycin with two 1-min 47 mM K^+ stimulations ($n = 8$). (**b**,**c**) Average mEPSC frequencies (**b**) and amplitudes (**c**) for the three recording conditions (± s.e.m., asterisks indicate $P < 0.01$, two-sample independent *t*-test against both other conditions).

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We are grateful to H. Taschenberger for extensive help with miniature analysis, M. Wienisch for advice in imaging analysis and E. Neher for critical reading of the manuscript. We thank all laboratory members for their support, M. Pilot for expert technical assistance and S. Gliem and T. Frank for participating in some of the initial experiments. This work was supported by a grant from the Human Frontier Science Project (J.K).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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