Inter-Bouton Variability of Synaptic Strength Correlates With Heterogeneity of Presynaptic Ca\(^{2+}\) Signals

SERGEI KIRISCHUK AND ROSEMARIE GRANTYN
Developmental Physiology, Johannes Müller Institute of Physiology, Humboldt University Medical School (Charité), 10117 Berlin, Germany

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The elevation of presynaptic calcium concentration is a crucial step in excitation-secretion coupling. However, the amplitudes of action-potential-induced presynaptic calcium transients can display high variability among different terminals. The aim of this study was to clarify whether, at individual boutons, synaptic strength correlates with the average amplitude of presynaptic calcium transients. Low-density collicular cultures were loaded with the calcium indicator Oregon Green bis-(o-aminophenoxo)-N,N',N''-tetraacetic acid (BAPTA) 1. Action potentials were blocked with tetrodotoxin. Presynaptic terminals were identified with FM4–64, a use-dependent vesicle marker. Presynaptic calcium influx was elicited by a focal electrical stimulation of single boutons. Whole cell patch-clamp and calcium imaging techniques were used to record GABAergic evoked inhibitory post synaptic currents (eIPSCs) and presynaptic fluorescence changes in the stimulated terminal. To make the eIPSCs from different boutons comparable, they were normalized to the mean value of miniature IPSCs (mIPSCs) of the postsynaptic cell. Records from 47 boutons showed that eIPSCs varied between 0.5 and 3.0 and presynaptic calcium transients varied between 0.1 and 1.3. However, there was a strong correlation between the mean amplitudes of eIPSCs and presynaptic calcium responses. The eIPSC-[Ca\(^{2+}\)]\(_{\text{pre}}\) relationship allows to use the amplitudes of presynaptic calcium transients as an indicator of release efficacy and, in a set of contacts made by one axon, to predict the relative impact of individual terminals.

INTRODUCTION

An action potential (AP)-induced depolarization of the plasma membrane results in a transient rise of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{pre}}\)). Domains of high [Ca\(^{2+}\)]\(_{\text{pre}}\) activate the exocytotic fusion of presynaptic vesicles with the plasma membrane and the release of neurotransmitter (for review, see Katz 1969; Neher 1998). Until recently, geometric constraints limited studies of presynaptic [Ca\(^{2+}\)]\(_{\text{pre}}\) dynamics in single CNS terminals to a few preparations with unusually large presynaptic boutons like mossy fiber terminals in the hippocampus (Liang et al. 2002; Regehr and Tank 1991) and the calyces of Held (Helmchen et al. 1997). However, by using high-resolution optical fluorescence microscopy, studies were extended to single axons of cortical neurons (Cox et al. 2000; Koester and Sakmann 2000; Mackenzie et al. 1996) and cerebellar basket cells (Llano et al. 1997). It was demonstrated that APs or bursts of APs reliably propagate through the axon arbor, which eliminates signal propagation failures as a major source of variability of synaptic responses, at least at those synapses. Surprisingly, AP-induced Ca\(^{2+}\) transients in different boutons varied over a wide range even when the release sites resided on the same axon collateral (Koester and Sakmann 2000; Llano et al. 1997). In individual terminals, there is a power relationship between Ca\(^{2+}\) influx and transmitter release (Dodge and Rahamimoff 1967). High-resolution optical fluorescence microscopy and a new generation of Ca\(^{2+}\) indicators (especially, genetically encoded Ca\(^{2+}\) dyes (Kerr et al. 2000)) provide a possibility to study presynaptic Ca\(^{2+}\) signals at the level of individual terminals in vivo. However, it is not yet known to what extent inter-bouton differences in the presynaptic Ca\(^{2+}\) signals also predict differences in synaptic strength. To answer this question is important because, if such a correlation existed, it would be possible to characterize spatial gradients in synaptic strength on the basis of presynaptic Ca\(^{2+}\) transients.

Unfortunately, simultaneous recording of postsynaptic responses and [Ca\(^{2+}\)]\(_{\text{pre}}\) measurements at individual synaptic contacts is often hampered by the small diameter of axon terminals in the CNS. However in low-density cultures, individual boutons can be activated by mimicking AP generation by a focal electrical pulse (Kirischuk et al. 2002). Presynaptic Ca\(^{2+}\) transients ([Ca\(^{2+}\)]\(_{\text{pre}}\)) and evoked inhibitory postsynaptic currents (eIPSCs) were recorded in parallel using digital imaging and whole cell patch-clamp techniques (Kirischuk et al. 1999b). By applying a standard low-frequency stimulation protocol to different boutons, we sought to determine whether a correlation exists between the average amplitudes of [Ca\(^{2+}\)]\(_{\text{pre}}\) and the average amplitudes of eIPSCs, a common indicator of synaptic strength.

METHODS

Culture preparation

Cell cultures were prepared as described previously (Perouansky and Grantyn 1989). Neonatal rats were anesthetized with halothane before decapitation. Superior colliculi of embryonic day 21 rats were removed, dissociated, and plated. The neurons were grown at low density collicular cultures were loaded with the calcium indicator Oregon Green bis-(o-aminophenoxo)-N,N',N''-tetraacetic acid (BAPTA) 1. Action potentials were blocked with tetrodotoxin. Presynaptic terminals were identified with FM4–64, a use-dependent vesicle marker. Presynaptic calcium influx was elicited by a focal electrical stimulation of single boutons. Whole cell patch-clamp and calcium imaging techniques were used to record GABAergic evoked inhibitory post synaptic currents (eIPSCs) and presynaptic fluorescence changes in the stimulated terminal. To make the eIPSCs from different boutons comparable, they were normalized to the mean value of miniature IPSCs (mIPSCs) of the postsynaptic cell. Records from 47 boutons showed that eIPSCs varied between 0.5 and 3.0 and presynaptic calcium transients varied between 0.1 and 1.3. However, there was a strong correlation between the mean amplitudes of eIPSCs and presynaptic calcium responses. The eIPSC-[Ca\(^{2+}\)]\(_{\text{pre}}\) relationship allows to use the amplitudes of presynaptic calcium transients as an indicator of release efficacy and, in a set of contacts made by one axon, to predict the relative impact of individual terminals.

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density (about 5,000 cells/cm²), on laminin-coated glass coverslips. Experiments were performed after 14–36 days in vitro. All experiments were carried out according to the guidelines laid down by the Animal Care and Use Committee.

**Imaging**

The detailed description of the method is given elsewhere (Kirischuk et al. 1999b). Briefly, cultures were loaded with Oregon Green bis-(o-aminophenoxy)-N,N′,N,N′-tetraacetic acid (BAPTA) 1 AM (OGB-1-AM, 5 μM, 20 min at 36°C). Synaptic vesicles were stained with a fluorescent marker FM4–64 in two steps: first, 1-min incubation in a solution containing high potassium (50 mM) and FM4–64 (50 μM) and then incubation in the standard extracellular solution containing only FM4–64 for another minute. The coverslip with the stained cultures formed the bottom of a recording chamber on the stage of an inverted microscope (Axiovert 100, Zeiss, Jena, Germany). A ×100 phase contrast oil-immersion objective (1.3 NA, Zeiss) was used. The excitation wavelength was controlled by a fast monochromator system, and fluorescence signals were recorded using a CCD camera (TILL Photonics, Munich, Germany). The probes were excited at 490 nm. The excitation and emission light was separated using a 510-nm dichroic mirror. The emitted light was filtered at 550 ± 30 nm for OGB-1 and at 600 nm for FM4–64. All [Ca²⁺] measurements were performed using 4 × 4 binning (1 pixel - 0.4 × 0.4 μm), the acquisition rate was set to 1 image/10 ms. Several phase contrast (binning 1 × 1) and FM4–64 images were captured at the beginning of each experiment. The former was used to calculate bouton cross-sections. The binary FM4–64 image was used as a mask to define the region of interest for subsequent OGB-1 images. The background fluorescence was determined from a region in the immediate vicinity of the stimulated bouton and subtracted. [Ca²⁺]pre is defined as the peak amplitude of individual fluorescence signals ΔF/F₀, where F₀ is the prestimulus intensity of OGB-1. When a high-affinity Ca²⁺ indicator such as ORG-1 is used for fluorescence measurements, amplitudes of individual Ca²⁺ transients could be underestimated due to the saturation of the dye. Moreover, with increasing Ca²⁺ influx, the underestimation will be larger. This problem could seriously interfere with our current task. Therefore we examined the relationship between the average (ΔF/F₀) and maximal [(ΔF/F₀)max] Ca²⁺ transients, as induced by a single pulse or a pulse train at 10 Hz. However, even in the case of the largest single pulse-induced Ca²⁺ transient (ΔF/F₀ = 1.1) in a set of 10 boutons, the fluorescence change was only 50% of the level reached by the 10-Hz stimulation. We therefore concluded that the amplitudes of presynaptic Ca²⁺ transients are not distorted by saturation of ORG-1.

**Recording**

Whole cell patch-clamp recordings were performed using glass pipettes containing (in mM): 100 K-gluconate, 50 KCl, 5 NaCl, 2 MgCl₂, 1 CaCl₂, 10 EGTA, and 20 HEPES-KOH (pH 7.2). Signals were acquired at 10 kHz using an EPC-7 patch-clamp amplifier. Measurement amplitudes of individual Ca²⁺ transients could be underestimated due to the saturation of the dye. Moreover, with increasing Ca²⁺ influx, the underestimation will be larger. This problem could seriously interfere with our current task. Therefore we examined the relationship between the average (ΔF/F₀) and maximal [(ΔF/F₀)max] Ca²⁺ transients, as induced by a single pulse or a pulse train at 10 Hz. However, even in the case of the largest single pulse-induced Ca²⁺ transient (ΔF/F₀ = 1.1) in a set of 10 boutons, the fluorescence change was only 50% of the level reached by the 10-Hz stimulation. We therefore concluded that the amplitudes of presynaptic Ca²⁺ transients are not distorted by saturation of ORG-1.

![Image of presynaptic [Ca²⁺] and evoked inhibitory postsynaptic current (eIPSC) recordings. A: phase contrast and Oregon Green bis-(o-aminophenoxy)-N,N′,N,N′-tetraacetic acid (BAPTA) 1 AM (OGB-1-AM, 5 μM, 20 min at 36°C). Synaptic vesicles were stained with a fluorescent marker FM4–64 in two steps: first, 1-min incubation in a solution containing high potassium (50 mM) and FM4–64 (50 μM) and then incubation in the standard extracellular solution containing only FM4–64 for another minute. The coverslip with the stained cultures formed the bottom of a recording chamber on the stage of an inverted microscope (Axiovert 100, Zeiss, Jena, Germany). A ×100 phase contrast oil-immersion objective (1.3 NA, Zeiss) was used. The excitation wavelength was controlled by a fast monochromator system, and fluorescence signals were recorded using a CCD camera (TILL Photonics, Munich, Germany). The probes were excited at 490 nm. The excitation and emission light was separated using a 510-nm dichroic mirror. The emitted light was filtered at 550 ± 30 nm for OGB-1 and at 600 nm for FM4–64. All [Ca²⁺] measurements were performed using 4 × 4 binning (1 pixel - 0.4 × 0.4 μm), the acquisition rate was set to 1 image/10 ms. Several phase contrast (binning 1 × 1) and FM4–64 images were captured at the beginning of each experiment. The former was used to calculate bouton cross-sections. The binary FM4–64 image was used as a mask to define the region of interest for subsequent OGB-1 images. The background fluorescence was determined from a region in the immediate vicinity of the stimulated bouton and subtracted. [Ca²⁺]pre is defined as the peak amplitude of individual fluorescence signals ΔF/F₀, where F₀ is the prestimulus intensity of OGB-1. When a high-affinity Ca²⁺ indicator such as ORG-1 is used for fluorescence measurements, amplitudes of individual Ca²⁺ transients could be underestimated due to the saturation of the dye. Moreover, with increasing Ca²⁺ influx, the underestimation will be larger. This problem could seriously interfere with our current task. Therefore we examined the relationship between the average (ΔF/F₀) and maximal [(ΔF/F₀)max] Ca²⁺ transients, as induced by a single pulse or a pulse train at 10 Hz. However, even in the case of the largest single pulse-induced Ca²⁺ transient (ΔF/F₀ = 1.1) in a set of 10 boutons, the fluorescence change was only 50% of the level reached by the 10-Hz stimulation. We therefore concluded that the amplitudes of presynaptic Ca²⁺ transients are not distorted by saturation of ORG-1.

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(HEKA, Lambrecht, Germany). Series resistance was compensated ≤70%.

**Stimulation**

All experiments were carried out on well-isolated GABAergic axo-dendritic boutons (Fig. 1A). A glass stimulation pipette filled with the standard extracellular solution (8–12 MΩ) was placed close (~1 μm) to a FM4–64-labeled spot. Presynaptic calcium transients and eIPSCs were evoked by a depolarizing electrical pulse. An isolated stimulation unit was used to generate electrical stimuli. Stimulation frequency was 0.2 Hz. The duration of stimuli was set to 2 ms. The values of [Ca\(^{2+}\)]\(_{\text{pre}}\) displayed a bell-shaped dependency on the stimulus intensity (Fig. 1B) (Kirischuk et al. 1999a). In any given bouton, we therefore first determined the pulse intensity giving the maximal value of [Ca\(^{2+}\)]\(_{\text{pre}}\) (usually between 1.5 and 2 μA) and then used this intensity for the rest of the protocol (Fig. 1C). Unfortunately, [Ca\(^{2+}\)]\(_{\text{pre}}\) decreased due to bleaching, whereas the average amplitude of eIPSCs remained stable throughout the experiment (Fig. 1D). Therefore to determine the mean [Ca\(^{2+}\)]\(_{\text{pre}}\), only the first 40 Ca\(^{2+}\) transients were used. The distribution of individual [Ca\(^{2+}\)]\(_{\text{pre}}\) was close to Gaussian (Fig. 1E). The mean coefficient of variation (CV) was 0.24 ± 0.02 (mean ± SD; range from 0.11 to 0.41, n = 47). The eIPSCs fluctuated more strongly than [Ca\(^{2+}\)]\(_{\text{pre}}\) (CV between 0.26 and 1.9, Fig. 1F). To make the values obtained from different boutons comparable, eIPSCs were normalized to the mean amplitude of miniature IPSCs (mIPSCs) from the same postsynaptic cell. The mean amplitude of mIPSCs varied from cell to cell (between 12 and 56 pA).

Amplitude histograms of mIPSCs were only slightly skewed to the right (CV: between 0.35 and 0.6, Fig. 1G), large-amplitude mIPSCs from the same postsynaptic cell. The mean amplitude quantal content) can then be calculated in two ways, as the number of observations divided by the number of failure traces (Korn and Faber 1987). Figure 2E shows that the m values determined either ways nearly coincided (linear slope of 0.98 ± 0.04, r = 0.94, P < 0.0001). As m was mostly more than 1, these results indicate that the release was multiquantal even in physiological [Ca\(^{2+}\)]. A strong correlation (Fig. 2E) between the m values obtained from the eIPSC/mIPSC ratio and the failure rate (which is not dependent on the mean amplitude of mIPSCs) renders additional support to our normalization method, which requires a reliable estimate of the average mIPSC amplitude.

Next we asked whether mean [Ca\(^{2+}\)]\(_{\text{pre}}\) correlated with mean eIPSC amplitudes. Indeed, the slope of the regression line was positive, and the correlation was significant (r = 0.54, P < 0.0001, Fig. 3A). To clarify whether the correlation could also be described as a power function, we replotted the graph in logarithmic coordinates. The slope of the regression line was positive (0.4 ± 0.1) and the correlation was significant (r = 0.47, P < 0.0001, Fig. 3B). Neither the fluorescence change (Fig. 3C) nor the mean eIPSC/mIPSC ratio, i.e., the mean quantal content (Fig. 3D), correlated

**RESULTS**

The study was performed on 47 axo-dendritic boutons. There was a high inter-bouton variability in the average [Ca\(^{2+}\)]\(_{\text{pre}}\). It ranged from 0.06 to 1.3 (mean: -0.54 ± 0.08, CV = 0.56, Fig. 2A). The average amplitudes of eIPSCs and the fraction of failures were also highly variable (Fig. 2, B and C). The values for the total population (mean, CV, range) were: 1.4 ± 0.1, 0.49, and 0.1–3.1 and 0.33 ± 0.03, 0.55, and 0.02–0.73 for eIPSCs and failure rates, respectively. As the mean amplitude of eIPSCs (excluding failures) was significantly larger than 1 (average: 1.95, Fig. 2D), we concluded that boutons were released more than one single vesicle in response to a standard pulse. This could be further tested by applying Poisson’s statistics (Isaacson and Walmsley 1995; Sahara and Takahashi 2001). The functionally relevant parameter m (average quantal content) can then be calculated in two ways, as the mean eIPSC/mIPSC ratio or as the natural logarithm of the number of observations divided by the number of failure traces (Korn and Faber 1987). Figure 2E shows that the m values correlated with the mean eIPSC amplitudes. Indeed, the slope of the regression line was positive, and the correlation was significant (r = 0.54, P < 0.0001, Fig. 3A). To clarify whether the correlation could also be described as a power function, we replotted the graph in logarithmic coordinates. The slope of the regression line was positive (0.4 ± 0.1) and the correlation was significant (r = 0.47, P < 0.0001, Fig. 3B). Neither the fluorescence change (Fig. 3C) nor the mean eIPSC/mIPSC ratio, i.e., the mean quantal content (Fig. 3D), correlated

**Superfusion**

A slow super-fusion system (0.5 ml/min) was used. The extracellular solution contained (in mM) 140 NaCl, 3 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 20 HEPES, and 30 glucose plus 1 μM tetrodotoxin, 50 μM DL-2-amino-5-phosphonopentanoic acid (APV), and 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) (pH = 7.4). OGB-1-AM, OGB-1-M6, M6,7-Di2-AM, and FM4–64 were obtained from Molecular Probes (Eugene, OR); all other chemicals were from Sigma-Aldrich (Deisenhofen, Germany). All experiments were performed at room temperature (23–25°C). All results are presented as means ± SE unless otherwise stated.

**FIG. 2.** Variability of pre- and postsynaptic parameters between boutons. Distribution of mean [Ca\(^{2+}\)]\(_{\text{pre}}\) (A), mean amplitudes of eIPSCs (B), failure rates (C), and mean amplitudes of eIPSCs excluding failures (D) in the population of boutons. In B and D, eIPSCs were normalized to the mean amplitude of miniature IPSCs (mIPSCs). E: correlation between m’s (Poisson’s distribution parameter) calculated in 2 different ways. - - -, linear fit; the line slope was 0.98.

**FIG. 3.** Variability of pre- and postsynaptic parameters between boutons. Distribution of mean [Ca\(^{2+}\)]\(_{\text{pre}}\) (A), mean amplitudes of eIPSCs (B), failure rates (C), and mean amplitudes of eIPSCs excluding failures (D) in the population of boutons. In B and D, eIPSCs were normalized to the mean amplitude of miniature IPSCs (mIPSCs). E: correlation between m’s (Poisson’s distribution parameter) calculated in 2 different ways. - - -, linear fit; the line slope was 0.98.
with the resting fluorescence level \((P > 0.05)\). Thus the mean amplitude of presynaptic \(\mathrm{Ca}^{2+}\) transients is an indicator of synaptic strength.

**DISCUSSION**

In a population of 47 boutons, we investigated the inter-bouton variability of eIPSCs and presynaptic \(\mathrm{Ca}^{2+}\) responses and tested for a correlation between these two parameters. It was found that both eIPSCs and \([\mathrm{Ca}^{2+}]_{\text{pre}}\) displayed a high inter-bouton variability and that boutons with larger average \([\mathrm{Ca}^{2+}]_{\text{pre}}\) values generated larger mean eIPSCs amplitudes.

As we measured time- and volume-averaged \([\mathrm{Ca}^{2+}]_r\), several factors can lead to a variability of \([\mathrm{Ca}^{2+}]_{\text{pre}}\). First, boutons may differ in their surface-to-volume ratio. However, to produce the observed differences in \([\mathrm{Ca}^{2+}]_{\text{pre}}\), the radius has to vary over the same wide range because the surface-to-volume ratio changes with \(r^{-1}\) (\(r\): radius). We did not see such a wide range of radiiues. Bouton diameters ranged from 11 to 21 pixels, and no correlation was found between mean \([\mathrm{Ca}^{2+}]_{\text{pre}}\) and the area of bouton cross-section. Second, \([\mathrm{Ca}^{2+}]_{\text{pre}}\) can depend on the resting level of \([\mathrm{Ca}^{2+}]_r\). Unfortunately, using the one-wavelength \(\mathrm{Ca}^{2+}\) indicator (OGB-1), we could not determine \([\mathrm{Ca}^{2+}]_r\). However, the resting fluorescence level \((F_0)\) did not correlate with the amplitudes of the \(\mathrm{Ca}^{2+}\) responses. Therefore, the high inter-bouton variability of \([\mathrm{Ca}^{2+}]_{\text{pre}}\) seems to reflect the functional heterogeneity of the tested bouton population.

The use of a \(\mathrm{Ca}^{2+}\) indicator may interfere with synaptic transmission. We therefore estimated the intra-terminal OGB-1 concentration by comparing the bouton fluorescence with the fluorescence produced by droplets of either intracellular solution or calcium calibration buffer kit solution (100 nM calcium, component E from Molecular Probes) containing OGB-1-\(K_\text{eq}\) at defined concentration. The OGB-1 concentration was only 30–70 \(\mu\text{M}\), which is below the level affecting the release of neurotransmitter (Rozov et al. 2001). Furthermore, if the presence of OGB-1 had an effect on transmitter release, one could expect that brighter boutons, which presumably contain higher concentration of the indicator, would display higher failure rates. Although there was a slightly negative slope relationship between the failure rates and the resting fluorescence, a significant correlation was absent \((r = -0.11, P = 0.36)\). In addition, the average quantal content was independent on the resting fluorescence level. We therefore concluded that OGB-1 had no effect on transmitter release.

In the present population of boutons, the relationship between the mean amplitudes of eIPSCs and \([\mathrm{Ca}^{2+}]_{\text{pre}}\) was sublinear. At individual collicular terminals average eIPSCs displayed an approximately third power dependency on \([\mathrm{Ca}^{2+}]_{\text{pre}}\) (Kirischuk et al. 1999a). However, this relationship must not necessarily be reproduced by a set of different boutons. If large inter-bouton differences exist in the exponents of the individual power functions, the power function relationship can be lost. Several mechanisms could account for the observed inter-bouton variability, including differences in \(\mathrm{Ca}^{2+}\) channel subtypes (Taschenberger and Grantyn 1995) and \(\mathrm{Ca}^{2+}\) channel clustering (Meinrenken et al. 2002), endogenous \(\mathrm{Ca}^{2+}\) binding ratio, the presynaptic vesicle pool size or organization (Mozhayeva et al. 2002) and local signals from the postsynaptic cells (Rozov et al. 2001). Although the eIPSC-[\(\mathrm{Ca}^{2+}]_{\text{pre}}\) dependency needs further investigation, we conclude that in a heterogeneous population of GABAergic terminals the amplitude of presynaptic \(\mathrm{Ca}^{2+}\) influx is an indicator of the synaptic strength.

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