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DOMINANCE OF P/Q-TYPE CALCIUM CHANNELS IN DEPOLARIZATION-INDUCED PRESYNAPTIC FM DYE RELEASE IN CULTURED HIPPOCAMPAL NEURONS ☆

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Abstract—Neurotransmitter release probability is related by high power to the local concentration of calcium in presynaptic terminals, which in turn is controlled by voltage-gated calcium channels. P/Q- and N-type channels trigger synaptic transmission in the majority of neurons of the central nervous system. However, whether and under which conditions both channel types act cooperatively or independently is still insufficiently understood. Previous studies suggested either a dominance of N- or P/Q-type channels, or a synergistic action of both channels, depending on the experimental paradigms. Thus, to provide insight into the properties of neurotransmitter release in cultured mouse hippocampal neurons, we used quantitative analysis of FM dye release from presynaptic boutons induced by high potassium membrane depolarization. Increasing extracellular potassium concentrations revealed a sigmoid dependence of FM dye release to the stimulation strength. Individual and combined application of the P/Q- and N-type channel-specific blockers ω-agatoxin-IVA and ω-conotoxin-GVIA, respectively, allowed us to specifically isolate the contribution of both channel types to release triggered with 40 mM KCl. Analysis of the release kinetics and the fractional release amplitude demonstrate that, whereas in only 15% of the synapses release depended exclusively on P/Q-type channels, the majority of synapses (85%) contained both N- and P/Q-type channels. Nevertheless, the kinetics of FM dye release in synapses containing both channel types was determined by the P/Q-type channels. Together, our data suggest a more direct coupling of P/Q-type channels to synaptic release compared to N-type channels, which may explain the high prevalence of neurological P/Q-type channelopathies.

Key words: voltage-gated Ca^{2+} channels, synapse function, N-type, P/Q-type, neurotransmitter release, calcium channel physiology.

INTRODUCTION

Transmitter release is tightly regulated by the presynaptic Ca^{2+} transient generated by voltage-gated Ca^{2+} channels (Ca_V) (Katz and Miledi, 1965; Schneggenburger and Neher, 2005). In fact, synaptic vesicle fusion depends to the fourth to fifth power on the local Ca^{2+} concentration (Schneggenburger and Neher, 2000), which in turn is directly related to the number of presynaptic Ca_Vs (Schweizer et al., 2012). The three members of the high voltage-activated Ca_V2 family, pharmacologically defined as P/Q- ($Ca_V2.1$), N- ($Ca_V2.2$) and R-type ($Ca_V2.3$) channels, are the major presynaptic pore-forming subunits triggering synaptic release (Reid et al., 1998). In the majority of neurons of the central nervous system synaptic transmission depends cooperatively on both P/Q- and N-type (Olivera et al., 1994; Dunlap et al., 1995) channels. However, whether both channel types contribute to transmitter release in a synergistic manner or whether synapses utilize either N- or P/Q-type channels is a matter of intense and ongoing research (Reid et al., 2003).

In specific neuronal cell types or, even more precisely, synaptic connections presynaptic functions may depend on a single Ca_V type. For example transmitter release at the inhibitory basket cell–granule cell synapse in rat hippocampus is exclusively triggered by P/Q-type channels (Bucurenciu et al., 2010). However, for the majority of glutamatergic neurons three different scenarios have been reported. First, synapses can contain both channel types and synaptic transmission depends on the synergistic action of both channels. Indeed the application of selective N- and P/Q-type channel antagonists has revealed an additive contribution of both channels to the presynaptic Ca^{2+} signal (Wheeler et al., 1996; Wu et al., 1999; Reid et al., 2003) and thus transmitter release. Second, while hippocampal neurons express both channels at comparable levels (Schlick et al., 2010) individual synapses may differ in containing either N- or P/Q-type channels as the major presynaptic Ca_V type. This is supported by the observation that subpopulations of synapses are fully blocked by specific antagonists (Reuter, 1995; Reid et al., 2003). Finally, the third scenario assumes the coexistence of both channel types in synapses, yet their contribution to synaptic release may differ with respect to their coupling

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Abbreviations: A, release amplitude; Aga, ω-agatoxin-IVA; Ca_V, voltage-gated Ca^{2+} channel; CTx, ω-conotoxin GVIA; DIV, days in vitro; $[K^+]$, extracellular potassium concentration; R_f, fractional release; τ, release time constant.

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efficiency. This possibility is suggested by findings showing that in defined synapses neurotransmitter release was less sensitive to selective N-type inhibition when compared to P/Q-type inhibition, although the amount of N-type Ca^{2+} current was comparable (Qian and Noebels, 2001). Furthermore, analysis of evoked EPSC recordings in hippocampal neurons suggested the existence of P/Q-type preferring channel slots, which impose a ceiling on the synaptic efficacy (Cao et al., 2004). On the contrary, N-type channels clearly dominated when release was triggered by single action potentials and analyzed using fluorescent imaging of synaptic boutons (Ariel et al., 2012). Together this indicates a great variability in the contribution of presynaptic N- and P/Q-type channels to synaptic release. This heterogeneity of findings may originate from interactions of axons with specific postsynaptic targets determining presynaptic properties (Branco et al., 2008). Moreover, species differences between mouse and rat neurons and different experimental techniques and stimulation protocols used for the analysis of presynaptic function may also contribute to these inconsistencies.

Hence, to characterize the channel-type composition in nerve terminals of low-density cultured mouse hippocampal neurons, a frequently used model system (Kaech and Banker, 2006), we used quantitative analysis of FM dye destaining induced by sustained membrane depolarization (Hoopmann et al., 2012). Individual and combined application of the pharmacological blockers ω-agatoxin-IVA (Aga) and ω-conotoxin-GVIA (CTx), allowed us to specifically characterize the contribution of voltage-activated P/Q- and N-type channels, respectively. Our results suggest a more direct coupling of P/Q-type channels to synaptic release although the majority of synapses contained both N- and P/Q-type channels.

EXPERIMENTAL PROCEDURES

Ethical approval

Mice were bred and maintained at the central laboratory animal facility of the Medical University Innsbruck according to national and EU regulations and approved by the Austrian Science ministry (BM.W_F[a]). The number of animals used to obtain cells for this project was annually reported to the Austrian Science ministry (BM.W_F[a]).

Cultured hippocampal neurons

Low-density cultures of hippocampal neurons were prepared from timed pregnant BALB/c mice on day 16.5 of gestation as previously described (Obermair et al., 2003, 2004, 2010; Kaech and Banker, 2006; Szabo et al., 2006). Briefly, pregnant mice were anesthetized in CO_2, decapitated and the pups were removed from the uterus and decapitated. Isolated hippocampi were dissociated by trypsin treatment and trituration and plated on poly-L-lysine-coated glass coverslips in 60 mm culture dishes at a density of ~5300 cells/cm^2. After plating, cells were allowed to attach for 3–4 h before transferring the coverslips neuron-side-down into a 60 mm culture dish with a glial feeder layer. For maintenance neurons and glial feeder layer were cultured in serum-free Neurobasal medium (Invitrogen GmbH, Karlsruhe, Germany) supplemented with Glutamax and B27 supplements (Invitrogen GmbH, Karlsruhe, Germany). Ara-C (5 μM) was added 3 days after plating to stop the proliferation of non-neuronal cells.

Imaging and analysis of FM dye release

18 mm cover glasses with cultured hippocampal neurons (days in vitro (DIV) 17–31) were mounted in a Ludin chamber in a modified tyrode solution (130 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 30 mM glucose, 10 μM CNQX, 50 μM DL-AP5, pH 7.4 with NaOH). Distinct high KCl concentrations ($[K^+]$) were generated by equimolar replacement of NaCl by KCl. Synaptic vesicles were loaded by bath perfusion of 60 mM $[K^+]$ Tyrode solution containing 15 μM FM1–43 for 90 s (Fig. 1A). After 5–10 min of continuous rinsing, synaptic release was induced by applying varying concentrations of $[K^+]$ through a microperfusion applicator for 90 s. After inducing FM dye release with varying $[K^+]$ the overall responsiveness of the synapses in all experiments was confirmed by a second depolarization step using 60 mM $[K^+]$, which resulted in a robust FM dye release independent of presynaptic N- and P/Q-type channels. Twelve-bit grayscale images were recorded at 0.5 Hz using an inverted Zeiss Axiovert 200 M epifluorescence microscope (Carl Zeiss Inc., Jena, Germany) equipped with a cooled CCD camera (SPOT; Diagnostic Instruments, Stirling Heights, MI, USA), Metavue image processing software (Universal Imaging, Corp., West Chester, PA, USA), and a 63×1.4 NA Zeiss Plan Apochromat oil immersion objective. Recordings were performed at 26 °C. For quantification of FM dye destaining, circular regions of interest (ROI, 3×3 pixels) were located manually over the center of FM dye fluorescence of spatially separated synapses. Their average intensity was measured using the ImageJ software package (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The background-corrected intensity was normalized to the average intensity of the last five frames before initiation of high $[K^+]$ stimulation. The FM dye release curves of single synapses were fitted mono-exponentially from 2 to 90 s using Clampfit 10.2 (Molecular Devices, Downingtown, PA, USA). A successful mono-exponential fit was defined by positive values for amplitude (A), time constant (τ), and offset. Fits not fulfilling all criteria were excluded from further analyses. In order to obtain an additional and unbiased measure of release efficacy including all release traces (also the traces which did not fulfill the mono-exponential fitting criteria), we determined the fractional release (R_f) of each synapse in each condition. R_f is defined as the percentage of total release determined at the time constant (τ) of the respective control condition. Standard errors are in the order of 1% and lower and thus not presented in the release diagrams.