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ScienceDirect
Neuroscience Letters 412 (2007) 239–242
Neuroscience Letters
www.elsevier.com/locate/neulet

Reduced aspartate release from rat hippocampal synaptosomes loaded with Clostridial toxin light chain by electroporation: Evidence for an exocytotic mechanism

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Received 8 August 2006; received in revised form 24 October 2006; accepted 3 November 2006

Abstract

Aspartate can be released from certain hippocampal pathways along with glutamate or GABA. Although aspartate immunoreactivity has been localized to synaptic vesicles and aspartate release is Ca^{2+}-dependent, there has been no clear evidence favoring an exocytotic mechanism. In particular, pretreatment with Clostridial toxins has not consistently inhibited aspartate release, even when release of glutamate from the same tissue samples was markedly inhibited. To address this issue directly, rat hippocampal synaptosomes were permeabilized transiently by electroporation in the presence of active or inactivated Clostridial toxin light chains. Loading rat hippocampal synaptosomes with the active light chain of tetanus toxin or of botulinum neurotoxins A, B or C reduced the K^+-evoked release of aspartate at least as much as that of glutamate. These results confirm that aspartate is released by exocytosis in rat hippocampus.

Keywords: Aspartate; Excitatory amino acids; Transmitter release; Hippocampus; Exocytosis; Clostridial toxins

Although hippocampal preparations have long been known to release aspartate along with the recognized transmitters glutamate and GABA [18,19], the mechanism and physiological significance of aspartate release remain unclear. Aspartate immunoreactivity has been localized to synaptic vesicles of certain excitatory [10,11] and inhibitory [12] hippocampal pathways. In those pathways, aspartate immunoreactivity was associated with synaptic vesicles to the same degree as glutamate or GABA immunoreactivity. Aspartate is coreleased with glutamate from the Schaffer collateral-commissural and dentate gyrus associational-commissural pathways in a Ca^{2+}-dependent manner [2,3,19] and could serve as a co-transmitter through its selective activation of NMDA receptors [4,22]. However, the mechanism of aspartate release appears to differ in some respects from that of the recognized amino acid transmitters. In our previous study of rat hippocampal synaptosomes, aspartate release was more sensitive than glutamate release to increases in intracellular $[Ca^{2+}]$ outside the presynaptic active zones, was reduced by KB-R7943, an inhibitor of Na^+/Ca^{2+} exchange, was much less sensitive than glutamate release to block of P/Q-type voltage-dependent Ca^{2+} channels, and resisted both bafilomycin A_1, an inhibitor of vacuolar H^+-ATPase, and Clostridial toxins [1]. Importantly, (±) threo-3-methylglutamate, a competitive but non-transportable inhibitor of excitatory amino acid transport, did not reduce aspartate release. This result and the distinct pharmacologies of aspartate and glutamate release processes excluded the possibility that aspartate was released by heteroexchange with released glutamate [20]. Our results suggested that aspartate is released in a neuropeptide-like fashion at sites distinct from those of glutamate release. Previous work by ourselves [28] and others [6,15] also favored independent mechanisms of aspartate and glutamate release.

Despite its Ca^{2+}-dependence, there has been no clear evidence favoring an exocytotic mechanism for aspartate release. Pretreatment of neocortical synaptosomes [17], whole brain synaptosomes [6] or hippocampal slices [10,11] with Clostridial toxins inhibited chemically evoked aspartate release, as expected for an exocytotic process, but either a high concentration of toxin was used or the possibility of release by heteroexchange

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doi:10.1016/j.neulet.2006.11.006

with released glutamate was not excluded. In our study of rat hippocampal synaptosomes, pretreatment with Clostridial toxins reduced glutamate release markedly, but did not reduce the simultaneous release of aspartate from the same tissue samples [1]. This result could be viewed as evidence against aspartate exocytosis. If the sites of aspartate release are distinct from the glutamate release sites, however, then internalized toxins may have failed to inhibit aspartate release because they did not reach an inhibitory concentration at the aspartate release sites. Native Clostridial toxins are composed of a heavy chain, which is required for internalization by synaptic terminals, and a light chain, which is endowed with proteolytic activity against one or more of the proteins (SNAREs) required for neuronal exocytosis. Internalization of botulinum neurotoxins (BoNTs) involves binding of the heavy chain to synaptic vesicle proteins expressed on the cell surface, incorporation into the vesicle, and translocation of the toxin light chain into the synaptic terminal cytoplasm as the vesicle interior acidifies [24–26]. The light chain then cleaves its target SNARE protein(s), inhibiting exocytosis. Toxin internalization is expected to take place primarily at and near the presynaptic active zones, where synaptic vesicles are recaptured for reuse. If the aspartate-containing vesicles undergo exocytosis at some distance from the active zones, restricted diffusion and degradation of toxin light chain may limit toxin-induced hydrolysis of SNARE proteins at those sites. We tested this hypothesis with rat hippocampal synaptosomes preloaded with Clostridial toxin light chains by electroporation. Electroporation allows the introduction of exogenous molecules into cells that are otherwise impermeable to those molecules by creating transient pores in the plasma membrane. Because this technique creates these pores at random locations, the concentration of toxin light chain was expected to be about equal at all release sites.

For each experiment, synaptosomes were prepared from the hippocampi of one female Sprague–Dawley rat (100–125 g; Zivic Laboratories, Pittsburgh, PA, USA) as described by Dunkley et al. [5]. Animal protocols were approved in advance by the Duke University Animal Care and Use Committee according to guidelines of the National Institutes of Health. Every effort was made to minimize the number of rats used, as well as their pain and suffering.

The preparation was suspended in an aerated HEPES-buffered medium that consisted of 122 mM NaCl, 3.1 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM KH_2PO_4, 10 mM D-glucose, 25 mM HEPES, 2 mM Na_2ATP, and 0.4 mM L-glutamine, pH 7.4. ATP was added to replace ATP lost during permeabilization [23] and glutamine was added as a substrate for resynthesis of any lost glutamate and aspartate. Preliminary studies showed that including glutamine in the electroporation medium maintained the normal K^+-evoked release of aspartate and glutamate without changing basal efflux. The preparation was divided into equal 175 μl portions. Clostridial toxin light chain (List Biological Laboratories, Campbell, CA, USA) in a volume of 12 μl was added to one portion and an equal amount of inactivated light chain to the other. Inactivation was achieved by heating the toxin light chain in a boiling water bath for 10–30 min. The tissue protein concentration was 1.5–2.0 μg/ml. Each synaptosome suspension was electroporated with a single pulse from a Gene Pulser Transfection Apparatus (Bio-Rad Laboratories, Hercules, CA, USA). An equal portion of each preparation was transferred to a disk of Whatman GF/A filter paper (Whatman International, Maidstone, UK) in six identical superfusion chambers. For each experiment, three of the tissue samples had been preloaded with active light chain and three with inactivated light chain. Release experiments were carried out and amino acids in the superfusate were quantitated by HPLC as previously described [1]. Briefly, the tissue samples were superfused dropwise from above at 32 °C and a flow rate of 1 ml/min. The superfusion medium was the same as the electroporation medium, except that HEPES was replaced by an equal concentration of $NaHCO_3$ (pH 7.4 by gassing with water-saturated 95% O_2/5% CO_2), the glucose concentration was reduced to 1 mM, and ATP and glutamine were omitted. The medium was supplemented with 16 μM fatty acid-free bovine serum albumin (BSA) for the first 56 min to reduce oxidative damage and then BSA was removed. After collecting a sample of effluent for determination of basal amino acid efflux, synaptosomes were challenged with a 2 min exposure to 25 mM KCl (with commensurate reduction of NaCl). Basal and evoked releases of aspartate and glutamate from the same tissue samples were quantitated simultaneously. K^+-evoked release was the difference between values obtained during and before exposure to 25 mM KCl normalized to the amount of tissue protein in the sample. Results are expressed as mean ± S.E.M.

Preliminary experiments determined the optimal electroporation voltage. Capacitance was maintained at 960 μF and the voltage was varied. Voltages of 300 V or above essentially abolished the K^+-evoked release of aspartate and glutamate, suggesting irreversible tissue damage. Reducing the voltage to 250 V inhibited the release of glutamate by 46 ± 14% ($P < 0.02$, Student's *t*-test, compared with non-electroporated synaptosomes from the same preparations, $n = 10$), with variable effects on aspartate release. The highest voltage that did not reduce K^+-evoked release was 200 V (aspartate release, 112 ± 27% of control, glutamate release, 95 ± 19% of control, $n = 9$). Thus, the Gene Pulser was set at 200 V and 960 μF (0.19 C) for these experiments.

Loading rat hippocampal synaptosomes with the active light chain of tetanus toxin (TeNT) or of BoNT/A, B or C reduced the K^+-evoked releases of both aspartate and glutamate (Fig. 1). Analysis of variance revealed no significant difference in the effects of Clostridial toxin light chains on release of the two amino acids; if anything, aspartate release was inhibited to a greater degree. When synaptosomes were permeabilized in the presence of 228 nM light chain, BoNT/B light chain was the most efficacious (68 ± 10 and 56 ± 8% reductions in aspartate and glutamate release, respectively, $n = 6$) and TeNT light chain was the least efficacious (28 ± 9 and 35 ± 10% reductions in aspartate and glutamate release, respectively, $n = 8$). For BoNT/C light chain, the inhibitory effect on amino acid release was clearly concentration-dependent. BoNT/B light chain was more potent; a concentration of 45.6 nM reduced both aspartate and glutamate release as effectively as a concentration of 228 nM.