LETTER TO THE EDITOR

Electrophysiological evidences of interaction between calcium channels and PA of anthrax

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Tripartite anthrax toxin is composed of nontoxic edema factor (EF, calmodulin/Ca\(^{2+}\)-dependent adeny- late cyclase), lethal factor (LF, a zinc-dependent metallopeptidase cleaving mitogen activated protein kinases) and non-toxic PA (83 kDa). On release from the anthrax bacillus as monomers, they assemble into toxic complexes on the surface of host cells. According to the established mechanism of toxic effect, PA binds to the cell surface receptors and facilitates the translo- cation of a “lethal toxin” composed of LF and EF.\(^1\)

Two PA receptors have been previously identified, including ATR/TEM8 (anthrax toxin receptor/tumor endothelial marker 82) and CMG2 (capillary morpho- genesis protein 2\(^3\)). Mechanism of toxic effect is not well known, except that PA binds to the cell surface receptors and forms heptameric pores that are involved in translocation of the “lethal toxin.” The formation of PA channels on the surface of a host target cell is a key step in the pathogenesis of anthrax.

PA requires for binding a common von Willebrand factor A (integrin-like) inserted domain that contains MIDAS composed of D\(_{x}S\_S\_x\_9\_79\_T\_12\_23\_D\), where \(x\) is any amino acid. The MIDAS domain is present in the accessory \(\alpha_{2}\delta-1\) and \(\alpha_{2}\delta-2\) subunits of the \(\alpha_{\text{a}}1\) and \(\alpha_{\text{a}}2\) families of the calcium channel. Common features of \(\alpha_{\text{a}}\delta\), ATR/TEM8 and CMG2 include an extracellular von Willebrand factor A domain and a single-pass transmembrane region. Because calcium channels are clustered in the plasma membrane,\(^4\) they suite well to co-localize the PA pores to allow for sufficient entry of anthrax toxin into the cells. Unlike ATR/TEM8 and CMG2, \(\alpha_{\text{a}}1\) and \(\alpha_{\text{a}}2\) calcium channels are present in a wide variety of cells of the body except cells of immune system and blood cells (although \(\alpha_{\text{a}}\delta\) is found in lymphocytes). Importantly, they are expressed in endothelial cells,\(^5\) keratinocytes,\(^6\) and fibroblasts,\(^7\) which represent the primary locations for bacterial entry. Given the wide distribution of calcium channel \(\alpha_{\text{a}}\delta\) proteins, we hypothesize that the \(\alpha_{\text{a}}\delta\) subunit may interact with PA. This study presents the first experimental evidence suggesting such an interaction.

The patch clamp study was carried out essentially as described earlier \(^8\) with the recombinant human Cav1.2 calcium channels composed of the fluorescently labeled vascular/fibroblast pore-forming ECFP\(_{N}\-\alpha_{\text{a}}1\_C\_77\_\) (z34815) subunit and accessory vascular \(\beta_{3}\) (X76555) subunit co-expressed with \(\alpha_{\text{a}}\delta-1\) (AAA51903) in Cos1 cells. In these studies we used PA-U7,\(^9\) the mutant of PA where the furin cleavage site was deleted. PA-U7 retains ability to bind to receptors but cannot be proteolytically activated. This property of PA-U7 is particularly useful for patch clamp experiments because PA-U7 is unable to form leak channels that would otherwise compromise the specificity of recordings and stability of patch clamp by generating a large non-selective leak current. It was found that PA-U7 inhibited the Ca\(^{2+}\) current in a nM range (Fig. 1A). Although there are data that PA-U7 may eventually dissociate from receptors, we did not observe a significant reversal of the calcium channel inhibition after wash-out for 10 min with the PA-free buffer. Longer wash-out was incompatible with the stability of whole-cell patch clamp. Under the same conditions, the inactive PA mutant PA-3M that contains 3 mutations in domain 4 preventing binding to a receptor, did not induce notable inhibition of the Ca\(^{2+}\) current (open circles). Slow linear decrease of the
Figure 1. (A) Inhibition of calcium current by PA in Cos1 cells expressing human recombinant Ca\textsubscript{a1.2} composed of ECFP\textsubscript{N}\textsubscript{-}\textalpha_{1C77/3/2} subunits. Peak calcium currents were elicited by a stepwise depolarization to +30 mV applied from the holding potential $V_h = -90$ mV before (left trace) and after (right trace) application of PA-U7 (500 ng/ml, closed circles; mean ± SEM, $n = 3$). A 90% inhibition occurred within 5–7 min at 20–22 °C. Under the same conditions, the inactive mutant of PA, PA-3M (500 ng/ml), did not induce notable inhibition of the Ca\textsuperscript{2+} current (open circles; $n = 3$). Slow linear decrease of the normalized current amplitude in control is due to rundown typically seen with the Ca\textsubscript{a1.2} calcium channels (see sample Ca\textsuperscript{2+} current trace, arrow). (B) Protective effect of gabapentin. When applied at 10 $\mu$M, gabapentin inhibited 60% of the calcium current in 10 min, the effect reaching apparent saturation (G). Subsequent application of PA (500 ng/ml) in the presence of 10 $\mu$M gabapentin (G+PA) for 10 min did not cause a significant additional inhibition of the calcium current amplitude, suggesting protection by gabapentin against the PA-dependent inhibition of the channel. Subsequent removal of gabapentin in the presence of PA (black bar PA) did not recover the PA-dependent block of the calcium current (dotted line) measured in the absence of gabapentin (white bar PA). Mean ± SEM; $n = 5$. 

E. KOBIRNSKY AND N. M. SOLDATOV
normalized current amplitude in control is due to rundown typically seen with the Ca_{1.2} calcium channels. \( \alpha_2 \delta \) specifically interacts with gabapentin,\(^{10-13}\) a Pfizer’s evolving drug class that is used to treat several clinical disorders, including epilepsy, pain from diabetic neuropathy, postherpetic neuralgia and fibromyalgia, and generalized anxiety disorder. The \( \alpha_2 \delta \)-mediated modulation of neurotransmission by these drugs is due to attenuation of the Ca\(^{2+}\) current without changing its kinetics and voltage-dependence. Given the specificity of gabapentin to \( \alpha_2 \delta \), we have tested the effect of this drug at physiological concentrations on PA-induced inhibition of the calcium current. Patch clamp experiments (Fig. 1B) revealed that gabapentin significantly protects the calcium channel current from the inhibition by PA-U7.

Taken together, from the presented patch clamp experiments we conclude that PA interacts with calcium channels with high affinity (K\(_{0.5}\) in a nM range) in an \( \alpha_2 \delta \)-specific and gabapentin-sensitive manner. Although the mechanism of the observed effects is not clear, these data show that \( \alpha_2 \delta \) represents a new potential molecular target for anthrax.

**Abbreviations**

MIDAS metal ion-dependent adhesion site
PA protective antigen

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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