Pharmacological Comparison of Two Different Insect Models Using the Scorpion α-Like Toxin BmK M1 from *Buthus martensii* Karsch

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Abstract: In this study, the effect of the scorpion α-like toxin BmK M1 was investigated on isolated DUM neurons from *Locusta migratoria* and compared with the effect on para/tipE voltage-gated Na^+ channels (VGSC), cloned from *Drosophila melanogaster*. The two insects display different pharmacological properties regarding α-like toxins. Moreover, with the aid of the α-like toxin BmK M1 and 5 of its mutants, the importance of aromatic residues for the interaction of the toxin with the VGSC in *L. migratoria* and *D. melanogaster*, is shown.

Keywords: BmKM1, voltage-gated Na^+ channels, locust DUM neurons, para/tipE.

1. INTRODUCTION

Scorpion venom contains a class of neurotoxins called α-toxins which mainly cause a slowing of the inactivation process of voltage-gated Na^+ channel (VGSC) currents and a prolongation of the action potential [1, 2]. By binding to VGSC receptor site 3 the α-toxins prevent the normal gating movements of the S4 segment of domain IV and thus uncouple Na^+ channel activation from inactivation [3]. They can be further divided into three subgroups, classical α-, α-like and insect α-toxins [4].

In vertebrates VGSCs are composed of a pore forming α subunit (260 kDa) and one to three auxiliary β subunits (21–23 kDa) [5, 6]. Insect VGSCs have only been cloned from invertebrate organisms like the German cockroach and the housefly, so little is known about the structure-function relationships of these sodium channels. The gene encoding a VGSC β subunit in *Drosophila melanogaster*, para, has been cloned and the cDNA sequence determined [7]. Cloning and functional analysis of tipE, a membrane protein of 50 kDa that enhances para Na^+ channel function, has led to the use of this protein as a β subunit for para [8]. Despite their similarity in electrophysiology and ion conductance, primary structure and allocation of all functional domains, the insect and vertebrate VGSCs are pharmacologically distinguishable, as revealed by the responsiveness of the heterologously expressed para/tipE clone to channel modifiers and blockers [9].

Dorsal unpaired median (DUM) neurons are among the most extensively studied neurons in the insect central nervous system [10]. Comprehensive patch clamp studies demonstrated that DUM neurons in primary cell culture express a wide variety of ion channels that are involved in regulating the firing pattern and the resting membrane potential [11, 12]. Especially the VGSCs play a key role in generating action potentials.

The α-like toxin BmK M1 from the venom of the scorpion *Buthus martensii* Karsch, which is endemic to eastern Asia, is composed of 64 amino acids cross-linked by 4 disulphide bridges [13, 14]. BmK M1 has been the subject of different studies: its 3D structure was determined by X-ray crystallography at 1.7 Å resolution [15], the pharmacological properties on $Na_v1.5$ channels and DUM neurons have been studied [16, 17], the gene cloning and expression of the wild type BmK M1 have been carried out [18, 19] and the functional site of BmK M1 has been extensively studied by site-directed mutagenesis [20, 21].

Electrophysiological studies on para/tipE have shown the existence of very potent and efficacious α-toxins [22]. Until today however, not much attention has been paid to parallel electrophysiological comparisons between different insect-models. Gilles et al. did perform binding studies with ^{125}I-labeled α-atracotoxin Hv1a on mammal and several insect neuronal membranes [23]. In our attempt for a better understanding of insect-selectivity, 5 mutants of BmK M1, being a typical α-like toxin, were studied in 2 different insect models. Due to a very limited number of cloned insect-VGSCs, we compared on the one hand the insect VGSC (para) from the fruit fly and on the other hand DUM neurons from the locust. Using the scorpion α-like toxin BmK M1 as a suitable pharmacological tool, 5 of its highly conserved aromatic residues (Y14, Y21, W38, Y35 and Y42) were mutated to glycine and expressed in *S. cerevisae* S-78 cells. These aromatic amino acids were chosen because of their high degree of conservation in the primary structure after

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alignment of several known α- and α-like toxins [20]. Large differences in toxin-induced effect were observed when applying the mutants within the same insect-model but also between the two insect-models.

2. EXPERIMENTAL PROCEDURES

2.1 Primary Cell Culture of locust DUM Neurons

Isolated DUM neuron cell bodies from the metathoracic ganglion of adult migratory locusts (*Locusta migratoria*) were prepared as described previously [17]. Briefly, the dorsal median region from ganglia was removed and subjected to a collagenase/dispase (2 mg/ml) treatment. The cells were centrifuged and subsequently washed three times. The cells were dissociated by repetitive suction through a pipette tip and they were finally plated on Nunc petri dishes and incubated overnight at 28 °C and 5% CO_2.

2.2 Expression in Xenopus Oocytes

For the expression in *Xenopus laevis* oocytes, the para/pGH19-13-5 vector [7] and tipE/pGH19 vector [8] were linearized with *Not*I and transcribed with the T7 mMESSAGE-mMACHINE kit (Ambion, U.S.A.).

The harvesting of oocytes from anaesthetized female *Xenopus laevis* frogs was as previously described [24]. Oocytes were injected with 50 nl of cRNA at a concentration of 1 ng nl^{-1} using a Drummond micro-injector (U.S.A.). The solution used for incubating the oocytes contained (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2 and HEPES 5 (pH 7.4), supplemented with 50 mg l^{-1} gentamycin sulphate.

2.3 Synthesis, Purification and Verification of BmK M1 Mutants

Plasmid pVT 102U/α, *E. coli* TG1 strain and *S. cerevisiae* S78 (Leu2, Ura3, Rep4) yeast strain were used. Restriction endonucleases and T4 DNA ligase were obtained from Roche (Germany). Primers were synthesized by Sangon (Shanghai, China). Taq DNA polymerase and Klenow fragment were obtained from MBI (U.S.A.). Cation exchanger CM C-32 cellulose and reversed-phase column Sephasil® Peptide C18 (12µm ST 4.6/250) were from Whatman (U.K.) and Pharmacia Biotech (Sweden), respectively. All other reagents were analytical grade and were purchased from Merck (Germany) or Sigma (U.S.A.).

The cDNA of BmK M1 was previously cloned [18] and inserted into pVT 102U/α [19]. The substitute residue for all aromatic residues was glycine. All mutants were produced using a previously described procedure [20]. Reversed-phase chromatography used for purification of the mutants was carried out on an *ÄKTA* Purifier chromatography system (Pharmacia Biotech, Sweden). The molecular mass of the purified mutants was obtained by using a Finnigan LCQ ion-trap mass spectrometer (ThermoQuest, San Jose, CA, USA) equipped with an ESI. Circular dichroism spectra were recorded as previously described [20].

2.4 Electrophysiological Experiments

The patch clamp technique in the whole cell configuration [25] was used to measure the Na^+ currents of isolated DUM neurons as previously described [17]. The electrodes were filled with a solution containing (in mM) NaCl 5, CsCl 75, CsF 65, $CaCl_2$ 1, $MgCl_2$ 1, MgATP 2, EGTA 10 and HEPES 10 (pH 6.65). The bath solution contained (in mM): NaCl 40, CholineCl 90, $CaCl_2$ 3, $CdCl_2$ 2, $MgCl_2$ 7, tetraethylammonium (TEA)-Cl 40, HEPES 10 and 4-aminopyridine (4-AP) 5 (pH 6.80). The composition of the solutions was chosen in such a way that the Ca^{2+} and K^+ currents were minimized and the Na^+ currents remained unaffected. The extracellular Na^+ concentration (40 mM) was low in order to reduce the inward Na^+ current and to diminish voltage clamp errors. Moreover, tetrodotoxin (TTX) was added to the medium at the end of the experiment to block the voltage-gated Na^+ current. All Na^+ currents of locust DUM neurons shown in the present paper are TTX-sensitive currents obtained by subtracting the current traces in the presence of TTX from the traces in the absence of TTX.

Patch clamp experiments and data acquisition were performed with a PC controlled EPC 9 or EPC 10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). The resistance of the patch electrodes was in between 600 and 900 kΩ. Liquid junction potentials were taken into account [26]. The cells were clamped at a holding potential of –90mV. Capacitive and leak currents were compensated for automatically by the Pulse program (HEKA Elektronik, Lambrecht, Germany) and residual capacitances and leak currents were eliminated by means of a P/6 protocol. The series resistance was compensated for in order to be lower than the double of the tip resistance. Data were filtered at 2.9 kHz and sampled at 50 kHz.

Two-electrode voltage-clamp recordings on *Xenopus laevis* oocytes expressing para/tipE were performed at room temperature (18° – 22° C) using a GeneClamp 500 amplifier (Axon instruments, U.S.A.) controlled by a pClamp data acquisition system (Axon instruments, U.S.A.). Whole-cell currents from oocytes were recorded 4 days after injection. Voltage and currents electrodes were filled with 3 M KCl. Resistances of both electrodes were kept as low as possible (< 0.5 MΩ). Bath solution composition was (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2 and HEPES 5 (pH 7.4). Using a four-pole low-pass Bessel filter, currents were filtered at 1 kHz and sampled at 10 kHz. Leak and capacitance subtraction were performed using a P/4 protocol. Current traces were evoked in an oocyte expressing Para/tipE by depolarisations between -70 to 40 mV, using 10 mV increments, from a holding potential of -90 mV.

2.5 Fast Inactivation Analysis

Para/tipE: The degree of fast inactivation is assayed by measuring the $I_{5\ ms}/I_{peak}$ ratio, which gives the optimal estimate of the probability for the channels not to be inactivated after 5 ms [27]. A test voltage is chosen so that $I_{5\ ms}/I_{peak}$ is close to zero under control conditions. $I_{5\ ms}/I_{peak}$ is measured at the same test voltage after addition of the toxin. Toxin-induced removal of fast inactivation is measured by plotting $I_{5\ ms}/I_{peak}$ as a function of toxin concentrations. The following equation is used:

$$I_{5\ ms}/I_{peak} = a_0 + \{a_1/[1+(EC_{50}/[toxin])^h]\}$$

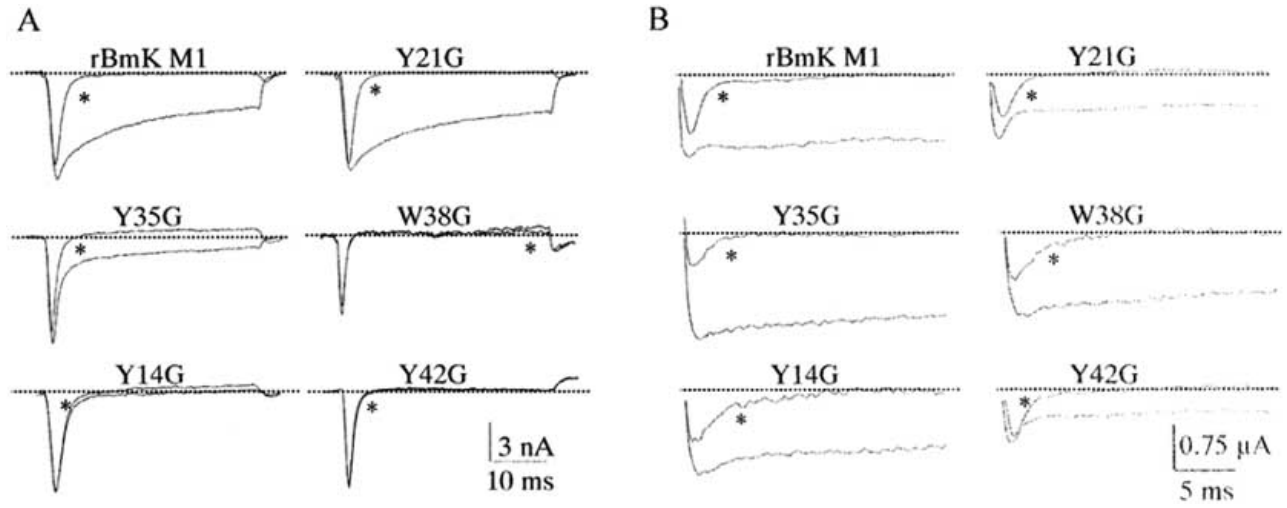

Figure 1. The effects of rBmK M1 and its aromatic amino acid mutants on insect Na^+ currents. The * shows the current trace in control conditions. **A.** The TTX-sensitive Na^+ currents in isolated DUM neurons of *Locusta migratoria* were evoked by depolarizing the cells from a holding potential of -90 mV to 0 mV during 40 ms. The toxic effect is always shown at a concentration of 1µM. **B.** The effect of the toxins on the inactivation kinetics of para/tipE channels expressed in *Xenopus laevis* oocytes. Current traces were evoked by a depolarization to 0 mV during 25 ms from a holding potential of -90 mV. The following concentrations are shown: rBmK M1 and Y21G; 0.1 µM, Y35G; 1 µM, W38G, Y14G and Y42G; 100 µM.

were h is the Hill coefficient, [toxin] the toxin concentration, a_0 the value of I_{5ms}/I_{peak} obtained at a chosen test voltage under control conditions, the sum of a_0 and a_1 equals the maximum value of $I_{5\ ms}/I_{peak}$ at the chosen test voltage indicating the expected maximum effect of the toxin on fast inactivation. Curve manipulations are performed using pClamp8 (Axon instruments, U.S.A.) and Origin software (Microcal, U.S.A.).

DUM neurons: Results are shown as mean ± standard error of the mean (SEM). Significance of differences between two means was calculated with the Wilcoxon matched pairs test using the software Prism (Graphpad Software, San Diego, USA). Data sets were fitted by mathematical expressions using Origin 6.0 (Microcal Software, Northampton, USA). Significance of the difference between parameters obtained by curve fitting (mean ± SEM) was calculated with a Student's t-test with Welch correction (Prism, Graphpad Software, San Diego, USA). Differences in the mean values were considered significant if $p \leq 0.05$.

The equation used for fitting dose response relationships by means of the software Origin 6.0 (Microcal Software, Northampton, USA) was the same as used for para/tipE except for the inactivation timeframe. Because of the slower inactivation of DUM neurons, we found that 10 ms was a more elegant timeframe to use.

3. RESULTS

3.1 Mutations, Expression and Purification

Tricine SDS-PAGE analyses of yeast cultures demonstrated that mutants (Y14G, Y21G, Y35G, W38G, Y42G) were expressed and secreted into the medium. Expression levels for the five mutants were about 1-2 mg per liter of culture medium, which was comparable to that of the unmodified rBmK M1 (~ 3mg/l). One liter of the YPD culture (in g/l: bacteriological peptone 20, yeast extract 10, glucose 20, pH after sterilization equals 6.5) was purified by chromatography on a CM C-32 cation exchange column followed by a C18 column. The molecular masses of the purified mutants were checked for accuracy with a Finnigan LCQ ion trap mass spectrometer.

Compared with the native BmK M1, the CD spectra of Y14G and Y35G have changed, indicating that there are apparent changes of secondary structures (J-700 for Windows Secondary Structure Estimation, Version 1.10.00) in these two mutants. For Y21G, W38G and Y42G the CD spectra show that the secondary structures have almost not changed as compared with that of the native toxin [20].

3.2 Electrophysiological Measurements

The effect of the BmK M1 mutants was tested on the native Na^+ currents of isolated metathoracic DUM neurons of the migratory locust (Fig. **1A**). In control conditions the TTX-sensitive Na^+ current of DUM neurons activated fast and inactivated completely. The recombinant toxin rBmK M1 slowed down and inhibited the inactivation process of the Na^+ current in DUM neurons at micromolar concentrations. The inhibition of inactivation by 1 µM rBmK M1 was only partial, i.e. at 10 ms after the start of the depolarization 65% of the inward current remained present. Y21G behaved like the wild-type rBmK M1. Although the dose response curve was shifted towards lower concentrations and the maximal effect was lower than for rBmK M1, no significant differences were found. In contrast Y35G caused a significant shift of the dose response relationship to higher concentrations while the maximal effect of Y35G was comparable to that of rBmK M1. We were not able to complete the dose response curve of the three other mutants, Y14G, W35G and Y42G due to the high concentrations necessary to achieve a maximal effect. Nevertheless, a shift of the dose response curve for Y14G to

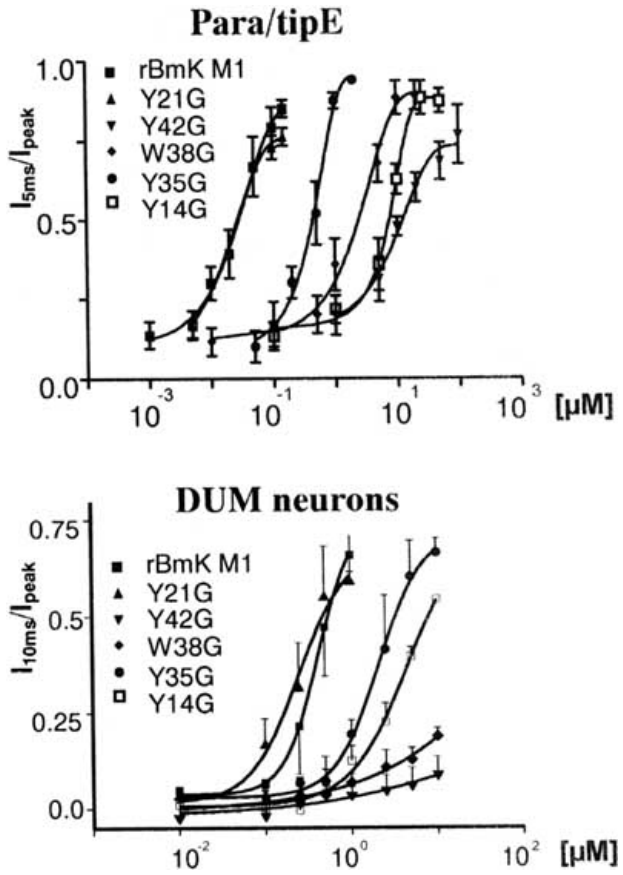

Figure 2. Upper panel shows the comparison of the dose-respons curves of rBmK M1 and its mutants on para/tipE. I_{5ms}/I_{peak} is shown in function of the concentration in μM. Data are mean ± SEM of at least three experiments. Lower panel shows the comparison of the dose-response curves (I_{10ms}/I_{peak}) of rBmK M1 and its mutants on DUM neurons. Data points are means of 5 DUM neurons for rBmK M1, 4-5 for Y21G, 4-8 for Y35G, 1-7 for Y14G, 2-6 for W38G and 2-6 for Y42G. The obtained EC_{50} values are discussed in the text.

Table 1. Overview of the obtained EC_{50} values of rBmK M1 and the aromatic amino acid mutants on DUM neurons and para/tipE.

Toxin	EC_{50} DUM	EC_{50} para/tipE
Y21G	246 ± 87 nM	22 ± 13 nM
rBmK M1	404 ± 38 nM	30 ± 6 nM
Y35G	2.1 ± 0.2 μM	506 ± 127 nM
W38G	> 10.0 μM	2.5 ± 0.9 μM
Y14G	-	7.9 ± 1.0 μM
Y42G	> 10.0 μM	10.6 ± 1.8 μM

higher concentrations and a reduction in effect of W38G and Y42G were obvious. Indeed, Fig. **1A**. A shows that 1μM Y42G or W38G didn't affect the inward Na^+ current at all while the application of Y14G induced a small inhibition of inactivation. The dose response curve of each toxin was fitted by a Boltzmann sigmoidal function. Only the data of rBmK M1, Y21G and Y35G could be fitted adequately, resulting in an EC_{50} of respectively 0.40 ± 0.04 μM, 0.25 ± 0.09 μM and 2.1 ± 0.2 μM (Fig. **2**, Table **1**).

Using the two-electrode voltage-clamp technique on *Xenopus laevis* oocytes, a pharmacological comparison was made regarding the effects of rBmK M1 and its mutants on para/tipE. Current traces were evoked using 25-ms-step depolarisations to a voltage of 0 mV from a holding potential of -90 mV. All of the BmK M1 mutants caused the inactivation to slow down (Fig. **1B**). All mutants except Y21G and Y42G showed a maximal efficacy on para/tipE with an almost complete removal of the inactivation process. The difference in efficacy of Y21G, situated in the α-helix, and rBmK M1 was significant (*t*-test: $p<0.05$). The potency of the aromatic mutants differed significantly from the native toxin except Y21G which corresponded to rBmK M1. Fig. **2** shows the concentration dependence of the slowing of inactivation induced by rBmK M1 and its mutants on para/tipE. EC_{50} values were obtained after a Boltzmann sigmoidal fit of the data. The following EC_{50} values were obtained (in increasing order); Y21G: 22 ± 13 nM ≈ rBmK M1: 30 ± 6 nM << Y35G: 506 ± 127 nM < W38G: 2.5 ± 0.9 μM < Y14G: 7.9 ± 1 μM < Y42G: 10.6 ± 1.8 μM (see also table **1**). rBmK M1 and Y21G are almost identical in potency but show a slight, but significant, difference in efficacy. Y42G has the highest EC_{50} value and does not reach the efficacy level of the other aromatic mutants.

As shown in table **1**, the EC_{50} values of rBmK M1 and its mutants are 5 or more times higher on DUM neurons than on para/tipE. Fig. **1** and **2** clearly show that the maximum toxin-induced effect (efficacy) is significantly higher on para/tipE than on DUM neurons.

4. DISCUSSION

The possibility to gain insight in insect-selective toxins is very important in order to design insecticides for specific plagues and hence reduce the probability of resistance. In this study, 5 mutants of the α-like toxin BmK M1, a widely used pharmacological tool, were used to study and compare 2 different insect models: the *D. melanogaster* VGSC para/tipE and DUM neurons from the *L. migratoria*.

The results within the same insect model clearly show the importance of the conserved aromatic residues for interaction with the VGSCs. The potencies, represented respectively by their EC_{50} values, show large differences, indicating a major role for hydrophobic interactions (Fig. **2**). These data are in concordance with our previous study on the vertebrate VGSC $Na_v1.5$ [20]. Regarding para/tipE, the efficacies are more or less the same except Y21G and Y42G which reveal a slightly, but significantly lower efficacy. In another study, the EC_{50} value of BmK M1 was determined on $Na_v1.5$ [16]. The results showed an EC_{50} value of 200 nM which is more than 6 times higher than on para/tipE, indicating its preference for insects.

VGSC receptor site 3 in the extracellular linker between transmembrane segments S3 and S4 of domain IV is almost identical in insect and mammal VGSCs [28]. When the composition of the entire outer pores of both VGSCs is compared, only 46 % of the amino acids are identical and $Na_v1.5$ contains more hydrophobic amino acids than para/tipE (data not shown). Consequently, it seems plausible to argue that hydrophobic interactions are not the crucial determinants in the VGSC specificity of toxins. On the other

hand, the aromatic amino acid mutants studied in this work do cause large differences in EC_{50} values in one insect model indicating an important role in potency and efficacy on one specific VGSC. Due to the limited amount of cloned insect VGSCs, we wanted to explore to what extent DUM neurons from locusts and the para/tipE VGSC from the fruit fly can be used in a pharmacological comparison in search for species-selective insecticides. The results of the comparison of the two insect models (Table **1**) indicate large inter-species differences between the locust and the fruit fly. In general, the EC_{50} values of rBmK M1 and its mutants are more than 5 times higher on DUM neurons than on para/tipE. The efficacy of all the mutants on DUM neurons is less than on para/tipE. When Y21 is mutated to a glycine, BmK M1 Y21G becomes slightly more potent in both insect models. Our results also show that the mutants have the same ranking according to potency (EC_{50}) in both insect models. This could indicate that the interaction mechanism toxin-VGSC is alike. Meanwhile, the effects that are evoked are quite different.

Insects, just like vertebrates, have a huge diversity of VGSCs. Para/tipE is well known and studied but our data support the fact that the VGSCs in DUM neurons have different pharmacological properties. In order to improve our insights in species-selective toxins, cloning of more insect VGSCs is indispensable.

According to the classical definition of α-like toxins they have an equally high toxicity towards both mammals and insects, they are toxic in the mouse brain but they do not bind or compete with classical α-toxins on binding to rat synaptosomes [4]. In contrast, our work shows that this group of toxins possesses very different potencies and efficacies towards their targets. BmK M1 seems to have a clear preference for the insect-VGSC. Although the application of α-like toxins as insecticides is questionable (due to the close similarity of the corresponding receptor sites in vertebrates), we believe that this study can also encourage further research into "species-selective" insecticides.

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