Calmodulin inhibits calcium influx current in vascular endothelium

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Received 17 May 1996

Abstract Utilizing the whole-cell configuration of the patch-clamp technique the effect of calmodulin (CaM) on thapsigargin-induced Ca^{2+} current has been studied. Addition of several concentrations of CaM to the patch pipette induced concentration-dependent inhibition of thapsigargin-induced Ca^{2+} current in bovine aortic endothelial cells. The effect of CaM was Ca^{2+} dependent and was not observed when the intracellular Ca^{2+} was buffered to 1 nM with EGTA. CaM produced two major effects on the thapsigargin-induced Ca^{2+} current. First CaM slow down activation of the current by thapsigargin from a control value of 16 ± 5 to 31 ± 6 s with 1 μM CaM in the pipette solution. The second effect of CaM was to reduce the current amplitude in a concentration-dependent manner. The inhibition of Ca^{2+} current was observed at the peak of the current and at the sustained current level. The reduction of current at the sustained level was observed 15–20 s after onset of the thapsigargin response. The half inhibitory concentration determined from these experiments was 0.1 μM. These results indicate that CaM can modulate thapsigargin-induced Ca^{2+} current in this endothelium, suggesting a possible role for CaM in the regulation of store-operated Ca^{2+} influx.

Key words: Calcium influx; Calmodulin; Endothelium; Store-operated current

1. Introduction

Depletion of intracellular Ca^{2+} compartments with agonists or inhibitors of the microsomal Ca-ATPase such as thapsigargin (TG) activates a Ca^{2+}-selective current in many non-excitable cells [1]. This Ca^{2+} pathway known as 'store-operated Ca^{2+} entry' appears to be a widely distributed mechanism for Ca^{2+} influx in many cell types [1,2]. The channel or family of channels responsible for this Ca^{2+} pathway have been termed 'store-operated channels' (SOC) [2].

In vascular endothelium depletion of the endoplasmic reticulum (ER) activates a Ca^{2+}-selective current whereas refilling of the ER inactivates the current [3]. The identity of the signal communicating the intracellular Ca^{2+} compartment with the SOC remains largely unknown.

Several attempts have been made to identify activators of SOC [4–6]; however, little information exists about inhibitors of this channel. Recent reports indicate that intracellular Ca^{2+} inhibits SOC in mast cells [7,8]. Similar results were obtained in vascular endothelium from bovine aorta [3,9].

In this report evidence is presented indicating that intracellular Ca^{2+} and calmodulin (CaM) are inhibitors of a TG-induced current in bovine aortic endothelial cells. These results suggest that CaM might down-regulate SOC after depletion of the ER. This inhibition of SOC might play an important role in preventing excessive influx of Ca^{2+} which could result in cell damage. Future experiments with SOC from other cell types might help to elucidate if this mechanism is conserved in other cells.

2. Materials and methods

2.1. Solutions and reagents

All salts used are analytical grade (Sigma Chemical Co., St. Louis, MO). Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra acetic acid (EGTA) and CaM from bovine heart were purchased from (Sigma Chemical Co., St. Louis, MO). Thapsigargin was purchased from Calbiochem. All the solutions used contained 100 mM Na^+ gluconate and 10 mM HEPES, pH 7.2. Two intracellular solutions were prepared: one with 1 nM free Ca^{2+} and the other with 100 nM free Ca^{2+}. The Ca^{2+} was buffered by addition of EGTA. The extracellular solution contained in addition 2 mM Ca^{2+} gluconate.

2.2. Cell culture

Bovine aortic endothelial cells (BAEC) were purchased from the American Type Culture Collection (ATCC) and maintained in culture at 37°C in a humidity controlled incubator with 5% CO_2. The media used is Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 100 μg/ml streptomycin. When monolayers reached confluency the cells were mechanically dispersed with a plastic pipette. Cells were then placed on 35 mm Petri dishes and mounted on the stage of the patch-clamp microscope. Only passages 5–15 were used for the experiments to be described.

2.3. Patch-clamp experiments

The whole-cell configuration of the patch-clamp technique was used to study single BAECs under voltage clamp. Patch pipettes were made of 7052 or 8161 glass (Garner Glass), pulled and fire polished to obtain pipette resistances in the range of 4–6 MΩ when measured in symmetrical 100 mM Na^+ gluconate solution. The AxoPatch 200 A (Axon Instruments) was used as the patch-clamp amplifier. The reference electrode was an Ag-AgCl plug connected to the bath solution via a 150 mM KCl agar bridge. Voltage-clamp data was stored on tape and digitized later for analysis using the Digidata 1200 (Axon Inst.) analog-digital interface and pClamp software (Axon Instruments). The data was filtered at 1 kHz using an 8-pole Bessel filter (Frequency Devices) and digitized at 2 kHz. The binary data was converted to ASCII and multiple experiments were combined to obtain mean and standard deviations of the current amplitude using the Sigmaplot software (Jandel Corporation). For illustration purposes data was plotted every 10th point. The holding potential in all experiments was maintained at 0 mV which is the reversal potential for Na^+. Any inward current would be carried by Ca^{2+} ions. Calmodulin was included in the patch pipette at the concentrations indicated in the text and figures. In all the experiments to be described n = number of experiments performed for each condition. Each experiment was performed on a single cell.

3. Results

3.1. Activation of a calcium current with thapsigargin

Several mechanisms that deplete the ER in vascular endothelium also activate a Ca^{2+} current [3,9]. One of the mechanisms frequently used is the blockade of the microsomal Ca-ATPase with specific agents such as TG or 2′,5′-di(tert-butyl)-1,4-benzohydroquinone (BHQ). Blockade of the microsomal

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PII S0014-5793(96)00675-8

Ca-ATPase results in the depletion of the ER with concomitant influx of Ca^{2+} from the extracellular space via SOCs [1,2]. Fig. 1A shows the activation of a Ca^{2+} current after application of 200 nM TG to the bath solution of a voltage-clamped single BAEC. The upper panel shows a single experiment and the lower panel shows the mean current ± standard deviation of 15 experiments. Fig. 1B shows the current–voltage relationship obtained at the voltages indicated in the figure for the 15 experiments shown in Fig. 1A. The solutions used in these experiments were selected to minimize the influence of K^+ and Cl^- channels present in this endothelium.

As illustrated in this figure, TG activates an inward current at a holding potential of 0 mV. Under these experimental conditions any inward current would be carried by Ca^{2+} ions since the reversal potential for Na^+ is 0 mV (symmetrical 100 mM Na^+ gluconate). The current reached its maximum at 15 ± 5 s after application of TG and remained stable for the duration of the experiments (in the continuous presence of TG). The TG-activated current reversed direction at potentials more positive than +40 mV, indicating that the current is more selective for Ca^{2+} than Na^+ (Fig. 1B).

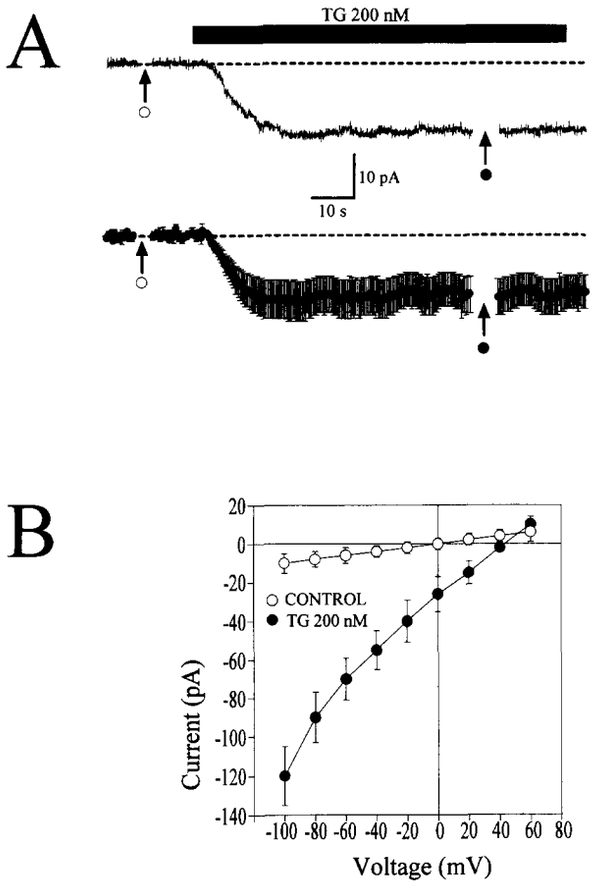

Fig. 1. Thapsigargin activates an inward current carried by calcium ions. (A) Activation of an inward current with 200 nM TG in a single, voltage-clamped BAEC. The solid bar indicates the time of application of 200 nM TG to the bath (extracellular) solution using a rapid perfusion system. The upper panel shows a single experiment and the lower panel shows the mean current and standard deviations of 15 experiments. The holding potential was maintained at 0 mV. Extracellular solution contained 100 mM Na^+ gluconate and 2 mM Ca^{2+} gluconate. Pipette (intracellular) solution contained 100 mM Na^+ gluconate and the Ca^{2+} was buffered to 100 nM with the addition of EGTA. Downward deflections indicate inward current. Under these experimental conditions inward current is carried by Ca^{2+} ions. Dotted horizontal lines show the zero current level. The vertical arrows indicate the point in time where the current–voltage relationships shown in (B) were obtained. The scale shows 10 pA and 10 s. (B) Current–voltage relationship obtained at the voltages indicated in the figure for the 15 experiments shown in (A). Each point was obtained 250 ms after giving a voltage pulse from the holding potential of 0 mV to each one of the potentials indicated in the figure. The pulse duration was 500 ms.

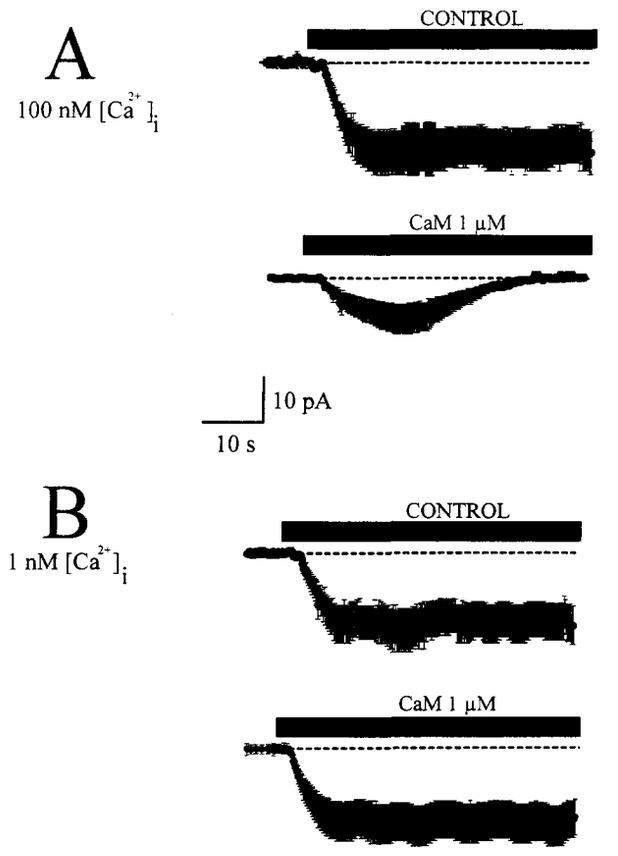

Fig. 2. Effect of CaM and calcium on the TG response. (A) Intracellular (pipette) solution was buffered to 100 nM free Ca^{2+} with EGTA. (B) Intracellular (pipette) solution was buffered to 1 nM free Ca^{2+} with EGTA. Solid bar indicates time of application of 200 nM TG. Control indicates no CaM added to the pipette solution. Extracellular solution contained 100 mM Na^+ gluconate and 2 mM Ca^{2+} gluconate. Holding potential maintained at 0 mV. Downward deflections indicate inward current. Under these experimental conditions inward current is carried by Ca^{2+} ions. Dotted horizontal lines show the zero current level. The scale shows 10 pA and 10 s.

Approximately 83% of the cells responded to the stimulation with TG (25/30 cells), the remaining cells either did not respond or the response was too small and transient and therefore was not included in the present study.

Current fluctuations were frequently observed during the sustained phase of the TG response, these fluctuations in current resemble the response previously reported for BHQ in this endothelium [3].

3.2. *Effect of calmodulin and calcium on the thapsigargin-induced current*

Addition of 1 μM CaM to the patch pipette changed the

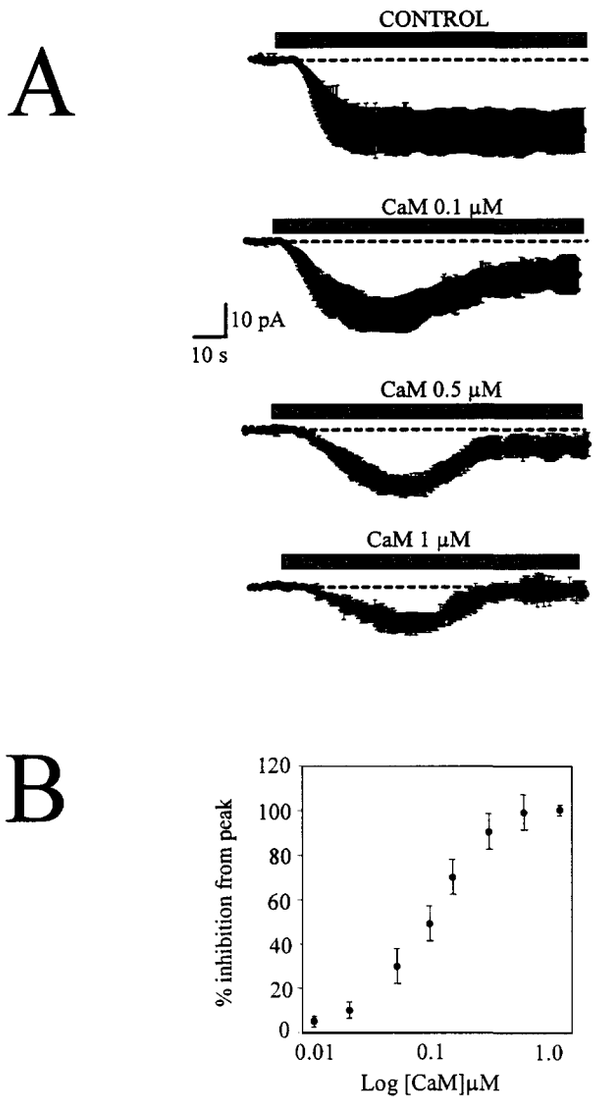

Fig. 3. Effect of various CaM concentrations on the amplitude of the TG-induced inward current. (A) Effect 3 CaM concentrations on the TG response. Solid bar indicates time of application of TG. Holding potential maintained at 0 mV. Downward deflections indicate inward current. Under these experimental conditions inward current is carried by Ca^{2+} ions. Dotted horizontal lines show the zero current level. The scale shows 10 pA and 10 s. (B) Concentration–response curve illustrating the effect of CaM on the sustained current induced by TG. Percent inhibition was obtained from the sustained currents measured 100 s after application of TG. Pipette (intracellular) solution contained 100 mM Na^+ gluconate and the Ca^{2+} was buffered to 100 nM with the addition of EGTA. Extracellular solution contained 100 mM Na^+ gluconate and 2 mM Ca^{2+} gluconate.

profile of the response to TG as illustrated in Fig. 2A. First, CaM reduced by about 50% the amplitude of the TG response from 20 ± 6 pA (control, $n = 12$) to 10 ± 3.5 pA (1 μM CaM, $n = 14$) when measured at the peak of the current at a holding potential of 0 mV. CaM also reduced the amplitude in the sustained phase of the TG response. Approximately 30 s after the application of 200 nM TG the current amplitude returned to control values (before addition of TG) in all the cells explored with 1 μM CaM in the patch pipette.

The effect of CaM on the Ca^{2+} current was dependent on intracellular Ca^{2+} since buffering the intracellular Ca^{2+} to 1 nM with EGTA abolished the effect of CaM on the inward current (Fig. 2B).

3.3. Calmodulin reduces the amplitude of the thapsigargin-induced current

Fig. 3 shows the effect of various concentrations of CaM on the TG-induced inward current. CaM produced a concentration-dependent inhibition of the peak inward current and the sustained current (Fig. 3A). The peak current amplitudes obtained at 0 mV were 26 ± 8 pA (without CaM, $n = 12$), 26 ± 6 pA (0.1 μM CaM, $n = 11$), 20 ± 4 pA (0.5 μM CaM, $n = 9$) and 12 ± 3.5 pA (1 μM CaM, $n = 8$).

Fig. 3B shows the effect of CaM on the sustained current measured at 100 s after activation of the inward current with 200 nM TG. In this figure, the peak current from each experiment was considered 100% of the response and the percentage of inhibition in the sustained current was measured from this value for each experiment. These measurements indicated that 0.1 μM CaM inhibited 53 ± 9% and 1 μM CaM inhibited 96 ± 7% of the sustained current when compared to the peak of the response (Fig. 3B).

3.4. Calmodulin delays the activation of the thapsigargin-induced current

The time to reach the peak of the response was increased by CaM in a concentration-dependent manner. The maximum effect was obtained with 1 μM CaM. The slope of the current activated by TG can be fitted by a single exponential function. As illustrated in Fig. 4, under control conditions (no CaM added) the current reached its peak with a time constant of 16 ± 5 s ($n = 12$). This time was almost doubled in the presence of 1 μM CaM with a time constant of 31 ± 6 s ($n = 10$). Intermediate CaM concentrations produced equivalent changes in the slope of the current. The time constant obtained with

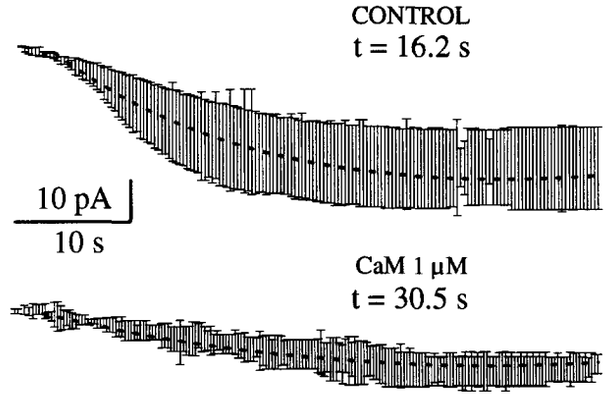

Fig. 4. CaM increases the time for activation of the TG-induced inward current. Control indicates no CaM added. 1 μM CaM was included in the pipette (intracellular) solution in the lower panel. Dotted lines indicate the fit to a single exponential decay with time constants (t) of 16.2 and 30.5 s for the control ($n = 12$) and 1 μM CaM ($n = 10$), respectively. The error bars in the current traces represent the standard deviations of the cells explored. The holding potential was maintained at 0 mV. Pipette (intracellular) solution contained 100 mM Na^+ gluconate and the Ca^{2+} was buffered to 100 nM with the addition of EGTA. Extracellular solution contained 100 mM Na^+ gluconate and 2 mM Ca^{2+} gluconate. Downward deflections indicate inward current. Under these experimental conditions inward current is carried by Ca^{2+} ions. The scale shows 10 pA and 10 s.

0.1 μM CaM was 23 ± 6 s and 28 ± 4 s with 0.5 μM CaM (data not shown).

4. Discussion

4.1. Calmodulin as a modulator of channel activity

Several reports have shown that CaM modulates a variety of ion channels. For example, the skeletal muscle Ca^{2+} release channel (ryanodine receptor) [10], the cyclic nucleotide-activated channel of olfactory receptor neurons [11] and K^+ and Na^+ channels in *Paramecium* [12]. CaM activates the ryanodine receptor at low Ca^{2+} (15 nM) and inhibits the channel at high Ca^{2+} (> 150 nM) [10]. In the nucleotide-activated channel, CaM reduces the apparent affinity of the channel for cAMP by up to 20-fold [11].

The finding of several mutants in the *Paramecium* showing a 'backward swimming' phenotype helped to demonstrate the role of CaM in the ciliary motion of this unicellular organism [12]. All mutants showed mutations in the *CaM* gene. The Ca^{2+}–CaM complex is a regulator of Na^+ and K^+ channels which are essential for ciliary motion in the *Paramecium* [12].

Using labelled CaM to probe an expression library of *Drosophila* head, several clones expressing CaM-binding fragments were retrieved [13]. From these clones two proteins essential for the phototransduction in the *Drosophila* photoreceptor have been isolated. The first isolated protein was named TRP for transient receptor potential [14] and the second protein TRPL for TRP-Like [13]. Sequence analysis indicated that TRP has one and TRPL has two putative CaM binding domains.

Expression of TRP using the baculovirus sf9 insect expression system showed that TRP is a Ca^{2+}-selective channel regulated by depletion of intracellular Ca^{2+} stores [15], while TRPL is a non-selective cation channel regulated by D-myo-inositol 1,4,5-trisphosphate [16].

4.2. Calmodulin as a possible modulator of store-operated calcium influx

The finding that TRP is a SOC stimulated the search for mammalian TRP homologues. Recently several independent groups reported the presence of TRP homologues expressed in several mammalian tissues including human [17,18]. Although there is no evidence indicating that the SOC found in vascular endothelium belongs to the TRP family of channels, the fact that these channels are modulated by the depletion of intracellular Ca^{2+} stores suggests the presence of a conserved domain or region responsible for communicating the internal Ca^{2+} organelle with the plasmalemmal channel. Another important similarity is the modulation of channel activity by D-myo-inositol 1,4,5-trisphosphate in vascular endothelium [19] and in TRPL [16]. Finally, the CaM modulation of the endothelial channel demonstrated here and the identification of two functional CaM binding domains present in TRPL are worth mentioning [20]. Further experiments will be required to determine the effect of CaM on TRP or TRPL channel activity.

4.3. Mechanism of action of the CaM inhibition of thapsigargin-induced calcium current

The effect of CaM on the TG-induced Ca^{2+} current is complex. The slowing down of the activation of the current can be explained by at least two mechanisms. First, if CaM affects the rate of depletion of the ER that would also affect the activation of the current. As previously shown, the communication between the ER and SOC is fast and reversible in such a way that depletion of the ER activates the current whereas refilling of the ER inactivates the current [3]. Second, the effect of CaM could be on the SOC itself (without affecting the depletion rate of the ER). If this is the case, then CaM could delay the activation of SOC or reduce the number of channels available for activation in a given time.

The time constants for current activation after depletion of the ER obtained here are comparable to time constants previously described for mast cells [7]. In the mast cell study, time constants of 18–44 s were reported, depending on the method used to deplete the intracellular Ca^{2+} stores (e.g. agonists, TG, D-myo-inositol 1,4,5-trisphosphate, etc.).

A recent report describes the fast inactivation of the depletion-activated Ca^{2+} current in T lymphocytes due to local Ca^{2+} feedback [8]. In this study Ca^{2+} induced a biexponential decline of the current with time constants of 8–30 ms and 50–150 ms. The fast inactivation induced by Ca^{2+} ions contrasts with the slow inactivation induced by CaM in the present study where the time constants obtained are in seconds. We have previously reported fast inactivation due to intracellular Ca^{2+} on SOC single channels in this endothelium [9].

CaM also reduced the peak current. This effect could be produced by reducing the channel open probability (P_o) of SOC or the number of channels 'available' for activation. A reduction on channel P_o could result in a smaller TG-induced current, however, a reduction on channel P_o would not slow down the activation of the current.

Finally, CaM produced a decline on the sustained current level during the TG response. This effect was time-dependent, that is, the decline of the current was observed 15–20 s after onset of the TG response. This result is more difficult to explain but could involve activation of second-messenger cascades which require time to exert its effect, such as Ca^{2+}–CaM-dependent phosphorylation of the channel.

Some of the complex effects of CaM on the TG-induced current could also be explained by a sequential binding of Ca^{2+} and CaM to the channel. Such a mechanism has been postulated for *Paramecium* Na^+ channels [12]. Complex effects on channel gating could be obtained in a channel with multiple binding sites for Ca^{2+} and CaM. Further experiments at the single channel level will be required to determine the mechanism of action of CaM on the endothelial SOC. However, these experiments have been difficult to performed so far, because channel activity runs down rapidly in excised patches [9].

Acknowledgements: This work was supported by grants from the Dirección General de Asuntos del Personal Académico (DGAPA-UNAM) No. IN-209495 and from the Consejo Nacional de Ciencia y Tecnología (CONACyT) No. 0103-PN to Luis Vaca.

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