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Biochimica et Biophysica Acta 1240 (1995) 196–200

BBA Biochimica et Biophysica Acta

Inhibition of L-type calcium current by genistein, a tyrosine kinase inhibitor, in pregnant rat myometrial cells

Masumi Kusaka *, Nicholas Sperelakis

Department of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, P.O. Box 670576, Cincinnati, OH 45267-0576, USA

Received 15 March 1995; accepted 17 July 1995

Abstract

Possible regulation of L-type Ca^{2+} channels by tyrosine kinase was examined in freshly isolated uterine smooth muscle cells obtained from late pregnant (18–19 day) rat, using whole-cell voltage clamp. Bath application of genistein, an inhibitor of tyrosine kinase, decreased L-type Ca^{2+} current ($I_{Ca(L)}$) dose-dependently. The maximal inhibition of $I_{Ca(L)}$ was 46% and the concentration for half-maximal inhibition (IC_{50}) was 50 μM (at a holding potential of -60 mV). The effect of genistein was reversible. Daidzein, an inactive analog of genistein, had no inhibitory effect on $I_{Ca(L)}$ at concentrations as high as 300 μM. The steady-state inactivation curve for $I_{Ca(L)}$ was shifted to the left by genistein (15 mV at 100 μM), whereas the activation curve was not affected, suggesting that genistein exerts a voltage-dependent block. These results suggest that the L-type Ca^{2+} channels in myometrial cells may be modulated by endogenous tyrosine kinase, i.e., they are in a tonically stimulated state due to tyrosine kinase activity. This modulatory mechanism may play a role on the regulation of Ca^{2+} influx and uterine contraction during normal labor and preterm labor.

Keywords: Tyrosine kinase; Genistein; Daidzein; Myometrium; Calcium ion current; Whole-cell voltage clamp; (Uterine smooth muscle); (Pregnant rat)

1. Introduction

Voltage-dependent Ca^{2+} channels are known to play a key role in excitation-contraction coupling of uterine smooth muscle. Opening of the channels produces membrane depolarization and Ca^{2+} entry that are needed for uterine contraction. Protein phosphorylation (on serine and/or threonine groups) is a major regulatory process for the modulation of Ca^{2+} channels in neuronal cells [1], myocardial cells [2], and vascular smooth muscle cells [2,3]. In uterine smooth muscle cells, however, cAMP and cGMP (and their respective protein kinases) do not appear to modulate the activity of the L-type Ca^{2+} channels [4]. We found that protein kinase C, in contrast, does modulate the Ca^{2+} channels: addition of phorbol esters increased the L-type Ca^{2+} current ($I_{Ca(L)}$) by 37% [5], and this stimulatory action depended on intracellular Ca^{2+} level (Kusaka, M. and Sperelakis, N., unpublished observations). To further clarify the modulation of Ca^{2+} channels in myometrial cells, we wanted to examine the possible role of tyrosine-specific protein kinase.

Tyrosine kinase has been shown to be correlated with the signal transduction for several growth factors, such as epidermal growth factor (EGF) [6,7], fibroblast growth factor (FGF) [8,9], platelet-derived growth factor (PDGF) [10,11]. The insulin receptor also has tyrosine kinase activity [12]. However, only a few reports have been published about the regulation of ion channels, including Ca^{2+} channels, by tyrosine kinase. In neuronal cells, basal FGF was reported to increase $I_{Ca(L)}$, but the participation of tyrosine kinase was not proposed [13,14]. Tyrosine kinase inhibitors, such as genistein, were reported to block the Ca^{2+} current in vascular smooth muscle cells [15], and to activate a non-selective cation channel [16]. However, no information is available for uterine smooth muscle.

In this study, we investigated the possible modulation of the slow Ca^{2+} channels in rat myometrial cells by studying the effect of tyrosine kinase inhibitors on $I_{Ca(L)}$ using the whole-cell patch clamp. We found that $I_{Ca(L)}$ is

* Corresponding author. Fax: +1 (513) 5585738.

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SSDI 0005-2736(95)00191-3

inhibited by the tyrosine kinase inhibitor, genistein. Thus, endogenous tyrosine kinase activity may be involved in the regulation of Ca^{2+} channel activity in myometrial cells, namely to exert tonic stimulation.

2. Materials and methods

2.1. Cell preparation

Freshly-isolated single smooth muscle cells were prepared from the longitudinal layer of late pregnant (18–19 day) rat (Sprague-Dawley, Zivic-Miller, USA) uterus as previously described [17]. The rat was anesthetized by CO_2 and decapitated. Distal part of uterine horn was excised and the longitudinal layer was carefully isolated by removing the endometrium and circular muscle layer under a binocular microscope. The tissues were cut into small pieces and incubated in nominally calcium-free solution containing (in mM) 140 NaCl, 6 KCl, 10 glucose, 10 Hepes (pH 7.35) for 20 min and then with 0.2–0.3% collagenase (Worthington Biochemical Co., Freehold, NJ, USA) for 80 min at 37°C. After digestion, the tissues were rinsed twice and agitated in the fresh calcium-free solution. Cell suspension was stored at 4°C and used for experiments within 6 h after cell dispersion. Most of the obtained cells were spindle-shaped, 50–200 μm long and 20–30 μm width, as previously reported [18].

2.2. Patch-clamp recording

Whole-cell voltage clamp recording was carried out with a suction pipette and a patch clamp amplifier (Axopatch-1D, Axon Instruments, Foster City, USA) using standard techniques [19]. The patch electrodes (2–5 MΩ) were made from borosilicate glass capillary tubing (World Precision Instruments, USA). The cell suspension was placed into a small chamber (0.2 ml) on the stage of an inverted microscope (TMD-Diaphoto, Nikon, Tokyo, Japan). To isolate the $I_{Ca(L)}$, the pipette was filled with high Cs^+ solution of the following composition (in mM): 110 CsOH, 20 CsCl, 110 glutamic acid, 5.2 $MgCl_2$, 10 Hepes, 5 Na_2ATP, 10 EGTA (pH 7.2). The bath solution contained (in mM): 140 tetraethylammonium chloride, 2 $CaCl_2$, 10 glucose, 10 Hepes, 3 4-aminopyridine (4-AP) (pH 7.35). Series resistance was partly (ca. 80%) compensated electrically. Leak current and residual capacitive current were subtracted using P/4 protocol. Current and voltage signals were filtered at 2 kHz and digitized by an A/D converter (TL-1, Axon Instruments) and analyzed on an IBM-AT personal computer using the pCLAMP software (Axon Instruments). The membrane capacitance was determined from the current amplitude elicited in response to a hyperpolarizing voltage ramp pulse of 0.2 V/s from a holding potential of 0 mV to avoid interference by any time-dependent ionic currents. All experiments were carried out at room temperature (22–24°C).

Data are presented as means ± S.E. Theoretical curves were fitted to the data with the least-squares method.

2.3. Drugs

Genistein, daidzein and tyrphostin-A25 were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). These agents were dissolved in dimethylsulfoxide (DMSO) for a stock solution, prepared in aliquots and frozen until use. Final maximal concentration of DMSO was 0.1% and had no effect on $I_{Ca(L)}$.

3. Results

The mean membrane capacitance of uterine smooth muscle cells was 125.0 ± 4.8 pF ($n = 27$) and was not affected by application of genistein or other drugs used in this study. Unless stated otherwise, the $I_{Ca(L)}$ was elicited by 300 ms depolarizing step pulses from a holding potential (HP) of −60 mV every 20 s. The study of the effect of genistein was started after the $I_{Ca(L)}$ became stabilized after breaking into the cell. The current was completely inhibited by 1 μM nifedipine (data not shown), and the kinetic properties including inactivation time course and current/voltage relationship (see Fig. 1) were consistent with those of $I_{Ca(L)}$ we reported previously for uterine smooth muscle cells [17,18].

Bath application of genistein (100 μM) reduced $I_{Ca(L)}$ by 44.7 ± 2.3% ($n = 8$). Fig. 1A shows superimposed current traces before (Aa) and 5 min after (Ab) genistein application. Note the marked inhibition of the peak inward current produced by genistein. The current/voltage relationship for peak $I_{Ca(L)}$ was not significantly shifted by

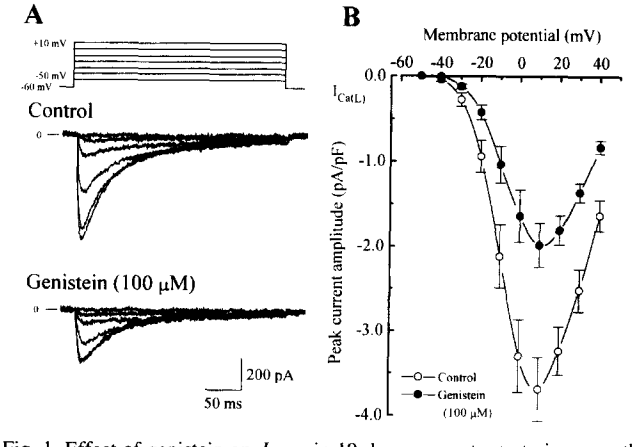

Fig. 1. Effect of genistein on $I_{Ca(L)}$ in 19-day pregnant rat uterine smooth muscle cells. (A) Superimposed current traces elicited by 300 ms depolarizing pulses (−50 mV to +10 mV in 10 mV increments) from a holding potential (HP) of −60 mV, before and 5 min after bath application of 100 μM genistein. (B) Current/voltage relationships of $I_{Ca(L)}$ obtained before (open circles) and 5 min after (closed circles) genistein application. The pipette solution contained Cs^+ to inhibit K^+ current and the bath solution contained 2 mM Ca^{2+}. Data were obtained from five cells.

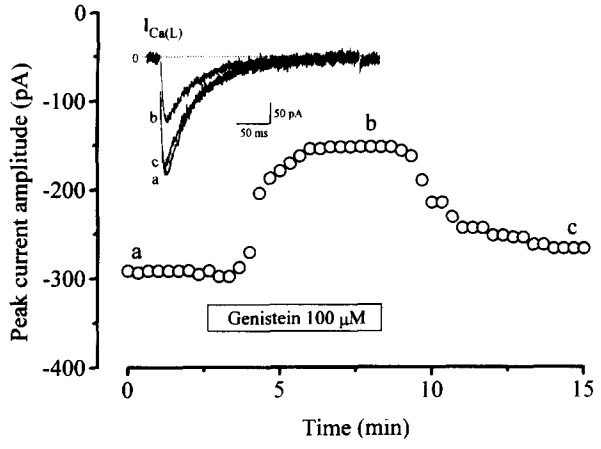

Fig. 2. Time-course of the inhibition of $I_{Ca(L)}$ by 100 μM genistein. The effect reached the stable level within 5 min after application of genistein, was reversed by washout. Currents were elicited from a HP of −60 mV to a step potential of +10 mV every 20 s. (Inset) Superimposed current traces showing the $I_{Ca(L)}$ recorded before (a) and 6 min after (b) application, and after washout (c) of genistein.

genistein; there was no obvious change in voltages for threshold or peak current (Fig. 1B).

Fig. 2 shows the time course of the effect of genistein in one representative experiment. $I_{Ca(L)}$ was evoked by depolarizing pulses to +10 mV (HP of −60 mV, every 20 s), and a peak current amplitude was measured. The decrease in $I_{Ca(L)}$ started within a few minutes after bath application of genistein and reached a stable level within 5 min. The effect of genistein on $I_{Ca(L)}$ was almost completely reversed by washout.

Fig. 3 shows the dose/response curve for the inhibitory effect of genistein at a HP of −60 mV. Peak $I_{Ca(L)}$ amplitude in the presence of genistein was plotted against the various concentrations of the drug as a percent of control. Data points were fitted to the Hill equation:

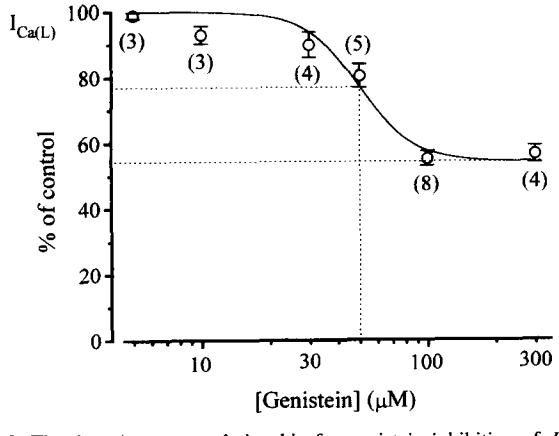

Fig. 3. The dose/response relationship for genistein inhibition of $I_{Ca(L)}$. Peak $I_{Ca(L)}$ amplitude was measured before and 5 min after application of various concentrations of genistein in the bath. Pulse protocol was the same as Fig. 2. Numbers in parentheses indicate the number of cells for each data points. The maximum inhibition, IC_{50} and Hill coefficient (n_H) were 46%, 50 μM and 3.7, respectively.

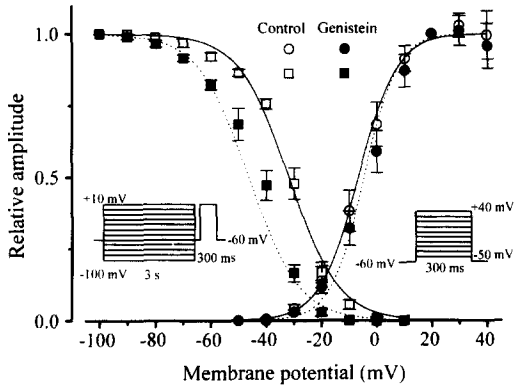

Fig. 4. The activation and inactivation curves for $I_{Ca(L)}$ before and after genistein (100 μM) application. Steady-state inactivation (h_∞) curves were obtained from five cells using a double-pulse protocol. Pre-conditioning pulses of 3 s duration were applied in 10 mV steps between −100 mV and +10 mV from a HP of −60 mV, and then the test pulse of 300 ms duration was applied to +10 mV (interpulse duration was 5 ms). The inactivation curve in the presence of genistein shifted towards a negative potential by about 15 mV, as compared with the control. Activation curves were derived from the current/voltage relationships obtained from five cells, and the voltage dependence was not changed by genistein. Inset shows the pulse protocol and experimental condition was the same as in Fig. 2.

$I_{genistein}/I_{control}$ (%) $= E_{max}/(1 + ([genistein]/IC_{50})^{nH}) + (100 - E_{max})$, where E_{max} is the maximum inhibitory effect, n_H is the Hill coefficient, IC_{50} is the concentration of the drug causing 50% of the maximal inhibitory effect. The n_H value was 3.65 and the IC_{50} value was 49.9 μM. The maximum inhibitory effect was calculated to be 45.6%, and was almost attained at the concentration above 100 μM.

To investigate the effects of genistein on the voltage dependence of activation and inactivation, steady-state activation and inactivation (h_∞) curves were obtained before and after genistein application (Fig. 4). Activation curves were derived from the current/voltage relationships (Fig. 4, right inset). Conductance (G) was calculated from the equation: $G = I_{Ca}/(V_m - V_{rev})$, where I_{Ca} is the peak current elicited by depolarizing test pulses to the various potentials, and V_{rev} is the reversal potential (+60.6 ± 1.3 mV, $n = 18$; obtained from the extrapolated current/voltage curves (see Fig. 1)). G_{max} is the maximum Ca^{2+} conductance (calculated at potentials above +10 mV). The points for G/G_{max} were plotted against the membrane potential as a relative amplitude. These data were fitted to the Boltzmann equation: $1/(1 + \exp((V_m - V_{1/2})/k))$, where V_m is the conditioning potential, $V_{1/2}$ is the potential required for half-activation of current, and k is the slope factor. $V_{1/2}$ and k were −6.7 mV and −7.0 mV in control, and −4.7 mV and −6.0 mV ($n = 5$) after genistein application, respectively. There is no apparent difference among these values.

The h_∞ curves were obtained by a double-pulse protocol (Fig. 4, left inset). The peak current elicited by test pulses was normalized by the maximum current (evoked

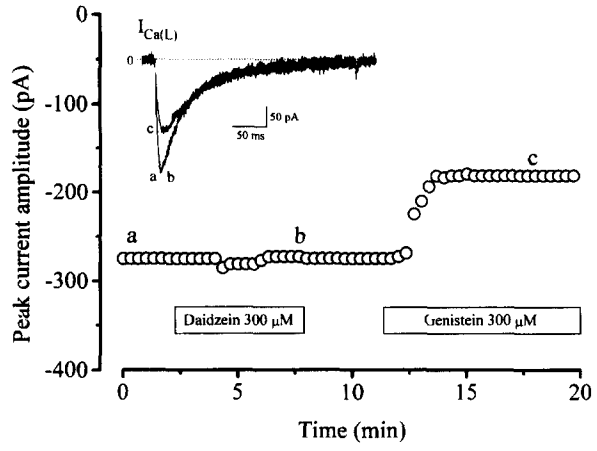

Fig. 5. Lack of the effect of daidzein, an inactive genistein analog. Bath application of daidzein (300 μM) did not affect $I_{Ca(L)}$. Subsequent application of genistein (300 μM) markedly reduced the current. Experimental condition was the same as in Fig. 2. (Inset) Superimposed current traces recorded during the control period (a), in the presence of daidzein (b) and genistein (c).

from a pre-pulse of −100 mV) and plotted against the conditioning potential. Resulting inactivation curves were also fitted to the Boltzmann equation. The values of $V_{1/2}$ (half-inhibition potential) and k were −31.9 mV and 9.0 mV in control, −46.4 mV and 9.1 mV in the presence of genistein ($n = 5$). The inactivation curve was shifted in the negative direction by 14.5 mV, thereby suggesting that genistein has a voltage-dependent inhibitory action.

In order to examine whether the effect of genistein on $I_{Ca(L)}$ was mediated by tyrosine kinase, experiments were performed using daidzein, which has similar structure to genistein but has no inhibitory effect on tyrosine kinase [20]. Bath application of 300 μM daidzein did not affect $I_{Ca(L)}$ (Fig. 5). Subsequent application of genistein (300 μM) decreased the current. The peak $I_{Ca(L)}$ measured at 5 min after daidzein (300 μM) application was 96.7 ± 1.1% of control ($n = 4$).

We also tested the effect of tyrphostin-A25, another tyrosine kinase inhibitor that has a different structure. However, bath application of tyrphostin-A25 (100 μM for 10 min) did not affect $I_{Ca(L)}$ (peak $I_{Ca(L)}$ of 103.9 ± 3.1% of control; $n = 4$, data not shown).

4. Discussion

In the present study, we found that a selective tyrosine kinase inhibitor, genistein, inhibits $I_{Ca(L)}$ dose-dependently in freshly-isolated uterine smooth muscle cells obtained from late pregnant rat. The maximal inhibition was 46%, and the IC_{50} value was 50 μM. The onset of the inhibition of $I_{Ca(L)}$ produced by genistein was fast (within a few minutes), and the effect could be quickly washed out. Genistein shifted the inactivation curve of $I_{Ca(L)}$ in the negative direction by 15 mV, whereas the activation curve was not affected. The genistein analog, daidzein, which is inactive as a tyrosine kinase inhibitor, did not produce a decrease of the current. These results suggest that $I_{Ca(L)}$ inhibition by genistein may be mediated by inhibition of endogenous tyrosine kinase activity.

The concentration range of genistein that affected $I_{Ca(L)}$ in this study ($IC_{50} = 50$ μM) is comparable to that needed for the inhibition of tyrosine kinases [20,21]. The fact that the maximal inhibitory effect (46%) was not 100% might be explained by several possible mechanisms. Genistein may decrease either single-channel conductance or open probability of the Ca^{2+} channels to about the half of the normal level. Another possibility is that genistein may completely block the Ca^{2+} channels, but only a fraction of the channels are sensitive to the agent, i.e., are normally phosphorylated by tyrosine kinase. The Hill coefficient for the dose/response curve is 3.7 (see Fig. 3), suggesting that there may be multiple tyrosine kinase phosphorylation sites on the Ca^{2+} channels.

Genistein shifts the steady-state inactivation curve to the left, indicating that the effect of genistein is voltage-dependent, and therefore should be more prominent when the membrane is held at depolarized potentials (see Fig. 4). The leftward shift of the steady-state inactivation curve may be one of the mechanisms of the inhibition of $I_{Ca(L)}$ by genistein, but is insufficient to account for the 46% inhibition; the predicted inhibitory effect due to the shift is ca. 15% at a HP of −60 mV (see Fig. 4).

All of these findings suggest that phosphorylation by tyrosine kinase regulates the basal activity of the L-type Ca^{2+} channels in myometrial cells. Our findings are consistent with the reported block of $I_{Ca(L)}$ in vascular smooth muscle cells [15]. The IC_{50} value for the inhibitory effect of genistein (36 μM) was similar to that in our study (50 μM). However, there are some differences between our findings and those in the previous report on vascular smooth muscle. In our study, genistein had the maximal inhibitory effect (ca. 50%) at a concentration of 100 μM; i.e., 300 μM did not produce greater inhibition. In contrast, in vascular smooth muscle cells, genistein and tyrphostin-23 completely blocked $I_{Ca(L)}$ at 300 μM. One possible explanation for this difference is that the L-type Ca^{2+} channels in these two types of smooth muscles are regulated to different degrees by endogenous tyrosine kinases.

Although genistein had significant inhibitory effect on $I_{Ca(L)}$, another tyrosine kinase inhibitor, tyrphostin-A25, did not affect the current when added to the bath. The lack of the effect of tyrphostin-A25 could be due to its difficulty in penetrating through the cell membrane. It was reported that the onset of inhibitory effect of tyrphostin derivatives is slow and many hours of incubation is required [22]. Alternatively, these two agents may act on different tyrosine kinase isotypes. Myometrial cells may have an isotype of tyrosine kinase that is sensitive to

genistein, but not to tyrphostin-A25. The concentration of tyrphostin-A25 used in this study (100 μM) was reported to be sufficient for the inhibition of tyrosine kinase activity of the EGF receptor (IC_{50} = 15 μM) [23].

The possibility of a direct blocking action of genistein on the Ca^{2+} channels can not be excluded. For example, the rapid onset of the effect of genistein and the rapid offset (washout recovery) are consistent with a direct action on the outer surface of the channel. In addition, both active and inactive tyrphostin derivatives had similar potent inhibitory effects on $I_{Ca(L)}$ in vascular smooth muscle cells [15]; this provides evidence for a direct action of these agents on the Ca^{2+} channel proteins. One argument against a direct action on the channel itself is that the maximum degree of inhibition of $I_{Ca(L)}$ was only to about 50% (Fig. 3); if the site of action was on the channel protein, one would expect 100% inhibition to be attained at high dose, unless there were two types of Ca^{2+} channels.

A physiological role of tyrosine kinase in the modulation of L-type Ca^{2+} channels by in myometrial cells is a distinct possibility. The inhibition of $I_{Ca(L)}$ by genistein suggests that tyrosine phosphorylation stimulates the basal activity of the Ca^{2+} channels in myometrial cells. Since tyrosine kinase may be anchored to receptors imbedded in the cell membrane, this enzyme is unlikely to be lost from the cytosol after breaking into the cell, and therefore rundown of $I_{Ca(L)}$ should not occur during whole-cell recording. Because the L-type Ca^{2+} channels play an important role in Ca^{2+} influx and thereby uterine contraction, the phosphorylation of the channels by tyrosine kinase could affect normal labor and preterm labor. For example, it was reported that angiotensin II, which is known to augment uterine contraction, stimulates tyrosine phosphorylation in vascular smooth muscle cells [24]. Although angiotensin II did not increase $I_{Ca(L)}$ in myometrial cells (Kusaka, M. and Sperelakis, N., unpublished observations), it is possible that, because the hormone induces Ca^{2+} release from SR, the resulting increase in intracellular Ca^{2+} may inhibit the Ca^{2+} channels. In neuronal cells, basic FGF, whose actions are known to be associated with tyrosine kinase, was reported to increase $I_{Ca(L)}$ [13,14]. These findings support a possible role of tyrosine kinase in agonist-induced contraction of uterine muscle.

In conclusion, genistein inhibits $I_{Ca(L)}$ in uterine smooth muscle cells. This effect may be mediated by inhibition of tyrosine kinase, but a direct action on the Ca^{2+} channels cannot be excluded. Possible modulation of the L-type Ca^{2+} channels by tyrosine kinase could contribute to the regulation of basal and agonist-induced contractility of uterine muscle during pregnancy.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (HL-26170).

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