Intracellular calcium release is more efficient than calcium influx in stimulating mitochondrial NAD(P)H formation in adrenal glomerulosa cells

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We compared the effect on mitochondrial NAD(P)H formation of calcium release from intracellular stores with that of calcium influx from the extracellular space. Simultaneous measurements of cytoplasmic free calcium ion concentration and mitochondrial NAD(P)H were performed on fura-PE3-loaded single rat adrenal glomerulosa cells. The effects of equipotent stimuli in terms of the evoked Ca^{2+} response were compared. Angiotensin II (AII; 1 nM) induced a higher amplitude NAD(P)H response than K^+ (5.6–7.6 mM). Vasopressin (1 μM) also induced a greater initial NAD(P)H formation than K^+, although the Ca^{2+} signal evoked by the two agonists had similar amplitude. To examine the effect of Ca^{2+} release from internal stores we applied AII in Ca^{2+}-free medium. We compared the effect on NAD(P)H formation of Ca^{2+} release with Ca^{2+} influx induced by K^+, and with capacitative Ca^{2+} influx induced by AII. NAD(P)H formation in response to Ca^{2+} release was greater than that induced by Ca^{2+} influx, irrespective of whether induced by K^+ or AII. Our results indicate that Ca^{2+}, presumably released in the vicinity of mitochondria, activates mitochondrial dehydrogenases more efficiently than Ca^{2+} entering through the plasma membrane. These data confirm the biological significance of previous observations showing that Ca^{2+} released from inositol 1,4,5-trisphosphate-sensitive internal stores increases mitochondrial matrix $[Ca^{2+}]$ to a greater extent than extracellular Ca^{2+}.

INTRODUCTION

Cytoplasmic Ca^{2+} signalling plays an important role in the control of aldosterone secretion by adrenal glomerulosa cells [1,2]. It mediates the effect of angiotensin II (AII), K^+ and the paracrine factor vasopressin [1]. The stimulation of aldosterone production by free cytoplasmic Ca^{2+} is achieved by multiple mechanisms. Most of the enzymic steps of the conversion of cholesterol into aldosterone take place in the mitochondria. The calmodulin-dependent action of Ca^{2+} [3] may be attributed to the enhanced transfer of cholesterol from the cytoplasm to the mitochondrial inner membrane [4]. Just as in other cell types, elevated free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) raises $[Ca^{2+}]$ also in the mitochondrial matrix [5]; an elevated mitochondrial matrix Ca^{2+} concentration ($[Ca^{2+}]_m$) was found to play an important role in the control of steroid biosynthesis [5,6]. One way that mitochondrial Ca^{2+} can stimulate aldosterone production is the formation of NAD(P)H. Elevation of $[Ca^{2+}]_m$ stimulates three metabolic enzymes, oxoglutarate dehydrogenase, isocitrate dehydrogenase and pyruvate dehydrogenase [7]. All three enzymes catalyse the formation of NADH. Transhydrogenase enzymes reduce $NADP^+$ at the expense of NADH, thus NADPH is also formed [8]. Mitochondrial NADPH is a cofactor of several mitochondrial steps in steroid synthesis, thus Ca^{2+}-induced reduction of pyridine nucleotides increases the cofactor supply of these enzymes. We have previously shown that, in glomerulosa cells, NADPH formed in response to Ca^{2+} is utilized by aldosterone synthesis [9].

More and more data support the idea that hormonal elevation of $[Ca^{2+}]_i$ is not uniformly distributed in the cytoplasm [10]. High $[Ca^{2+}]$ microdomains in the vicinity of different Ca^{2+} release and influx channels have been described in several cell types [10–12]. Stimuli operating with different modes of action may raise $[Ca^{2+}]$ in different compartments of the cytoplasm [13,14]. We have previously found that the rate of Ca^{2+}-mediated aldosterone production was dependent on the source of Ca^{2+} [1]. This dependence may be accounted for by the compartmentalization of Ca^{2+} signalling. It is, however, difficult to correlate aldosterone production and Ca^{2+} signalling, as presently there is no method available to measure the two phenomena on the same cells and in the same time resolution. Therefore we examined the effect of different Ca^{2+} sources on mitochondrial NAD(P)H formation. Mitochondrial NAD(P)H formation supports aldosterone production [9], correlates with steroid production [15] and can be measured simultaneously with $[Ca^{2+}]_i$ in the same cell [9,16,17]. In order to evaluate the biological significance of different Ca^{2+} transport routes into the cytoplasm we compared the efficiency of inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release and Ca^{2+} influx in inducing mitochondrial NAD(P)H formation in rat glomerulosa cells.

EXPERIMENTAL

Materials

Materials used for cell isolation have been described previously [18]. AII (Ile^5-AII) and arginine vasopressin were obtained from Serva (Heidelberg, Germany), fura-PE3 acetoxymethyl ester was from TEFLABS (Austin, TX, U.S.A.).

Cell isolation and incubation

Glomerulosa cells were obtained from the adrenal capsular tissue of male Wistar rats by collagenase digestion, as described previously [18]. The cells were plated on poly-L-lysine-coated glass coverslips in a mixture (38:62, v/v) of modified Krebs–Ringer/bicarbonate/glucose solution and M199 (medium A). The mixture contained: 3.6 mM K^+, 1.2 mM Ca^{2+}, 0.5 mM

Abbreviations used: IP_3, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, free cytoplasmic Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial matrix Ca^{2+} concentration; AII, angiotensin II.

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Mg^{2+}, 5 mM Hepes and 20 mM HCO_3^-. The cells were kept for 3–9 h at 37 °C under 5% CO_2 (pH 7.4).

Measurement of mitochondrial NAD(P)H and $[Ca^{2+}]_i$

Simultaneous measurements of NADH plus NADPH, designated as NAD(P)H, and $[Ca^{2+}]_i$ were performed in single glomerulosa cells, as described previously [9]. Briefly, the cells plated on the coverslips were mounted on the stage of an inverted microscope (Zeiss) in a modified Krebs–Ringer/bicarbonate solution, containing 3.6 mM K^+, 1.2 mM Ca^{2+}, 0.5 mM Mg^{2+}, 2 mM HCO_3^- and 10 mM Hepes, pH 7.4, at 37 °C (medium B). The microscope was coupled to a photomultiplier-based microspectrofluorimeter (PTI, South Brunswick, NJ, U.S.A.). The cells were loaded with fura-PE3 acetoxymethyl ester (0.5–1 μM) for 15–30 min at 37 °C in medium A. Fura-PE3 has the same spectral properties as fura-2 [19], with smaller leakage from the cells [20]. For simultaneous measurement of changes in $[Ca^{2+}]_i$ and NAD(P)H the cells were excited with 360 and 395 nm light and the emitted light was recorded at 470 nm. The intrinsic fluorescence of the pyridine nucleotides was measured at an excitation wavelength of 360 nm, the isosbestic point of fura-PE3, where the fluorescence of the dye does not depend on $[Ca^{2+}]$. At an excitation wavelength of 395 nm the fluorescence of NAD(P)H is negligible, and the Ca^{2+}-sensitive dye responds to Ca^{2+} with a decrease in fluorescence intensity. The gradual decrease in baseline fluorescence in both dye-loaded and unloaded cells was eliminated by division of the curves by a negative exponential function. The *y*-axes of the Figures show the fluorescence intensity relative to the control period. In dye-loaded cells, the fluorescence intensity at 360 nm is determined both by the light emitted from NAD(P)H and the light emitted, Ca^{2+}-independently, from fura-PE3. Owing to this dual origin of fluorescence in dye-loaded cells, the relative changes at the 360 nm excitation wavelength underestimate the changes in NAD(P)H concentration. For the sake of clarity the curves obtained at 395 nm are inverted on the Figures.

For measurement of $[Ca^{2+}]_i$ with the ratio method [without monitoring NAD(P)H] the cells were loaded with 2 μM fura-PE3 for 30 min in medium A at 37 °C. Fluorescence was measured at excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm in medium B. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence intensities at excitation wavelengths of 340 and 380 nm, using the method of Grynkiewicz et al [19].

Statistical analysis

Statistical data are presented as means ± S.E.M. Significance was estimated by Student's *t*-test or analysis of variance. All the curves shown in the Figures are representative of at least six measurements.

RESULTS

Repeated Ca^{2+} signals sensitize mitochondrial NAD(P)H formation

To test the reproducibility of repetitive stimulation, we first examined the effect of repeated stimulation with K^+. Potassium concentration was elevated from 3.6 mM, the physiological resting level, to 6.6 mM for a short period (13–43 s), then lowered to the control level. This protocol was repeated four times. NAD(P)H response increased gradually, reaching its plateau usually by the fourth pulse (results not shown). When these experiments were performed in cells loaded with the Ca^{2+}-sensitive fluorescent dye fura-PE3, the successive Ca^{2+} spikes had comparable amplitude, but the NAD(P)H response again displayed a gradual increase. In eight experiments there was no significant alteration in the

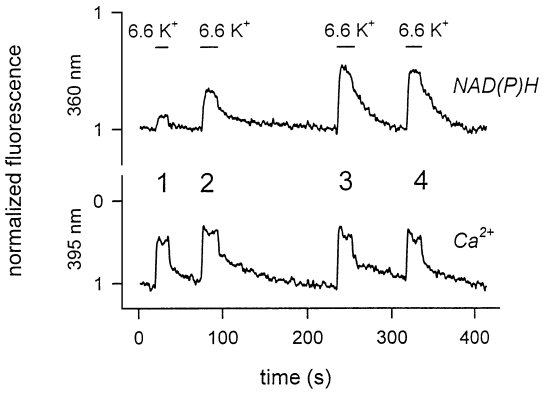

Figure 1 Effect of repeated stimulation of a glomerulosa cell with 6.6 mM K^+

A single glomerulosa cell was stimulated four times with elevation of extracellular $[K^+]$ from 3.6 to 6.6 mM, as shown by the horizontal bars. Simultaneous measurement of $[Ca^{2+}]_i$ (395 nm) and NAD(P)H (360 nm) was performed, as described in the Experimental section. Representative curves are shown for eight measurements performed on five separate cell preparations.

Ca^{2+} responses (first pulse, 104 ± 7%; second pulse, 98 ± 5%, third pulse, 95 ± 6% of the response to the fourth pulse). In contrast, the NAD(P)H response to the first pulse attained only 43 ± 7%, to the second pulse it attained 80 ± 6%, and finally, it amounted to 94 ± 5% in response to the third pulse (regarding the responses to the fourth pulse as 100%). A representative experiment is shown in Figure 1. The difference between the first and second as well as between the second and fourth NAD(P)H response was highly significant ($P < 0.001$). Sensitization of the NAD(P)H response to successive Ca^{2+} spikes has been observed previously in glomerulosa cells stimulated with a submaximal concentration of AII [9,16]. On the other hand, no sensitization was induced by repeated stimulation with the maximally effective 8.4 mM K^+ [9]. In order to avoid sensitization problems, in all subsequent experiments the cells were prepulsed several times with K^+ until no further increase in the response was observed.

Since $[Ca^{2+}]_i$ cannot be quantified in experiments where NAD(P)H is also monitored, we performed separate experiments with the ratio method to obtain absolute values for $[Ca^{2+}]_i$. Basal $[Ca^{2+}]_i$ was 91.1 ± 7.1 nM ($n = 13$). Potassium (5.6 mM) elevated $[Ca^{2+}]_i$ by 37.2 ± 6.3 nM ($n = 13$), 6.6 mM K^+ by 119 ± 21.8 nM ($n = 13$) and 7.6 mM K^+ by 344.1 ± 72.4 ($n = 7$). In nominally Ca^{2+}-free medium, AII induced a transient Ca^{2+} response of 204.8 ± 49.9 nM ($n = 6$).

AII stimulates NAD(P)H formation more efficiently than K^+

Next we compared the effect of angiotensin II and K^+ on mitochondrial NAD(P)H formation. First the cells were prepulsed several times with K^+ until no further sensitization was observed. Then we compared the effect of 1 nM AII with that of K^+ applied at equipotent concentration in terms of Ca^{2+} response. In order to compare equipotent concentrations of K^+ and AII (or later, vasopressin), we examined the effect of 5.6 mM, 6.6 mM and 7.6 mM K^+ on $[Ca^{2+}]$ and then chose to analyse the concentration, or an interpolated value, that was most similar to that evoked by AII. The effect of AII (or vasopressin) is expressed as a percentage of that evoked by the selected K^+ concentration. AII (1 nM) was equipotent with 5.6 mM K^+ in two experiments, with 6.6 mM K^+ in two experiments and with ≈ 6 mM K^+ again in two experiments.

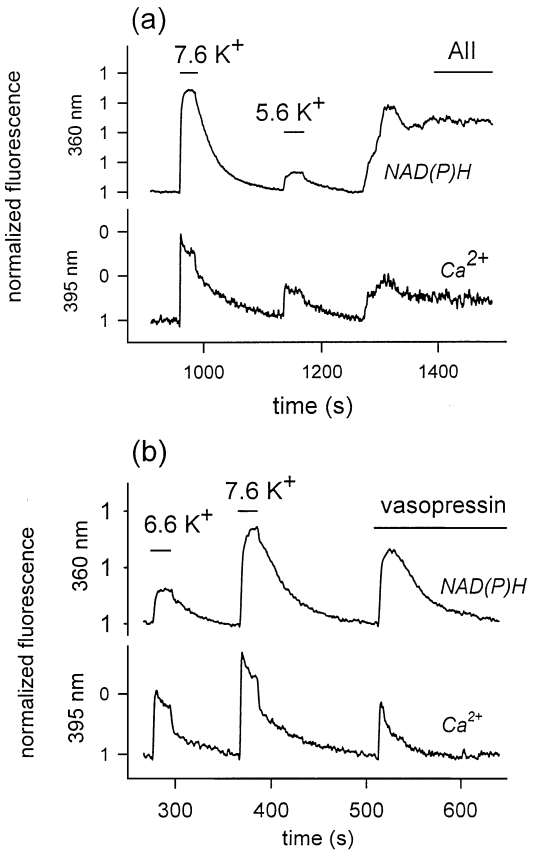

Figure 2 Effect of K^+, AII and vasopressin on $[Ca^{2+}]_i$ and NAD(P)H

Effect of 1 nM AII (**a**) or 1 μM vasopressin (**b**) and of two different concentrations of K^+ (all shown by horizontal bars) on simultaneously measured $[Ca^{2+}]_i$ (395 nm) and NAD(P)H (360 nm) in single glomerulosa cells is shown. Before the period shown, the cells were prepulsed with K^+ several times until no further sensitization was observed. Taking the baseline fluorescence as 1, the curves are representative of six (**a**) and nine (**b**) measurements performed on two and three separate cell preparations respectively.

Table 1 Comparison of the effect of Ca^{2+} release and Ca^{2+} influx on the reduction of pyridine nucleotides

Ca^{2+} release was induced by AII or vasopressin in control or Ca^{2+}-free medium. Ca^{2+} influx was induced by raising K^+ concentration or by adding Ca^{2+} after exposing the cell to AII in Ca^{2+}-free medium (capacitative Ca^{2+} influx). The effect of AII or vasopressin is expressed as a percentage of the effect evoked by K^+. The agonists were applied at equipotent concentrations in terms of $[Ca^{2+}]_i$ (see Results section for details). Significance of the difference between K^+- and AII- or vasopressin-induced NAD(P)H response: $^*P < 0.05$, $^{**}P < 0.01$.

Stimulus	Ca^{2+} (%)	NAD(P)H (%)	No. of cells	Shown in Figure
K^+	100	100	–	2, 3
AII	93 ± 5	263 ± 34**	6	2(a)
Vasopressin	98 ± 8	210 ± 41*	9	2(b)
AII (Ca^{2+}-release)	94 ± 8	237 ± 41**	9	3
AII (capacitative Ca^{2+} influx)	104 ± 7	117 ± 19	9	3

As shown in Figure 2(a), AII induced a significantly higher NAD(P)H increase than K^+ applied at equipotent concentration in terms of Ca^{2+} response. The difference between the AII-induced $[Ca^{2+}]_i$ and NAD(P)H increases, expressed as the percentage of the K^+-induced increases (Table 1) was statistically

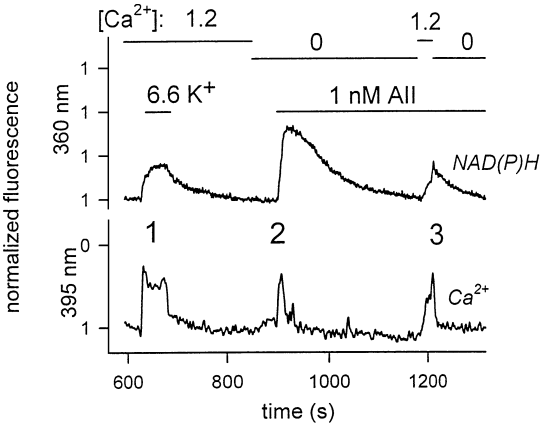

Figure 3 Comparison of Ca^{2+} influx and Ca^{2+} release on NAD(P)H response

Effect of 6.6 mM K^+ and 1 nM AII on simultaneously measured $[Ca^{2+}]_i$ (395 nm) and NAD(P)H (360 nm) is shown in a single glomerulosa cell, prepulsed several times with K^+. The horizontal bars indicate the addition of agonists as well as the sequential addition of medium containing 1.2 mM Ca^{2+} and Ca^{2+}-free medium containing 0.1 mM EGTA. Representative curves for nine measurements performed on five separate cell preparations are shown.

significant ($P < 0.01$). Similar results have been obtained with 300 pM AII (results not shown).

Vasopressin induces a greater increase in NAD(P)H than K^+

The greater peak amplitude of the AII-induced NAD(P)H response suggests that IP_3-induced Ca^{2+} release is a more effective stimulus of mitochondrial metabolism than Ca^{2+} influx. To evoke IP_3-induced Ca^{2+} release with a stimulus other than AII, we applied vasopressin. In glomerulosa cells, vasopressin too is a Ca^{2+} mobilizing agonist acting through IP_3 [21,22], it enhances the reduction of mitochondrial NAD(P) [9] and stimulates aldosterone production [21,23]. As shown in Figure 2(b), 1 μM vasopressin induced a transient increase in both $[Ca^{2+}]_i$ and NAD(P)H. The NAD(P)H response was compared with that induced by K^+. Again, in each experiment we compared the effect on NAD(P)H of vasopressin with that of K^+ applied at equipotent concentration in terms of the Ca^{2+} reponse. This was 5.6 mM K^+ in one experiment, 6.6 mM in five experiments and 7.6 mM in three experiments. As shown in Table 1, vasopressin-induced NAD(P)H production was significantly higher than that induced by K^+ ($P < 0.05$). This observation confirms that Ca^{2+} release evokes mitochondrial formation of NAD(P)H more efficiently than Ca^{2+} influx.

Comparison of Ca^{2+} release with Ca^{2+} influx

To confirm that the higher efficiency of AII and vasopressin is due to Ca^{2+} release from internal stores, we applied AII in a Ca^{2+}-free medium. This way the transient rise in $[Ca^{2+}]_i$ is due solely to IP_3-induced Ca^{2+} release. Under such conditions AII induced a transient $[Ca^{2+}]_i$ and NAD(P)H response (Figure 3, pulse 2). Before the application of AII the cells were exposed to different K^+ concentrations in the Ca^{2+}-containing control medium (pulse 1). The NAD(P)H response to AII in Ca^{2+}-free medium (pulse 2) was significantly greater than the response to K^+ applied at equipotent concentration (in terms of Ca^{2+} response) in control medium (pulse 1). After the extinction of AII-induced Ca^{2+} and NAD(P)H response in the Ca^{2+}-free

medium, a transient Ca^{2+} influx was induced by applying a short Ca^{2+} pulse (pulse 3). In order to obtain a Ca^{2+} spike with duration similar to that of the release-induced Ca^{2+} spike (pulse 2), the Ca^{2+} pulse was administered for various times between 15 and 54 s. The amplitude of the NAD(P)H response to AII in Ca^{2+}-free medium (pulse 2) was higher than that induced by the re-addition of Ca^{2+} (pulse 3), although the amplitude of the Ca^{2+} responses was comparable.

For statistical analysis (Table 1) the data were again expressed as a percentage of the K^+-induced response. AII (1 nM in Ca^{2+}-free medium) was equipotent (in terms of Ca^{2+} response) with 6.6 mM K^+ in eight experiments and with ≈ 7 mM K^+ in one experiment. NAD(P)H formation induced by AII in Ca^{2+}-free medium was significantly higher than that induced by K^+ or by the re-addition of Ca^{2+} ($P < 0.01$). Potassium-induced NAD(P)H formation was not significantly different from that induced by the re-addition of Ca^{2+}. These observations further confirm that Ca^{2+} released from internal stores induces mitochondrial NAD(P) reduction more efficiently than Ca^{2+} entering through the plasma membrane, either via voltage-dependent Ca^{2+} channels or by a capacitative mechanism. Moreover, they also demonstrate that the difference between the effect of AII and K^+ was not due to the order of addition of the agonists or a further sensitization of the mitochondrial response.

DISCUSSION

Various cellular organelles participate in the control of cytoplasmic Ca^{2+} homoeostasis [24]. The endoplasmic reticulum, or its specialized part, the so called calciosome, functions as the sole or the major IP_3-sensitive intracellular Ca^{2+} store. Ca^{2+} release from these stores plays an important role in regulating $[Ca^{2+}]_i$. Mitochondria, on the other hand, take up Ca^{2+} when $[Ca^{2+}]_i$ is elevated [5,25]. A special relationship exists between these two organelles. Measurement of $[Ca^{2+}]_m$ with the recombinant Ca^{2+}-sensitive luminescent photoprotein aequorin, targeted to the mitochondria, revealed that, with the exception of the insulin-producing cell line INS-1 [26], IP_3-induced Ca^{2+} release evoked a much higher increase in $[Ca^{2+}]_m$ than Ca^{2+} influx from the extracellular space. This phenomenon was demonstrated in different cell types, such as HeLa cells [11], cell lines of osteosarcoma and fibroblast origin [27] and bovine glomerulosa cells [5]. The effect of Ca^{2+} release and Ca^{2+} influx on $[Ca^{2+}]_m$ was hitherto compared only with recombinant aequorin targeted to the mitochondria. The superiority of Ca^{2+} release over Ca^{2+} influx was explained as being due to the juxtaposition of IP_3 receptor-channels and mitochondria, resulting in high $[Ca^{2+}]$ microdomains between the two [11,27]. It should, however, be recalled that the function of light emission by aequorin versus $[Ca^{2+}]$ is sigmoidal. Therefore the luminescence intensity may reflect the formation of microdomains of high $[Ca^{2+}]$ rather than changes in the average $[Ca^{2+}]_m$. High local $[Ca^{2+}]$ domains in small compartments thus may lead to the overestimation of the average $[Ca^{2+}]$ increase [28]. So it becomes questionable whether elevated $[Ca^{2+}]_m$, as detected by aequorin, correctly reflects enhanced function of the total mitochondrial population of the cell.

The main physiological consequence of the elevation of $[Ca^{2+}]_m$ is the enhanced activity of the Krebs cycle [7]. We studied the activity of mitochondrial dehydrogenases by examining NAD(P)H formation in situations previously documented to augment $[Ca^{2+}]_m$. The single cell fluorimetry applied has been validated by previous observations. β-Hydroxybutyrate, known to reduce mitochondrial NAD^+, as well as the electron-chain inhibitors amytal and rotenone, increased the fluorescence intensity [9,15] as expected. Similarly, when permeabilized cells were studied, raising extra-mitochondrial $[Ca^{2+}]$ was followed by increased NAD(P)H fluorescence, and this response could be prevented by Ruthenium Red, an inhibitor of the mitochondrial Ca^{2+} uniporter [9].

Our present observations, showing that Ca^{2+} release from IP_3-sensitive intracellular stores is more efficient than Ca^{2+} influx in changing the redox state of mitochondria, is in accordance with the aforementioned literary data showing that Ca^{2+} release is more efficient than Ca^{2+} influx in augmenting $[Ca^{2+}]_m$. Our results thus validate the biological significance of the $[Ca^{2+}]$ measurements obtained when applying aequorin.

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