Cytoplasmic Ca²⁺ signalling and reduction of mitochondrial pyridine nucleotides in adrenal glomerulosa cells in response to K⁺, angiotensin II and vasopressin

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We have examined the mitochondrial formation of NAD(P)H in rat adrenal glomerulosa cells. A short-term elevation of the K⁺ concentration from 3.6 to 8.4 mM induced a reversible increase in the formation of reduced pyridine nucleotides. Potassium applied after the addition of rotenone had no further effect, confirming that the redox signal was of mitochondrial origin. Inhibition of aldosterone synthesis by aminoglutethimide in K⁺-stimulated cells decreased the rate of decay of the NAD(P)H signal upon the termination of stimulation, indicating that the NADPH formed was consumed in aldosterone synthesis. When the NAD(P)H signal was measured simultaneously with the cytoplasmic free Ca²⁺ concentration ([Ca²⁺]$_i$), elevation of the K⁺ concentration to 6.6 or 8.4 mM induced parallel increases in [Ca²⁺]$_i$ and NAD(P)H formation. The rates of increase and decrease of NAD(P)H were lower than for [Ca²⁺]$_i$, confirming that the redox signal was secondary to the Ca²⁺ signal. Angio-

tensin II (100 pM–1 nM) induced an oscillatory NAD(P)H signal which usually returned to a lower baseline concentration, while a sustained signal with superimposed oscillations was observed at higher concentrations. Simultaneous measurements showed that NAD(P)H levels followed the [Ca²⁺]$_i$ pattern evoked by angiotensin II. Vasopressin (100 nM) also induced parallel oscillations of [Ca²⁺]$_i$ and NAD(P)H. A sustained rise in the extra-mitochondrial Ca²⁺ concentration to 1 μM induced a sustained elevation of the intramitochondrial Ca²⁺ concentration in permeabilized cells, as measured with rhod-2. A sustained rise in [Ca²⁺]$_i$ evoked by long-term stimulation with 8.4 mM K⁺ or 2.5 nM angiotensin II resulted in sustained NAD(P)H production. These Ca²⁺-dependent changes in the mitochondrial redox state support the biological response, i.e. aldosterone secretion by glomerulosa cells.

INTRODUCTION

Cytoplasmic Ca²⁺ signalling plays a major role in regulating aldosterone production by adrenal glomerulosa cells. The two physiologically most important secretagogues, angiotensin II (AII) and extracellular K⁺, as well as the paracrine factor vasopressin, induce aldosterone secretion by elevating the free cytoplasmic Ca²⁺ concentration ([Ca²⁺]$_i$). The signal transduction mechanisms of adrenal glomerulosa cells have been reviewed [1,2].

It has been shown using different techniques [3–6] that elevation of [Ca²⁺]$_i$ leads to a rise in the Ca²⁺ concentration in the mitochondrial matrix. The sustained elevation of [Ca²⁺]$_i$ generally results in a transient increase in the mitochondrial Ca²⁺ concentration [4,5,7,8] which, at least in hepatocytes, may be due to a lack of cytoplasmic Ca²⁺ oscillations [5]. An increased mitochondrial Ca²⁺ concentration results in the activation of three metabolic enzymes, i.e. pyruvate dehydrogenase, isocitrate dehydrogenase and oxoglutarate dehydrogenase [6,9,10]. The resulting reduction of pyridine nucleotides has been revealed by microfluorimetry in adrenal glomerulosa cells [11,12], pancreatic B-cells and hepatocytes [12]. A more detailed study on hepatocytes [5] showed that the sustained formation of NAD(P)H in the mitochondria occurred in response to an oscillatory cytoplasmic Ca²⁺ signal only, whereas a maintained (plateau-like) cytoplasmic Ca²⁺ signal failed to induce a long-lasting mitochondrial response.

In the present study we show that, in rat adrenal glomerulosa cells, mitochondrial NAD(P)H follows the pattern of [Ca²⁺]$_i$, irrespective of whether it is evoked by AII, vasopressin or a transient or sustained increase in the extracellular K⁺ con-

centration. In contrast to hepatocytes [5], a sustained increase in [Ca²⁺]$_i$ evokes a sustained NAD(P)H signal in cells stimulated with either K⁺ or a high concentration of AII, indicating sustained activation of the Ca²⁺-sensitive mitochondrial dehydrogenases. This activation seems to be a consequence of a sustained rise in the mitochondrial Ca²⁺ concentration. We provide evidence for the biological importance of the increased formation of reduced pyridine nucleotides in adrenal glomerulosa cells, and discuss the biological significance of the kinetics of the NAD(P)H signal in these cells.

EXPERIMENTAL

Materials

Materials used for cell isolation have been described previously [13]. AII ([Ile⁵]AII), vasopressin and rotenone were from Serva (Heidelberg, Germany), fura-2 acetoxymethyl ester (fura-2 AM), fura-PE3 AM and rhod-2 AM were from TEFLABS (Austin, TX, U.S.A.), aminoglutethimide was from Ciba-Geigy (Basle, Switzerland), and β-hydroxybutyrate was from Sigma (St. Louis, MO, 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 [13]. 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 Ca²⁺, 0.5 mM Mg²⁺,

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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 $[Ca^{2+}]_i$ in intact cells

For measuring the fluorescence of reduced pyridine nucleotides, the cells plated on 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). All measurements were performed at 37 °C. The microscope was coupled to a photomultiplier-based microspectrofluorimeter (PTI, South Brunswick, NJ, U.S.A.). Fluorescence was followed at excitation and emission wavelengths of 360 and 470 nm respectively. Since NADH and NADPH have identical fluorescence spectra, we cannot separate the two signals and the measured signal is referred to as NAD(P)H.

For simultaneous measurements of $[Ca^{2+}]_i$ and NAD(P)H, the cells were loaded with fura-PE3 AM (0.5–1 μM) for 15–30 min at 37 °C in medium A until comparable fluorescence changes were obtained for Ca^{2+} and NAD(P)H. Fura-PE3 has the same spectral properties as fura-2 [14], with less leakage from the cells [15]. A few measurements were performed on cells loaded with fura-2. As there was no qualitative difference between the results obtained with the two dyes, the data were pooled. At an excitation wavelength of 360 nm, the isosbestic point of the two Ca^{2+}-sensitive dyes [14], the fluorescence of reduced pyridine nucleotides can be measured. At 395 nm the fluorescence of NAD(P)H is negligible, and the Ca^{2+}-sensitive dyes respond to Ca^{2+} with a decrease in fluorescence intensity. For the sake of clarity the curves obtained at 395 nm were turned upside-down on the Figures. The y-axes of Figures show the relative change in fluorescence intensity related to the control period. In dye-loaded cells, the fluorescence intensity at 360 nm is determined by both Ca^{2+}-dependent emitted light from NAD(P)H and Ca^{2+}-independent emitted light from fura-PE3 or fura-2. Due to this dual origin of fluorescence in dye-loaded cells, the relative changes in fluorescence intensity at an excitation wavelength of 360 nm underestimate the changes in the NAD(P)H concentration. The continuous decrease in the baseline fluorescence in both dye-loaded and unloaded cells was eliminated by division of the curves by a negative exponential function.

For $[Ca^{2+}]_i$ measurements, the cells were loaded with 1 μM fura-2 for 30 min in medium A at 37 °C. The excitation wavelengths were 340 and 380 nm, and emission was detected at 510 nm. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence intensities at excitation wavelengths of 340 and 380 nm by the method of Grynkiewicz et al. [14], including viscosity correction.

Measurement of mitochondrial Ca^{2+} concentration and NAD(P)H in permeabilized cells

For measuring the mitochondrial Ca^{2+} concentration, the cells were loaded with 2 μM rhod-2 AM for 30 min at 37 °C in medium A. This dye has a net positive charge that causes it to accumulate in the mitochondria. Residual dye in the cytoplasm was removed by permeabilizing the cells with 25 μg/ml digitonin in an intracellular-like solution containing 115 mM K^+, 35 mM Na^+, 2 mM Mg^{2+}, 1 mM ATP, 4 mM succinate and 4 mM pyruvate. Ca^{2+} was buffered with 2 mM EGTA. To achieve 100 nM and 1 μM free Ca^{2+}, 0.45 mM and 1.49 mM total Ca^{2+} was added respectively at pH 7.1. Free Ca^{2+} in the medium was estimated using fura-2 free acid. The fluorescence of single cells was detected at excitation and emission wavelengths of 535 and 605 nm respectively.

NAD(P)H in permeabilized cells was measured in cells not loaded with fluorescent dye. Permeabilization was performed with the same protocol. Fluorescence was detected at excitation and emission wavelengths of 360 and 470 nm respectively.

Statistical analysis

Statistical data are presented as means $\pm$ S.E.M. Significance was estimated by Student's t test, and correlation coefficients were calculated. All the curves shown in Figures are representative of least for four measurements, mostly performed on separate cell preparations.

RESULTS

The NAD(P)H signal is formed in the mitochondria

Elevation of the K^+ concentration in the medium from the control value of 3.6 mM to 6.6 mM induced an increase in NAD(P)H fluorescence. The NAD(P)H signal was also induced with β-hydroxybutyrate (Figure 1a). This substrate is metabolized by the mitochondrial enzyme β-hydroxybutyrate dehydrogenase to acetoacetate, with the generation of NADH. β-Hydroxybutyrate did not induce a cytoplasmic Ca^{2+} signal (results not shown). The effect of β-hydroxybutyrate shows that the applied microfluorimetric technique is appropriate to detect changes in the mitochondrial pyridine nucleotide redox state.

To test whether the increased NAD(P)H fluorescence intensity and the associated cytoplasmic Ca^{2+} signal originated from the mitochondria, we examined the effects of the mitochondrial electron chain inhibitor rotenone, both alone and applied together with K^+. Short-term exposure to 8.4 mM K^+ induced a transient reduction of pyridine nucleotides. Rotenone also generated an NAD(P)H signal, after which K^+ had no further effect (Figure 1b). Stimulation with 6.6 and 8.4 mM K^+ increased the fluorescence intensity by $12 \pm 1\%$ ($n = 13$) and $21 \pm 1\%$ ($n = 18$) of the control respectively. The responses to β-hydroxybutyrate and rotenone were $7 \pm 1\%$ ($n = 6$) and $40 \pm 4\%$
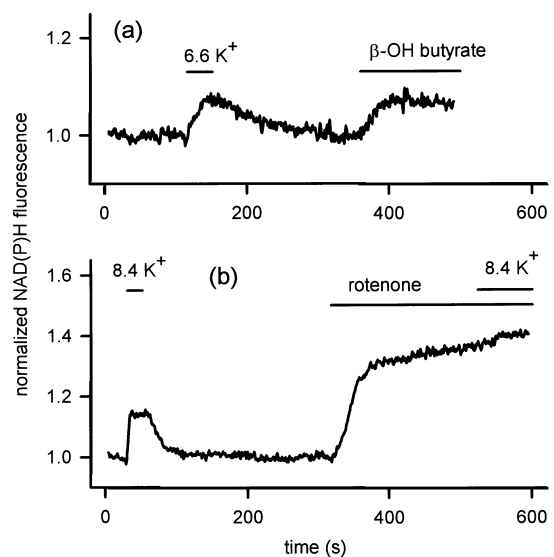

Figure 1   Mitochondrial localization of the NAD(P)H signal

The fluorescence of NAD(P)H was measured as described in the Experimental section. Fluorescence is related to that during the control period (= 1.0). (**a**) Increasing the K^+ concentration from 3.6 to 6.6 mM and the addition of 20 mM β-hydroxybutyrate (β-OH butyrate) are indicated by the horizontal bars. (**b**) Increasing the K^+ concentration from 3.6 to 8.4 mM and the addition of 1 μM rotenone are indicated by the horizontal bars.
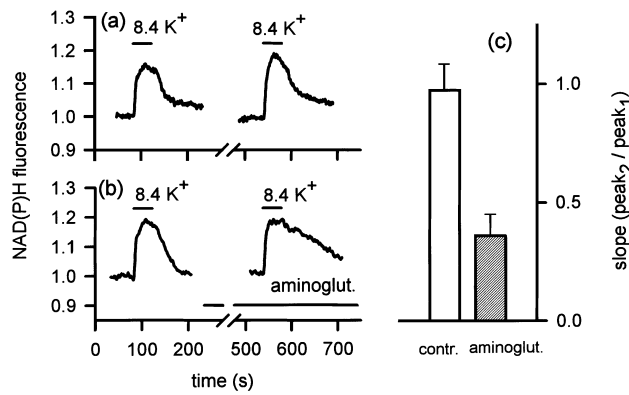
Figure 2   Effect of an inhibitor of steroid synthesis on the kinetics of the NAD(P)H signal

The fluorescence of NAD(P)H was measured as described in the Experimental section. (**a**) Representative trace from a single glomerulosa cell stimulated twice with 8.4 mM K$^+$, as indicated by the horizontal bars. (**b**) Traces from another cell which was first stimulated with 8.4 mM K$^+$, then treated with 300 μM aminoglutethimide (aminoglut.) for 5 min and finally stimulated with K$^+$ again. (**c**) Linear fits of 60 s were fitted on the declining phase of the signal after the return to the basal K$^+$ concentration. The slope after the second stimulation was divided by the slope after the first stimulation for each cell. The columns show this ratio for control (contr.) and aminoglutethimide-treated cells.

($n = 11$) respectively ($P < 0.005$ for all four stimuli). Similar data have been obtained with antimycin (results not shown) and amytal [11]. These observations provide evidence that changes in NAD(P)H fluorescence as monitored under the present experimental conditions are of mitochondrial origin.

Relationship between steroid synthesis and kinetics of the NAD(P)H signal

The measured NAD(P)H signal reflects activation of the Krebs cycle. To provide evidence that this activation in glomerulosa cells is important not only because of increased ATP formation but also because of increased reduction of NADP$^+$, we examined the effect of the rate of aldosterone synthesis on the kinetics of the NAD(P)H signal. The cells were stimulated for 40 s with 8.4 mM K$^+$, the maximally effective concentration with respect to aldosterone production [11]. Potassium induced a reversible NAD(P)H signal (Figure 2a). When, 5 min after the cessation of the NAD(P)H signal, a second 40 s K$^+$ stimulation was applied, the signal generated was almost identical to the first one. The ratio of the slope of the decay after the second stimulation to that after the first stimulation was 0.98 ± 0.11 ($n = 4$). The decay slope was, however, decreased if steroid synthesis was inhibited during the second stimulation. In four cells, 300 μM aminoglutethimide was applied for 5 min before the second stimulation. This drug inhibits the first step of steroid synthesis, i.e. the conversion of cholesterol into pregnenolone, and therefore decreases the consumption of NADPH. In previous experiments 300 μM aminoglutethimide decreased AII- and K$^+$-stimulated aldosterone production below non-stimulated control values in superfused glomerulosa cells [16]. In aminoglutethimide-treated cells the second 40 s stimulus again induced an NAD(P)H signal. However, as re-oxidation of NADPH in steroid synthesis was inhibited, the decay of the signal was substantially slower after termination of the stimulus (Figure 2b). The ratio of the slope of the decay after the second stimulation to that after the first stimulation was 0.36 ± 0.09 (Figure 2c).

Simultaneous measurement of [Ca^{2+}]$_i$ and NAD(P)H in K$^+$-stimulated cells

The spectral overlap of the Ca^{2+}-sensitive dye and reduced pyridine nucleotides allows the monitoring of [Ca^{2+}]$_i$ and NAD(P)H simultaneously [5,12]. It was observed previously that K$^+$ increased both [Ca^{2+}]$_i$ and formation of mitochondrial NAD(P)H in rat glomerulosa cells; however, the two parameters were not systematically compared [11]. Therefore, in order to study their causal relationship, we first analysed the correlation between changes in [Ca^{2+}]$_i$ and NAD(P)H. The short-term application of 6.6 or 8.4 mM K$^+$ (instead of 3.6 mM) induced parallel changes in [Ca^{2+}]$_i$ and NAD(P)H. The [Ca^{2+}]$_i$ response, however, reached its peak earlier and decayed faster than NAD(P)H signal (Figures 3a–3d). We calculated the times required to reach half the peak amplitude for both [Ca^{2+}]$_i$ and NAD(P)H. The other kinetic parameter that we used for the characterization of the two signals was the time required for the fluorescence intensity to decrease to half of the peak amplitude. Both the half-peak and half-decay time were significantly shorter for Ca^{2+} than for NAD(P)H (Figures 3e and 3f). This kinetic difference suggests that NAD(P)H formation occurs as a consequence of the Ca^{2+} signal.

We performed separate experiments on fura-2-loaded cells to estimate basal and stimulated values of [Ca^{2+}]$_i$ by the ratio method. An increase in the K$^+$ concentration to 6.6 mM increased [Ca^{2+}]$_i$ from 107.6 ± 9.7 to 214.6 ± 23.8 nM ($n = 5$), whereas 8.4 mM K$^+$ increased [Ca^{2+}]$_i$ from 108.5 ± 9.1 to 411.8 ± 52.1 nM ($n = 6$).

Effects of AII on pyridine nucleotide reduction

To characterize the effects of AII on NAD(P)H formation, we first examined three different concentrations of AII (100, 300 and 1000 pM). In order to avoid any artifact potentially caused by Ca^{2+} chelation, the cells were not loaded with Ca^{2+}-sensitive dyes in these experiments. Nearly all of the cells responded to AII; however, even at any given concentration of the agonist, the response pattern differed from cell to cell. This heterogeneity resembled the well known heterogeneity of the cytoplasmic Ca^{2+} signal induced by Ca^{2+}-mobilizing agents in several cell types [17,18]. Nevertheless, some dependence of the response pattern on hormone concentration could be observed. Figure 4 shows two representative curves for each concentration. AII at 100 pM usually induced an oscillatory pattern, while at higher concentrations of AII the oscillations became more and more confluent. The higher the concentration of AII, the shorter the lag time of the NAD(P)H response became (Figure 4g). The negative correlation between the log of the AII concentration and the lag time was statistically significant ($r = -0.79$, $P < 0.001$). At 300 pM AII the spiking frequency (0.63 ± 0.09 min^{-1}) was somewhat higher than that at 100 pM (0.48 ± 0.06 min^{-1}); however, this difference was not statistically significant. Yet the time-averaged amplitude of the NAD(P)H response, reflecting the difference in the formation and consumption of reduced pyridine nucleotides throughout the stimulation, showed concentration dependence (Figure 4h; $r = 0.56$, $P < 0.01$). The increased area/time values at higher AII concentrations are due to the confluence of the single spikes as well as to higher response amplitudes.

Simultaneous measurement of [Ca^{2+}]$_i$ and mitochondrial NAD(P)H in cells stimulated with AII

To analyse the correlation between Ca^{2+} and NAD(P)H signals in AII-stimulated cells, we performed experiments on cells loaded with Ca^{2+}-sensitive dye. The lag time of the Ca^{2+} signal depended
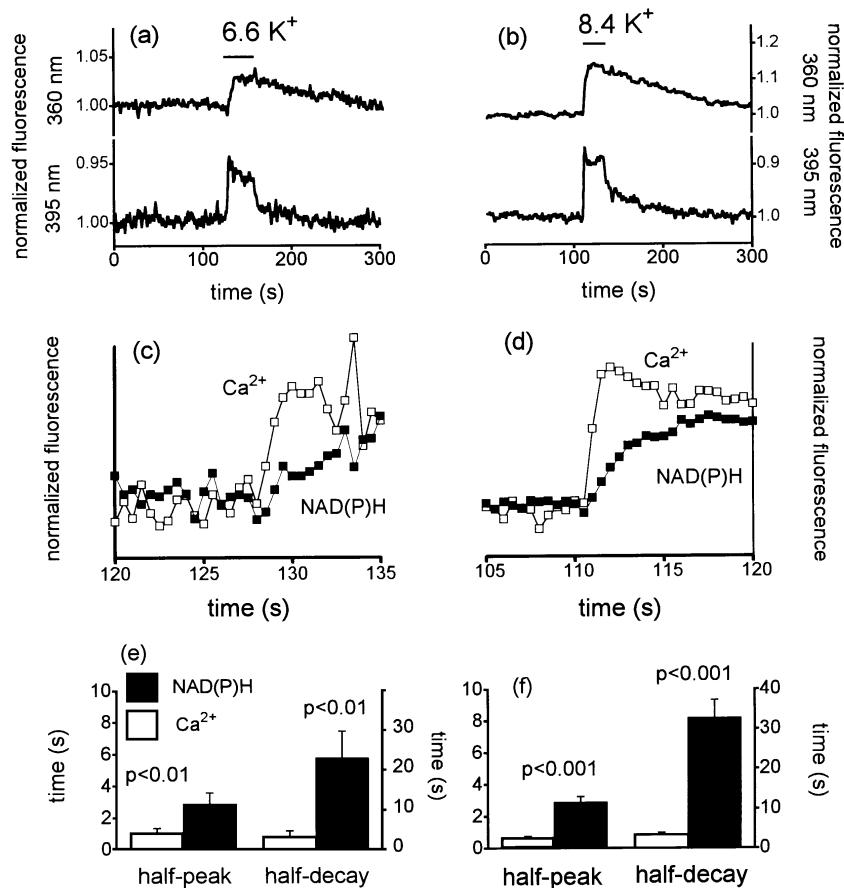
Figure 3   Simultaneous measurement of [Ca^{2+}]$_i$ and NAD(P)H in K$^+$-stimulated cells

Simultaneous measurements of [Ca^{2+}]$_i$ and NAD(P)H were performed as described in the Experimental section. (**a**) Upper trace (360 nm), NAD(P)H signal in response to a 20 s pulse of 6.6 mM K$^+$; lower curve (395 nm), Ca^{2+} response in the same cell. (**b**) The same protocol as in (**a**) but with 8.4 mM K$^+$. In (**c**) and (**d**) the initial periods of (**a**) and (**b**) respectively are shown with an enlarged time scale. (**e**, **f**) Kinetics of Ca^{2+} and NAD(P)H signals in cells stimulated with 6.6 mM (**e**) and 8.4 mM (**f**) K$^+$. 'Half-peak' is the time required to reach half the amplitude of the peak of the Ca^{2+} or NAD(P)H signal; 'half-decay' refers to the time required to decrease to half the amplitude of the peak Ca^{2+} or NAD(P)H signal upon termination of stimulation ($n = 9$ for each concentration). Note the different time scales for half-peak and half-decay.

on the concentration of AII; it was 63.0 ± 9.8 s ($n = 13$) and 28.8 ± 9.7 s ($n = 11$) at 300 pM and 1 nM AII respectively ($P < 0.05$). The change in oscillation frequency (0.78 ± 0.09 min^{-1} at 300 pM AII and 0.90 ± 0.14 min^{-1} at 1 nM) was not statistically significant.

Figure 5 shows representative curves obtained from cells stimulated with AII at the physiological concentration of 300 pM. In five out of 13 cells relatively regular oscillations (Figure 5a), while in other cells less regular Ca^{2+} spikes (Figures 5b and 5c), coincided with changes in NAD(P)H fluorescence. This, however, did not mean identical kinetics, since both the increasing and decreasing phases of the NAD(P)H spikes were slower than those of the Ca^{2+} spikes. The rising and decaying phases exhibited similar kinetic differences in cells stimulated with 1 nM AII (results not shown). In two out of 13 cells, the re-oxidation of NAD(P)H between the Ca^{2+} spikes was too sluggish for the return of the NAD(P)H signal to baseline levels (Figure 5c). When extracellular Ca^{2+} was chelated with 1.5 mM EGTA, both the Ca^{2+} and NAD(P)H signals were abolished (Figures 5b and 5c). EGTA also abolished the AII-induced NAD(P)H signal in cells not loaded with Ca^{2+}-sensitive dye (results not shown). The similar patterns of the corresponding Ca^{2+} and NAD(P)H curves, the slower kinetics of the NAD(P)H response and the effects of

EGTA indicate a primary role for Ca^{2+} in evoking the reduction of pyridine nucleotides; moreover, the effect of Ca^{2+} chelation also shows that Ca^{2+} influx plays an essential role in maintaining both the Ca^{2+} and NAD(P)H signals in AII-stimulated cells.

AII at 1 nM again induced parallel changes in [Ca^{2+}]$_i$ and NAD(P)H (Figure 6b). In cells where the frequency of Ca^{2+} spikes exceeded three per min, or the Ca^{2+} signal was confluent, the associated NAD(P)H signal was also confluent (results not shown).

Effect of a sustained [Ca^{2+}]$_i$ signal on NAD(P)H

Next we examined whether a long-lasting Ca^{2+} signal evokes a sustained NAD(P)H signal, or a transient one as observed in hepatocytes [5]. In order to induce a sustained Ca^{2+} signal the cells were exposed for 10 min to 2.5 nM AII or 8.4 mM K$^+$ (Figure 7). These concentrations of agonists were maximally effective with respect to aldosterone production (results not shown). Both agents induced a plateau-like Ca^{2+} signal, associated with a sustained NAD(P)H response. The sustained Ca^{2+} signal induced by AII was superimposed with low-amplitude, very-high-frequency oscillations. We terminated the Ca^{2+} signal by the addition of 1.5 mM EGTA. Upon chelation of
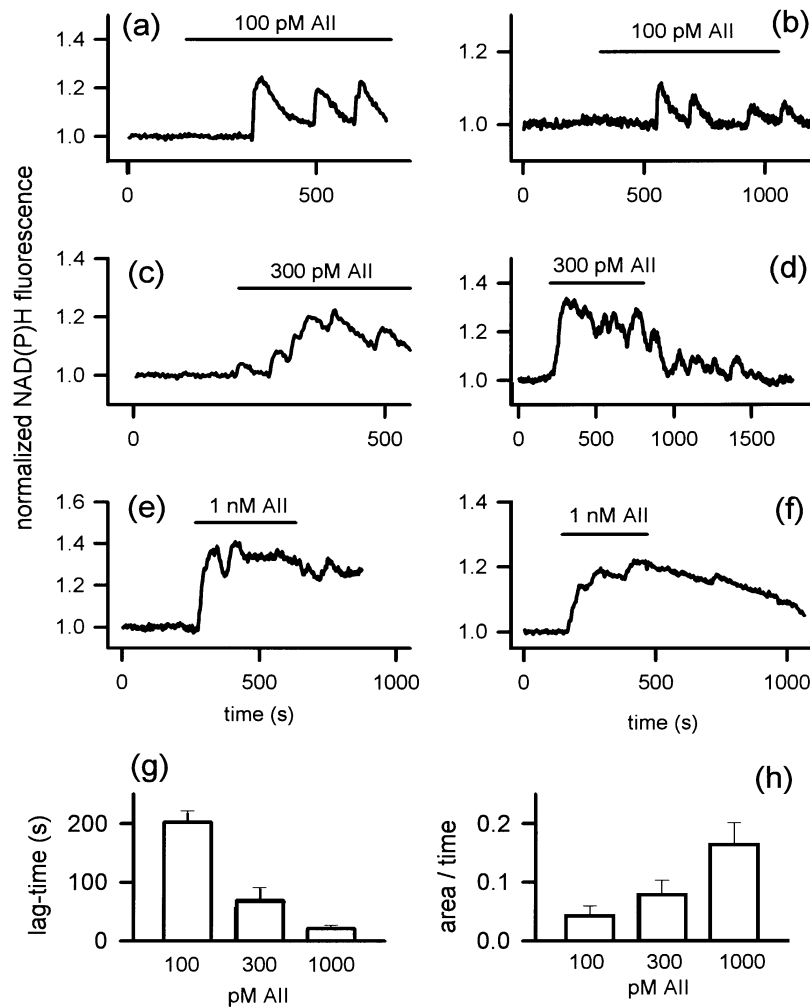
Figure 4   NAD(P)H signal in response to AII

The NAD(P)H signal in cells not loaded with fluorescent dye is shown in response to 100 pM (**a**, **b**), 300 pM (**c**, **d**) and 1 nM (**e**, **f**) AII. Each trace shows the response of a separate cell. (**g**) Relationship between the lag time of the NAD(P)H response ($n = 6$ for 100 pM, $n = 9$ for 300 pM and $n = 7$ for 1 nM). (**h**) Relationship between the time-averaged amplitude of the NAD(P)H response and the concentration of AII. The curves were integrated and the area was divided by the stimulation time. The lag period was excluded from the calculation.

extracellular Ca^{2+}, both the Ca^{2+} signal and the NAD(P)H signal returned to basal values (Figure 7a). The Ca^{2+} signal induced by 8.4 mM K^+ was terminated on restoring the K^+ concentration to 3.6 mM. Upon termination both $[Ca^{2+}]_i$ and NAD(P)H returned to basal levels (Figure 7b). Both agonists also induced a sustained NAD(P)H response in cells not loaded with Ca^{2+}-sensitive dye (results not shown).

Effect of a sustained rise in the extramitochondrial Ca^{2+} concentration on mitochondrial Ca^{2+} and NAD(P)H

The sustained reduction of pyridine nucleotides may reflect a long-lasting mitochondrial Ca^{2+} signal. We monitored mitochondrial Ca^{2+} with the fluorescent dye rhod-2, a dye that accumulates in the highly negative mitochondrial matrix [6,19]. Short-term stimulation of the cell with 8.4 mM K^+ induced a reversible rise in fluorescence intensity (Figure 8a). Permeabilization of the cell with digitonin induced a large decrease in fluorescence, indicating that a significant fraction

of the dye had been in the cytoplasm. Raising the extra-mitochondrial free Ca^{2+} concentration from 100 nM to 1 μM resulted in an elevation of fluorescence intensity, which persisted as long as the Ca^{2+} concentration was high in the medium. The fluorescence showed an immediate decrease upon the addition of Ruthenium Red, an inhibitor of the mitochondrial Ca^{2+} uniporter (Figure 8a). These data indicate that a sustained rise in the extramitochondrial Ca^{2+} concentration induces a sustained in-crease in intramitochondrial Ca^{2+}. The rise in fluorescence in response to K^+ in the intact cell before permeabilization probably reflects a combination of cytoplasmic and intramitochondrial Ca^{2+} signals.

To demonstrate that the elevation of mitochondrial Ca^{2+} in the experiment shown in Figure 8(b) is accompanied by changes in mitochondrial metabolism, we measured the NAD(P)H fluorescence of cells under the same conditions. Elevation of the Ca^{2+} concentration in the medium from 100 nM to 1 μM for 10 min evoked a sustained rise of NAD(P)H fluorescence in permeabilized cells (Figure 8b). Ruthenium Red (5 μM) pre-vented the Ca^{2+}-induced NAD(P)H response (results not shown).
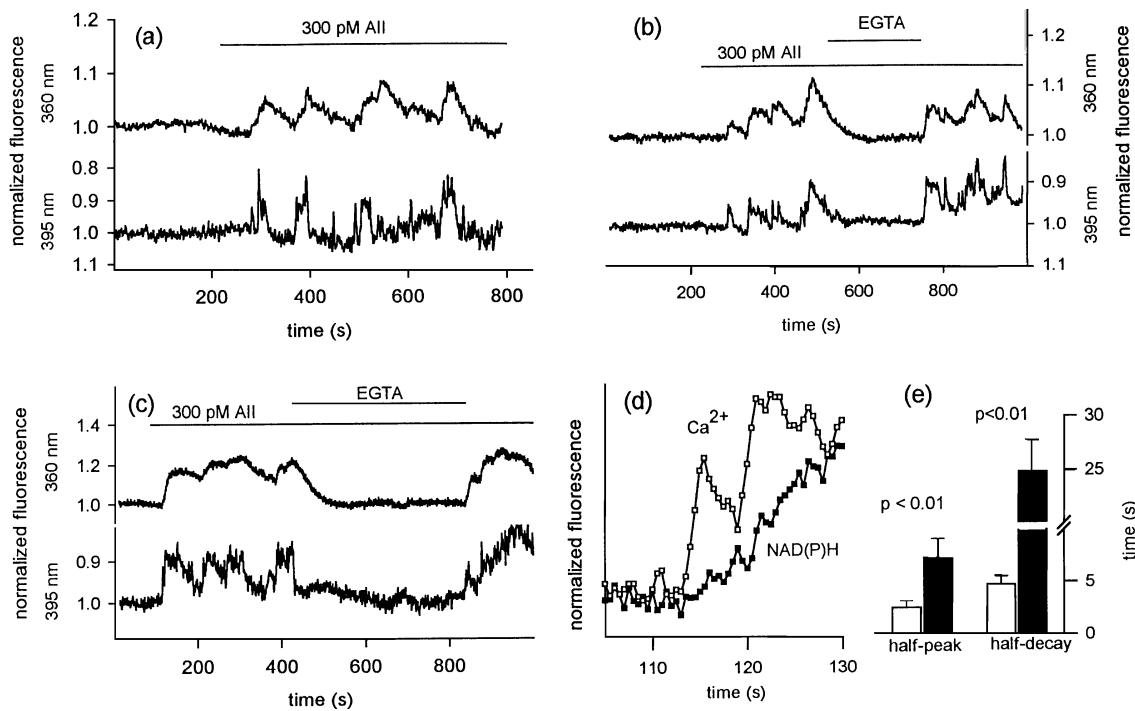
Figure 5   The NAD(P)H signal follows the pattern of the Ca²⁺ signal in cells stimulated with AII

Simultaneous measurements of $[Ca^{2+}]_i$ (395 nm) and NAD(P)H (360 nm) were performed as described in the Experimental section. Three cells (**a**, **b** and **c**) were stimulated with 300 pM AII, as shown by the horizontal bars. In (**b**) and (**c**), EGTA (1.5 mM) was added during AII stimulation. (**d**) The onset of stimulation from (**c**) is shown with an enlarged time scale. (**e**) Kinetics of the Ca²⁺ and NAD(P)H signals calculated from seven cells stimulated with 300 pM AII (see the legend to Figure 3).
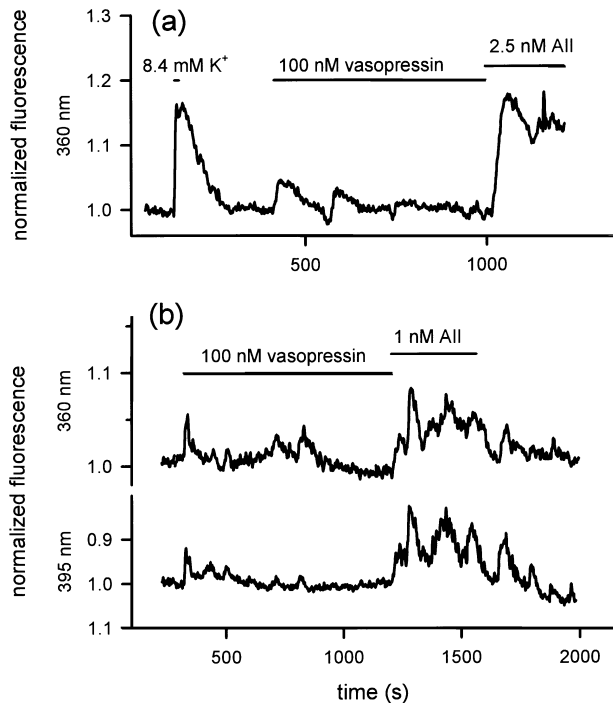

Figure 6   Vasopressin induces oscillations of $[Ca^{2+}]_i$ and NAD(P)H

(**a**) Measurement of NAD(P)H in a cell not loaded with Ca²⁺-sensitive dye, successively stimulated with 8.4 mM K⁺, 100 nM vasopressin and 2.5 nM AII. (**b**) Simultaneous NAD(P)H (360 nm) and Ca²⁺ (395 nm) measurements in a cell stimulated first with 100 nM vasopressin and then with 1 nM AII.

Effects of vasopressin on $[Ca^{2+}]_i$ and NAD(P)H

Vasopressin stimulates aldosterone production. It exerts its effect through V_1 receptors, which are coupled to the phosphoinositide signalling pathway [20–22]. We examined whether vasopressin also induces the reduction of pyridine nucleotides. Figure 6(a) shows the NAD(P)H response to the addition of 8.4 mM K⁺, 100 nM vasopressin and 2.5 nM AII in a cell not loaded with dye. Vasopressin is known to be much less effective than AII or K⁺ in stimulating aldosterone production [22,23]. The vasopressin-induced NAD(P)H signal was also much smaller than those induced by K⁺ or AII. Despite the continuous presence of the hormone, the vasopressin-induced oscillatory NAD(P)H signal decayed rapidly. Although vasopressin induces the sustained formation of inositol phosphates [21,22], its effects on Ca²⁺ signalling [23] and on aldosterone production [20] are only transient. AII (2.5 nM) stimulated NAD(P)H formation when added after vasopressin, showing the maintained ability of the mitochondria to reduce pyridine nucleotides. To examine whether the transient nature of the NAD(P)H signal in response to vasopressin was due to the cessation of the $[Ca^{2+}]_i$ signal, we performed experiments in fluorescent-dye-loaded cells. Figure 6(b) shows simultaneous measurement of $[Ca^{2+}]_i$ and NAD(P)H. Vasopressin induced parallel $[Ca^{2+}]_i$ and NAD(P)H signals in 11 of 19 cells tested. The oscillatory Ca²⁺ signal was transient and accompanied by a similarly transient NAD(P)H signal. The lag time of the response was between 0 and 13 s. AII (300 pM, 1 nM or 2.5 nM) induced Ca²⁺ and NAD(P)H signals after vasopressin treatment in both vasopressin-responsive and non-responsive cells, indicating that the absence or the transient nature of the vasopressin effect is not due to the desensitization of the Ca²⁺ or the mitochondrial signal.
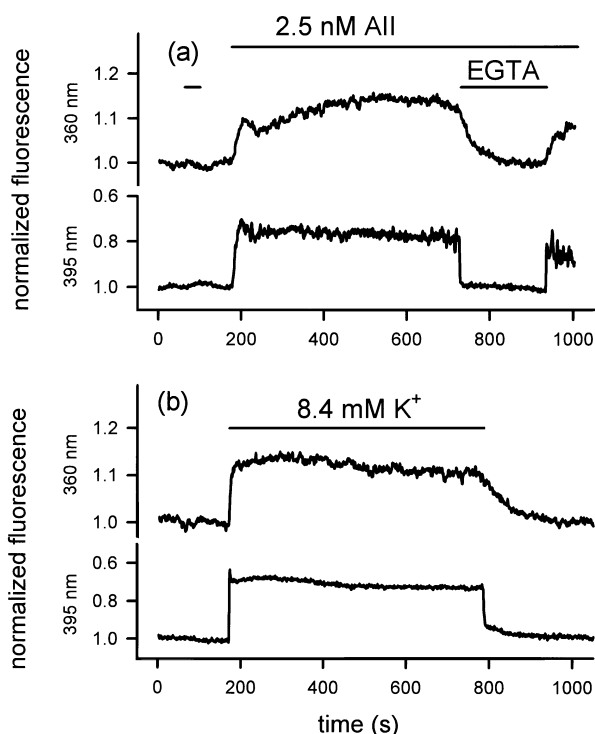
Figure 7   A sustained Ca^{2+} signal is accompanied by a sustained NAD(P)H signal

Simultaneous measurements of [Ca^{2+}]$_i$ and NAD(P)H were performed as described in the Experimental section. (**a**) Stimulation for 10 min with 2.5 nM AII. The addition of 1.5 mM EGTA is indicated. (**b**) Stimulation for 10 min with 8.4 mM K$^+$.

Figure 8   Effect of Ca^{2+} on the mitochondrial Ca^{2+} concentration and NAD(P)H in permeabilized cells

Measurement of the mitochondrial Ca^{2+} concentration and NAD(P)H was performed as described in the Experimental section. (**a**) A cell loaded with rhod-2 was stimulated with 8.4 mM K$^+$ for 30 s, and then 2 mM EGTA was added. The cell was permeabilized with 25 μg/ml digitonin (arrow) in an cytosol-like solution (free Ca^{2+} 100 nM). The addition of 1 μM Ca^{2+} and 5 μM Ruthenium Red (RR) is indicated. (**b**) Measurement of NAD(P)H in another cell performed using a similar protocol as in (**a**). The addition of different solutions is indicated with horizontal bars and the addition of digitonin by an arrow.

DISCUSSION

Elevation of the extracellular K$^+$ concentration, a physiological stimulus for adrenal glomerulosa cells, induces a [Ca^{2+}]$_i$ signal. The mechanism involves both the activation of voltage-dependent Ca^{2+} channels [24,25] and a recently described non-voltage-operated Ca^{2+} current (I_{g1}) [26]. It has been shown previously that physiological elevation of the extracellular K$^+$ concentration also enhances the formation of mitochondrial NAD(P)H in adrenal glomerulosa cells [11]. Physiological concentrations of AII induce oscillating Ca^{2+} signals [18], and these oscillations are associated with parallel mitochondrial NAD(P)H oscillations [12]. In the present study we confirm and extend these data by presenting results of simultaneous [Ca^{2+}]$_i$ and NAD(P)H measurements in single adrenal glomerulosa cells stimulated with AII, K$^+$ and vasopressin. In spite of considerable intercellular heterogeneity, the patterns of the [Ca^{2+}]$_i$ and mitochondrial NAD(P)H signals showed striking similarity.

The NAD(P)H signal as examined in the present study gives information on the activity of the mitochondrial Ca^{2+}-sensitive dehydrogenases. The activation of mitochondrial metabolism has multiple consequences in glomerulosa cells. As in every cell type, the ATP formed obviously meets the increased energy demands upon stimulation. On activation of the Krebs cycle, GTP is also formed, which acts in synergism with the steroidogenesis-activating polypeptide, thus facilitating the transfer of cholesterol into the mitochondria [27,28].

Another effect of activated mitochondrial oxidation, the enhanced reduction of NADP$^+$, requires a more detailed discussion. NADH formed by the dehydrogenase enzymes redu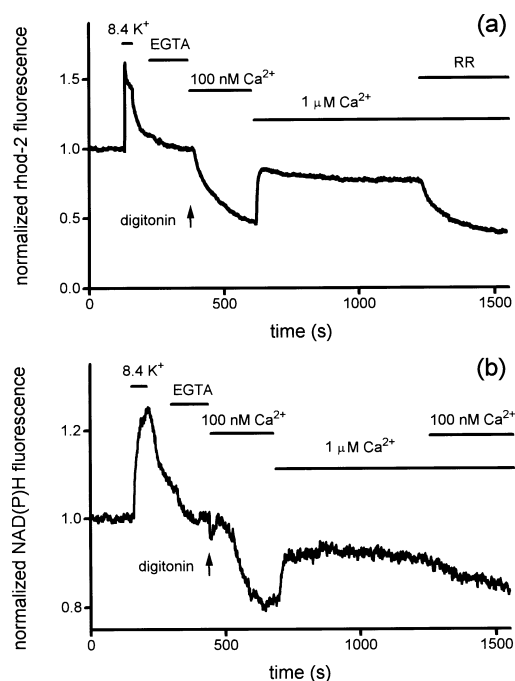ces NADP$^+$ via transhydrogenase enzymes [29]. NADPH is a cofactor of three steps in aldosterone biosynthesis, namely side-chain cleavage, 11β-hydroxylation and the conversion of cortico-sterone into aldosterone. Thus the increased reduction of pyridine nucleotides increases the cofactor supply for these three enzymes of the aldosterone synthetic pathway. In principle, the increased cofactor supply may increase the rate of steroid hydroxylation. Yet, the question arises as to whether NADPH at resting levels limits steroid hydroxylation at all. In bovine glomerulosa cells treated with ionomycin, an increase in [Ca^{2+}]$_i$ up to 1 μM failed to affect NAD(P)H-dependent intramitochondrial hydroxylation [30]. This observation would argue against a role for Ca^{2+}-dependent NADPH formation in the control of steroid hydroxylation. However, the intramitochondrial Ca^{2+} concentration during stimulation by physiological factors reaches values much higher than the average [Ca^{2+}]$_i$, and may far exceed 1 μM [6–8]. The presence of ionomycin in the aforementioned study [30] may have decreased the accumulation of Ca^{2+} by the mitochondria, and the intramitochondrial Ca^{2+} concentration may not have attained a level sufficient to enhance the reduction of pyridine nucleotides. At variance with that report, the idea that the formation of NADPH influences the rate of steroid production is supported by the observation that, in isolated adrenocortical mitochondria, the rate of side-chain cleavage of endogenous cholesterol correlates with the NADH/NAD$^+$ ratio. Moreover, rotenone also stimulates side-chain cleavage [31]. It was also reported that a decrease in NADPH levels can limit steroid synthesis; in thyroidectomized rats the diminished NADPH formation was accompanied by decreased activity of the adrenocortical steroid hydroxylase enzymes [32]. Further indirect evidence in favour of a controlling role for NADPH in

hormone secretion is the observation that the *in vivo* depletion of the reducing compound ascorbate inhibits the conversion of deoxycorticosterone into aldosterone by surviving guinea pig adrenal cells [33].

We planned to assess the significance of the increased reduction of NADP$^+$ by examining the effect of rotenone on aldosterone production. However, because this drug increased not only NAD(P)H formation and hormone production but $[Ca^{2+}]_i$ as well, the role of enhanced NADPH formation could not be evaluated (P. Enyedi, G. Szabadkai, T. Rohács and A. Spät, unpublished work). Next we assumed that, even if NADPH does not limit hydroxylation under resting conditions, its rapid consumption during hypersecretion may render it limiting. We found that inhibition of aldosterone synthesis by amino-glutethimide substantially decreased the rate of re-oxidation of NAD(P)H. This observation indicates that changes in NAD(P)H formation as measured in our study are not only an indicator of mitochondrial activity but also reflect the availability of a biologically significant cofactor of steroid synthesis.

In the present study we found that the pattern of the $[Ca^{2+}]_i$ signal was identical with that of the mitochondrial NAD(P)H signal. Low-frequency $[Ca^{2+}]_i$ oscillations evoked by low concentrations of AII or by vasopressin were followed by similar low-frequency NAD(P)H oscillations. Due to the slow re-oxidation of NAD(P)H, at a higher Ca^{2+} spiking frequency the redox spikes fused into a sustained NAD(P)H signal, similar to that described in hepatocytes [5,12]. The frequency at which fusion occurs is much higher in glomerulosa cells than in hepatocytes. This difference may be attributed to the rapid consumption of NADPH by the stimulated aldosterone production. There is, however, a more important difference between the two cell types. While a sustained rise in $[Ca^{2+}]_i$ failed to induce sustained mitochondrial Ca^{2+} and NAD(P)H responses in hepatocytes, it resulted in sustained mitochondrial NAD(P)H formation in glomerulosa cells stimulated either with K$^+$ or with higher concentrations of AII. Moreover, a sustained elevation of the extramitochondrial Ca^{2+} concentration from 100 nM to 1 μM induced long-lasting mitochondrial Ca^{2+} and NAD(P)H signals in permeabilized cells. The kinetics of the NAD(P)H response in intact cells thus probably reflect the kinetics of changes in the mitochondrial Ca^{2+} concentration. Whether the behaviour of glomerulosa cells or that of hepatocytes is characteristic of most cell types remains to be examined. Certainly, the type response shown by glomerulosa cells is not unique, since a sustained mitochondrial Ca^{2+} signal in response to a sustained increase in $[Ca^{2+}]_i$ has also been reported in the ECV304 endothelial cell line [34].

When the functions of hepatocytes and glomerulosa cells are compared, it should be recalled that, while in the former cells Ca^{2+}-mobilizing agonists at physiological concentrations all induce oscillatory Ca^{2+} signals, one of the major physiological stimuli of glomerulosa cells, i.e. extracellular K$^+$, induces non-oscillating, long-lasting increases in $[Ca^{2+}]_i$. The ability of these cells to maintain increased activation of mitochondrial metabolism, even in the presence of the continuous elevation of $[Ca^{2+}]_i$, is a prerequisite for the hypersecretion of aldosterone during hyperkalaemia, a basic protective mechanism in vertebrate species.

In conclusion, Ca^{2+} signalling induces a mitochondrial redox response which follows the pattern of the $[Ca^{2+}]_i$ signal, irrespective whether it is sustained or oscillating. In adrenal glomerulosa cells the biological significance of this signal is not only the increased formation of ATP and GTP but also the

enhanced reduction of NADP$^+$, which may contribute to the increase in hormone production. The kinetics of the mitochondrial Ca^{2+} and NAD(P)H signals are cell-specific and are in harmony with the cells' physiological function.

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