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Pyridine nucleotide redox state parallels production of aldosterone in potassium-stimulated adrenal glomerulosa cells

[NAD(P)H fluorescence/cytosolic Ca^{2+}/rat glomerulosa cells/steroidogenesis]

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ABSTRACT **Extracellular potassium ions (K^+) raise the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) by gating voltage-dependent Ca^{2+} channels and stimulate aldosterone production in adrenal glomerulosa cells. The pathway leading from calcium influx to increased steroid synthesis has not been completely elucidated. In the present study we demonstrate that the reduction of pyridine nucleotides known to be required for steroid hydroxylation is enhanced by K^+ (4.1–8.4 mM) in single rat glomerulosa cells. The action of K^+ was strictly dependent on the presence of extracellular Ca^{2+}. Amytal, a blocker of site I of the mitochondrial respiratory chain, abolished the K^+ effect, indicating a mitochondrial origin for the recorded changes. Supraphysiological K^+ concentration (18 mM) resulted in a further increase in $[Ca^{2+}]_i$, while steroidogenesis was decreased as measured in cell suspensions. However, a possible explanation for this dichotomy is provided by the finding that the level of reduced pyridine nucleotides also decreased at supraphysiological K^+ concentration.**

Secretion of aldosterone is stimulated by K^+ both *in vivo* and *in vitro* (for review see ref. 1). The effect is secondary to the rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) mediated by Ca^{2+} influx through voltage-dependent Ca^{2+} channels (2–4). In rat glomerulosa cells, the nifedipine-sensitive Ca^{2+} influx was already detected at 3.6 mM K^+ (5), the physiological plasma concentration in this species (6, 7). Ca^{2+} influx elicits maximal aldosterone production at around 8 mM K^+, and steroidogenesis is reduced at higher concentrations (8–10) in spite of further increases in $[Ca^{2+}]_i$ (11). The reason for the dichotomy between $[Ca^{2+}]_i$ and aldosterone production at supraphysiological K^+ concentrations has not been elucidated. Here we have examined the formation of reduced pyridine nucleotides involved in cholesterol hydroxylation (1) and aldosterone production in K^+-stimulated rat glomerulosa cells. We found that K^+-induced elevation of $[Ca^{2+}]_i$ exerts similar effects on the reduction of mitochondrial pyridine nucleotides and the rate of aldosterone production. These data suggest that the Ca^{2+} modulation of redox state in the glomerulosa cell is a critical determinant of steroidogenesis.

MATERIALS AND METHODS

Glomerulosa cells were obtained from the adrenal capsular tissue of male Wistar rats by digestion with collagenase, as described (5). The harvested cells were resuspended in a mixture of modified Krebs–Ringer–glucose solution and medium 199 (38:62, vol/vol) containing 142 mM Na^+, 3.6 mM K^+, 1.2 mM Ca^{2+}, 0.5 mM Mg^{2+}, 25 mM HCO_3^-, 135 mM Cl^-, and 2 g of human serum albumin (fraction V) per liter. For aldosterone studies, the cell suspension was preincubated at 37°C for 3 hr under a mixture of 95% O_2 and 5% CO_2. After centrifugation, the cells were resuspended in the same medium (completed with KCl if required) and incubated for 2 hr under conditions as above. [Hyperosmosis induced by KCl had no effect on hormone production (unpublished observations).] At the end of incubation, the cells were pelleted and the supernatants were analyzed for aldosterone by radioimmunoassay (5).

For measuring $[Ca^{2+}]_i$, after the 3-hr preincubation period the cells (10^6 per ml) were loaded with 1 μM fura-2 acetoxymethyl ester (Calbiochem) for 30 min at 24°C and 15 min at 37°C. After repeated washings about 10^6 cells were added to 2 ml of the same medium containing 20 μM *N,N,N',N'*-tetrakis(2-pyridymethyl)ethylenediamine (TPEN, Sigma) but no albumin. Fluorescence was monitored at 35°C in a PTI Deltascan spectrofluorimeter, using the ratio mode at 340 and 380 nm for excitation and 500 nm for emission wavelength. Calibration and calculation of $[Ca^{2+}]_i$ were carried out by conventional methods, assuming 224 nM as the dissociation constant of fura-2 (12).

For measuring the fluorescence of reduced pyridine nucleotides in single glomerulosa cells, freshly digested cells (5×10^4 per Petri dish) were plated onto polyornithine-coated glass coverslips in 50 μl of incubation medium containing 100 international units of penicillin and 100 μg of streptomycin per ml. The plates were incubated in a thermostated CO_2 (5%) incubator for 3 hr; 2 ml of the same incubation medium was then added and the incubation was continued for another 18–28 hr. Thereafter the medium was replaced by a modified Krebs–Ringer solution, containing 142 mM Na^+, 3.6 mM K^+, 1.5 mM Ca^{2+}, 0.5 mM Mg^{2+}, 2 mM HCO_3^-, 135 mM Cl^-, and 10 mM Hepes, pH 7.4. The reduced pyridine nucleotides were measured as described (13). In short, a SPEX modular fluorimeter coupled to an inverted microscope (Diaphot-TMD, Nikon) was used in the epifluorescence mode (objective: Nikon F100) at 37°C. For excitation 360 nm and 390 nm (the latter as reference) were applied; emission wavelength was fixed by an interference filter at 470 nm. When $[Ca^{2+}]_i$ and NAD(P)H fluorescence were measured simultaneously, the cells were preloaded with 200 nM fura-2 acetoxymethyl ester for 15 min at 37°C in the presence of bovine serum albumin at 0.2 mg/ml. Excitation wavelengths of 340 and 363 nm were selected for fura-2 and NAD(P)H fluorescence, respectively; emission was measured at 470 nm. Amytal was from Serva. The quenching property of amytal was evaluated by comparing the increase in NAD(P)H after amytal (1 mM) with that after NaCN (1 mM). Similar qualitative increases (not shown) were obtained for the two metabolic inhibitors, indicating no significant quenching by amytal.

RESULTS

The rate of NAD(P) reduction appeared to be remarkably controlled by ambient K^+ concentrations (Fig. 1). When

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Abbreviation: $[Ca^{2+}]_i$, intracellular concentration of free Ca^{2+}.

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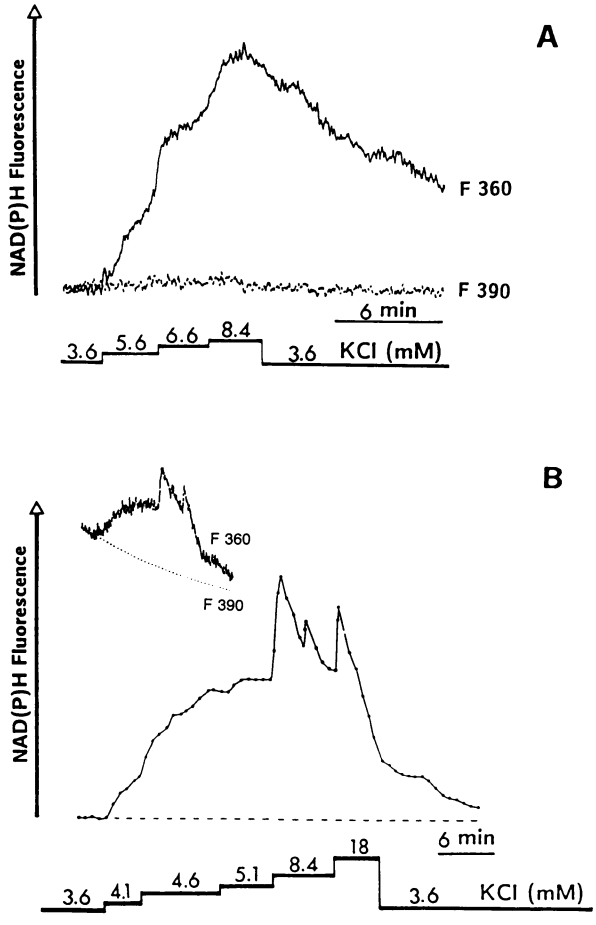

FIG. 1. Fluorescence of reduced pyridine nucleotides, NAD(P)H, as a function of extracellular K^+ measured in single adrenal glomerulosa cells. The traces are representative of 10 independent experiments. (*A*) Recorded fluorescence (470 nm) for the two excitation wavelengths (360 and 390 nm). The 390-nm recording is shown close to the 360-nm for comparative purposes and does not correspond to the relative fluorescence recorded at that wavelength. The actual values of the 390-nm trace represent no more than 25% of the intensity of the 360-nm trace. (*B*) Recalculated trace from *Inset* (360-nm divided by 390-nm).

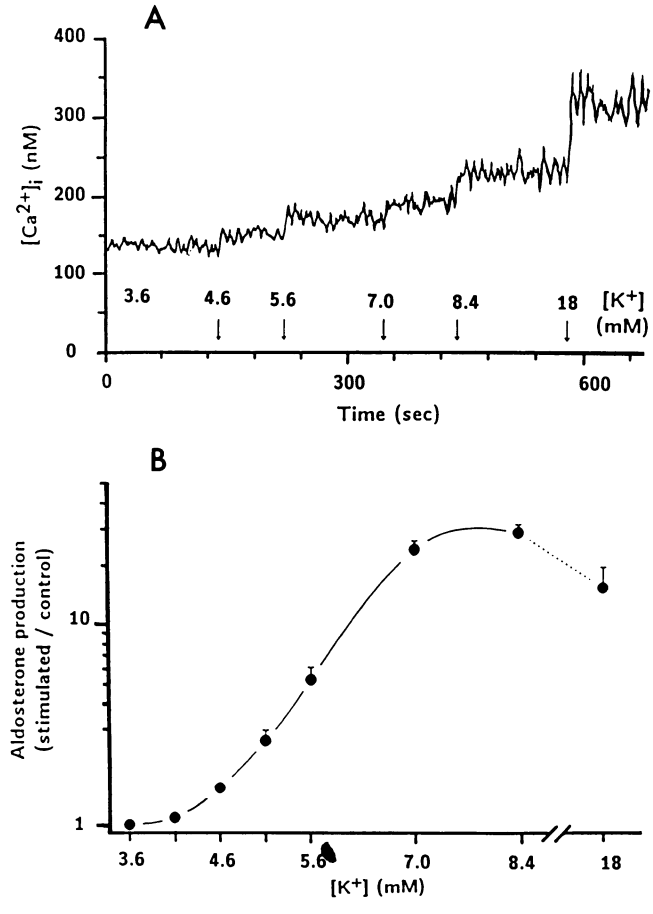

FIG. 2. $[Ca^{2+}]_i$ (*A*) and aldosterone production (*B*) as a function of extracellular K^+ concentrations in suspensions of rat adrenal glomerulosa cells. The trace in *A* is representative of four similar experiments. In *B*, mean ± SEM is shown for the ratio of stimulated to control production in nine independent experiments (control: 2.69 ± 1.82 pmol per 10^5 cells per 2 hr). Aldosterone production in the presence of 18 mM KCl is only half of that obtained at 8.4 mM ($P < 0.05$, $n = 4$). Note logarithmic ordinate scale.

gradual increases in K^+ were tested on the same single glomerulosa cell, NAD(P)H progressively rose to new levels (Fig. 1) as did $[Ca^{2+}]_i$ in cell populations (Fig. 2*A*). It is noteworthy that the NAD(P)H fluorescence signal was enhanced when K^+ was increased from 3.6 to 4.1 mM (Fig. 1*B*), although a detectable rise in $[Ca^{2+}]_i$ was observed only at 4.6 mM K^+ (Fig. 2*A*). The highest sustained NAD(P)H fluorescence (corresponding to an increase of about 30–40% of basal) was observed at 8.4 mM K^+, a concentration where maximal aldosterone production was also attained (Fig. 2*B*). When the cells were stimulated with 18 mM K^+ ($n = 3$), an initial sharp increase in NAD(P)H fluorescence was followed by an abrupt fall, which was terminated only upon switching to the control K^+ concentration (Fig. 1*B*). This concentration of K^+ also reduced aldosterone production, as compared with the effect of 8.4 mM K^+ (Fig. 2*B*). The inhibition of the NAD(P)H and aldosterone response took place in spite of further increase in $[Ca^{2+}]_i$ (Fig. 2*A*).

NAD(P)H fluorescence "on response" showed more rapid kinetics than the "response reversal." Both appeared to be related to the K^+ concentration. In addition, the latter also depended on the duration of stimulus application (Fig. 3). NAD(P)H fluorescence often showed a biphasic pattern in that a rapidly attained peak was followed by a gradual decrease to a plateau level (Figs. 1*B* and 3*C*). In Fig. 3 *A* and *B*, the same glomerulosa cell was stimulated for 100 s with 8.4 or 5.6 mM K^+, respectively. The NAD(P)H fluorescence level reached at the end of the stimulation with 8.4 mM was

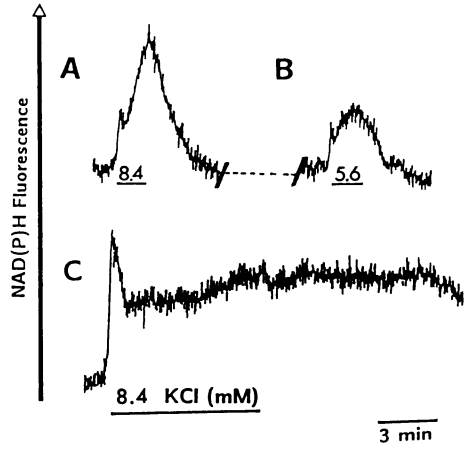

FIG. 3. Effect of K^+ concentration on the rate of NAD(P)H formation and oxidation upon removal of the stimulus after different exposure times in single glomerulosa cells. The traces are expressed as the ratio of fluorescence with 360-nm excitation to that with 390-nm excitation and are representative of at least three independent experiments. Bars indicate duration and concentration (mM) of KCl.

more than twice that obtained with 5.6 mM K^+. Furthermore, after short K^+ exposure (Fig. 3 *A* and *B*), NAD(P)H returned to basal in about 2–3 min. In contrast, prolonged stimulations (more than 5 min) often resulted in recovery times longer than 10 min (Fig. 3*C*).

The effect of K^+ was dependent on the presence of extracellular Ca^{2+} (Fig. 4). Depletion of extracellular Ca^{2+} abolished the effect of high K^+ on NAD(P)H fluorescence (Fig. 4*A*). Readdition of Ca^{2+} restored the effect. When Ca^{2+} was chelated with EGTA after the onset of stimulation (Fig. 4*B*), NAD(P)H fluorescence returned towards prestimulatory values, although the rate of reversal varied from cell to cell. Reintroduction of Ca^{2+} again increased the rate of NAD(P) reduction, largely restoring the original plateau level.

To determine whether the response occurs in the cytosolic or mitochondrial pyridine nucleotide pool, we used amytal. This agent prevents the oxidation of NADH by site I of the mitochondrial respiratory chain. Amytal (1 mM) induced an immediate elevation of NAD(P)H fluorescence similar to that obtained after the first stimulation with 8.4 mM K^+ (Fig. 5*A*). The presence of amytal completely obliterated the response to 8.4 mM K^+ (Fig. 5*A*). Conversely, when amytal was added after K^+, no additional increase was detected (Fig. 5*B* and legend). It is noteworthy that the NAD(P)H level reached with 8.4 mM K^+ is close to that induced by amytal (1 mM) (10 independent determinations).

In one cell preparation, five of eight K^+-stimulated (5.6 mM) single cells displayed oscillations in NAD(P)H fluorescence (Fig. 6*A*). One batch of these cells was loaded with the Ca^{2+} indicator fura-2. Under these conditions we observed oscillatory changes superimposed on a progressive increase in fluorescence at both excitation wavelengths, 340 and 363 nm, indicating changes in $[Ca^{2+}]_i$ and in the redox state of the cell, respectively (Fig. 6*B*; for further explanations see figure legend). The onset of the Ca^{2+} spikes just preceded that of the NAD(P)H spikes, reflecting the close correlation in time between the two parameters. Similar oscillations have been observed in two additional preparations at intermediate KCl (5.6 mM).

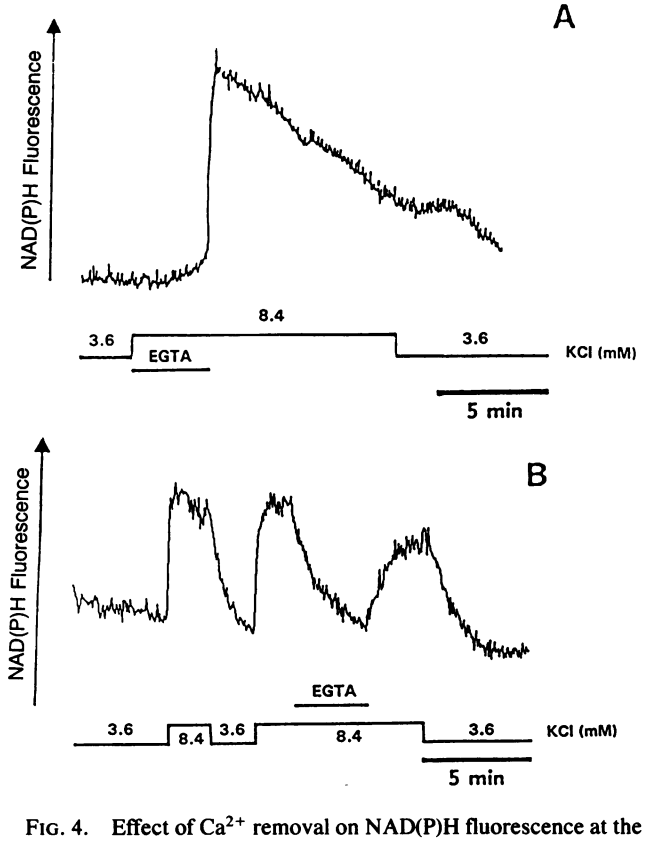

FIG. 4. Effect of Ca^{2+} removal on NAD(P)H fluorescence at the time of (*A*) and after (*B*) exposure of single adrenal glomerulosa cells to 8.4 mM K^+. EGTA (3 mM) was added to the buffer containing 1.5 mM Ca^{2+}. The two traces are expressed as in Fig. 3 and are representative of six (*A*) and five (*B*) separate experiments.

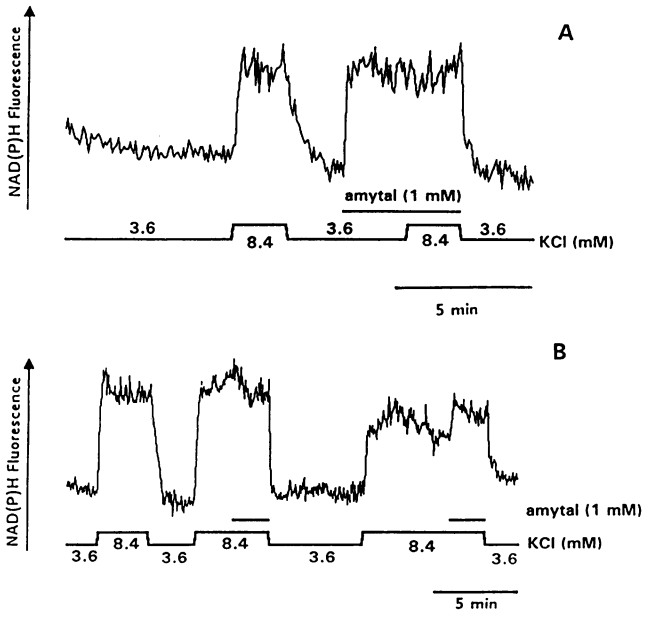

FIG. 5. Effect of amytal on pyridine nucleotide fluorescence before (*A*) and after (*B*) application of 8.4 mM K^+ in single rat adrenal glomerulosa cells. In *B*, the cell shows a diminished response to the second K^+ stimulation most likely due to the former amytal treatment. However, the reduction obtained by the second amytal is similar to that consecutive to the first application of the inhibitor. The traces are expressed as in Fig. 3 and are representative of 10 separate experiments.

DISCUSSION

Aldosterone secretion depends essentially on *de novo* synthesis, since steroid hormones are not packaged and stored in secretory granules. In the processing of steroid hormones, cholesterol and its C_{21} derivatives undergo several hydroxylation steps requiring NADPH. The mitochondria are the sites for all except one (21-hydroxylation) of the hydroxylation steps. These reactions, including cholesterol side-chain cleavage (14), depend essentially on NADPH that is generated by mitochondrial transhydrogenases at the expense of NADH (14, 15). The cytoplasmic NADPH, utilized for 21-hydroxylation, originates from the pentose phosphate pathway.

In the present study, we applied fluorimetric methods to monitor whether changes in pyridine nucleotide redox state follow those in $[Ca^{2+}]_i$ in intact glomerulosa cells stimulated with K^+. Due to its high resolution, a single-cell approach was selected (13). This method measures total NADH and NADPH fluorescence, in both the intra- and extramitochondrial compartments.

Exposure of the cells to K^+, at concentrations occurring *in vivo*, increased both $[Ca^{2+}]_i$ (Figs. 1 and 6; refs. 1 and 16) and the formation of NAD(P)H within a few seconds. Since we observed a certain degree of heterogeneity in the NAD(P)H response at intermediate K^+ (5.6 mM), most experiments were performed at optimal KCl (8.4 mM), where this was less apparent.

As for $[Ca^{2+}]_i$ (16), the K^+-induced NAD(P)H response was strictly dependent on the presence of extracellular Ca^{2+}. These observations, as well as the close correlation between

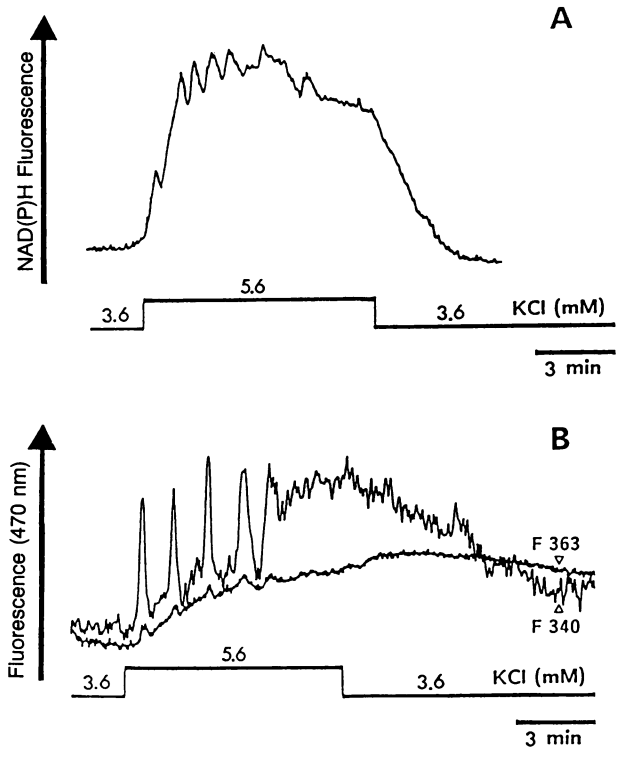

FIG. 6. Oscillatory pattern of NAD(P)H fluorescence in a non-fura-2-loaded single glomerulosa cell stimulated with 5.6 mM K^+ (*A*) and combined recording of $[Ca^{2+}]_i$ and NAD(P)H in a fura-2-loaded single glomerulosa cell (*B*). The trace in *A* is expressed as in Fig. 3. The 363-nm excitation wavelength, which is just above the isosbestic point of the indicator (12), was chosen so that any small contamination of the fura-2 signal would be in the opposite direction to the NAD(P)H signal. This is indeed the case, as attested by the small increase in fluorescence observed upon the removal of K^+ on the 363-nm recording in the face of a marked decrease in the 340-nm trace, indicating lowering of $[Ca^{2+}]_i$.

oscillatory patterns of $[Ca^{2+}]_i$ and NAD(P)H observed in the present study, point to a cause-and-effect relationship between $[Ca^{2+}]_i$ and NAD(P)H level.

Which are the possible mechanisms leading to the increased pyridine nucleotide reduction after $[Ca^{2+}]_i$ elevation? It is now well accepted that mitochondrial dehydrogenases, including pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase, can be activated by Ca^{2+} in various cell types to generate reducing equivalents (17). This process may well be relevant in rat glomerulosa cells because mitochondria occupy as much as 25–30% of the cytoplasmic volume of this cell (18). However, it should be kept in mind that in glomerulosa cells only 50% of total cellular pyridine nucleotides has been reported to be present in the mitochondrial compartment (19). Therefore it was important to test in which compartment (cytosolic or mitochondrial) the recorded changes took place. To this end, amytal, an inhibitor of the NADH–coenzyme-Q reductase complex (site I of the mitochondrial respiratory chain) was applied. Blockade of this site results in the accumulation of NADH in the mitochondria. As the amytal-induced elevation of NAD(P)H fluorescence either was obliterating the K^+ effect or, conversely, was not further enhanced by K^+, it is suggested that the recorded signal is predominantly of mitochondrial origin. It is noteworthy that blockade of site I in isolated mitochondria of adrenal cortex, incubated in the presence of citric acid cycle intermediates, was shown to support side-chain cleavage (14). Furthermore, in the same study, the inhibition of the NADH–coenzyme-Q reductase resulted not only in an increased NADH-to-NAD ratio but also in an augmented NADPH-to-NADP ratio. This was taken as evidence that the NADPH used for side-chain cleavage could be generated from NADH by transhydrogenation. The same process could underlie the increased NAD(P)H signal in response to either K^+ or amytal observed in the present study. In line with our findings, it should be recalled that Ca^{2+}-induced aldosterone production in permeabilized bovine glomerulosa cells was dependent on the presence of NADP and could be inhibited by ruthenium red (20), a known blocker of Ca^{2+} uptake by mitochondria (17).

Maximal NAD(P)H formation and aldosterone production in response to K^+ were reached at 8.4 mM. Stimulation with 18 mM K^+, a concentration incompatible with life, although further increasing $[Ca^{2+}]_i$, depressed both NAD(P)H levels and aldosterone production. In this situation, the aldosterone production was, however, less depressed than the NAD(P)H signal. There may be several mechanisms leading to the decrease in pyridine nucleotide redox state and aldosterone production under high K^+ conditions. On the one hand, augmented oxidation of NADH used for the synthesis of ATP required for the homeostasis of Ca^{2+} and other ions would diminish the availability of reduced pyridine nucleotides for the mitochondrial hydroxylation steps. This could in part explain the apparent discrepancy between the NAD(P)H signal and aldosterone production under such nonphysiological conditions. Indeed, the NAD(P)H signal indicates only the pyridine nucleotide redox state and may not proportionally reflect the flux through either the transhydrogenases and the hydroxylations steps or the respiratory chain. On the other hand, high $[Ca^{2+}]_i$ is known to be deleterious for mitochondrial functions with time (21, 22), uncoupling the oxidative flux and finally damaging the organelle. In this context Quinn *et al.* (16) have reported $[Ca^{2+}]_i$ levels in the range of 700 nM after stimulation with high K^+. It is known that $[Ca^{2+}]_i$ measured in single responsive cells generally gives higher values than in cell suspensions (Fig. 2*A*).

The kinetics of K^+-induced changes in NAD(P)H fluorescence are reminiscent of both the kinetics of $[Ca^{2+}]_i$ changes in single glomerulosa cells previously reported (16) and those of aldosterone production with the same stimulus in cell populations (11). Reoxidation of NAD(P)H, when control K^+ concentration was restored, depended on the duration and concentration of the K^+ stimulation. The fluorescence signal decreased instantaneously after short-term exposure but had a time lag of several minutes after long-term stimulation. This latter phenomenon may be due to the time-dependent actions of intracellular Ca^{2+} leading to hysteresial enzyme activations, to the accumulation of metabolic intermediates, and/or to a change in the kinetics of Ca^{2+} reequilibration (16) between the different intracellular compartments.

Thus by favoring Ca^{2+} influx, K^+ may stimulate steroid production by different means: (*i*) By stimulating mitochondrial dehydrogenases (17), Ca^{2+} may directly increase the availability of reduced pyridine nucleotides necessary for the hydroxylation steps and side-chain cleavage (14, 15); indeed, Krebs cycle intermediates have been shown to support the reduction of pyridine nucleotides (14, 23) and steroid hydroxylation in adrenocortical cells (19, 24–26), including glomerulosa cells (27). Along the same lines, it should be recalled that corticotropin also enhances the activity of the Krebs cycle in the adrenal cortex (28). (*ii*) In addition, Ca^{2+}, in activating citric acid cycle flux, may also favor steroidogenesis by an increased formation of GTP by succinyl-CoA synthetase occurring simultaneously to the reduction of pyridine nucleotides. Indeed, it has been reported recently that GTP and the steroidogenesis activator polypeptide (SAP) stimulate cholesterol side-chain cleavage synergistically (29). (*iii*) By eliciting protein phosphorylation either via Ca^{2+}–calmodulin- (30) or cAMP-dependent protein kinase [secondary to activation of adenylate cyclase (31, 32)], Ca^{2+}

may also favor steroidogenesis by promoting cholesterol transport into the mitochondria (33).

In summary, the amplitude and kinetics of NAD(P)H formation paralleled those of aldosterone production at various K^+ concentrations. Both parameters displayed bell-shaped dose responses to K^+ stimulation with a maximum at 8.4 mM. These observations demonstrate that the modulation of mitochondrial pyridine nucleotide redox state is one of the earliest events triggered by Ca^{2+} in the activation of adrenal glomerulosa cells by K^+. The fall in redox state observed at high K^+ levels offers an attractive explanation for the dichotomy between $[Ca^{2+}]_i$ and aldosterone production occurring in intact rat adrenal glomerulosa cells (11). These data suggest that during the process of stimulus–secretion coupling, a critical level of reduced pyridine nucleotides may be one of the factors governing steroid production in the adrenal glomerulosa cell. Similar mechanisms could be relevant in other steroid-producing cells whose steroidogenesis is increased after stimulation with Ca^{2+}-raising agonists. Furthermore, the present study shows that intermediate K^+ concentrations may induce oscillations in both $[Ca^{2+}]_i$ and NAD(P)H fluorescence in adrenal glomerulosa cells.

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