Quantitative analysis of the cytosolic-free-Ca\textsuperscript{2+}-dependency of aldosterone production in bovine adrenal glomerulosa cells

Different requirements for angiotensin II and K\textsuperscript{+}

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Angiotensin II (AII) and K\textsuperscript{+} raise the cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and stimulate aldosterone production in isolated bovine adrenal glomerulosa cells. The mechanisms leading to an elevation of [Ca\textsuperscript{2+}]\textsubscript{i} were analysed with the fluorescent Ca\textsuperscript{2+} probe quin 2. (1) Whereas [Ca\textsuperscript{2+}]\textsubscript{i} rose transiently and returned to basal values within 5 min in response to AII, the effect of K\textsuperscript{+} was sustained for at least 15 min. (2) AII released Ca\textsuperscript{2+} from intracellular stores, whereas the [Ca\textsuperscript{2+}]\textsubscript{i} response to K\textsuperscript{+} depended solely on extracellular [Ca\textsuperscript{2+}]\textsubscript{i}. (3) When added after K\textsuperscript{+} stimulation, AII provoked a dramatic decrease in [Ca\textsuperscript{2+}]\textsubscript{i} to below the resting value. The role of [Ca\textsuperscript{2+}]\textsubscript{i} in stimulating steroidogenesis was determined by manipulating the concentration of this cation. (4) In a cell superfusion system, the aldosterone response to AII is biphasic. Suppressing the transient [Ca\textsuperscript{2+}]\textsubscript{i}, elevation triggered by AII resulted in the disappearance of the initial secretory peak, but the final production rate was similar to that of control cells. (5) Normal basal [Ca\textsuperscript{2+}]\textsubscript{i} levels were, however, necessary to maintain continuous AII-induced steroidogenesis. (6) When added after AII, the antagonist analogue [Sar\textsuperscript{1},Ala\textsuperscript{8}]AII suppressed steroidogenesis without affecting [Ca\textsuperscript{2+}]\textsubscript{i} levels. (7) In contrast, continuously elevated [Ca\textsuperscript{2+}]\textsubscript{i} values were required for the initiation and the maintenance of K\textsuperscript{+}-stimulated aldosterone production. These results demonstrate important differences in the mechanisms through which AII and K\textsuperscript{+} activate the Ca\textsuperscript{2+} messenger system. Moreover, functional correlations have shown that K\textsuperscript{+}, but not AII, depends solely on a sustained [Ca\textsuperscript{2+}]\textsubscript{i} response for its steroidogenic effect. However, the AII-induced effect is also a Ca\textsuperscript{2+}-requiring process: the initial [Ca\textsuperscript{2+}]\textsubscript{i} transiently accelerates the onset of steroidogenesis, which is subsequently extremely sensitive to [Ca\textsuperscript{2+}]\textsubscript{i}, decreases below normal basal levels.

INTRODUCTION

It is now generally accepted that the two stimulators of aldosterone production in adrenal zona-glomerulosa cells, namely angiotensin II (AII) and K\textsuperscript{+}, trigger steroidogenesis by a mechanism involving changes in [Ca\textsuperscript{2+}]\textsubscript{i}. Concentration. The early hypotheses on the involvement of Ca\textsuperscript{2+} [1–9] have been recently verified by means of the direct measurement of [Ca\textsuperscript{2+}]\textsubscript{i} in intact glomerulosa cells [10,11] with the fluorescent probe quin 2 [12]; AII and K\textsuperscript{+} both induce rises of [Ca\textsuperscript{2+}]\textsubscript{i}. In parallel, studies based on more indirect approaches ([Ca\textsuperscript{2+}]\textsubscript{i} fluxes, Ca\textsuperscript{2+} channel blockers) have led to similar conclusions [13–20]. In another target tissue for AII, vascular smooth muscle, AII has been shown to raise [Ca\textsuperscript{2+}]\textsubscript{i}, by inducing release of Ca\textsuperscript{2+} from intracellular stores and Ca\textsuperscript{2+} influx through nifedipine-insensitive channels [21,22].

However, the final demonstration of the role of [Ca\textsuperscript{2+}]\textsubscript{i} in triggering steroidogenesis depends on the establishment of a quantitative relationship between [Ca\textsuperscript{2+}]\textsubscript{i} and secretion, as has been shown, for example, in human neutrophils [23]. We have therefore manipulated [Ca\textsuperscript{2+}]\textsubscript{i} at various times before or after cell stimulation and correlated the levels of this cation with steroidogenesis. The results reported here indicate not only that AII and K\textsuperscript{+} activate the Ca\textsuperscript{2+} messenger system by entirely different mechanisms, but also that Ca\textsuperscript{2+} plays different roles for these two agonists in the activation of bovine adrenal glomerulosa cells.

MATERIALS AND METHODS

Materials

[Ile\textsuperscript{5}]AII and [Sar\textsuperscript{1},Ala\textsuperscript{8}]AII were purchased from Bachem. Ionomycin was purchased from Calbiochem. Quin 2 tetra-acetoxyethyl ester (quin 2/AM) and quin 2 free acid were purchased from Amersham. Collagenase (type I) was from Sigma, and medium 199 was from Seromed. Nifedipine was kindly donated by Bayer.

Preparation of bovine adrenal glomerulosa cells

Isolated bovine adrenal glomerulosa cells were prepared by collagenase digestion and mechanical dispersion in medium 199 containing 3.5 mM-K\textsuperscript{+} as previously described [10]. Erythrocytes were separated from adrenal cells on a Percoll gradient, as reported elsewhere [24].

Loading with quin 2, fluorescence measurements and calibration

The loading procedure was similar to that previously described [10], except that a lower concentration of quin 2/AM (30 μM) was used. Fluorescence of quin 2-loaded

Abbreviations used: [Ca\textsuperscript{2+}]\textsubscript{i}, cytosolic free Ca\textsuperscript{2+} concentration; AII, angiotensin II; [Ca\textsuperscript{2+}]\textsubscript{extracellular}, extracellular Ca\textsuperscript{2+} concentration; EC\textsubscript{90}, concentration producing 50% of maximal stimulation.
and non-loaded cells was measured, as described elsewhere [10], in a Perkin–Elmer LS-3 fluorescence spectrometer. The calibration of $[Ca^{2+}]$ as a function of quin 2 fluorescence was performed as described previously [10,24], as originally described by Tsien et al. [12], using digitonin (50 μM) to release quin 2 from the cells. Assuming an intracellular water space of 0.1 μl/10⁶ cells, the calculated quin 2 loadings were 0.91 ± 0.2 mM ($n = 10$).

**Measurement of aldosterone production**

The functional response of glomerulosa cells was assessed either in static incubations or in a dynamic cell superfusion system as previously described [10], and the aldosterone produced was determined by direct radioimmunoassay as described [10].

**RESULTS**

**Duration, source and cross-effect of the $[Ca^{2+}]$, response induced by receptor binding (AII) or by plasma-membrane depolarization (K⁺)**

Sustained $[Ca^{2+}]$, response induced by K⁺. The interaction of AII with its receptor on adrenal glomerulosa cells led to a rapid and transient $[Ca^{2+}]$, elevation, followed by a return to basal levels within 5 min (Fig. 1a and [10]).

By contrast, the rise of $[Ca^{2+}]$ induced by K⁺ was sustained for at least 15 min. As can be seen in Fig. 1(b), the addition of the Ca²⁺ channel blocker nifedipine (10⁻⁴ M) induced a decrease in $[Ca^{2+}]$, to values of about 50 nM in non-stimulated glomerulosa cells. By comparison, the decrease in $[Ca^{2+}]$, produced by nifedipine in glomerulosa cells 15 min after stimulation with K⁺ was much larger, indicating that $[Ca^{2+}]$, fell from values substantially higher than resting values. The steady-state $[Ca^{2+}]$, reached after addition of nifedipine was similar for control and K⁺-stimulated cells.

Role of extracellular Ca²⁺ in the $[Ca^{2+}]$, response induced by AII and K⁺. When quin 2-loaded adrenal glomerulosa cells were resuspended in a Ca²⁺-free medium containing 1 mM-EGTA ([Ca²⁺]₀ = 10⁻⁸ M), resting $[Ca^{2+}]$, levels fell within 10 min to extremely low values (close to 20 nM) (Fig. 2b). A challenge with AII immediately after EGTA had buffered all the extracellular Ca²⁺ induced a rapid rise of $[Ca^{2+}]$, (Fig. 2a), indicating release of Ca²⁺ from intracellular stores. However, as the cells were rapidly becoming deprived of intracellular Ca²⁺, a challenge with AII at later times, when resting $[Ca^{2+}]$, levels had reached very low values, only triggered a markedly decreased Ca²⁺ release (Fig. 2b).

In the absence of extracellular Ca²⁺, K⁺ (12 mM) had no effect on $[Ca^{2+}]$, in isolated adrenal glomerulosa cells (Fig. 2c and 2d). Varying the extracellular Ca²⁺ concentration from 20 to 2000 μM was accompanied by concentration-dependent increases in resting and K⁺-stimulated $[Ca^{2+}]$, with an EC₅₀ of about 300 μM.

**Effect of AII on the $[Ca^{2+}]$, rise induced by K⁺.** When AII (10⁻⁸ M) was added after the $[Ca^{2+}]$, rise induced by K⁺ had reached its plateau, a biphasic effect was observed (Fig. 3). First, a small transient peak in $[Ca^{2+}]$, occurred, presumably due to release of Ca²⁺ from intracellular pools. Secondly, a dramatic decrease in $[Ca^{2+}]$, took place, reaching steady-state values somewhat lower than resting levels.

**Fig. 1. Duration of the $[Ca^{2+}]$, response induced by AII (a) and K⁺ (b)**

The stippled trace shows resting levels in control cells. The broken line in (b) indicates that the lamp was turned off to avoid photobleaching.

**Fig. 2. Role of $[Ca^{2+}]$, in the $[Ca^{2+}]$, response induced by AII and K⁺**

Quin 2-loaded cells were placed in a Ca²⁺-free medium containing EGTA and stimulated with either AII (a and b) or K⁺ (c and d). Each trace was obtained on a separate cell suspension from one representative experiment.

**Role of $[Ca^{2+}]$, elevations induced by AII and K⁺ in the stimulation of aldosterone production**

Suppression of the initial $[Ca^{2+}]$, rise induced by AII. The effect on the steroidogenic response of suppressing the initial $[Ca^{2+}]$, peak induced by AII is shown in Fig. 4. The cells were equilibrated for 20 min either in standard
Cytosolic free Ca\^{2+} concentration

Fig. 3. Effect of AII on the [Ca\^{2+}]i rise induced by K+

(a) Effect of K\textsuperscript{+} alone. (b) Effect of K\textsuperscript{+} plus AII. The traces were obtained on separate cell suspensions from one of four similar experiments.

Ca\textsuperscript{2+}-containing medium (treatment A) or in Ca\textsuperscript{2+}-free medium (treatment B). Whereas AII elicited the expected [Ca\textsuperscript{2+}i] transient in the cells which had been preincubated in the presence of Ca\textsuperscript{2+}, the hormone induced only a very small [Ca\textsuperscript{2+}i] response in cells incubated in Ca\textsuperscript{2+}-free medium (Fig. 4a). At 5 or 6 min after stimulation, extracellular [Ca\textsuperscript{2+}] was restored to 1 mm for the cells incubated in Ca\textsuperscript{2+}-free medium; a rapid influx of Ca\textsuperscript{2+} re-established normal [Ca\textsuperscript{2+}i] values, which, however, did not increase beyond basal values. The kinetics of aldosterone production were monitored in parallel. On stimulation with AII in a standard Ca\textsuperscript{2+}-containing medium, the cells produced aldosterone in a biphasic manner: an initial secretory peak was followed by a sustained production at an intermediary level (Fig. 4b). By contrast, the cells in which AII had been unable to induce an adequate [Ca\textsuperscript{2+}i] transient were incapable of displaying the initial secretory response. The final production rates, however, were similar (Fig. 4b). Finally, if the absence of Ca\textsuperscript{2+} was maintained, no steroidogenic response could be elicited by AII (Fig. 4b).

Fig. 4. Effect on steroidogenesis of suppressing the initial [Ca\textsuperscript{2+}i] transient induced by AII

(a) [Ca\textsuperscript{2+}]i: cells were equilibrated for 15 min in regular Ca\textsuperscript{2+}-containing medium (treatment A, trace A) or in Ca\textsuperscript{2+}-free medium (treatment B, trace B) and then stimulated with AII (10\textsuperscript{-4} M). At 5 min after stimulation, extracellular [Ca\textsuperscript{2+}] was restored to 1 mm in Ca\textsuperscript{2+}-deprived cells. (b) The kinetics of aldosterone production after AII stimulation in a superfusion system. The cells were equilibrated for 30 min by superfusion with either Ca\textsuperscript{2+}-free Krebs–Ringer medium (B, ○) or standard Ca\textsuperscript{2+}-containing medium (A, ■) and were then stimulated with AII. At 6 min after stimulation, [Ca\textsuperscript{2+}]i was restored to 1 mm for Ca\textsuperscript{2+}-depleted cells (open arrow). The effect of AII in the constant absence of Ca\textsuperscript{2+} is shown by the closed circles (●, bottom trace). Fractions corresponding to 2 min were collected. Each point is the mean for three superfusion columns from two separate experiments.

Effect of blockade with [Sar\textsuperscript{1},Ala\textsuperscript{8}]AII. We have previously shown [10] that the specific antagonist analogue [Sar\textsuperscript{1},Ala\textsuperscript{8}]AII prevents both the [Ca\textsuperscript{2+}]i rise and aldosterone production induced by AII in adrenal glomerulosa cells. In contrast, when added immediately after the AII-induced [Ca\textsuperscript{2+}]i transient had vanished, [Sar\textsuperscript{1},Ala\textsuperscript{8}]AII had no effect on [Ca\textsuperscript{2+}]i levels (Fig. 5a). However, the antagonist peptide inhibited AII-induced aldosterone production when added at any time during a 1 h incubation period (Fig. 5b). Interestingly, however, the later the antagonist was added, the less effective was the blockade.

Role of [Ca\textsuperscript{2+}]i levels during the sustained phase of AII-induced aldosterone production. The above experiment suggested that [Ca\textsuperscript{2+}]i is not important for the maintenance of AII-stimulated steroidogenesis. The importance for cell function of maintaining [Ca\textsuperscript{2+}]i at resting levels after the transient [Ca\textsuperscript{2+}]i rise induced by AII had vanished was therefore tested by decreasing [Ca\textsuperscript{2+}]i with EGTA, 8 min after AII challenge. EGTA induced a concentration-dependent decrease in [Ca\textsuperscript{2+}]i basal levels, which was accompanied by a concentration-dependent decrease in aldosterone production, as measured over a 1 h incubation period (Fig. 6a). It was necessary for glomerulosa cells to maintain adequate resting [Ca\textsuperscript{2+}]i levels during the whole stimulation period, since addition of EGTA (2 mm) at any time during this period practically stopped AII-induced aldosterone production (results not shown).

Role of elevated [Ca\textsuperscript{2+}]i levels on aldosterone production induced by K\textsuperscript{+}. By contrast, the maintenance of elevated [Ca\textsuperscript{2+}]i values was a much more important event when aldosterone production was stimulated with K\textsuperscript{+}. The decrease in [Ca\textsuperscript{2+}]i obtained by addition of various amounts of EGTA also 8 min after K\textsuperscript{+} challenge was followed by corresponding decreases of [Ca\textsuperscript{2+}]i, which remained, however, above or at resting [Ca\textsuperscript{2+}]i values up to 0.5 mm-EGTA (Fig. 6b). Yet, even though [Ca\textsuperscript{2+}]i was still higher than in non-stimulated cells, aldosterone production was already significantly decreased when the
stimulated \([\text{Ca}^{2+}]_i\) levels (220 nM) were decreased by as little as 20 nM (0.1 mM-EGTA). Higher EGTA concentrations induced a more marked inhibition of steroidogenesis. The stimulated aldosterone production detected at or below basal \([\text{Ca}^{2+}]_i\) levels occurred during the 8 min preceding the addition of EGTA.

DISCUSSION

The direct measurement and manipulation of \([\text{Ca}^{2+}]_i\) in quin 2-loaded bovine adrenal glomerulosa cells has allowed us to analyse and quantify the role of the \([\text{Ca}^{2+}]_i\) elevations elicited by the two physiological stimulators of aldosterone production, AII and \(K^+\). Our results show important differences in the \(\text{Ca}^{2+}\) requirements of these two steroidogenic factors.

In the absence of extracellular \(\text{Ca}^{2+}\), adrenal glomerulosa cells were unable to maintain adequate \([\text{Ca}^{2+}]_i\) levels, which fell rapidly to extremely low values. This behaviour of glomerulosa cells contrasts with that of vascular-smooth-muscle cells, which are much less sensitive to variations of extracellular \(\text{Ca}^{2+}\) [21]. It appears, therefore, as we have already suggested previously [10] and has been confirmed by others in the rat [18], that the adrenal glomerulosa cell is constantly undergoing \(\text{Ca}^{2+}\) influx from the extracellular medium to adjust its \([\text{Ca}^{2+}]_i\), even in the resting state. This \(\text{Ca}^{2+}\) influx occurs presumably through voltage-sensitive \(\text{Ca}^{2+}\) channels which are partially operative under basal conditions [10,18].

Various important features distinguish the \([\text{Ca}^{2+}]_i\) response triggered by \(K^+\) from that induced by AII. Firstly, in contrast with the transient \([\text{Ca}^{2+}]_i\), change elicited by AII (about 5 min), the \([\text{Ca}^{2+}]_i\), rise induced by \(K^+\) was sustained and lasted for at least 15 min. This continuous \(\text{Ca}^{2+}\) influx apparently leads to an increase in total cell \(\text{Ca}^{2+}\) [25]. Secondly, when added in a \(\text{Ca}^{2+}\)-free medium before the cells became depleted of \(\text{Ca}^{2+}\), AII was able to induce an important \([\text{Ca}^{2+}]_i\), transient, a result which indicates release of \(\text{Ca}^{2+}\) from intracellular stores. In contrast, no response to \(K^+\) was observed in the absence of extracellular \(\text{Ca}^{2+}\). At close to physiological concentrations (5–10 mM), \(K^+\) is able to depolarize the cell membrane in isolated adrenal glomerulosa cells, and this depolarization is accompanied by a concomitant rise
of [Ca\textsuperscript{2+}], [10]. We have also shown that nifedipine does not alter the kinetics nor the amplitude of the AII-induced [Ca\textsuperscript{2+}], transient [10]. The [Ca\textsuperscript{2+}], response to K\textsuperscript{+} displayed an absolute requirement for extracellular Ca\textsuperscript{2+}, and Ca\textsuperscript{2+} influx occurred most certainly through voltage-dependent channels, since the [Ca\textsuperscript{2+}], response to K\textsuperscript{+} could be blocked with nifedipine [10].

Thirdly, K\textsuperscript{+}-induced Ca\textsuperscript{2+} influx can be interrupted by AII. Several mechanisms could account for this effect of angiotensin II. Firstly, the additional transient rise in [Ca\textsuperscript{2+}], induced by AII, would be sufficient to activate the Ca\textsuperscript{2+} pump (Ca\textsuperscript{2+/Mg\textsuperscript{2+}}-ATPase) in the plasma membrane [26], thus counteracting the sustained increase of [Ca\textsuperscript{2+}]i consequent to the activation of voltage-dependent Ca\textsuperscript{2+} channels by K\textsuperscript{+}. Secondly, these channels could be themselves inactivated as a consequence of AII action. Protein kinase C has been reported to phosphorylate the voltage-dependent Ca\textsuperscript{2+} channel [27]. Thirdly, an inhibitory coupling between the AII receptor and the Ca\textsuperscript{2+} channels could be exerted through a GTP-binding protein [28]. Whatever mechanism or combination of mechanisms is involved, our data clearly show that AII counteracts Ca\textsuperscript{2+} influx rather than triggering it, as suggested by others [25,29]. A similar finding has been reported for thyrotropin-releasing hormone, which can drastically decrease the K\textsuperscript{+}-induced rise in [Ca\textsuperscript{2+}], [30]. Moreover, its effect on voltage-dependent Ca\textsuperscript{2+} channels, if any, would result in a closing of these channels, even though AII action leads to a slow depolarization of glomerulosa cell membrane [10]. These conclusions contradict previous reports. One explanation for these discrepancies might lay in the indirect methodologies applied by the other authors [13–17]. In some cases, species differences might also be invoked [18].

In the second part of this work we exploited the exquisite sensitivity of the glomerulosa cell to changes in [Ca\textsuperscript{2+}],. This property enabled us to manipulate [Ca\textsuperscript{2+}], and thus to examine the role of these [Ca\textsuperscript{2+}], changes in the steroidogenic response of glomerulosa cells. The initial transient peak of [Ca\textsuperscript{2+}],, that occurs on stimulation of glomerulosa cells by AII, on generation of inositol 1,4,5-trisphosphate and on release of Ca\textsuperscript{2+} from a non-mitochondrial pool [31], is not a necessary event, but is responsible for the initial secretory peak of aldosterone production, a finding analogous to what has been reported in polypeptide-hormone-secreting cells such as GH cells [32,33] or other secretory cells. It is possible, however, that the transient [Ca\textsuperscript{2+}], spike is required to ‘prime’ the protein kinase C system, causing a translocation or binding to the cell membrane of the enzyme [29,34]. Indeed, it has been shown in our laboratory that AII induced a translocation of protein kinase C activity from a soluble to a membrane-bound compartment in bovine adrenal glomerulosa cells [35].

The results obtained with the antagonist [Ca\textsuperscript{2+},Ala\textsuperscript{2+}]AII demonstrate that continuous receptor occupancy is necessary to maintain steroidogenesis. They also provide evidence for an event in activation of steroidogenesis which outlasts the hormone-receptor occupancy. Such a ‘short-term memory’ might be represented by a sustained activation of protein kinase C [36]. Indeed, in hepatocytes, AII elicits a prolonged increase in the levels of 1,2-diacylglycerol [37], a metabolite that is thought to activate the Ca\textsuperscript{2+}-sensitive phospholipid-dependent protein kinase C. In bovine adrenal glomerulosa cells, AII stimulates the incorporation of [\textsuperscript{3}H]arachidonic acid into 1,2-diacylglycerol [14]. These results stress the importance of the C kinase branch in the maintenance of AII-induced steroidogenesis.

Even though, after the [Ca\textsuperscript{2+}], transient, the control of AII-stimulated steroidogenesis appears to be taken over by the protein kinase C branch of the messenger system, this does not mean that [Ca\textsuperscript{2+}], levels are not important any more. In fact, our experiments clearly show that it is necessary for adrenal glomerulosa cells to maintain adequate resting [Ca\textsuperscript{2+}], values during the whole period of the sustained steroidogenic response.

The situation appears entirely different for K\textsuperscript{+} ion. [Ca\textsuperscript{2+}], must remain elevated above basal levels during the whole period of the sustained steroidogenic response. Data from our laboratory [35] indicate that K\textsuperscript{+} has no effect on protein kinase C activity in bovine adrenal glomerulosa cells. These results suggest that, in the case of K\textsuperscript{+}-induced steroidogenesis, the triggering mechanisms depend exclusively on the Ca\textsuperscript{2+} branch of the signal-transduction process.

In summary, the direct measurement of [Ca\textsuperscript{2+}], has revealed fundamental differences in the mechanisms of the Ca\textsuperscript{2+} elevations induced by AII and K\textsuperscript{+} in adrenal zona-glomerulosa cells: (1) AII, but not K\textsuperscript{+}, releases Ca\textsuperscript{2+} from intracellular pools; (2) K\textsuperscript{+}, but not AII, elicits a sustained [Ca\textsuperscript{2+}], rise; (3) AII inhibits K\textsuperscript{+}-induced Ca\textsuperscript{2+} influx.

In addition, functional correlations allowed us to demonstrate that K\textsuperscript{+}, but not AII, depends mainly, if not solely, on a sustained [Ca\textsuperscript{2+}], response for its steroidogenic effect. However, although AII-induced continuous steroidogenesis appears to be dependent much more on protein kinase C activation than on [Ca\textsuperscript{2+}],, it is nevertheless a Ca\textsuperscript{2+}-requiring process, being extremely sensitive to [Ca\textsuperscript{2+}], decreases below basal levels.

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