The site of action of Ca^{2+} in the activation of steroidogenesis: studies in Ca^{2+} -clamped bovine adrenal zona-glomerulosa cells

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The Ca^{2+} -messenger system plays a crucial role in the regulation of steroid production in adrenal zona-glomerulosa cells, as it is known to mediate the action of both angiotensin II and K^+ . In the present study we used intact isolated glomerulosa cells in which the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) was clamped at various levels with the Ca^{2+} ionophore ionomycin in order to locate the site(s) of action of Ca^{2+} . By measuring in parallel steroid synthesis and $[\text{Ca}^{2+}]_c$, we show that Ca^{2+} levels (50–860 nM) regulate the production of both pregnenolone (up to $669 \pm 71.1\%$ of the basal production) and aldosterone (up to $301 \pm 42.2\%$; $\text{EC}_{50} = 303$ nM). By contrast, Ca^{2+} did not stimulate the conversion of 11-deoxycorticosterone into aldosterone. Ca^{2+} modulation did not affect the formation of pregnenolone from freely diffusible analogues of cholesterol, indicating that

Ca^{2+} acts at a step upstream of cholesterol side-chain cleavage. Moreover cycloheximide, an inhibitor of protein translation and of adrenocorticotropin-induced facilitation of intramitochondrial cholesterol transport, the rate-limiting step in steroidogenesis, also blocked Ca^{2+} -triggered pregnenolone formation. This is consistent with a model in which Ca^{2+} promotes cholesterol transfer between mitochondrial membranes. In addition, agents using the cyclic AMP pathway as well as angiotensin II potentiated the steroidogenic response to increases in $[\text{Ca}^{2+}]_c$ by augmenting both the efficacy and the potency of Ca^{2+} . This effect of angiotensin II did not involve protein kinase C. These results establish a direct link between agonist-induced $[\text{Ca}^{2+}]_c$ rises and a specific step of the steroidogenic pathway.

INTRODUCTION

Angiotensin II (AngII) and K^+ are two crucial factors in the regulation of aldosterone production in adrenal zona-glomerulosa cells [1,2]. A common characteristic of AngII and K^+ is the mobilization of the Ca^{2+} -messenger system as an intracellular transducer, although through different mechanisms. In bovine glomerulosa cells, AngII receptor activation leads, on the one hand, to protein kinase C (PKC) activation [3], and, on the other hand, to the release of Ca^{2+} from internal stores and to the stimulation of Ca^{2+} influx across the plasma membrane [4–6]. An elevation of extracellular K^+ is believed to open voltage-operated Ca^{2+} channels and hence to trigger Ca^{2+} influx into the cytosol [7].

The importance of Ca^{2+} in the regulation of aldosterone synthesis has been firmly established [1,2]. However, in most approaches, either the demonstration of this role was indirect [8–10] or the effective Ca^{2+} concentrations used to stimulate steroidogenesis were far from physiological [11,12]. Our laboratory has provided direct evidence for the involvement of physiological concentrations of cytosolic Ca^{2+} in the stimulation of the mitochondrial steps of aldosterone biosynthesis: in electro-permeabilized glomerulosa cells, Ca^{2+} was alone able to stimulate aldosterone output, an effect that was dependent upon the entry of Ca^{2+} into the mitochondrial matrix [13]. Nevertheless, the site(s) of action of this ion along the complex pathway of aldosterone synthesis, their Ca^{2+} -sensitivity and a potential modulation by additional factors remained to be determined.

Aldosterone synthesis is functionally divided into three consecutive stages: (1) the early mitochondrial steps, consisting of the transport of cholesterol into the mitochondrial inner membranes, which is the rate-limiting step for the whole steroidogenic

pathway [14] and its conversion into pregnenolone by the cytochrome *P*-450 side-chain-cleavage enzyme (P450_{sc}); (2) the intermediate steps, located on the endoplasmic reticulum and involving the conversion of pregnenolone into progesterone and then into 11-deoxycorticosterone (DOC); (3) the late mitochondrial steps, including the formation of corticosterone and, eventually, its conversion into aldosterone.

The present work had a dual goal: first, we wanted to establish a model to investigate the direct regulation of aldosterone production by physiological concentrations of cytosolic Ca^{2+} in intact glomerulosa cells. Secondly, we wished to identify the step(s) of aldosterone synthesis affected by Ca^{2+} . We show here that ionomycin can be effectively used to clamp the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) at various levels in intact glomerulosa cells and that this paradigm constitutes a valuable model to study Ca^{2+} -sensitive processes. We provide the first direct demonstration that $[\text{Ca}^{2+}]_c$ stimulates both aldosterone and pregnenolone synthesis in the submicromolar range in intact cells. Moreover, our results strongly suggest that Ca^{2+} affects the intramitochondrial transport of cholesterol. Finally, we report that AngII and cyclic AMP enhance the stimulatory effect of Ca^{2+} on aldosterone and pregnenolone production.

MATERIALS AND METHODS

Materials

Ionomycin and phorbol 12-myristate 13-acetate (PMA) were purchased from Calbiochem (Lucerne, Switzerland), $[\text{Ile}^5]\text{AngII}$ from Bachem (Bubendorf, Switzerland) and forskolin from LC Laboratories (Woburn, MA, U.S.A.). 25-hydroxycholesterol (25-OHChol), (22*R*)-hydroxycholesterol (22*R*-OHChol), cyclohex-

Abbreviations used: ACTH, adrenocorticotrophic hormone; AngII, angiotensin II; $[\text{Ca}^{2+}]_c$, cytosolic free Ca^{2+} concentration; $[\text{Ca}^{2+}]_t$, total extracellular Ca^{2+} concentration; PKC, protein kinase C (Ca^{2+} -sensitive phospholipid-dependent protein kinase); Bt_2cAMP , dibutyryl cyclic AMP; P450_{sc} , cytochrome *P*-450 side-chain-cleavage enzyme; CHX, cycloheximide; DOC, 11-deoxycorticosterone; 25-OHChol, 25-hydroxycholesterol; (22*R*)-OHChol, (22*R*)-hydroxycholesterol; PMA, phorbol 12-myristate 13-acetate; fura-2/AM, fura-2 acetoxymethyl ester.

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Figure 1 Establishment of the Ca^{2+} -clamp model in adrenal glomerulosa cells

Fura-2-loaded bovine glomerulosa cells were incubated in the presence of ionomycin ($2\ \mu\text{M}$) and increasing extracellular Ca^{2+} concentrations. (a) A representative recording of fura-2 fluorescence in glomerulosa cells exposed to increments of extracellular Ca^{2+} concentration (arrows) in the presence of ionomycin ($2\ \mu\text{M}$). (b) The measured $[\text{Ca}^{2+}]_i$ was expressed as a function of total extracellular Ca^{2+} concentration (mean $\pm$ S.E.M. for 22–25 independent experiments for each $[\text{Ca}^{2+}]_t$). The S.E.M. bars have been omitted when they are smaller than the symbols. Each value is significantly different ($P < 0.05$) from all the others. In all subsequent Figures, this $[\text{Ca}^{2+}]_i$ -versus- $[\text{Ca}^{2+}]_t$ relationship has been used as a calibration curve in order to express steroid production as a function of $[\text{Ca}^{2+}]_i$.

imide (CHX), aminoglutethimide and dibutyl cyclic AMP (Bt_2cAMP) were obtained from Sigma (St. Louis, MO, U.S.A.). Fura-2 acetoxymethyl ester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR, U.S.A.). $[\text{H}]\text{Pregnenolone}$ was obtained from Amersham (Zürich, Switzerland). WIN 19'578 (cyanoketone) was kindly donated by Sterling-Winthrop (Rensselaer, NY, U.S.A.) and losartan (DuP 753) was generously given by Dr. R. Smith from du Pont (Wilmington, DE, U.S.A.).

Bovine adrenal zona-glomerulosa-cell preparation

Bovine adrenal zona-glomerulosa cells were obtained by enzymic dispersion as described in detail elsewhere [15]. Glomerulosa cells were purified on Percoll density gradients as described in [16], resuspended at a density of 10^7 cells/ml in a modified Krebs–Ringer buffer (NaCl , 136 mM; NaHCO_3 , 5 mM; KH_2PO_4 , 1.2 mM; MgSO_4 , 1.2 mM; KCl , 1.8 mM; EGTA, 0.2 mM; CaCl_2 , 1.2 mM; D-glucose, 5.5 mM; Hepes, 20 mM; pH 7.4) and preincubated at 37°C for 60 min. Purified glomerulosa cells were loaded for 30 min at 37°C with $2\ \mu\text{M}$ fura-2/AM. After being washed in fresh Krebs–Ringer buffer, the cells were resuspended in the same medium (10^6 cells/ml) and left at room temperature until use for either $[\text{Ca}^{2+}]_i$ -monitoring or steroid-production experiments.

$[\text{Ca}^{2+}]_i$ measurements

The $[\text{Ca}^{2+}]_i$ was monitored in purified glomerulosa cells with the Ca^{2+} -sensitive fluorescent probe fura-2 using a Perkin–Elmer LS-5 fluorimeter, which was calibrated as described previously [16]. Excitation and emission wavelengths were 340 nm and 505 nm respectively. Just before use, the cells were centrifuged, resuspended in a modified Krebs–Ringer buffer containing no CaCl_2 and the cell suspension was placed in a fluorescence cuvette. EGTA (0.2 mM) was present in the modified Krebs–Ringer buffer in order to obtain very low $[\text{Ca}^{2+}]_i$ levels.

Steroid production

Purified fura-2-loaded glomerulosa cells were centrifuged, resuspended (10^6 cell/ml) in the Ca^{2+} -free Krebs–Ringer buffer and incubated for 180 min at 37°C in the presence of $2\ \mu\text{M}$ iono-

mycin, various concentrations of total extracellular Ca^{2+} ($[\text{Ca}^{2+}]_t$) and modulators of steroid production. For the assessment of pregnenolone production and the estimation of DOC conversion into aldosterone, WIN 19'758 ($1\ \mu\text{M}$) was included in the incubation medium in order to prevent conversion of pregnenolone into progesterone and to avoid endogenous DOC formation [17]. At the end of the incubation period the cells were sedimented and the incubation medium was collected for the determination of steroid production. The relationship between $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_t$ was determined in parallel in the same cell preparation.

Steroid measurements

The aldosterone content of the incubation media was measured by a direct radioimmunoassay, using a commercially available kit (DPC, Los Angeles, CA, U.S.A.). Pregnenolone was determined directly in the medium by radioimmunoassay [18], using $[\text{H}]\text{pregnenolone}$ and an antibody kindly donated by Professor P. Vescei (University of Heidelberg, Germany).

Analysis of data

Results are expressed as means $\pm$ S.E.M. unless otherwise stated. The means were compared by analysis of variance using the Fisher or Scheffé's *F* test when comparing balanced or unbalanced groups respectively. A value of $P < 0.05$ was considered as statistically significant. Where appropriate, concentration–response data were fitted to a four-parameter logistic function for the calculation of EC_{50} values.

RESULTS

Ca^{2+} clamping in bovine adrenal glomerulosa cells

In the absence of extracellular Ca^{2+} , ionomycin ($2\ \mu\text{M}$) triggered a transient rise of $[\text{Ca}^{2+}]_i$, followed by a rapid decline to values below basal level (results not shown). This transient elevation, while the cells were placed in Ca^{2+} -free medium, was probably due to the release of Ca^{2+} from intracellular stores, as indicated by the lack of effect of a subsequent addition of AngII or thapsigargin, an inhibitor of the microsomal Ca^{2+} -ATPase

Figure 2 Ca^{2+} stimulates steroid production in Ca^{2+} -clamped glomerulosa cells

Glomerulosa cells were incubated for 3 h in the presence of ionomycin ($2 \mu\text{M}$) and various concentrations of extracellular Ca^{2+} . Steroid production and $[\text{Ca}^{2+}]_e$ were determined in each experiment. (a) Aldosterone output is expressed as a percentage of basal production in the absence of Ca^{2+} and presented as a function of $[\text{Ca}^{2+}]_e$ (see the legend to Figure 1). Each point is the mean $\pm$ S.E.M. for 24 separate experiments. (b) Glomerulosa cells were incubated for 3 h as in (a), in the additional presence of WIN 19'758 ($1 \mu\text{M}$), a blocker of pregnenolone conversion into progesterone. Pregnenolone accumulation (% of basal production in the absence of Ca^{2+} ; mean $\pm$ S.E.M., $n = 36$) is expressed as a function of $[\text{Ca}^{2+}]_e$. (c) The effect of Ca^{2+} on DOC conversion into aldosterone was assessed as described in (a), except that the incubation was performed in the presence of $10 \mu\text{M}$ DOC and $1 \mu\text{M}$ WIN 19'758, the latter being added in order to avoid endogenous DOC formation ($n = 4$). DOC conversion into aldosterone is expressed in ng/h per 10^6 cells. * and ***, significantly different from the value in the absence of Ca^{2+} ($P < 0.05$ and $P < 0.001$ respectively).

(results not shown). When $[\text{Ca}^{2+}]_e$ was increased stepwise, however, there was a gradual rise in $[\text{Ca}^{2+}]_e$ in the submicromolar range (Figure 1a) that was sustained for at least 1 h (results not shown). In Figure 1(b), $[\text{Ca}^{2+}]_e$ in ionomycin-clamped bovine glomerulosa cells is represented as a function of $[\text{Ca}^{2+}]_i$. Similar relationships were established in the presence of various modulators of steroid production such as AngII, K^+ , thapsigargin, the dihydropyridine Ca^{2+} -channel blocker nicardipine, the PKC activator PMA, 25-OHChol, Bt_2cAMP or forskolin, and no

Figure 3 Ca^{2+} does not affect hydroxycholesterol-supported pregnenolone formation in Ca^{2+} -clamped glomerulosa cells

The effect of Ca^{2+} on hydroxycholesterol-supported pregnenolone formation was assessed as in Figure 2(b) in the presence of either 25-OHChol ($\square$; $1 \mu\text{M}$, $n = 12$) or (22R)-OHChol ($\blacksquare$; $0.1 \mu\text{M}$, $n = 4$). Pregnenolone production is expressed in ng/3 h per 10^6 cells. Inset: glomerulosa cells were clamped with ionomycin in the absence of Ca^{2+} and the incubation was performed in the presence of WIN 19'758 ($1 \mu\text{M}$) and of various concentrations of 25-OHChol. Each point is the mean $\pm$ S.E.M. for three separate experiments.

significant differences with the control condition were observed (see, e.g., the inset to Figure 5b below).

Ca^{2+} stimulates aldosterone biosynthesis

The clamp of $[\text{Ca}^{2+}]_e$ in glomerulosa cells allowed us to study the $[\text{Ca}^{2+}]_e$ -dependency of aldosterone production in intact cells by measuring aldosterone output during incubation in the presence of ionomycin ($2 \mu\text{M}$) and of various concentrations of extracellular Ca^{2+} . Basal ($[\text{Ca}^{2+}]_e = 50 \pm 6.5 \text{ nM}$) aldosterone production was $133 \pm 24 \text{ pg/h per } 10^6 \text{ cells}$. Figure 2(a) shows that submicromolar $[\text{Ca}^{2+}]_e$ stimulated aldosterone secretion in a concentration-dependent manner, with an EC_{50} of 303 nM . The maximal effect of Ca^{2+} was attained at about 600 nM , with an aldosterone output reaching $301 \pm 42.2\%$ of the basal production. Aldosterone secretion was practically linear over the 3 h incubation period, both under basal and maximally stimulatory conditions (results not shown). It is noteworthy that neither nicardipine nor K^+ had any effect on Ca^{2+} -induced aldosterone synthesis in Ca^{2+} -clamped glomerulosa cells (results not shown).

Ca^{2+} stimulates the early steps of aldosterone synthesis

Since pregnenolone synthesis has been reported to be enhanced by K^+ in glomerulosa cells [19], the early mitochondrial steps are likely candidates for regulation by Ca^{2+} . We have tested this hypothesis in our Ca^{2+} -clamped model, avoiding the conversion of pregnenolone into progesterone by performing the experiments in the presence of the inhibitor of progesterone formation, WIN 19'758 [17]. The basal pregnenolone formation in the absence of Ca^{2+} was $1.29 \pm 0.26 \text{ ng/h per } 10^6 \text{ cells}$. As depicted in Figure 2(b), Ca^{2+} in the submicromolar concentration range was a potent stimulator of pregnenolone formation, increasing its synthesis to $669 \pm 71.1\%$ of the basal value at 860 nM . The threshold for statistically significant stimulation was attained at $200 \text{ nM } [\text{Ca}^{2+}]_e$. By contrast, when the late steps of aldosterone synthesis were investigated by measuring DOC conversion into aldosterone, no detectable effect of Ca^{2+} was observed at $[\text{Ca}^{2+}]_e$ effective for pregnenolone synthesis. We have observed, however,

Figure 4 Cycloheximide inhibits the Ca^{2+} -induced stimulation of pregnenolone synthesis from endogenous cholesterol, but does not affect pregnenolone formation from exogenously added 25-OHChol

(a) The effect of Ca^{2+} on pregnenolone production from endogenous cholesterol was assessed as in Figure 2(b), except that the incubation was performed in the absence or in the presence of 1 mM CHX ($n = 3$). *Significantly different from the respective control at the same $[\text{Ca}^{2+}]_e$ ($P < 0.05$). (b) The effect of Ca^{2+} on 25-OHChol-supported pregnenolone formation was assessed as in Figure 3(b), except that the incubation was performed in the absence ($n = 12$) or in the presence of CHX (1 mM, $n = 2$).

Figure 5 AngII, but not PMA, potentiates the Ca^{2+} -induced stimulation of steroid synthesis in Ca^{2+} -clamped glomerulosa cells

(a) The effect of Ca^{2+} on aldosterone synthesis was assessed as in Figure 2(a), except that the incubation was performed in the absence ($n = 24$) or in the presence of AngII (10 nM, $n = 11$) or PMA (0.1 μM , $n = 7$). (b) The effect of Ca^{2+} on pregnenolone production was assessed as in Figure 2(b), except that the incubation was performed in the absence ($n = 36$) or in the presence of AngII (10 nM, $n = 23$) or PMA (0.1 μM , $n = 7$). *, ** and ***, significantly different from the respective control at the same $[\text{Ca}^{2+}]_e$ ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively). Inset: $[\text{Ca}^{2+}]_e$ -versus- $[\text{Ca}^{2+}]_i$ relationship in the presence ($n = 5-7$) and in the absence ($n = 22-25$) of AngII (10 nM) in Ca^{2+} -clamped glomerulosa cells.

a slight ($38.4 \pm 4.9\%$) but significant reduction of DOC conversion into aldosterone at the highest, and probably supra-physiological, $[\text{Ca}^{2+}]_e$ tested (Figure 2c).

In order to assess the activity of the P450_{sec} enzyme in intact cells, it is necessary to by-pass the barrier constituted by the mitochondrial intermembranous space. This is achieved by using hydrophilic derivatives of cholesterol such as 25-OHChol or (22R)-OHChol. These compounds are readily metabolized by P450_{sec} into pregnenolone in intact cells [20]. In the presence of concentrations of 25-OHChol that overcome the endogenous cholesterol supply (1 μM), Ca^{2+} did not affect pregnenolone formation in Ca^{2+} -clamped glomerulosa cells (Figure 3). Similar results were obtained with (22R)-OHChol. This lack of effect of Ca^{2+} was not because the maximal steroidogenic capacity had

already been attained, since a further increase in 25-OHChol concentration to 10 μM led to an enhanced pregnenolone accumulation (see the inset to Figure 3). It should be noted that pregnenolone production in Ca^{2+} -clamped glomerulosa cells (in the presence or in the absence of 25-OHChol) truly represented the activity of the P450_{sec} , since it could be completely blocked (results not shown) by aminoglutethimide, an inhibitor of the latter enzyme [17].

Ca^{2+} -stimulated pregnenolone synthesis is sensitive to CHX

The adrenocorticotrophic-hormone (ACTH)-triggered increase in the kinetics of cholesterol transfer from the mitochondrial outer into the mitochondrial inner membrane is sensitive to inhibition

Figure 6 Cyclic AMP enhances the Ca^{2+} -induced stimulation of steroid synthesis in Ca^{2+} -clamped glomerulosa cells

(a) The effect of Ca^{2+} on aldosterone synthesis was assessed as in Figure 2(a), except that the incubation was performed in the absence ($n = 24$) or in the presence of Bt_2cAMP (1 mM, $n = 3$). (b) The effect of Ca^{2+} on pregnenolone production was assessed as in Figure 2(b), except that the incubation was performed in the absence ($n = 36$) or in the presence of Bt_2cAMP (1 mM, $n = 8$) or forskolin (10 μM , $n = 9$). *, ** and ***, significantly different from the respective control at the same $[\text{Ca}^{2+}]_c$ ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively).

by CHX [21,22]. In addition, it has been reported that AngII-induced pregnenolone synthesis can be blocked by the same agent [23]. We have therefore tested whether CHX may also inhibit Ca^{2+} -stimulated pregnenolone formation from endogenous cholesterol or from exogenously added 25-OHChol in Ca^{2+} -clamped glomerulosa cells. We have observed that CHX partially, but significantly, diminished the accumulation of pregnenolone stimulated by the rise in $[\text{Ca}^{2+}]_c$ (Figure 4a). By contrast, 25-OHChol-supported pregnenolone formation was not affected by CHX, as expected for a drug blocking the ACTH-induced facilitation of cholesterol transport (Figure 4b).

AngII and cyclic AMP modulate Ca^{2+} -stimulated steroidogenesis

AngII and K^+ both stimulate influx of Ca^{2+} across the plasma membrane, although the maximal influx achieved with AngII is much weaker than with K^+ [4–6]. Yet, in bovine glomerulosa cells, AngII is at least as potent in stimulating aldosterone production as K^+ . In order to test whether AngII could alter the Ca^{2+} -sensitivity of aldosterone synthesis, we performed Ca^{2+} -clamping of glomerulosa cells and measured aldosterone output in the presence or in the absence of AngII (10 nM). The results depicted in Figure 5(a) indicate that AngII potentiated the aldosterone response to increases in $[\text{Ca}^{2+}]_c$, while having no effect on the $[\text{Ca}^{2+}]_c$ -versus- $[\text{Ca}^{2+}]_i$ relationship (see the inset to Figure 5b). AngII enhanced both the potency (maximal induction: $559 \pm 149.3\%$ of basal output versus $301 \pm 42.2\%$ in the absence of AngII, $P < 0.05$) and the efficacy of the stimulation by Ca^{2+} , the EC_{50} for Ca^{2+} being shifted towards lower concentrations ($\text{EC}_{50} = 205$ nM versus 303 nM in the absence of AngII). Similarly, AngII also potentiated the effect of Ca^{2+} on pregnenolone biosynthesis (Figure 5b). This effect of AngII appears to be concentration-dependent ($\text{EC}_{50} = 6.4$ nM) and to be mediated by the AT1 receptor subtype [24], since it was prevented by the non-peptidic AT1 receptor antagonist losartan (results not shown). The potentiating effect of AngII may not be mediated by an activation of PKC, since a pharmacological activator of PKC, PMA, did not mimic the action of the hormone (Figures 5a and 5b).

ACTH is a potent steroidogenic agent whose action is thought to be mediated by the cyclic AMP messenger system [25].

Figure 7 Potentiating action of AngII and forskolin on Ca^{2+} -stimulated pregnenolone production is not additive to that of Bt_2cAMP

The effect of Ca^{2+} on pregnenolone production was assessed as in Figure 2(b), but in the presence of Bt_2cAMP (1 mM), and in the absence ($n = 8$) or in the presence of AngII (10 nM, $n = 3$) or forskolin (10 μM , $n = 3$).

Bt_2cAMP , a membrane-permeable surrogate of cyclic AMP, has been widely used to study the importance of this pathway in the regulation of steroid secretion. We observed that, in Ca^{2+} -clamped glomerulosa cells, a maximally stimulating concentration of Bt_2cAMP (1 mM) markedly enhanced the stimulatory effect of Ca^{2+} on aldosterone and pregnenolone production (Figures 6a and 6b). A similar result was obtained with forskolin, a direct activator of adenylyl cyclase (Figure 6b). Surprisingly, pregnenolone formation in the concomitant presence of AngII and of a maximally stimulating concentration of Bt_2cAMP was no different from the production observed in the presence of Bt_2cAMP alone (Figure 7). By contrast, the lack of additivity between Bt_2cAMP and forskolin was expected, since both of

these agents are known to activate the cyclic AMP/protein kinase A pathway.

DISCUSSION

Ca^{2+} ionophores, including ionomycin, have been widely used as tools for the study of the involvement of Ca^{2+} in a variety of physiological phenomena. However, the actual $[\text{Ca}^{2+}]_i$ attained under ionophore treatment in the presence of high concentrations of extracellular Ca^{2+} have usually not been monitored, thus weakening the value of observations obtained with this type of protocol. Ionomycin is a Ca^{2+} ionophore of the carrier type [26] that is able to transport Ca^{2+} along its chemical gradient across the plasma membrane. It may also translocate H^+ out of the cell, provided that the pH gradient is favourable, thus allowing the recycling of the ionophore. In intact cells, low steady-state $[\text{Ca}^{2+}]_i$ levels are maintained in the presence of high extracellular Ca^{2+} concentrations through the action of a Ca^{2+} -ATPase. When intact cells are exposed to ionomycin in the presence of extracellular Ca^{2+} , the ionophore causes an important influx of Ca^{2+} into the cytosol. Concomitantly, the Ca^{2+} ATPase is driven to extrude more Ca^{2+} ions. After a while, as Ca^{2+} influx is maintained constant through the recycling of ionomycin, a new steady state is attained, at a higher $[\text{Ca}^{2+}]_i$. This level depends upon both the extracellular free Ca^{2+} concentration and the ionophore concentration. Provided that the ionomycin concentration is constant, it is therefore possible to clamp $[\text{Ca}^{2+}]_i$ at various levels by submitting the cells to various $[\text{Ca}^{2+}]_e$. Such a model has been used [27] to demonstrate the role of Ca^{2+} in the regulation of early gene expression in HL-60 cells. The usefulness and necessity of determining the exact $[\text{Ca}^{2+}]_i$ -sensitivity of biological processes is clearly illustrated in that paper [27]: a maximal transcriptional stimulation was attained at approx. 300 nM, while it was barely changed at 700–1250 nM. The indiscriminate use of an ionophore and of a high Ca^{2+} concentration would have led to the erroneous conclusion that Ca^{2+} does not affect early gene expression.

Another advantage of Ca^{2+} -clamping with ionomycin is that the cells remain intact, in contrast with permeabilized cell systems in which the cell content is dialysed into the medium. For example, in order to demonstrate the Ca^{2+} -sensitivity of aldosterone production in electroporabilized glomerulosa cells, it is necessary to supply NADP^+ , a cofactor of steroidogenesis [13]. In addition, the integrity of the cells allows the investigator to study the modulation of the response to Ca^{2+} by other hormones or factors whose action is frequently mediated by soluble messengers.

In the present study, we have applied the ionomycin-mediated Ca^{2+} clamp model to bovine adrenal zona glomerulosa cells. As exemplified in Figure 1(a), an efficient and stable Ca^{2+} clamp was established that spanned the entire physiological domain of $[\text{Ca}^{2+}]_i$ (50–1000 nM). Ionomycin was used at the lowest concentration (2 μM) allowing complete coverage of this domain. In spite of the presence of the ionophore, the Ca^{2+} gradient across the plasma membrane was not abolished, but was set at a lower level. The relationship between extracellular and intracellular Ca^{2+} concentration obtained under these conditions (Figure 1b) is comparable with the results obtained by others [27,28]. Although AngII and K^+ both induce Ca^{2+} influx in intact glomerulosa cells, they did not appear to perturb $[\text{Ca}^{2+}]_i$ in ionomycin-clamped cells (see the inset to Figure 5b), presumably because these influx pathways were negligible compared with the ionomycin-mediated Ca^{2+} entry.

Although a role for Ca^{2+} in the regulation of aldosterone biosynthesis has been suspected for a long time, conclusive evidence is scarce and based either on indirect demonstrations

[8–10] or on effective Ca^{2+} concentrations exceeding by far the physiological range [11,12]. A direct demonstration of a stimulatory effect of Ca^{2+} has been provided in electroporabilized glomerulosa cells [13]. In the latter study, Ca^{2+} induced a maximal 226% stimulation of aldosterone output with an EC_{50} of approx. 500 nM, values that are comparable with those obtained in the present study. An important question is whether the stimulation of steroidogenesis in Ca^{2+} -clamped glomerulosa cells is a direct effect of Ca^{2+} or whether it reflects a rise in cyclic AMP, secondary to the stimulation of a Ca^{2+} -sensitive adenylyl cyclase [29]. Our demonstration that, in the presence of a maximally stimulating concentration of Bt_2cAMP , Ca^{2+} was still able to markedly increase pregnenolone production, suggests a direct effect of Ca^{2+} itself on steroidogenesis (Figures 6a and 6b).

The global rate-limiting step in steroidogenesis is the transport of cholesterol into a steroidogenic pool (i.e. accessible to P450_{sec} and metabolizable into pregnenolone), possibly located in the mitochondrial inner membrane [14,30]. Agents that stimulate the cyclic AMP pathway, such as ACTH, have been shown to exert a double effect on steroidogenesis: first, they activate cholesterol ester hydrolase, which mobilizes free cholesterol from its stored esters [31]. Secondly, they increase the kinetics, otherwise very slow, of cholesterol transfer from a presteroidogenic pool, possibly located in the mitochondrial outer membrane, into the steroidogenic pool [9,21,22]. Interestingly, the latter effect of cyclic AMP can be blocked by CHX, an inhibitor of protein translation. It is therefore thought that cholesterol availability to P450_{sec} is the major determinant for steroid synthesis. A prediction of this model is that a rise of NADPH supply or a stimulation of P450_{sec} intrinsic activity may not influence steroid production, unless saturating metabolizable cholesterol levels are achieved.

In earlier studies, various indirect arguments had suggested that the early mitochondrial steps are a target of Ca^{2+} regulation: (1) K^+ stimulates pregnenolone accumulation [19]; (2) calmodulin inhibitors block AngII- and K^+ -stimulated pregnenolone production [32]; (3) calmodulin inhibitors prevent ACTH-induced accumulation of cholesterol in the mitochondrial inner membrane [9]. The results of the present study provide the first direct evidence that physiological cytosolic Ca^{2+} can itself increase pregnenolone formation in intact cells. Interestingly, the availability of reducing equivalents (NADPH) and the intrinsic P450_{sec} activity did not appear to constitute limiting factors, since glomerulosa cells were able to convert large amounts of 25-OHChol or (22R)-OHChol into pregnenolone, even at very low $[\text{Ca}^{2+}]_i$ (50 nM; Figure 3). In addition, in the same experiments, an elevation of $[\text{Ca}^{2+}]_i$ did not lead to an increased level of pregnenolone output, in spite of the fact that the maximal steroidogenic capacity was not attained with 1 μM 25-OHChol (see the inset to Figure 3). Thus the reported rise of mitochondrial redox potential triggered in rat glomerulosa cells by K^+ [33] may not be responsible for the stimulation of pregnenolone synthesis. It is noteworthy that the conversion of 25-OHChol into pregnenolone is a specific process, since it can be blocked by aminoglutethimide, an inhibitor of $\text{P-450}_{\text{sec}}$.

Moreover, the sensitivity of Ca^{2+} -stimulated pregnenolone synthesis towards CHX (Figure 4a) is consistent with a model in which Ca^{2+} increases the transfer of cholesterol from the outer membrane to the steroidogenic pool, possibly in the mitochondrial inner membrane or a subfraction of it [21,22]. In this respect it is important to remember that the Ca^{2+} -induced enhancement of steroidogenesis in permeabilized glomerulosa cells requires the influx of Ca^{2+} into the mitochondrial matrix [13]. We are therefore led to conclude that the supply of cholesterol to the P450_{sec} , and in particular cholesterol transfer

from the mitochondrial outer to the mitochondrial inner membrane is the most likely Ca^{2+} -sensitive step in the early steroidogenic pathway.

We observed that high Ca^{2+} reduced DOC conversion into aldosterone (Figure 2c). However, the $[\text{Ca}^{2+}]_i$ required for this inhibition were much higher than those required for the induction of pregnenolone formation. This modest ($38.4 \pm 4.9\%$) inhibition at high $[\text{Ca}^{2+}]_i$ may account for the fact that Ca^{2+} -triggered aldosterone biosynthesis reached a plateau or even declined at about 600 nM (Figure 2a), although pregnenolone production was further increased at higher $[\text{Ca}^{2+}]_i$ (Figure 2b). Interestingly, a reduction of aldosterone production at high K^+ concentrations (and hence high $[\text{Ca}^{2+}]_i$) was reported both in bovine [19] and in rat glomerulosa cells [33]. In the latter study, this phenomenon was accompanied by a fall in the redox potential, in spite of a further increase in $[\text{Ca}^{2+}]_i$. On the other hand, high concentrations of Ca^{2+} are known to provoke an increase in mitochondrial-inner-membrane permeability, a fall of the membrane potential and to lead eventually to severe mitochondrial damage [34].

A striking feature of the present study was the marked potentiation by AngII of the Ca^{2+} -mediated stimulation of steroid production. A potentiation between K^+ , which acts through a rise in $[\text{Ca}^{2+}]_i$, and AngII, at low concentrations of both agonists has been reported [35–37]. Moreover, the $[\text{Ca}^{2+}]_i$ requirement for the stimulation of aldosterone synthesis is different according to whether the cells are challenged with K^+ or with AngII [4]. In the case of AngII, a $[\text{Ca}^{2+}]_i$ near the basal level is sufficient for a full steroidogenic effect; by contrast, continuously elevated $[\text{Ca}^{2+}]_i$ values are required for the initiation and the maintenance of K^+ -stimulated aldosterone production. The potentiating effect reported here is reminiscent of this lower requirement of Ca^{2+} in AngII-stimulated steroidogenesis. With respect to the mode of action of AngII, it is unlikely that a perturbation of $[\text{Ca}^{2+}]_i$ would be responsible for this action, since the hormone did not affect ionomycin-clamped Ca^{2+} levels (see the inset to Figure 5b).

PKC is another possible mediator of the potentiating effect of AngII, since AngII activates PKC in bovine glomerulosa cells [3]. However, the role of PKC in aldosterone synthesis is a matter of debate [24], one group claiming that PKC is positively involved in the maintenance of AngII-induced aldosterone synthesis [38], while the majority of the studies, including our own, have concluded that an inhibitory effect of the PKC-stimulating phorbol ester PMA [37,39–41] is involved. We report here that PMA had no effect on Ca^{2+} -induced steroid synthesis (Figures 5a and 5b). Moreover, PMA did not modify the AngII-triggered potentiation (results not shown). This lack of effect of PKC activators in Ca^{2+} -clamped cells may indicate that, in non-clamped cells, PMA-activated PKC negatively affects $[\text{Ca}^{2+}]_i$. In Ca^{2+} -clamped cells, voltage-dependent Ca^{2+} channels no longer play a role in $[\text{Ca}^{2+}]_i$, as indicated by the lack of effect of K^+ or dihydropyridines on both $[\text{Ca}^{2+}]_i$ and steroid output. A likely explanation for the effect of PMA in non-clamped cells could be provided by recent results from our laboratory showing that PKC inhibits voltage-dependent Ca^{2+} -channels (M. F. Rossier, C. P. Python, H. B. C. Aptel, M. B. Vallotton and A. M. Capponi, unpublished work). This hypothesis is consistent with the blockade of the Ca^{2+} response to K^+ by AngII in both bovine and rat adrenal glomerulosa cells [4,42]. Alternatively, we cannot exclude the possibility that the potentiation of steroid synthesis by AngII is mediated by a PKC isoform that is insensitive to PMA such as the ζ isoform [43].

The second messenger of ACTH, cyclic AMP, is another important steroidogenic agent that appeared to modulate Ca^{2+} -induced steroid production in ionomycin-clamped glomerulosa cells. In the presence of Bt_2cAMP or forskolin, steroid output

was dramatically augmented, even at very low $[\text{Ca}^{2+}]_i$. At all $[\text{Ca}^{2+}]_i$ tested, the effects of cyclic AMP agents were more than additive to that of Ca^{2+} (Figures 6a and 6b). We conclude that Ca^{2+} and cyclic AMP appear as distinct but convergent mediators that are each on their own able to increase steroidogenesis and which are able to reinforce each other's action. Such an interaction of cyclic AMP and Ca^{2+} was expected, since Fakunding et al. [8] have reported that aldosterone synthesis supported by 8-bromo cyclic AMP, another membrane-permeant analogue of cyclic AMP, did not in the strict sense depend on, but was modulated by, the extracellular Ca^{2+} concentration. Kojima et al. [10] similarly concluded from indirect evidence that Ca^{2+} and cyclic AMP act synergistically.

With respect to cyclic AMP, an interesting point has been recently raised by Baukal and Hunyady [44]: in contrast with rat glomerulosa cells, in which AngII has been shown to decrease cyclic AMP levels through a GTP-binding protein (G_i)-dependent mechanism [45,46], AngII enhanced the ACTH-induced cyclic AMP response in bovine glomerulosa cells, as it has also been reported in bovine adrenal fasciculata-reticularis cells [47,48]. This phenomenon may explain the potentiating effect of AngII in ionomycin-clamped cells. In addition, this is consistent with the fact that the AngII effect is not additive to that of Bt_2cAMP (Figure 7). Thus AngII might trigger the activation of adenylyl cyclase through a Ca^{2+} -independent mechanism that remains to be determined. Elevated cyclic AMP levels would then stimulate steroid production in conjunction with Ca^{2+} . The weak effect of AngII at low $[\text{Ca}^{2+}]_i$ (50 nM), as compared with the AngII response at higher $[\text{Ca}^{2+}]_i$ (600 nM) (Figure 5) could be explained by the fact that some members of the adenylyl cyclase family require a minimal $[\text{Ca}^{2+}]_i$ to be active [29]. However, the isoform(s) present in bovine glomerulosa cells has (have) not yet been identified.

In conclusion, we have shown that ionomycin-clamped cells are a model of choice for the study of the regulation of steroidogenesis by Ca^{2+} and of its modulation by other physiological factors. Using this paradigm, we have demonstrated that elevations of $[\text{Ca}^{2+}]_i$ equivalent to the levels attained during stimulation with AngII or K^+ themselves activate steroidogenesis in intact bovine glomerulosa cells. Ca^{2+} appears to affect the early mitochondrial steps of aldosterone synthesis, probably at the level of intramitochondrial cholesterol transport. Finally, we have provided direct evidence indicating that AngII and cyclic AMP markedly enhance the effect of Ca^{2+} .

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