Capacitative calcium entry is involved in steroidogenesis in bovine adrenocortical fasciculata cells

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Abstract

Capacitative Ca^{2+} entry into bovine adrenocortical fasciculata cells was investigated by using the mobilization of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and Ca^{2+} -induced steroidogenesis as the indicators. Bovine adrenocortical fasciculata cells on a glass coverslip were loaded with fura-2. The $[\text{Ca}^{2+}]_i$ mobilization was detected by a change of fura-2 fluorescence intensity. In the intracellular Ca^{2+} store depleted cells, the addition of Ca^{2+} to the incubation medium elicited a marked and sustained increase in $[\text{Ca}^{2+}]_i$. In the intracellular Ca^{2+} store non-depleted cells, the addition of thapsigargin, an endoplasmic reticulum Ca^{2+} -ATPase inhibitor, in the absence of extracellular Ca^{2+} , induced a slight and transient increase in $[\text{Ca}^{2+}]_i$, but an extensive and sustained increase in $[\text{Ca}^{2+}]_i$ was obtained by adding Ca^{2+} to the incubation

medium after the thapsigargin treatment. The sustained increase induced by thapsigargin was not inhibited by nifedipine, but was inhibited by Zn^{2+} and Cd^{2+} in a concentration-dependent manner. The effect of Zn^{2+} was more potent than that of Cd^{2+} . Thapsigargin stimulated steroidogenesis in the presence of extracellular Ca^{2+} . The steroidogenic effect of thapsigargin was inhibited by Zn^{2+} and Cd^{2+} but not by nifedipine.

These results suggest that there is, in bovine adrenocortical fasciculata cells, a steroidogenesis-linked Ca^{2+} entry process other than that involving voltage-operated Ca^{2+} channels and that the process might be capacitative Ca^{2+} entry.

Journal of Endocrinology (2000) **167**, 473–478

Introduction

Adrenocorticotrophic hormone (ACTH) stimulates adrenocortical fasciculata cells and facilitates glucocorticoid biosynthesis (steroidogenesis). It is well known that the steroidogenic effect of ACTH involves cyclic AMP production (Schimmer 1980). However, it was also proposed that Ca^{2+} was required for ACTH-induced steroidogenesis (Yanagibashi 1979). We previously showed that bovine adrenocortical fasciculata cells (BAFC) possess a voltage-operated calcium channel (VOC) and that ACTH activates the channel to enhance steroidogenesis (Yanagibashi *et al.* 1990). We also reported that extracellular adenosine 5'-triphosphate (ATP) facilitates steroidogenesis in BAFC via P2Y receptors (Kawamura *et al.* 1991). Niitsu (1992) has suggested that ATP induces Ca^{2+} influx via a dihydropyridine-insensitive pathway. Thus, ATP-induced steroidogenesis might be associated with a particular type of Ca^{2+} influx.

Recently, it has been reported that capacitative Ca^{2+} entry (i.e. Ca^{2+} entry triggered by depletion of intracellular Ca^{2+} stores) occurs in diverse non-excitable cells (Berridge 1995). Gq protein-coupled receptor agonists stimulate inositol 1,4,5-trisphosphate (IP_3) production and

subsequent Ca^{2+} release from intracellular stores. Consequently, Ca^{2+} entry from an extracellular pool may arise (Putney & Bird 1993).

In steroidogenic cells, it was reported that angiotensin II, which activates phospholipase $\text{C}\beta$ to increase intracellular IP_3 , stimulates aldosterone production via capacitative Ca^{2+} entry activation in bovine adrenocortical glomerulosa cells (Burnay *et al.* 1994). From these reports, we surmised that a capacitative Ca^{2+} entry was also involved in BAFC and was linked to glucocorticoid production.

In the present study, we investigated the involvement of a capacitative Ca^{2+} entry process in BAFC steroidogenesis.

Materials and Methods

Isolation and primary culture of BAFC

Isolated adrenocortical fasciculata cells were prepared aseptically from bovine adrenal cortex by use of collagenase and deoxyribonuclease I (DNase I) as described previously (Matsui 1991). Briefly, a minced adrenal cortex was incubated with collagenase (0.1%) and DNase I (0.005%) in Krebs–Ringer bicarbonate buffer (123.4 mM NaCl,

5.9 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.2 mM CaCl_2 , 25 mM NaHCO_3 , 2 mg/ml glucose, and 3 mg/ml bovine serum albumin; pH 7.4) for 1 h at 37 °C under 95% O_2 –5% CO_2 as a gas phase. The cells were isolated by pipetting. The isolated cells were cultured in Ham's F-10 medium supplemented with 5% fetal calf serum, 10% newborn calf serum, 2.5% horse serum, and antibiotics. The 3-day cultured cells were used for the experiments.

Measurement of intracellular concentration of Ca^{2+}

The determination of intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) in the monolayered BAFC on a glass coverslip coated with collagen was performed with the fluorescent probe fura-2 as described by Matsui (1991). Briefly, the cells were incubated with acetoxymethylester of fura-2 (5 μM) for 1 h at 37 °C in cremophor EL (0.02%) containing Krebs–Ringer HEPES buffer (123.4 mM NaCl, 5.9 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.2 mM CaCl_2 , 10 mM HEPES, 0.01 mM EGTA and 2 mg/ml glucose; pH 7.4). The intracellular Ca^{2+} stores in BAFC could be diminished during the culture period, because Ham's F-10 medium contained 0.3 mM Ca^{2+} . The Ca^{2+} stores, however, might be refilled during the fura-2 loading process (i.e. Krebs–Ringer HEPES buffer is supplemented by 1.2 mM Ca^{2+}). Therefore, to diminish the intracellular Ca^{2+} stores in BAFC markedly, Krebs–Ringer HEPES buffer (pH 7.4) which is not supplemented with Ca^{2+} was applied to the fura-2 loading period (the Ca^{2+} -depleted cells). Fura-2-loaded BAFC were transferred to a quartz cuvette containing Ca^{2+} (–) HEPES buffer (123.2 mM NaCl, 3 mM KCl, 1.2 mM NaH_2PO_4 , 1.2 mM MgSO_4 , 0.5 mM EGTA, 10 mM HEPES and 2 mg/ml glucose; pH 7.4) and the concentrated CaCl_2 and/or agents were added with continuous stirring. Fluorescence of the fura-2 loaded cells was monitored by Hitachi F-2000 fluorescence spectrophotometer (Hitachi, Japan) at 30 °C with continuous stirring at an emission wavelength of 510 nm, while the excitation wavelength altered every 0.5 s between 340 and 380 nm. $[\text{Ca}^{2+}]_i$ was shown as the ratio of the fluorescence intensity excited at 340 nm to that of 380 nm (I_{340}/I_{380}).

Measurement of steroidogenesis

Cortisol production by monolayered BAFC on a 24-well type dish ($15\text{--}20 \times 10^4$ cells/well) was measured. BAFC were incubated in the low K^+ -Krebs–Ringer bicarbonate buffer (123.4 mM NaCl, 3 mM KCl, 1.2 mM NaH_2PO_4 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 , 0.01 mM EGTA, 2 mg/ml glucose and 3 mg/ml bovine serum albumin; pH 7.4) with or without 1.2 mM Ca^{2+} for 1 h at 37 °C in a humidified atmosphere at 5% CO_2 in air. Steroid in the incubation medium was determined fluorometrically by use of cortisol as a standard (Salvinski *et al.* 1976). The

sensitivity of the method was 1 ng, and the intra- and inter-assay coefficient variations were 2.1% and 3.1% respectively.

Statistics

Analysis of variance and Student's *t*-test were used for the statistical analysis of the data. The value of $P < 0.05$ was considered to be statistically significant.

Materials

The materials were purchased from the following: collagenase from Funakoshi (Tokyo, Japan); DNase I, nifedipine and thapsigargin (TG) from Sigma Chemical Co. (St Louis, MO, USA); fura-2/AM from Dojindo (Kumamoto, Japan); Ham's F-10 medium from Gibco Laboratories Inc. (New York, USA). All the other chemicals were of reagent grade.

Results

Effect of the intracellular Ca^{2+} store depletion on Ca^{2+} entry

We first examined the effect of the extracellular addition of Ca^{2+} on Ca^{2+} entry in fura-2 loaded BAFC. Fura-2 was loaded in BAFC in the absence (the Ca^{2+} -depleted cells) and presence (the Ca^{2+} -non-depleted cells) of 1.2 mM Ca^{2+} for 1 h at 37 °C. After that $[\text{Ca}^{2+}]_i$ in the fura-2 loaded cells on a glass coverslip was determined in Ca^{2+} (–) HEPES buffer (pH 7.4). In the Ca^{2+} -non-depleted cells, the addition of 2.4 mM Ca^{2+} caused only a slight increase in $[\text{Ca}^{2+}]_i$ (Fig. 1A). However, in Ca^{2+} -depleted BAFC, the addition of 2.4 mM Ca^{2+} triggered a significant and immediate increase in $[\text{Ca}^{2+}]_i$ (Fig. 1B). The effect of added Ca^{2+} was concentration-dependent, and reached a maximum at 1.2 mM Ca^{2+} (data not shown). The results suggest that the depletion of intracellular Ca^{2+} stores elicits a Ca^{2+} flux from the extracellular pool in BAFC.

As shown in Fig. 2A, 30 mM K^+ induced a rapid increase in $[\text{Ca}^{2+}]_i$ and an ensuing sustained rise in $[\text{Ca}^{2+}]_i$. Nifedipine (10 μM), a VOC-selective antagonist, inhibited the sustained phase. However, 10 μM nifedipine inhibited the Ca^{2+} entry induced by 2.4 mM Ca^{2+} only slightly in the Ca^{2+} -depleted cells (Fig. 2B). However, because the low K^+ (3 mM) buffer was used in the experiment to avoid the contribution of VOC to $[\text{Ca}^{2+}]_i$ mobilization, the result suggests that VOC may be involved in a small part of the Ca^{2+} entry produced by the addition of extracellular Ca^{2+} .

Effect of TG on Ca^{2+} entry

In order to deplete intracellular Ca^{2+} stores extensively, we treated BAFC with 2 μM TG, which inhibits the

Figure 1 Effect of the extracellular addition of Ca^{2+} on $[\text{Ca}^{2+}]_i$ mobilization in Ca^{2+} -depleted BAFC. (A) The Ca^{2+} -non-depleted cells. (B) The Ca^{2+} -depleted cells. Each trace is representative of five to ten experiments, yielding qualitatively similar results.

Figure 2 Effect of nifedipine on $[\text{Ca}^{2+}]_i$ mobilization induced by 30 mM K^+ and Ca^{2+} store depletion in BAFC. (A) BAFC on a glass coverslip were loaded with fura-2/AM in Krebs-Ringer HEPES buffer (pH 7.4) supplemented with 1.2 mM Ca^{2+} at 37 °C for 1 h. KCl was added 50 s after the beginning of fluorescence determination. (B) BAFC on a glass coverslip were loaded with fura-2/AM in Krebs-Ringer HEPES buffer (pH 7.4) in the absence of Ca^{2+} at 37 °C for 1 h. CaCl_2 was added 50 s after the beginning of fluorescence determination. Each trace is representative of three to five experiments, yielding qualitatively similar results.

Ca^{2+} -ATPase on endoplasmic reticulum (ER) (Thstrup *et al.* 1989). As shown in Fig. 3A, adding 2 μM TG in Ca^{2+} (-) HEPES buffer caused a transient rise in $[\text{Ca}^{2+}]_i$ and thereafter $[\text{Ca}^{2+}]_i$ declined gradually to basal level. The addition of 2.4 mM Ca^{2+} extracellularly after TG treatment caused a significant sustained rise in $[\text{Ca}^{2+}]_i$ (Fig. 3). This effect of Ca^{2+} addition was not inhibited by 10 μM nifedipine (data not shown) but was inhibited markedly by Zn^{2+} and Cd^{2+} (Fig. 3A,B). The effects of Zn^{2+} and Cd^{2+} were concentration-dependent and the effect of Zn^{2+} was more potent than that of Cd^{2+} (Fig. 4). Ni^{2+} showed only a weak inhibitory effect on the $[\text{Ca}^{2+}]_i$ mobilization (data not shown).

The dependence of steroidogenesis on extracellular Ca^{2+}

We previously reported that steroidogenesis was facilitated by the entry of Ca^{2+} from an extracellular pool as well as by cyclic AMP (Yanagibashi *et al.* 1990). We therefore examined the steroidogenic effect of TG treatment in BAFC. Though the addition of 1.2 mM Ca^{2+} stimulated cortisol production only slightly in the absence of TG, the addition of 1.2 mM Ca^{2+} to the TG-treated cells caused marked steroidogenic activity (Fig. 5). The effect of Ca^{2+} in the TG-treated cells was inhibited significantly by 1 mM Zn^{2+} and 1 mM Cd^{2+} , but not by 1 μM nifedipine (Fig. 6). The tested concentration of nifedipine inhibited

Figure 3 Effects of Zn^{2+} and Cd^{2+} on Ca^{2+} entry in TG-treated BAFC. TG (2 μM) was added 50 s after the beginning of fluorescence determination. (A) Effect of 50 μM Zn^{2+} . (B) Effect of 100 μM Cd^{2+} . Each trace is representative of four experiments, yielding qualitatively similar results.

Figure 4 Effects of Zn^{2+} and Cd^{2+} on TG (2 μM)-induced increase in $[\text{Ca}^{2+}]_i$ in BAFC. The increase in $[\text{Ca}^{2+}]_i$ was determined as the distance from the basal level at 600 s, and represented the percentage of the value in the absence of each antagonist. Each column represents the mean $\pm$ s.d. from four separate experiments. *Significantly different from the control value ($P < 0.01$).

high K^{+} -induced steroidogenesis completely in isolated bovine adrenocortical cells (Yanagibashi *et al.* 1990).

Discussion

The present experiments strongly suggest capacitative Ca^{2+} entry occurs in BAFC and that this process participates in the regulation of glucocorticoid production.

So far, a selective antagonist of capacitative Ca^{2+} entry is not yet available. However, Hoth & Penner (1993) reported a Ca^{2+} current associated with store depletion (a Ca^{2+} release-activated Ca^{2+} current; I_{CRAC}) in rat peritoneal mast cells different from other plasma membrane Ca^{2+} entry pathways. They showed that I_{CRAC} was inhibited by divalent cations with a potency order of $\text{Zn}^{2+} > \text{Cd}^{2+} > \text{Ni}^{2+}$. In the Ca^{2+} store-depleted BAFC,

Figure 5 Effects of the extracellular Ca^{2+} and TG (2 μM) on steroidogenesis in BAFC. BAFC were incubated in low K^+ -Krebs–Ringer bicarbonate buffer (pH 7.4) either supplemented with or without 1.2 mM Ca^{2+} in the presence or absence of TG at 37 °C for 1 h. Each column represents the mean $\pm$ S.D. from three separate experiments. *, **Significantly different from steroidogenesis at the Ca^{2+} (-) condition (* P <0.05, ** P <0.01).

Figure 6 Effects of nifedipine, Zn^{2+} and Cd^{2+} on TG (2 μM)-induced steroidogenesis in BAFC. The incubation medium was low K^+ -Krebs–Ringer bicarbonate buffer (pH 7.4) supplemented with 1.2 mM Ca^{2+} . The values represent per cent of 2 μM TG-induced cortisol production (control). Each column represents the mean $\pm$ S.D. from three separate experiments. *Significantly different from the control value (P <0.01).

Zn^{2+} , Cd^{2+} and Ni^{2+} inhibited a significant sustained rise in $[\text{Ca}^{2+}]_i$ induced by the addition of extracellular Ca^{2+} and TG at the same potency order as reported by Hoth & Penner (1993). Our results of the inhibitory effects of these cations on $[\text{Ca}^{2+}]_i$ mobilization are in agreement with

those of Hoth & Penner (1993), and suggest a similar mechanism is involved in BAFC.

Yanagibashi *et al.* (1990) showed that the steroidogenic effect of ACTH in the presence of extracellular Ca^{2+} was completely abolished by dihydropyridines in freshly isolated bovine adrenocortical cells. They proposed the existence of a VOC which was activated by ACTH in BAFC. Our result also suggests the presence of VOC in BAFC. However, we used the low K^+ concentration buffer in the experiments to avoid the contribution of VOC to $[\text{Ca}^{2+}]_i$ mobilization. Therefore, in our experimental conditions, a capacitative Ca^{2+} entry process must have been activated in BAFC.

The TG treatment accelerated cortisol production remarkably in the presence of extracellular Ca^{2+} but did not in its absence. TG-induced steroidogenesis is inhibited by Zn^{2+} and Cd^{2+} , but a dihydropyridine derivative did not attenuate the steroidogenic effect of TG. This effect of TG on steroidogenesis was consistent with the observation of TG-induced Ca^{2+} entry. Steroidogenesis due to the TG-induced Ca^{2+} entry in BAFC was not involved in VOC. As described above, TG activates Ca^{2+} influx from an extracellular pool via capacitative Ca^{2+} entry in BAFC. These results indicate that BAFC contain a capacitative Ca^{2+} entry process which is linked to steroidogenesis. However, the physiological role of the capacitative Ca^{2+} entry in glucocorticoid biosynthesis remains obscure. In BAFC, extracellular ATP stimulates the release of Ca^{2+} from the intracellular Ca^{2+} stores to cause Ca^{2+} influx from an extracellular pool (Matsui 1991) and facilitates steroidogenesis via P2Y receptors (Kawamura *et al.* 1991), which is not inhibited by dihydropyridine (Niitsu 1992). Therefore ATP-induced steroidogenesis might be activated by capacitative Ca^{2+} entry. In bovine adrenal glomerulosa cells, the major part of aldosterone biosynthesis by angiotensin II involves capacitative Ca^{2+} entry initiated by angiotensin II-induced release of Ca^{2+} from intracellular stores (Burnay *et al.* 1994). ATP and angiotensin II both activate phospholipase C β and release IP₃ and diacylglycerol via P2Y and angiotensin II receptors respectively (Bird *et al.* 1989, Bollag *et al.* 1991, Boarder *et al.* 1995).

These results suggest that there are at least two pathways involved in Ca^{2+} entry in BAFC: one is VOC and the other is capacitative Ca^{2+} entry. The mechanism of capacitative Ca^{2+} entry in BAFC still remains to be elucidated.

Acknowledgements

We thank Drs S G Hillier and Ashraf Ahmed for critical reading of this manuscript, Dr Haruhisa Nishi for his statistical analysis of the data, Ms Miyuki Kagata for technical assistance and typing the manuscript, and also Ms Chieko Gido for technical assistance.

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Received 20 December 1999

Revised manuscript received 21 July 2000

Accepted 15 August 2000