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 ([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 [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 [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 [Ca\(^{2+}\)]\(_i\), but an extensive and sustained increase in [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.


Introduction
Adrenocorticotropic 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 C\(_B\) 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 (Matsumi 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 KH₂PO₄, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 25 mM NaHCO₃, 2 mg/ml glucose, and 3 mg/ml bovine serum albumin; pH 7.4) for 1 h at 37 °C under 95% O₂–5% CO₂ 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% horse serum, and antibiotics. The 3-day cultured cells were used for the experiments.

Measurement of intracellular concentration of Ca²⁺

The determination of intracellular concentration of Ca²⁺ ([Ca²⁺]) 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 KH₂PO₄, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 10 mM HEPES, 0.01 mM EGTA and 2 mg/ml glucose; pH 7.4). The intracellular Ca²⁺ stores in BAFC could be diminished during the culture period, because Ham’s F-10 medium contained 0.3 mM Ca²⁺. The Ca²⁺ stores, however, might be refilled during the fura-2 loading process (i.e. Krebs–Ringer HEPES buffer is supplemented by 1.2 mM CaCl₂). Therefore, to diminish the intracellular Ca²⁺ stores in BAFC markedly, Krebs–Ringer HEPES buffer (pH 7.4) which is not supplemented with Ca²⁺ was applied to the fura-2 loading period (the Ca²⁺-depleted cells). Fura-2-loaded BAFC were transferred to a quartz cuvette containing Ca²⁺ (−) HEPES buffer (123.4 mM NaCl, 3 mM KCl, 1.2 mM NaH₂PO₄, 1.2 mM MgSO₄, 0.5 mM EGTA 10 mM HEPES and 2 mg/ml glucose; pH 7.4) and the concentrated CaCl₂ 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. [Ca²⁺], was shown as the ratio of the fluorescence intensity excited at 340 nm to that of 380 nm (F₃₄₀/F₃₈₀).

Measurement of steroidogenesis

Cortisol production by monolayered BAFC on a 24-well type dish (15–20 × 10⁶ 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 NaH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 0.01 mM EGTA, 2 mg/ml glucose and 3 mg/ml bovine serum albumin; pH 7.4) with or without 1.2 mM Ca²⁺ for 1 h at 37 °C in a humidified atmosphere at 5% CO₂ 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 Ca²⁺ store depletion on Ca²⁺ entry

We first examined the effect of the extracellular addition of Ca²⁺ on Ca²⁺ entry in fura-2 loaded BAFC. Fura-2 was loaded in BAFC in the absence (the Ca²⁺-depleted cells) and presence (the Ca²⁺-non-depleted cells) of 1.2 mM CaCl₂ for 1 h at 37 °C. After that [Ca²⁺], in the fura-2 loaded cells on a glass coverslip was determined in Ca²⁺(−) HEPES buffer (pH 7.4). In the Ca²⁺-non-depleted cells, the addition of 2.4 mM Ca²⁺ caused only a slight increase in [Ca²⁺], (Fig. 1A). However, in Ca²⁺-depleted BAFC, the addition of 2.4 mM Ca²⁺ triggered a significant and immediate increase in [Ca²⁺], (Fig. 1B). The effect of added Ca²⁺ was concentration-dependent, and reached a maximum at 1.2 mM Ca²⁺ (data not shown). The results suggest that the depletion of intracellular Ca²⁺ stores elicits a Ca²⁺ flux from the extracellular pool in BAFC.

As shown in Fig. 2A, 30 mM K⁺ induced a rapid increase in [Ca²⁺], and an ensuing sustained rise in [Ca²⁺]. Nifedipine (10 µM), a VOC-selective antagonist, inhibited the sustained phase. However, 10 µM nifedipine inhibited the Ca²⁺ entry induced by 2.4 mM Ca²⁺ only slightly in the Ca²⁺-depleted cells (Fig. 2B). However, because the low K⁺ (3 mM) buffer was used in the experiment to avoid the contribution of VOC to [Ca²⁺], mobilization, the result suggests that VOC may be involved in a small part of the Ca²⁺ entry produced by the addition of extracellular Ca²⁺.

Effect of TG on Ca²⁺ entry

In order to deplete intracellular Ca²⁺ stores extensively, we treated BAFC with 2 µM TG, which inhibits the
Ca\(^{2+}\)-ATPase on endoplasmic reticulum (ER) (Thatrup et al. 1989). As shown in Fig. 3A, adding 2 µM TG in Ca\(^{2+}\) (-) HEPES buffer caused a transient rise in [Ca\(^{2+}\)]\(_i\) and thereafter [Ca\(^{2+}\)]\(_i\) declined gradually to basal level. The addition of 2·4mM Ca\(^{2+}\) extracellularly after TG treatment caused a significant sustained rise in [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 [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
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 Zn\(^{2+}\) > Cd\(^{2+}\) >> Ni\(^{2+}\). In the Ca\(^{2+}\) store-depleted BAFC,
Zn\textsuperscript{2+}, Cd\textsuperscript{2+} and Ni\textsuperscript{2+} inhibited a significant sustained rise in [Ca\textsuperscript{2+}]\textsubscript{i} induced by the addition of extracellular Ca\textsuperscript{2+} and TG at the same potency order as reported by Hoth & Penner (1993). Our results of the inhibitory effects of these cations on [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{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\textsuperscript{+} concentration buffer in the experiments to avoid the contribution of VOC to [Ca\textsuperscript{2+}]\textsubscript{i} mobilization. Therefore, in our experimental conditions, a capacitative Ca\textsuperscript{2+} entry process must have been activated in BAFC.

The TG treatment accelerated cortisol production remarkably in the presence of extracellular Ca\textsuperscript{2+} but did not in its absence. TG-induced steroidogenesis is inhibited by Zn\textsuperscript{2+} and Cd\textsuperscript{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\textsuperscript{2+} entry. Steroidogenesis due to the TG-induced Ca\textsuperscript{2+} entry in BAFC was not involved in VOC. As described above, TG activates Ca\textsuperscript{2+} influx from an extracellular pool via capacitative Ca\textsuperscript{2+} entry in BAFC. These results indicate that BAFC contain a capacitative Ca\textsuperscript{2+} entry process which is linked to steroidogenesis. However, the physiological role of the capacitative Ca\textsuperscript{2+} entry in glucocorticoid biosynthesis remains obscure. In BAFC, extracellular ATP stimulates the release of Ca\textsuperscript{2+} from the intracellular Ca\textsuperscript{2+} stores to cause Ca\textsuperscript{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\textsuperscript{2+} entry. In bovine adrenal glomerulosa cells, the major part of aldosterone biosynthesis by angiotensin II involves capacitative Ca\textsuperscript{2+} entry initiated by angiotensin II-induced release of Ca\textsuperscript{2+} from intracellular stores (Burnay et al. 1994). ATP and angiotensin II both activate phospholipase C/PI, 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\textsuperscript{2+} entry in BAFC: one is VOC and the other is capacitative Ca\textsuperscript{2+} entry. The mechanism of capacitative Ca\textsuperscript{2+} entry in BAFC still remains to be elucidated.

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