Possible Contribution of 2-Aminoethoxydiphenyl-borate-sensitive Ca^{2+} Mobilization to Adrenocorticotropic-induced Glucocorticoid Synthesis in Rat Adrenocortical Cells

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Abstract. Cytoplasmic calcium ([Ca^{2+}]_{i}) provided through voltage-dependent Ca^{2+} channels (VdCC) plays an important role in adrenocorticotropin (ACTH)-induced steroidogenesis in adrenocortical cells. To identify alternative mechanisms for [Ca^{2+}]_{i} supply, we investigated the 2-aminoethoxydiphenyl borate (2APB)-sensitive pathway as one of the possible signaling pathways involved in [Ca^{2+}]_{i} supply for ACTH-induced steroidogenesis. In monolayers of cultured rat adrenal fasciculate and reticularis cells, ACTH at 10^{-11} M stimulated corticosterone synthesis without increasing intracellular cAMP, and corticosterone synthesis was decreased by 10 µM 2APB by 51.8% (6.71 ± 0.97 vs. 3.23 ± 0.05 ng/mL/4 hours; p<0.05). Furthermore, 2APB significantly decreased the 10^{-11} M ACTH-stimulated [Ca^{2+}]_{i}. ACTH increased the intracellular inositol-1,4,5-trisphosphate (IP3) content with a peak at 10^{-11} M ACTH, which illustrates the possibility that ACTH activates IP3/diacylglycerol-dependent protein kinase C signal transduction. However, the difference in ACTH concentrations between that responsible for the IP3 increase and steroidogenesis without elevated cAMP, suggest a hypothesis that IP3 is not required for steroidogenesis, but does involve an unknown messenger, which stimulates the release of Ca^{2+} from the ER or the subsequent store-operated Ca^{2+} entry (SOCE). The pregnenolone concentration in the culture medium was increased by ACTH, which was significantly suppressed by 2APB, showing that the 2APB-sensitive Ca^{2+} supply affects cholesterol transport into the mitochondrial membrane via steroidogenic acute regulatory protein. Therefore, the SOCE may contribute to ACTH-induced steroidogenesis in the mitochondrial region. In conclusion, the [Ca^{2+}]_{i} used for steroidogenesis may be derived from a 2APB-sensitive pathway and via VdCCs, particularly at physiological concentrations of ACTH. We suggest that ACTH receptors activate steroidogenesis via inositol triphosphate, or an unknown downstream messenger, which could be inhibited by 2APB.

Key words: Corticosterone, Adrenocorticotropin, Steroidogenic acute regulatory protein, Steroidogenesis

IN ADRENAL zona fasciculate (AZF) cells, adrenocorticotropic (ACTH) stimulates glucocorticoid synthesis by activating adenylate cyclase signal transduction and increasing intracellular adenosine 3', 5'-cyclic monophosphate (cAMP) [1, 2]. In turn, cAMP inhibits a background K^{+} channel (bTREK-1), which maintains the resting membrane potential of AZF cells, and induces membrane depolarization, and subse-

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Bor qently increases the intracellular calcium concentra-
tion ([Ca^{2+}]_{i}) through voltage-dependent Ca^{2+} channels (VdCC) [3]. [Ca^{2+}]_{i} is essential for steroidogenesis, particularly for the activation of adenylate cyclase and transport of cholesterol into the mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein following activation of cholesterol side-chain cleavage [4, 5].

The possibility that ACTH at physiological concentrations may act on steroidogenesis via pathways other than cAMP has been discussed for a long time, and is based on the fact that picomolar concentrations of ACTH may induce glucocorticoid synthesis without increased intracellular cAMP accumulation.
Recent studies have shown that ACTH can activate or inhibit various intracellular signaling molecules including inositol phosphates [9, 10], mitogen-activated protein kinase (MAPK) and phosphodiesterase [11]. These suggested that two or more signaling pathways regulate steroidogenesis induced by ACTH. For example, it was reported that low and high concentrations of ACTH might activate $[Ca^{2+}]_i$, through a phospholipase A2-dependent process in steroidogenesis in bovine AZF cells[6]. Accordingly, it seems that $[Ca^{2+}]_i$ is an indispensable messenger in ACTH-mediated steroidogenesis.

In this study, we used 2-aminoethoxydiphenyl borate (2APB), which does not act directly on VDCCs, to investigate possible signaling pathways for $[Ca^{2+}]_i$, supply independent of VDCCs for ACTH-induced steroidogenesis at physiological conditions. It was thought that this signal transduction occurred independently of cAMP, by which $[Ca^{2+}]_i$ is mobilized from intracellular stores or through store-operated $Ca^{2+}$ entry (SOCE).

Materials and Methods

**Chemical Reagents**

ACTH 1-39 was purchased from Bachem AG (Bubendorf, Switzerland). 2-aminoethoxydiphenyl borate (2APB), which is thought to inhibit SOCE by inhibition of inositol-1,4,5-trisphosphate (IP3) receptor inhibition, and thapsigargin, an inhibitor of endoplasmic reticulum (ER) $Ca^{2+}$ ATPase, were purchased from Sigma-Aldrich (St. Louis, USA). Fluo-4 acetoxymethyl (AM) solution in DMSO was purchased from Invitrogen (Tokyo, Japan). Other chemicals used in this study were obtained from Sigma-Aldrich and WAKO (Osaka, Japan).

**Preparation of monolayer culture of rat adrenal fasciculate and reticularis (AZFR) cells**

Male Sprague-Dawley rats, weighing 150–250 g, were housed for 1–2 weeks in a temperature-controlled (24°C) room with lights on from 0700 to 1900 h daily, and given free access to standard rat chow and water. The rats were killed by decapitation on the morning that the cells were prepared. Only AZFR cells were used for this study and were separated from the zona glomerulosa by peeling the adrenal gland. After isolation by dispersion in HEPES dissociation buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na$_2$HPO$_4$, 25 mM HEPES, 10 mM glucose and 0.1% BSA, pH 7.3) containing 0.1% collagenase and 10 μg/mL DNase, the cells were plated at 10$^5$ cells/well for corticosterone and cAMP determination in 0.5 mL culture medium (medium 199 containing 10 mM HEPES, 10 mM NaHCO$_3$, 0.1% BSA, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% FCS), and maintained for 3 days in a humidified incubator under 5% CO$_2$/95% air at 37°C. On the day of experiment, the AZFR cells were washed twice with serum-free culture medium and preincubated for 30 min with serum-free culture medium containing vehicle or 10 μM 2APB. The medium was then changed to 0.5 mL of serum-free medium containing each test-substance. To measure corticosterone secretion, the cells were incubated for 4 h and the supernatant was then taken and centrifuged at 400 g for 10 min, and stored at −20°C. To measure the cAMP content, the cells were incubated without phosphodiesterase inhibitors for 15 min. The culture medium was removed by aspiration at the end of incubation and the remaining cells were incubated with 1 mL of 0.01 M acetic acid for 60 min at 4°C to extract intracellular cAMP. The extract was lyophilized and stored at −20°C until acetylation.

**Measurement of corticosterone by radioimmunoassay (RIA)**

Antiserum against corticosterone was raised in a rabbit immunized with corticosterone 3-(O-carboxymethyl)oxime conjugated to bovine serum albumin. The characteristics of the corticosterone RIA using this antibody have been described elsewhere [12]. $^{125}$I-corticosterone was purchased from MP Biomedicals (Solon, United States). The minimum detection limit of the assay was 0.1 ng/assay tube.

**Measurement of cAMP accumulation by RIA**

The cAMP RIA was performed using rabbit antisera against cAMP raised by immunization with cAMP-succinimide conjugated to albumin. The antiserum did not cross-react with cyclic GMP or other nucleotides, and the assay detection limit was 4 fmol/assay tube. Acetylation was performed before the assay according to the method of Steiner [13].

**Measurement of $[Ca^{2+}]_i$ in cultured AZFR cells**

To measure $[Ca^{2+}]_i$, using fluorescence, we prepared a primary culture of 10$^5$ AZFR cells in a 35-mm poly-L lysine coated glass bottomed dish (Matsunami...
co. Osaka, Japan). After culture for 1 day, the cells were washed with warmed HEPES dissociation buffer (37°C, pH 7.3) containing bovine albumin. The cells were then incubated in loading medium, containing 5 μM AM ester forms of fluo-4 [14] in the presence of 0.01% Pluronic F-127, and stirred by sonication for 15 min at 37°C. The loading medium was removed and the cells were washed twice in HEPES dissociation buffer. The change in fluo-4 fluorescence (excitation, 485 nm; emission, 535 nm) following administration of each test reagent was recorded through a fluorescent filter with a spectrometer, which was attached to Biostation IM (Nikon Co. Tokyo, Japan). Test substances were 10^{-11} M ACTH, 1 μM thapsigargin and 10 μM 2APB, which were dissolved in Hanks’ balanced salt solution (140 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 25 mM HEPES, 10 mM glucose and 0.1% BSA, pH 7.3). We confirmed the viability of the tested cells by the additive effects of 50 mM KCl solution, which was sufficient to cause membrane depolarization.

**Measurement of inositol-1,4,5-trisphosphate in cultured AZFR cell by radioreceptor assay**

Cells (3 × 10⁵/well) were placed in 1.0 mL culture medium, as described above. After incubation in medium in the presence or absence of 10^{-11}–10^{-9} M ACTH for 1 min, the cell suspensions were extracted by the addition of 0.2 volumes of ice-cold 20% perchloric acid, and incubated on ice for 20 minutes. The precipitate was removed by centrifugation (2000 × g for 15 min at 4°C). The supernatant was transferred to a test tube and neutralized to pH 7.5 by titrating with 10 M KOH containing 60 mM HEPES buffer. Sedimentary KClO₄ was removed by centrifugation and the supernatant was collected to a test tube for the assay. Aliquots (100 μL) of the samples were used for the ³H-IP₃ radioreceptor assay (PerkinElmer Life Sciences, Inc., Boston, MA) [15]. The minimum detection limit of the assay was 0.12 pmol/assay tube.

**Measurement of intermediate products of steroidogenesis in the medium**

Cells were incubated with 10^{-11} M ACTH with or without 10 μM 2APB for 4 h. Intermediate products of corticosterone synthesis including progesterone, pregnenolone and deoxycorticosterone (11-DOC) in the medium were measured by liquid chromatography-tandem mass spectrometry at ASKA Pharma Medical Co Ltd. (Kawasaki, Japan) [16]. To determine the effects of the SOCE inhibitor on steroidogenic enzymes, we considered the concentration of pregnenolone to indicate P450scc activity, the ratio of progesterone to pregnenolone as 3β-hydroxysteroid dehydrogenase (3β-HSD) activity and the ratio of 11-DOC to progesterone for P450c11.

**Statistical analysis**

All results are expressed as means ± SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Scheffe’s multiple comparison test and two-group t-tests as appropriate. Statistical significant was defined at p<0.05.

**Results**

**Effects of ACTH on corticosterone synthesis and cAMP accumulation**

Fig. 1 shows the effect of ACTH at concentrations ranging from 10^{-14} to 10^{-9} M on corticosterone synthesis and intracellular cAMP accumulation in rat cultured AZFR cells. ACTH dose-dependently increased corticosterone synthesis and intracellular cAMP accumulation. Corticosterone synthesis was increased significantly by ACTH at concentrations exceeding 10^{-11} M (Fig. 1A), whereas the accumulation of cAMP was increased significantly by ACTH at concentrations exceeding 10^{-10} M (Fig. 1B). ACTH at 10^{-11} M stimulated corticosterone synthesis without an increase in intracellular cAMP accumulation.

**Effects of 2APB on unstimulated corticosterone synthesis**

Fig. 2 shows the effect of either 2APB at concentrations from 1 µM to 100 µM on corticosterone synthesis in rat AZFR cells in vitro. 2APB had no effects on basal corticosterone synthesis.

**Effects of 2APB on ACTH-induced corticosterone synthesis and intracellular cAMP accumulation**

We examined the effects of 2APB on corticosterone synthesis and cAMP accumulation. Ten micromolar 2APB inhibited corticosterone synthesis induced by ACTH at 10^{-11}–10^{-9} M significantly (Fig. 3A). 2APB decreased corticosterone synthesis induced by 10^{-11} M ACTH by 51.8% (6.71 ± 0.97 vs. 3.23 ± 0.05 ng/mL/4 h, p<0.05). On the other hand, 10 μM 2APB did not inhibit ACTH-induced cAMP accumulation (Fig. 3B).
completely prevented the thapsigargin-induced \([\text{Ca}^{2+}]_i\) signals, including the initial rise in \([\text{Ca}^{2+}]_i\) and the secondary sustained \([\text{Ca}^{2+}]_i\) (data not shown).

Effects of 2APB on ACTH-induced \([\text{Ca}^{2+}]_i\) change in cultured AZFR cells

It has been reported that a rapid increase in \([\text{Ca}^{2+}]_i\), induced by thapsigargin, which depletes the internal \(\text{Ca}^{2+}\) store, is required to maintain the \(\text{Ca}^{2+}\) influx through SOCE in several cell types \[17\]. We confirmed that thapsigargin caused a biphasic change in \([\text{Ca}^{2+}]_i\), in AZFR cells. Pretreatment with 10 \(\mu\)M 2APB completely prevented the thapsigargin-induced \([\text{Ca}^{2+}]_i\) signals, including the initial rise in \([\text{Ca}^{2+}]_i\) and the secondary sustained \([\text{Ca}^{2+}]_i\). (Data not shown).

Fig. 2. Effect of 2APB on corticosterone secretion. Rat AZF cells (10⁵ cells/well) were incubated with 1–100 \(\mu\)M 2APB for 4 h. Cells incubated with medium alone as the vehicle served as controls. Each column represents the mean of six determinations of corticosterone secretion during the incubation; bars indicate SEM.

Effects of 2APB on thapsigargin-induced \([\text{Ca}^{2+}]_i\), in cultured AZFR cells

Stimulation with 10⁻¹¹ M ACTH caused a bimodal increasing in \([\text{Ca}^{2+}]_i\), in AZFR cells. The first burst occurred within 120 s with the peak of 176.6 ± 9.1% of the basal level at 50 s. The \([\text{Ca}^{2+}]_i\), experienced a secondary increase, which was greater than the unstimulated fluorescence basal level (Fig. 4, line A). The changes in \([\text{Ca}^{2+}]_i\), (both the first and secondary rises) induced by ACTH were mostly abolished by pretreatment with 10 \(\mu\)M 2APB (423.06 ± 49.78 vs. 128.34 ± 36.17, AUC during the 420-s stimulation period, which was calculated as fluorescence relative to the unstimulated basal level as 1.00, \(p<0.05\) AUC) (Fig. 4, line B). Line B, which represents the \([\text{Ca}^{2+}]_i\), signals in cells pretreated with 2APB and stimulated with ACTH, was shifted upward compared with that of the non-pretreated non-stimulated control cells (line C). However, the increase was not significant indicated that unknown effects of 2APB may affect the basal \([\text{Ca}^{2+}]_i\) signals.

Effect of ACTH on intrinsitol-1,4,5-trisphosphate content

Fig. 5 shows the dose-dependent effects of ACTH on intracellular IP3 content in rat AZFR cells \textit{in vitro}.
Fig. 3. Effect of 2APB on ACTH-induced corticosterone secretion and cAMP accumulation.

Rat AZF cells (10⁵ cells/well) were incubated with 10⁻¹⁴–10⁻⁹ M ACTH in the presence or absence of 10 µM 2APB for 4 h for corticosterone secretion (panel A) or for 15 min for cAMP accumulation (panel B). Each column represents the mean of four determinations of corticosterone secretion during the incubation; bars indicate SEM. Each column represents the mean of six determinations of corticosterone secretion during the incubation; bars indicate SEM. *, p<0.05 compared with vehicle alone by one-way ANOVA followed by Scheffe’s multiple comparison test.

Fig. 4. Effect of 2APB on ACTH-stimulated \([\text{Ca}^{2+}]_i\).

The time-course of changes in fluorescence intensity of fluo-4 was measured in single cells. The vertical scale represents the ratio of fluorescence intensity to the average of the 300-s unstimulated basal intensity [F/F₀]. Fluorescence intensity was recorded every 10 s, for 420 s after adding 10⁻¹¹ M ACTH at time zero. Cells were pretreated with 2APB 30 min before adding ACTH. Line A/solid circles, cells incubated with ACTH alone (n=7); Line B/solid squares, cells incubated with 2APB plus ACTH (n=6); Line C/solid triangles, cells incubated with vehicle alone (n=6). Values represent means; bars indicate SEM.

Fig. 5. Effect of ACTH on intracellular inositol-phosphate content.

Rat AZFR cells (3 × 10⁵ cells/well) were incubated with vehicle or 10⁻¹⁴–10⁻¹⁰ M ACTH for 1 min. Each column represents the mean of four determinations of intracellular inositol phosphate content during the incubation; bars indicate SEM. *, p<0.05 compared with vehicle alone by one-way ANOVA followed by Scheffe’s multiple comparison test.
At $10^{-13}$ M, ACTH increased the intracellular IP3 content by 66.5% ($39.43 \pm 3.40$ vs. $26.75 \pm 3.23$ pmol/1 min; $p < 0.05$). At the concentration of $10^{-11}$ M, at which ACTH stimulated corticosterone synthesis without increasing intracellular cAMP accumulation, ACTH did not affect intracellular IP3 content ($24.55 \pm 3.72$ vs. $27.11 \pm 2.42$ pmol/1 min; $p > 0.05$). Meanwhile, at $10^{-9}$ M, at which ACTH stimulated corticosterone synthesis mainly via the cAMP-mediated signal pathway, ACTH did not increase the intracellular IP3 content.

**Effects of 2APB on intermediate products of ACTH-stimulated steroidogenesis in AZFR cells**

ACTH at $10^{-11}$ M significantly increased the concentration of pregnenolone and the ratio of corticosterone to DOC in the culture medium. 2APB significantly decreased the concentration of pregnenolone, by 60.7% ($144.92 \pm 19.64$ vs. $88.00 \pm 13.70$ ng/mL/4 h; $p < 0.05$), which presumably reflects the enzyme activity of P450$_{sc}$ or the rate of transport of cholesterol into the mitochondrial membrane. The ratio of corticosterone to DOC was not changed by 2APB. ACTH had no effect on the ratios of progesterone to pregnenolone or DOC to progesterone, which reflect the activities of 3β-HSD and P450$_{21}$ in ER, respectively. (Table 1 and Fig. 6).

**Discussion**

$[\text{Ca}^{2+}]_{i}$, which is supplied by $\text{Ca}^{2+}$ entry from the extracellular region via several $\text{Ca}^{2+}$ channels located in the plasma membrane or by $\text{Ca}^{2+}$ mobilization from intracellular stores, is involved in various signal transduction systems in many kinds of excitable and nonexcitable cells. $\text{Ca}^{2+}$ channels are principally divided into three kinds, namely, VDCCs, receptor-operated $\text{Ca}^{2+}$ channels and agonist-activated nonselective $\text{Ca}^{2+}$-permeable cation channels [18]. VDCCs are opened by membrane depolarization and increase $[\text{Ca}^{2+}]_{i}$, following the activation of cell-surface receptors in excitable cells. Receptor-operated $\text{Ca}^{2+}$ entry increases $[\text{Ca}^{2+}]_{i}$, by mobilizing stored $\text{Ca}^{2+}$ in the ER or sarcoplasmic reticulum (SR), and is involved signaling of receptors coupled to the activation of inositol 1,4,5-triphosphate (IP3) or ryanodine in excitable or nonexcitable cells [19]. A typical example of agonist-activated nonselective $\text{Ca}^{2+}$-permeable cation channels is the glutamate receptor, which allows excitatory synaptic transmission between neurons in the central nervous system.

It is evident that $[\text{Ca}^{2+}]_{i}$ plays an important role in ACTH-induced steroidogenesis. Here, we investigated whether the 2APB-sensitive mechanism is a possible source of $[\text{Ca}^{2+}]_{i}$, supply, particularly at physiological concentrations of ACTH. 2APB was originally reported to inhibit the IP3 receptor without affecting VDCCs or arachidonic acid-gated $\text{Ca}^{2+}$ channels [20, 21]. In this study, the increase in $[\text{Ca}^{2+}]_{i}$, induced by thapsigargin was inhibited by 2APB, confirming the previously reported pharmacological characteristics of 2APB [22]. In the recent studies, 2APB appeared to show antagonistic effects on $\text{Ca}^{2+}$ entry following $\text{Ca}^{2+}$ release from stores, rather than on $\text{Ca}^{2+}$ release itself [23, 24].

In this study, we demonstrated that ACTH at $10^{-11}$ M stimulates corticosterone synthesis without a corresponding increase in intracellular cAMP accumulation, which is in agreement with previous reports [5, 7, 8]. Adrenal steroidogenesis induced by $10^{-10}$ M ACTH was significantly decreased, by about 50%, by pretreatment with 2APB. Likewise, at ACTH concentrations over $10^{-10}$ M, 2APB decreased corticosterone synthesis without affecting ACTH-induced cAMP accumulation. These results suggest that the blockade of adrenal steroidogenesis by 2APB does not involve changes in adenylate cyclase activity. From these findings, we propose that the $[\text{Ca}^{2+}]_{i}$ required for glucocorticoid synthesis may be derived from a supplier other than VDCCs, and may include a 2APB-sensitive pathway, particularly at physiological concentrations of ACTH (<$10^{-10}$ M) because VDCCs are activated subsequent to an increase in cAMP. At ACTH concentrations exceeding $10^{-10}$ M, 2APB partially inhibited corticosterone secretion, suggesting that $[\text{Ca}^{2+}]_{i}$, derived through the 2APB-sensitive pathway is partly involved in ACTH-stimulated corticosterone synthesis.

The change in $[\text{Ca}^{2+}]_{i}$, caused by ACTH, with or without 2APB, was also examined using fluorescent methods. Omura et al. previously reported that low concentrations of ACTH $(10^{-11}-10^{-12}$ M) increase $[\text{Ca}^{2+}]_{i}$, without a corresponding increase in cAMP accumulation [6]. It seems possible that the bimodal increase in $[\text{Ca}^{2+}]_{i}$, induced by $10^{-11}$ M ACTH might represent, at least in part, the sum of $\text{Ca}^{2+}$ mobilization from the ER and the $\text{Ca}^{2+}$ supply derived from SOCE. Based on the finding that 2APB completely suppressed both phases, it was speculated that the $[\text{Ca}^{2+}]_{i}$, required for ACTH-induced corticosterone synthesis
### Fig. 6. Effect of 2APB on steroidogenic enzyme activity.

For each panel, cells were incubated with 10^{-11} M ACTH in the absence or presence of 10 µM 2APB. Panel A, concentration of pregnenolone to represent P450scc activity. Panel B, ratio of progesterone to pregnenolone to represent 3β-HSD activity. Panel C, ratio of 11-DOC to progesterone to represent P450c21 activity. Panel D, ratio of corticosterone to DOC to represent P450c11 activity. Each column represents the mean of four determinations; bars indicate SEM. *, p<0.05 compared, as indicated, by one-way ANOVA followed by Scheffe’s multiple comparison test.

The figure shows the schema for steroidogenesis. Mit, mitochondria; ER, endoplasmic reticulum; 11-DOC, deoxycorticosterone; P450_{scc}, cholesterol side chain cleavage; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450_{c21}, 21 hydroxylase; P450_{c11}, 11β-hydroxylase.

### Table 1. Intermediate products of corticosterone synthesis in adrenal zona fasciculata cell

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>ACTH 10^{-11} M</th>
<th>ACTH 10^{-11} M+2APB 10 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>pregnenolone (ng/mL)</td>
<td>82.80 ± 10.15</td>
<td>144.92 ± 19.64 *</td>
<td>88.00 ± 13.70 **</td>
</tr>
<tr>
<td>progesterone/pregnenolone</td>
<td>10.07 ± 0.94</td>
<td>12.32 ± 1.03</td>
<td>9.51 ± 0.70</td>
</tr>
<tr>
<td>DOC/progesterone</td>
<td>7.97 ± 0.93</td>
<td>8.37 ± 1.22</td>
<td>7.56 ± 1.14</td>
</tr>
<tr>
<td>corticosterone/DOC</td>
<td>0.121 ± 0.003</td>
<td>0.408 ± 0.082 *</td>
<td>0.473 ± 0.061</td>
</tr>
</tbody>
</table>

DOC; deoxycorticosterone
All results are expressed as mean ± SEM of four separate experiments. Statistical significance was defined at p<0.05
* p<0.05 vs. vehicle
** p<0.05 vs. ACTH alone
at low concentrations of ACTH is partly derived from [Ca$^{2+}$], supply either from the intracellular Ca$^{2+}$ store or through SOCE, which does not conflict with the other results in monolayer culture.

On the basis that 2APB is thought to have direct inhibitory effects on either Ca$^{2+}$ release from storage or through SOCE, we tried to investigate whether the 2APB-sensitive [Ca$^{2+}$], supply is involved in Ca$^{2+}$ release from storage. The physiological mediators that regulate the release of Ca$^{2+}$ from the ER or SR are IP3 and ryanodine [20]. However, there is little evidence that IP3 participates in [Ca$^{2+}$], for ACTH-induced steroidogenesis. In rat adrenal cells, one report described the activation of IP3 by ACTH, that induced steroidogenesis. In rat adrenal cells, one report described the activation of IP3 by ACTH, that induced steroidogenesis. In rat adrenal cells, one report described the activation of IP3 by ACTH, that induced steroidogenesis. This difference prompts the hypothesis that ACTH-
inhibited by 2APB, was observed at 10$^{-11}$ M ACTH. This difference prompts the hypothesis that ACTH-stimulated IP3 levels may not be required for steroidogenesis, and that Ca$^{2+}$ release from ER or the SOCE is regulated by an unknown messenger [9].

To identify the step(s) at which 2APB acts on ACTH-induced steroidogenesis, we measured the concentration and ratio of intermediate products of corticosterone synthesis. Yamazaki et al. have reported that ACTH exceeding 10$^{-11}$ M could stimulate pregnenolone synthesis in bovine AZF cells [26]. Nishikawa et al. reported that ACTH at concentrations as low as 10$^{-11}$ M significantly elevated StAR protein synthesis [27]. Our data revealed that 2APB inhibited ACTH-induced pregnenolone production, which represents combined activity of StAR and P450$_{sec}$. Because the rate-limiting step in steroidogenesis is StAR activity rather than P450$_{sec}$ activity, pregnenolone synthesis is thought to represent the activity of StAR rather than P450$_{sec}$. On the other hand, 2APB did not inhibit the activities of 3β-HSD or P450$_{c11}$, which are located in the cytoplasm. These results suggest that 2APB-sensitive [Ca$^{2+}$], supply might contribute to ACTH-induced glucocorticoid synthesis in the mitochondrial region.

In conclusion, the increase in [Ca$^{2+}$], through the 2APB-sensitive pathway may play an important role in modulating ACTH-induced glucocorticoid synthesis, particularly at physiological concentrations of ACTH, in the mitochondria of AZFR cells. Because 2APB acts on multiple steps in [Ca$^{2+}$], supply, further studies are needed to determine the precise source of the [Ca$^{2+}$], that is actually involved in steroidogenesis by cooperation with a cAMP-mediated signal transduction system.

### References

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