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Cytoskeletons do not Involve the Synergistic Effect of UTP on ACTH-induced Cyclic AMP Productions in Bovine Adrenocortical Cells

Masahiro KAWAMURA, Miyuki KAGATA, Chikara OHTSUBO, and Takashi UDAGAWA

Department of Pharmacology (I), The Jikei University School of Medicine

ABSTRACT

We examined the participation of actin microfilaments and microtubules in the potentiation effect of uridine 5′-triphospate (UTP) on adrenocorticotropic hormone (ACTH)-induced cyclic AMP (cAMP) production by using the cytoskeletons disrupting agents in bovine adrenocortical fasciculata cells (BAFCs). The 3-day primary cultured BAFCs were used for the experiment. Total cAMP concentration was determined by radioimmunoassay. As the results ; 1) Neither cytochalasin D, an inhibitor of actin polymerization, nor latrunculin B, which disrupts actin microfilaments affected on the effect of UTP on cAMP production by ACTH. 2) ML-9, a myosin-light chain kinase inhibitor, slightly inhibited the effect of UTP on cAMP production, but the difference was not significant. 3) Colchicine, which disrupts microtubules, did not inhibit the effect of UTP. These results suggest that at least, actin-network and microtubules may not involved in the potentiating effect of UTP on the ACTH-induced cAMP production.

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Key words : adrenal cortex, ACTH, cyclic AMP, UTP, cytoskeleton

INTRODUCTION

There are at least 2 pathways to trigger glucocorticoid production (steriodogenesis) in bovine adrenocortical fasciculata cells (BAFCs). One is the cyclic AMP (cAMP)-mediated cascade and the other is the Ca^{2+}-mediated cascade. Adrenocorticotropic hormone (ACTH) produces cAMP via the activation of adenylyl cyclase (AC) and results in stimulation of steroidogenesis in the presence of the extracellular Ca^{2+} [1,2]. On the other hand, extracellular adenosine 5′- phosphate (ATP) or uridine 5′-phosphate (UTP) enhances steroidogenesis by stimulation of Ca^{2+} influx from the extracellular pool via $P2Y_2$ receptors[3,4].

Recently, it has been mentioned that the importance of cross-talk on the cellular function between 2 biological active substances in various cell types. In the case of nucleotides, extracellular ATP potentiates the insulin secretion by acetylcholine in perfused rat pancreas *in vitro*[5], and the nucleotide also enhances the activity of parathyroid hormone in response to intracellular Ca^{2+} in rat osteoblastic cells[6]. We also reported that the extracellularly added ATP exhibited the synergistic effect on ACTH-induced steroidogenesis and cAMP production[7]. And we suggested that coupling process between ACTH receptor-linked Gs protein and AC might be affected by extracellularly added ATP [7]. After that Nishi et al. proposed that ATP, not UTP, itself stimulates cAMP production via novel type of P2Y in BAFCs[8]. Although ATP has considerably lower efficacy than ACTH on cAMP production to avoid the influence of

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川村　将弘，利田　美幸，大坪　主税，宇田川　崇
Mailing address : Masahiro KAWAMURA, Department of Pharmacology (I), The Jikei University School of Medicine, 3-25-8, Nishi-shimbashi, Minato-ku, Tokyo 105-8461, Japan.

ATP on AC, we examined whether UTP potentiates the ACH-elicited cAMP synthesis or not in BAFCs.

The cytoskeltons, actin microfilaments, intermediate tubules and microtubules, play an important role in the regulation of many cellular functions via the intracellular signaling pathways[9]. In AC activation, the involvement of actin cytoskeleton was reviewed[10]. From preliminary study, we found that extracellular UTP also potentiate ACTH-induced cAMP production in BAFCs. Therefore, in the present study, we examine whether the potentiating effect of UTP on ACTH-induced cAMP production is influenced by cytoskeltons, using cytochalsin D[11,12] and latrunculin B[13], actin microfilament disrupting agents.

Materials and Methods

1. Primary culture of BAFCs

BAFCs were isolated aseptically using 0.1% collagenase and 0.005% deoxyribonuclease I as previously described[7]. 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 in a 24-well type plate ($10\text{-}15\times10^5$ cells/well) at 37°C under 5% CO_2 in the air as a gas phase (CO_2 incubator)[7]. The 3-day primary cultured monolayer cells were used for the experiments.

2. Determination of cAMP production

Total cAMP, which was induced by ACTH, was determined as previously described[7]. In brief, the cultured monolayer cells were incubated in the Krebs-Ringer bicarbonate buffer (pH 7.4 ; 125 mM NaCl, 5.9 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 25.3 mM $NaHCO_3$, 2 mg/ml glucose and 3 mg/ml bovine serum albumin) in the presence of 3-isobutyl-methyl-xanthine and ACTH with on without UTP in a total incubation medium of 0.5 ml for 15 min in CO_2 incubator. The reagents, which affect the cytoskeletal structures, were pretreated for 30 min (cytochalasin D and ML-9), 1 hr (latrunculin B) and 2 hr (colchicine). Adding ice-cold ethanol terminated the reactions and the extracted cyclic AMP was determined by a commercially available radioimmunoassay kit.

3. Statistics

The statistical analysis of data was carried out by using Student's t-test and analysis of variance. The software we used was Stat View 5.0 (SAS Institute Inc. Cary, NC). The value of $P<0.01$ was considered to be statistically significant.

4. Materials

The materials were purchased from the following companies : cytochalasin D, colchicine, 3-isobutyl-methyl-xanthine, Ham's F-10 medium and deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO, USA) ; collagenase (Funakoshi Co., Tokyo) ; latrunculin B (Biomol Research Laboratories Inc., Plymouth, PA, USA) ; ML-9 (Wako Pure Chemical Industries, Ltd, Osaka) ; UTP and cyclic AMP assay kit (Yamasa Co., Chiba). All the other chemicals were of reagent grade.

Results

1. Effect of UTP on ACTH-induced cAMP production in BAFCs

As shown in Fig. 1, extracellular UTP showed a potentiating effect on cAMP production by 100 pM ACTH concentration-dependent manner. The maxi-

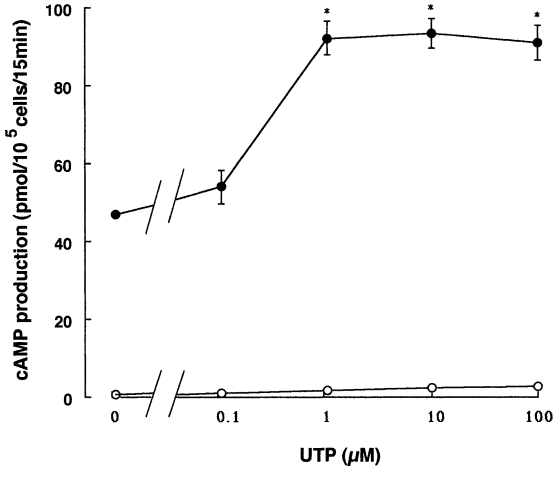

Fig. 1 Effect of UTP on ACTH-induced cAMP production in BAFCs. BAFCs were incubated with UTP (0～100 μM) in the presence (-●-) or absence (-○-) of ACTH (100 pM) as described in Materials and Methods. Each value represents the mean+S.E. of triplicate determinations. *$P<0.01$

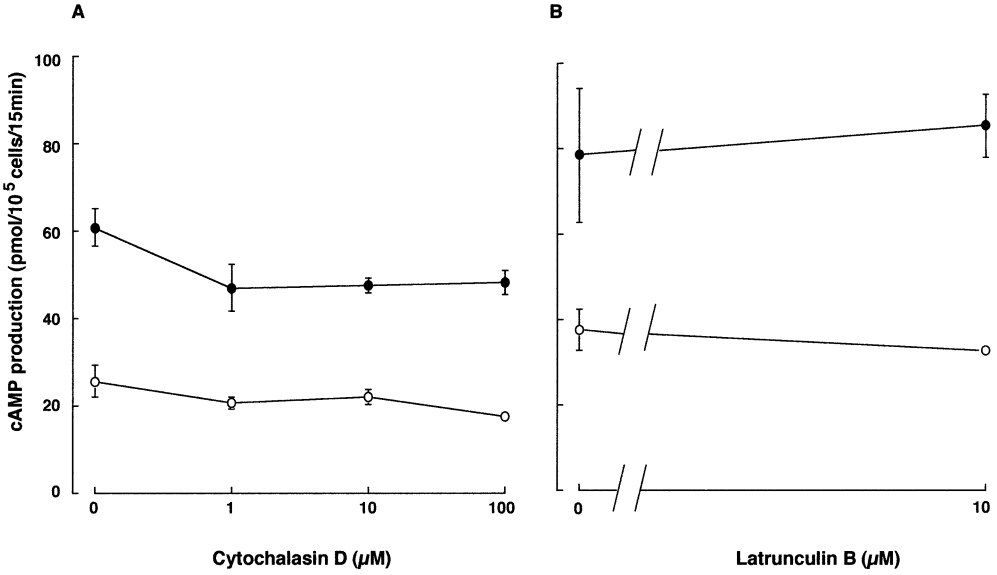

Fig. 2 Effect of actin microfilament disrupting agents on the potentiating effect of UTP on ACTH-induced cAMP production in BAFCs. BAFCs were pretreated with (A) cytochalasin D (0～100 μM) for 30 min or (B) latrunculin B (0～10 μM) for 1 hr followed by the 15 min incubation with 100 pM ACTH in the presence (-●-) or absence (-○-) of UTP (10 μM) as described in Materials and Methods. Each value represents the mean+S.E. of triplicate determinations.

mum effect of UTP was observed between 1 and 100 μM. And UTP itself did not enhance intracellular cAMP concentration. From the results, we used 10 μM UTP in the following experiments.

2. Effects of cytochalasin D and latrunculin B on ACTH-induced cAMP production in the absence or presence of extracellular UTP in BAFCs

As shown in Fig. 2 (A) and (B), 100 pM ACTH stimulated cAMP biosynthesis and the addition of 10 μM UTP potentiated the effect of ACTH. And the pretreatment of cytochalasin D (1～100 μM) showed a tendency to inhibit the potentiating effect of UTP. However, the effect of cytochalacin D was not significant by different from the control value (A). We previously reported that the pretreatment of 25 μM cytochalasin D for 30 min disrupt the actin filament in BAFCs[12]. The pretreatment of 10 μM latrunculin B did not show any inhibitory effect on the effect of UTP on ACTH-induced cAMP biosynthesis (B). Although we did not study the effect of latrunculin B on the actin filaments in BAFCs, it was reported that treatment of 1 μM latrunculin B for 10-30 min perturbed actin cytoskeleton in Dictyostelium[14].

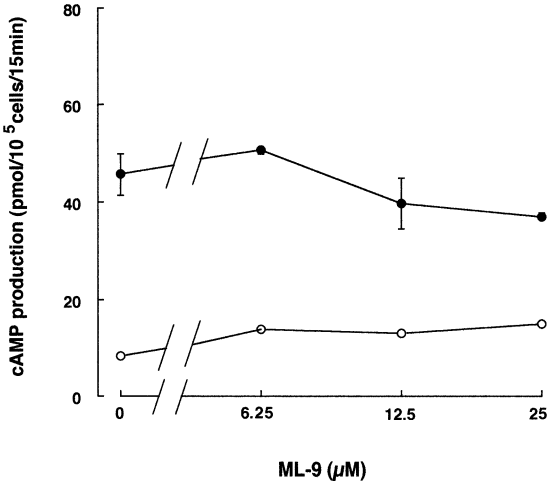

Fig. 3 Effect of ML-9 on the potentiating effect of UTP on ACTH-induced cAMP production in BAFCs. BAFCs were pretreated with ML-9 (0～25 μM) for 30 min followed by the 15 min incubation with 100 pM ACTH in the presence (-●-) or absence (-○-) of UTP (10 μM) as described in Materials and Methods. Each value represents the mean+S.E. of triplicate determinations.

3. Effect of ML-9 on ACTH-induced cAMP production in the absence or presence of UTP in BAFCs

We examined the effect of ML-9, a MLCK inhibitor[15], on the cAMP production by ACTH in the

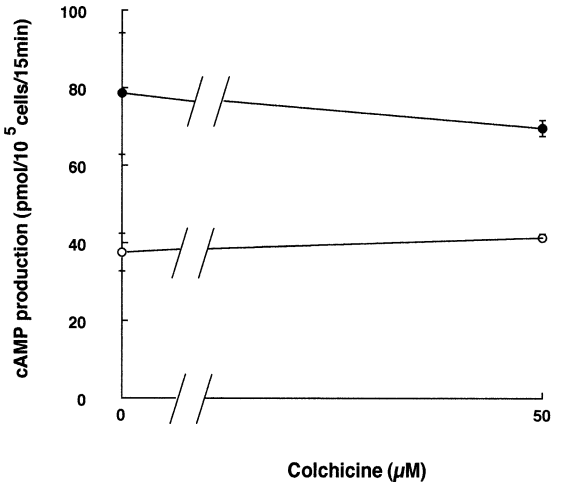

Fig. 4 Effect of colchicine on the potentiating effect of UTP on ACTH-induced cAMP production in BAFCs. BAFCs were pretreated with colchichine (0～50 μM) for 2 hr followed by the 15 min incubation with 100 pM ACTH in the presence (-●-) or absence (-○-) of UTP (10 μM) as described in Materials and Methods. Each value represents the mean+S.E. of triplicate determinations.

absence or presence of UTP.

As shown in Fig. 3, 10 μM UTP potentiated the 100 pM ACTH-elicited cAMP biosynthesis, and the pretreatment of ML-9 (6.25～25 μM) did not inhibit the potentiating effect of UTP.

4. Effect of colchicine on ACTH-induced cAMP production in the absence or presence of UTP in BAFCs

As shown Fig. 4, 50 μM colchicine pretreatment did not affect on ACTH-elicited cAMP biosynthesis both in the absence and presence of 10 μM UTP.

DISCUSSION

We reported that the extracellular ATP synergistically accelerated ACTH-induced cAMP production in BAFCs[7]. Extracellular ATP and UTP induce steroidogenesis in BAFCs by stimulating Ca^{2+} influx via $P2Y_2$ receptors[3,4]. Furthermore, ATP, not UTP, also elicits cAMP production via novel P2Y receptors (the non-cloning receptors) in BAFCs[4,8]. Therefore in this study, we used UTP instead of ATP to remove the action of nucleotides on AC. In our experimental condition, UTP (over 0.1 μM) potentiated ACTH-induced cAMP production (Fig. 1). UTP activates Gq protein linking $P2Y_2$ receptors and forms inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol from phosphatidylinositol 4, 5-bisphosphate by the activation of phospholipase Cβ via Gqα subunits[16]. IP_3 acts on IP_3 receptors in endoplasmic reticulum followed by release Ca^{2+} from the luminal space and results to induced Ca^{2+} influx from the extracellular pool by store-operated Ca^{2+} entry[17] to regulate the functions in nonexcitable cells.

On the other hand, it is not clear that how do the Gq$\beta\gamma$ subunits play roles in the regulation of cellular functions. We previously suggested that ATP-$P2Y_2$ receptor complex affects on the ACTH receptor-AC interaction to potentiate the AC activity in BAFCs[7]. At present, it is reported that there are 9 subtypes in AC (type I～IX)[18]. Tang and Gilman suggested type-specific regulation of AC by G$\beta\alpha$ subunits[19]. And Taussig et al. indicated that activity of Gsα-linked type II AC was potentiated by G$\beta\gamma$ subunits[20]. In preliminary study, we found that BAFCs expressed type II AC (data not shown). From above we hypothesize the possible involvement of Gq$\beta\gamma$ subunits in the potentiating effect of UTP on ACTH-induced cAMP production.

As stated above, it was reported that the cortical actin filament might participate in the interaction between G$\beta\gamma$ subunits and AC[9]. Therefore effects of actin filament disturbing agents on the effect of UTP was examined in BAFCs. The pretreatment of cytochalasin D and latrunculin B did not inhibit the effect of UTP. Myosin-light chain kinase (MLCK) plays an obligatory role in the actin polymerization[21]. We previously reported that the treatment of less than 50 μM ML-9, MLCK inhibitor, for 10-30 min disrupt the actin cytoskeleton in BAFCs[12]. ML-9 also did not attenuate the effect of UTP on ACTH-induced cAMP production. One of the cytoskeltal components is microtubule. We examined the effect of colchicine, which disrupts microtubules[22], on the cAMP production by ACTH in the absence or presence of UTP. The results indicated that microtubules also were not involved in the UTP action. These results suggest that actin filaments do not participate in the development of synergistic accelerative effect of UTP on the

ACTH-induced cAMP production, and Gq$\beta\gamma$ subunits could not influence the UTP action. However, these are conjectures obtained by the indirect experimental results by use of cytoskeletal disrupting agents. Thus, the further studies are necessary to have the above evidence by use of the direct methods, i.e. using RNAi of Gq$\beta\gamma$ subunits.

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