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ORIGINAL PAPER

Inhibition of ATP-induced Ca^{2+} Influx by Corticosterone in Dorsal Root Ganglion Neurons

Xiaohong Liu · Junwei Zeng · Yandong Zhao · Zhi Xiao · Chuanqing Fang · Huaizhen Ruan

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Abstract In addition to the classic genomic effects, it is well known that glucocorticoids also have rapid, nongenomic effects on neurons. In the present study, the effect of corticosterone (CORT) on ATP-induced Ca^{2+} mobilization in cultured dorsal root ganglion (DRG) neurons were detected with confocal laser scanning microscopy using fluo-4/AM as a calcium fluorescent indicator that could monitor real-time alterations of intracellular calcium concentration ($[Ca^{2+}]i$). ATP, an algesic agent, caused $[Ca^{2+}]i$ increase in DRG neurons by activation of P2X receptor. Pretreatment with CORT (1 nM–1 μM for 5 min) inhibited ATP-induced $[Ca^{2+}]i$ increase in DRG neurons. The rapid inhibition of ATP-induced Ca^{2+} response by CORT was concentration-dependent, reversible and could be blocked by glucocorticoid receptor antagonist RU38486 (10 μM). Furthermore, the inhibitory effect of CORT was abolished by protein kinase A inhibitor H89 (10 μM), but was not influenced by protein kinase C inhibitor Chelerythrine chloride (10 μM). On the other hand, membrane-impermeable bovine serum albumin-conjugated corticosterone had no effect on ATP-induced $[Ca^{2+}]i$ transients. These observations suggest that a nongenomic pathways may be involved in the effect of CORT on ATP-induced $[Ca^{2+}]i$ transients in cultured DRG neurons.

Keywords Corticosterone · ATP · P2X Receptor · Dorsal root ganglion neurons

Introduction

It has been recognized that glucocorticoids (GC) play an important role in the developmental organization and ongoing activities of the nervous system [1]. Classically, GC are thought to induce genomic effects through genomic mechanism where the complex of GC and their receptors acts as nuclear transcription factor and leads to new protein synthesis. However, some recent studies provide evidences that GC may also act on specific membrane receptors to generate multiple rapid effects on various tissues and cells [2]. For example, Liu et al. [3] found that corticosterone (CORT, a natural glucocorticoid hormone) instantaneously inhibited NMDA receptor current in cultured hippocampal neurons by using patch-clamp techniques. In addition, dexamethasone, a synthetic glucocorticoid, rapidly enhanced ATP-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) increase in cochlear spiral ganglion neurons [4]. These early actions are not dependent upon the gene expression, which suggest that nongenomic mechanism may be participated in these events.

A number of studies have shown that GC regulate neuropeptide synthesis and may modulate neurogenic inflammation and sensory perception in DRG neurons through genomical pathway [5–8]. In addition, nongenomic actions have been reported regarding the role of GC. For

X. Liu and J. Zeng have contributed equally to this study.

X. Liu · J. Zeng · Y. Zhao · Z. Xiao · H. Ruan (✉)
Department of Neurobiology, College of Medicine, Third Military Medical University, 30 Gaotanyan Street, 400038 Shapingba District, Chongqing, People's Republic of China
e-mail: hzruan61@yahoo.com; hzruan79@sohu.com

X. Liu · J. Zeng · Z. Xiao
Department of Physiology, Zunyi Medical College, 563003 Zunyi, People's Republic of China

C. Fang (✉)
Department of Neurology, Daping Hospital, Third Military Medical University, 400038 Chongqing, People's Republic of China
e-mail: chongqingfcq@163.com

example, He et al. [9] found a rapid inhibitory effect of CORT on high K^+-induced $[Ca^{2+}]i$ elevation and calcium channel current in DRG neurons. ATP, an algesic agent, is implicated in peripheral sensory transduction of noxious stimuli by activating P2X receptors, a superfamily of ligand-gated, non-selective cation channels [10]. Reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry demonstrate that P2 receptors, including $P2X_{1-6}$, $P2Y_1$ and $P2Y_4$, are expressed in DRG neurons [11–14]. ATP-induced $[Ca^{2+}]i$ transients in DRG neurons have been used to model the response of nociceptors to painful stimuli [15]. Our previous study indicates that CORT rapidly inhibits ATP-induced currents through activating PKA in DRG neurons [16]. However, whether GC can modulate ATP-induced $[Ca^{2+}]i$ transients in DRG neurons remains unclear. The aim of this study is to explore whether an nongenomic mechanism and some downstream molecules are involved in the effect of CORT on ATP-induced $[Ca^{2+}]i$ increase in cultured DRG neurons by using single-cell Ca^{2+} fluorescence recording technique.

Experimental Procedure

Cell Culture

DRG neurons were obtained from 2 to 3-week-old Sprague-Dawley rats. All experiments conformed to local and international guidelines on ethical use of animals and all efforts were made to minimize the number of animals used and their suffering. The dissected lumbar DRG were desheathed, cut and incubated in 4 ml Dulbecco's modified Eagle's medium (DMEM, GIBCO, Rockville, MD, USA) containing 0.2% collagenase (Class I, Sigma, St Louis, MO, USA) for 60 min at 37°C. The tissue was then incubated in 4 ml Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 0.1% trypsin (Sigma) at 37°C for 15 min. The solution was replaced with 1 ml growth medium comprising of DMEM supplemented with 10% bovine serum, 50 ng/ml nerve growth factor, 200 IU/ml penicillin and 200 IU/ml streptomycin. The ganglia were then dissociated into single neurons by gentle trituration. DRG cells were plated onto sterile glass coverslips pre-coated with 10 μg/ml poly-d-lysine (Sigma) followed by 10 μg/ml Laminin-I (Sigma) and maintained in a 95% air and 5% CO_2 humidified incubator at 37°C, and used within 48 h.

$[Ca^{2+}]i$ Measurement

$[Ca^{2+}]i$ measurement was carried out according to the methods previously described [17]. Changes of $[Ca^{2+}]i$ in DRG neurons were detected with confocal laser scanning microscopy using fluo-4/AM (Dojindo, Japan) as a calcium fluorescent indicator that could monitor real-time alterations of $[Ca^{2+}]i$. All fluorescence measurements were made from subconfluent areas of the dishes so that individual DRG neurons could be readily identified. Prior to recording, DRG neurons were loaded with the Ca^{2+}-sensitive fluorescent dye fluo-4/AM (2 μM) for 30 min at 37°C in the incubator at 37°C in a humidified 5%CO_2–95% air atmosphere. Subsequently, the coverslips were thoroughly rinsed with Hanks' balanced salt solution (HBSS, GIBCO) lacking fluo-4/AM to remove extracellular traces of the dye and to complete de-esterification. At last, the coverslips were mounted cell-side up in the free bottom of the chamber, placed on the stage of the confocal microscope (Leica TCS-SP5, Germany). The dye in the selected cytoplasmic part of cells was excited by wavelength at 494 nm and fluorescence images were captured at 516 nm with an intensified charge coupled device camera controlled by a computer. All fluorescence measurements were made at room temperature (20–22°C). Image data were analyzed off-line. The change in $[Ca^{2+}]i$ was represented by relative fluorescence intensity [F1/F0 %] (F0, control; F1, administration of drugs).

Drug Application

ATP (Sigma), 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP, Sigma), CORT (Sigma), CORT-BSA (Sigma), chelerythrine (CHE, Sigma) and H-89 (Sigma) were made with 0.01 M PBS. RU38486 (Sigma) were dissolved in ethanol. Fluo-4/AM was dissolved in dimethyl sulfoxide (DMSO). Ca^{2+}-free solution was made up of 0.2 mM calcium with the addition of 1 mM EGTA. All drugs were added directly to bath solutions. The presence of dimethyl sulfoxide (<0.1%) alone did not affect the Ca^{2+} fluorescence intensity.

Statistical Analysis

All data was presented as mean ± SD. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by LSD post hoc test when multiple comparisons were made (SPSS13.0, USA). IC50 were obtained by using GraphPad Prism 4 Software (USA). Differences at the $P < 0.05$ level were considered statistically significant.

Results

The Effect of CORT on ATP-Induced $[Ca^{2+}]i$ Increase in Rat DRG Neurons

We observed the effect of 100 μM ATP on small- to medium-size neuronal $[Ca^{2+}]i$ by using confocal laser scanning microscopy. 100 μM ATP caused $[Ca^{2+}]i$

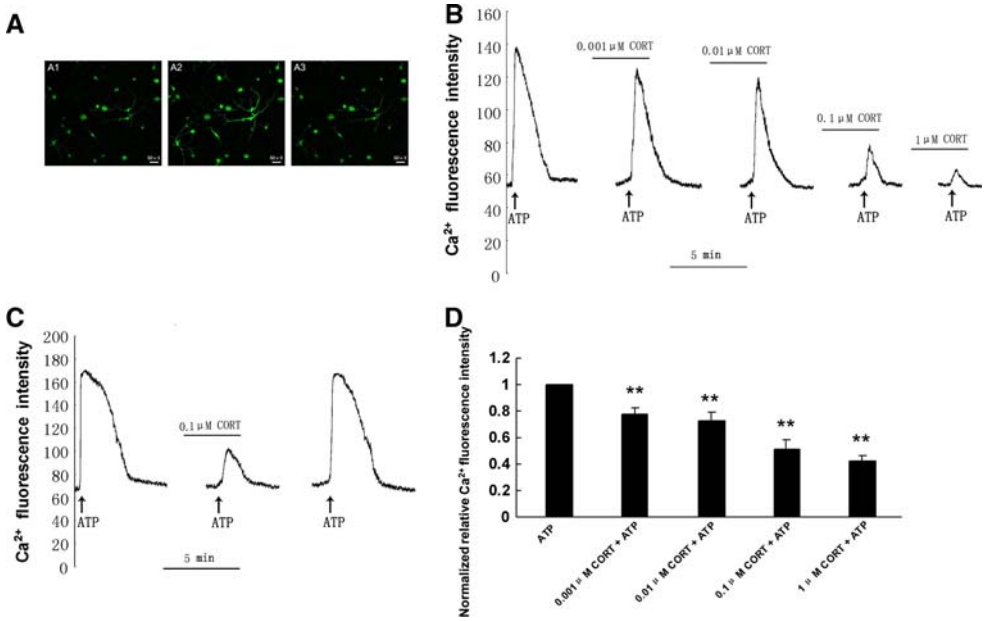

Fig. 1 Concentration-dependent inhibitory effect of CORT on ATP-induced Ca^{2+} response in DRG neurons. **a** Fluorescence images of $[Ca^{2+}]i$ change in cultured DRG neurons. (*A1*) Control: Ca^{2+} fluorescence image of neurons; (*A2*) The effect of 100 μM ATP on neurons; (*A3*) The effect of 0.1 μM CORT on ATP-induced $[Ca^{2+}]i$ increase in neurons. **b** The trace is from a single DRG neuron and shows features of ATP-induced $[Ca^{2+}]i$ increase, with a rapid rise after 100 μM ATP application (*arrow*) followed by a slower recovery. CORT (0.001–1 μM) added 5 min prior to ATP (*arrow*) gradually decreased ATP-induced $[Ca^{2+}]i$ increase. **c** The trace is from another single DRG neurons and shows that 100 μM ATP-induced Ca^{2+} response (*arrow*) was reversibly attenuated by extracellular application of 0.1 μM CORT. Time indicated by bars. **d** CORT (0.001–1 μM) inhibited 100 μM ATP-stimulatory action on $[Ca^{2+}]i$ in DRG neurons with a dose-dependent manner. (** $P < 0.01$ compared with 100 μM ATP, mean ± SD, $n = 10$ cells)

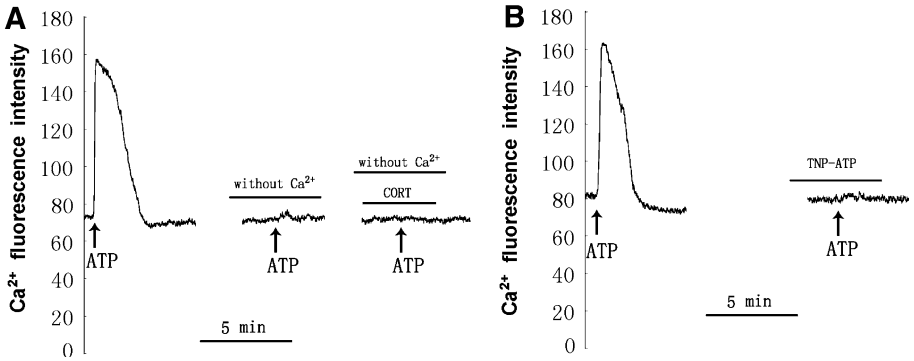

Fig. 2 The influence of Ca^{2+}-free solution and TNP-ATP on ATP-induced Ca^{2+} responses in DRG neurons. **a** The trace is from a single neuron and shows that 100 μM ATP increased $[Ca^{2+}]i$ in DRG neurons. Ca^{2+}-free solution eliminated $[Ca^{2+}]i$ increase response to ATP. CORT treatment could not influence the effect of Ca^{2+}-free solution on ATP-induced Ca^{2+} responses. **b** Pretreatment with TNP-ATP (10 μM for 10 min) completely blocked the ATP-induced Ca^{2+} response

increase in 61% of tested DRG neurons (Fig. 1a, b). Repeated ATP application could produce equal Ca^{2+} responses after a 10 min washout with HBSS.

In order to identify whether CORT regulate ATP-evoked Ca^{2+} responses, DRG neurons were perfused with CORT for 5 min prior to ATP application. CORT (1 μM) by itself had no effect on basal Ca^{2+} fluorescence intensity in neurons. As shown in Fig. 1b, CORT (1 nM–1 μM) inhibited ATP-induced Ca^{2+} responses with a dose-dependent manner. CORT inhibited ATP-induced $[Ca^{2+}]i$ increase to 77.83 ± 4.75 %, 72.5 ± 6.8%, 51.33 ± 6.86%, 42.17 ± 3.92% at 1, 10, 100 nM and 1 μM, respectively (Fig. 1d). The IC50 of the CORT effect was 42.1 ± 4.6 nM. Upon wash out of CORT for 10 min, ATP-evoked

Ca^{2+} responses mostly recovered (Fig. 1c). In addition, chelating extracellular Ca^{2+} with EGTA (NP-ethyleneglycoltetraacetic acid) eliminated ATP-induced $[Ca^{2+}]i$ responses in both control conditions and CORT treatment (Fig. 2a). Pretreatment with P2X receptor antagonist TNP-ATP (10 μM for 10 min) completely blocked the ATP-induced $[Ca^{2+}]i$ increase (Fig. 2b).

Involvement of Glucocorticoid Receptor and Protein Kinase A in CORT-Related Inhibition

To ascertain the role of glucocorticoid receptor (GR) in the effects of CORT on ATP-induced $[Ca^{2+}]i$ elevation, RU38486, a GR antagonist, was applied. 10 μM RU38486 alone had no effect on Ca^{2+} fluorescence intensity in cultured DRG neurons. Calcium-imaging data demonstrated that RU38486 (10 μM for 20 min) significantly abolished the inhibitory effect of 0.1 μM CORT on 100 μM ATP-induced $[Ca^{2+}]i$ elevation (Figs. 3a, 4), which suggests that the inhibitory effect is mediated by GR.

It has been suggested that protein kinases A (PKA) or protein kinases C (PKC) may be activated by GC in its rapid nongenomic effects in various neuronal tissues [3, 9, 18]. We checked this hypothesis by applying PKA inhibitor H89 and PKC inhibitor CHE, respectively. Addition of 10 μM H89 and 10 μM CHE alone failed to affect ATP-induced $[Ca^{2+}]i$ elevation. In the presence of H89 (10 μM for 20 min), the inhibitory effect of 0.1 μM CORT on ATP-induced $[Ca^{2+}]i$ elevation was abolished (Figs. 3b, 4). In contrast, the inhibition of CORT was not influenced after CHE (10 μM for 20 min) administration (Figs. 3c, 4). This result suggests that CORT inhibits ATP-induced $[Ca^{2+}]i$ increase by suppressing the activity of PKA.

Effect of CORT-BSA on ATP-Induced $[Ca^{2+}]i$ Increase in Rat DRG Neurons

Extracellular application of 100 nM CORT significantly reduced ATP-induced $[Ca^{2+}]i$ elevation. Then, to examine whether CORT acts on membrane binding sites, we tested the effects of bovine serum albumin-conjugated corticosterone(CORT-BSA), which is impermeable to cell membrane. Extracellular application of a membrane-impermeable CORT-BSA (0.1 μM for 5 min) did not attenuate the Ca^{2+} response to ATP, which means that CORT-BSA could not mimic the effect of CORT in DRG neurons (Figs. 3d, 4).

Discussion

ATP exerts its effects via two general classes of purinergic receptors, P2X and P2Y. P2X is an iontotropic receptor that allows the passage of cations including Na^{+} and Ca^{2+}. P2X receptor activation causes the influx of extracellular Ca^{2+} [10]. On the other hand, stimulation of P2Y metabotropic receptor lead to Ca^{2+} release from intracellular Ca^{2+} stores and the following $[Ca^{2+}]i$ increase, which are not affected by the removal of extracellular Ca^{2+} [19]. In the present experiments, chelating extracellular Ca^{2+}

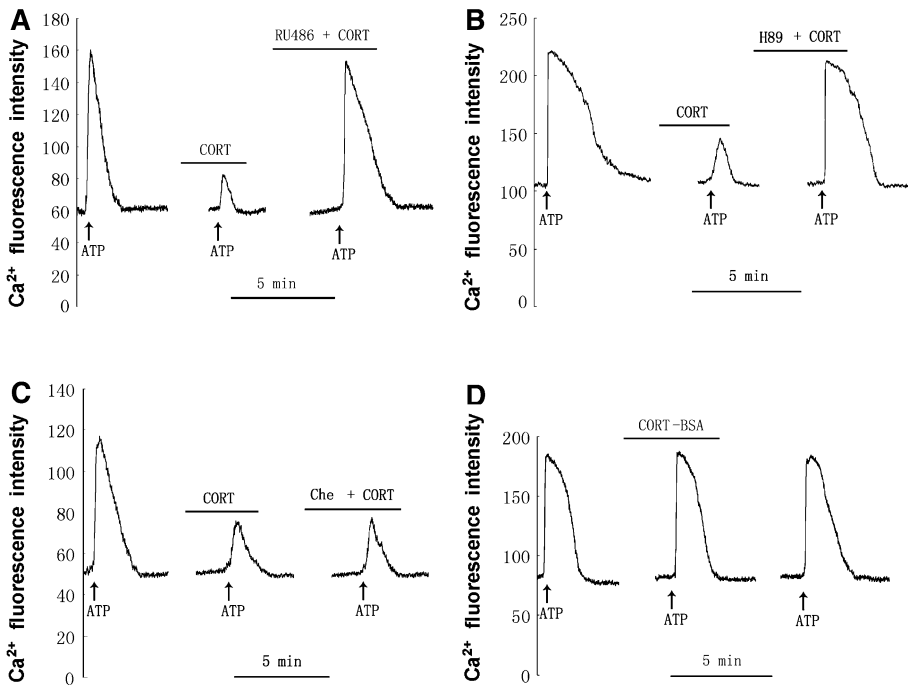

Fig. 3 The inhibitory effect of CORT on ATP-induced $[Ca^{2+}]i$ increase was blocked by RU38486 and H89, but was not mimicked by CORT-BSA. **a** Application of RU38486 (10 μM for 20 min) blocked the inhibitory effect of CORT. **b** Pre-treated with RU38486 (10 μM for 20 min) eliminated the inhibitory effect of CORT. **c** CHE (10 μM for 20 min) failed to block the inhibitory effect of of CORT. **d** CORT-BSA (0.1 μM for 5 min) failed to inhibit ATP-induced Ca^{2+} elevation in DRG neuron. Time indicated by *bars*

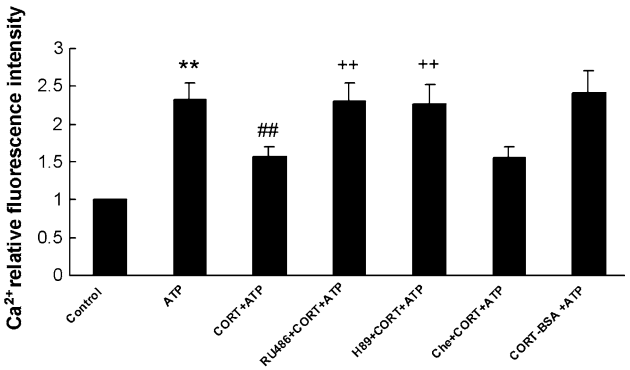

Fig. 4 The pharmacological profile of the effect of CORT on ATP-induced Ca^{2+} response in DRG neurons. 100 μM ATP caused $[Ca^{2+}]i$ increase in cultured DRG neurons (** $P < 0.01$, compare with control, $n = 12$ cells). Addition of CORT (0.1 μM for 5 min) significantly decreased 100 μM ATP-induced Ca^{2+} response ($^{\#\#} P < 0.01$, compare with ATP, $n = 12$ cells). GR antagonist RU38486 (10 μM for 20 min) treatment significantly suppressed CORT-stimulatory action on ATP-induced Ca^{2+} response ($^{++} P < 0.01$, compare with CORT + ATP, $n = 8$ cells). The inhibitory effects of CORT was blocked by PKA inhibitor H89 (10 μM for 20 min), but was unaffected by PKC inhibitor CHE (10 μM for 20 min) ($^{++} P < 0.01$, compare with CORT + ATP, $n = 8$ cells). Application of 0.1 μM CORT-BSA failed to impair Ca^{2+} response induced by ATP

blocked ATP-evoked $[Ca^{2+}]i$ increase. Moreover, pre-treatment with TNP-ATP, the selective antagonist for P2X receptors, completely blocked the ATP-evoked Ca^{2+} responses, which largely confirm that ATP-induced $[Ca^{2+}]i$ elevation in DRG neurons is mediated by P2X receptor. Previous studies suggest that the predominant purinergic receptors in DRG neurons are $P2X_1$ and $P2X_3$ forms and the $P2X_3$ subtype is the predominant receptor in DRG small neurons [20]. Taken together, these results indicate that ATP-induced Ca^{2+} responses in medium- to small-diameter DRG neurons are probably mediated by $P2X_3$ receptor. This result is in agreement with the previous observation of Petruska et al [21].

More recent studies convince that all classes of steroids can rapidly change physiological processes through non-genomic mechanisms that are typically associated with the rapid effects of neurotransmitters and peptide hormones [3, 4, 19, 22]. In the present study, we find that CORT rapidly attenuated ATP-induced $[Ca^{2+}]i$ transients with does-dependent manner in cultured DRG neurons. It is known that nongenomic effects have several features that can distinguish them from genomic effects: dose-dependent, rapid onset and equally fast recovery after removing steroid [23]. Indeed, upon wash out of CORT, ATP-induced Ca^{2+} influx was almost fully recovered in a short time, which suggests that nongenomic mechanisms is involved. On the other hand, in the nucleus GR interacts with glucocorticoid response elements (GRE) on promoters of regulated genes and recruits various co-activators or co-repressors to regulate the expression of target genes and protein synthesis. It is clear that gene transcription and protein synthesis will take some times. For example, in hippocampal slices, protein synthesis was first detectable following a 30-min exposure of slices to CORT [24]. In addition, in neuronal PC12 cells, a high dose of corticosterone-evoked transcriptional responses was detected 1 h after GR activation [25]. However, in this report, we show that ATP-induced $[Ca^{2+}]i$ increase is inhibited by CORT in a very short time, vanish rapidly. This rapid onset and offset indicates that transcription and gene expression is not involved in the action of CORT on ATP-induced $[Ca^{2+}]i$ increase. On the basis of above results, we speculate that the inhibitory effect of GC is through nongenomic pathways.

It is noted that responses to GC are considered to be mediated by four different mechanisms of action: the classical genomic mechanism of action caused by the cytosolic glucocorticoid receptor (cGCR); secondary non-genomic effects which are also initiated by the cGCR; membrane-bound glucocorticoid receptor (mGCR)-mediated non-genomic effects; non-specific, non-genomic effects caused by interactions with cellular membranes [26]. Being large and predominantly hydrophilic molecules, CORT-BSA conjugates are thought to be membrane-impermeable within 30 min. In our experiment, CORT-BSA failed to mimic the action of CORT. It appears that non-genomic effects, initiated by the cGCR, is involved in the action of CORT. However, stimulation of GR lead to activation of the PKA, which imply that mGCR may be involved in the action of CORT. A possible alternative mechanism for the inhibition caused by CORT is that GR may be located entirely within the plasma membrane bilayer and have no extracellular domain. It seems that ligand binding domain of GR may be located in the intracellular side of the plasma membrane.

It is known that RU38486 is supposed to prevent the association of GC with cGCR. We show that RU38486 counteracted the CORT suppression of the Ca^{2+} responses to ATP. At the same time, CORT-BSA cannot mimic the effect of CORT. It is a reasonable explanation that mGCR and cGCR have similar ligand binding domain. This is further supported by some previous observations that the binding site of CORT and estrogen (another kind of steroids) are located in the plasma membrane and these membrane receptor have similar pharmacology with those in nucleus [27–30].

Previous researches indicate that mGCR is linked to the activation of PKA or PKC intracellular signaling cascades [3, 18]. Recently, CORT was found to inhibit the voltage-dependent Ca^{2+} channel current through the activation of PKC in DRG neurons [9]. On the other hand, Han et al. reported that GC modulated P2X receptor-medicated Ca^{2+}

influx through a membrane-initiated, non-genomic and PKA-dependent pathway in HT4 cells [31]. We observed that the inhibitory effect of CORT was abolished by PKA inhibitor H89, but was unaffected by PKC inhibitor CHE in our study. The observation suggests that the rapid inhibitory effect of CORT on ATP-induced $[Ca^{2+}]i$ increase in DRG neurons is mediated by GR through activating PKA but not PKC signal pathway.

In a short, pain transduction begins with DRG neurons and trigeminal neurons, the primary sensory neurons. Our data suggests that CORT can inhibit ATP-induced $[Ca^{2+}]i$ increase in DRG neurons through a nongenomic mechanism and this effect probably involves mGCR pathway that activates PKA. It is possible that CORT inhibits nociceptive information transmission by blocking ATP-induced $[Ca^{2+}]i$ increase at the level of the primary afferent neuron, thereby decreasing the sensitivity to painful stimuli in the periphery. The next important question as to whether CORT inhibits nociceptive information transmission by blocking ATP-induced $[Ca^{2+}]i$ increase in situ awaits resolution.

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