Effects of dexamethasone on intracellular Ca^{2+} in its sensitive cells from neonatal mouse hippocampus and cultured cortical neurogliocytes

WANG Yu[1], WANG Jin-Xi[2], HUANG Hai-Dong, WANG Hong-Yi, ZHAO Xiao-Ning, ZHANG Zu-Xuan (*Medical School*, [2]*The State Key Laboratory of Coordination Chemistry*, *Nanjing University*, *Nanjing 210093*, *China*)

KEY WORDS dexamethasone; hippocampus; neuroglia; calcium; Fura-2; mifepristone; tetrodotoxin

ABSTRACT

AIM: To investigate the effects of dexamethasone (Dex) on intracellular free calcium ($[Ca^{2+}]_i$) in the single neuron or neurogliocyte. **METHODS**: Neonatal mouse hippocampal cells (NMHC) and cultured cortical neurogliocytes (CCN) were loaded with Fura 2-AM. The $[Ca^{2+}]_i$ was measured with AR-CM-MIC-cation measurement system. **RESULTS**: Most of freshly isolated NMHC exhibited a rapid and concentration-dependent $[Ca^{2+}]_i$ increase after administration of Dex 40 - 200 $\mu mol \cdot L^{-1}$. Only 10 % of NMHC showed their $[Ca^{2+}]_i$ decreases in total 96 tested cells. Dex-triggered $[Ca^{2+}]_i$ rise was prevented by incubating the cells with Mg^{2+}-free solution and reduced by adding $LaCl_3$. Suspended NMHC in Ca^{2+}-free solution or pretreated cells with mifepristone or tetrodotoxin prevented the initial $[Ca^{2+}]_i$ increases caused by Dex 40 - 90 $\mu mol \cdot L^{-1}$, but only diminished the later $[Ca^{2+}]_i$ rises by Dex 200 $\mu mol \cdot L^{-1}$. About 50 % of tested single CCN showed a rapid and concentration-related $[Ca^{2+}]_i$ increase due to Dex 90 - 270 $\mu mol \cdot L^{-1}$ exposure. This effect was partially inhibited under extracellular Ca^{2+}- or Mg^{2+}-free and mifepristone pretreatment conditions. **CONCLUSION**: Dex produces the rapid $[Ca^{2+}]_i$ changes in both neurons and glia cells. Reactions among most cells include a Mg^{2+}-dependent and glucocorticoid receptor-related extracellular Ca^{2+} influx and a high concentration of Dex-mediated intracellular Ca^{2+} release.

[1] Correspondence to Dr WANG Yu. Phn 86-25-359-3192. Fax 86-25-330-2728. E-mail yingfeng@nju.edu.cn
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INTRODUCTION

Recently the fast membrane effects of glucocorticoids (GC) attracted a lot of research interest[1]. GC evoked rapid intracellular biochemical changes and Ca^{2+} channel activities[2,3], the GC receptor existed in the membrane of hippocampal neurons[4,5]. GC and related drugs increased KCl-stimulated calcium influx in the synaptosomes from rat cerebral cortex[5]. However, it is almost neglected to verify if GC receptor antagonist mifepristone exerts influence on the neuronal responses to GC. Although calcium-associated action potential was increased in GC-exposed hippocampal neurons[6], it is still lack of evidence that GC-enhanced Ca^{2+} conductance likely accounts for neuronal intracellular Ca^{2+} accumulation. On the other hand, contradictory experimental results showed that cortisol inhibited voltage-activated Ca^{2+} current in guinea pig hippocampal cells[7]. These different results might be explained by the different experimental methods or animal species, but that the disparity in cell types affected GC effects could not be ruled out. Up to date it is still unclear whether and how GC possess direct effects on intracellular free calcium ($[Ca^{2+}]_i$) in

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hippocampus.

To explore further the $[Ca^{2+}]_i$ response after GC receptor activation in different brain cells, dexamethasone (Dex)-evoked changes of $[Ca^{2+}]_i$ between neonatal mouse hippocampal cells (NMHC) and cultured cortical neurogliocytes (CCN) were studied compatively by the present study, the effects of mifepristone (Mif), a GC receptor blocker and different cations on Dex-induced $[Ca^{2+}]_i$ rise were investigated so as to more precisely understand the effects of GC in CNS.

MATERIALS AND METHODS

NMHC preparation and Fura 2-AM loading Hippocampal CA1 regions were acutely isolated from 2 to 4-d-old mice. The tissues were incubated in trypsin 2.5 $g \cdot L^{-1}$ dissolved in Ca^{2+}- and Mg^{2+}-free Hanks' solution at 37 ℃ for 15 min. The single NMHC were obtained after gently dispersing tissues with a tip-polished glass pippet, and then filtrated through a nylon mesh (φ 95 μm) following wash with ice-cold Hanks' solution, which contained NaCl 137, KCl 5, $MgSO_4$ 0.5, KH_2PO_4 0.44, Na_2HPO_4 0.38, $NaHCO_3$ 2.62, $CaCl_2$ 1.3, glucose 5.6 $mmol \cdot L^{-1}$, pH 7.2 – 7.4. Suspended in Eagle's Minimum Essential Medium (DMEM, Gibco) supplemented with 10 % heat-inactivated bovine serum which contained fluorescence dye Fura 2-AM 5 $\mu mol \cdot L^{-1}$, NMHC were further incubated for 35 min, then were washed twice by centrifugation at $1000 \times g$ for 5 min and finally resuspended in medium solution according to experimental designs. Ninety percent of the cells were negative to trypan blue staining.

Cortical neurogliocyte culture Cerebral cortex of 16 – 18 d fetal mice were dissected in aseptic condition and dissociated by trypsin 0.6 $g \cdot L^{-1}$. Washed cortical cells were suspended in culture medium and seeded in plastic chambers. The cultures were incubated at 37 ℃ in 5 % CO_2 environment and refed with fresh medium every 2 d. On d 7 they were digested again with trypsin 1.25 $g \cdot L^{-1}$ contained edetic acid 0.5 $mmol \cdot L^{-1}$. Initially passed cells were transplanted on thin glass coverslip and continued incubation for a week to allow CCN growth.

$[Ca^{2+}]_i$ measurement The $[Ca^{2+}]_i$ was measured by testing the ratio of the fluorescence emission intensities (λ 505 nm) excited both at λ 340 and 380 nm, using AR-CM-MIC cation measurement system (Spex Industries Inc, USA). Ca^{2+}-signal in a randomly picked cell was continually recorded before and after administration of each drug. The maximal and minimal fluorescence parameters requisite for $[Ca^{2+}]_i$ calculation were obtained through adding ionomycin (Sigma) and egtazic acid. All data were analyzed with software in the measurement system.

Drugs and chemicals Dexamethasone Natrii Phosphat (Dex), an aqueous injection, was made by the Fourth Pharmaceutical Factory of Wuxi, Jiangsu, China. Mif, supplied by the Research Institute of Family Planning of Shanghai, was dissolved in ethanol and diluted with Hanks' solution. Fura 2-AM was provided by Shanghai Institute of Physiology, Chinese Academy of Sciences. All other chemicals were AR and purchased from Shanghai Chemical Cooperation.

Statistical methods All data were presented as $\bar{x} \pm s$. The paired t test or one-way ANOVA was used for quantitative data analysis. The EC_{50} were estimated by fitting log dose-response curve with Bliss method.

RESULTS

Resting $[Ca^{2+}]_i$ and solvent effect When extracellular medium contained Ca^{2+} 1.3 $mmol \cdot L^{-1}$, the resting $[Ca^{2+}]_i$ of NMHC was (173 ± 21) $nmol \cdot L^{-1}$ ($n = 96$). Ethanol 0.5 % – 1 % used as solvent produced a

negligible $[Ca^{2+}]_i$ change to this value, for the resting $[Ca^{2+}]_i$ was (187 ± 23) nmol·L^{-1} after ethanol exposure ($n = 8$).

Effects of Dex on $[Ca^{2+}]_i$ in NMHC Dex-evoked different $[Ca^{2+}]_i$ reactions were observed. About 10 % NMHC showed their remarkable $[Ca^{2+}]_i$ decreases from (174 ± 36) to (121 ± 19) nmol·L^{-1} (n = 10 cells from 96 NMHC, $P < 0.01$) after Dex 90 μmol·L^{-1} exposure, and 10 % – 15 % other cells were unresponsiveness. However, applying Dex to most of NMHC increased their $[Ca^{2+}]_i$ concentration-dependently after 20 s or less latency (Fig 1).

The process was metablism-related because it rarely occurred at 25 ℃ or below. The EC_{50} was 105 μmol·L^{-1}(95 % confidence limits 95 – 121 μmol·L^{-1}). Similar $[Ca^{2+}]_i$ rise was produced after administration of hydrocortisone. This excitatory effect was Mg^{2+}-dependent since suspended NMHC into Mg^{2+}-free Hanks' solution prevented the Dex-induced $[Ca^{2+}]_i$ increase though accompanied with resting $[Ca^{2+}]_i$ decreases. Additional application of lanthanum chloride ($LaCl_3$) 2 mmol·L^{-1} wholly inhibited the Dex (90 and 200 μmol·L^{-1})-induced $[Ca^{2+}]_i$ increase from (250 ± 33), (317 ± 68) nmol·L^{-1} to (134 ± 36)($n = 8$, $P < 0.01$) and (190 ± 85) nmol·L^{-1} ($n = 8$, $P < 0.01$), respectively. Removal of Ca^{2+} from Hanks' medium decreased the resting $[Ca^{2+}]_i$ value and blocked initial $[Ca^{2+}]_i$ increase activated by Dex 40 to 90 μmol·L^{-1}, but only slightly decreased the $[Ca^{2+}]_i$ rise due to Dex 200 μmol·L^{-1}, so the different reactions to Dex between the cells remained distinguishable in extracellular Ca^{2+}-free condition. Pretreatment of NMHC with LiCl 2 – 10 mmol·L^{-1} had no effect on Dex-induced $[Ca^{2+}]_i$ increases (Tab 1).

Effects of Mif and TTX on Dex-evoked $[Ca^{2+}]_i$ increase Preincubated NMHC in Hanks' solution containing Mif 10 μmol·L^{-1}, did not change the resting $[Ca^{2+}]_i$ but inhibited

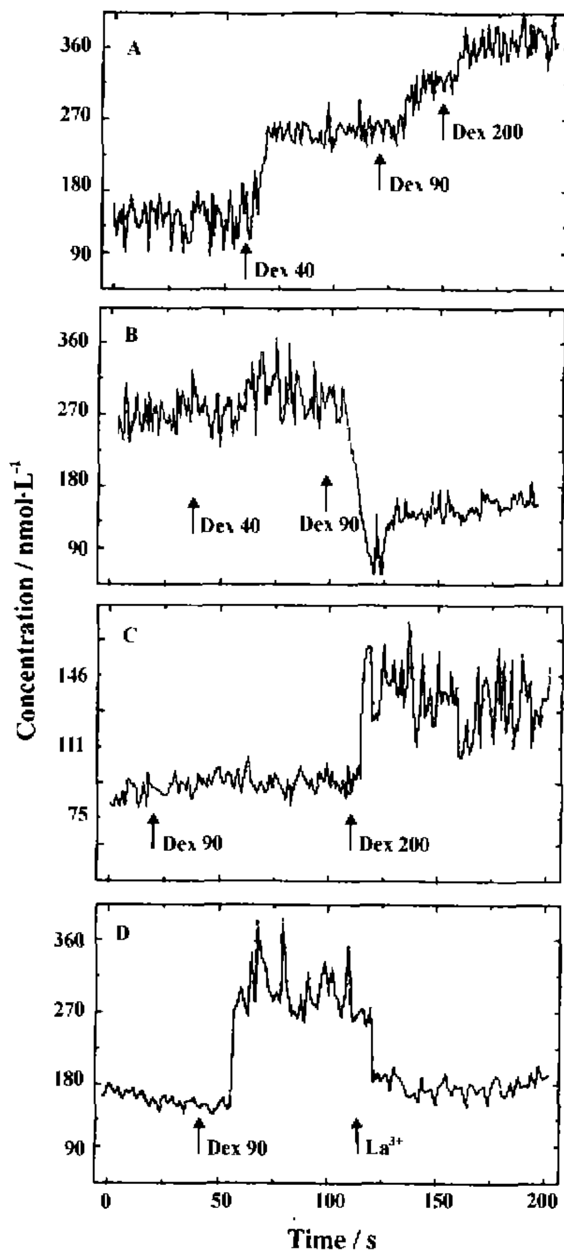

Fig 1. Fast $[Ca^{2+}]_i$ changes triggered by Dex (μmol·L^{-1}) in NMHC. A) The concentration-dependent $[Ca^{2+}]_i$ rises in Dex-exposed NMHC. B) Dex induced abrupt $[Ca^{2+}]_i$ decrease in some NMHC. C) Dex-induced $[Ca^{2+}]_i$ rises were partially prevented by extracellular Ca^{2+}-free. D) Adding $LaCl_3$ 2 mmol·L^{-1} attenuated Dex-induced $[Ca^{2+}]_i$ rise.

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Tab 1. Effects of some cations, Mif and TTX on Dex-evoked $[Ca^{2+}]_i$ rise in hippocampal cells from neonatal mice. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* Dex exposure before. $^eP < 0.05$, $^fP < 0.01$ *vs* control. Ca^{2+}-free Hanks' solution contained egtazic acid 0.2 mmol·L^{-1}.

Pretreatment/ mmol·L^{-1}	$[Ca^{2+}]_o$/ mmol·L^{-1}	n	$[Ca^{2+}]_i$/ nmol·L^{-1}	Dexamethasone/μmol·L^{-1} 40	90	200
Control	1.3	8	187 ± 23	217 ± 31	259 ± 48^c	325 ± 78^c
Ca^{2+}-free	0	8	89 ± 11^f	89 ± 16	95 ± 23	155 ± 58^b
Mg^{2+}-free	1.3	9	134 ± 26^f	133 ± 21	139 ± 36	141 ± 39
LiCl/4	1.3	10	179 ± 35	213 ± 40	251 ± 50^c	365 ± 85^c
Mifepristone/0.01	1.3	8	182 ± 27	190 ± 29	199 ± 37	229 ± 62^b
Mifepristone/0.01	0	8	87 ± 17^f	88 ± 24	97 ± 30	136 ± 49^b
Tetrodotoxin/0.001	1.3	8	141 ± 42^e	153 ± 36	158 ± 40	217 ± 73^b

Dex (40 – 90 μmol · L^{-1})-evoked $[Ca^{2+}]_i$ increases in extracellular Ca^{2+} 1.3 mmol · L^{-1} conditions. However, $[Ca^{2+}]_i$ elevation reactions in NMHC after adding Dex amount to 200 μmol · L^{-1} were unchanged regardless of existance of Mif. When TTX 1 μmol · L^{-1} was used to block sodium channels on the surface of NMHC, accompanied with the decrease of resting $[Ca^{2+}]_i$, the initial $[Ca^{2+}]_i$ increase to Dex 40 – 90 μmol · L^{-1} was abolished, whereas the effect of Dex 200 μmol·L^{-1} remained (Tab 1).

Effects of Dex on the $[Ca^{2+}]_i$ in CCN The resting $[Ca^{2+}]_i$ of CCN suspended in Hanks' solution was (130 ± 21) nmol·L^{-1} (n = 40), which was lower than that in NMHC. Usually, applying monosodium glutamate 1 mmol ·L^{-1} induced rapid $[Ca^{2+}]_i$ decrease in CCN from (120 ± 17) to (86 ± 13) nmol·L^{-1} (n = 11, $P < 0.01$). However, application of Dex rapidly increased $[Ca^{2+}]_i$ in 22 CCN among total 40 tested cells, the rest had no fast reaction to Dex until 270 μmol·L^{-1}. The concentration-dependent $[Ca^{2+}]_i$ rise in CCN was seen in 12 cells which were exposed to increasing concentrations of Dex with the EC_{50} estimated at 159 μmol·L^{-1} (95 % confidence limits 109 – 178 μmol·L^{-1}).

Incubated CCN in Ca^{2+}- or Mg^{2+}-free medium decreased their resting $[Ca^{2+}]_i$ and diminished their $[Ca^{2+}]_i$ elevative changes caused by Dex. Pretreatment of CCN with Mif for 30 min prevented the Dex (90 – 180 μmol·L^{-1})-induced $[Ca^{2+}]_i$ changes but the actions of Dex 180 – 270 μmol·L^{-1} were unaffected (Tab 2).

DISCUSSION

The present study demonstrated that application of Dex concentration-dependently

Tab 2. Effects of Dex on their $[Ca^{2+}]_i$ in cultured cortical neurogliocytes under several pretreatment conditions. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* the resting $[Ca^{2+}]_i$.

Pretreatment/ μmol·L – 1	n	$[Ca^{2+}]_i$/umol·L^{-1} resting	Dexamethasone/μmol·L^{-1} 90	180	270
Control	12	113 ± 18	154 ± 36	203 ± 38^b	261 ± 48^c
Ca^{2+}-free	8	78 ± 25	87 ± 29	108 ± 36^b	140 ± 40^b
Mg^{2+}-free	7	106 ± 20	118 ± 35	154 ± 39^b	176 ± 61^b
Mifepristone/10	7	124 ± 19	135 ± 26	160 ± 28	190 ± 38^c
Mifepristone/40	7	119 ± 18	128 ± 22	135 ± 28	164 ± 35^b

evoked $[Ca^{2+}]_i$ elevative responses in most of NMHC. That the effect had rapid onset with a less than 1 min latency was consistent with the fast membrane effects of glucocorticoids (GC)[1]. Moreover, CCN exhibited similar $[Ca^{2+}]_i$ elevation after Dex exposure with the EC_{50} approximated to the value of NMHC. Thus, intracellular free calcium ($[Ca^{2+}]_i$) as a biochemical messenger involved in the central effects of GC is definitly proposed. Besides, even though NMHC and CCN belong to different cell types most of them were sensitive to Dex, therefore, some fundamental biochemical processes, shared by neurons and glial cells, may be associated with Dex-triggered fast $[Ca^{2+}]_i$ increases.

Recently contradictory reactions of neurons to GC were reported when brains of rats or guinea pigs were used in experiments[6,7]. However, Dex-triggered different $[Ca^{2+}]_i$ changes and unresponsiveness were coexisted in the present observations. These different reactions may not attribute to any animal species difference because the cells used at the present study were absolutely obtained from mice. In addition, the effect disparity between mineralocorticoids and GC could be ruled out since Dex actually lacks of mineralocorticoid-like actions. Thus, present disparite results confirm that different reactions to GC may exist as a cytophysiologic phenomenon.

Suspending NMHC and CCN in Ca^{2+}-free Hanks' solution abolished their $[Ca^{2+}]_i$ rises caused by Dex 40 – 90 $\mu mol \cdot L^{-1}$, while the effect of Dex 200 $\mu mol \cdot L^{-1}$ remained, it revealed that the mechanisms resultant with these $[Ca^{2+}]_i$ rises were different according to the applied concentration of Dex. Extracellular Ca^{2+} influx induced by Dex 40 – 90 $\mu mol \cdot L^{-1}$ was blocked by GC receptor antagonist Mif or Na^+ channel blocker TTX (in NMHC only), which indicated that Dex-mediated neuronal Ca^{2+} influx belonged to a receptor and depolarized-dependent process. The intracellular Ca^{2+} release triggered by Dex 200 $\mu mol \cdot L^{-1}$ was resistant to Mif and TTX, it reveals that high concentration of Dex had more direct effects on intracellular fast biological processes.

The present study also found that Mg^{2+}, a cofactor of G protein, was necessary to Dex-induced $[Ca^{2+}]_i$ elevation, which happened only at a suitable temperature. These phenomena may suggest some linkage between this $[Ca^{2+}]_i$ rise and their intracellular biochemical metablism. However, pretreatment of NMHC or CCN with LiCl, an inhibitor of phosphoinositide cycle, had no effects on the $[Ca^{2+}]_i$ rise. Therefore, the precise relationship between GC and other second messengers requires further investigation.

A considerable number of experiments demonstrate that GC increase the hippocampal neuronal vulnerability to Ca^{2+}-related excitotoxin and impair the primate hippocampus[8,9,11]. GC-involved Ca^{2+} dysregulation was hypothesized as a mechanism of the neuron loss during brain aging[9,10]. This study showed that Dex rapidly modulated $[Ca^{2+}]_i$ among different brain cell types, suggesting that membrane effects of GC were directly and rapidly involved in sequential cellular events in brain. In addition, its Mg^{2+} dependence and Mif and TTX sensitive behavior gain some insight into the mechanism following GC receptor activation.

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地塞米松对新生小鼠海马和培养的皮层神经胶质中敏感细胞内钙浓度的影响

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王 瑜[1], 王金晞[2], 黄海东, 汪红仪, 赵晓宁, 张祖暄 (南京大学医学院, [2]南京大学配位化学国家重点实验室, 南京 210093, 中国)

关键词 地塞米松; 海马; 神经胶质; 钙; Fura-2; 米非司酮; 河鲀毒素

目的: 研究地塞米松(Dex)对神经元和胶质细胞内钙浓度($[Ca^{2+}]_i$)的影响. **方法**: Fura 2-AM 负载小鼠海马细胞(NMHC)和培养的胶质细胞(CCN). 单细胞内$[Ca^{2+}]_i$由 AR-CM-MIC 检测系统测定. **结果**: Dex 使多数 NMHC$[Ca^{2+}]_i$浓度依赖地迅速升高, 96 个 NMHC 中仅 10 % 出现$[Ca^{2+}]_i$降低. $[Ca^{2+}]_i$升高被无镁细胞外液阻滞、被氯化镧逆转, 但不受氯化锂影响. 无钙 Hanks' 液悬浮、米非司酮(Mif)或河鲀毒素均可阻断 Dex 40 - 90 μmol·L⁻¹的升$[Ca^{2+}]_i$效应, 而 Dex 200 μmol·L⁻¹的效应仍被保持. 40 个 CCN 中 50 % 对 Dex 产生浓度依赖的$[Ca^{2+}]_i$升高, 并被无钙或无镁的细胞外液和 Mif 预处理抑制. **结论**: Dex 快速改变海马神经元和胶质细胞内$[Ca^{2+}]_i$. $[Ca^{2+}]_i$的这种改变是由 Mg^{2+}和受体相关的外钙内流及高浓度 Dex 诱发的内钙释放介导的. (责任编辑 李 颖)