Cell Calcium 53 (2013) 256–263

Contents lists available at SciVerse ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Glucocorticoids reduce intracellular calcium concentration and protects neurons against glutamate toxicity

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ARTICLE INFO

Article history:
Received 3 October 2012
Received in revised form
14 December 2012
Accepted 24 December 2012
Available online 20 January 2013

Keywords:
Calcium
Neuron
Astrocyte
Glucocorticoid
Corticosterone
Dexamethasone
Glutamate

ABSTRACT

Glucocorticoids are steroid hormones which act through the glucocorticoid receptor. They regulate a wide variety of biological processes. Two glucocorticoids, the naturally occurring corticosterone and chemically produced dexamethasone, have been used to investigate the effect of glucocorticoids on Ca^{2+}-signalling in cortical co-cultures of neurons and astrocytes. Dexamethasone and to a lesser degree corticosterone both induced a decrease in cytosolic Ca^{2+} concentration in neurons and astrocytes. The effect of both compounds can be blocked by inhibition of the plasmamembrane ATPase, calmodulin and by application of a glucocorticoid receptor antagonist, while inhibition of NMDA receptors or the endoplasmic reticulum calcium pump had no effect. Glucocorticoid treatment further protects against detrimental calcium signalling and cell death by modulating the delayed calcium deregulation in response to glutamate toxicity. At the concentrations used dexamethasone and corticosterone did not show cell toxicity of their own. Thus, these results indicate that dexamethasone and corticosterone might be used for protection of the cells from calcium overload.

1. Introduction

Glucocorticoids (GCs) are a class of steroid hormones that bind to the glucocorticoid receptor (GR), which is expressed on mammalian cells. Due to their anti-inflammatory activity they are widely used in medicine. A variety of synthetic glucocorticoids, some far more potent than cortisol, have been created for therapeutic use. Glucocorticoids have a wide range of physiological activities but for many of them the underlying mechanism is still unknown [1].

One such activity is the maintenance of calcium homeostasis within the cell. Calcium ions are universal regulators of cellular processes, from cell division to cell death. There is a vast amount of publications which suggest a role for glucocorticoids in calcium homeostasis. It has been shown that GC-reduced calcium absorption can result from reduced expression of calcium-processing genes [2]. Many studies have demonstrated that GC changes the intracellular calcium concentration under both physiological and pathological conditions [3]. Exogenous application of a high dose of GC-induced a decrease in $[Ca^{2+}]_i$ in hypothalamic neurons [3]. This is opposite to what Reul et al. show, where increased corticosterone level enhance Ca^{2+} influx into CA1 pyramidal neurons [4]. High corticosterone modulated Ca^{2+} influx leads to altered physiological properties of the cell and network function [5,6]. Despite the apparent interest in this phenomenon the role of glucocorticoids in this process remains controversial. This might be due to the different cell types used by different groups as well as different glucocorticoids. To shed light on this disparity we used two different commonly used glucocorticoids, endogenous mammalian glucocorticoid corticosterone (CORT) produced in the rodent adrenal gland and a more potent synthetic preparation, dexamethasone (DEX) to investigate the effect of glucocorticoids on calcium homeostasis.

Dexamethasone (DEX) is a potent synthetic GC agonist, 25–30 times more potent than the natural steroid [7], known to cross the blood–brain barrier [8], whereas corticosterone (CORT) is a principal glucocorticoid synthesised in rodent adrenal cortex. DEX and CORT exerts their biochemical function mainly by binding to the glucocorticoid receptor (GR), which is expressed in almost all cell types but has varying effects in different cells [9]. Both are widely used therapeutically for many diseases such as neurological, neonatal respiratory distress syndrome, inflammatory, rheumatologic and autoimmune diseases. However, administration of GCs has many side effects, such as reduced growth and body weight [10], loss of memory and impaired logical thinking [11], disrupted hypothalamic-pituitary-adrenal (HAP) axis function [12] and reduction in calcium absorption [13].

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http://dx.doi.org/10.1016/j.ceca.2012.12.006

Therefore it remains important to understand the exact mechanisms by which GCs exert their protective as well as detrimental effects.

Here we show the action of DEX and CORT on the cytosolic calcium concentration. They act through the GR, which modulates the cytosolic calcium concentrations through the plasmalemmal ATPase in a calmodulin dependent manner. We also demonstrate that glucocorticoids reduce the calcium signal activated by physiological (5 μM) and pathological (100 μM) concentrations of glutamate. Finally, this leads to a protective effect of DEX and CORT against glutamate induced excitotoxicity in primary neuronal cultures.

2. Methods

2.1. Cell culture

Mixed cultures of cortical neurones were prepared as described previously [14,15] with modifications, from Sprague–Dawley rat pups 2–4 days post-partum (UCL breeding colony). Hippocampi, cortex and midbrain were removed into ice-cold HBSS (Ca^{2+}, Mg^{2+}-free, Gibco-Invitrogen, Paisley, UK). The tissue was minced and trypsinised (0.1% for 15 min at 37 °C), triturated and plated on poly-D-lysine-coated coverslips and cultured in Neurobasal A medium (Gibco-Invitrogen, Paisley, UK) supplemented with B-27 (Gibco-Invitrogen, Paisley, UK) and 2 mM L-glutamine. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, media changed twice a week. To avoid the age dependence of the delayed calcium deregulation, we used cells after 12–15 days in vivo in all experiments. Neurons were easily distinguishable from glia: they appeared phase bright, had small smooth rounded somata and distinct processes, and lay just above the focal plane of the glial layer.

2.2. Imaging $[Ca^{2+}]_c$

Hippocampal, cortical and midbrain neurons were loaded for 30 min at room temperature with 5 μM fura-ff AM, 5 μM fura-2 AM or 5 μM fluo-4 AM and 0.005% pluronic in a HEPES-buffered salt solution (HBSS) composed (mM): 156 NaCl, 3 KCl, $2MgSO_4$, 1.25 KH_2PO_4, 2 $CaCl_2$, 10 glucose and 10 HEPES, pH adjusted to 7.35 with NaOH. For simultaneous measurement of $[Ca^{2+}]_c$ and $\Delta\psi_m$, Rh123 (10 μM, Molecular Probes) was added into the cultures during the last 15 min of the fura-2 loading period, and the cells were then washed.

Fluorescence measurements were obtained on an epifluorescence inverted microscope equipped with a 20× fluorite objective. $[Ca^{2+}]_i$ and $\Delta\psi_m$ were monitored in single cells using excitation light provided by a Xenon arc lamp, the beam passing sequentially through 10 nm band pass filters centred at 340, 380 and 490 nm housed in computer-controlled filter wheel (Cairn Research, Kent, UK). Emitted fluorescence light was reflected through a 515 nm long-pass filter to a cooled CCD camera (Retiga, QImaging, Canada). All imaging data were collected and analysed using software from Andor (Belfast, UK). The fura-2 or fura-ff data have not been calibrated in terms of $[Ca^{2+}]_i$ because of the uncertainty arising from the use of different calibration techniques. Fluo-4 signal was excited by 490 nm and measured above 515 nm. Accumulation of Rh123 in polarised mitochondria quenches the fluorescent signal; in response to depolarisation the fluorescence signal is dequenched; an increase in Rh123 signal therefore indicates mitochondrial depolarisation. We have normalised the signals between resting level (set to 0) and a maximal signal generated in response to the uncoupler FCCP (1 μM; set to 100%).

2.3. Toxicity experiments

For toxicity assays cells were exposed to 5 μM propidium iodide (PI) and 5 μM Hoechst 33342 (Molecular Probes, Eugene, OR) for 30 min prior to imaging. The PI is excluded from viable cells and exhibits a red fluorescence following a loss of membrane integrity, while the Hoechst 33342 labels all nuclei blue. This allows expression of the number of dead (red stained) cells as a fraction of the total number of nuclei counted. Using phase contrast optics, a bright field image allowed identification of neurones, which look quite different to the flatter glial cells and also lie in a different focal plane, above the glial layer. A total number of 100–300 neurons were counted in 4–5 fields of each coverslip. Each experiment was repeated four or more times using separate cultures.

2.4. Statistical analysis

Statistical analysis was performed with the aid of Origin 8 (Microcal Software Inc., Northampton, MA, USA) software. Means expressed ±the standard error of the mean (S.E.M.).

3. Results

3.1. Effect of glucocorticoids on $[Ca^{2+}]_c$ in neurons and astrocytes

The application of DEX (1 μM) to co-cultured neurons and astrocytes loaded with the fluorescent calcium indicator fura-2, induced a significant decrease of cytosolic calcium concentration $[Ca^{2+}]_c$ in both cell types (0.93 ± 0.06 vs 1.21 ± 0.07; $P < 0.01$, $n = 104$ neurons and 93 astrocytes 0.94 ± 0.01 vs 1.15 ± 0.03; $P < 0.05$; Fig. 1A and B). To avoid any artefacts of quenching the fura-2 signal by DEX, we also used another calcium indicator fluo-4. Adding of DEX (1 μM) to fluo-4 loaded neurons and astrocytes had the same Ca^{2+}-reducing effect ($n = 20$ neurons and 22 astrocytes; Fig. 1C). Corticosterone (1 μM) was slightly less potent in reducing the basal $[Ca^{2+}]_c$ in cells, but effectively decrease calcium in stimulated cells ($n = 99$ neurons and 96 astrocytes; Fig. 1D). Thus, CORT decreased $[Ca^{2+}]_c$ in $46.4 \pm 3.9\%$ of neurons and $37.2 \pm 3.1\%$ of astrocytes compare $94.7 \pm 5.4\%$ and $87.7 \pm 4.6\%$ in neurons and astrocytes in response to DEX; Fig. 1E.

DEX and corticosterone are both glucocorticoids and can bind glucocorticoid receptors [16]. We used a specific antagonist (GR antagonist) to check whether the $[Ca^{2+}]_c$-reducing effect of these compounds is indeed through binding of glucocorticoid receptors. Pre-incubation of cells with the GR antagonist completely prevented the effect of DEX and corticosterone on $[Ca^{2+}]_c$ ($n = 54$ neurons and 58 astrocytes for DEX and $n = 57$ neurons and 61 astrocytes for CORT) (Fig. 2A–D; see also summary histogram Fig. 4G).

3.2. Mechanism of glucocorticoid-induced reduction of $[Ca^{2+}]_c$

Glucocorticoids are known to suppress NMDA receptors [17]. In order to investigate whether the decreased $[Ca^{2+}]_c$ seen after DEX treatment was NMDA receptor mediated, we used a selective inhibitor of this receptor, MK801 (5 μM). Application of MK801 did not change the level of cytosolic calcium in neurons and astrocytes and also had no effect on the response to DEX ($n = 34$ neurons and 42 astrocytes; Figs. 3A and 4G).

The effect of glucocorticoids on $[Ca^{2+}]_c$ in neurons and astrocytes can be induced by activation of active ion transport or buffering of Ca^{2+} by mitochondria. Mitochondrial calcium uptake is directly dependent on the mitochondrial membrane potential ($\Delta\psi_m$). Application of corticosterone or DEX to neurons and astrocytes induced a mild depolarisation of $\Delta\psi_m$ in both cell types ($21.42 \pm 2.18\%$ for corticosterone and $25.65 \pm 7.43\%$ for DEX; Fig. 3B). These data strongly suggests that both glucocorticoids do