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Corticosterone acutely prolonged *N*-methyl-D-aspartate receptor-mediated Ca^{2+} elevation in cultured rat hippocampal neurons

Taiki Takahashi, Tetsuya Kimoto, Nobuaki Tanabe, Taka-aki Hattori, Nobuaki Yasumatsu and Suguru Kawato

Department of Biophysics and Life Sciences, Graduate School of Arts and Sciences, University of Tokyo at Komaba, Meguro, Tokyo, Japan

Abstract

This work reports the first demonstration that corticosterone (CORT) has a rapid and transient effect on NMDA receptor-mediated Ca^{2+} signaling in cultured rat hippocampal neurons. Using single cell Ca^{2+} imaging, CORT and agonists of glucocorticoid receptors were observed to modulate the NMDA receptor-mediated Ca^{2+} signals in a completely different fashion from pregnenolone sulfate. In the absence of steroids, 100 μM NMDA induced a transient Ca^{2+} signal that lasted for 30–70 s in 86.1% of the neurons prepared from postnatal rats (3–5 days old). After pre-treatment with 0.1–100 μM CORT for 10–20 min, NMDA induced extremely prolonged Ca^{2+} elevation. This prolonged Ca^{2+} elevation was terminated by the application of MK-801 and followed by washing out of CORT. The proportion of CORT-modulated neurons within the NMDA-responsive cells increased from 25.1 to 95.5% when the concentration of CORT was raised from 0.1 to 50 μM. Substitution of BSA-conjugated CORT produced essentially the same results. When hippocampal neurons were preincubated with 10 μM cortisol and 1 μM dexamethasone for 20 min, a very prolonged Ca^{2+} elevation was also observed upon NMDA stimulation. The CORT-prolonged Ca^{2+} elevation caused a long-lasting depolarization of the mitochondrial membrane, as observed with rhodamine 123. In contrast, incubation with 100 μM pregnenolone sulfate did not considerably alter the time duration of NMDA-induced transient Ca^{2+} elevation, but caused a significant increase in the peak amplitude of Ca^{2+} elevation in hippocampal neurons. These results imply that high levels of CORT induce a rapid and non-genomic prolongation of NMDA receptor-mediated Ca^{2+} elevation, probably via putative membrane surface receptors for CORT in the hippocampal neurons.

Keywords: acute effect, Ca^{2+} signals, corticosterone, glucocorticoids, NMDA receptor, hippocampus.

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Corticosterone (CORT) is a principal glucocorticoid synthesized in the rodent adrenal cortex and secreted in response to stress. There are a series of studies about the chronic and genomic effects of corticosteroids in the hippocampus (Reagan and McEwen 1997; Nair *et al*. 1998). The stress-induced increase of CORT secretion is known to produce neuronal cell damage. Exogeneous application of a high dose of CORT has been shown to elicit the neuronal atrophy in the hippocampus (Woolley *et al*. 1990). Rats exposed to restraint stress for 3 weeks exhibited neuronal atrophy identical to that seen in rats treated with a high-dose of CORT for 3 weeks (Watanabe *et al*. 1992). In addition to these classical genomic effects, which are actuated via intracellular steroid receptors, glucocorticoids act acutely on neuronal excitability (Landfield and Pitler 1984; Lupien and McEwen 1997). Stress levels of glucocorticoids have been demonstrated to acutely suppress within 20 min the long-term potentiation (LTP) of primed burst potentiation (Diamond *et al*. 1992).

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Address correspondence and reprint requests to Suguru Kawato, Department of Biophysics and Life Sciences, Graduate School of Arts and Sciences, University of Tokyo at Komaba, Meguro, Tokyo 153, Japan. E-mail: kawato@phys.c.u-tokyo.ac.jp

Abbreviations used: BSS, balanced salt solution; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; CORT, corticosterone; DMSO, dimethyl sulfoxide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; GABA, γ-aminobutyric acid; LTP, long-term potentiation; MEM, minimum essential medium; PREGS, pregnenolone sulfate; n_i, the number of independent experiments.

The LTP of the population spike amplitude was also acutely (within 1 h) suppressed by a high concentration of glucocorticoids (Vidal *et al*. 1986; Diamond *et al*. 1992). CORT, injected just before the tetanic stimulation, was found to have an acute depressant effect on potentiation of both the field excitatory postsynaptic potentials (EPSPs) and the population spike amplitude in the dentate gyrus (Dubrovsky *et al*. 1993). Neurosteroids such as pregnenolone sulfate (PREGS), which are synthesized in the mammalian brain, have been observed to affect cell surface receptors very acutely (Paul and Purdy 1992; Baulieu 1997). PREGS' mode of modulation on NMDA receptors and γ-aminobutyric acid $(GABA)_A$ receptors has been extensively investigated (Wong and Moss 1994; Mienville and Vicini 1989). PREGS potentiated the opening probability of NMDA receptors, but suppressed that of $GABA_A$ receptors, in cultured neurons (Wu *et al*. 1991; Irwin *et al*. 1992; Bowlby 1993; Fahey *et al*. 1995). In behavioral studies, the various influences of steroids on memory formation in the hippocampus have been attracting much attention. Peripheral steroids have been shown to have profound influences, either damaging or beneficial, on the memory performances exhibited in a wide variety of tasks (Vaher *et al*. 1994; Dohanich *et al*. 1994; Luine 1997; Oitzl *et al*. 1998). Steroids may influence memory processes by modulating LTP in the hippocampus, which has recently been shown to be inhibited by high stress-induced levels of glucocorticoids and to be enhanced by low endogenous levels (Pavlides *et al*. 1993, 1996; Rey *et al*. 1994; McEwen 1996, 1999; McEwen and Sapolsky 1995; Kim and Yoon 1998). Recently, stress hormones have been found to impair learning and memory function rapidly (within several tens of minutes), via unknown neuronal mechanisms (Mesches *et al*. 1999).

The rapid effects of CORT (appearing within 30 min) on NMDA-induced signal transduction have not been well elucidated in the cultured hippocampal neurons. We therefore examined the rapid effects of CORT on NMDA receptor-mediated Ca^{2+} signals in hippocampal neurons in comparison with those of the neurosteroid PREGS. CORT resulted in extreme prolongation of the time duration of NMDA receptor-mediated Ca^{2+} signals, resulting in Ca^{2+} neurotoxicity. On the other hand, PREGS did not change the time duration of Ca^{2+} signals, although the amplitude of NMDA-induced Ca^{2+} signals was increased. Agonists of glucocorticoid receptors such as dexamethasone and cortisol were investigated. These agonists also showed extremely prolonged NMDA receptor-mediated Ca^{2+} signals.

Materials and methods

Chemicals

Fura-2/AM was purchased from Dojindo Laboratory (Kumamoto, Japan). Cycloheximide was purchased from Wako pure chemicals (Osaka, Japan). PREGS, CORT, NMDA, nicardipine DNase I, cytosine arabinoside, rhodamine 123, MK-801, dexamethasone, cortisol and antineurofilament 200 kDa polyclonal antibodies were purchased from Sigma (St Louis, MO, USA). Dispase I was purchased from Boehringer Mannheim (Tokyo, Japan). Minimum essential medium (MEM) was from Gibco (Rockville, MD, USA). RU38486 and RU28632 were obtained from Roussel Uclaf (Romainville, France). All other chemicals were of the highest purity commercially available.

Preparation of rat hippocampal neurons and cell culture

Postnatal hippocampal tissue was taken from 3- to 5-day-old Wistar rats purchased from SLC (Japan). After exposure to an overdose of ether anesthesia, the rats were decapitated and the brains were removed using a sterile technique. The brains were placed in ice-cold MEM (–) (MEM supplemented with 2 mM glutamine, 22 mM glucose, 20 μM kanamycin and 26 mM $NaHCO_3$) which was continuously bubbled with 95% O_2–5% CO_2 gas. The hippocampi were then removed and rinsed once with ice-cold MEM (–). Hippocampal tissues were incubated in MEM (–) containing 3 U/mL of dispase I for 10 min at 37°C and triturated after the addition of 0.1% DNase I. The tissue was then filtered with a 100-μm cell strainer and suspended in MEM (–). The cell suspensions were centrifuged for 5 min at 120 *g*. Centrifugation was performed three times. Finally, neuronal cells were suspended in MEM (+) (MEM (–) supplemented with 10% heat-inactivated fetal calf serum), and seeded on poly L-lysine-coated glass-bottom dishes (35 mm, MatTek, Ashland, MA, USA) at the density of 10^6 cells/mL. Neurons were cultured for 7–10 days under 5% CO_2–95% ambient air at 37°C. Half of the culture medium was replaced every other day, in order to provide sufficient glucose for neuronal growth. The neuronal samples were treated on day 2, in culture with 10 μM cytosine arabinoside for 24 h, to prevent the glial proliferation. In the case of the preparation of embryonic neurons, E19–E20 rat pups were used. The culture medium and preparation methods were the same as those used for the postnatal neuron culture. The purity of cultured neuronal cells was determined by the immunostaining of the cells against neurofilament 200 kDa. More than 85 of cells were identified as neurons on day 7–10. All experiments using animals were conducted in accordance with the institutional guidelines.

Loading with fluorescent indicators

Measurement of cytoplasmic calcium concentration $[Ca^{2+}]_i$ was performed using the Ca^{2+}-sensitive indicator fura-2 (Grynkiewicz *et al*. 1985). Prior to Ca^{2+} signal measurements, neuronal cells were loaded for 30 min at 37°C with 5 μM fura-2/AM [from 1 mM stock solution in dimethyl sulfoxide (DMSO)] in the presence of 0.03% cremophore EL in 1 mL of a Mg^{2+}-free balanced salt solution (BSS), consisting of 130 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 5.5 mM glucose, 10 μM glycine and 10 mM Hepes (pH 7.3). Active mitochondria in hippocampal neurons were labeled with 0.2 μM rhodamine 123, a fluorescent dye which indicates mitochondrial membrane potential (Bellomo *et al*. 1991; Sureda *et al*. 1997) for 5 min at 37°C in BSS. After the neurons were labeled with fluorescent indicators, they were washed three times with BSS and incubated with chemicals for pre-treatment. Solutions of CORT, PREGS and thapsigargin were prepared at the appropriate dilution with BSS from the stock solution in DMSO, the final concentration of DMSO being less than 0.01% in each case.

Measurement of $[Ca^{2+}]_i$ and mitochondrial potential

Prior to the application of 100 μM NMDA, the neurons were incubated for 10–20 min at 37°C, in the presence of steroids in BSS. Control samples were preincubated in BSS alone for the same duration, at 37°C in the absence of steroids, before the application of NMDA. The neurons were perfused at a rate of 1.5 mL/min with O_2-bubbled BSS with or without steroids, NMDA and inhibitors. For fluorescence measurements, a digital fluorescence microscope system, consisting of an inverted microscope (Nikon TMD 300, Japan) equipped with a xenon lamp for excitation and a CCD camera (Hamamatsu Photonics C2400-77, Japan) was used. The inverted microscope is equipped with a temperature chamber which maintained the air around the sample at 37°C and high humidity, using a warm air-supplying system. In all measurements, from 12 to 24 neurons fell within the microscope field. For fura-2 measurements, the excitation wavelength varied discretely between 340 and 380 nm, every 1.15 s with a step motor. Fluorescence was measured above 520 nm with an IF excitation filter, a DM510 dichroic mirror, and a BA 520 emission filter. $[Ca^{2+}]_i$ in each cell was expressed as F_{340}/F_{380}, which is the ratio of the fluorescence intensity at 340 nm excitation (F_{340}) to that at 380 nm excitation (F_{380}) (Vergun *et al.* 1999). Fluorescence of rhodamine 123 was measured by the same microscope system with excitation at 470 nm and emission at 490 nm. Because rhodamine 123 is a single-wavelength dye and its initial fluorescence intensity may vary between cells, the fluorescence data sets were normalized by adjusting the resting fluorescence intensity to be 100%, to facilitate comparison. The fluorescence image analysis was performed with ARGUS-50 system (Hamamatsu Photonics, Japan). The acquired images were stored in a hard disk with 512 × 483 pixels resolution with 16-bit depth. For generation of the time dependent curves, data in each area of 10 × 10 pixels were averaged with a 2.3-s time resolution.

Statistical analysis

Statistical analysis was performed with the aid of Excel software (Microsoft, USA). Data expressed as mean ± SEM. An unpaired, two-tailed *t*-test, under the assumption of unequal variances, was utilized to test the significance of observed differences between groups. The number of independent experiments (n_i) was used to determine the parameters of *t*-distribution for the test.

Results

Characteristics of NMDA receptor – mediated Ca^{2+} signals

The response of cultured neurons to continuous NMDA exposure was characterized by a transient elevation in $[Ca^{2+}]_i$ followed by a decay to a plateau as NMDA receptors were inactivated (Fig. 1), as reported previously (Nicholls and Budd 1998; Nicholls *et al.* 1999; Vergun *et al.* 1999). It is noteworthy that the plateau level depended significantly on whether the hippocampal neurons were prepared from postnatal 3–5-day-old rats or from embryonic E19–E20 rats. Upon application of 100 μM NMDA in Mg^{2+}-free and 10 μM glycine-containing BSS, a complete decay of the Ca^{2+} elevation to the resting level was typically observed within 30–70 s, in the neurons which were prepared from postnatal 3–5-day-old rats and cultured for 7–10 days (94.6 ± 2.3% of the NMDA-responsive neurons, where the number of independent experiments was $n_i = 7$, and each contained

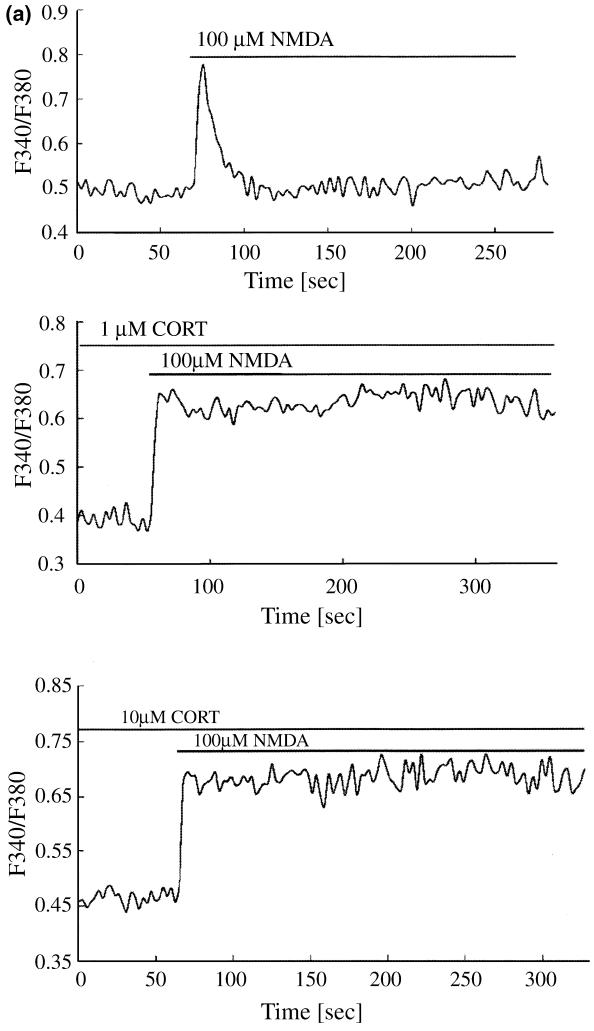

Fig. 1 Effect of corticosterone on the time course of NMDA-induced Ca^{2+} signals in the hippocampal neurons. (a) The time course of transient Ca^{2+} elevation induced upon application of 100 μM NMDA alone in a Mg^{2+}-free BSS (curve a). Pre-incubation with 1 μM (curve b) and 10 μM (curve c) CORT, before the application of 100 μM NMDA prolonged the Ca^{2+} elevation considerably. We performed between five and seven independent experiments (12–24 cells for each experiment) under each different condition and essentially the same results were obtained. The vertical scale (F340/F380) is the ratio of the fluorescence intensity of fura-2 excited at 340 nm and 380 nm, and the horizontal axis is the time in seconds. (b) Pseudo-colored images demonstrate that the neurons show a transient Ca^{2+} elevation upon 100 μM NMDA stimulation at $t = 55$ s (b1), Ca^{2+} signals in the presence of 1 μM CORT upon 100 μM NMDA stimulation at $t = 55$ s (b2). The ratio of fura-2 fluorescence is indicated with a color bar from blue (low $[Ca^{2+}]_i$) to red/white (high $[Ca^{2+}]_i$). Scale bar = 50 μm.

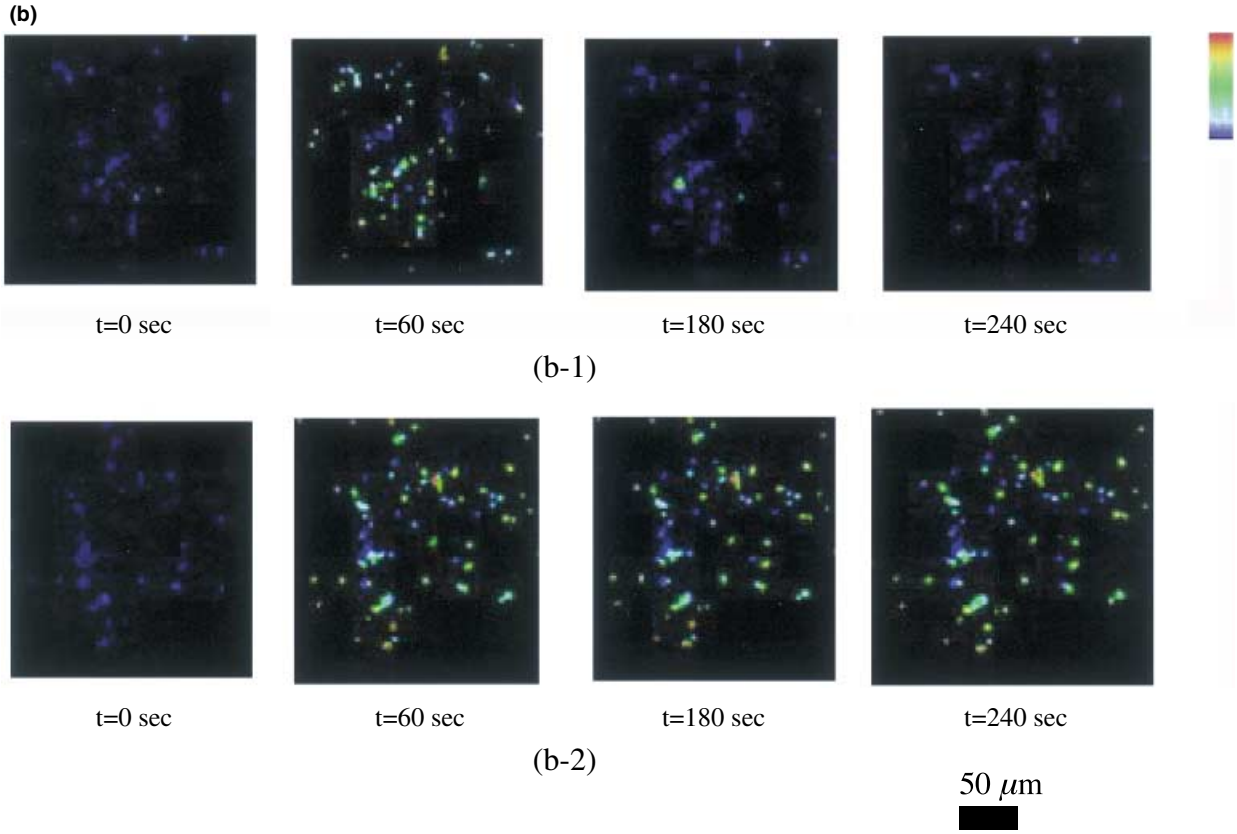

Fig. 1 (Continued)

12–24 neurons). On the other hand, the Ca^{2+} responses in most hippocampal neurons prepared from E19–E20 rats were not followed by a complete decay to resting levels after 100 μM NMDA stimulation, within the experimental time range of 300 s. $[Ca^{2+}]_i$ also remained at high plateau levels of approximately 74 ± 4% of the maximal peak values (76.5 ± 2.4% of the NMDA-responsive neurons; $n_i = 6$ experiments; 22–25 neurons/experiment). That the application of MK-801, alone induced no change in the neuronal $[Ca^{2+}]_i$ ($n_i = 3$) suggests that the MK-801 acted by blocking the NMDA-induced Ca^{2+} signal, rather than by directly inducing a change in $[Ca^{2+}]_i$.

Rapid effects of CORT on NMDA receptor-mediated Ca^{2+} signals

We examined the effect of CORT on hippocampal neurons prepared from postnatal 3–5 days and cultured for 7–10 days. Analyzed parameters of the Ca^{2+} signals are summarized in Fig. 2 and Table 1. Most of the postnatal neurons showed a rapid elevation in $[Ca^{2+}]_i$ upon application of 100 μM NMDA. The proportion of NMDA- responsive neurons {= 100 × [(the number of the NMDA-responsive neurons)/(the total number of the neurons)]} was 86.1 ± 3.6% ($n_i = 7$). The peak amplitude of $[Ca^{2+}]_i$ elevation, indicated as the increase in F_{340}/F_{380} from the resting level [$-(F_{340}/F_{380})$], was 0.26 ± 0.02 ($n_i = 5$). Most of the NMDA-responsive neurons showed a decrease in $[Ca^{2+}]_i$, towards the resting levels within 30–70 s, as described above.

After pre-treatment with 0.1–100 μM CORT for 20 min, an extremely prolonged $[Ca^{2+}]_i$ elevation was observed in a

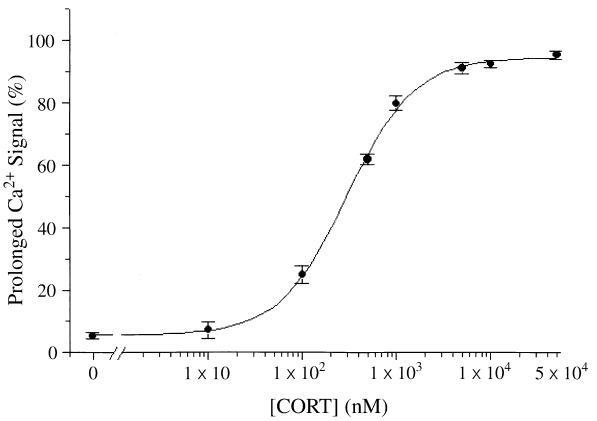

Fig. 2 Dose-dependency of the rapid prolongation effect of CORT on $[Ca^{2+}]_i$ elevation induced upon NMDA-stimulation. Vertical scale is the proportion of the neurons showing prolonged Ca^{2+} elevation [= 100 × (the number of neurons exhibiting prolonged Ca^{2+} elevation)/(the number of NMDA-responsive neurons)]. Horizontal axis indicates logarithmic concentration of CORT.

Table 1 Characteristics of Ca^{2+} signals in the postnatal hippocampal neurons stimulated by NMDA after short-term exposure to CORT or other modulators

Modulators	% Proportion of the of prolonged Ca^{2+} signals'[a] elevation[b]	Maximal amplitude $[Ca^{2+}]_i$
No modulator (NMDA alone) (n_i[c] = 7)	5.4 ± 1.1	0.26 ± 0.02
10 μM CORT ($n_i = 7$)	92.4 ± 1.2**	0.31 ± 0.04
10 μM CORT + 10 μM nicardipine ($n_i = 5$)	89.1 ± 2.2**	0.21 ± 0.02
10 μM CORT + 2 μM thapsigargin ($n_i = 5$)	94.2 ± 3.1**	0.24 ± 0.03
10 μM CORT + 10 μM cycloheximide ($n_i = 5$)	91.2 ± 3.3**	0.27 ± 0.02
1 μM CORT ($n_i = 5$)	80.3 ± 2.4**	0.28 ± 0.03
1 μM CORT + 10 μM nicardipine ($n_i = 5$)	79.8 ± 3.0**	0.23 ± 0.02
1 μM CORT + 2 μM thapsigargin ($n_i = 5$)	81.3 ± 2.5**	0.25 ± 0.01
1 μM CORT + 10 μM cycloheximide ($n_i = 5$)	79.4 ± 3.2**	0.29 ± 0.01
1 μM[d] BSA-CORT ($n_i = 5$)	78.3 ± 3.1**	0.27 ± 0.02
1 μM dexamethasone ($n_i = 4$)	94.2 ± 3.2**	0.28 ± 0.04
10 μM cortisol ($n_i = 5$)	88.4 ± 2.3**	0.27 ± 0.03
10 μM RU28362 ($n_i = 4$)	14.3 ± 3.3	0.22 ± 0.01
1 μM RU28362 ($n_i = 4$)	12.5 ± 3.0	0.25 ± 0.03
1 μM CORT + 1 μM RU38486 ($n_i = 4$)	84.3 ± 2.2**	0.25 ± 0.03
10 μM CORT + 10 μM progesterone ($n_i = 5$)	4.8 ± 1.3	0.27 ± 0.02
10 μM progesterone ($n_i = 5$)	5.8 ± 1.4	0.25 ± 0.03
100 μM PREGS ($n_i = 5$)	2.3 ± 0.8	0.36 ± 0.01**
100 μM PREGS + 10 μM progesterone ($n_i = 3$)	3.4 ± 2.2	0.34 ± 0.02**

After the preincubation of hippocampal neurons for 20 min with or without the modulators indicated in the table, neurons were stimulated with 100 μM NMDA. Between 12 and 24 neurons were analyzed in each independent experiment. Maximal amplitude of NMDA-induced $[Ca^{2+}]_i$ elevation is represented as the maximal increase in the ratio of fura-2 fluorescence intensities, excited at 340 nm and 380 nm [$\Delta(F_{340}/F_{380})$]. Values are means ± SEM. [a]The elevated $[Ca^{2+}]_i$ did not decay within the experimental time range of 300 s. [b]Maximal sustained amplitude in the case of prolonged Ca^{2+} signals, or peak amplitude in the case of transient Ca^{2+} elevation. [c]n_i is the number of independent experiments. [d]Approximately 20 CORT molecules per one BSA molecule. **Significantly larger than the value with no modulators ($p < 0.01$).

portion of the NMDA-responsive neurons upon the application of 100 μM NMDA (Figs 1 and 2). This sustained elevation of $[Ca^{2+}]_i$ did not decay in the presence of CORT, within the experimental time range of 300 s (Fig. 1). The proportion of CORT-modulated cells, which exhibited sustained $[Ca^{2+}]_i$ elevation increased with increasing CORT concentration, as shown by a sigmoidal dose-dependence curve (Fig. 2). The CORT dosage which yielded the half-maximal number of modulated cells (EC_{50}) was approximately 0.53 μM. The lowest effective dose was 0.1 μM. A dosage of 0.01 μM of CORT caused no significant effect. In particular, 1, 5, 10 and 50 μM of CORT induced the modulation in 80.3 ± 2.4% ($n_i = 5$), 91.2 ± 1.9% ($n_i = 5$), 92.4 ± 1.2% $n_i = 7$ and 95.5 ± 1.4% ($n_i = 6$) of treated neurons, respectively. Pre-treatment of the neurons by CORT had no significant effect on the proportion of NMDA responsive neurons, for example, remaining at 85.4 ± 3.4% ($n_i = 7$) with 10 μM CORT treatment. The presence of CORT did not cause an excessive change in the maximal amplitude of the $[Ca^{2+}]_i$ elevation.

The impact of inhibitors was generally investigated using 1–10 μM CORT, because concentrations in this range induced a prolonged $[Ca^{2+}]_i$ elevation in a sufficiently large proportion of neurons for the effects of the inhibitor to be precisely analyzed. The application of 10 μM MK-801 terminated the prolonged $[Ca^{2+}]_i$ elevation which was induced by 100 μM NMDA in the presence of 1 μM CORT, and a rapid $[Ca^{2+}]_i$ decay towards the resting level ($n_i = 6$; Fig. 3) was observed. Removal of CORT, by switching the perfusion solution from 1 μM CORT-containing BSS to a CORT-free BSS with 100 μM NMDA, also terminated the prolonged $[Ca^{2+}]_i$ elevation (Fig. 3). From these results, we conclude that the present effect of CORT was not due to the irreversible impairment of Ca^{2+} extrusion from the neurons. That the subsequent application of 100 μM NMDA (alone), after the removal of CORT, re-induced transient, typical NMDA-type $[Ca^{2+}]_i$ elevations ($n_i = 5$), also supports this conclusion.

Pre-treatment of neurons with 2 μM thapsigargin for 20 min, in the presence and absence of 1–10 μM CORT, did not change either the proportion of neurons that demonstrated a prolonged Ca^{2+} signal or the maximal amplitude of the NMDA-induced Ca^{2+} signal (see Table 1). This excludes the possibility that intracellular Ca^{2+} stores

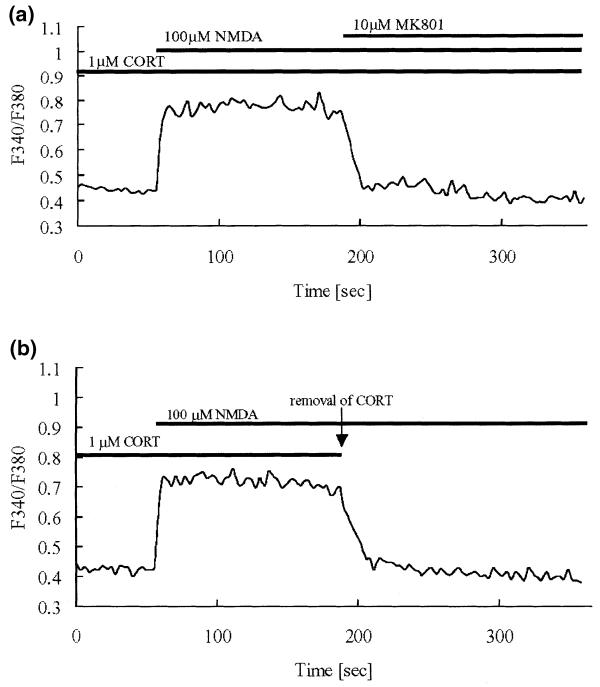

Fig. 3 Application of MK-801 or removal of CORT terminates the prolonged $[Ca^{2+}]_i$ elevation observed in the presence of CORT. (a) Change in the time course of Ca^{2+} signaling upon the application of 10 μM MK-801 during the prolonged Ca^{2+} signaling induced by 100 μM NMDA in the presence of 1 μM CORT. The vertical scale (F340/F380) is the ratio of the fluorescence intensity of fura-2, excited at 340 nm and 380 nm, and the horizontal axis is the time in seconds. (b) Change in the time course of Ca^{2+} signaling upon replacement of an external solution by a Mg^{2+}, CORT-free solution containing 100 μM NMDA. The prolonged $[Ca^{2+}]_i$ elevation was induced by 100 μM NMDA in the presence of 1 μM CORT.

significantly contributed to the prolonged $[Ca^{2+}]_i$ elevation induced by the CORT treatment. Pre-treatment with 10 μM nicardipine for 20 min did not significantly decrease the proportion of the neurons in which Ca^{2+} signals were prolonged by 1–10 μM CORT (see Table 1). This implies that a Ca^{2+} influx via L-type voltage sensitive Ca^{2+} channels was not necessary for the appearance of the present CORT effect. In order to activate all types of voltage-sensitive Ca^{2+} channels, 30–50 mM KCl in isotonic BSS was applied to the neurons. Upon the application of KCl alone, the neurons were depolarized, and a sustained $[Ca^{2+}]_i$ elevation was observed during the presence of KCl ($n_i = 3$). The KCl-induced Ca^{2+} signals observed in the absence and presence of CORT were not significantly different. The sustained level of $[Ca^{2+}]_i$ induced by 50 mM KCl was $\Delta(F_{340}/F_{380}) = 0.19 \pm 0.02$ (without CORT) and 0.20 ± 0.02 (with 1 μM CORT), respectively.

In the absence of NMDA, the application of CORT (alone) at 1–10 μM failed to induce a Ca^{2+} signal. This implies that the present CORT effect requires the activation of NMDA receptors. In order to examine whether new protein synthesis is involved in the present CORT effect, the neurons were subjected to a 40-min preincubation with cycloheximide, a protein synthesis inhibitor, prior to the NMDA application, and then the preincubation of neurons with CORT was additionally performed for 20 min prior to the NMDA application. The resulting concentrations of cycloheximide and CORT were maintained at the same level during the time course of the $[Ca^{2+}]_i$ measurements. When the neurons were coincubated with 10 μM cycloheximide and 1–10 μM CORT in this way, the NMDA application again induced a very prolonged $[Ca^{2+}]_i$ elevation in 85.1% of the neurons (see Table 1). This indicates that the prolongation by CORT did not require the synthesis of new proteins, which is necessary for the classical genomic effects of glucocorticoids, via intracellular steroid receptors.

Rapid effect of agonists and antagonist of glucocorticoid receptors on NMDA receptor-mediated Ca^{2+} signals

To examine whether the present rapid CORT effect appears via surface CORT receptors, membrane non-permeable BSA-conjugated CORT was substituted for CORT. After pre-treatment with 1 μM BSA-CORT (approximately 20 CORT molecules per BSA molecule) for 20 min, an extremely prolonged $[Ca^{2+}]_i$ elevation was observed in 78.3% of the NMDA-responsive neurons upon application of 100 μM NMDA (see Table 1). The maximal amplitude of NMDA-induced $[Ca^{2+}]_i$ elevation was the same for BSA-CORT and CORT. Dexamethasone, cortisol and RU 28362, which are known as potent agonists of glucocorticoid receptors were used to investigate the glucocorticoid effect on NMDA-induced $[Ca^{2+}]_i$ elevation (see Table 1). The pre-treatment of neurons with either 1 μM dexamethasone or 10 μM cortisol for 20 min induced an extremely prolonged $[Ca^{2+}]_i$ elevation upon NMDA stimulation, with essentially the same characteristics as that induced by CORT, with respect to both the proportion of neurons which demonstrated a prolonged $[Ca^{2+}]_i$ elevation, and the maximal amplitude of the $[Ca^{2+}]_i$ elevation. On the other hand, the preincubation with 1 or 10 μM RU28362 (a synthetic agonist of intracellular glucocorticoid receptor (GR, type-2 receptor)) had only a small effect on the prolongation of the NMDA-induced $[Ca^{2+}]_i$ elevation (see Table 1). The proportion of neurons which demonstrated a prolonged $[Ca^{2+}]_i$ elevation was 12.5 and 14.3% in the presence of RU28362, at 1 and 10 μM, respectively. The coapplication of 10 μM progesterone with 10 μM CORT completely prevented the modulating effect of CORT, while the pre-treatment of neurons with only 10 μM progesterone without CORT had no modulating effect on the $[Ca^{2+}]_i$ elevation induced by NMDA (see Table 1). The presence of an equimolar concentration of RU38486 (a synthetic antagonist of intracellular glucocorticoid receptors) with CORT did not significantly change the CORT-modulated, prolonged $[Ca^{2+}]_i$ elevation (see Table 1).

Effect of CORT on mitochondrial membrane potential upon NMDA stimulation

We examined the toxic effect of the prolonged $[Ca^{2+}]_i$ elevation induced by CORT and NMDA on mitochondria. Upon a 100-μM NMDA stimulation without CORT ($n_i = 4$), a transient decrease in the rhodamine 123 fluorescence was observed, which indicates that NMDA stimulation induced only a transient depolarization of the mitochondrial membrane (see Fig. 4). Pre-treatment with 1–10 μM CORT resulted in an extreme prolongation of the decreased phase in the fluorescence of rhodamine 123, upon 100 μM NMDA stimulation ($n_i = 5$). The time profile of the change of mitochondrial membrane potential coincided with that of the $[Ca^{2+}]_i$ elevation. It should be noted that when the mitochondrial membrane is depolarized, the sequestration of rhodamine 123 molecules within mitochondria is reduced, which results in a decrease in the fluorescence of rhodamine 123 in the concentration range of 0.1–1 μM (Sureda *et al.* 1997; Yang *et al.* 1997; Seo *et al.* 1999). In fact, the application of 1 μM carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), a mitochondrial uncoupler, decreased the fluorescence of rhodamine 123 ($n_i = 5$; Fig. 3).

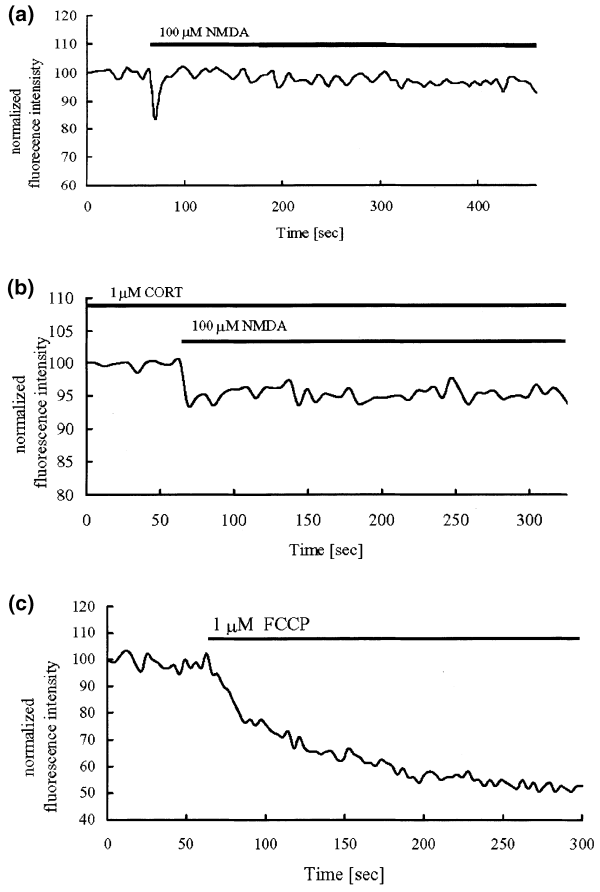

Fig. 4 Effect of CORT on the fluorescence of rhodamine 123, following NMDA stimulation in the hippocampal neurons. (a) The time course of rhodamine 123 fluorescence, following 100 μM NMDA application in the absence of CORT in a Mg^{2+}-free BSS. The initial fluorescence intensity was adjusted to 100%. The vertical scale is the normalized fluorescence intensity of rhodamine 123, and the horizontal axis is the time in seconds. (b) The time course of rhodamine 123 fluorescence upon 100 μM NMDA application in the presence of 1 μM CORT. (c) The time course of rhodamine 123 fluorescence upon the application of 1 μM FCCP.

PREGS-induced potentiation of NMDA receptor-mediated Ca^{2+} signals

By means of preincubation of the neurons with PREGS for 10–20 min, a significant potentiation was generated in the peak amplitude of the transient $[Ca^{2+}]_i$ elevation induced by NMDA (Fig. 5). In the presence of 100 μM PREGS, the peak amplitude of the $[Ca^{2+}]_i$ elevation was 0.36 ± 0.01 [expressed in terms of $\Delta(F_{340}/F_{380})$; $n_i = 5$, see Table 1]. The proportion of NMDA-responsive neurons was $92.1 \pm 2.2\%$ ($n_i = 7$) in the presence of PREGS. It should be emphasized that PREGS did not change the time duration of the NMDA-induced transient Ca^{2+} elevation (which lasted

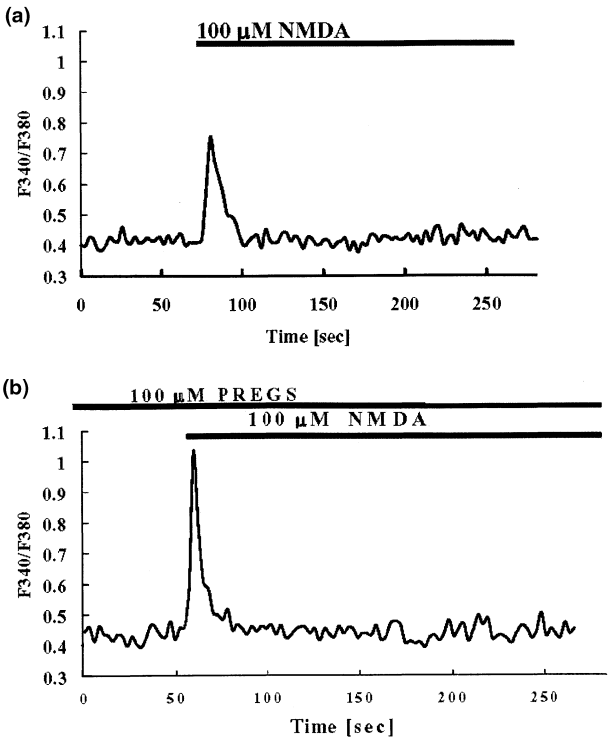

Fig. 5 Effect of pregnenolone sulfate on the magnitude of NMDA-induced Ca^{2+} signaling in the hippocampal neurons. The time course of transient $[Ca^{2+}]_i$ elevation in fura-2-loaded hippocampal neurons in the absence (a) and the presence (b) of 100 μM PREGS induced by the application of 100 μM NMDA in a Mg^{2+}-free BSS. The vertical scale F340/F380 is the ratio of fura-2 fluorescence excited at 340 nm and 380 nm, and the horizontal axis indicates the time in seconds.

for 30–70 s), although it did cause a significant increase in the mean peak magnitude of Ca^{2+} elevation. These results may correlate with the fact that PREGS increased the frequency of channel opening, although PREGS did not considerably enhance the mean duration of channel opening, or the current flow through a single NMDA receptor (Bowlby 1993; Wong *et al*. 1994).

Discussion

Characteristics of NMDA-induced Ca^{2+} signals in hippocampal neurons

Three distinct phases of Ca^{2+} dynamics (i.e. rise, decay, and plateau) were observed in the neurons, after NMDA application. This behavior may result from the difference in the time dependences of Ca^{2+} influx and extrusion. That the internal Ca^{2+} store did not contribute to the observed dynamics was established using thapsigargin (see Results and Table 1). In the rise phase, Ca^{2+} influx via the NMDA receptors exceeded Ca^{2+} extrusion. Conversely, in the following decay phase, Ca^{2+} extrusion exceeded Ca^{2+} influx via the NMDA receptors, probably due to an inactivation of the NMDA receptors as well as Ca^{2+} extrusion. In the plateau phase, influx and extrusion were balanced. Ca^{2+} influx depends on the activity of Ca^{2+}-permeable receptors/channels. Ca^{2+} extrusion, on the other hand, is sensitive to intracellular $[Ca^{2+}]_i$. It should be noted that Ca^{2+} extrusion may occur even before the inactivation of the NMDA receptors, because the rate of Ca^{2+} extrusion is a function of the level of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ plateau level following the rise and decay phases was the same as the initial resting level for the neurons obtained from postnatal 3–5-day-old rats, and 74% for the neurons obtained from embryonic E19–E20 rats (see Results). This difference in $[Ca^{2+}]_i$ decay may be due to differences in the level of Ca^{2+} extrusion by Ca^{2+}-ATPases or Ca^{2+} transporters (Garcia and Strehler 1999).

It was also confirmed that 10 μM MK-801, alone had no effect on $[Ca^{2+}]_i$, which suggests that MK-801 had no effect on either Ca^{2+} pumps or Ca^{2+} transporters.

Characteristics of the acute effect of glucocorticoids on NMDA-induced Ca^{2+} signals

Although there are many reports indicating that neurosteroids such as PREGS and dehydroepiandrosterone sulfate might rapidly alter the excitability of neurons via the modulation of ligand-gated receptors such as NMDA and $GABA_A$ receptors (Wu *et al*. 1991; Irwin *et al*. 1992; Bowlby 1993), the rapid effects of glucocorticoids and mineralocorticoids have not been well elucidated, particularly on transmitter-gated receptors (Joels 1997; Lupien and McEwen 1997). The present study demonstrates both the rapid and transient effects of CORT (within 20 min) on the NMDA receptor-mediated Ca^{2+} signaling, in postnatal hippocampal neurons at stress-induced high concentrations. The presence of CORT caused the extreme prolongation of NMDA receptor-mediated $[Ca^{2+}]_i$ elevation, resulting in a loss of mitochondrial membrane potential. Both dexamethasone (a strong agonist of glucocorticoid receptors) and cortisol (a dominant stress steroid in primates and cattle) caused similar acute prolongation effect on the NMDA receptor-mediated Ca^{2+} signals. On the other hand, PREGS increased the Ca^{2+} signal amplitude without essentially affecting the time duration of transient $[Ca^{2+}]_i$ elevation. Because CORT and PREGS, in the same concentration range, modulated NMDA receptor-mediated Ca^{2+} signaling very differently, the observed effects should not be non-specific due to, for example, the membrane-solvation of steroids or membrane-disordering by steroids. The existence of a specific mechanism of steroid-modulation for NMDA receptor-mediated Ca^{2+} signaling is also supported by our observations that progesterone did not affect NMDA-induced Ca^{2+} signaling, and that the coapplication of progesterone with CORT completely prevented the modulatory effect of CORT.

The blocking of NMDA receptors with MK-801 completely abolished Ca^{2+} signals, even in the presence of CORT. Because the net rate of change in $[Ca^{2+}]_i$ is equal to the difference between the influx and the extrusion of Ca^{2+}, the observed decay in the Ca^{2+} signal indicates that Ca^{2+} extrusion exceeded Ca^{2+} influx, after the blockage of Ca^{2+} influx via NMDA receptors by MK-801 (again, note that MK-801 itself did not change $[Ca^{2+}]_i$). Because (i) blocking L-type Ca^{2+} channels with nicardipine did not abolish the prolongation effect of CORT and (ii) the KCl-induced Ca^{2+} signal was not affected by CORT, voltage-sensitive Ca^{2+} channels are probably not involved in the CORT-induced prolongation of Ca^{2+} signals. These findings imply that the observed CORT effect is probably caused by modulation of the NMDA receptor-mediated Ca^{2+} influx.

The efficiency of these steroids on NMDA receptor-mediated Ca^{2+} signaling may not be lower than for $GABA_A$ receptors, as a PREGS concentration in excess of 1 μM was also necessary to modulate $GABA_A$ receptors. PREGS and dehydroepiandrosterone are known to have specific, high-affinity binding sites on $GABA_A$ receptors, whose modulation efficiencies are not altered by the agonists or antagonists of the benzodiazepine or barbiturate sites (Majewska *et al*. 1990; Demirgoren *et al*. 1991; Le Foll *et al*. 1997).

Possible mechanism of the acute glucocorticoid actions

According to the classical view of the steroid action, CORT is thought to penetrate into the cytoplasm of neurons, bind to intracellular receptors and induce genomic effects through new protein synthesis, resulting in the modulation of Ca^{2+} signals. The possibility of these classical genomic mechanisms can be excluded as an explanation for the present CORT effect however, for the following reasons. Cycloheximide, an inhibitor of protein synthesis, did not

abolish the effect of CORT on NMDA-induced Ca^{2+} signals. In addition, the present CORT effect was transient and immediately disappeared when CORT was removed (see Fig. 3), which is also distinct from the behavior expected from a genomic effect. The incubation time with CORT of 20 min is probably too short for the associated genomic effects via intracellular steroid receptors to appear (Sapolsky 1999), and even BSA-conjugated CORT was observed to induce a rapid prolongation of NMDA-induced Ca^{2+} signals without penetrating into the cytoplasm. The rapid CORT effect should therefore be due to a non-genomic effect via membrane surface receptors. This conclusion is further supported by the absence of a pronounced effect by specific agonists and antagonists of intracellular glucocorticoid receptors on the CORT-induced modulation of NMDA-induced Ca^{2+} signals. RU28362 (a synthetic agonist) caused no significant prolongation of NMDA-induced Ca^{2+} signals, and RU38486 (a synthetic antagonist) caused no significant inhibition of the CORT-induced prolongation of NMDA-induced Ca^{2+} signals.

The rapid decay of Ca^{2+} signals after the removal of CORT is probably due to a rapid inactivation of NMDA receptors, which occurred after the rapid release of CORT from its binding site. We used a 20-min preincubation for loading CORT, to account for likelihood that correct insertion of CORT to the binding sites on the cell surfaces probably requires a number of trial-and-error binding attempts, in contrast with the removal of CORT, which needs only one release process. Because the membrane non-permeable BSA-CORT was also observed to induce a rapid effect, it is likely that CORT binds to putative surface CORT receptors. Candidates for surface CORT receptors are unknown CORT receptors, NMDA receptors, and classical intracellular glucocorticoid receptors (e.g. GR, a type-2 receptor), which are bound to plasma membranes. This idea is supported by reports that the immunoreactivity of antibodies against GR was associated with plasma membranes from hippocampal and hypothalamic neurons (Liposits and Bohn 1993) and that specific CORT binding to neuronal membranes occurred in different brain areas with moderate affinity (K_d = 120 nM) (Towle and Sze 1983; Guo *et al*. 1995). Classical GRs are expressed in the cytoplasm of cultured hippocampal neurons (Packan and Sapolsky 1990). These glucocorticoid receptors are functional in genomic pathways (Nishi *et al*. 2001). Cultured hippocampal neurons seem to have both non-genomic corticosteroid action pathways and classical genomic pathways.

We speculate here on the existence of a possible protein kinase-dependent signaling pathway, from surface CORT receptors to NMDA receptors. CORT has recently been found to acutely and non-genomically modulate protein kinase C-dependent cascades via putative membrane binding sites (Qiu *et al*. 2001). It is also known that Ca^{2+}-dependent protein kinase C modulates NMDA receptor activation via two types of pathways: (i) the direct phosphorylation of NMDA receptors (NR1 subunit), or (ii) the indirect protein kinase C-dependent pathway, which involves the activation of other kinases (e.g. tyrosine kinases), and results in the phosphorylation of different NMDA receptor subunits. The direct pathway induces the NMDA receptor inactivation, while the indirect pathway activates NMDA receptors via the reduction of Ca^{2+} dependent inactivation (MacDonald *et al*. 2001). Taken together, our results could be explained by the binding of CORT to putative membrane glucocorticoid receptors, which induces the activation of the indirect protein kinase C-dependent pathways, resulting in the prolonged activation of the NMDA receptors. The decay of the prolonged Ca^{2+} elevation after CORT removal can be interpreted as the termination of this indirect protein kinase C-dependent process.

The counteracting effect of progesterone on the CORT action could be due to the competitive inhibition of CORT by progesterone at membrane glucocorticoid binding sites. Actually, progesterone has been demonstrated to bind to the membrane glucocorticoid binding sites with an affinity higher than that of dexamethasone (Guo *et al*. 1995). A sigma receptor-dependent pathway might be another possible candidate for the mechanism of the present counteracting effect of progesterone on the CORT action, because progesterone is known to suppress sigma receptor-mediated activation of the NMDA response in neurons (Bergeron *et al*. 1996; Maurice *et al*. 2001). If this is the case, CORT should act as an agonist of sigma receptors. This is, however, probably not the case, because (i) both CORT and progesterone are reported to inhibit the binding of sigma-1 agonist (e.g. SKF-10 047) to the sigma receptors (Su *et al*. 1990), and (ii) in contrast to the action of stress levels of CORT, most agonists of sigma receptors (sigma-1 and sigma-2 agonists) have antistress, antidepressant, and anti-amnesic actions (Maurice and Privat 1997; Maurice *et al*. 2001), indicating that CORT does not act as an agonist of sigma receptors.

It is also improbable that the present CORT effect on NMDA-induced Ca^{2+} signals is due to the reported inhibitory action of CORT on sigma receptors (Su *et al*. 1990), because progesterone, an antagonist of sigma receptors (Maurice *et al*. 2001), did not induce the prolongation of the NMDA-induced Ca^{2+} signals (see Table 1).

Neurophysiological consequences of the acute effect of glucocorticoids on NMDA-induced Ca^{2+} signal

Investigations of dose dependency showed that CORT was effective at a minimal dose of 0.1 μM and became considerably more effective in the range, 0.5–1 μM (Fig. 2). Rats which were subjected to immobilization-stress for 1 h showed high plasma CORT concentrations (e.g. 800 ng/mL (approximately 2 μM)), implying that CORT at 0.5–1 μM may be naturally secreted under stressful conditions

(Marinesco *et al*. 1999). The present CORT effect is therefore likely to be physiologically significant.

Demonstrations of rapid, non-genomic glucocorticoid action on neuronal excitability have accumulated in recent years (Lupien and McEwen 1997). In addition, extensive investigations have been conducted to investigate inhibitory effects on LTP-induction and learning, which result from chronic exposure (1–24 h) to high levels of glucocorticoids (McEwen 1996; Pavlides *et al*. 1996; Kim and Yoon 1998).

We have demonstrated further that the pre-treatment of 10 μM CORT for 20 min significantly suppresses the development of LTP in the CA1 region of the hippocampus upon high frequency tetanic stimulation with 100 Hz in 1 mM Mg^{2+} medium (Kawato *et al*. 2001). There are previous reports showing the CORT-induced suppression of the magnitude of LTP in the CA1 region and CA3 region, upon incubation with a high concentration of CORT and its agonists for 1 h (Kerr *et al*. 1994; Rey *et al*. 1994). In addition, because (i) prolonged Ca^{2+} elevation causes a permeability transition of the mitochondrial membranes, resulting in mitochondrial dysfunction (Nicholls *et al*. 1999), and (ii) the dysfunction of mitochondria is known to suppress LTP induction (Albensi *et al*. 2000), the CORT-induced suppression of LTP may be due, in part, to a dysfunction of the mitochondria induced by a prolonged decrease in the mitochondrial membrane potential.

We have recently discovered the neurosteroidogenic systems in pyramidal and granule neurons in the rat hippocampal formation, where synthesis of pregnenolone and PREGS from endogenous cholesterol is driven by Ca^{2+} influx through NMDA receptors (Kawato *et al*. 2000). CORT synthesis from [^{3}H]deoxycorticosterone has also been demonstrated in the rat hippocampus (Gomez-Sanchez *et al*. 1997). These novel observations led to the hypothesis that stress-induced CORT production may be performed not only in adrenal glands but also within the hippocampus, resulting in the acute and effective modulation of NMDA receptors and LTP.

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