Korean J Physiol Pharmacol
Vol 9: 363–369, December, 2005

Calcium Ion Dynamics after Dexamethasone Treatment in Organotypic Cultured Hippocampal Slice

Hee-Jung Chae, Tong-Ho Kang, and Ji-Ho Park

Department of Medical Science, Graduate School of East-West Medical Science, Kyunghee University, Gyeonggi 449–701, Korea

It is imperative to analyse brain injuries directly in real time, so as to find effective therapeutic compounds to protect brain injuries by stress. We established a system which could elucidate the real time Ca^{2+} dynamics in an organotypic cultured hippocampal slice by the insults of artificial stress hormone, dexamethasone. The real time Ca^{2+} dynamics could continuously be detected in cornus ammonis 3 (CA3) of the organotypic hippocampus for 8 hours under confocal microscopy. When dexamethasone concentration was increased, the Ca^{2+} was also increased in a dose dependent manner at 1~100 μM concentrations. Moreover, when the organotypic cultured hippocampal slice was treated with a glutamate receptor antagonist together with dexamethasone, the real time Ca^{2+} dynamics were decreased. Furthermore, we confirmed by PI uptake study that glutamate receptor antagonist reduced the hippocampal tissue damage caused by dexamethasone treatment. Therefore, our new calcium ion dynamics system in organotypic cultured hippocampal slice after dexamethasone treatment could provide real time analysis method for investigation of brain injuries by stress.

Key Words: Calcium dynamics, Hippocampus, Stress, Dexamethasone, Glutamate antagonist

INTRODUCTION

Ca^{2+} acts as a universal intracellular messenger to modulate many processes such as neurotransmission, enzyme and hormone secretion as well as many biological processes (Berridge et al, 1998). Accordingly, measurement of intracellular Ca^{2+} level would be useful to detect the condition of a cell. To study the dynamics of Ca^{2+} in the cytosol, optical techniques in combination with Ca^{2+}-sensitive fluorescent dyes have become the most frequently employed method (Takahashi et al, 1999). A variety of fluorescent Ca^{2+} indicators are available, most of which simply increase their fluorescence intensity through binding to Ca^{2+} (Thomas et al, 2000). The principal biological Ca^{2+} indicators in current use include fluorescent compounds that undergo large fluorescence enhancements or spectral shifts upon binding to Ca^{2+} (Thomas et al, 2000). The success of these dyes in reporting accurate time-dependent [Ca^{2+}] requires not only properly controlled studies, but also appropriate choices of dyes for the specific intracellular conditions in which the Ca^{2+}-probe will encounter (David, 1999). Key characteristics of fluorescent Ca^{2+}-indicators that govern their usefulness include excitation and emission wavelengths (λex and λem), fluorescence enhancement induced by Ca^{2+}-binding, Ca^{2+} dissociation constants (K_d (Ca^{2+})) and ease of use (David, 1999).

Corresponding to: Tong-Ho Kang and Ji-Ho Park, Department of Medical Science, Graduate School of East-West Medical Science, Kyunghee University, 1 Seocheon-ri, Giheung-eup, Yongin-si, Gyeonggi-do 449-701, Korea. (Tel) 82-31- 201-2187, (Fax) 82-31-201-2180, (E-mail) jihopark@khu.ac.kr

Fluo-4 used in the present study exhibits high fluorescence emission, a high rate of cell permeation, and a large dynamic range for reporting [Ca^{2+}] at around 350 nM K_d (Ca^{2+}) (Haugland, 2002). Because of its high fluorescence emission intensity, Fluo-4 could be used at low concentrations (David, 1999), and it is well suited for photometric and imaging applications that make use of confocal laser scanning microscopy, flow cytometry, or spectrofluorometry and also in fluorometric high-throughput microplate screening assays (Gee et al, 2000). Real-time confocal microscopy has been a powerful tool to observe functional changes of cells in living condition (Haugland et al, 2002). The confocal microscopy for Ca^{2+} imaging has permited to calibratie the imaging system and calculate of absolute Ca^{2+} concentrations in the cell (Christian, 2003). During the past decade, an increasing number of investigators have used confocal laser scanning microscopy to study Ca^{2+} signaling in a cell (Berridge et al, 1998), however, a cell provides only a limited insight into the mechanisms that operate in tissue. More useful information can be obtained by using intact tissue (Saino & Satoh, 2004), but there are limitations for the recording of spatio-temporal physiological change in tissue preparation. One of the most difficult problems is to maintain a condition of living tissue for several hours. Because of this reason, we developed a system which includes an organotypic cultured hippocampal slice and techniques to maintain a tissue to overcome such problems.

Dexamethasone, a synthetic corticosteroid, can induce a

ABBREVIATIONS: CA3, cornu ammonis 3; DG, dentate gyrus; AP-3, DL-2-Amino-3-Phosphonopropionic acid; PI, propidium iodide; ROI, region of interest; aCSF, artificial cerebrospinal fluid.

neuronal damage in CA3 and DG regions of hippocampus (Haynes, 2001). Glucocorticoid is secreted from the adrenal gland (Chrels & Andrew, 2003), and the stress-related hormone targets the brain, including hippocampus (McEwen & Sapolsky, 1995). In the present study, the accumulation of Ca^{2+} in CA3 was found to significantly increase after 100 μM dexamethasone treatment. It is most likely due to cell death induced by Ca^{2+} overload (Schuster et al, 1999). Furthermore, the effect of dexamethasone could be ameliorated by treatment with some antagonists of glutamate receptor (Supko, 1994).

The newly developed system successfully obtained the spatio-temporal functional change of Ca^{2+} dynamics in the cell of hippocampus for several hours. Employing this system, we tried to apply a full repertoires of stress related glutamate mechanisms in view of Ca^{2+} dynamics of hippocampal cultured tissue.

METHODS

Organotypic hippocampal culture

Hippocampal slice cultures were performed by an interface culture method, originally developed by Stoppini et al (Stoppini et al, 1991). Sprague-Dawley rats (7 days old) were decapitated, and their brains were quickly removed under sterile condition. After isolation of the hippocampi, their dorsal halves were sectioned transversely at 350 μm using a tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK). Slices were placed in chilled HBSS-medium (Sigma Co., St. Louis, MO, USA) with 20 mM HEPES (Sigma Co., St. Louis, MO, USA). The slice was placed on a membrane insert (polytetrafluorethylene membranes, 0.4 μm, Millicell-CM, Millipore Co., Bedford, MA, USA), which was set into the 6-well plates filled with 750 μl of culture medium containing 50% MEM-medium (LM 007-01, JBI, Daegu, South Korea), 25% inactivated horse serum (S 104-01, JBI, Daegu, South Korea), 25% Hank's balanced salt solution (LB 003-1, JBI, Daegu, South Korea), 6 g/L D-glucose (G 7528, Sigma Co., St. Louis, MO, USA), 1 mM L-glutamine (G-8540, Sigma, St. Louis, MO, USA), 20 mM HEPES (H-4034, Sigma, St. Louis, MO, USA) and 1% penicillin-streptomycin (Gibco BRL, USA) at pH 7.3. The medium was changed every 3 or 4 days, and cultures were used after 14 days in an incubator at 36°C with 5% CO_2.

Calcium sensitive dye loading

Slices cultured for 2 weeks were stabilized in an artificial cerebrospinal fluid (aCSF; in mM; 135 NaCl, 3.5 KCl, 1 $MgCl_2$, 15 $NaHCO_3$, 1.5 Glucose, 2 $CaCl_2$, 20 HEPES, 30 Sucrose) for 60 min at 36°C and then incubated with calcium indicator dye Fluo-4 AM (acetoxymethyl) ester (4.5 μM; 28C2-3, Molecular Probes, Eugene, OR, USA) in aCSF for 90 min at 36°C in dark. The dye was dissolved in DMSO (final concentration: 0.1%, v/v). After dye loading, the slice was rinsed three times and reincubated in aCSF for 4 hour at 36°C to allow de-esterification of the dye and clearing of background. All slices were gassed with 5% CO_2 to maintain pH at physiological level.

Acquisition of calcium image

For confocal imaging, the tissue with attached insert membrane was placed in an open perfusion microincubator (MPS-2000, SEC). The contained tissue microincubator which was placed in a Petri dish with small cuts and mounted on the microscope stage. It was perfuse with aCSF that contained the reagents (1, 10 and 100 μM dexamethasone, and 30 μM AP-3P) using microperfusion system (MPS-2000, SEC). Slice was maintained in 5% CO_2 (CTI-controller 3700 digital, Zeus, Oberko, Germany), physiological pH and 33°C during the measurement of Ca^{2+} dynamics. Under these conditions, Ca^{2+} fluorescence levels in tissue were stable for up to 8 hours.

Measurements of Ca^{2+} flow in CA3 region's cells of cultured hippocampal slice were performed with a confocal laser scanning microscope (Zeiss LSM 510, Zeiss, Oberko, Germany) at 33°C. The Ca^{2+}-sensitive dye Fluo-4 was excited by 488 nm line of a krypton-argon laser. (Excitation and emission signals were separated by a diachronic mirror at a wavelength of 488 nm, and emission signal was turned by a 505~550 nm optic band pass filter) (Andreas et al, 2004).

The Ca^{2+} images were taken with 30 frames at a frequency of 6 Hz every hour, using 10 X neofluor objectives (sequence of fluorescence images was sampled in one focal plane). Avoidance of photobleaching and cytotoxic effects of the laser illumination made it possible to continuously monitor cytosolic Ca^{2+} concentration over several minutes at fast acquisition rates (491 msec for a 512×512 pixel scan field).

A region of interest (ROI) was defined in the whole area of image obtained. All settings of the laser-, optical filter- and microscope as well as date acquisition were controlled by PC software (confocal microscopy software Release 3.2, Zeiss, Oberko, Germany).

Treatment of chemicals

All reagents were applied by using a perfusion system (MPS-2000, SEC). Dexamethasone (D4902, Sigma, St. Louis, MO, USA) at 1, 10, and 100 μM concentrations was used. Tissues were treated with glutamate receptor antagonists shortly after capturing control image, and treated with dexamethasone after 1 hour (Fig. 1). Thirty mM metabotrophic glutamate receptor I antagonist, AP3 (A-4910, Sigma Co., St. Louis, MO, USA) was used. The concentration was determined, based on other works (Tocris cookson Ltd, 2004).

Calibration of Ca^{2+} concentration

The fluorescence of Fluo-4 was calibrated to estimate the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), using the formula 1. (Thomas et al, 2000; Takahashi et al, 1999).

$[Ca^{2+}]_i = K_d (F- F_{min}) / (F_{max} -F)$ ← Formula 1. (Table 1)

Where F is the fluorescence measured experimentally, F_{min} is the fluorescence for Ca^{2+}-free and F_{max} for Ca^{2+}-saturated condition. F_{min} was determined in a Ca^{2+}free aCSF containing 10 mM EGTA, and F_{max} was determined in an aCSF containing 10 mM $CaCl_2$. The K_d of 350 nM was taken from the manufacture (Haugland, 2002) (Fig. 2).

PI uptake

PI is a polar compound that enters only dead or dying cells with damaged membranes (Macklis and Madison, 1990). Once inside cells, PI binds to nucleic acids, inducing

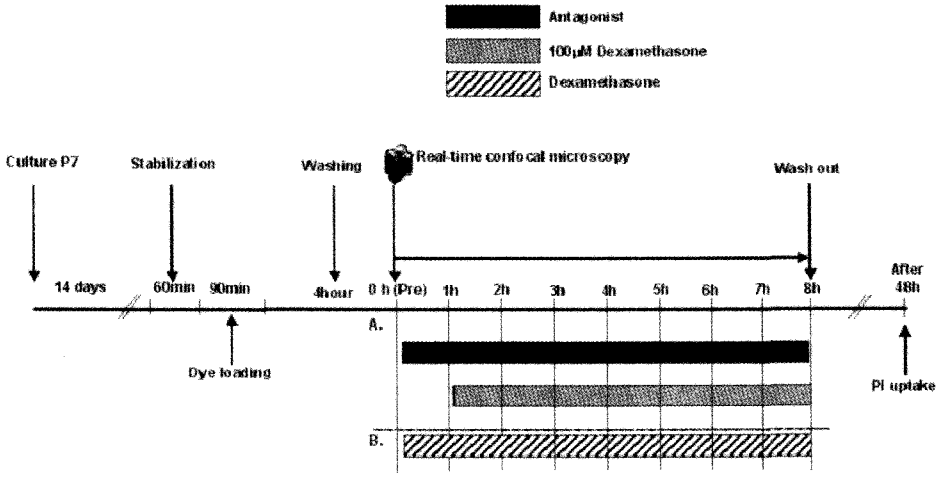
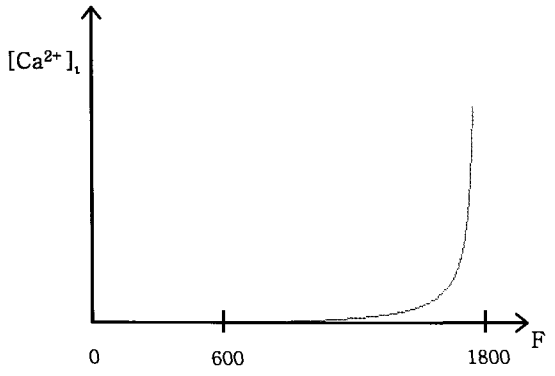


Fig. 1. Repertories of experiments. (A) The experimental procedure for antagonists treatment, (B) the experimental procedure for dexamethasone (0, 1, 10, and 100 μM).

Table 1. Formulat 1 for calculation of calcium concentration in the tissue

$[Ca^{2+}]_i=K_d*(F-F_{min})/(F_{max}-F)$
$F_{max}=Ca^{2+}$ – saturated (aCSF containing 10 mM $CaCl_2$)
$F_{min}=Ca^{2+}$ – free (aCSF containing 10 mM EGTA)
F=Examined value, Kd=350 nM
(Only single wavelength indicator)

(Thomas et al, 2000; Takahashi et al, 1999).



Standard curve of Ca^{2+} concentration in the tissue

intense red fluorescence when excited by green light (Macklis and Madison, 1990). At a concentration of 5 μg/ml, PI is basically non-toxic to neurons (Laake et al, 1999). Tissues were treated with the regents under the same condition of calcium image except calcium indicator after 8 hours. PI (5 μg/ml) was added to each well and PI stained images were captured, using a fluorescence inverted microscope (Axiovert S 100, Zeiss, Oberko, Germany) attached with a digital CCD camera (Axiocam, Zeiss, Oberko, Germany).

The observed PI uptake areas were measured using density slice' within image analysis program (Scion image beta 4.02 win, Scion Co., Maryland, USA), and the percent of

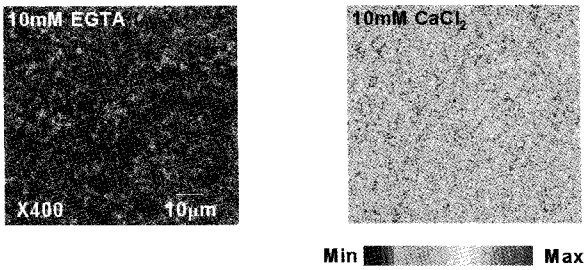


Fig. 2. Images of the F_{min} and F_{max}. The F_{min} value was 609.5± 15.02, and the F_{max} value was 1823.5±27.04. F_{min} is the fluorescence for Ca^{2+}-free and F_{max} for Ca^{2+}-saturated condition. F_{min} was determined in a Ca^{2+}-free aCSF containing 10 mM EGTA, and F_{max} was determined in an aCSF containing 10 mM $CaCl_2$.

neuronal death in each subfield was calculated.

Statistic analysis

All data whose background was subtracted by using the fluorescence emission originating from a region on the insert containing no tissue (Hua et al, 2004).

Data analysis was performed using confocal microscopy software Release 3.2 (Zeiss), Excel 2002 (Microsoft) and Sigma plot (SPSS inc, V6.0). The Ca^{2+}concentration was obtained from 30 frames each hour and these values were averaged. Data were expressed as mean±SEM with n indicating the number of tissues. Statistical notice was evaluated using one-way analysis of variance (ANOVA).

RESULTS

In order to observe changes of real time calcium dynamics after dexamethasone treatment, hippocampus was loaded with the Ca^{2+} indicator Fluo-4 AM, and intracellular Ca^{2+} levels were monitored using the quantitative confocal microscopy for up to 8 hours. All color images were pseudo-

colored. The blue color revealed the area of inactivation, while the more bright green color indicated the area of more activation by Ca^{2+}. Fig. 3 indicates that Ca^{2+} positive area was diffused from CA3 to CA1 with the laps of time (n=6, Fig. 3), therefore, measuring the Ca^{2+} dynamics at pyramidal layer of CA3.

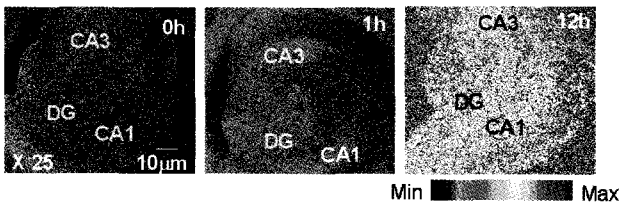


Fig. 3. Ca^{2+} dynamics after 100 μM dexamethasone treatment. The high Ca^{2+} area was gradually expanded from CA3 to CA1 after 100 μM dexamethasone treatment (the pseudocolor image). CA1: cornus ammonis 1, CA3: cornus ammonis 3, DG: dentate gyrus.

We examined whether Ca^{2+} dynamics affected dexamethasone concentration. As seen in Fig. 4A, Ca^{2+} intensities were remarkably increased with 100 mM dexamethasone, and Ca^{2+} intensity seemed to be not changed in control case [n=6, Fig. 4(A)]. This result indicates that photobleaching and cytotoxic effects of the laser illumination seemed not to affect the pyramidal cells of hippocampal tissue.

The treatment of 1 μM dexamethasone caused an increase of Ca^{2+} concentration up to 2 hours [182.46$\pm$4.68 nM, n=5; Fig. 4B] and then decreased afterward to the control level. In contrast, 10 and 100 μM dexamethasone treatment increased gradually calcium concentration until 4 hours [510.86$\pm$6.53 nM, n=5; 499.37$\pm$7.23 nM, n=5 and 1011.86$\pm$12.30 nM, n=5. Fig. 4B], and they did not return to the control level. Next, we determined destination of the damaged hippocampal cell, and the results showed that 10 and 100 μM dexamethasone treatments seemed to induce permanent cell damage, however, 1 μM dexamethasone appeared not to damage hippocampus (Fig. 6).

To test whether glutamate plays any role in Ca^{2+}

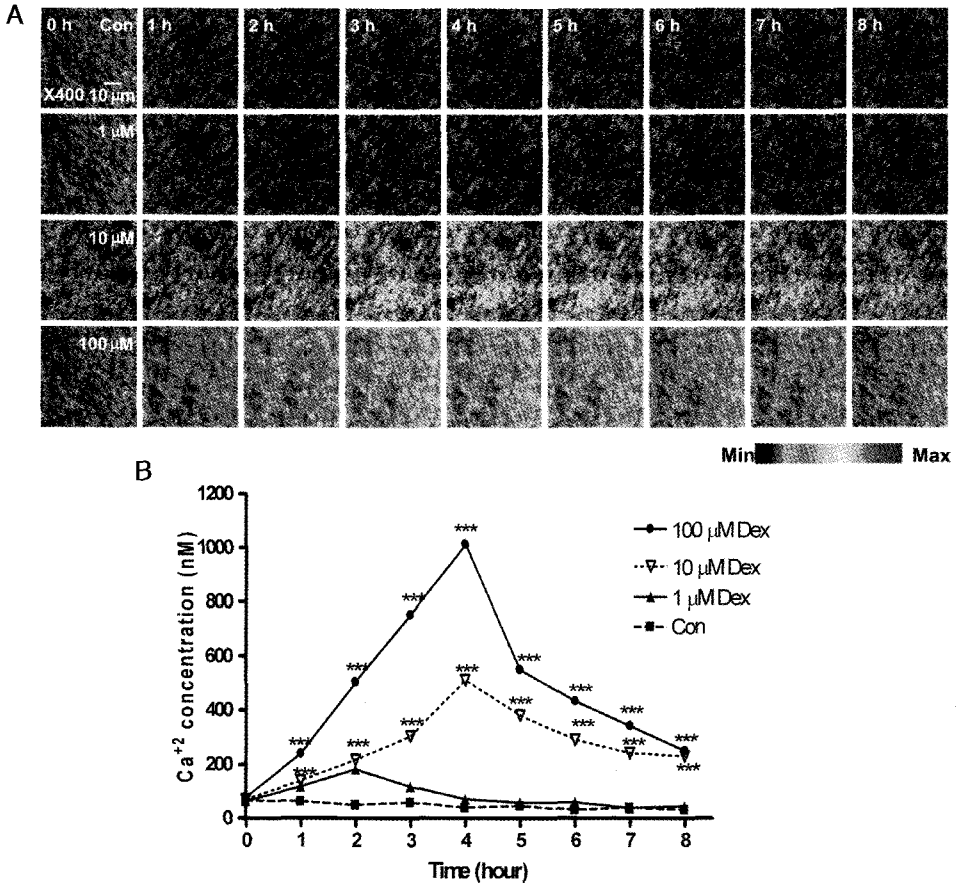


Fig. 4. (A) The change of Ca^{2+} intensities after dexamethasone treatment. The series of frame indicates the dynamics change of Ca^{2+} intensities in CA3 region of hippocampus. The blue color reveals area of un-activated, while the more bright green color indicates the more activation of cell by free Ca^{2+}. Con: control. (B) The change of Ca^{2+} concentration by dexamethasone treatment (1, 10, and 100 μM). The Ca^{2+} dynamics at CA3 by the treatment of 1 μM dexamethasone caused an increase of calcium intensity up to 2 hours and decreased to the control level afterward. In contrast, 10 and 100 μM dexamethasone (n=5) treatment caused gradual increase of calcium intensity till 4 hours, and they did not return to the control level. Dex; dexamethasone. Control: n=6 (Values of each group are expressed as mean$\pm$s.e.m. ***P$<$0.001 vs control group).

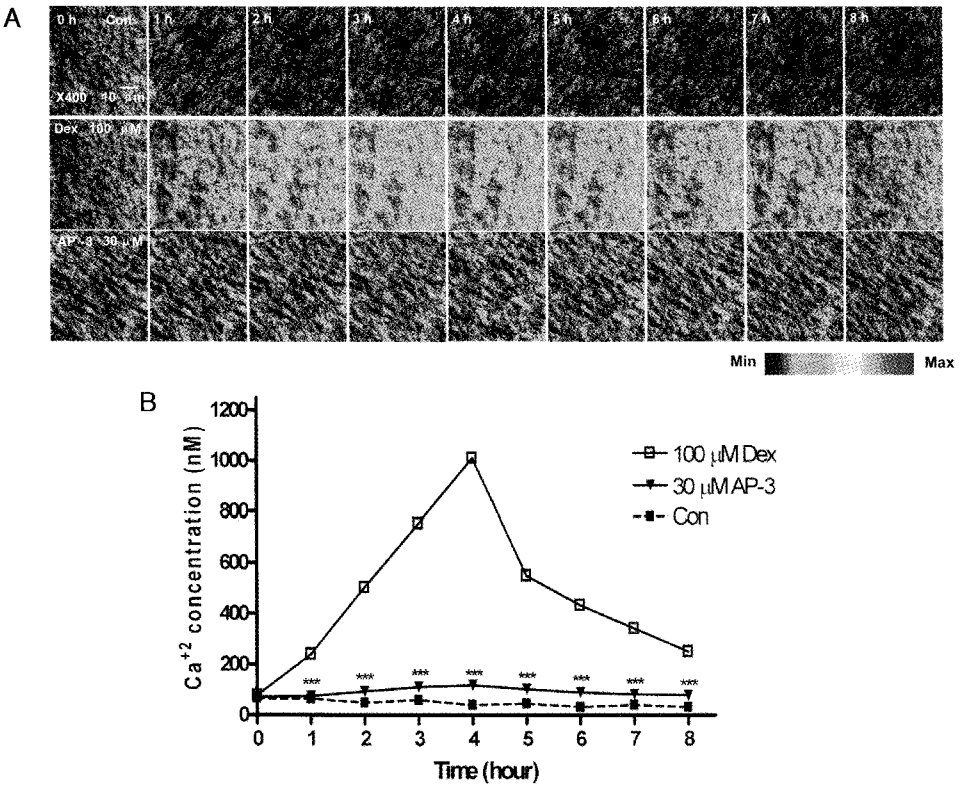


Fig. 5. (A) The change of Ca^{2+} intensities after glutamate receptor antagonist. The series of frame indicates the dynamics change of Ca^{2+} intensities in CA3 region of hippocampus. The blue color reveals area of un-activated, while the more bright green color indicates the more activation of cell by free Ca^{2+}. Con: control(n=6), Dex: Dexamethasone 100 μM (n=6), 30 μM AP-3: metabotrophic glutamate receptor I antagonist (n=3). (B) The change of Ca^{2+} concentration after glutamate receptor antagonist treatment. 30 μM AP-3 (metabotrophic glutamate receptor I antagonist, n=3) seemed to block the accumulation of Ca^{2+} induced by dexamethasone. Control: n=6. Dex:; Dexamethasone 100 μM (n=6), (Values of each group are expressed as mean±s.e.m. ***P<0.001 vs 100 μM dexamethasone group).

Table 2. The change of Ca^{2+} concentration after glutamate receptor antagonist treatment

	Con	100 μM Dex	30 μM AP-3
0	66.55±2.21	77.85±1.93	72.11±3.73
1	63.29±1.97	239.07±3.85	74.73[a]±3.69
2	48.71±2.23	503.38±6.39	92.93[a]±3.27
3	59.23±2.13	753.06±12.73	110.95[a]±4.15
4	39.28±1.38	1011.86±12.30	118.05[a]±4.69
5	45.75±1.50	550.59±11.32	102.21[a]±3.98
6	31.95±1.45	433.66±8.05	90.17[a]±3.40
7	39.17±2.04	342.36±6.79	82.29[a]±3.16
8	31.42±1.51	250.48±5.20	78.02[a]±2.43

Values of each group are expressed as mean±s.e.m. [a]significant difference (P<0.001) compared with 100 mM dexamethasone group. 30 mM AP-3 (metabotrophic glutamate receptor I antagonist, n=3) seemed to block the accumulation of Ca^{2+} induced by dexamethasone. Con: Control (n=6), Dex: 100 mM Dexamethasone (n=6).

dynamics during dexamethasone treatment, tissues was treated with a glutamate receptor antagonist together with 100 μM dexamethasone. The treatment of type I metabotrophic glutamate receptor antagonist AP-3 (30 μM, n=3) seemed to attenuate the accumulation of Ca^{2+} induced by dexamethasone (Fig. 5, Table 2). Therefore, as seen in Fig. 6, the results of PI uptake and glutamate receptor antagonist (30 μM AP-3) indicate that the hippocampal tissue damage induced by dexamethasone was reduced by the antagonist.

DISCUSSION

In this study, we tried to elucidate the real-time Ca^{2+} dynamics after dexamethasone treatment using confocal microscopy. Fluorescence microscopy imaging has been developed for the study of cell structure and function in cell biology. This technique makes it possible to characterize, localize and qualitatively quantify free ions, messengers, pH, voltage and other molecules in living cells (Haugland et al, 2002).

In the present study, the intracellular Ca^{2+} dynamics in CA3 were found to change after dexamethasone treatment (Fig. 3). However, it is not certain whether Ca^{2+} dynamics in the cell originated from extracellular space or intracellular store. The fine image could not be obtained because the equipment processed in this laboratory has low resolution, and the origin of Ca^{2+} dynamics after dexamethasone

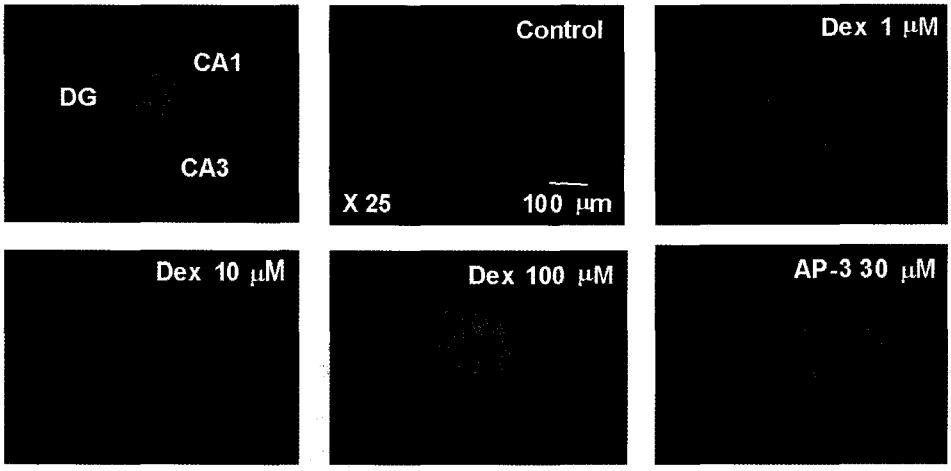


Fig. 6. Regional distribution and propidium iodide (PI) uptake after 48 hours. Neuronal cell death was photographed by PI fluorescence imaging that was performed with a fluorescence microscope at 514 nm. 10 and 100 μM dexamethasone treatment seemed to induce permanent cell damage, but 1 μM dexamethasone seemed not to damage hippocampus. Glutamate receptor antagonist (30 μM AP-3) treatment reduced the hippocampal tissue damage induced by dexamethasone treatment. Bar=100 μM.

treatment needs to be studied in future.

Dexamethasone treatment caused change of Ca^{2+} dynamics in CA3 region of hippocampus. It has been suggested that there are two different features of response: the cases of low and high concentrations treatment. It was remarkable that the calcium dynamics in CA3 increased up to 2 hours after the treatment of 1 μM and 5 μM dexamethasone and then decreased to the control level (experiment about 5 μM dexamethasone treatment is not shown). In contrast, however, 10 and 100 μM dexamethasone treatment gradually increased the calcium intensity till 4 hours, and they did not return to the control level (Figs. 4A and Fig. 6). It is quite likely that low concentration of dexamethasone could recover the damage, while high concentration of dexamethasone treatment might cause a permanent damage on the cell. As evidenced by PI stain results (Fig. 6), The accumulation of Ca^{2+} seems to lead tissue to cell death.

It has been hypothesized that the elevation of Ca^{2+} by dexamethasone is associated with glutamate receptor (Supko, 1994) and stress hormone-related receptor (Elicia et al, 1993). Therefore, the glutamate receptor antagonists were tried in this new image system. As shown in Fig. 5, the accumulation of Ca^{2+} was blocked by metabotrophic glutamate receptor I antagonist.

Stress related-hormones increase free cytosolic calcium concentrations in cultured hippocampal neuron (Elicia et al, 1993) and voltage-dependent calcium conductance and prolong calcium spike duration in the CA1 cell of hippocampus (Lee et al, 2002). The dexamethasone and glucocorticoids are bound to the glucocorticoid receptor (GR) with high affinity (Kloet, 2003). When glucocorticoids exist at low level, they are mediated by a high affinity corticosteroid receptors that are heavily concentrated in the hippocampus. In contrast, however, when glucocorticoid level is raised, these steroids have deleterious effects on the hippocampus and they are mediated by a low affinity receptor (McEwen & Sapolsky, 1995; Lee et al, 2002).

Based on the above considerations, MR appears to be important for calcium increase at high concentration of dexamethasone. In the treatment with low concentration (1 μM and 5 μM) of dexamethasone, Ca^{2+} dynamics was decreased after 2 hours. It seemed that this is the underlying mechanism to reduce the GR activation. The mechanisms of GR and MR are still poorly understood, however, it seems quite possible that MR activation affects the maintenamce of GR activation (Meijer et al, 2004).

Taken together, these results led us to conclude that chronic Ca^{2+} image obtained through this system is useful in investigation of structural and functional changes of cells in living tissues, therefore, this technique could be applicable to various studies.

ACKNOWLEDGEMENT

This work was supported by grants of Brain Korea 21 projects (Ministry of Education, Korea) and by the SRC/ERC program of MOST/KOSEF (R11-2002-103).

REFERENCES

Andreas B, Robin ZN, Hans-Peter S, Joachim WD. Calcium release from intracellular stores in rodent astrocytes and neurons in situ. *Cell Calcium* 35: 47–58, 2004

Berridge MJ, Bootman MD, Lipp P. Calcium - a life and death signal. *Nature* 395: 645–648, 1998

Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signaling. *Nat Rev Mol Cell Biol* 1: 11–21, 2000

Christian L. Monitoring neuronal calcium signaling using a new method for ratiometric confocal calcium imaging. *Cell Calcium* 34: 295–303, 2003

Chrles LR, Andrew HM. When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. *Am J Psychiatry* 160: 1554–1565, 2003

Ciriza I, Azcoitia I, Garcia-Segura LM. Reduced progesterone metabolites protect rat hippocampal neurones from kainic acid excitotoxicity in vivo. *J Neuroendocrinol* 16: 58–63, 2004

Colin B. Cellular calcium imaging: so, what's new? *Trend In Cell Biology* 10: 451–457, 2000

David GL. Calcium signaling protocols. 1st ed. *Humana Press,* Leicester UK, 1999

Elicia ME, Robert MS. Corticosterone impairs hippocampal neuronal calcium regulationpossible mediating mechanisms. *Brain Res* 602: 84–90, 1993

Fabienne R, Anne-Marie B, Pierre B. Dynamic study of cell mechanical and structural responses to rapid changes of calcium level. *Cell Motility and Cytoskeleton* 45: 93–105, 2000

Gee KR, Brown KA, Chen W-NU, Bishop-Stewart J, Gray D, Johnson I. Chemical and physiological characterization of fluo-4 Ca^{2+}-indicator dyes. *Cell Calcium* 27: 97–106, 2000

Haugland RP. Handbook of fluorescent probes and research products. 9th ed. *Molecular Probes,* USA, 2002

Hua W, Erik BM, Sunjara V, Steven ER, Wen-hong L. Ca^{2+} dependent glutamate release involves two classes of endoplasmic reticulum Ca^{2+} stores in astrocytes. *J Neurosci Res* 76: 86–97, 2004

Joëls M. Corticosteroid actions in the hippocampus. *J Neuroendo* 13: 657–669, 2001

Kloet ER. Hormones, brain and stress. *Endocrine Regulations* 37: 51–68, 2003

Kobayashi T, Yamada T, Okada Y. The levels of adenosine and its metabolites in the guinea pig and rat brain during complete ischemia-in vivo study. *Brain Res* 787: 211–219, 1998

Laake JH, Haug FM, Wieloch T, Ottersen OP. A simple in vitro model of ischemia based on hippocampal slice cultures and propidium iodide fluorescence. *Brain Res Protoc* 4: 173–184, 1999

Lee AL, Ogle WO, Sapolsky RM. Stress and depression: possible links to neuron death in the hippocampus. *Bipolar Disord* 4: 117–128, 2002

Macklis JD, Madison RD. Progressive incorporation of propidium iodide in cultured mouse neurons correlates with declining electrophysiological status: a fluorescence scale of membrane integrity. *J Neurosci Methods* 31: 43–46, 1990

McEwen BS, Sapolsky RM. Stress and cognitive function. *Curr Opin Neurobiol* 5: 205–216, 1995

Meijer OC, Kalkhoven E, Laan S, Steenbergen PJ, Houtman SH, Dijkmans TF, Pearce D, de Kloet ER. Steroid receptor coactivator-1 splice variants differentially affect corticosteroid receptor signaling. *Endocrinology* 24: 1–48, 2004

Saino T, Satoh Y. Application of real-time confocal laser scanning microscopy to observe living cells in tissue specimens. *J Electron Microsc* 53: 49–56, 2004

Schuster S, Marhl M, Hofer T. Modeling of simple and complex calcium oscillations. *Eur J Biochem* 269: 1333–1355, 2002

Stephen AS, Whitaker M. Confocal laser scanning microscopy of calcium dynamics in living cells. *Microscopy Reseach and Technique* 46: 356–369, 1999

Stoppini LP, Buchs A, Muller D. A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* 37: 173–193, 1991

Supko DE, Johnston MV. Dexamethasone potentiates NMDA receptor-mediated neuronal injury in the postnatal rat. *Eur J Pharmacol* 270: 105–113, 1994

Takahashi A, Camacho P, Lechleiter JD, Herman B. Measurement of intracellular calcium. *Physiol Rev* 79: 1089–1125, 1999

Thomas D, Tovey SC, Collins TJ, Bootman MD, Berridge MJ, Lipp P. A comparison of fluorescent Ca^{2+} indicator properties and their use in measuring elementary and global Ca^{2+} signals. *Cell Calcium* 28: 213–223, 2000