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Astrocytes Modulate Thapsigargin-induced Changes in Calcium Concentration and Neuronal Survival

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ABSTRACT

When mature cerebellar granule neurons (CGN) grown in high K^+ (25 mM K^+, HK)-serum containing medium are subjected to the HK/serum deprivation, they are destined for neuronal death. In this study, we attempted to elucidate the roles of endoplasmic reticular (ER) Ca^{2+}-store and co-cultured astrocytes in HK/serum deprivation induced neuronal death. Thapsigargin (TG), an inhibitor of ER Ca^{2+}-ATPase was simultaneously applied with normal K^+ (5 mM K^+, NK) serum free medium, and its effects on neuronal death in either astrocyte-poor or astrocyte-rich culture were examined. By means of the fura-2 microfluorimetric technique, we monitored the changes of the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, associated with neuronal death under various treatments. The results obtained showed that in astrocyte-poor cultures of mature CGN (10 days *in vitro*, DIV), the basal level of $[Ca^{2+}]_i$ markedly decreased from 184 ± 5 to 89.7 ± 5 nM 24 h after HK/serum deprivation. Although treatment with TG slightly increased the $[Ca^{2+}]_i$ to 117.6 ± 4 nM, the survival rate of the neurons was even worse; it was reduced from 49 ± 4% to 28 ± 2%. In the astrocyte-rich cultures, HK/serum deprivation also caused a profound reduction of neuronal $[Ca^{2+}]_i$ from 166 ± 3 to 90.2 ± 6 nM, accompanied by even more serious neuronal death (95.5 ± 1%). On the other hand, treatment with TG in astrocyterich cultures further lowered the $[Ca^{2+}]_i$ to 65 ± 2 nM but markedly improved the neuronal survival rate from 4.5 ± 1% to 60 ± 2% in a concentration-dependent manner. The strong implication of these findings is that ER Ca^{2+}-store and astrocytes participate in modulating the responses of neurons to stress stimulation.

Key Words: cerebellar granule neurons, thapsigargin, astrocyte, intracellular Ca^{2+}, survival

I. Introduction

In vivo, cerebellar granule neurons (CGN) develop closely with astrocytes. It is accepted that neurons *in vivo* are more resistant to neurotoxicants than those *in vitro*, possibly owing to the protective effect of astrocytes (Beaman-Hall *et al*., 1998). For example, glial cells can release nutrients like glutamine and uptake toxins like excessive glutamate, thus improving neuronal survival *in vivo* (Rosenberg and Aizenman, 1989). In addition, glial cells can uptake excess detrimental glutamate and possess catalase activity, which decomposes harmful H_2O_2. Thus, glial cells can prevent glutamate- and H_2O_2-mediated toxicity in cultured neurons (Desagher *et al*., 1996). Moreover, the neuronal environment can influence the response of neuron to excitatory amino acids (Vernadakis, 1996). We attempted to elucidate the influence of astrocytes on the survival of mature CGN subjected to HK/serum deprivation, a paradigm commonly used to investigate the mechanism underlying neuronal death in CNS (Chang and

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Abbreviations used: **ACSF**, artificial cortical spinal fluid; **AMPA**, alpha-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid; **CGN**, cerebellar granule neurons; **CNS**, central nervous system; **DIV**, days in vitro; **DMEM**, Dulbecco's modified Eagle's medium; **DMSO**, dimethyl sulfoxide; **EGTA**, ethylene glycol bis(beta-aminoethyl ether)-N,N,N'N'-tetraacetic acid; **ELISA**, enzyme linked immunosorbent assay; **ER**, endoplasmic reticular; **HEPES**, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; **HK**, high extracellular K^+ (25 mM), **NK**, normal extracellular K^+ (5 mM); **MTT**, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium); **NMDA**, N-methyl-D-aspartate; **PBS**, phosphate buffered saline; **TG**, thapsigargin.

Wang, 1997; D'Mello *et al*., 1998; Levick *et al*., 1995; Skaper *et al*., 1998; Yao *et al*., 1999).

By omitting the cytosine arabinoside at the beginning of the neuron culture, astrocytes were allowed to proliferate together with the differentiation of CGN in culture and to create astrocyte-rich conditions after 10 days *in vitro* (10 DIV). Unexpectedly, we found that the HK/serum deprivation induced neuronal death was even more serious in astrocyte-rich cultures than in astrocyte-poor cultures. The survival of CGN in culture was very closely associated not only with their $[Ca^{2+}]_i$ levels (Gallo *et al*., 1987; Franklin and Johnson, 1992), but also with endoplasmic reticular (ER) Ca^{2+}-store (Yao *et al*., 1999). Therefore, in this study, we attempted to reduce the death of CGN by using thapsigargin (TG), an inhibitor of ER Ca^{2+}-ATPase, and evaluated its effects on $[Ca^{2+}]_i$ both in astrocyte-rich and astrocyte-poor neuronal cultures. The results obtained showed that TG could exert opposing effects on massive neuronal death accompanied by a dramatic decrease in $[Ca^{2+}]_i$ induced by HK/serum deprivation, depending mainly on the presence or absence of co-cultured astrocytes. The implications of this newly discovered effect of ER Ca^{2+}-store coupled with astrocytes on neuronal survival will be discussed.

II. Materials and Methods

1. Preparation of Cultured CGN

Rat CGN were prepared from 7-day-old Wistar rats as previously described (Levi *et al*., 1984). In briefly, neurons were dissociated from freshly dissected cerebelli by means of mechanical disruption in the presence of trypsin and DNase and then seeded onto poly-L-lysine (Sigma, St. Louis, MI, U.S.A.)-coated 22 mm diameter coverslips. Cells were seeded at a density of 2×10^6 cells/coverslip or 1.8×10^5 cells/well (96 well culture plate) (Nunc Rochester, New York, NY, U.S.A.) in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, New York, NY, U.S.A.) supplemented with 10% fetal calf serum (FCS) (Gibco), 25 mM KCl, penicillin G (100 IU/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37 ± 0.5˚C in a humidified atmosphere of 95% air/5% CO_2. In astrocyte-poor cultures, cytosine arabinoside (10 μM) was added to the culture medium 18-24 h after plating in order to arrest the growth of non-neuronal cells. In the case of astrocyte-rich cultures, cytosine arabinoside was omitted.

2. Treatment of Cultures

Thapsigargin (RBI, Natick, MA, U.S.A.) was dissolved in DMSO and diluted in artificial cortical spinal fluid (ACSF) to a final 0.025% DMSO concentration. Mature neurons (10 DIV) were treated with TG in original HK serum containing DMEM for 24 h. In the experiment on HK/serum deprivation induced neuronal death, granule neurons were shifted to serum-free DMEM containing 5 mM KCl with or without TG 500 nM for 24 h either in astrocyte-rich or astrocyte-poor cultures.

3. Measurement of $[Ca^{2+}]_i$ of Granule Neurons

Measurement of basal $[Ca^{2+}]_i$ levels was carried out by using microspectrofluorimetry and Ca^{2+}-sensitive indicator fura-2/AM (Sigma) as described previously (Grynkiewicz *et al*., 1985; Yan *et al*., 1995; Lin *et al*., 1998). After treatment, granule neurons grown on poly-L-lysine-coated 22 mm diameter coverglass were loaded with fura-2/AM (5 μM) for 30 min at 37 ± 0.5˚C in ACSF buffer solution containing HK or NK, depending on the original culture concentration. The ACSF solution contained (mM): 143 NaCl, 25 KCl, 10 HEPES, 1.8 $CaC1_2$, 0.8 $MgSO_4$, 1 NaH_2PO_4, and 5.6 glucose, pH 7.4. The cells were then washed three times with the same buffer solution and placed in a thermostatted (37 ± 0.5˚C) stage (TC-202 and PDMI-2, Medical Systems, Greenvale, NY, U.S.A.) on an up-right fixed stage microscope (Olympus BXWI-50, Tokyo, Japan). Excitation of fura-2 was conducted at 340 and 380 nm with emitted light monitored at 510 nm. Cell-derived fluorescent images were visualized using a 40×, 0.8NA water-immersion objective and amplified with an intensifier (VideoScope VS-2525, Washington, D.C., U.S.A.) before there were recorded by a camera (VideoScope VS-200E). The digitized images were then proce-ssed on an IBM PC (486DX2-66) to obtain ratio images and area measurements using an image processing package (Optimas 5.1a, Bothell, MA, U.S.A.). The calcium concentration was calculated using the following formula (Grynkiewicz *et al*., 1985): $[Ca^{2+}]_i = K_d[(R-R_{min})/(R_{max}-R)]$ (S_{f2}/S_{b2}), where $K_d = 285$ nM (Groden *et al*., 1991; Yan *et al*., 1995) and S_{f2}, S_{b2} are the emitted fluorescent intensity (510 nm) excited by 380 nm at R_{min} and R_{max}, respectively. The values of R_{max} and R_{min} were the averaged values obtained using a calibration procedure with 10 μM ionomycin (Sigma) and 5 mM EGTA (Sigma).

4. Neuronal Survival

Viable granule neurons were quantified by means of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) tests. Yellowish MTT (Sigma) was dissolved in PBS. Mitochondrial dehydrogenase of viable cells selectively cleaved the tetrazolium ring to yield blue/purple formazan crystals. After treatments, aliquots of MTT (final concentration 1 mg/ml) were added, and then the cells were incubated in a CO_2 incubator at 37 ± 0.5˚C for 3 h. The plates were then shaken for 15 min to dissolve the blue/purple formazan crystals in DMSO. The optical densities were read at 570 nm using an ELISA reader (Dyna-

tech MR7000, Ashford, Middlesex, U.K.). A decrease in optical density when compared with control cells made possible quantitative assessment of cell damage.

In the case of the co-culture experiment (astrocyte-rich cultures), the viable neurons were distinguished and counted directly under the microscope. Viable neurons in three randomly selected fields of a 40× objective lens were counted and averaged. The data were expressed as % of control group.

5. Morphological Methods

Cerebellar granule neurons were observed using a 40× objective lens adapted for use with an Olympus IMT-2 inverted microscope.

6. Statistical Analysis

All data are shown as mean ± standard error (SE). Statistical significance ($p < 0.05$) was assessed using ANOVA, followed by Dunnett's t-tests.

III. Results

1. Effects of TG on Survival Rate and $[Ca^{2+}]_i$ Level of HK/Serum Deprived Neurons in Astrocyte-poor Culture

In astrocyte-poor cultures, treatment with TG 200 nM for 24 h had no significant effect on the neuronal survival of mature CGN maintained in HK serum-containing medium (Fig. 1), and the basal level of $[Ca^{2+}]_i$ was only slightly reduced from 193 ± 1 to 177 ± 3 nM (Fig. 2). When these 10 DIV mature CGN were shifted to NK serum-free medium for 24 h, the basal level of $[Ca^{2+}]_i$ decreased from 184 ± 5 to 89.7 ± 5 nM (Fig. 2), resulting in the death of 51 ± 4% CGN (Fig. 1). Although treatment with TG (500 nM) for 24 h slightly increased the reduced $[Ca^{2+}]_i$ level to 117.6 ± 4 nM (Fig. 2), it did not improve but further reduced the survival rate of HK/serum deprived CGN from 49 ± 4% to 28 ± 2% (Figs. 1 and 3).

2. Effects of Astrocyte Co-culture on TG-Induced Changes in Neuronal Survival and $[Ca^{2+}]i$ Level of HK/Serum Deprived Neurons

As shown in Fig. 4, HK/serum deprivation-induced death of CGN was even more serious in astrocyte-rich cultures (95.5 ± 1%) than in astrocyte-poor cultures (51 ± 4%). Unlike the astrocyte-poor cultures, treatment with TG 500 nM in astrocyte-rich cultures for 24h significantly improved the survival rate of HK/serum deprived CGN from 4.5 ± 1% to 60 ± 2% (Fig. 4). Owing to the MTT reduction activity of viable astrocytes, the survival rate of all cells (astrocytes + CGN) examined using the MTT test revealed significant improvement in the survival rate (from 57 ± 1% to 71 ± 4%) induced by 500 nM TG (Fig. 4). When viable neurons were directly counted under the microscope (Fig. 5) the survival of HK/serum deprived CGN was found to be even more significantly

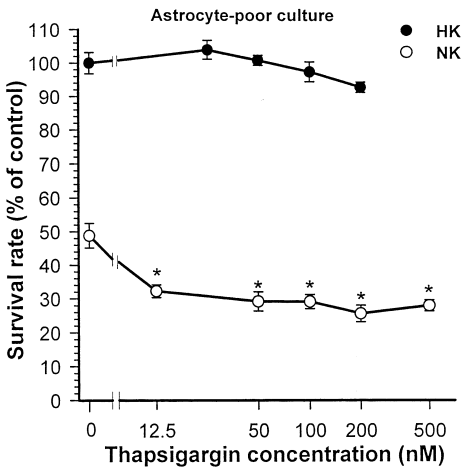

Fig. 1. Concentration-dependent effects of TG on the survival of mature granule neurons grown in astrocyte-poor cultures. The neurons (10 DIV) were treated with various concentrations of TG for 24 h in original HK control DMEM (-●-), or immediately after they were shifted to NK serum free DMEM (-▫-). The viable neurons were assayed using the MTT test as described in Methods. Data are presented as mean ± SE ($n = 3$). *$p < 0.05$, as compared with the respective TG free control group.

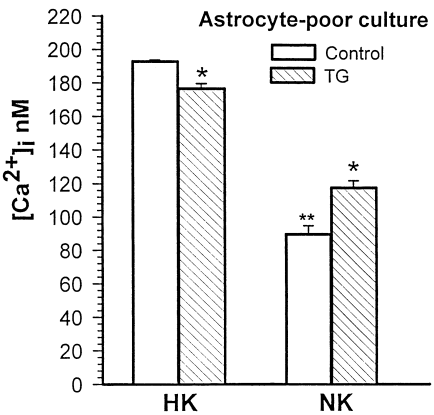

Fig. 2. TG-induced changes in $[Ca^{2+}]_i$ of mature granule neurons grown in astrocyte-poor cultures. Mature granule neurons (10 DIV) were treated with TG (200 nM) in the original HK-serum containing control DMEM or 500 nM TG immediately after they were shifted to serum-free NK DMEM. After 24 h of treatment, the basal levels of $[Ca^{2+}]_i$ were measured using the fura-2 microfluorimetric technique as described in Methods. Data are presented as mean ± SE (n = 64-103). *$p < 0.05$, as compared with the respective TG free control group. **$p < 0.05$, as compared with the respective HK serum containing group.

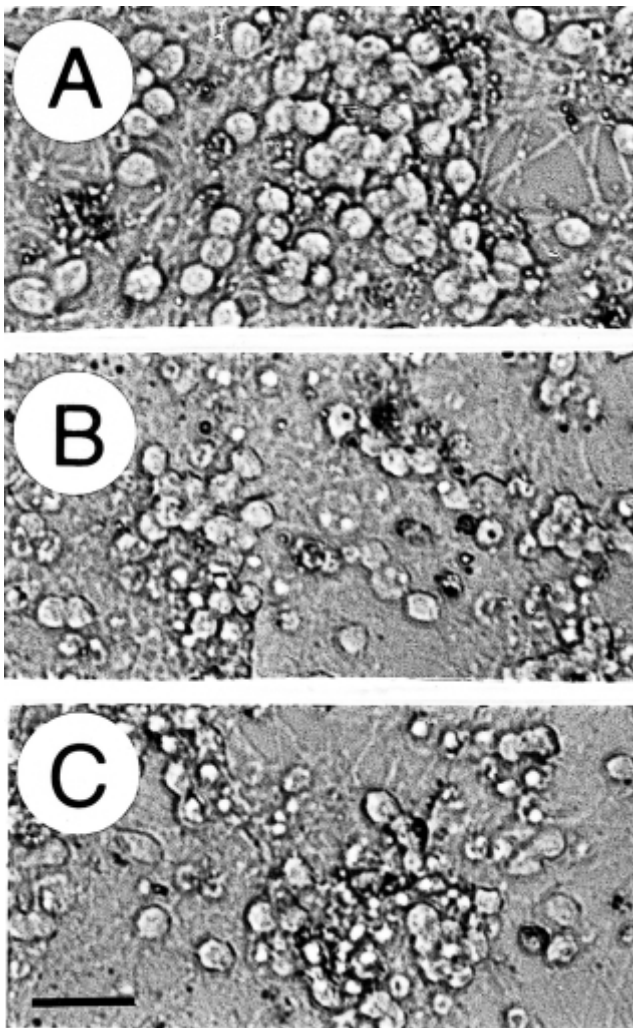

Fig. 3. TG-induced morphological changes of mature granule neurons in NK/serum-free medium. Cellular morphology was observed using a 40× objective lens adapted for use with an Olympus IMT-2 inverted microscope. Mature granule neurons (10 DIV) were grown in control HK serum containing DMEM (A) and shifted to NK serum free DMEM without (B), or with (C) TG 500 nM for 24 h. Note the massive neuronal death caused by shifting to NK-serum free medium, which appeared to be exacerbated by the additional TG treatment. Bar = 20 µm.

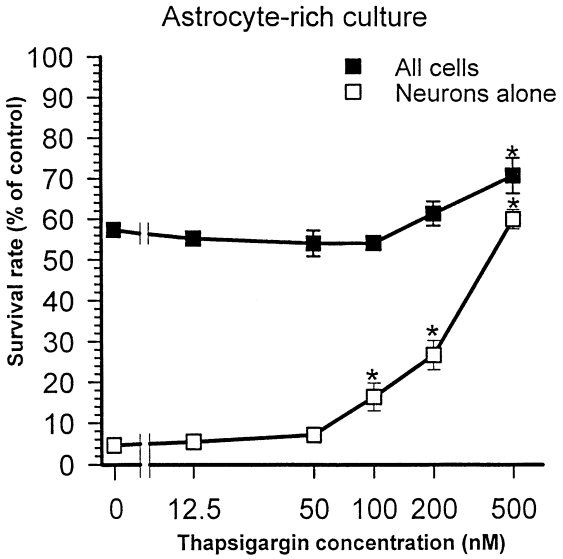

Fig. 4. Concentration-dependent effect of TG on mature granule neurons co-cultured with astrocytes in NK serum free medium. Mature granule neurons (10 DIV) co-cultured with astrocytes were switched to NK serum-free DMEM containing various concentrations of TG for 24 h. Viability of all cells (astrocytes + neurons) was assayed using the MTT test (-■-), and the number of viable neurons (-□-) was estimated directly based on their morphology in the field of vision under a microscope as described in Methods. Note that massive neuronal death was found in NK serum free medium, which could be prevented by TG in a concentration-dependent manner. Data are presented as mean ± SE. $*p < 0.05$, as compared with the respective TG free control group.

improved by TG in a concentration-dependent manner (Fig. 4). HK/serum deprivation for 24 h caused the basal $[Ca^{2+}]_i$ level to decrease from 166 ± 3 to 90.2 ± 6 nM in neurons co-cultured with astrocytes (Fig. 6). However, in contrast to the astrocyte-poor cultures, TG had the opposite effect on the $[Ca^{2+}]_i$ level of HK/serum deprived CGN which it reduced from 90.2 ± 6 nM to 65 ± 2 nM (Fig. 6). This phenomenon on $[Ca^{2+}]_i$ of CGN could also be observed after 3 h and 9 h of incubation following HK/serum deprivation in the respective astrocyte-poor (Fig. 6(a)) and astrocyte-rich cultures (Fig. 6(b)). This finding may account for the contradictory effects of TG on the survival of HK/serum deprived CGN cultured with or without astrocytes.

IV. Discussion

In vivo, granule neurons grow with the surrounding astrocytes. The primary role of astrocytes *in vivo* is to protect the integrity of the CNS (Banati and Graebar, 1994). Astrocytes can diminish neurotoxicity induced by excitotoxic compounds like glutamate or NMDA in CNS neurons (Beaman-Hall *et al*., 1998). In this study, we tested the influence of astrocytes on neuronal death induced by HK/serum deprivation in mature CGN, a paradigm commonly used to investigate the mechanism underlying neuronal death in CNS (Levick *et al*., 1995; Chang and Wang, 1997; D'Mello *et al*., 1998; Skaper *et al*., 1998). Unlike the protective effects of the astrocytes described above, HK/ serum deprivation induced even more serious death of mature CGN in astrocyte-rich cultures as compared with that in astrocyte-poor cultures. The actual mechanism of this astrocyte enhanced harmful effect is still not known. We propose that astrocytes may be activated by HK/serum deprivation induced injury and may behave like inflammatory cells releasing excess neurotox-

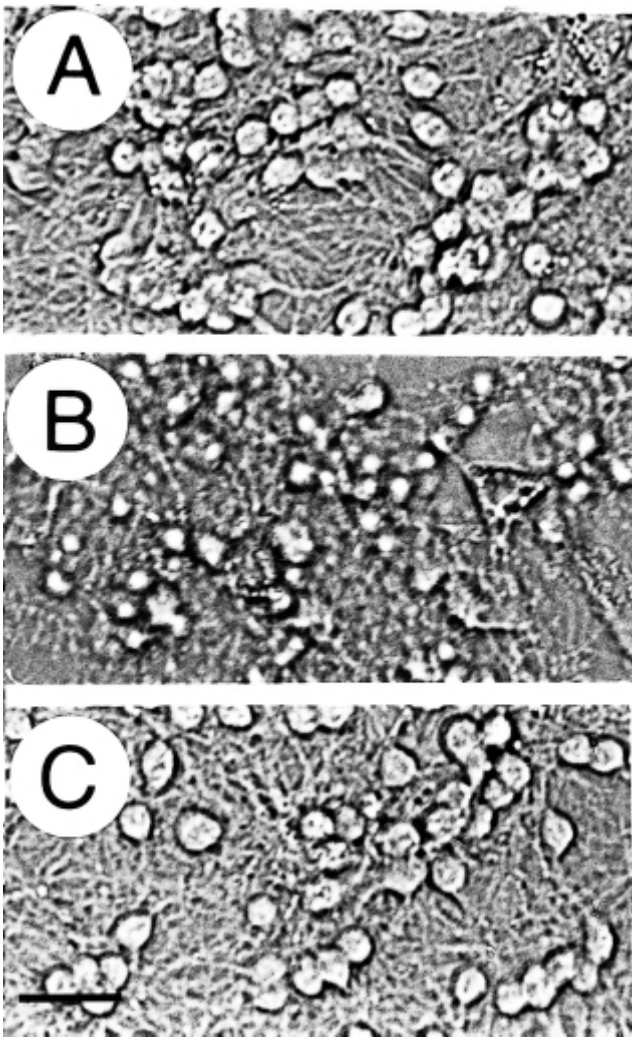

Fig. 5. TG induced morphological changes of mature granule neurons cocultured with astrocytes in NK/serum-free medium. Cellular morphology was observed using a 40× objective lens adapted for use with an Olympus IMT-2 inverted microscope (bar = 20 μm). Mature granule neurons (10 DIV) were grown in cytosine arabinoside-free HK serum containing DMEM (A) and shifted to NK serum-free DMEM without (B), or with (C) TG 500 nM for 24 h. Note that denser neurite networks and extracellular matrices were found in these co-cultures as compared with those in astrocyte-poor neuronal cultures, and that TG had a profound neuroprotective effect on the detrimental effect of NK serum-free DMEM (C).

ic pro-inflammatory cytokines, similar to the activated glial cells found in many neurodegenerative disorders (Epstein, 1998; Hu *et al*., 1998). The detrimental role of astrocytes *in vitro* has also been observed in AMPA-induced neurotoxicity. Dugan *et al*. (1995) reported that AMPA-induced neurotoxicity was exacerbated in astrocyte-rich cultures versus astrocyte-poor cultures, possibly owing to AMPA receptor-mediated glial glutamate release. Thus, it is suggested that, depending on the kind of stimulation, astrocytes can attenuate or exacerbate the damage inflicted on the neurons around them.

However, in comparison with the detrimental role of astrocytes in HK/serum deprivation induced neuronal death, the influence of astrocytes on the TG-mediated effects on neuronal survival appears to be different. In this study, TG was found to prevent neuronal death induced by HK/serum deprivation in astrocyte-rich cultures but to exacerbate it in astrocyte-poor cultures. It is well established that Ca^{2+} plays a pivotal role in manipulating neuronal survival and development (Gallo *et al*., 1987; Collins *et al*., 1991; Franklin and Johnson 1992; Yao *et al*., 1999). Scientists are investigating drugs which possess definite $[Ca^{2+}]_i$ modulating properties that prevent neuronal death under various circumstances. In this study, we found that TG, an inhibitor of ER Ca^{2+}-ATPase, exerted completely different effects in astrocyte-poor and astrocyte-rich cultures. Although the short-term effect of TG on $[Ca^{2+}]_i$ of CGN was not significant enough to be detected, as we and others reported previously (Kiedrowski and Costa, 1995; Yao *et al*., 1999), measurement of the basal $[Ca^{2+}]_i$ level after prolonged TG treatment revealed that TG altered the neuronal $[Ca^{2+}]_i$ under these two different conditions (Fig. 6). This was perhaps due to lower neuronal $[Ca^{2+}]_i$ in the astrocyte-rich cultures, such that TG was able to interrupt the Ca^{2+} transacting death signal. On the other hand, TG treatment increased the level of $[Ca^{2+}]_i$ associated with more serious neuronal death in astrocyte-poor cultures, probably due to failure of the cellular Ca^{2+} extrusion or sequestration system. An alternative explanation is that astrocytes may alter neuronal development/phenotype such that the response to TG is quite different from that in astrocyte-poor cultures. Thus, TG had the opposite effect on $[Ca^{2+}]_i$ of HK/serum deprived mature CGN in the respective astrocyte-poor and astrocyte-rich cultures.

In addition to the protection provided by catalase activity and the glutamate transport system against neurotoxic damage (Rosenberg and Aizenman 1989; Desagher *et al*., 1996), we can not rule out the possibility that astrocytes which grow around the neurons possess some properties such as the ability to balance the ions, especially as a K^+-sink, and to produce trophic factors which modulate neuronal characteristics and responsiveness to stimulation (Abiru *et al*., 1998). In agreement with this concept, it has been found that astrocytes can decrease both NMDA and glutamate-induced toxicity in cerebellar granule neurons (Beaman-Hall *et al*., 1998).

In conclusion, in addition to agents that directly or indirectly affect intracellular $[Ca^{2+}]_i$ status, the pivotal role of astrocytes in modulating the growth and function of granule neurons can not be neglected. The findings of this study indicate that TG-sensitive Ca^{2+}-store exhibits a neuroprotective effect on the mature neurons grown in astro-

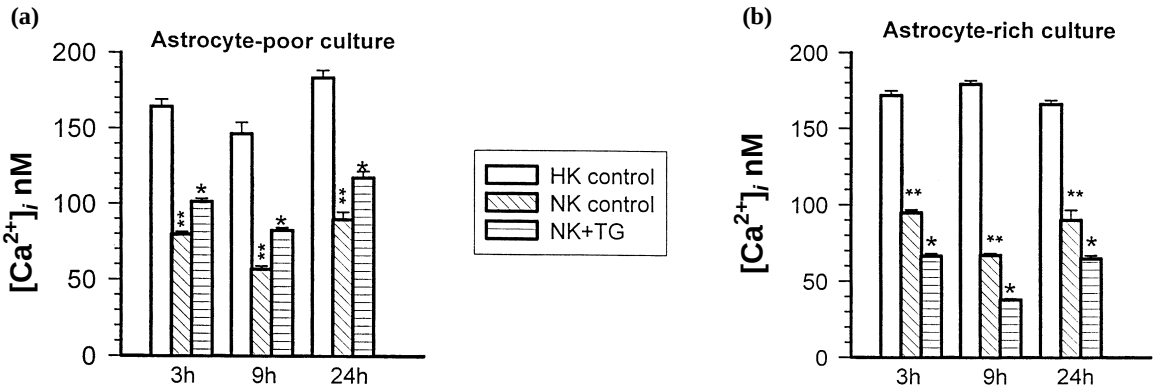

Fig. 6. Time course of TG-induced changes in $[Ca^{2+}]_i$ of HK/serum deprived mature granule neurons in astrocyte-poor (A) and astrocyte-rich cultures (B). Mature granule neurons (10 DIV) were treated with TG (500 nM) immediately after they were shifted to NK serum-free DMEM. After the time interval as indicated, the basal levels of $[Ca^{2+}]_i$ were measured by Fura-2 microfluorimetric technique as described in Methods. Data are presented as mean ± SE (n = 64-103). *$p < 0.05$, as compared with the respective TG-free control group. **$p < 0.05$, as compared with the respective HK serum containing group.

cyte-poor culture but has an opposite detrimental effect in astrocyte-rich culture. Therefore, the implication of these findings is that TG sensitive ER Ca^{2+}-store plays a pivotal role in regulating neuronal survival whether beneficial or detrimental, depending at least in part on the presence or absence of astrocytes.

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神經膠細胞調控Thapsigargin對神經細胞的內鈣濃度及生存的作用

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摘 要

在體外培養於高鉀（25 mM K^+）含血清培養液的小腦顆粒形神經細胞在成熟後，若將其移至正常生理鉀（5 mM K^+）濃度無血清的培養液時，24 小時後神經細胞內鈣濃度會劇烈下降而且大量死亡，此乃常用於研究中樞神經細胞的死亡模式。本實驗發現若有與神經膠細胞共同培養的神經細胞，此種因失去高鉀及血清刺激的神經細胞的死亡會加重，生存的神經細胞由49 ± 4% 降至4.5 ± 1% 。然而在此種神經膠細胞與神經細胞共同培養的狀態下，以內質網鈣離子幫浦阻斷劑thapsigargin（TG）處理，不但會使神經細胞內鈣濃度更加排空降低（由90.2 ± 6 nM 至65 ± 2 nM），而且可以有效降低神經細胞死亡，生存率由4.5 ± 1% 升至60 ± 2% 。若是在無神經膠細胞存在的狀態下，TG 雖可略微提升因失去高鉀及血清而下降的神經細胞內鈣濃度由89.7 ± 5 nM 升至117.6 ± 4 nM ，但是卻會加重神經細胞的死亡，生存率由49 ± 4% 降至28 ± 2% 。本實驗的結果顯示神經細胞周圍的神經膠細胞會顯著地影響神經細胞對外來刺激的反應，此種影響又可因神經細胞所受不同的刺激，而產生保護或加劇傷害神經細胞的相反作用。本研究所發現的意義是指出神經細胞內質網鈣儲存池可與神經膠細胞協同作用，對神經細胞的生存具有顯著的調控作用。