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Polychlorinated biphenyls disrupt the actin cytoskeleton in hippocampal neurons

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Abstract

It is well known that developmental exposure to polychlorinated biphenyls (PCBs) could cause learning and memory deficits, but the underlying mechanisms are not clear. Actin cytoskeleton is directly involved in synaptic plasticity which is considered critical to learning and memory formation by LIM kinase 1 (LIMK-1)/cofilin pathway. To determine whether PCBs could alter actin cytoskeleton, we exposed the cultured hippocampal neurons to PCBs mixture Aroclor 1254 (A 1254). By biochemical measurement, fluorimetric assay and fluorescence microscopy, we found that A 1254 elicited a loss of filamentous actin, which preceded cytotoxicity. Western blots showed that a concentration-dependent decrease in the phosphorylation of cofilin and a decrease in LIMK-1 were induced by A 1254. We concluded that PCBs induced actin depolymerization in hippocampal neurons, probably by inhibiting the LIMK-1/cofilin signaling pathway. The above findings offer new perspectives for the understanding of PCBs-induced learning and memory deficits.

Keywords: Aroclors; Polychlorinated biphenyls; LIM kinase 1; Cofilin; F-actin

1. Introduction

Polychlorinated biphenyls (PCBs) are a class of widely dispersed and environmentally persistent organic compounds. Epidemiologic studies have indicated that developmental exposure to PCBs leads to learning and memory deficits (Schantz et al., 2003). Children who are exposed during development exhibit neuropsychologic deficits such as lower full-scale IQ (intelligence quotient), reduced visual recognition memory, attention and motor deficits (Ayotte et al., 2003; Darvill et al., 2000; Walkowiak et al., 2001). Previous studies in rodents and monkeys supported these findings (Berger et al., 2001; Roegge et al., 2000; Widholm et al., 2001). A number of potential mechanisms of PCBs neurotoxicity have been proposed, comprising alterations in LTP (Gilbert et al., 2000; Sharma and Kodavanti, 2002), neurotransmitter levels (Bemis and Seegal, 1999), calcium channels (Pessah et al., 2006), apoptosis (Sanchez-Alonso et al., 2004) and perturbation in intracellular second messenger systems (Kodavanti and Tilson, 1997) including Ca^{2+} disposition (Sharma et al., 2000), inositol phosphates (Shafer et al., 1996), and protein kinase C (Yang and Kodavanti, 2001). The cellular mechanisms responsible for the neurotoxicity of PCBs are, however, not well understood and have been the subject of intensive investigations in recent years.

Our preliminary experiment used cDNA microarrays to screen genes that might be involved in PCBs-induced learning and memory deficits. We found that the expression of LIMK-1 was significantly decreased in PCBs-treated hippocampal neurons as compared to untreated ones (unpublished data). LIMK-1 is a key component of a signal transduction network that connects extracellular stimuli to changes in cytoskeletal

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structure. LIMK-1 regulates actin filament dynamics through inhibition of actin depolymerizing factor (ADF)/cofilin. The actin cytoskeleton is important for many cellular processes, including cytokinesis, endocytosis, chemotaxis and neurite outgrowth (Mitchison and Cramer, 1996). Furthermore, the actin network is directly involved in synaptic regulation at mature synapses, including hippocampal long term potentiation (LTP), an important form of synaptic plasticity in learning and memory formation (Smart and Halpain, 2000; Rao and Craig, 2000). In addition, LIMK-1 knockout mice have deficits in spatial learning, alterations in LTP, and abnormalities in hippocampal dendritic spine structure (Meng et al., 2002). Surprisingly, abnormal expression of LIMK-1 is associated with Williams syndrome, a mental disorder with profound deficits in visuospatial cognition (Frangiskakis et al., 1996). The findings are consistent with a critical role for actin cytoskeleton by LIMK-1/cofilin pathway in the formation and maintenance of memory and learning. Based on these reports, we wanted to investigate whether the neurotoxic effects of PCBs may involve the actin cytoskeleton.

The hippocampus has been implicated in learning and memory function in human beings and animals, and the cultured hippocampal neuron is attractive as a model system for exploring learning and memory mechanisms in vitro. The present study was undertaken to investigate the in vitro effects of Aroclor 1254 (A 1254), a complex mixture of more than 80 environmentally relevant PCB isomers and congeners, on the actin network in rat hippocampal neuronal cultures. To elucidate neurotoxic mechanisms, we evaluated the dynamic equilibrium between monomeric and polymerized actin by using quantitative biochemical and fluorimetric assays for the determination of cellular actin. Also, LIMK-1 and cofilin were assessed in order to determine the possible involvement of this signal pathway.

2. Materials and methods

2.1. Cell culture

Hippocampal neurons were prepared from 18-day-old embryonic SD rat and grown in Neurobasal medium containing B27 supplement, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5 mM L-glutamine, 1 mM sodium pyruvate, and 1 mM L-serine (all from Gibco), with some minor modifications, as described previously (Rae et al., 2000; Willets et al., 2004). Briefly, chopped pieces of hippocampus were treated with pronase E and thermolysin (0.5 mg/mL each, Sigma) for 30 min, before being re-suspended in HBSS (Invitrogen) supplemented with 40 Ag/mL DNase (Gibco) and triturated through a fire-polished glass pipette. Cells were then re-suspended in Neurobasal medium containing 10% heat-inactivated fetal calf serum (Invitrogen) and plated onto poly-D-lysine-coated plates. After 24 h, this medium was replaced with serum-free neurobasal medium, and 5 μM cytosine arabinoside was added to the cultures to inhibit glial cell proliferation. All cultures were maintained in a humidified 37 °C incubator with 5% CO_2.

2.2. PCBs treatments

For some experiments, A 1254 (technical grade purity, Supelco) was dissolved in dimethyl sulfoxide and added to the cultures at the final concentration of 0.1, 1.0, 10.0 and 100.0 μM. Cultures were treated beginning on the sixth day in vitro for 12 h. Molarity of A 1254 solutions was based on the average molecular weight (326.4). Samples with DMSO without PCBs were used as controls (final concentration of DMSO in the plates 0.3%).

2.3. Cytotoxicity assays

Cells were seeded onto six-well plates at the concentration of 5×10^4 cells/mL and exposed to A 1254 for 12 h. The mitochondrial activity that cleaves MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was used to assess cell survival in a quantitative colorimetric assay. Cytotoxicity was also evaluated by measuring the release of lactate dehydrogenase (LDH) in the culture medium, utilizing a commercially available kit (Nanjing Jianchen Bioengineering Institute, China). To further determine the effects of treatments on cell survival, cells were treated with trypan blue and counted by using a hemocytometer.

2.4. Fluorescence measurement of F-actin content

F-actin was measured as described previously (Wysolmerski and Lagunoff, 1988). Hippocampal neurons were grown in plates, washed with buffer A (pH 7.2, 75 mM KC1, 3 mM $MgSO_4$, 1 mM EGTA, 0.2 mM dithiothreitol, 10 mM imidazole, 10 μg/mL aprotinin, 0.1 mM PMSF), and permeabilized with 0.03% saponin in buffer A for 10 min at room temperature. Cells were fixed in freshly prepared 3% formaldehyde in buffer A for 20 min at room temperature, washed, and stained in the dark with 0.175 μg/mL fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma) for 30 min. After another wash extraction of phalloidin was initiated by addition of ice cold HPLC-grade methanol and continued overnight at −20 °C. Methanol extraction resulted in a quantitative removal of phalloidin bound to actin. Cells in methanol were removed from the plates with a rubber policeman. The methanol suspension was aspirated, and centrifuged at 8000 × *g*. The fluorescein isothiocyanate in the supernatants was determined using an Aminco-Bowman spectrophotofluorometer (Colora, Lorch, FRG). Excitation and emission wavelengths were 495 and 520 nm, respectively. Matched cells were washed with buffer A, lysed with 0.1% triton X-100, scraped from the plate, and used for determination of protein according to Bradford (1976). Data were expressed as micrograms phalloidin per milligram cell protein.

2.5. Visualization of F-actin

The cells were washed with Hanks' balanced salt solution. The reaction was terminated by the addition of paraformaldehyde (final 3%), and F-actin in the cells was stained as described previously (Nagaishi et al., 1999) using fluorescein isothiocyanate (FITC)-labeled phalloidin. The stained cells were observed by a fluorescence microscope.

2.6. Measurement of G-actin and total actin

Actin was determined as inhibitor of DNase I (Sigma) from bovine pancreas under standard assay conditions, as described by Malicka-Blaszkiewicz and Roth (1981). The concentration of G-actin was estimated by DNase I inhibition, directly in a crude cytosol sample. Total actin (T) content was measured after dilution of the samples with buffer A until maximal inhibition of DNase I was reached (Malicka-Blaszkiewicz, 1986). Actin concentration was expressed in units of DNase I inhibitor per milligram of sample protein.

2.7. Immunoblotting

Changes in the levels of LIMK-1, cofilin and phosphorylated cofilin (p-cofilin) were analyzed by Western blotting as described previously (Arber et al., 1998; Meberg et al., 1998). Briefly, equal amounts of samples were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes. The blots were dried, washed several times and blocked for 1 h in TBS containing 5% bovine serum albumin. The blots were incubated overnight at 4 °C with the primary antibodies in TBS containing 1% bovine serum albumin, and then washed three times in TBS containing 0.05% Tween 20. Goat or rabbit polyclonal antibodies specific for LIMK-1 (Santa Cruz), cofilin (Cell singaling techonology)

and phosphorylated cofilin (p-cofilin) (Santa Cruz, sc-12912-R) were used. They were then incubated with a secondary horseradish peroxidase-conjugated antibody for 1 h. After five washes with TBS and 0.05% Tween 20, the blots were developed by using a chemiluminescence detection kit (Amersham Biosciences, UK). GAPDH (Kangchen, China) was used as an internal control for protein loading. To compare the relative degree of phosphorylation, the staining intensities were analyzed using the MacBas image system (MacBAS, Fuji Film Co., Tokyo, Japan). Data were expressed as a ratio of the protein of interest to GAPDH (Kots et al., 1992).

2.8. Statistics

The data was analyzed by one way analysis of variance followed by Student–Newman–Keuls' multiple comparison test. The significance was set at $P < 0.05$.

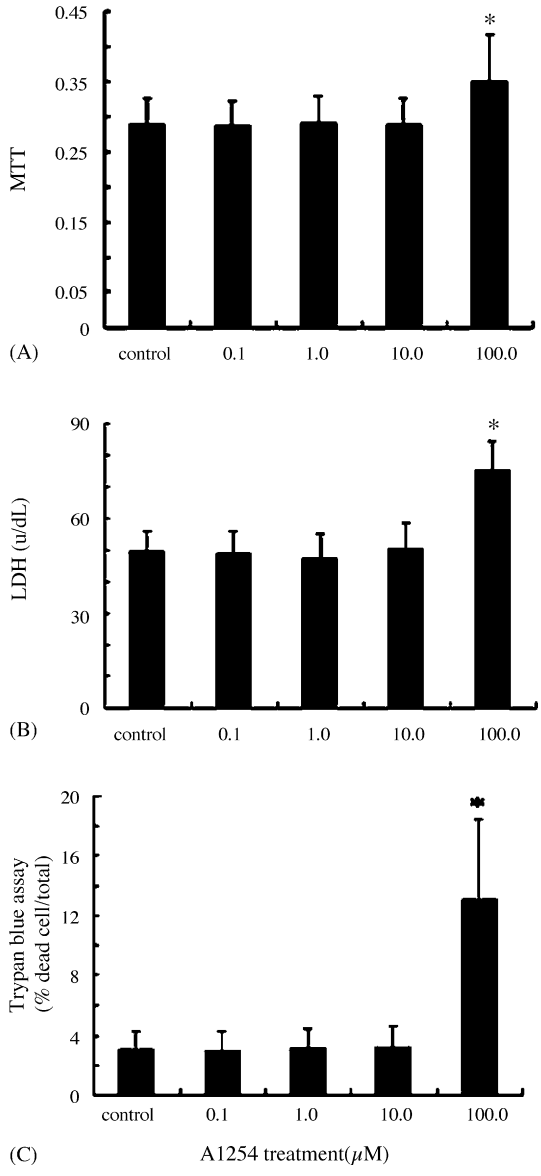

Fig. 1. Effects of A 1254 on cell viability in hippocampal neurons. Effects of A 1254 treatment upon the cellular viability of hippocampal neuronal cell cultures were estimated by MTT, LDH release, and trypan blue assay. Cells were incubated in absence (DMSO) or presence of several A 1254 concentrations (0.1–100.0 μM) for 12 h. Bars represent the mean ± S.D. for data obtained from six independent experiments. *Significantly different from control at $P < 0.05$.

3. Results

3.1. Effects of A 1254 on cell viability

To determine whether A 1254 affected cell viability, hippocampal neuronal cultures were treated with 0.1–100.0 μM of A 1254 for 12 h. The cytotoxicity of A 1254 in hippocampal neuron was assessed by measuring MTT, LDH release (an indicator of membrane integrity) and by direct cell counting with trypan blue. As shown in Fig. 1, A 1254 at low concentrations (0.1–10.0 μM) did not have any effects on all indexes above. However, both MTT and trypan blue assay showed that A 1254 caused significant cell death at the concentration of 100.0 μM (Fig. 1A and C). Similarly, there was a significant elevation in LDH leakage after exposure to A 1254 (100.0 μM) for 12 h (Fig. 3B).

3.2. Effects of A 1254 on LIMK-1

To determine the changes of LIMK-1 following exposure of A 1254, cells were immunoblotted against LIMK-1 antibody. Fig. 2A showed that A 1254 significantly decreased LIMK-1 at the concentrations of 10.0 and 100.0 μM. The reduction of LIMK-1 was in a concentration-dependent manner by A 1254 (Fig. 2B).

3.3. Effects of A 1254 on phosphorylated states of cofilin

To determine the changes of phosphorlated states of cofilin following exposure of A 1254, cells were immunoblotted against

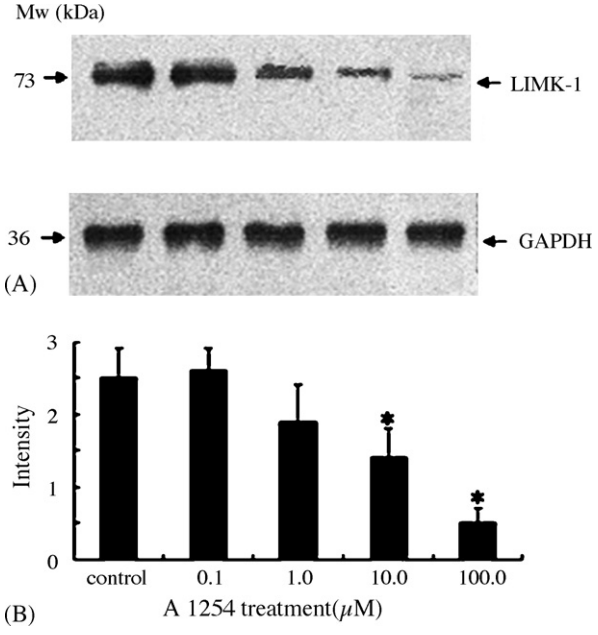

Fig. 2. Effects of A 1254 on LIMK-1 in hippocampal neurons. Cells were incubated in absence (DMSO) or presence of several A 1254 concentrations (0.1–100.0 μM) for 12 h. Immunoblots of LIMK-1 were conducted using polyclonal antibody as described under Section 2 (A). GAPDH was used as an internal control for protein loading. The intensity of immunoblots was quantitated by the image system and the values shown are mean ± S.D. of three independent experiments (B). *Significantly different from control at $P < 0.05$.

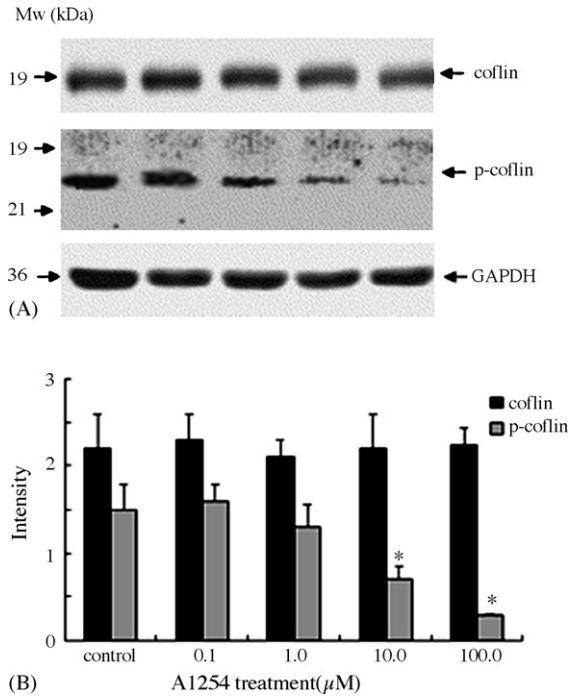

Fig. 3. Effects of A 1254 on phosphorylated states of cofilin in hippocampal neuron. Cells were incubated in absence (DMSO) or presence of several A 1254 concentrations (0.1–100.0 μM) for 12 h. Immunoblots of cofilin and phosphorylated cofilin (p-cofilin) were conducted using polyclonal antibody as described under Section 2 (A). GAPDH (Kangchen, China) was used as an internal control for protein loading. The intensity of immunoblots was quantitated by the image system and the values shown are mean ± S.D. (B) of three independent experiments. *Significantly different from control at $P < 0.05$.

coflin or phosphorylation of cofilin (p-cofilin) antibody. As shown in Fig. 3A, cofilin in all treatment groups had no significant difference compared to the control, but the p-cofilin in 10.0, 100.0 μM of A 1254 treated cells was significantly decreased in a concentration-dependent manner (Fig. 3B).

3.4. Effects of A 1254 on the polymerization state of actin

Because F-actin-depolymerizing and severing activities of cofilin are dependent on the degree of its phosphorylation, dynamic changes in F-actin in the A 1254 cells were investigated. As shown in Fig. 4A, the fluorescent phalloidin-stained intracellular F-actin was significantly decreased in 10.0, 100.0 μM of A 1254 treated cells. In the control group, hippocampal neurons after 6 days in culture exhibited extensive staining for F-actin throughout all cells, including dendrites and axons. After exposure to A 1254 (10.0, 100.0 μM), the fluorescent phalloidin-stained F-actin was significantly decreased in the whole cells, particularly in the dendrites and axons (Fig. 4A). The tendency was quantitatively confirmed by fluorescence measurement of F-actin content (Fig. 4B). Further, the decreased F-actin induced by A 1254 was concentration-dependent (Fig. 4B).

To confirm the above findings we further performed direct measurements of G-actin and total actin. As shown in Fig. 5A and B, the cellular G-actin level was significantly increased in cells treated with A 1254 (10.0, 100.0 μM), whereas no statistically significant differences were observed for total actin levels. In addition, A 1254 (10.0, 100.0 μM) increased actin depolymerization in a concentration-dependent manner, as indicated by the increased G/total actin ratios (Fig. 5C).

4. Discussion

PCBs are known, even at low doses, to be toxic to nerve system. Extensive evidence from animal studies shows that the learning and memory deficits induced by PCBs are partially due to alterations in synaptic plasticity. The underlying mechanisms by which PCBs alter synaptic plasticity, however, are not completely understood.

The organization of the actin cytoskeleton is essential for many cellular functions including cell survival, differentiation, proliferation, cytokinesis, apoptosis and motility. Actin exists either in an unassembled (G-actin) form, or in a filamentous (F-actin) form. The functionality of the actin cytoskeleton depends on a dynamic equilibrium between F-actin and G-actin. Actin remodeling may be particularly important for the establishment and structural modification of dendritic spines on which the great majority of excitatory synapses are formed in the mammalian CNS. Changes in spine are controlled by changes in actin filament structure and have been implicated in synaptic plasticity and LTP (Krucker et al., 2000). Actin is also required for LTP-induced synaptic growth (Fukazawa et al., 2003). Consequently, the actin dynamics are intimately involved in regulating receptor complexes and in the formation of functional synapses. Therefore, in the present work we considered the possibility that PCBs could induce rapid changes in the actin cytoskeleton, which may be involved in PCBs-induced learning and memory deficits. This hypothesis would imply polymerization or depolymerization of the filamentous actin network.

Our results showed a concentration-dependent increase of G/total-actin ratio in hippocampal neurons treated with A 1254. This effect was detectable at concentrations of 10.0–100.0 μM. It was due to a significant increase of the G-actin content, while the total actin level was not signigicantly affected by A 1254. Associated direct fluorimetric measurements showed decreased F-actin content in FITC-phalloidin-labelled cell preparations treated with A 1254. From these results we concluded that A 1254 induced actin depolymerization in hippocampal neurons. The changes in the depolymerizaton using biochemical and fluorimetric assays were corroborated by morphological observations with fluorescence microscopy.

Actin dynamics are tightly regulated at multiple levels. Recent studies have indicated that cofilin, an ubiquitously expressed actin-binding protein which depolymerizes and severs actin filaments, appears to play a central role in actin filament turnover and reorganization (Bierne et al., 2001). Activities of cofilin are abolished by phosphorylation at Ser-3. Cofilin is phosphorylated by LIM kinase (LIMK-1), which inhibits cofilin function and stabilizes actin reorganization (Arber et al., 1998; Yang et al., 1998). LIMK-1, which is highly expressed in neural tissue and accumulates at high levels at mature synapses, and other tissues as well, presents in the growth cones of cultured neurons (Foletta et al., 2004; Endo et al., 2003). LIMK-1 can

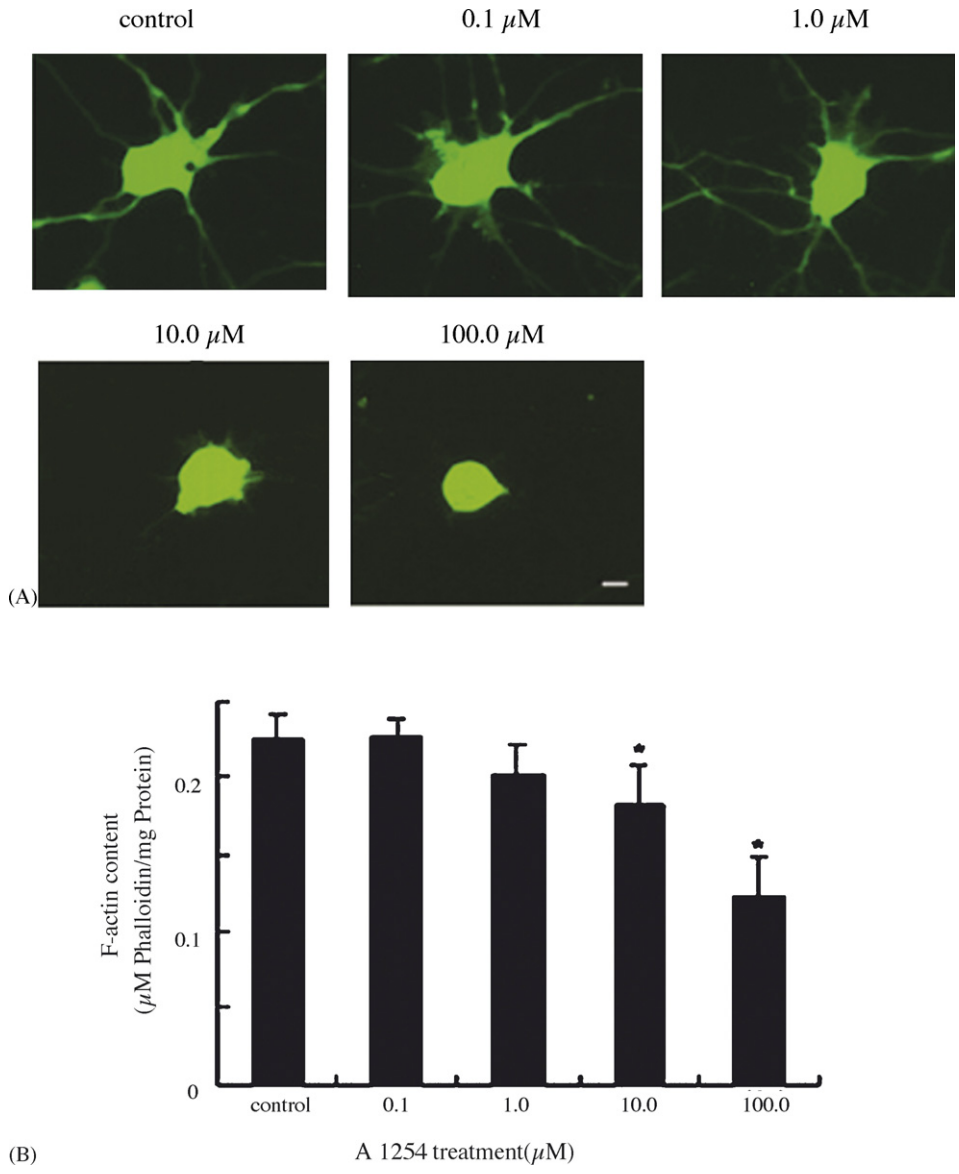

Fig. 4. Effects of A 1254 on F-actin in hippocampal neurons. Cells were incubated in absence (DMSO) or presence of several A 1254 concentrations (0.1–100.0 μM) for 12 h. Then the cells were fixed with paraformaldehyde, and the intracellular F-actin was stained with fluorescent FITC-phalloidin (A). The graph shows that changes in F-actin content induced by different concentrations of A 1254 was determined by quantitating actin-bound FITC phalloidin as outlined in Section 2 (B). Scale bars = 10 μm. The values presented are mean ± S.D. of five separate experiments. *Significantly different from control at $P < 0.05$.

directly interact with protein kinase C and neuregulins (Kuroda et al., 1996; Wang et al., 1998), both of which are known to play a critical role in the learning and memory processes and formation. In addition to abnormalities in synaptic structure and function, the LIMK-1 knockout mice were altered in LTP and certain behavioral responses, which included heightened locomotor activites and impaired spatial learning (Meng et al., 2002). In humans, hemizygotic deletion of the LIMK-1 gene is linked to impairment of visuospatial constructive cognition in patients with Williams syndrome (Frangiskakis et al., 1996; Belluji et al., 1999). LTP also causes the phosphorylation of cofilin; the block of this phosphorylation inhibits LTP (Fukazawa et al., 2003). The actin dynamics, by the LIMK-1cofilin pathway, play an important role in spine formation and are highly associated with learning and memory behavior and hippocampal function. Therefore, disruption of actin filament dynamics in hippocampal neurons by the LIMK-1/cofilin pathway may have severe consequences on learning, memory, and development.

By using hippocampal neuronal culture model, we have demonstrated for the first time that A 1254 inhibited actin polymerization. This finding was accompanied by a decreased LIMK-1 level and an inhibited phosphorylation of cofilin. A 1254 exerted a concentration-dependent inhibitory effect in the range of 0.1–100.0 μM. An important finding is that A 1254 disrupted the actin polymerization dynamics in a concentration-dependent manner in the hippocampal neuron. This action seems to be mediated via LIMK-1/cofilin signaling pathway.

Although the disruption of the actin network was observed after 10.0 μM A 1254 exposure, cytotoxicity was not detected until 100.0 μM A 1254 exposure. These findings demonstrate

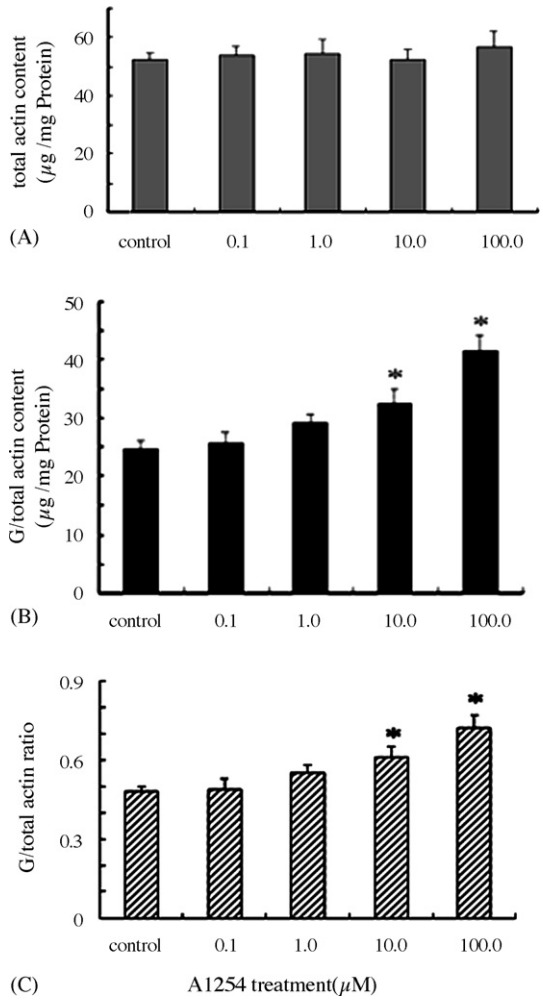

Fig. 5. Effects of A 1254 on G-actin and total actin in hippocampal neurons. Cells were incubated in absence (DMSO) or presence of several A 1254 concentrations (0.1–100.0 μM) for 12 h. The graph shows that changes in total actin (A), G-actin (B) and G/total-actin ratios (C) induced by different concentration of A 1254 were determined as described in Section 2. The values presented are mean ± S.D. of six separate experiments. *Significantly different from control at $P < 0.05$.

that actin network disruption preceded overt cytotoxicity after exposure of A 1254, and suggest that the demise of these hippocampal neurons could be attributed in part to alterations in the actin cytoskelton. Therefore, the decrease in cell viability, as evidenced by an increase in LDH release, MTT and typan blue assay, could be a result of an excessive destruction of the cytoskeleton induced by PCBs at higher concentrations.

It has been reported that PCBs perturb Ca^{2+} homeostasis, protein kinase C and neuregulins, and increase cyclic AMP (cAMP)-responsive element-binding protein (CREB) phosphorylation in the formation of learning and memory deficits. Inglefield et al. showed that A 1254-induced CREB phosphorylation is dependent on synaptic activity and mediated by Ca^{2+}-dependent pathways in neuron (Inglefield et al., 2002). Coincidentally, LIMK-1 has been proved to interact with PKC and neuregulins directly (Kuroda et al., 1996; Wang et al., 1998). Further, LIM kinase 1 activates CREB during the neuronal differentiation of immortalized hippocampal progenitor cells (Yang et al., 2004). In addition, LIMK-1 expression was regulated in an opposing manner by two second messengers, cAMP and Ca^{2+}, that is, cAMP increased LIMK-1 and Ca^{2+} decreased it (Tojima et al., 2003). From above, the mentioned neurobiologic substrates, namely, Ca^{2+}, CREB, PKC and neuregulins, interact with LIMK-1 to participate in the pathologic and physiologic process of PCBs-induced learning and memory deficits.

Interestingly, PCBs have been shown to inhibit or decrease LTP, while genetic deletion of LIMK-1 in mice results in enhanced LTP. However, the reason for this contradiction is unknown. Also, the mechanisms whereby PCBs block LTP are presently unknown. Developmental exposure to PCBs have alterations in many neurobological substrates. The alterations in some neurobological substrates, such as NO, NMDA receptor (Sharma and Kodavanti, 2002; Altmann et al., 2001), may contribute to the decrement in LTP induced by PCBs, but the alterations in other neurobological substrates, such as LIMK-1, lead to enhance LTP. So we presume the counteraction between the opposites effects finally leads to the decrement in LTP after exposure to PCBs. Therefore, this contradiction may be possible due to a counteraction of different effects of neurobological substrates induced by PCBs in the formation of LTP.

In conclusion, our results show that PCBs modify actin polymerization dynamics in hippocampal neurons at concentrations that did not produce overt cytotoxicity. The effect is probably mediated via an inhibition of the LIMK-1/cofilin signaling pathway. These findings offer new perspectives for the understanding of PCBs-induced learning and memory deficits.

Acknowledgements

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