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# Competitive binding of postsynaptic density 95 and Ca<sup>2+</sup>-calmodulin dependent protein kinase II to *N*-methyl-*D*-aspartate receptor subunit 2B in rat brain<sup>1</sup>

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**KEY WORDS** *N*-methyl-*D*-aspartate receptors; Ca<sup>2+</sup>-calmodulin dependent protein kinase; brain ischemia

## ABSTRACT

**AIM:** To investigate the interactions among postsynaptic density 95 (PSD-95), Ca<sup>2+</sup>-calmodulin dependent protein kinase II $\alpha$  (CaMKII $\alpha$ ), and *N*-methyl-*D*-aspartate receptor subunit 2B (NR2B) during ischemia and reperfusion in hippocampus of rats. **METHODS:** Brain ischemia was induced by four-vessel occlusion procedure in rats. Immunoprecipitation and immunoblotting were performed to study the interactions and phosphorylation of proteins. The association-dissociation of PSD-95 and CaMKII $\alpha$  to and from *N*-methyl-*D*-aspartate (NMDA) receptor induced by ischemia and reperfusion and the effects of 1-[*N,O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-*L*-tyrosyl]-4-phenyl-piperazine (KN-62, a selective inhibitor of CaMKII) on these protein interactions were investigated. Coimmunoprecipitation and immunoblotting were performed for the studies of interactions among proteins. **RESULTS:** The alternations of the binding level of PSD-95 and CaMKII $\alpha$  to NR2B during ischemia and reperfusion demonstrated the negative correlation to each other. Pre-administration of KN62 through both cerebral ventricles inhibited the 10 min ischemia-induced increase of the binding of PSD-95 to NR2B and, on the contrary, promoted the binding of CaMKII $\alpha$  to NR2B. **CONCLUSION:** PSD-95 competes with CaMKII to bind to NR2B during ischemia and reperfusion in rat hippocampus.

## INTRODUCTION

Postsynaptic density-95 (PSD-95/SAP-90), a major protein constituent of postsynaptic density (PSD), participates in assembly of the PSD<sup>[1]</sup>. PSD-95, Synapse-Associated Protein 97 (SAP97/hDlg), chapsyn-110/PSD-93, and Synapse-Associated Protein 102 (SAP102) belong to the membrane-associated guanylate kinase

(MAGUK) superfamily of proteins, which share the same molecular structure, namely, three PSD-95/Dlg/Zo-1 domains, one SH<sub>3</sub> domain and one Guanylate kinase (GK) domain<sup>[2]</sup>. Since PSD-95 associates with many kinds of signal proteins, such as synaptic ras-GTPase activating protein (SynGAP)<sup>[3]</sup>, neuronal nitric oxide synthase (nNOS)<sup>[4]</sup>, and *N*-methyl-*D*-aspartate receptors, it is believed that PSD-95 may be crucial for the synapse functions by recruiting postsynaptic proteins. Multiple previous studies demonstrated that both the PDZ<sub>1</sub> and PDZ<sub>2</sub> domains of PSD-95 potently interact with the conserved sequence (S/T)XV in COOH-terminal of NMDA receptor subunit 2B<sup>[5]</sup>. Because of the critical role of NMDA receptors in neu-

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ronal functions such as synaptic transmission and synaptic plasticity<sup>[6]</sup>, PSD-95 may play an important role by clustering NR2B in the PSD and coupling NMDA receptors to cytoplasmic signaling pathways.

NR2B does not solely bind to PSD-95, NMDA receptors have been shown to bind to Ca<sup>2+</sup>-calmodulin dependent protein kinase II (CaMKII) which is a threonine/serine protein kinase and abundant in PSD with broad substrate specificity<sup>[7,8]</sup>. It is well known that autophosphorylation of CaMKII $\alpha$  following influx of Ca<sup>2+</sup> through NMDA receptor is essential for hippocampal dependent long term potentiation (LTP) and this Thr<sup>286</sup> autophosphorylation of  $\alpha$  subunit of CaMKII could be inhibited by KN62, a selective inhibitor of CaMKII. KN62 could depress the activity of CaMKII by competing with calmodulin to bind to the protein kinase. Recently, studies showed that Ca<sup>2+</sup> influx through NMDA receptor induced the translocation and targeting of CaMKII to NR2B<sup>[9]</sup> and PSD-95 also was identified as one of the substrates of CaMKII<sup>[10]</sup>. However, the interactions among these three proteins during ischemia and reperfusion remain unclear until now. Since NMDA receptors is the dominant ions channel on the membranes and plays an important role in calcium overload and neuronal damage during brain ischemia, what is more, CaMKII is the most sensitive protein kinase to the cellular calcium concentration fluctuation and PSD-95 is a widely studied skeleton protein, so to illustrate the interactions among them will provide fundamental knowledge for the prevention and treatment of brain ischemia.

## MATERIALS AND METHODS

**Animal surgical procedures** In animal model preparation, four-vessel occlusion was used to induce forebrain ischemia of male Sprague-Dawley rats (250-300 g, Grade II, Certificate No D02-49-2, Shanghai Experimental Animal Center, Chinese Academy of Sciences). Surgical procedures were conducted following the principles described by Pulsinelli, *et al*<sup>[11]</sup>. Under anesthesia with choral hydrate (300 mg/kg, ip), vertebral arteries were occluded by electrocautery. After 24 h, both carotid arteries were occluded to induce a 15-min ischemia and rats were required to meet the criteria such as dilated pupils, losing of righting reflex, 37 °C-37.5 °C rectal temperature, flat electroencephalogram during ischemia. Carotid artery blood was restored for reperfusion by releasing the clips. When

necessary, KN62 (Sigma, St Louis, USA) was administered through both cerebral ventricles by a micro-injector 20 min before the ischemia.

**Tissue preparation** Rats were decapitated immediately after different ischemia or reperfusion time and the hippocampuses were separated and dipped into liquid nitrogen quickly at -80 °C for storing. Tissues were homogenized in 1:10 (w/v) ice-cold homogenization buffer (HB) consisting of 3-(*N*-morpholino) propanesulfonic acid 50 mmol/L (MOPS; pH 7.4), KCl 100 mmol/L, MgCl 320 mmol/L, dithiothreitol 0.2 mmol/L, phosphatase and protease inhibitors ( $\beta$ -glycerophosphate 20 mmol/L, sodium pyrophosphate 20 mmol/L, NaF 50 mmol/L, EDTA 0.5 mmol/L, EGTA 1 mmol/L, phenylmethylsulfonyl fluoride 1 mmol/L, sodium orthovanadate 1 mmol/L, leupeptin 10 mg/L and pepstatin 10 mg/L) with a Teflon-glass homogenizer. Following description by Shamloo *et al*<sup>[12]</sup>, the homogenates were spun at 800 $\times$ g for 15 min at 4 °C. The resulting supernatant was centrifuged at 100 000 $\times$ g for 30 min at 4 °C in a TL100.2 rotor (Beckman Palo Alto, USA); The pellet (crude membranes fraction) was re-suspended in HB containing 0.1 % Triton X-100 for 10 s by sonication for use.

**Immunoprecipitation and immunoblotting** As described previously<sup>[13]</sup>, tissue homogenates (400  $\mu$ g of protein) were diluted 4-fold with Hepes buffer 50 mmol/L, pH 7.4, containing 10 % glycerol, NaCl 150 mmol/L, 1 % Triton X-100, 0.5 % Nonidet P-40 and inhibitors of phosphatase and protease as that described above. Samples were pre-incubated for 1 h with 20  $\mu$ L protein A then centrifuged to remove any protein adhered nonspecifically to protein A. The supernatants were incubated with proper antibodies overnight at 4 °C. The incubation continued for another 2 h with 20  $\mu$ L protein A/G-sepharose. Samples were centrifuged at 10 000 $\times$ g and the pellet were washed for three times with Hepes buffer. Bound proteins were eluted by adding 4 $\times$ SDS-PAGE sample buffer (10  $\mu$ L) and boiled at 100 °C for 10 min for immunoblotting. Proteins were separated by 10 % SDS-PAGE and electrotransferred onto nitrocellulose membrane (NC, pore size, 0.45  $\mu$ m) by the method of Towbin *et al*<sup>[14]</sup>. The membrane was probed with relevant antibodies at 4 °C overnight. Detection was performed using alkaline phosphatase conjugated IgG and developed using BCIP/NBT color substrate. The bands on the membrane were scanned and analyzed with an image analyzer (Labworks Software, UVP Inc Upland, CA, USA). The intensit were

determined by absorbance.

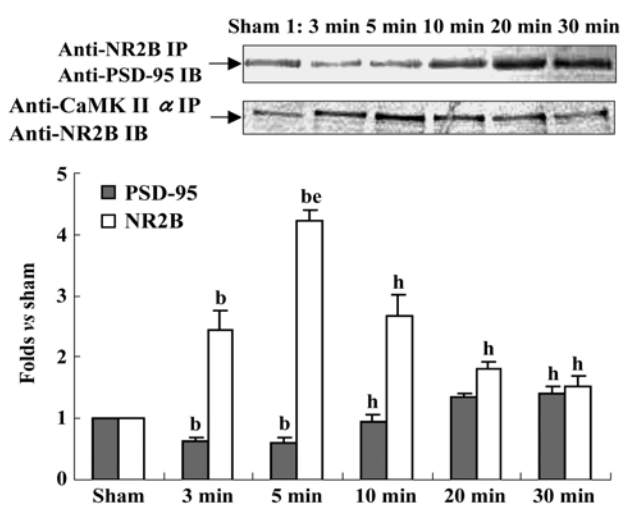
**Antibodies** Anti-CaMKII $\alpha$  antibody and Anti-PSD95 antibody were from Sigma (St Louis, MO, USA); Anti-NR2B antibody was from BD transduction laboratories (Franklin lakes, NJ, USA);

**Drug treatment** KN-62 was prepared by dissolving in Me<sub>2</sub>SO at a concentration of 1 g/L. Drug infusion was performed using a microinjector through both cerebral ventricles (from the bregma: antero-posterior, -0.8 mm; lateral, 1.5 mm; depth, 3.5 mm). A volume of 5  $\mu$ L each was infused over 5 min. Infusion of Me<sub>2</sub>SO served as vehicle control. KN-62 was given 20 min before ischemia.

**Statistics** Values were expressed as mean $\pm$ SD from more than 3 independent animals at each time point for western blotting. Statistical analysis of the result was carried out by one way analysis of variance (ANOVA) followed by the least significant difference (LSD) test or Newman-Keuls test.  $P < 0.05$  was considered significant.

**RESULTS**

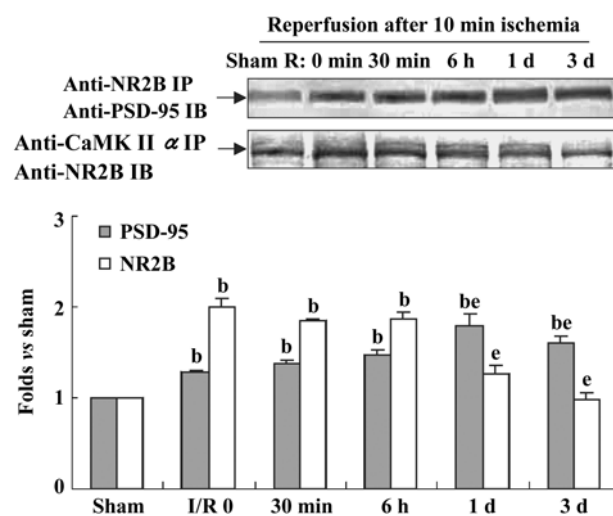
**Effects of ischemia on the association of PSD-95 and CaMKII $\alpha$  with NR2B** Ischemia of 3 or 5 min induced remarkable decreases of the coimmunoprecipitation of PSD-95 and NR2B (upper panel of Fig 1). But the association of CaMKII $\alpha$  with NR2B increased dramatically after 3 or 5 min ischemia and the binding level



**Fig 1.** The interaction of PSD-95 with NR2B (upper panel) and CaMKII $\alpha$  with NR2B (bottom panel) after 3, 5, 10, 20, and 30 min ischemia. I, ischemia.  $n=3$ . Mean $\pm$ SD. <sup>b</sup> $P < 0.05$  vs sham operation, <sup>c</sup> $P < 0.05$  vs 3 min ischemia, <sup>h</sup> $P < 0.05$  vs 5 min ischemia (ANOVA followed by LSD).

of CaMKII $\alpha$  to NR2B reached its peak after 5 min ischemia (bottom panel of Fig 1). Correspondingly, the association of PSD-95 and NR2B showed the lowest level after 3 min ischemia compared with other operation groups. As ischemia time prolonged to 10, 20, and 30 min, there were remarkable increase of the binding level of PSD-95 to NR2B (upper panel of Fig 1) and decrease of the association of CaMKII $\alpha$  with NR2B (bottom panel of Fig 1).

**The alternations of the association of PSD-95 and CaMKII $\alpha$  with NR2B during reperfusion** The associations of PSD-95 and CaMKII $\alpha$  with NR2B were examined by coimmunoprecipitation, our findings showed that the 10 min induced high binding level of PSD-95 to NR2B kept high during 30 min and 6 h reperfusion and became even higher as reperfusion episodes extended to 1 and 3 d (upper panel of Fig 2). Conversely, 10 min ischemia induced association of CaMKII $\alpha$  with NR2B remained the similar levels during 30 min and 6 h reperfusion but diminished after 1 and 3 d reperfusion (bottom panel of Fig 2).



**Fig 2.** The interaction of PSD-95 and CaMKII $\alpha$  with NR2B during 0, 30 min, 6 h, 1 d, and 3 d reperfusion after 10 min ischemia. R, reperfusion.  $n=3$ . Mean $\pm$ SD. <sup>b</sup> $P < 0.05$  vs sham operation, <sup>e</sup> $P < 0.05$  vs 10 min/R0 (ANOVA followed by LSD).

**The total protein levels of PSD-95, CaMKII $\alpha$ , and NR2B during ischemia and reperfusion** To investigate the total protein expression of PSD-95, CaMKII $\alpha$ , and NR2B, we performed the immunoblotting with different antibodies. There was no alternation of total protein levels of PSD-95, CaMKII $\alpha$ , and NR2B during 10 min ischemia and other reperfusion operation

groups (Fig 3).

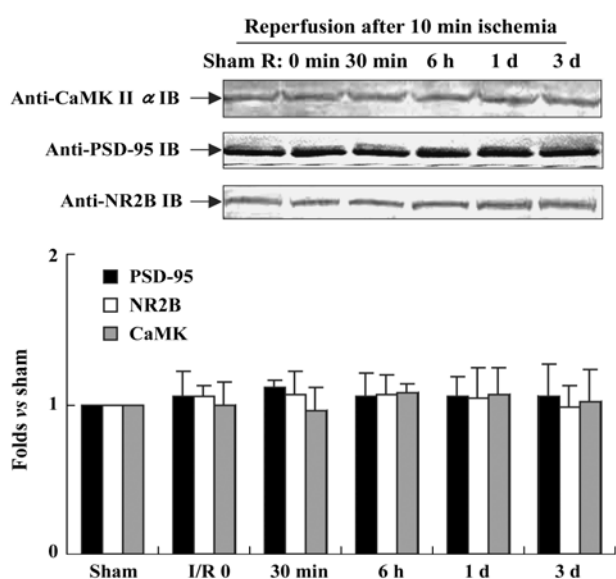


Fig 3. Immunoblotting analysis of the total protein levels of PSD-95, CaMKII $\alpha$ , and NR2B during 0, 30 min, 6 h, 1 d, and 3 d reperfusion after 10 min ischemia. R, reperfusion,  $n=3$ . Mean $\pm$ SD.

**Effects of KN62 on the ischemia induced binding of PSD-95 and CaMKII $\alpha$  to NR2B** Pre-administration of KN62 through cerebral ventricle 20 min before ischemia can obviously promote the ischemia-induced binding of PSD-95 to NR2B which was not affected by the same dosage of vehicle (upper panel of Fig 4). In contrast, ischemia-induced increase of the coimmunoprecipitation level of CaMKII $\alpha$  and NR2B was remarkably inhibited by KN62, but administration of vehicle had no effect on this interaction.

## DISCUSSION

Ischemia of 3 or 5 min induced the decrease of the interaction level of PSD-95 with NR2B, but as ischemia time prolonged to 10, 20, or 30 min the association levels rebounded (Fig 1), which is in line with the results of Takagi *et al*<sup>[15]</sup> who performed their studies with sodium deoxycholate (DOC), one kind of detergent. The 15 min ischemia-induced decrease in DOC solubility of the NMDA receptor might reflect an increasing association between NMDA receptor and PSD-95; During reperfusion after 10 min ischemia, our data clearly showed that reperfusion led to more severe increase of the interaction between PSD-95 and NR2B than that induced by 10 min ischemia (Fig 2). What

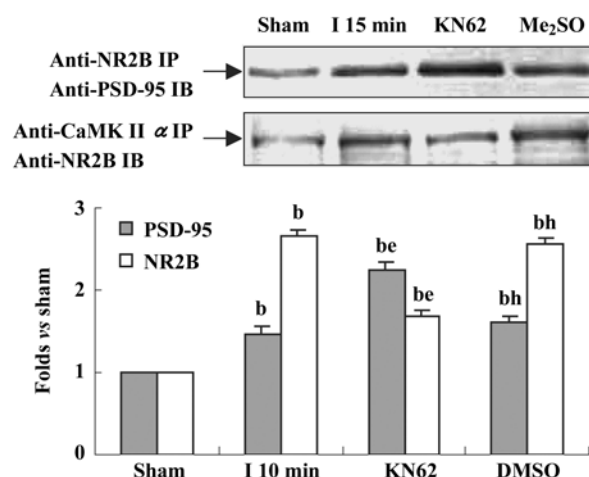


Fig 4. Effects of KN62 on the associations of PSD-95 and CaMKII $\alpha$  with NR2B, homogenates from rats pre-administered KN62 and Me<sub>2</sub>SO (the vehicle) by cerebral ventricle injection 20 min before ischemia were used for immunoprecipitation.  $n=3$ . Mean $\pm$ SD. <sup>b</sup> $P<0.05$  vs sham operation, <sup>c</sup> $P<0.05$  vs I10 min/R0, <sup>h</sup> $P<0.05$  vs I10 min+KN62 (ANOVA followed by LSD).

mechanisms underlie the alternations of the association between PSD-95 and NR2B described above is obscure. Our other data may provide us some implications. We found that the complex levels of CaMKII $\alpha$ -NR2B immediately increased after 3 min ischemia and reached its peak at 5 min ischemia; as ischemia time extended, the complex levels of CaMKII $\alpha$ -NR2B decreased dramatically (bottom panel of Fig 1). Thirty minute reperfusion after 10 min ischemia kept the interaction between CaMKII $\alpha$  and NR2B high and this interaction decreased as reperfusion time prolonged to 1 d or 3 d. CaMKII $\alpha$  competes with PSD-95 for the binding to C-terminal of NR2A<sup>[16]</sup>. The association-dissociation of CaMKII $\alpha$  and PSD-95 to and from NR2B were coincident in our studies (Fig 1, 2). In other words, increase or decrease of the binding levels of PSD-95 to NR2B always accompanied the inverse changes of the interaction between CaMKII and NR2B. The reasonable explanation of this phenomenon we can suppose is that PSD-95 also competes with CaMKII for binding to NR2B during ischemia and reperfusion. No change of total protein levels was detected in 10 min ischemia and all reperfusion operation groups in our study, which indicated that no protein of PSD-95, CaMKII $\alpha$ , or NR2B was synthesized and the genes of these proteins were dormant during ischemia and reperfusion within 3 d.

For further investigation, KN62, a kind of selective inhibitor of CaMKII which can inhibit the kinase

activity by directly binding to the calmodulin binding site of the enzyme<sup>[17]</sup> was used to illustrate whether it is true that there are some relationships between the bindings of CaMKII and PSD-95 to NR2B. Our results indicate that administration of KN62 depressed the 10 min ischemia induced binding of CaMKII $\alpha$  to NR2B, in contrast, the association of PSD-95 with NR2B induced by 10 min ischemia was promoted by pretreatment with KN62 (Fig 4). These data further indicate that CaMKII may compete with PSD-95 for the binding to NR2B during ischemia. Although many mediating mechanisms of the interaction between PSD-95 and NMDA receptor expect to be investigated in the future, one thing is clear: CaMKII plays an essential role directly or indirectly in mediating the association and dissociation of PSD-95 to NR2B during brain ischemia and reperfusion.

In conclusion, CaMKII $\alpha$  and PSD-95 compete with each other for binding to NMDA receptors during ischemia and reperfusion, which may benefit the coupling of NMDA receptors to different signal transduction pathways. Our studies of the dynamic interaction among the three proteins may offer some assistance for further understanding of the molecular mechanisms involved in brain ischemia and will be useful for finding some clinic way to cure the patients. However, the basis for the ischemia-induced interaction of PSD-95 with other proteins may be complex and multifaceted and molecular mechanisms of the interactions between these three proteins need to be further elucidated.

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