Glutamate-induced transient modification of the postsynaptic density

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Contributed by Thomas S. Reese, July 2, 2001

Depolarization of rat hippocampal neurons with a high concentration of external potassium induces a thickening of postsynaptic densities (PSDs) within 1.5–3 min. After high-potassium treatment, PSDs thicken 2.1-fold in cultured neurons and 1.4-fold in hippocampal slices compared with their respective controls. Thin-section immunoelectron microscopy of hippocampal cultures indicates that at least part of the observed thickening of PSDs can be accounted for by an accumulation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) on their cytoplasmic faces. Indeed, PSD-associated gold label for CaMKII increases 5-fold after depolarization with potassium. The effects of high-potassium treatment on the composition and structure of the PSDs are mimicked by direct application of glutamate. In cultures, glutamate-induced thickening of PSDs and the accumulation of CaMKII on PSDs are reversed within 5 min of removal of glutamate and Ca^{2+} from the extracellular medium. These results suggest that PSDs are dynamic structures whose thickness and composition are subject to rapid and transient changes during synaptic activity.

Postsynaptic densities (PSDs) are specialized protein complexes apposed to the postsynaptic membrane. These complexes contain and organize various components of the postsynaptic response to neurotransmitter release such as receptors and signal transduction molecules. A structural change in the PSD is likely to reflect an organizational change in the postsynaptic molecular machinery and, therefore, a functional modification of the synapse.

PSDs can thicken after cerebral ischemia (1, 2) and in cultured hippocampal neurons when ischemic energy depletion is mimicked by the inclusion of a mitochondrial uncoupler in the absence of glucose (3). The thickening of PSDs is accompanied by an increase in certain proteins, including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), in the PSD fraction (1), suggesting that these may have accumulated on the PSD structure. Immunogold labeling of CaMKII in hippocampal cultures confirms that CaMKII accumulates on the PSD under ischemia-like conditions (3). Ischemia is known to result in excessive glutamate release (4, 5), so it is possible that glutamate mediates the above-mentioned modification of PSDs.

Early studies (6) revealed that PSDs in unanesthetized animals are thicker than those in anesthetized animals, suggesting an influence of synaptic activity on PSD thickness. Thickening of the PSD also occurs in conditions expected to promote activation of glutamate receptors, such as rhythmic stimulation of the dentate gyrus in the guinea pig hippocampus (7), combined application of glutamate and acetylcholine in the sensorimotor region of the rat cerebral cortex (8), or chronic *N*-methyl-D-aspartate treatment of doubly innervated optic tecta in the frog (9). However, it is not clear whether the PSD thickening under these nonpathological conditions is also accompanied by an accumulation of CaMKII on the PSDs. Observations at the light microscopic level (10) do indicate colocalization of CaMKII with PSD-95 puncta after stimulation of hippocampal cultures with glutamate. However, immunocytochemical localization at the electron microscopic level is needed to show that CaMKII actually associates with the PSD.

To our knowledge, no changes in thickness of the PSDs after long-term potentiation (LTP) have been reported, although it is not clear whether this parameter has ever been systematically measured at different stages of LTP induction and maintenance. Considering that most of the studies on morphological correlates of LTP were designed to investigate relatively long-term (1 h to several days) changes after the cessation of the stimulus (11–17), it is possible that a transient PSD thickening during, or shortly after, induction may have escaped notice. Thus, it remains possible that PSD thickening is an acute and reversible consequence of synaptic activation.

The present electron microscopic study sets out to investigate acute changes in the PSD that occur after brief depolarization or exposure to glutamate. Immunolabeling for CaMKII, a protein considered to be a major component of the PSD and a necessary element for the induction of long-term synaptic modification, allows visualization of acute changes in the association of this molecule with the PSD. We demonstrate that PSDs markedly thicken during synaptic activity, at least in part because of translocation of CaMKII, and that these changes are reversible. These results point out a largely unrecognized feature of the PSDs: that they are dynamic structures capable of rapid, reversible modification.

Materials and Methods

Materials. Monoclonal antibody against α-CaMKII (clone 6G9–2) was from Boehringer Mannheim, monoclonal antibody against PSD-95 (MA1–046) was from Affinity Bioreagents (Golden, CO); and L-glutamate and glycine were purchased from Tocris (Ballwin, MO).

Hippocampal Cell Culture. Rat hippocampal cell cultures were prepared and maintained as described in Lu *et al.* (18). Hippocampal cells from 21-day-old embryonic Sprague–Dawley rats were dissociated, and cultures were grown on top of a feeder layer of glial cells. Cultures were maintained in an incubator under 90% air/10% CO_2 at 37°C. Most of the work in this study was performed with 3-week-old cultures grown in 35-mm dishes with or without a 20-mm glass coverslip. Occasionally, 4- to 5-week-old cultures were used. No significant differences were detected in these older cultures.

Stimulation of cultures with either high K^+ (90 mM KCl) or glutamate/glycine (100 μM glutamate/10 μM glycine) was performed with the dishes floating on a platform in a water bath at 37°C unless otherwise specified. In all experiments, cells were

Abbreviations: PSD, postsynaptic density; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; ACSF, artificial cerebrospinal fluid.

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 www.pnas.org/cgi/doi/10.1073/pnas.181336998

washed once with normal incubation medium (124 mM NaCl/2 mM KCl/1.24 mM KH_2PO_4/1.3 mM $MgCl_2$/2.5 mM $CaCl_2$/30 mM glucose in 25 mM Hepes at pH 7.4) before the addition of reagents. In all experiments, control cultures were processed in parallel, including all of the washing and medium-changing steps.

To examine recovery after exposure to high K^+, one group of samples was washed three times in normal incubation medium, then culture medium (modified MEM) was added and the cultures were returned to the incubator for another 30 min. After incubation with glutamate/glycine, one group of samples was washed three times in Ca^{2+}-free incubation medium (composition as in normal incubation medium except for the omission of $CaCl_2$ and addition of 1 mM EGTA, with the osmolarity compensated by the addition of sucrose). Cultures were subsequently left to recover in the same Ca^{2+}-free medium for 5 min on the floating platform in a 37°C water bath. The transfer to Ca^{2+}-free medium was intended to expedite the return to basal intracellular [Ca^{2+}] after the removal of glutamate.

Electron Microscopy. Cells were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 1 h. After fixation, cells were washed in buffer, treated with 1% osmium tetroxide, followed by *en bloc* mordanting with 0.25–0.5% uranyl acetate, dehydrated through a series of ethanol solutions, embedded in epoxy resin, and sectioned and stained conventionally.

Preembedding Immunocytochemistry. Cultures were processed as described in Tanner *et al.* (19). Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 45 min, washed with buffer, permeabilized with 0.1% saponin, and blocked with 5% normal goat serum in PBS for 1 h. They were then incubated with the primary antibody for 1 h, washed, incubated with the secondary antibody conjugated to 1.4-nm gold (Nanogold, Nanoprobes, Yaphank, NY) for 1 h, washed, and fixed with 2% glutaraldehyde in PBS. Samples were then washed and silver enhanced (HQ silver enhancement kit, Nanoprobes), treated with 0.2% osmium tetroxide in buffer for 30 min and with 0.25–0.5% uranyl acetate for 30–60 min or overnight, washed, dehydrated in ethanol, and finally embedded in epoxy resins. Specificity of antibody labeling was tested by omission of the primary antibody.

Hippocampal Slices from Adult Rat. Four-week-old male rats (Sprague–Dawley) were anesthetized with chloroform and rapidly decapitated, observing approved National Institutes of Health protocols and guidelines. Hippocampi were dissected and placed in ice-cold artificial cerebrospinal fluid (ACSF: 124 mM NaCl/2 mM KCl/1.24 mM KH_2PO_4/1.3 mM $MgSO_4$/17.6 mM $NaHCO_3$/2.5 mM $CaCl_2$/10 mM glucose). Transverse slices 400 μm thick were cut with a McIlwain tissue chopper and placed in a recovery chamber containing ACSF equilibrated with 95% O_2/5% CO_2 maintained at room temperature. After at least 2 h of recovery, slices were transferred to an interface-type recording chamber perfused with a continuous flow of ACSF at 34°C, and exposed to an atmosphere of 95% O_2/5% CO_2. Field excitatory postsynaptic potentials (EPSPs) were evoked in CA1 stratum radiatum by stimulation of Schaffer collaterals with a low-resistance stainless steel tetrode, and recorded with ACSF-filled glass pipettes (<5 MΩ) with an isolated bioamplifier (DAM80iP, World Precision Instruments, Sarasota, FL). Test stimuli consisted of monophasic 200-μs pulses of constant current delivered by stimulus isolation units (ISO-Flex, AMPI, Jerusalem, Israel) by using a Master-8 pulse generator (AMPI). Basal synaptic transmission was monitored by low-frequency stimulation (1 every 30 s). Only slices exhibiting EPSPs 2–3 mV in amplitude without superimposed population spikes were used for the following experiments. After establishing a stimulus intensity that evoked stable EPSPs 1–1.5 mV in amplitude, slices were exposed to either high K^+ for 90 s or maintained in normal ACSF (controls). Slices were then immediately fixed by immersion in fixative containing 6% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4, warmed to 40°C, and then microwaved for 10 s (20). The slices were left in the same fixative overnight at room temperature, and then washed extensively in cacodylate buffer. CA1 was dissected out and samples were treated with 1% osmium tetroxide for 1 h followed by 0.25% uranyl acetate overnight; further processing was as above.

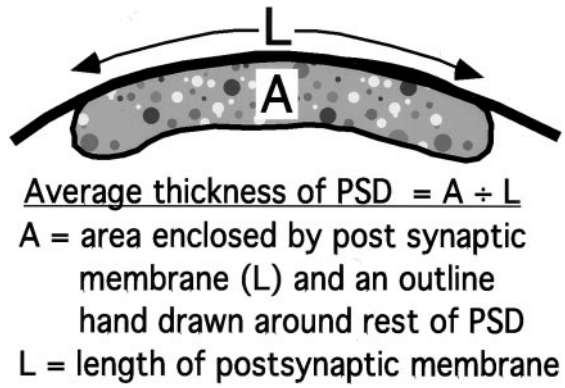

Fig. 1. Measurement of the thickness and the amount of gold label associated with PSDs. A cytoplasmic outline of the PSD was traced by hand. This area was then enclosed by tracing the postsynaptic membrane separately. The average thickness of the PSD was calculated by dividing the outlined area by the length of the postsynaptic membrane. The intensity of the gold label was estimated as the number of silver-enhanced gold particles within the outline around the PSD divided by the length of the corresponding postsynaptic membrane.

Morphometry. For hippocampal cultures, several grid openings were randomly selected to be photographed, but for brain slices, only CA1 stratum radiatum was sampled. Within these chosen areas, every synapse with a distinct cross-sectioned postsynaptic membrane was photographed for quantitative morphometry. Micrographs were enlarged to ×82,500 for measurements, which were done blind on coded images in random order with NIH IMAGE 1.61. To measure the average thickness of a PSD, its cytoplasmic outline, including the associated dense material, was traced, and this area was then enclosed by tracing the postsynaptic membrane separately (Fig. 1). The area was then divided by the length of the postsynaptic membrane to derive an average thickness for each PSD, for each synapse. To estimate the intensity of the label, all silver-enhanced gold particles within or contacting the contour line of each PSD were counted and their number was divided by the length of the corresponding postsynaptic membrane. Results are expressed as mean ± SEM. Control, stimulated, and recovered groups were compared for thickness and gold per μm by post hoc ANOVA with Bonferroni–Dunn correction (STATVIEW 4.5; SAS Institute, Cary, NC).

Results

Depolarization of cultured hippocampal neurons by application of 90 mM KCl for 3 min causes an increase in the cross-sectional thickness of the PSDs (Fig. 2 *A* and *B*). Measurements on electron micrographs from parallel sets of control and depolarized samples show an increase in thickness from 23.5 ± 0.8 nm (control) to 48.4 ± 8.6 nm (depolarized), a 2.1-fold increase ($P <$ 0.001). Similar changes occur in hippocampal slices exposed to 90 mM KCl for 90 s (Fig. 2 *C* and *D*). In control slices the mean thickness of PSDs of synapses within CA1 stratum radiatum was 32.4 ± 1.55 nm, which is higher than the corresponding value in

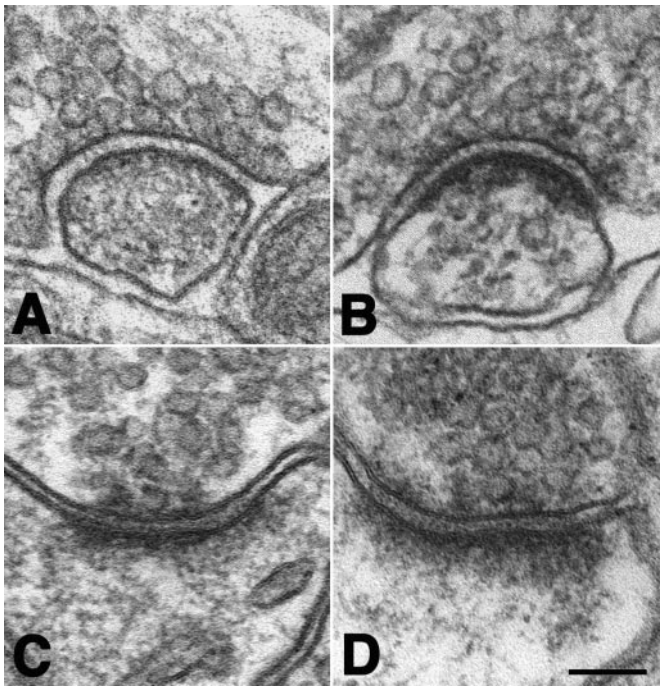

Fig. 2. Brief depolarization with high K^+ induces thickening of PSDs in hippocampal cultures (*A* and *B*) and hippocampal slices (*C* and *D*). Hippocampal cultures were incubated for 3 min either in normal incubation medium (*A*) or in medium containing 90 mM KCl (*B*) before fixation. After recovery hippocampal slices were perfused for 90 s in either normal ACSF (*C*) or in ACSF containing 90 mM KCl (*D*) and fixed immediately. In media containing 90 mM KCl, osmolarity was adjusted by an equivalent reduction in [NaCl]. (Bar = 100 nm.)

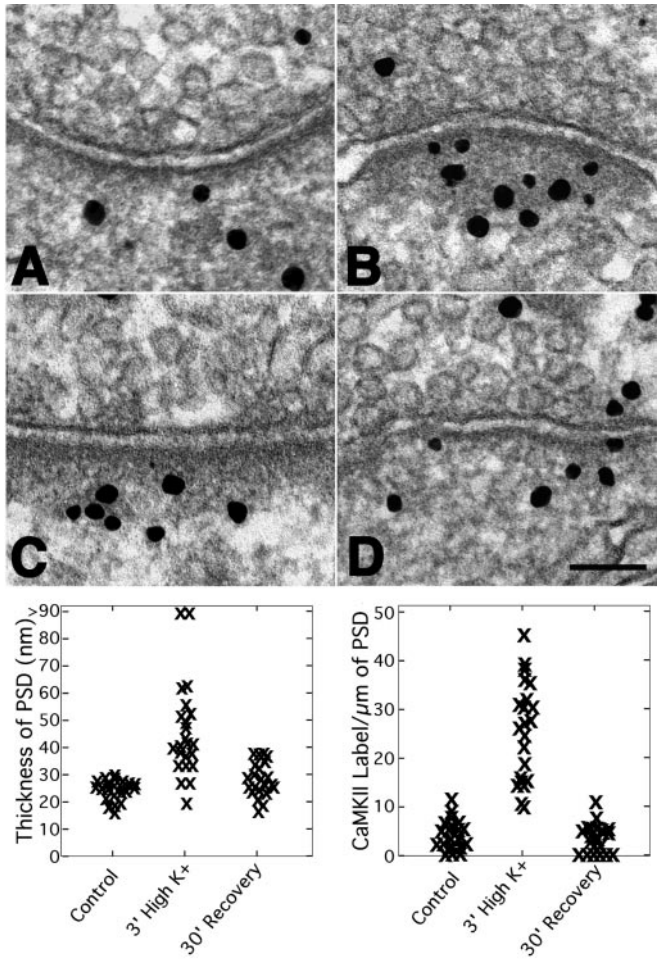

Fig. 3. Reversible thickening of PSDs induced by high K^+ is accompanied by a reversible increase in CaMKII labeling. Cultures were incubated in either normal incubation medium (*A*) or medium containing 90 mM KCl (*B* and *C*) and fixed immediately. One group of cultures was allowed to recover for 30 min after exposure to high K^+ (*D*). After fixation, samples were immunogold labeled with an antibody against CaMKII. Silver-enhanced gold particles appear as black grains of variable size. (Bar = 100 nm.) (*Bottom*) Scatter plots show measurements of the thickness (*Bottom Left*) and CaMKII label intensity (*Bottom Right*) of PSDs in parallel experiments. Each point corresponds to a measurement from an individual PSD. Between 22 and 24 PSDs were analyzed per group.

cultured neurons. Exposure to high K^+, however, induced further thickening of PSDs in adult hippocampal slices to 44.5 ± 3.98 nm, a 1.4-fold increase ($P < 0.01$, Student's t test)

Based on the response of PSDs to ischemic stress (3), it seems likely that the thickening of the PSDs upon depolarization with high K^+ is, at least in part, caused by an accumulation of CaMKII on PSDs. To test this possibility, immunocytochemical studies were conducted with hippocampal cultures. Dispersed cell cultures offer a good system for such studies because intact neurons and their synapses lie near the surface, thereby providing ready access for immunocytochemical reagents. Analysis of hippocampal cultures immunolabeled with an antibody against α-CaMKII indicates that, under resting conditions, CaMKII immunoreactivity within postsynaptic elements is generally dispersed in the dendritic cytoplasm. In parallel to the observed changes in PSD thickness, application of high K^+ for 3 min results in an increased accumulation of immunogold on the PSDs (Fig. 3 *B* and *C*). Analysis of electron micrographs from parallel control and high-K^+-treated cultures shows significant increases in the thickness of the PSD and in the density of gold labeling after K^+ treatment (control: 4.6 ± 0.7 gold particles per μm; depolarized: 24.9 ± 2.2 gold particles per μm; $P < 0.001$).

To test whether depolarization-induced changes in PSD thickness and CaMKII content are reversible, cultures were washed extensively after 3-min exposure to high K^+ and returned to the incubator in culture medium in the same series of experiments. Within 30 min of incubation under normal conditions, the thickening of the PSDs and the CaMKII accumulation were reversed (Fig. 3*D*). After a recovery period of 30 min, mean PSD thickness returned to 27.4 ± 1.2 nm, and CaMKII immunogold label density to 3.8 ± 0.7 gold particles per μm. These numbers do not differ significantly from the control values from parallel experiments.

The effects of depolarization by high K^+ on PSDs are similar to the effects of ischemia-like conditions (2, 3), in that both treatments result in thickening concomitant with accumulation of CaMKII on the cytoplasmic face of PSDs. Because both depolarization and ischemia promote neurotransmitter release, the action of neurotransmitter could modify PSDs in both instances. To test this possibility, hippocampal cultures were treated directly with the excitatory neurotransmitter glutamate and the changes in PSDs were measured as before (Fig. 4 *A* and *B*). Exposure of neurons to 100 μM glutamate and 10 μM glycine for 90 s promoted a 3.5-fold increase in PSD thickness (control: 16.6 ± 2.0 nm; glutamate-treated: 57.5 ± 3 0.1 nm). Concomitantly, the density of CaMKII immunogold labeling on the cytoplasmic side of the PSDs increased (control: 8.0 ± 1.7 gold particles per μm; glutamate-treated: 21.5 ± 2.6 gold particles per μm). The differences between control and glutamate-treated samples in PSD thickness as well as in CaMKII label intensity

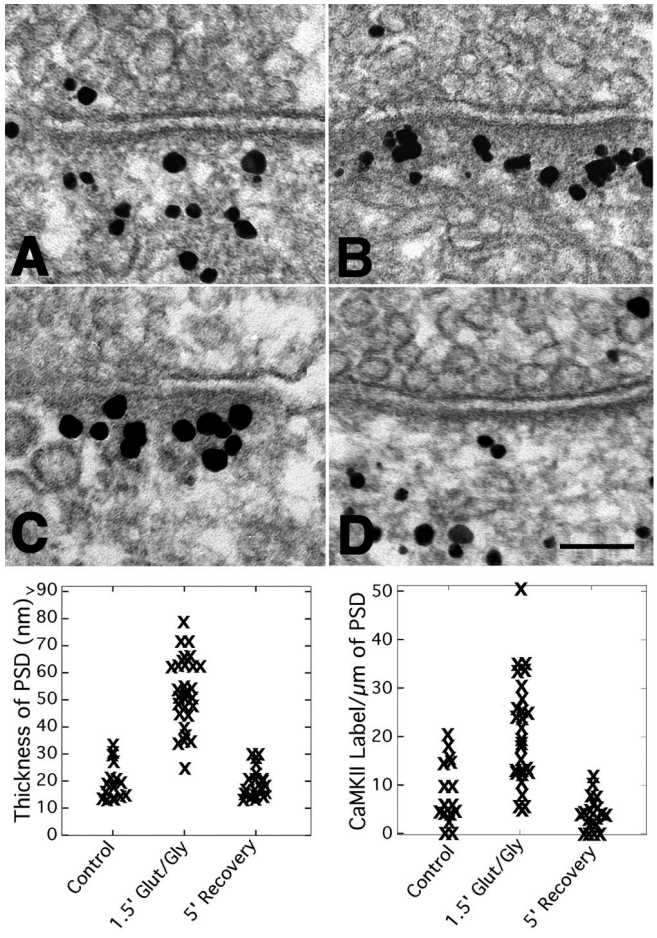

Fig. 4. Glutamate/glycine application mimics the effects of high-K^+ depolarization. Cultures were incubated in either normal incubation medium (*A*) or the same medium containing 100 mM glutamate/10 mM glycine for 90 s (*B* and *C*). After exposure to glutamate, one group of samples was washed and incubated for an additional 5 min in Ca^{2+}-free incubation medium (*D*). *A*, *B* and *C*, *D* represent pairs from two separate experiments. (Bar = 100 nm.) (Bottom) Measurements of the thickness of the PSDs (*Bottom Left*) and intensity of CaMKII gold label (*Bottom Right*) are presented as scatter plots. Each point corresponds to a measurement from an individual PSD. Between 15 and 20 PSDs were analyzed per group.

were significant (Fig. 4, scatter plots; $P < 0.001$ for both). These effects of glutamate were reversed within 5 min of incubation in Ca^{2+}-free medium (Fig. 4*D*). Indeed, 5 min after the addition of medium containing 1 mM EGTA in the absence of glutamate/glycine, the PSD thickness was 16.8 ± 6.8 nm and the density of CaMKII immunolabel was reduced to 4.5 ± 3.7 gold particles per μm.

The thickening of the PSD after exposure to either high K^+ or glutamate/glycine apparently is caused by the addition of loose material on the cytoplasmic face of the PSD. Immunogold labeling with an antibody against PSD-95 indicates that PSD-95 is localized within the compact region of the PSD next to the plasma membrane (Fig. 5*A*), and that stimulation with glutamate/glycine does not seem to promote any increase or change in distribution of PSD-95 labeling (Fig. 4*B*). In fact, a decrease in the PSD-95 labeling of the thickened PSDs is attributable to a decreased penetration of the antibodies through the thickened PSD. Comparison of the immunolabeling for PSD-95 and for CaMKII (Fig. 5 *A* and *B, C* and *D*, respectively) reveals marked differences in the localization of these two molecules within the PSD structure as well as in the lability of their association with

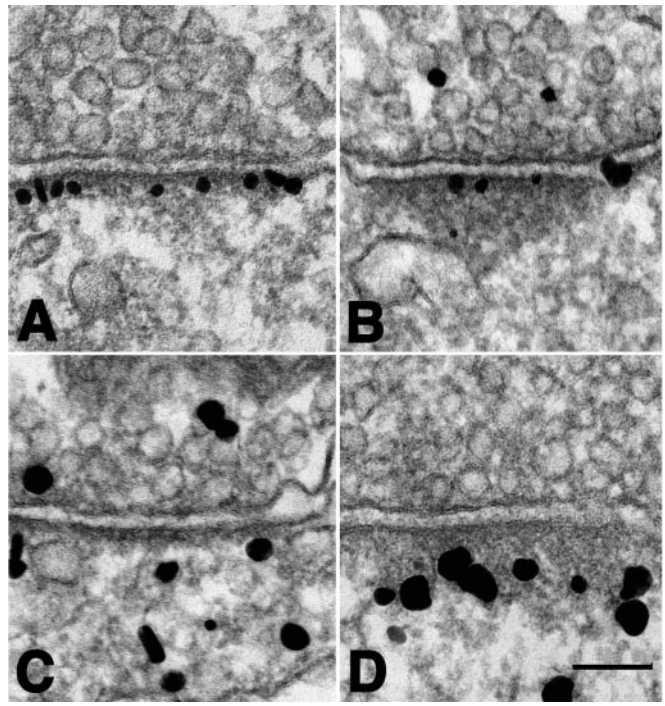

Fig. 5. Immunogold labeling pattern for PSD-95 is distinct from that for CaMKII. Cultures were labeled with an antibody against PSD-95 (*A* and *B*) or with an antibody against CaMKII (*C* and *D*) after 3-min exposure to either normal incubation medium (*A* and *C*) or medium containing 100 μM glutamate/10 μM glycine (*B* and *D*). (Bar = 100 nm.)

the PSD. PSD-95 is located within the compact region on the cleft side of the PSD and its association does not seem to change with stimulation. In contrast, CaMKII seems to be part of the looser material on the cytoplasmic side of the PSD and its association is strongly affected by stimulation.

Discussion

Thickening of the PSD has previously been observed after ischemia (1–3) and after various protocols designed to activate glutamate receptors (7–9). The present study shows that stimulation induced by application of high K^+ or glutamate for as little as 90 s to hippocampal neurons in culture is sufficient to induce a thickening of PSDs. Thickening of PSDs in response to depolarization also occurs in hippocampal slices, indicating that this phenomenon is not an artifact of dissociated cultures. However, the thickening in slices was small compared with that in dispersed cell cultures. A higher basal PSD thickness in slices possibly results from mild ischemic conditions inherent in the perfusion system, because perfusion with oxygenated medium is not as effective as delivery of oxygen and nutrients through capillaries *in vivo*.

Glutamate-induced thickening of PSDs apparently is not caused by an extension of the dense subsurface lamina that contains PSD-95. Instead, looser material is added to the cytoplasmic face of the PSD, giving it a more irregular contour. Parallel immunocytochemical studies show that the thickening of PSDs is accompanied by an increased labeling for CaMKII on the cytoplasmic face. Thus, at least part of the thickening must be caused by an accumulation of CaMKII on the PSD. Demonstration that the two events are reversed in parallel confirms this conclusion. At this stage, however, it is not clear whether CaMKII is the only protein that contributes to the PSD thickening.

Our observations are in agreement with a previous demonstration (10) that, on treatment of hippocampal cultures with glutamate, distribution of CaMKII tagged with green fluorescent protein (GFP) becomes punctate and coincides with PSD-95 staining. Moreover, this phenomenon was found to be reversible, to require Ca^{2+}/calmodulin, and to be regulated by autophosphorylation (10, 21). These results were thought to represent association/dissociation of CaMKII with the PSDs. The electron microscopic observations presented here provide direct evidence that glutamate, indeed, induces reversible association of CaMKII with the PSDs.

The results from the present study demonstrate that glutamate-induced thickening and association of CaMKII with the PSDs are rapidly reversed after the cessation of the stimulus. This conclusion opens the interesting possibility that under physiological conditions the thickness and the CaMKII content of the PSDs change continuously in response to the activity of the synapse. Thus, it is likely that, upon synaptic activation, certain proteins within the dendrite/spine cytoplasm, including CaMKII, are added to the postsynaptic complex and are subsequently dispersed after the cessation of the stimulus.

The observations that the glutamate-induced increase in CaMKII immunolabeling is reversible also indicate that this protein is a variable component of the PSD. Although, based on the analysis of isolated PSD fractions, CaMKII is considered to be "the major postsynaptic density protein" (22, 23) it is probable that PSDs do not contain high quantities of CaMKII under resting conditions. During subcellular fractionation, the unavoidable lag period of a few minutes between decapitation and homogenization would be enough to create ischemic conditions, which would in turn induce accumulation of CaMKII on the PSDs as well as formation of CaMKII clusters that contaminate the PSD fraction (3). The observation by Suzuki *et al.* (24) that the CaMKII content of the PSD fraction increases as the time taken to process brains is increased supports this conclusion.

In addition to an accumulation of CaMKII on PSDs, ischemic conditions cause clustering of cytoplasmic CaMKII into spherical structures ≈110 nm in diameter, apparently through self-association. We interpreted CaMKII clustering as a mechanism to decommission CaMKII during episodes of Ca^{2+} overload (3). Because formation of CaMKII clusters and thickening of PSDs occur under the same ischemic conditions, it is likely that accumulation of CaMKII on the PSDs and formation of CaMKII clusters have a common mechanism and similar functions. Under the relatively mild conditions used in the present studies with hippocampal cultures, very few CaMKII clusters were formed (data not shown), but the CaMKII labeling on the PSD increased to the same level as that observed under ischemia-like conditions (3). It is possible that a local rise of $[Ca^{2+}]$ above threshold levels within the confines of a dendritic spine after sustained synaptic activity causes clustering of CaMKII on the cytoplasmic face of the PSD.

In conclusion, our observations reveal an unexpected short-term plasticity of the PSD. PSDs appear as dynamic structures that constantly readjust themselves in response to the activity state of the synapse. These findings point to an involvement of the PSD in short-term activity-driven changes. Further studies should reveal the threshold level of synaptic activity required to trigger a change in the PSD. These observations will be helpful in understanding the functional significance of synaptic thickening.

We thank Virginia Tanner Crocker, Rita Azzam, John Chludzinski, and Pat Zerfas for assistance with electron microscopy. Jennifer Petersen performed image analyses. This work was supported by the National Institutes of Health Intramural Program and National Science Foundation Grant 9817317 (to A.D.).

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