17-β-Estradiol increases neuronal excitability through MAP kinase-induced calpain activation

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Contributed by Richard F. Thompson, October 30, 2009 (sent for review August 20, 2009)

17-β-Estradiol (E2) is a steroid hormone involved in numerous brain functions. E2 regulates synaptic plasticity in part by enhancing NMDA receptor function and spine density in the hippocampus, resulting in increased long-term potentiation and facilitation of learning and memory. As the calcium-dependent neutral protease, calpain, is also involved in these processes, we tested whether E2 could activate calpain and examined the functional consequences of E2-mediated calpain activation in hippocampus. Calpain activity was analyzed by a fluorescence resonance energy transfer (FRET)-based assay that allows both quantitative determination and spatial resolution. E2 rapidly activated calpain in cultured cortical and hippocampal neurons, prominently in dendrites and dendritic spines. E2-induced calpain activation was mediated through mitogen-activated protein kinase (MAPK), as it was completely blocked by MEK inhibitors. It was also calcium-independent, as it was still evident in presence of the calcium chelator, BAPTA-AM. Activation of ERα and ERβ receptors by specific agonists stimulated calpain activity. Finally, the rapid E2-mediated increase in excitability in acute hippocampal slices was prevented by a membrane-permeable calpain inhibitor. Furthermore, E2 treatment of acute hippocampal slices resulted in increased actin polymerization and membrane levels of GluR1 but not GluR2/3 subunits of AMPA receptors; both effects were also blocked by a calpain inhibitor. Our results indicate that E2 rapidly stimulates calpain activity through MAP kinase-mediated phosphorylation, resulting in increased membrane levels of AMPA receptors. These effects could be responsible for E2-mediated increase in neuronal excitability and facilitation of cognitive processes.

FRET | hippocampus | synaptic plasticity

Calpains are intracellular calcium-dependent neutral cysteine proteases that have been implicated in several brain processes, including synaptic plasticity [e.g., long term potentiation (LTP)] and learning and memory formation (1, 2). To account for the role of calpain in these processes, calpain activity has been proposed to be intricately involved in activity-dependent changes in dendritic spine morphology via truncation of a variety of cytoskeletal proteins, including spectrin, as well as of enzymes participating in regulation of spine morphology and function (3–6). Several studies have indicated that *m*-calpain could be rapidly activated by MAP kinase-mediated phosphorylation in various cell types (7–9), and we recently reported that a similar process occurs in neurons (10). Moreover, we showed that brain-derived neurotrophic factor (BDNF)-elicited increase in actin polymerization in cultured cortical neurons was blocked by a calpain inhibitor (10). These findings provided a link between several elements proposed to play a critical role in LTP, namely BDNF, calpain, MAP kinase, and actin polymerization.

17-β-Estradiol (E2) is a steroid hormone that is critical for the functions of multiple organs, including the brain. In particular, E2 plays a significant role in neuroprotection, neurogenesis, and synaptic plasticity in both males and females (11, 12). It has been shown that E2 facilitates LTP by activation of the Src/MAP kinase pathway and subsequent phosphorylation of NMDA receptors (13–15). Recent data indicate that the rapid effects of E2 on synaptic function and plasticity are mediated by alterations of the cytoskeletal network, especially by stimulation of actin polymerization (16–19). Furthermore, although the respective roles of the two types of E2 receptors, ERα and ERβ, are still debated, several lines of evidence indicate that the rapid effects of E2 on synaptic function are mediated by ERβ rather than ERα receptors (20). Therefore, in view of the relationship between MAP kinase, calpain, and actin polymerization, we examined whether E2 could induce calpain activation in neurons, and whether such activation could account for some of the effects of E2 on synaptic function. We also evaluated the effects of agonists for ERα and ERβ receptors on calpain activation.

Previous studies have used the accumulation of a selective calpain-mediated spectrin breakdown product (SBDP) as a quantitative assay for calpain activation (21). More recently, a fluorescence resonance energy transfer (FRET)-based calpain assay was described in ref. 22, which we also used to demonstrate that BDNF activates calpain in dendritic spines (10). This assay is based on the FRET kinetics of a synthetic substrate, consisting of a peptide with an optimized calpain-cleavage site coupled to the optimal FRET pair, DABCYL and EDANS. Our results obtained with this FRET-based calpain assay indicate that E2 rapidly activates calpain via MAPK in a calcium-independent manner through both ERα and ERβ receptors in dendritic spines of cultured hippocampal neurons. In addition, calpain activation is involved in E2-induced increased neuronal excitability, actin polymerization, and membrane levels of GluR1-containing AMPA receptors.

Results

Rapid Calpain Activation by E2 in Primary Neuronal Cultures. Hippocampal neuronal cultures at 14 days in vitro (DIV) were incubated with the FRET substrate for 2 h before the addition of E2 (10 nM). Confocal microscopy was used to confirm internalization of the FRET substrate and to perform live imaging analysis of calpain activation. E2 addition produced a rapid (within 1 min) decrease in FRET signal, indicating that the FRET substrate had been cleaved by calpain, thus decreasing acceptor FRET pair-mediated donor quenching. This translated into increased fluorescence that was localized in dendrites and dendritic spine-like structures in cultured hippocampal neurons (arrows in Fig. 1 *A* and *B*). Cortical neuronal cultures were also processed for spectrofluorometric analysis for a quantitative measure of calpain activity. Normalized fluorescence values were calculated by subtracting fluorescence values at time 0, before initiation of treatment, from fluorescence values recorded every 30 s for 50 min after application of 10 nM E2. Calpain activation reached maximal levels by 5 min of treatment with E2. Reactions were monitored for up to 50 min and did not show further increases in fluorescence. Accordingly, we chose the 5 min time point for all remaining experiments (Fig. 1*C*). To confirm that the

Author contributions: S.Z., M.R.F., R.T., and M.B. designed research; S.Z., Q.Q., H.Z., and Y.K. performed research; S.Z., X.B., Y.K., M.R.F., and M.B. analyzed data; and S.Z., X.B., R.T., and M.B. wrote the paper.

The authors declare no conflict of interest.

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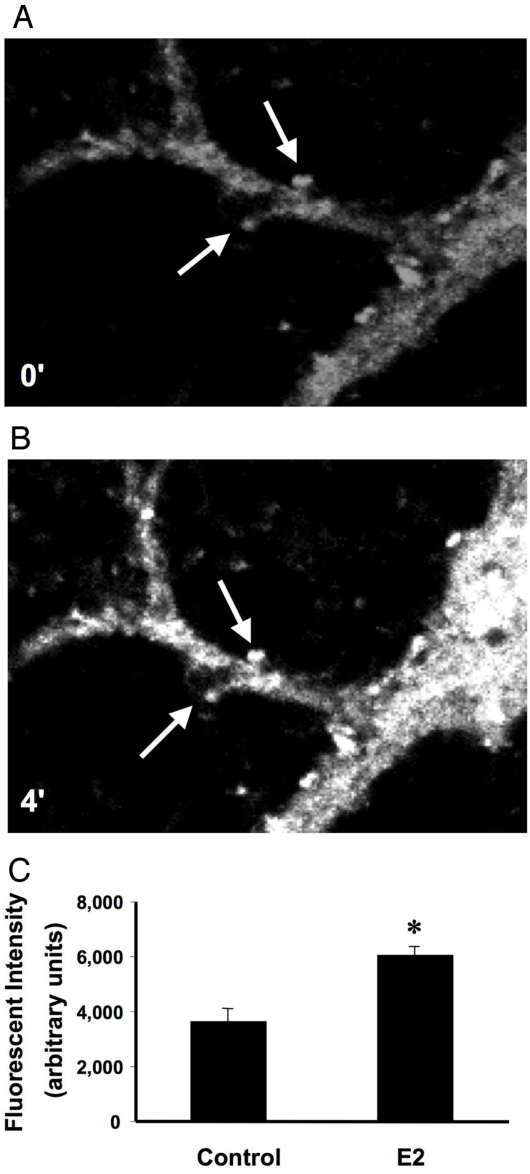

Fig. 1. E2-mediated calpain activation in primary hippocampal neuronal cultures. (*A* and *B*) Cultured hippocampal neurons were preloaded with the FRET substrate as described in *Materials and Methods*. E2 (10 nM) was applied at time 0 (*A*), and confocal images were recorded at various times after application. (*B*) The image shows increased fluorescence after 4 min of E2 application. Arrows indicate dendritic spine-like structures. (*C*) Kinetic analysis of the FRET signal analyzed by spectrofluorometry. Cortical neurons were preloaded with the FRET probe and were treated with E2 (10 nM) for various periods of times. Maximum increase in fluorescence was observed after 5 min. Fluorescence intensity was normalized by subtraction of values measured in untreated controls and the results represent means of ± SEM of eight experiments. *, $P < 0.05$ (Student's *t* test).

E2-dependent decrease in FRET was mediated by calpain, cultures were preincubated with the calpain inhibitor, calpeptin (10 μM), for 30 min before E2 addition. Calpeptin completely eliminated E2-induced decrease in FRET (Fig. 2 *A* and *C*).

E2-Induced Calpain Activation in Primary Neuronal Cultures Was MAPK-Dependent and Calcium-Independent. Since we recently showed that calpain could be activated by MAP kinase-mediated phosphorylation (10), and because E2 is known to activate this pathway (14, 15), we examined whether the MAPK pathway was involved in E2-induced calpain activation using a MAPK inhibitor. Hippocampal or cortical neurons were incubated with the FRET substrate and treated with a selective inhibitor of the MAPK pathway (PD98059; 10 μM), before the addition of E2 for 4 min (Fig. 2*B*) or 5 min (Fig. 2*C*), respectively. Under these conditions, no change in FRET kinetics was observed (Fig. 2 *B* and *C*), indicating that E2-induced calpain activation is mediated by MAP kinase activation. Moreover, when DIV 14 neuronal cultures were preloaded with the FRET substrate and treated with BAPTA-AM (10 μM), a calcium chelator, before application of E2, both confocal microscopy and spectrofluorometric analysis (Fig. 2*D*) indicated a decrease in FRET in E2-treated cultures, indicating that E2-induced calpain activation occurs independently of calcium.

E2-Mediated Activation Was ERα- and ERβ-Dependent. In view of the possible differences in signaling pathways activated by E2-induced stimulation of ERα or ERβ receptors, we tested the effects of specific agonists of these receptors on calpain activation in cultured neurons. Both PPT and DPN, agonists of ERα and of ERβ receptors respectively, activated calpain in cultured neurons (Fig. 3*D*). In addition to MAP kinase, E2 has been shown to activate a variety of signaling pathways, including the cAMP-dependent protein kinase (PKA) pathway (12). It was therefore of interest to determine whether this pathway contributed to E2-induced calpain activation. Interestingly, when neurons were treated with inhibitors of either adenylate cyclase (SQ22536) or PKA (KT5720), E2-induced calpain activation was greatly enhanced (Fig. 3 *A–C*), suggesting that, like in fibroblasts, PKA-mediated calpain phosphorylation results in inhibition of calpain activation (9).

E2-Induced Calpain Activation Increased Actin Polymerization and Membrane Insertion of GluR1-Containing AMPA Receptors in Acute Hippocampal Slices. We modified the rhodamine-phalloidin fluorescence enhancement previously described by Katanaev and Waymann (23) to analyze actin polymerization in cultured cells. When applied to cultured cortical neurons, E2 was found to produce an increase in actin polymerization, which was completely blocked by the calpain inhibitor, calpeptin (Fig. 4*A*). We then modified this assay to adapt it for acute hippocampal slices. The CA1 region was microdissected from acute slices after treatment with E2 (10 nM) for 30 min and actin polymerization was then determined with this assay. E2-treated slices exhibited a significant increase in actin polymerization as compared to vehicle-treated slices (Fig. 4*B*). This increase in actin polymerization was also completely blocked by calpeptin.

We next tested whether E2 could influence membrane levels of AMPA receptors. Acute hippocampal slices were treated with E2 (10 nM) by bath application for 5 min and then subjected to surface biotinylation. Area CA1 was dissected out and processed for Western blots of GluR1 and GluR2/3 subunits of AMPA receptors after purification of biotinylated proteins by avidin binding. E2-treated slices exhibited a significant increase in levels of GluR1 subunits (Fig. 5*A*). This increase was completely blocked when slices were pretreated with the calpain inhibitor, calpeptin. In contrast, no change in levels of GluR2/3 subunits was observed (Fig. 5*B*).

E2-Mediated Increase in Excitability in Hippocampal Slices Was Prevented by a Calpain Inhibitor. Numerous laboratories have reported that addition of E2 in the perfusion medium of acute hippocampal slices produces an increase in synaptic transmission (13, 15, 19, 20, 24). The mechanism underlying this increased synaptic transmission is not clear, although it has been proposed to be due to an increased insertion of AMPA receptors in postsynaptic membranes (19). In view of E2-induced rapid calpain activation in hippocampal neurons, we tested the effects of the calpain inhibitor calpeptin on E2-induced increase in synaptic transmission. Synaptic responses to stimulation of the Schaffer collateral pathway were recorded in field

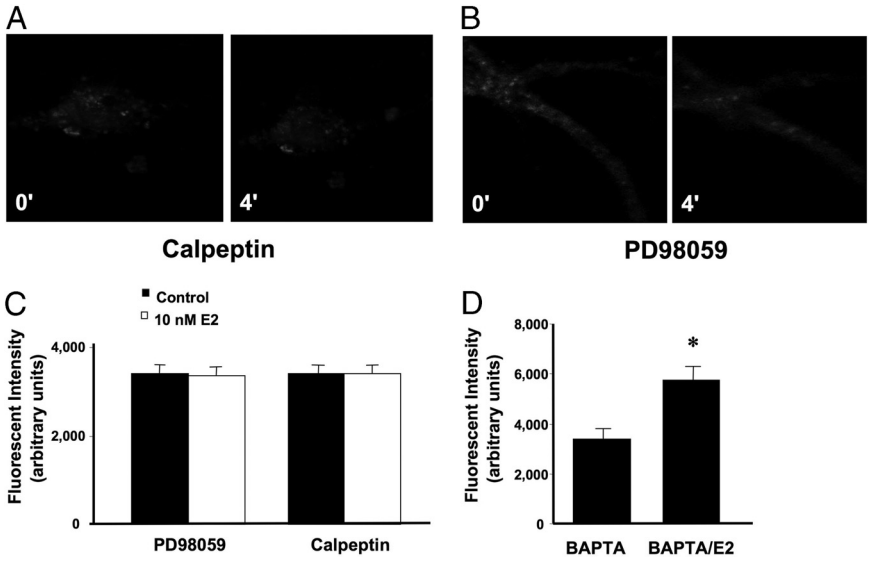

Fig. 2. E2-induced calpain activation in cultured neurons is MAPK-dependent and calcium-independent. (*A* and *B*) Cultured hippocampal neurons preloaded with the FRET substrate as described in *Materials and Methods* were pretreated with a calpain inhibitor, calpeptin (10 μM) or a MEK inhibitor, PD98059 (25 μM). E2 (10 nM) was added and confocal images taken at various times. Images taken after 4 min of E2 application illustrate the lack of changes in fluorescence. (*C* and *D*) Kinetic analysis of the FRET signal analyzed by spectrofluorometry. Cortical neurons were preloaded with the FRET probe and were preincubated with PD98059 (25 μM) or calpeptin (10 μM) or BAPTA-AM (50 μM). They were treated with E2 (10 nM) for 5 min. Fluorescence intensity was normalized by subtraction of values measured in untreated controls; data represent means of $\pm$ SEM of eight experiments. *, $P < 0.05$ (Student's *t* test).

CA1 of acute hippocampal slices. After 5 min of stable baseline, 10 μM calpeptin, a cell-permeable calpain inhibitor was added in the perfusion medium and responses were recorded for 10 min, before the addition of 10 nM E2, a concentration we previously showed to be effective in enhancing baseline responses in CA1 of rat hippocampal slices (15) (Fig. 6). In the absence of calpeptin, E2 treatment produced a small but significant increase in synaptic responses (12 $\pm$ 2% increase over baseline responses after 30 min; mean $\pm$ SEM of 10 experiments). This increase was completely eliminated when slices were preincubated and incubated in the presence of calpeptin (10 μM) (2 $\pm$ 2% after 30 min; mean $\pm$ SEM of 10 experiments). Incubation of slices in the presence of calpeptin alone had no effect on baseline synaptic transmission.

Discussion

Using a FRET-based assay to monitor calpain activation, we showed that E2 rapidly (within minutes) activated calpain in primary cultured neurons in a MAPK-dependent but calcium-independent manner. We further provided evidence that E2-induced calpain activation was involved in E2-mediated actin polymerization and increases in levels of membrane AMPA receptor subunit GluR1 and synaptic responses in acute hippocampal slices. Calpains belong to a large family of calcium-dependent cysteine proteases, and two isoforms, calpain 1 (a.k.a., μ-calpain) and calpain 2 (a.k.a., *m*-calpain) are ubiquitously distributed in mammalian brain (25). Both isoforms require calcium for activation, with μ-calpain requiring micromolar concentrations of cal-

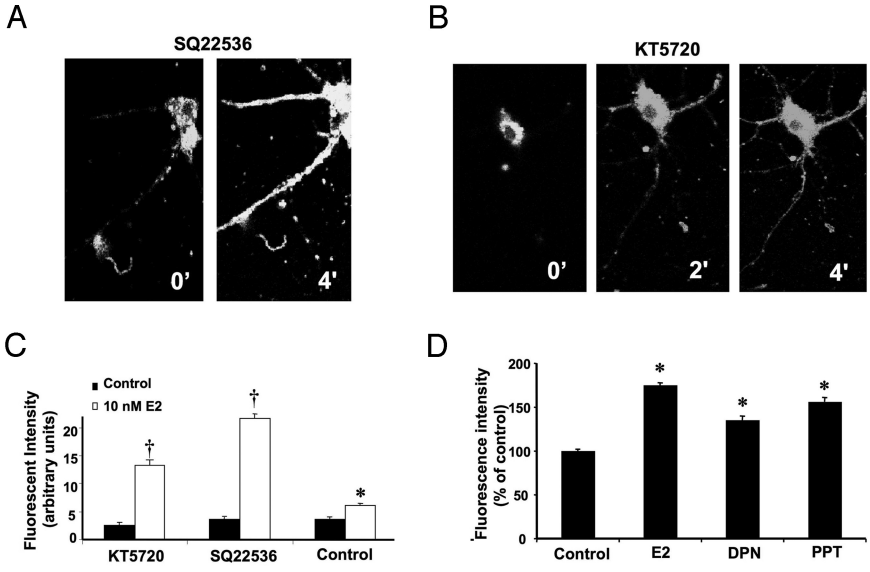

Fig. 3. PKA modulation of E2-mediated calpain activation in cultured neurons and effects of ERα and ERβ agonists. (*A* and *B*) Cultured hippocampal neurons were preloaded with the FRET substrate as described in *Materials and Methods* and treated with inhibitors of adenylate cyclase or PKA (SQ22536, 10 μM and KT5720, 10 μM, respectively) before application of 10 nM E2. Confocal images were taken at the indicated times. (*C*) Cortical neurons were preloaded with the FRET probe and pretreated with inhibitors of adenylate cyclase or PKA (SQ22536, 10 μM and KT5720, 10 μM, respectively); they were then treated with E2 (10 nM) for 5 min. FRET was analyzed by spectrofluorometry and fluorescence intensity was normalized by subtraction of values from untreated controls; data represent means $\pm$ SEM of eight experiments. *, $P < 0.01$, as compared to control; †, $P < 0.001$, as compared to E2 alone (ANOVA followed by Bonferroni test). (*D*) Cultured cortical neurons were preloaded with the FRET probe and treated for 5 min with agonists of ER-α or of ER-β, PPT (10 nM) and DPN (10 nM), respectively. FRET was analyzed by spectrofluorometry and fluorescence intensity was normalized by subtraction of values from untreated controls; data were then expressed as percentage of values found in untreated control and represent means of $\pm$ SEM of eight experiments. *, $P < 0.01$ as compared to control.

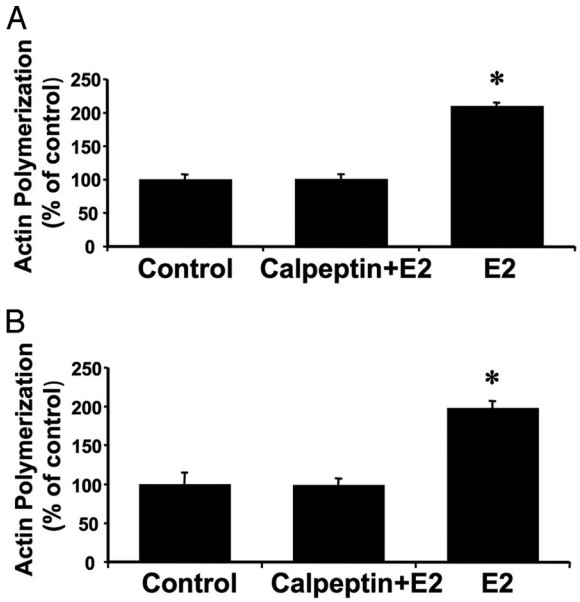

Fig. 4. E2-mediated increased actin polymerization is blocked by calpain inhibition in cultured neurons and acute hippocampal slices. (*A*) Cultured cortical neurons (DIV 14) were pretreated with or without the calpain inhibitor, calpeptin (10 μM) for 20 min before adding E2 (10 nM) for 5 min. At the end of incubation, cultures were subjected to the phalloidin fluorescent enhancement assay described under *Materials and Methods*. (*B*) Acute hippocampal slices (200-μm thick) were prepared from young male rats (2–4-month-old); area CA1 was dissected out and pretreated with or without a cell permeable calpain inhibitor, calpeptin (10 μM) for 20 min before adding vehicle of E2 (10 nM) for 5 min. At the end of incubation, actin polymerization was analyzed as described under *Material and Methods* with the rhodamine-phalloidin fluorescent enhancement assay. Results of the Alexa Fluor594-phalloidin fluorescence were normalized (subtraction of control fluorescence signal) and expressed as percentage of values found in untreated control slices and represent means ± SEM of 10 experiments. *, $P < 0.05$ (ANOVA followed by Bonferroni test).

cium and *m*-calpain millimolar concentrations (26). We previously proposed the hypothesis that calpain played a critical role in LTP of synaptic transmission elicited by high frequency stimulation of glutamatergic synapses and in learning and memory by participating in the modification of synaptic structure and function (4, 5). As studies conducted in μ-calpain knockout mice failed to provide evidence for LTP or learning and memory deficits, we suggested that *m*-calpain was critically involved in synaptic plasticity (27). However, the seemingly unphysiological levels of calcium required for *m*-calpain activation necessitate the existence of another mechanism for its activation. Recent studies in fibroblasts demonstrated that calpain activation by the growth factor, epidermal growth factor (EGF), was mediated by extracellular signal-regulated kinase (ERK) phosphorylation of *m*-calpain at Ser-50 (9). We recently reported that both EGF and BDNF activated *m*-calpain in neurons as a result of MAP kinase-mediated serine phosphorylation (10). These results were based on the inhibition of EGF- and BDNF-induced calpain activation by MAP kinase inhibitors, as well as by siRNAs for *m*-but not for μ-calpain. In the present study, we extended these results and found that E2 treatment of cultured neurons also stimulated calpain through MAPK activation and independently of calcium influx. E2-mediated calpain activation also occurred within minutes, eliminating the possibility that increased activity was the result of increased calpain protein expression.

Confocal analysis of the FRET signal after E2 application indicated that calpain was activated within dendrites and dendritic spine-like structures. Calpain has previously been localized in dendritic spines (28), and there is also evidence for the presence of E2 receptors in dendrites (29). These results further support a critical role for E2 and calpain in regulating structural changes at synapses after the induction and maintenance of LTP. They also fit well with the evidence that E2 participates in the regulation of synaptic contacts in hippocampus (17).

Both ERα and ERβ receptors are present in dendrites of hippocampal neurons, although their respective roles in mediating various effects of E2 in adult hippocampus are still debated. Our data indicated that E2-mediated calpain activation could be elicited by stimulation of either ERα or ERv receptors in cultured neurons. Activation of either ERα or ERβ receptors has been shown to lead to MAP kinase activation (14), a result consistent with the idea that calpain is activated by MAP kinase-dependent phosphorylation. In addition to MAP kinase, E2 has been shown to activate CaMKII as well as PKA. In fibroblasts, stimulation of adenylate cyclase or PKA inhibited calpain activation by phosphorylating calpain at serine/threonine 369–370 (7). Interestingly, when neurons were treated with inhibitors of either adenylate cyclase or PKA, E2-induced calpain activation in cultured neurons was greatly enhanced, suggesting that, like in fibroblasts, PKA-mediated calpain phosphorylation in neurons results in inhibition of calpain activation. Our results indicate that E2, by activating different intracellular signaling pathways, has complex effects on calpain. On one hand, by activating MAP kinase, E2 elicits calpain activation, while by activating PKA, it produces calpain inhibition. The location and balance between these two opposite effects of E2 on calpain activity is likely to be important to determine the location, extent and duration of calpain activation. In any event, these effects could participate in several of the reported cellular effects of E2, including regulation of synaptic plasticity and dendritic spine morphology. Further studies should also explore the possible interactions between E2 and PKA in regulating synaptic plasticity.

Several studies have shown that E2 participates in the regulation of dendritic spine morphology in area CA1 of hippocampus (18, 19). E2 has been shown to inactivate cofilin by stimulating LIM

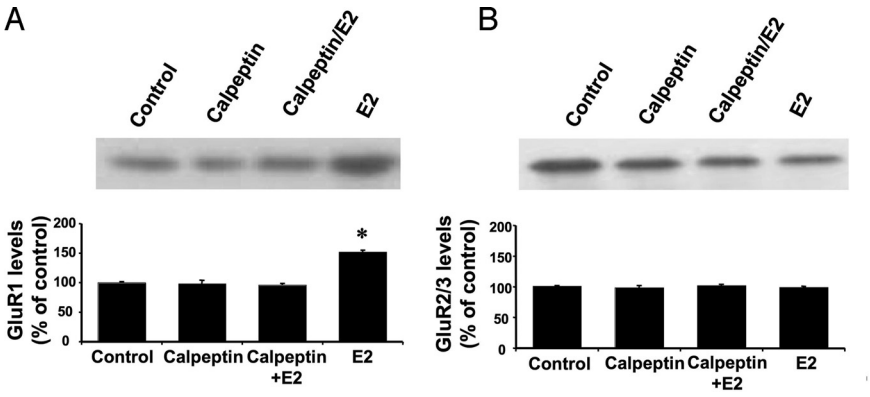

Fig. 5. E2-mediated increase in GluR1 subunits of AMPA receptors in acute hippocampal slices is blocked by calpain inhibition. Acute hippocampal slices pretreated with or without calpeptin (10 μM) and E2 (10 nM) were subjected to surface biotinylation and Western blot analysis with GluR1 (*A*) and GluR2/3 (*B*) antibodies as described in *Materials and Methods*. *Upper* Representative Western blots for GluR. *Lower* Quantitative analysis of Western blots similar to those shown on top. Levels of the GluR proteins were quantified and expressed as percentage values found in control slices. Results are means ± SEM of five independent experiments. *, $P < 0.05$, as compared to control levels.

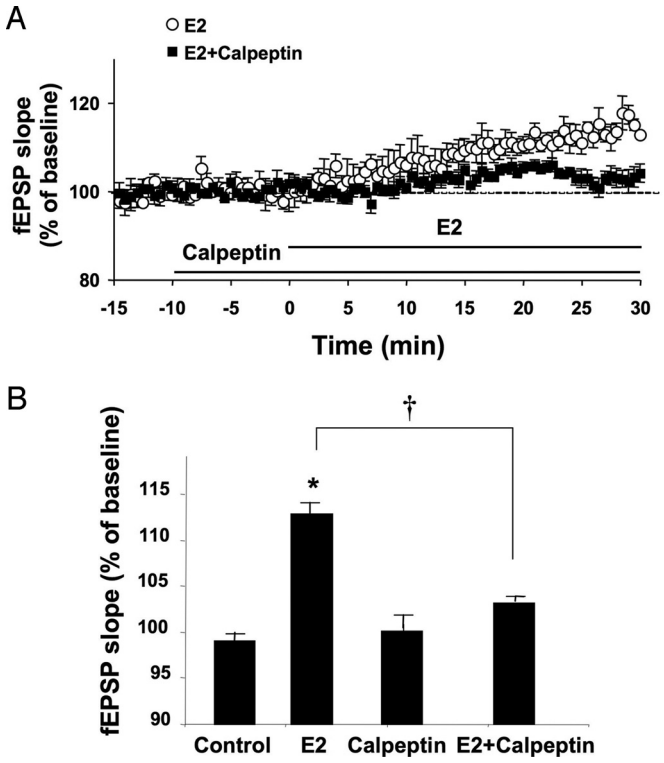

Fig. 6. E2-mediated increase in synaptic responses in hippocampal slices is blocked by calpain inhibition. Hippocampal slices were prepared from adult male rats. Extracellular postsynaptic potentials (EPSPs) evoked by stimulation of the Schaffer collateral pathway were recorded in CA1 stratum radiatum. (*A*) After 15 min of stable baseline recording, E2 (10 nM) was added and recording continued for 30 min. Alternatively, calpeptin (10 μM) was added after 5 min of baseline recording, and 10 min later E2 (10 nM) was added. Results represent the slopes of field EPSPs (fEPSP) and are expressed as percentage of the average values recorded during the 5 or 10 min baseline period, respectively. They are means ± SEM of eight to 10 experiments. (*B*) Summary of the data for fEPSPs measured after 30 min of E2 application (control values represent the average of all of the responses recorded during the 10 min baseline period in slices treated with ACSF alone). *, $P < 0.01$, as compared to baseline values; †, $P < 0.01$ as compared to E2 treated slices (ANOVA followed by Bonferroni test).

kinase, thereby leading to actin polymerization and increased formation of filopodia (18, 19). As calpain inhibition prevented E2-induced increase in actin polymerization, our results indicate that calpain activation is an important step leading to actin polymerization, possibly through cofilin phosphorylation/inactivation. Such a role for calpain has been clearly demonstrated in fibroblasts and other cell types (30). In addition, E2 treatment for 1 h has also been shown to increase the levels of GluR1 subunits of membrane AMPA receptors in hippocampal slices, suggesting that E2 increased the trafficking of AMPA receptors (20). Our results showed that 10 min of E2 treatment in acute hippocampal slices was sufficient to increase GluR1 levels in membranes prepared from CA1 region. Moreover, E2-mediated increase in GluR1 levels was completely blocked with a calpain inhibitor, indicating that calpain activation plays a critical role in the regulation of AMPA receptor membrane insertion. Because we did not find a similar increase in levels of GluR2/3 subunits, our results raise the possibility that E2 might modify the subunit composition of AMPA receptors at hippocampal synapses.

Finally, many laboratories have reported that E2 rapidly increased basal synaptic responses in field CA1 of hippocampal slices (13, 15, 19, 20, 25). This effect was also completely blocked by calpain inhibition. It has been proposed that E2-mediated increased excitability in CA1 was due to increase in actin polymerization possibly leading to increased surface expression of AMPA receptors (19, 20, 31). In other words, E2 would reproduce some of the events associated with LTP induction, with the major differences that these effects are reversible, whereas LTP induction leads to long lasting increase in synaptic efficacy. It has recently been demonstrated that LTP consolidation requires the activation of two separate cascades leading to cytoskeletal modifications (32), and it was proposed that E2 would activate only one of the two cascades, which would account for its reversible effects on cytoskeleton and synaptic function (19). This interpretation is completely consistent with our results indicating that E2-mediated increased actin polymerization, increased membrane levels of GluR1 subunits of AMPA receptors, and increased synaptic responses are all blocked by calpain inhibition.

In conclusion, our results demonstrate that E2 rapidly activates calpain in cultured neurons and in hippocampal slices. E2-mediated calpain activation resulted in increased actin polymerization and AMPA receptor surface insertion as well as enhanced synaptic responses. These findings shed light on the molecular mechanisms involved in E2-mediated processes involved in synaptic plasticity, learning, and memory. They also clearly indicate that calpain activation is a critical element in the sequence of events activated by stimulation of E2 receptors and leading to changes in synaptic structure and function.

Materials and Methods

Primary Neuronal Cultures. Whole cerebral neocortex of embryonic day 18–19 rats (Sprague–Dawley) or hippocampi of embryonic day 18 mice (BALB/c) were digested with trypsin before mechanical dissociation and plating onto polyD-lysine-coated dishes at low (100–300 cells/mm^2) density for imaging experiments or at medium density (600–800 cells/mm^2) for all other experiments and maintained in neurobasal medium supplemented with B27 nutrient mixture (B27-NBM) for 2 weeks.

Calpain Specific FRET Substrate. A cell-permeable calpain specific FRET substrate [RRRRRRRR-(EDANS)-GQQEVYGMMPRDG-DABCYL] composed of a peptide with an optimized calpain cleavage site and a hepta-arginine motif to facilitate cellular permeation was synthesized manually at 90% purity by solid phase methodology on a rink-amide resin by FMOC strategy. The substrate was subsequently coupled to the following FRET pair: FRET acceptor probe, DABCYL (N-{4-[4′(dimethylamino)phenylazo]benzoyloxy}succinimide; emission: none; absorption: 425 nm) and FRET donor probe, EDANS (5-(2-aminoethylamino)-1-naphtalenesulfonic acid; emission: 493 nm; absorption: 335 nm). The purity of the calpain specific FRET substrate was determined via mass spectrometry and HPLC (BioMer Technologies). The substrate was dissolved in 10 mM PBS (pH 7.4). Cultures were incubated with the FRET probe (100 μM) for 2 h; after incubation, cultures were washed three times in 10 mM PBS (pH 7.4) and provided with fresh medium. Cultures were then subjected to the indicated treatments; following treatments, cells were washed three times again and provided fresh medium before the addition of 10 nM E2 (Tocris Bioscience) for 5 min. All media was siphoned and cultures were trypsinized in 10 mM PBS buffer containing 5 mg/mL trypsin and 2 mg/mL EDTA for 10 min. Whole cell lysates were subjected to spectrofluorometric analysis in a 10 mm quartz cuvette (VWR) at room temperature, using a HORIBA Jobin Yvon FluoroMax-3 spectrofluorometer (Horiba) at excitation and emission wavelengths of $\lambda = 320$ nm and $\lambda = 480$ nm, respectively.

Confocal Image Acquisition and FRET Analysis. The FRET substrate was dissolved in 10 mM PBS and used in all imaging experiments at a final concentration of 100 μM. Primary hippocampal neuronal cultures kept for 14 days in vitro (DIV) were incubated for 2 h with the FRET substrate as described above. Uptake of the calpain specific FRET substrate by neurons was confirmed and analyzed via confocal microscopy (Nikon Eclipse TE-2000 with D-Eclipse C1 system). E2 (10 nM) was directly added from stock solution while FRET kinetics was continuously monitored. Images were acquired at various time points before and during treatments.

Phalloidin Immunostaining. Culture neurons preincubated with 100 μM FRET substrate, were washed and treated with 10 nM E2 before fixed in 4% paraformaldehyde for 20 min. Neurons were then washed three times in 10 mM PBS and incubated with phalloidin-Alexa Fluor594 (Invitrogen) for 50 min. Neurons were subsequently washed three times with 10 mM PBS and actin polymerization was analyzed and imaged via confocal microscopy (Nikon Eclipse TE-2000 with D-

Eclipse C1 system). Images were captured separately to include fluorescence signal of the calpain FRET substrate and of the Alexa Fluor 594 probe. Images were then superimposed to localize actin polymerization within dendrites via the D-Eclipse C1 System (Nikon).

Drug Treatments. Cultured neurons of DIV14 were treated with the following inhibitors: calpeptin (10 μM; calpain inhibitor), PD98059 (25 μM; MAPK pathway inhibitor), BAPTA (50 μM; calcium chelator), KT5720 (10 μM; PKA inhibitor), and SQ22536 (10 μM; adenyl cyclase inhibitor). They were also treated with DPN (10 nM; ERβ agonist) and PPT (10 nM, ERα agonist) for 30 min or with 10 nM E2 (Tocris Bioscience) for 5 min for FRET spectrofluometric and confocal analysis.

Membrane Biotinylation. After treatments, acute hippocampal slices were transferred to ice-cold artificial cerebrospinal fluid (aCSF) (125 mM NaCl, 2.5 mM KCL, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM d-glucose, and 22 mM $NaHCO_3$) for 2 min, followed by biotinylation in 1 mg/mL biotin (EZ-Link Sulfo-NHS-SS-Biotin; Pierce) for 45 min with slow agitation. After three rinses in quenching solution (Pierce) to quench free biotin, CA1 ''minislices'' were homogenized in cold 1% Triton X-100 homogenization buffer (150 μL/35-mm well) [50 mM NaCl, 10 mM EDTA, 10 mM EGTA, 1 mM NaF, 1$\times$ protease inhibitor mixture tablet (Calbiochem), and 50 mM Hepes] to prevent solubilization of postsynaptic densities. Homogenates from slices were centrifuged at 10,000 $\times$ *g* for 2 min. Biotinylated surface proteins in the supernatant were immunoprecipitated with 40 μL of 50% avidin-agarose beads (ImmunoPure immobilized Avidin; Pierce) for 1 h at 4 °C. The beads were pelleted and the supernatant was mixed and heated with 2$\times$ SDS sample buffer.

Actin Polymerization Assay. Actin polymerization was quantified by measurement of ''rhodamine-phalloidin fluorescent enhancement'' as described by Katanaev and Waymann (23). This assay was also applied to hippocampal slices. In brief, CA1 region of 200-μm thick hippocampal slices from male Sprague–Dawley rats (2–4 months) was microdissected and preincubated at room temperature in the absence or presence of calpeptin (10 μM) for 20 min; they were then treated with or without 10 nM E2 for 5 min. Slices were subsequently washed twice with fresh aCSF and fixed in 4% paraformaldehyde and 1% octyl-β-D-glucopyranoside for 30 min at room temperature. Slices were then homogenized and centrifuged at 1,000 $\times$ *g* for 1 min. Lysates were incubated with 50 nM phalloidin-Alexa Fluor594 (Invitrogen) for 1 h at room temperature. Lysates were collected in 1.0 mL of 10 mM PBS and normalized fluorescent intensity was recorded in a 10-mm cuvette using a spectrophotometer (excitation and emission wavelength were λ = 552 nm and λ = 580 nm, respectively).

Quantitative Western Blot Analysis. Primary antibodies were GluR1 and GlurR2/3 (rabbit polyclonal, Millipore). Biotinylated samples were subjected to 10% SDS/PAGE. Separated proteins were transferred to nitrocellulose membrane and immunoreactivity was detected with goat anti-rabbit (Millipore) conjugated to peroxidase (1:10,000) followed by chemiluminescence reaction combined with film exposure (Pierce).

Slice Preparation and Recording. Male Sprague–Dawley rats (2–4 months) were used for slice recording. Brains were quickly removed and placed in ice-chilled oxygenated dissection medium containing (in mM) 124 NaCl, 3 KCl, 1.25 KH_2PO_4, 5 $MgSO_4$, 3.4 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose. Transverse hippocampal slices (400 μm) were prepared and immediately transferred to an interface recording chamber containing aCSF of the following composition (in mM): 124 NaCl, 3 KCl, 1.25KH_2PO_4, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose and maintained at 32 °C (13). Slices were continuously perfused with this solution at a rate of 1.75–2 mL/min while the surface of the slices were exposed to warm, humidified 95% O_2/5% CO_2. Recordings began after at least 1.5 h of incubation. Field excitatory postsynaptic potentials (fEPSPs) were recorded from CA1b stratum radiatum using a single glass pipette (2–3 MΩ). Stimulation electrodes (twisted nichrome wires, 65 μm) were positioned at two sites (CA1a and CA1c) in the apical Schaffer collateral-commissural projections to provide convergent activation of CA1b pyramidal cells. After establishing a 5–10-min stable baseline, the indicated treatments were infused into the perfusion lines by switching from control aCSF to drug-containing aCSF. Extracellular postsynaptic potentials (EPSPs) were collected and digitized by WinLTP.

Statistical Analysis. Statistical analysis was performed using one way analysis of variance followed by the Bonferroni test (for multiple factors). Alternatively, Student's *t* test was applied to data with multiple variables and one factor. Normalized FRET results were obtained from multiple sister cultures and their means calculated; means from independently grown cultures were then expressed as means $\pm$ SEM. (n = 3–6 cultures with a total of 8–16 readings.)

ACKNOWLEDGMENTS. We thank Karole Rostamiani for culture preparation. This work was supported by National Institute of Aging Grant P01AG14751 (PI: Dr. C. Finch) and National Institute of Neurological Disorders and Stroke Grant P01NS045260-01 (PI: Dr. C.M. Gall).

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