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PDZ Proteins Interacting with C-Terminal GluR2/3 Are Involved in a PKC-Dependent Regulation of AMPA Receptors at Hippocampal Synapses

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Summary

We investigated the role of PDZ proteins (GRIP, ABP, and PICK1) interacting with the C-terminal GluR2 by infusing a ct-GluR2 peptide ("pep2-SVKI") into CA1 pyramidal neurons in hippocampal slices using whole-cell recordings. Pep2-SVKI, but not a control or PICK1 selective peptide, caused AMPAR-mediated EPSC amplitude to increase in approximately one-third of control neurons and in most neurons following the prior induction of LTD. Pep2-SVKI also blocked LTD; however, this occurred in all neurons. A PKC inhibitor prevented these effects of pep2-SVKI on synaptic transmission and LTD. We propose a model in which the maintenance of LTD involves the binding of AMPARs to PDZ proteins to prevent their reinsertion. We also present evidence that PKC regulates AMPAR reinsertion during dedepression.

Introduction

The majority of fast excitatory synaptic transmission in the mammalian CNS is mediated by the ionotropic glutamate receptors. Ionotropic glutamate receptors are divided into three subtypes based on their pharmacology and molecular biology: α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptors (AMPARs), *N*-methyl-D-aspartate receptors (NMDARs), and kainate receptors (KARs; Watkins and Evans, 1981; Hollmann and Heinemann, 1994). AMPARs mediate the majority of low-frequency transmission at glutamatergic synapses and are involved in the expression of long-term potentiation (LTP) and LTD (Bliss and Collingridge, 1993; Bear and Abraham, 1996; Malenka and Nicoll, 1999).

AMPARs are heteromeric assemblies made up of combinations of up to four subunits, GluR1–4, producing receptor complexes with diverse properties. The GluR2 subunit is of particular importance because it undergoes posttranscriptional editing in the pore-forming region at the so-called Q/R site (Hollmann and Heinemann, 1994), which leads to AMPAR complexes containing edited GluR2 subunits exhibiting low permeability to Ca^{2+}. The vast majority of AMPARs in the postnatal CNS are impermeable to Ca^{2+} because they contain edited GluR2 (Jonas et al., 1994; Geiger et al., 1995). GluR2 is highly regulated, and disruption in the cellular control of this subunit has been shown to cause altered synaptic plasticity and neurotoxicity (Jia et al., 1996; Feldmeyer et al., 1999).

Recent studies using the yeast two-hybrid technique have identified a number of proteins that interact with the C-terminal regions of GluR2 and GluR3 (Braithwaite et al., 2000). Two distinct interaction domains on the C-terminal of GluR2 (ct-GluR2) have so far been identified, an NSF binding site between residues 844 and 853 (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998) and an extreme ct-PDZ binding motif (ct-GluR2/3 PDZ; Dong et al., 1997; Srivastava et al., 1998; Dev et al., 1999; Xia et al., 1999). The PDZ binding motif has been shown to interact with three PDZ domain-containing proteins: GRIP (Dong et al., 1997) and ABP (one splice form is also known as GRIP2 [Srivastava et al., 1998; Brückner et al., 1999]), which are closely related (Srivastava et al., 1998; Brückner et al., 1999; Braithwaite et al., 2000), and PICK1 (Dev et al., 1999; Xia et al., 1999). Although the functional consequences of the GluR2-NSF interaction have received considerable attention (Nishimune et al., 1998; Song et al., 1998; Lüscher et al., 1999; Lüthi et al., 1999; Noel et al., 1999), only recently has progress been made in understanding the functional roles of the PDZ interactions (Li et al., 1999; Chung et al., 2000; Matsuda et al., 2000; Osten et al., 2000).

GRIP and ABP contain multiple PDZ domains through which they can form homo- and heteromultimers (Dong et al., 1997; Srivastava et al., 1998; Dong et al., 1999). As well as interacting with AMPARs, GRIP and ABP also interact with other proteins containing the PDZ binding motif, for example ephrin ligands and receptors (Torres et al., 1998). Therefore, it has been hypothesized that they are involved in the clustering of AMPARs or other PDZ-containing proteins with signaling molecules at the synapse, and in the trafficking of these proteins from the Golgi apparatus to the postsynaptic membrane (Dong et al., 1997; Srivastava et al., 1998; Braithwaite et al., 2000).

PICK1 was first identified as an interactor with the catalytic subunit of PKCα (Staudinger et al., 1995, 1997). It has a single PDZ domain that can interact with PKCα or GluR2/3. It can also dimerize via an interaction at a site distinct from the PDZ domain. Therefore, PICK1 can form dimers with two available PDZ domains and thus may be involved in clustering AMPARs at synapses and/or the regulation of PKC dependent phosphorylation of AMPAR subunits (McGlade-McCulloch et al., 1993; Roche et al., 1996; Dev et al., 1999; Matsuda et al., 1999; Xia et al., 1999; Chung et al., 2000; Matsuda et al., 2000).

Here we show that ct-GluR2/3 PDZ interactions are

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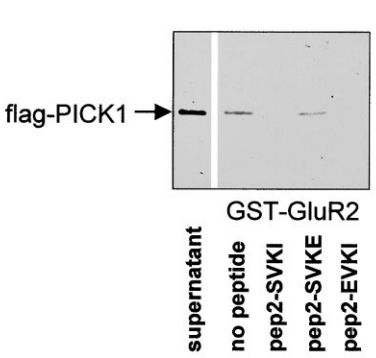
important for regulating the function of synaptic AMPARs in hippocampal CA1 neurons. Introduction of a peptide corresponding to the last 11 amino acids of ct-GluR2/3 (pep2-SVKI) into CA1 neurons caused a potentiation of basal synaptic transmission in approximately one-third of cells. In addition, it blocked LTD in all cells tested. Application of a PKC inhibitor prevented both the potentiation in basal transmission and the blockade of LTD caused by pep2-SVKI. Prior induction of LTD resulted in pep2-SVKI causing a potentiation of EPSCs in the majority of cells. Furthermore, dedepression (i.e., repotentiation of a pathway that had previously received LTD) was selectively blocked by PKC inhibitors. These data indicate that the ct-GluR2/3 PDZ interactions regulate synaptic transmission at CA1 synapses in an activity- and PKC-dependent manner.

Results

Biochemical Investigation of the Effects of ct-GluR2 Peptides on the PICK1-GluR2 Interaction

To study the functional role(s) of ct-GluR2/3 PDZ interactions in synaptic transmission and long-term synaptic plasticity, we used a peptide corresponding to the last 11 amino acids of GluR2 (YNVYGIESVKI; "pep2-SVKI" [Dev et al., 1999; Xia et al., 1999]) to disrupt ct-GluR2 PDZ interactions in neurons. It has already been shown that an almost identical peptide corresponding to the last ten amino acids of GluR2, "GluR2-SVKI" (sequence: NVYGIESVKI), blocks the GRIP-GluR2 interaction in a biochemical assay (Li et al., 1999). Two other peptides, "GluR2-SVKE" (NVYGIESVKE), which contains a single amino acid substitution in the PDZ binding motif, and "GluR2-EVKI" (NVYGIEEVKI), in which serine 880 is replaced with a glutamate, were found to be ineffective at blocking this interaction.

We tested the effects of the 11–amino acid equivalents of these three peptides, pep2-SVKI, pep2-SVKE, and pep2-EVKI on the PICK1-GluR2 interaction (Figure 1). Pep2-SVKI and pep2-EVKI blocked the retention of PICK1 by GST-GluR2, while pep2-SVKE had no effect. Thus, pep2-SVKE is an ideal control peptide for testing ct-GluR2 PDZ interactions, and pep2-EVKI, in agreement with another recent study (Chung et al., 2000), can be used to selectively block the PICK1-GluR2 interaction.



b

Peptide	Sequence	Interaction blocked
pep2-SVKI	^{873}YNVYGIESVKI^{883}	PICK1, GRIP, ABP
pep2-SVKE	YNVYGIESVK**E**	None
pep2-EVKI	YNVYGIE**E**VKI	PICK1

Figure 1. The Effects of ct-GluR2 Peptides on the In Vitro Binding of ct-GluR2 to PICK1

(a) Western blot with anti-PICK1 showing the levels of flag-PICK1 retained by GST-ct-GluR2 in the presence or absence of ct-GluR2 peptides: no peptide control, pep2-SVKI, pep2-SVKE, pep2-EVKI. Fusion protein GST-ct-GluR2, was isolated from *E. coli* strain BL21. Flag-tagged PICK1 was obtained from transfected COS-7 cells. GST-ct-GluR2 was bound to glutathione Sepharose beads and incubated with COS-7 cell lysate containing flag-PICK1 (that had been preincubated with or without peptides; in the illustrated experiment a peptide concentration of 133 μM was used). Beads were finally washed and examined by Western blotting for content of flag-PICK1. (b) Sequences of the synthetic peptides used in this study (single letter amino acid code) and the interactions that they block. pep2-SVKI is the sequence of the last 11 amino acids of wild-type GluR2. Bold underlined residues indicate differences from the wild-type GluR2 sequence.

Pep2-SVKI Causes an Increase in the Amplitude of AMPAR-Mediated EPSCs in a Subset of Experiments

To study the role of ct-GluR2/3 PDZ interactions in regulating synaptic AMPARs, we introduced ct-GluR2 peptides into CA1 pyramidal cells in hippocampal slices via the patch electrode during whole-cell recordings. In a first set of experiments, we studied the effects on basal synaptic transmission by infusing peptides into neurons while evoking AMPAR-mediated excitatory postsynaptic currents (EPSCs). In the majority of experiments two independent pathways were stimulated. When using an intracellular solution containing pep2-SVKI (100 μM), an increase in the amplitude of EPSCs was observed $\sim$5 min after the start of the recording in 36/99 (36%) pathways (EPSC amplitude average over a 5 min period from 25–30 min $>$ 130% of initial baseline at 0–5 min; Figure 2a). The increase in amplitude stabilized by $\sim$30 min and persisted for the duration of recordings (up to 90 min). In the remaining pathways (63/99, 64%), no change in EPSC amplitude was observed for the duration of recordings (up to 100 min; Figure 2b). This increase in EPSC amplitude in a subset of experiments was consistently observed with different batches of pep2-SVKI and by different investigators. When all data were pooled (increase and no increase in EPSC amplitude) there was a significant increase in EPSC amplitude at 25–30 min compared to the first 5 min of recordings (EPSC amplitude = 134% $\pm$ 7%, $p < 5 \times 10^{-4}$, n = 99; Figure 2c). We also separated and pooled the data on the basis of whether there was an increase in amplitude (Figure 2d). EPSC amplitude increased to 205% $\pm$ 15% ($p < 1 \times 10^{-5}$, n = 36) in the subset that exhibited the increase, while amplitude exhibited a small depression in the remainder (93% $\pm$ 2%, $p < 0.05$, n = 63).

This effect of pep2-SVKI on EPSC amplitude was pre-

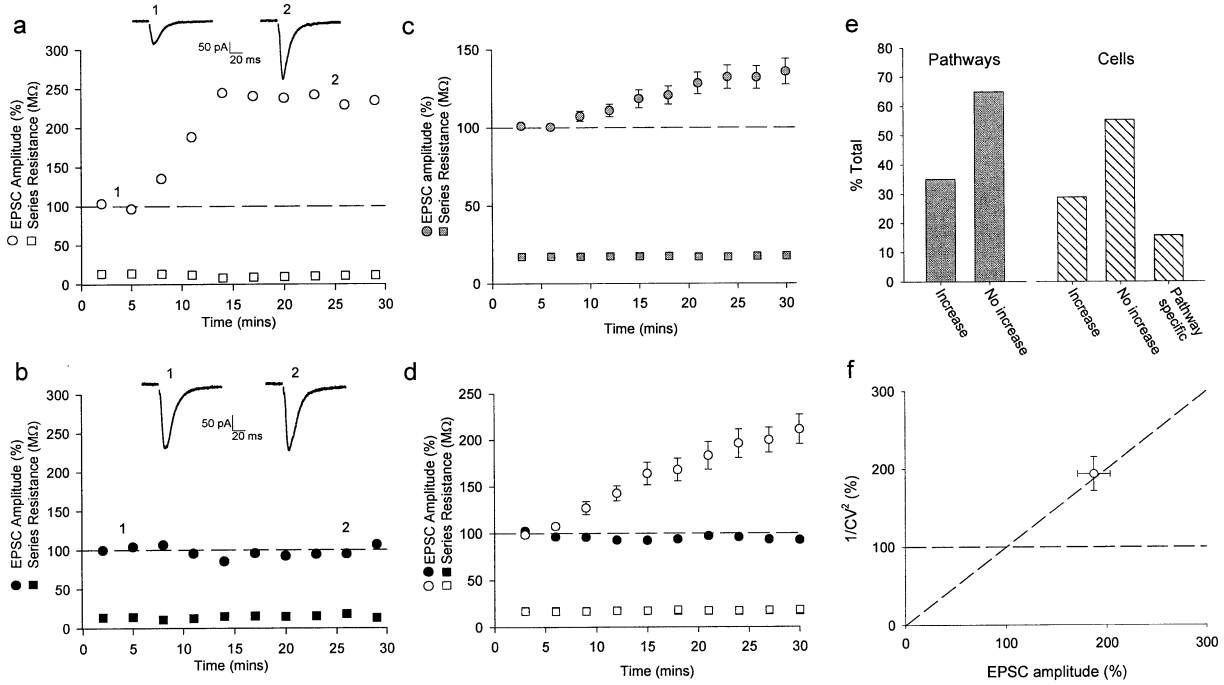


Figure 2. Pep2-SVKI Causes an Increase in AMPAR-Mediated EPSCs in Hippocampal CA1 Neurons in a Subset of Experiments

(a) Amplitude (normalized to first 5 min) versus time (relative to the start of the recording) for a single experiment with pep2-SVKI (100 μM) in the recording electrode showing an increase in EPSC amplitude.

(b) An example experiment with pep2-SVKI (100 μM) in the recording electrode in which no increase in EPSC amplitude was observed.

(c) Summary graph of mean amplitude versus time of all experiments with pep2-SVKI in the intracellular solution (n = 99 pathways, 59 cells; significant increase in EPSC amplitude when statistics performed on pathways $p < 5 \times 10^{-5}$, or on cells [data for both pathways onto the same cell averaged] $p < 0.001$).

(d) Summary graph of mean amplitude versus time for experiments with pep2-SVKI subdivided into experiments in which there was an increase (n = 36 pathways, 23 cells; significant increase for statistics based on pathways $p < 1 \times 10^{-5}$, or cells $p < 1 \times 10^{-5}$) and was not an increase (n = 63 pathways from 42 cells) in EPSC amplitude (N.B. in six cells, one pathway increased and the other did not). Open symbols: pathways in which average EPSC amplitude 25–30 min > 130% baseline; closed symbols: < 130% baseline. For this and following figures, series resistance (squares) is plotted on the same graph as EPSC amplitude. EPSC traces (average of three EPSCs, stimulus artifacts removed) were taken at the time points indicated.

(e) Analysis of all experiments showing the proportion of pathways or cells in which EPSC amplitude increased with pep2-SVKI ("pathway specific" indicates cells in which pep2-SVKI had different effects on the two pathways).

(f) Mean change in $1/CV^2$ plotted versus change in EPSC amplitude (values at 25–30 min compared to baseline of 0–5 min) for 11 of the pathways (randomly selected) that showed an increase with pep2-SVKI.

dominantly cell rather than pathway specific (Figure 2e). In the majority of cells in which data from two pathways were collected, pep2-SVKI had the same effect on both pathways (in 11/38 cells [29%] both pathways increased, in 21/38 cells [55%] there was no effect), while in only 6/38 cells (16%) the effects were pathway specific.

We also performed a coefficient of variation (CV) analysis (Faber and Korn, 1991; Kullmann, 1994) on 11 of the pathways that exhibited the increase with pep2-SVKI. This analysis showed that on average there was a large change in CV associated with the increase in amplitude. When change in $1/CV^2$ was plotted *versus* change in EPSC amplitude, the mean value was very close to the line of unity ($1/CV^2$ = 194% ± 22%; EPSC amplitude = 186% ± 16% n = 11; Figure 2f), indicating that the increase in EPSC amplitude was most likely due to a change in quantal content. Quantal content is composed of probability of release (Pr) and number of functional synapses (n). Since the peptide was introduced into the postsynaptic cell and specifically disrupted the interaction between ct-GluR2 and PDZ proteins, the change in quantal content was very likely due to a postsynaptic increase in the number of functional synapses. One possibility, therefore, is that pep2-SVKI causes the insertion of AMPARs at silent synapses producing a postsynaptic increase in n.

We also performed two types of controls, interleaved with the pep2-SVKI experiments. In one set of experiments, an intracellular solution that lacked pep2-SVKI, but was otherwise identical, was used (Figures 3a and 3b). In the other controls, the inactive control peptide pep2-SVKE was used (Figures 3c and 3d). There was no change in EPSC amplitude in the "no peptide" control (107% ± 5%, p = 0.1, n = 51) or in the presence of pep2-SVKE (103% ± 5%, p = 0.3, n = 44).

Pep2-SVKI does not distinguish between the GRIP/ABP-GluR2 and PICK1-GluR2 interactions. To investigate which interacting protein was important for this effect, we used pep2-EVKI, which blocks PICK1 but not GRIP/ABP binding to GluR2/3 (Figure 1; see also Li et al., 1999; Chung et al., 2000). Pep2-EVKI (100 μM) had

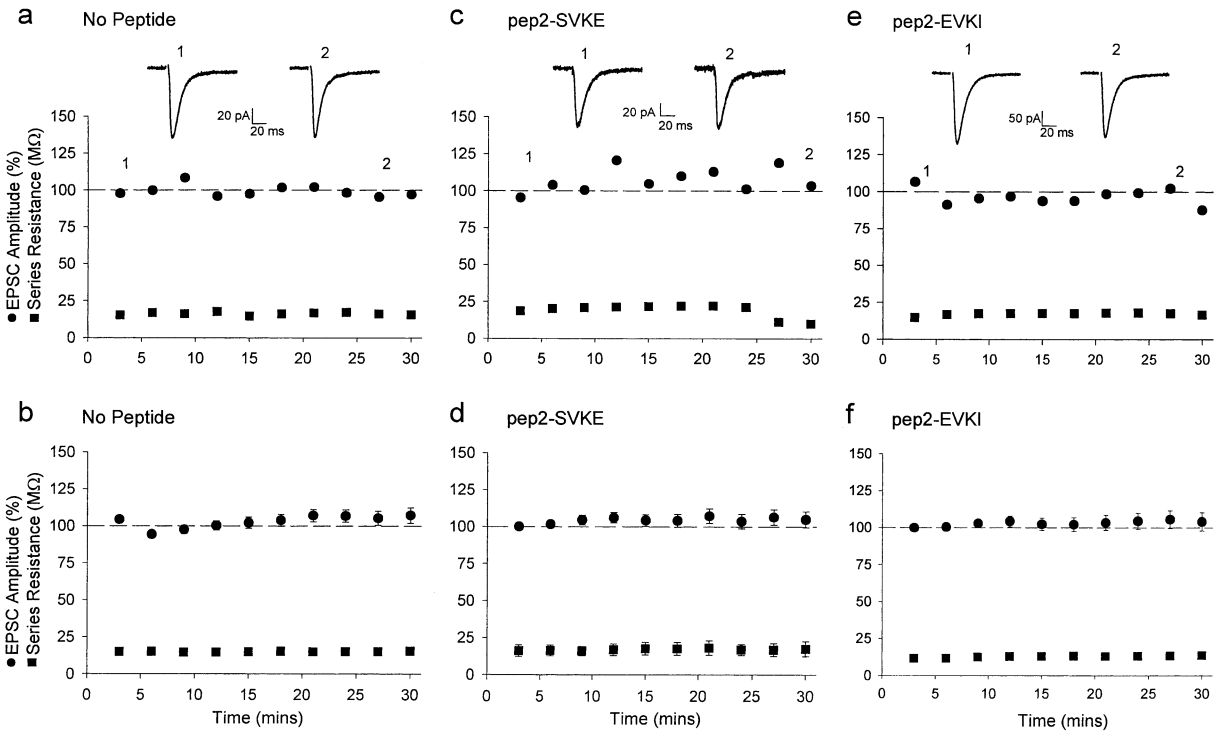


Figure 3. EPSCs Are Unaffected by a Control Peptide or a PICK1 Selective Peptide

Amplitude versus time plots for: (a) a single experiment without peptide, (b) summary graph of experiments without peptide (n = 51 pathways, 26 cells; no significant change in EPSC amplitude when statistics performed on pathways p = 0.1 or on cells p = 0.2), (c) a single experiment with pep2-SVKE (100 μM), (d) summary graph of experiments with pep2-SVKE (n = 44 pathways, 24 cells; no significant change in EPSC amplitude when statistics performed on pathways p = 0.3 or on cells p = 1.0), (e) an example experiment with pep2-EVKI (100 μM), and (f) summary data for pep2-EVKI (n = 44 pathways, 22 cells; no significant change in EPSC amplitude when statistics performed on pathways p = 0.6 or on cells p = 0.6).

no effect on basal synaptic transmission (103% ± 6%, p = 0.6, n = 44; Figures 3e and 3f) when included in the intracellular solution. This could suggest that PICK1 is not involved in this regulation of AMPARs and that rather it is the GRIP/ABP-GluR2/3 interaction that is important. Alternatively, it could mean that the GRIP/ABP and PICK1 are both involved but that they are interchangeable.

PKC Activity Is Required for the Pep2-SVKI Effect on Basal Synaptic Transmission

The experiments described above show that disruption of ct-GluR2/3 PDZ interactions causes an increase in AMPAR-mediated synaptic transmission in a subset of cells. The extreme C terminus of GluR2/3 contains a PKC phosphorylation consensus sequence (-SVK-) and the serine in this region (Ser^{880}) is phosphorylated by PKC (Matsuda et al., 1999; Chung et al., 2000; Matsuda et al., 2000). Therefore, we investigated the involvement of PKC in the effects of blocking ct-GluR2/3 PDZ interactions. First, we tested whether PKC activity is important for basal transmission under control conditions. Bath application of the selective PKC inhibitor bisindolylmaleimide I (BIS, 1 μM), which acts on the regulatory domain of PKC (Toullec et al., 1991; Nishizuka, 1995), had no effect on basal transmission in control cells (102% ± 4%, p = 0.6, n = 56; Figures 4a and 4b). We also tested BIS (1 μM) and another highly specific PKC inhibitor, Ro-31-8220 (1 μM), which also acts on the regulatory domain (Davis et al., 1989; Wilkinson et al., 1993), on basal synaptic transmission using extracellular field potential recordings. Neither inhibitor had any effect on basal transmission in these experiments (data not shown). These data suggest that during basal transmission AMPAR function at these synapses is not maintained by constitutive PKC activity. This is in agreement with other studies in which it was found that inhibitors acting on the regulatory domain of PKC did not affect basal AMPAR-mediated synaptic transmission (e.g., Malinow et al., 1988; Reymann et al., 1988; Colley et al., 1990; Bortolotto and Collingridge, 2000).

We then investigated the effects of infusing pep2-SVKI into neurons while PKC was blocked. In the presence of bath applied BIS (1 μM), there was no increase in EPSC amplitude with pep2-SVKI (103% ± 7%, p = 0.6, n = 34; Figures 4c and 4d), even though in interleaved experiments with pep2-SVKI alone, increases in EPSC amplitude were still consistently observed in approximately one third of experiments (data included in Figure 2). These data suggest that a PKC-dependent process is responsible for the increase in EPSC amplitude observed when ct-GluR2/3 PDZ interactions are disrupted.

Pep2-SVKI Prevents the Expression of LTD

Previous studies have demonstrated a role for the GluR2-NSF interaction in the expression of LTD (Lüscher et al., 1999; Lüthi et al., 1999). Therefore, we investigated whether the ct-GluR2/3 PDZ interactions are also in-

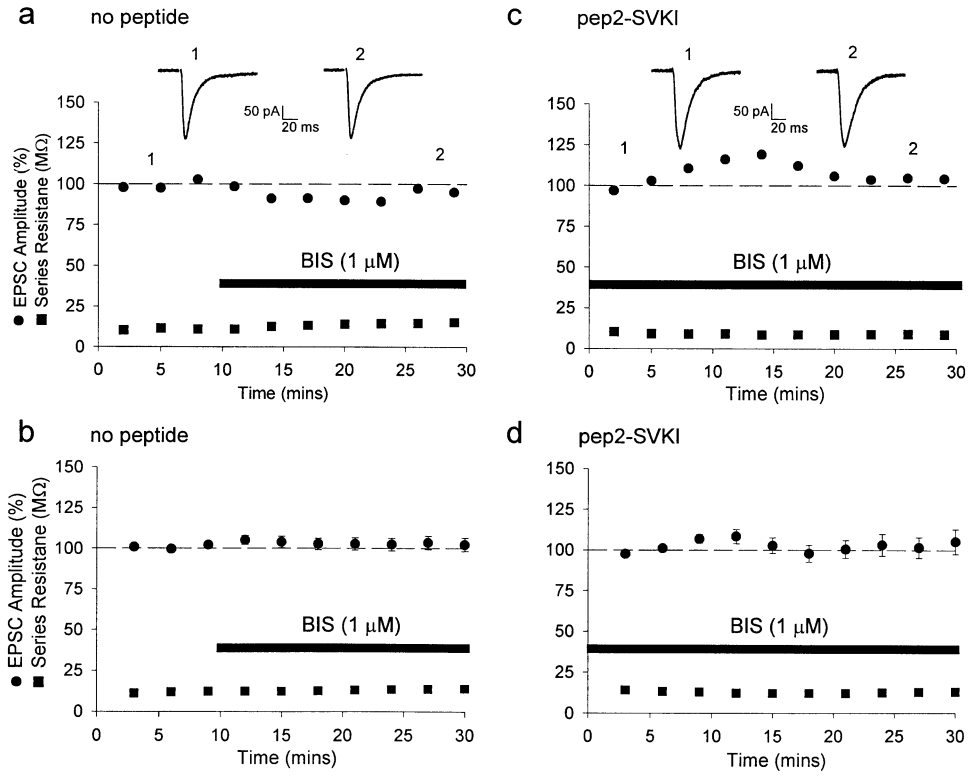


Figure 4. Bath Application of the PKC Inhibitor BIS Blocks the Increase in EPSC Amplitude Caused by Pep2-SVKI

(a) Amplitude versus time plot for an example experiment in which BIS (1 μM) was applied after 10 min of baseline recording without peptide in the recording electrode.

(b) Summary graph of these experiments (n = 56 pathways, 28 cells; no significant change in EPSC amplitude when statistics performed on pathways p = 0.6 or on cells p = 0.6).

(c) Example experiment with pep2-SVKI in the intracellular solution in the presence of bath applied BIS (1 μM).

(d) Summary graph of these pep2-SVKI experiments in the presence of BIS (n = 34 pathways, 17 cells; no significant change in EPSC amplitude when statistics performed on pathways p = 0.6 or on cells p = 0.8).

volved in LTD. Whole-cell recordings were made, and two independent pathways were monitored. Following at least 30 min of whole-cell dialysis, an LTD induction protocol of 300 stimuli at 1 Hz paired with a holding potential of −40 mV was applied to one pathway (test pathway). This protocol has previously been shown to induce homosynaptic NMDAR-dependent LTD (Hjelmstad et al., 1997; Lüthi et al., 1999). In the absence of peptide, LTD was reliably induced in the test pathway (EPSC amplitude 25–30 min after LTD induction = 63% ± 6% of pre-LTD baseline [0–10 min before induction], $p < 0.005$ versus pre-LTD baseline, n = 5; Figure 5a). However, in interleaved experiments, pep2-SVKI blocked LTD in all experiments, leaving only a transient depression in the test pathway (95% ± 6%, p = 0.4 versus pre-LTD baseline, n = 10; Figures 5c and 5d). The effect of pep2-SVKI on LTD was independent of whether or not it caused an increase in basal EPSC amplitude. Importantly, the control peptide pep2-SVKE did not block LTD (73% ± 9%, $p < 0.05$ versus pre-LTD baseline, n = 10; Figure 5b). We also tested whether pep2-EVKI, which selectively blocks the PICK1 interaction, prevented LTD. In the presence of pep2-EVKI, LTD was also reliably induced (81% ± 3%, $p < 0.001$ versus pre-LTD baseline, n = 8; Figure 5b). Thus, pep2-SVKI, but neither pep2-SVKE nor pep2-EVKI, blocked LTD. However, unlike with basal transmission, pep2-SVKI was effective at blocking LTD in all pathways. These data suggest that ct-GluR2/3 PDZ interactions are important both for the regulation of basal transmission and LTD.

PKC Activity Is Required for the Pep2-SVKI Block of LTD

We investigated whether there was an involvement of PKC in the pep2-SVKI block of LTD, since the PKC inhibitor, BIS blocked the effects of pep2-SVKI on basal transmission. In the presence of bath applied BIS (1 μM), pep2-SVKI did not block LTD (73% ± 4%, $p < 5 \times 10^{-5}$ versus pre-LTD baseline, n = 11; $p < 0.005$ versus pep2-SVKI LTD; Figures 6e and 6f). This suggests that, similar to the effects on basal transmission, PKC activity is required for the block of LTD to occur when ct-GluR2/3 PDZ interactions are disrupted by pep2-SVKI.

Prior Induction of LTD Modifies the Effects of Pep2-SVKI on Basal Transmission

We reasoned that the block of LTD by pep2-SVKI might be because LTD primes synapses to undergo the increase in transmission in the presence of the peptide. To test this idea, we performed two pathway experiments using extracellular recordings in which LTD was induced in one pathway by applying a low-frequency stimulation protocol (LFS; 900 stimuli, 1Hz; Figure 6a). This pro-

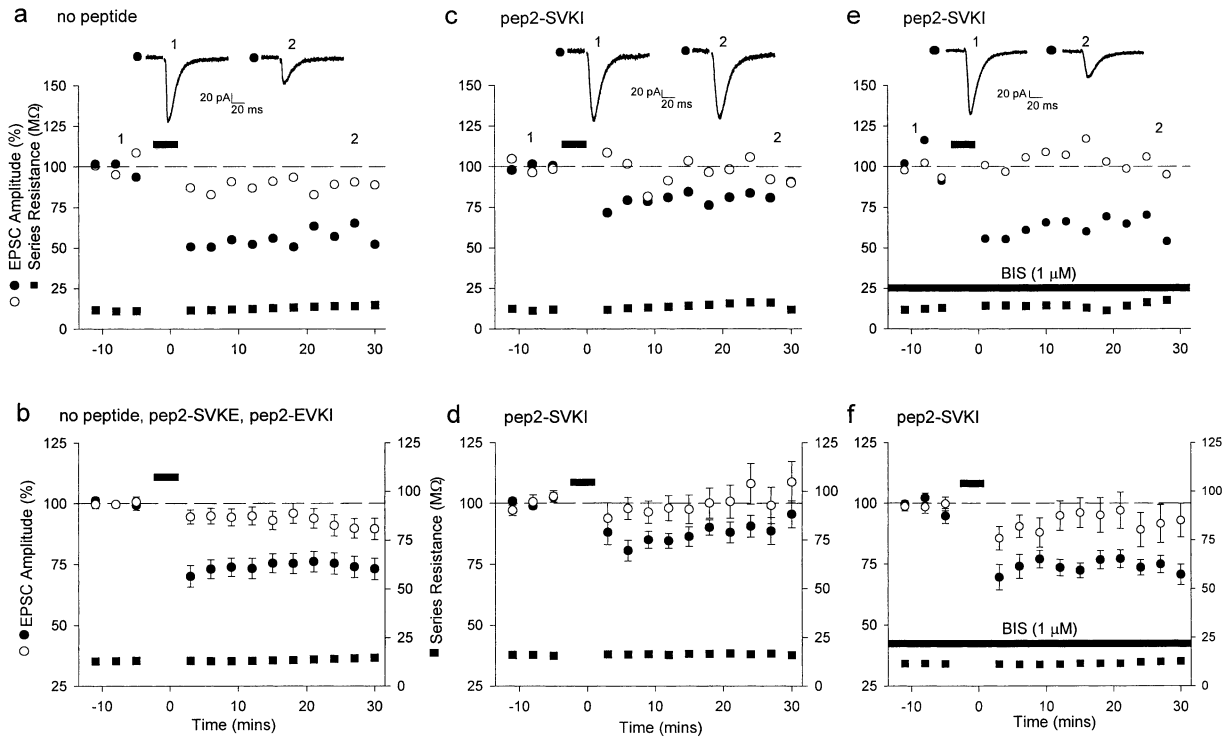


Figure 5. Pep2-SVKI Blocks LTD in a PKC-Dependent Manner

(a) EPSC amplitude (percent of pre-LTD baseline collected for 10 min before induction) versus time plot for an example LTD experiment with no peptide. For this figure, the paired pathway is represented by closed symbols and control pathway by open symbols. The black bar represents the pairing protocol for induction of LTD.

(b) Summary graph of pooled control LTD experiments (n = 23) either with no peptide (n = 5), with the inactive control peptide pep2-SVKE (n = 10) or pep2-EVKI (n = 8; all controls were interleaved with pep2-SVKI LTD experiments).

(c) Example LTD experiment with pep2-SVKI in the recording electrode.

(d) Summary graph of the pep2-SVKI LTD experiments (n = 18 at 20 min, n = 10 at 30 min; 8 cells were lost between 20 and 30 min).

(e) EPSC amplitude versus time for an example LTD experiment with pep2-SVKI in the recording electrode in the presence of bath applied BIS (1 μM).

(f) Summary graph of LTD experiments with pep2-SVKI and BIS (n = 12 at 20 min, n = 11 at 30 min; 1 cell lost between 20 and 30 min).

duced a homosynaptic depression in fEPSP slope to 71% ± 4% of baseline (n = 8). After induction of LTD, a whole-cell recording was obtained from a neuron in the same region of the slice, and EPSCs were evoked at the same two pathways using the same or lower stimulus intensity. Under these conditions, the frequency with which pep2-SVKI caused an increase in transmission was greatly increased (Figures 6b–6d). Surprisingly, this effect was not restricted to the pathway that had previously received LTD, but occurred in both the conditioned and nonconditioned pathways.

Since these experiments were performed at a higher temperature to facilitate the induction of LTD using LFS (28°C–30°C rather than room temperature), we also tested the effect of pep2-SVKI at this temperature on slices in which LTD had not been induced ("nonconditioned" slices). Similar to experiments performed at room temperature, pep2-SVKI caused an increase in EPSC amplitude only in a subset of experiments (Figures 6c and 6d), although the kinetics of the increase exhibited a somewhat different profile, presumably due to the higher temperature. These data suggest that prior induction of LTD primes synapses or receptors so that disruption of the ct-GluR2 PDZ interactions causes an increase in amplitude in the majority of experiments. The lack of pathway specificity of this particular effect suggests that the induction of LTD causes the production of some signal that can diffuse to other nonconditioned synapses causing the heterosynaptic effects.

Prior induction of LTD also had another effect. The onset of the increase in EPSC amplitude caused by pep2-SVKI was much more rapid following LTD induction, compared to nonconditioned slices that showed a characteristic onset delay of ~5 min (Figure 6c). This suggests that prior induction of LTD (i.e., in the absence of pep2-SVKI) primes synapses both homo- and heterosynaptically so that subsequent disruption of the ct-GluR2 PDZ interactions causes a very rapid insertion of receptors.

A Model for the Regulation of AMPAR Insertion by ct-GluR2 PDZ Interactions

Figure 7 shows a hypothesis based on the data presented so far, for the mechanism by which ct-GluR2/3 PDZ interactions regulate AMPARs at CA1 synapses. In this model, there are two intracellular pools of AMPARs at synapses: AMPARs containing GluR2 phosphorylated at serine 880 in a "constitutive pool," which rapidly and

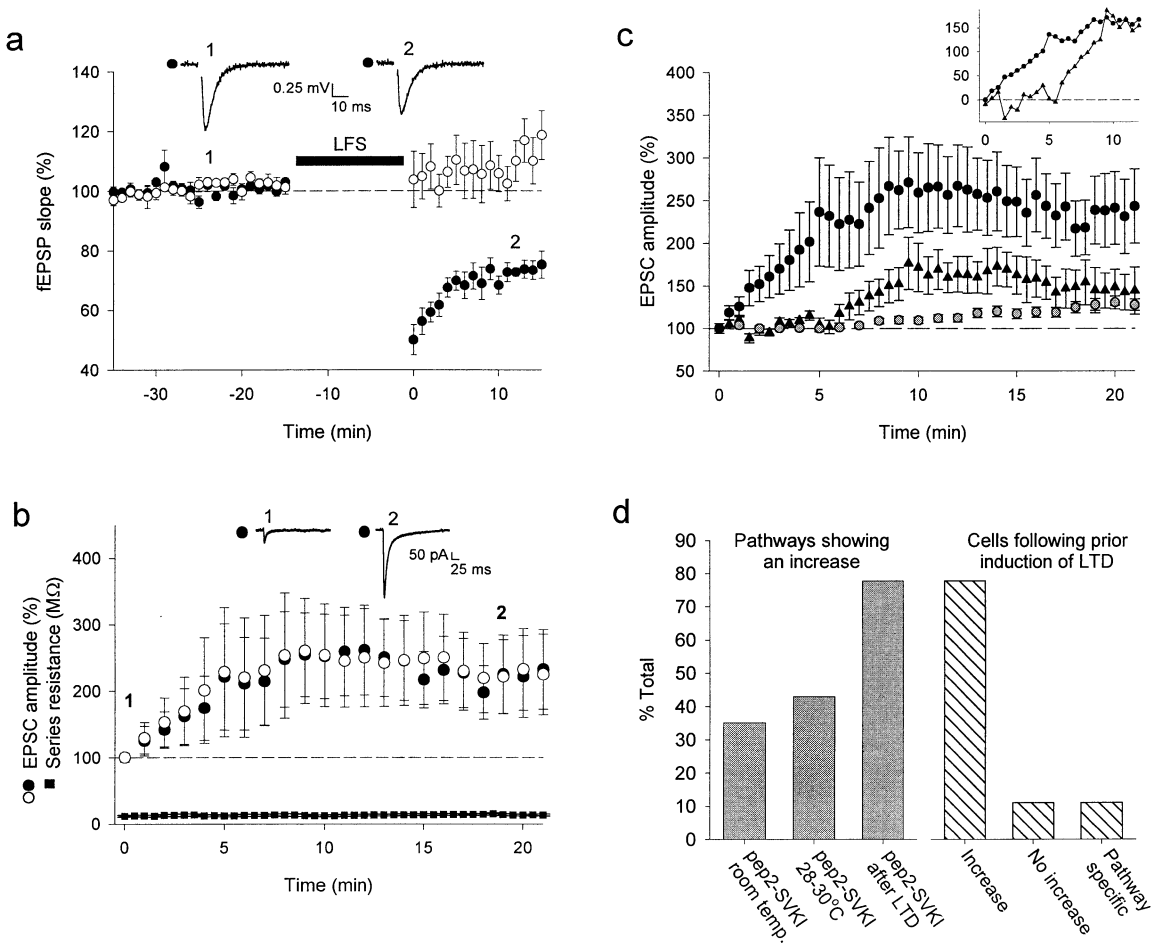


Figure 6. Prior Induction of LTD Causes EPSCs to Increase in the Majority of Pathways in the Presence of Pep2-SVKI

(a) Summary data from extracellular recordings for two pathway experiments in which LTD was induced in one pathway (closed circles; open circles control path; n = 8). Field EPSPs from an example experiment, collected at the times indicated for the LTD pathway.

(b) Pooled data (n = 9) of EPSC amplitude versus time for two pathway experiments in which pep2-SVKI was introduced into cells in slices in which LTD had been previously induced using extracellular recordings. EPSCs were evoked at the same two pathways (closed circles conditioned path, open circles control path; these experiments performed at 28°C–30°C).

(c) Summary data for all experiments in which pep2-SVKI was introduced into cells following LTD induction (closed circles, conditioned and nonconditioned pathway data pooled; n = 18 pathways from nine cells), effects of pep2-SVKI in nonconditioned slices (closed triangles; at 28°C–30°C; n = 14 pathways from seven cells) and pep2-SVKI in nonconditioned slices at room temperature (gray circles; data replotted from Figure 2c). Inset: data for effects of pep2-SVKI for prior induction of LTD (circles) and nonconditioned slices at 28°C–30°C (triangles) shown on an expanded time scale to illustrate the difference in the onset of the increase in EPSC amplitude for the two data sets. For this inset, data are replotted as percent change from baseline (i.e., no change = 0%) and nonconditioned slice data is scaled to the peak of the EPSC amplitude increase of the conditioned slice data (at 10–15 min).

(d) Summary data showing (gray bars) the frequency of observing the increase in EPSC amplitude for the three different conditions and (hatched bars) the lack of pathway specificity for pep2-SVKI following prior induction of LTD.

constitutively recycle via the NSF-GluR2-dependent mechanism previously described (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Lüscher et al., 1999; Lüthi et al., 1999; Noel et al., 1999). AMPARs that contain GluR2 not phosphorylated at serine 880 are in a "regulated pool" and require PKC phosphorylation of this site to enter the constitutive pool and be inserted into the membrane. In this scheme, proteins interacting at the ct-GluR2/3 PDZ binding motif bind to nonphosphorylated GluR2/3 and act as an "insertion clamp"; their binding blocks PKC-dependent phosphorylation of serine 880, thus preventing the receptors entering the constitutive pool.

Dedepression Is Blocked by PKC Inhibitors

This model predicts that one form of LTP, dedepression (DD; LTP following prior induction of LTD) should be PKC dependent. Lee et al. (2000) have recently reported that DD is blocked by a PKA inhibitor; however, there was a component of DD that was insensitive to this inhibitor. To investigate the possible involvement of PKC in DD, we used KN-62 (10 μM) and KT5720 (1 μM) to block CaMKII and PKA, respectively. Under these conditions in two pathway field potential experiments, LTP of naïve pathway was fully blocked; however, the same induction protocol caused DD of the input that had previously undergone LTD (Figure 8a). DD, however, was

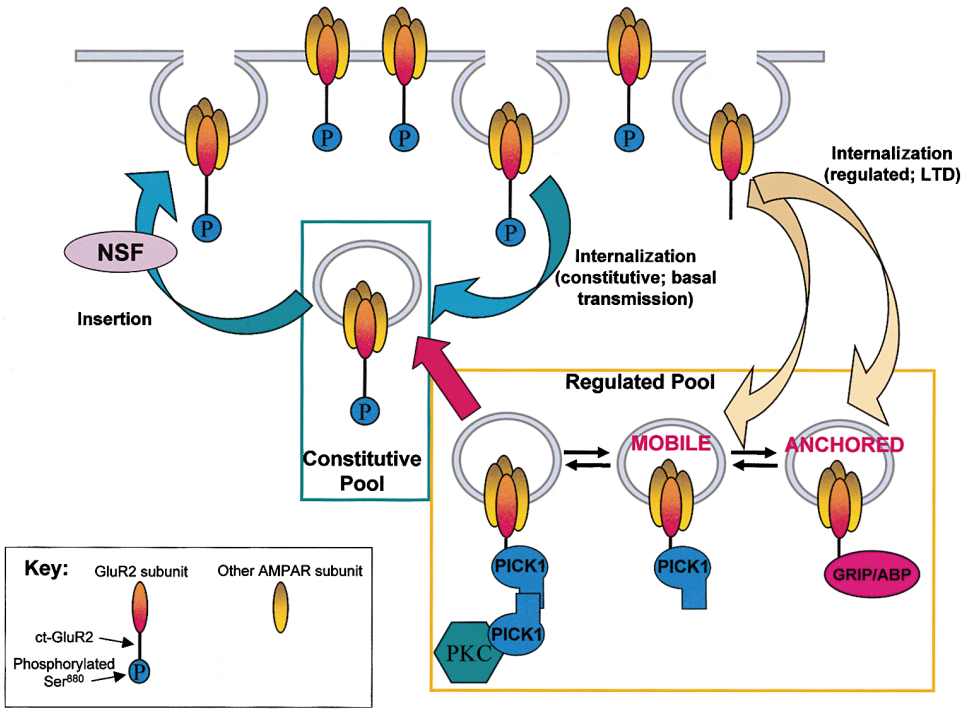


Figure 7. A Model for the Regulation of AMPARs by ct-GluR2/3 PDZ Interacting Proteins

PKC-dependent phosphorylation of ct-GluR2/3 at serine 880 causes insertion of AMPARs into the postsynaptic membrane. This process is regulated by ct-GluR2/3 PDZ interactions, which prevent the PKC-dependent phosphorylation.

blocked in a fully reversible manner when a PKC inhibitor ($n = 9$), either BIS (1 μM) or Ro-31-8820 (1 μM), was coapplied with KN-62 and KT5720 (Figures 8b and 8c). Therefore, these data show that DD is PKC dependent and suggests that the ct-GluR2 PDZ interactions can be disrupted physiologically during synaptic activity.

Discussion

In this study, we have shown that ct-GluR2/3 PDZ interactions are functionally important for regulating synaptic AMPARs in hippocampal CA1 neurons. Disruption of these interactions, with an 11–amino acid peptide corresponding to ct-GluR2/3 (pep2-SVKI), caused an increase in basal AMPAR-mediated transmission in approximately a third of cells and blocked the generation of LTD in all cells. These effects were not observed with control peptides and were blocked by a PKC inhibitor. Following prior induction of LTD, pep2-SVKI caused an increase in basal transmission at both conditioned and control pathways in most experiments. Finally, we demonstrated that DD was blocked by PKC inhibitors. We propose a model in which ct-GluR2 PDZ proteins are involved in the expression of LTD. In addition, we propose that these interactions are involved in a form of LTP, by regulating the PKC-dependent insertion of AMPARs so that DD can be triggered by an appropriate physiological stimulus.

Specificity of the Interaction

Using a biochemical assay, we have shown that pep2-SVKI blocks the PICK1-GluR2 interaction, and in a previous study an almost identical peptide, "GluR2-SVKI," corresponding to the last ten amino acids of GluR2, was shown to block GRIP-GluR2 interactions (Li et al., 1999). Since a single amino acid substitution (-SVKI to -SVKE) renders the peptide inactive at blocking both PICK1 (Figure 1; Chung et al., 2000) and GRIP/ABP (Li et al., 1999) interactions, and since pep2 -SVKE peptide is inactive in the physiological experiments, the effects of pep2-SVKI can be specifically attributed to the disruption of ct-GluR2/3 PDZ interactions.

A recent study has implicated a ct-GluR1 PDZ interaction in the regulated insertion of GluR1 homomers in hippocampal neurons (Hayashi et al., 2000) presumably involving the GluR1-SAP97 interaction (Leonard et al., 1998). One possibility, therefore, is that the effects of pep2-SVKI might involve the blockade of this interaction. However, this is unlikely because SAP97 does not interact with either GluR2 or GluR3 (Leonard et al., 1998) and the PDZ binding domain on GluR1 has a different sequence to pep2-SVKI. Furthermore, Hayashi and coworkers found that the GluR1 PDZ interaction was necessary for insertion, and therefore if blocked this would cause a depression in EPSC amplitude, contrary to the results in the present study.

One potential problem with the peptides used in this study is that there is a possibility that they could act as competitive inhibitors of PKC. However, in our study all the effects of pep2-SVKI were blocked by a PKC inhibitor, strongly suggesting that pep2-SVKI was not itself acting as a PKC inhibitor. Furthermore, the effects of the ten amino acid ct-GluR2 peptides on AMPARs in spinal cord neurons have been shown not to be due to direct inhibition of PKC (Li et al., 1999). A related consideration is that pep2-SVKI needs to be phosphory-

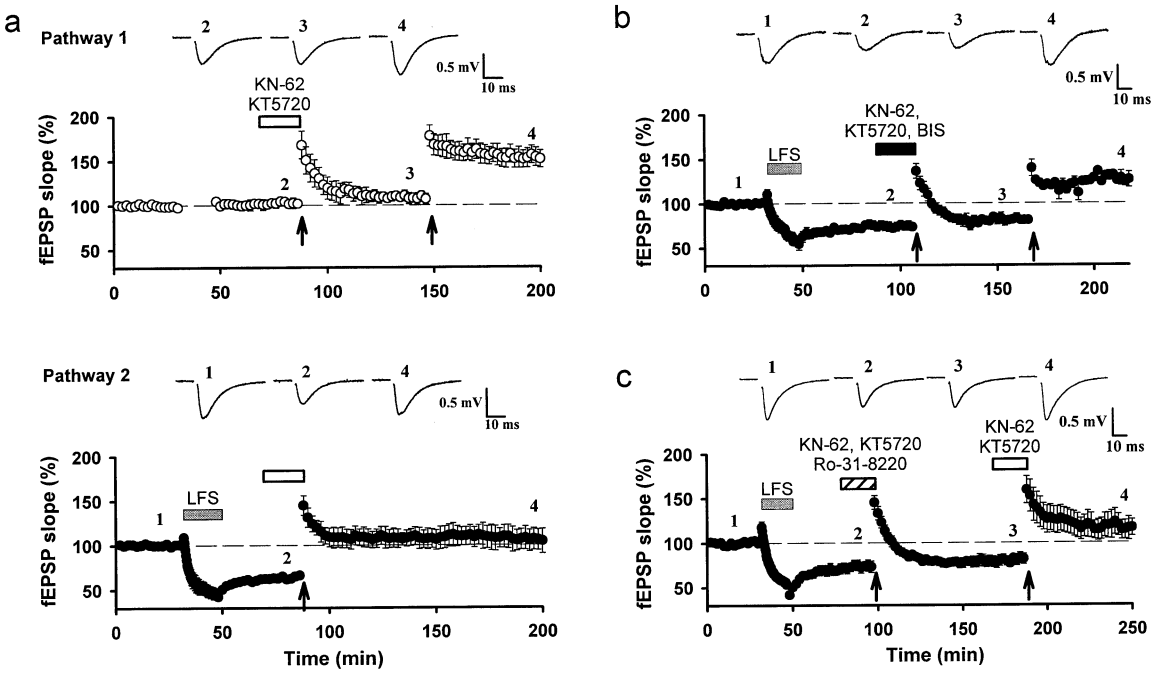


Figure 8. Dedepression Is Blocked by PKC Inhibitors

(a) Summary data of two pathway field potential experiments (n = 4) showing fEPSP slope (percent baseline) versus time in which an LTP induction protocol was applied, in the presence of KN-62 (10 μM; CaMKII inhibitor) and KT5720 (1 μM; PKA inhibitor), to a naïve pathway (open circles) and a pathway in which LTD had previously been induced (closed circles). Representative traces from a single experiment shown, taken at the times indicated.

(b) Summary data for experiments similar to those in (a) in which an LTP induction protocol was applied to the pathway that had undergone LTD in the presence of KN-62, KT5720, and BIS (1 μM; PKC inhibitor; n = 5).

(c) Summary data for experiments in which an LTP induction protocol was applied, in the presence of KN-62, KT5720, and Ro-31-8220 (1 μM; PKC inhibitor; n = 4), to the pathway that had undergone LTD.

lated before it becomes active and that the PKC inhibitors work in our study by blocking this phosphorylation rather than that of an endogenous substrate such as GluR2. Although we cannot completely exclude this possibility, evidence against this is provided by the lack of effect of pep2-EVKI in which the serine is substituted by a glutamate, a manipulation equivalent to the phosphorylation of serine 880 (at least with respect to preventing the interaction between GRIP/ABP and GluR2).

Functional Effects of Pep2-SVKI on AMPAR-Mediated EPSCs

There are a number of possible mechanisms by which pep2-SVKI could cause the enhancement of synaptic transmission. Given that we use a postsynaptically injected peptide, it is highly unlikely that the effects are presynaptic or via glutamate transporter mechanisms. The possible postsynaptic mechanisms are: an increase in single channel conductance (γ), an increase in the probability of the channel opening on binding ligand (P_{open}), a change in channel kinetics or an increase in the number of receptors available to bind L-glutamate. We think the latter is a plausible explanation for the changes in synaptic transmission we observe given the body of evidence that AMPARs can be rapidly targeted to and removed from the postsynaptic membrane of hippocampal neurons (Nishimune et al., 1998; Song et al., 1998; Carroll et al., 1999; Lüscher et al., 1999; Lüthi et al., 1999; Noel et al., 1999; Shi et al., 1999; Hayashi et al., 2000; Man et al., 2000) and cerebellar neurons (Liu and Cull-Candy, 2000; Matsuda et al., 2000; Wang and Linden, 2000). The finding that the increase in basal transmission with pep2-SVKI is associated with a large change in CV is consistent with this possibility and indicates that receptors may be preferentially inserted at silent synapses.

One surprising finding was that the effects of pep2-SVKI were primarily cell specific rather than pathway specific. Further evidence for a cell-specific effect was provided by the experiments in which LTD was induced prior to introducing the peptide into cells. Under these conditions, the incidence of the increase in transmission with pep2-SVKI was greatly increased, but this occurred at both the LTD and control pathways. This suggests that some signal is generated at the synapses that underwent LTD, which diffuses intracellularly to other synapses in the cell. This signal could be mobile PKC regulated AMPARs perhaps associated with PICK (Chung et al., 2000) or a second messenger such as release of Ca^{2+} from intracellular stores that stimulates the PKC-dependent regulation of AMPARs (B. Maher and P. T. Kelly, 2000, Soc. Neurosci. Abstr. *26,* 38.17).

PKC Modulation of AMPAR Function

There is considerable evidence for the PKC-dependent modulation of AMPAR function at synapses. A number of studies have provided support for a role for PKC in LTP (e.g., Lovinger et al., 1987; Malinow et al., 1988;

Reymann et al., 1988; Colley et al., 1990; Reymann et al., 1990; Klann et al., 1991; Wang and Feng, 1992; Sacktor et al., 1993; Hvalby et al., 1994; Osten et al., 1996); however, the conclusions of some of these studies have been challenged based on the specificity of the inhibitors used (Muller et al., 1992; Lopez-Molina et al., 1993; Roberson et al., 1996). Indeed, recently, we have provided evidence that potent PKC inhibitors, including Ro-31-8220 that was used in the present study, have no effect on LTP induction in the CA1 region of adult rat hippocampus (Bortolotto and Collingridge, 2000). However, it is possible that there is a developmental regulation of a PKC-dependent mechanism for LTP, and this could explain some of these conflicting findings.

PKC does not appear to be involved for the induction of NMDAR-dependent LTD (Oliet et al., 1997), but there is evidence that the constitutively active catalytic fragment of PKCζ (PKMζ) is involved in its expression (Hrabetova and Sacktor, 1996). In CA1 neurons, PKC activators cause an increase in both the amplitude and frequency of mEPSCs (Parfitt and Madison, 1993; Wang et al., 1994; Carroll et al., 1998). In spinal neurons, PKC potentiates synaptic transmission and this is dependent upon ct-GluR2/3 PDZ interactions (Li et al., 1999). Therefore, under certain but not all experimental conditions, PKC appears to be involved in the regulation of AMPAR function both during basal transmission and during synaptic plasticity. The precise mechanisms, however, for the PKC-dependent regulation of AMPAR function are not known.

In the present study, we used the PKC inhibitors BIS and Ro-31-8220, at a concentration that blocks several PKC isoforms, including PKCα (Davis et al., 1989; Toullec et al., 1991; Wilkinson et al., 1993). We found no involvement of these PKC isoforms in maintaining basal transmission; however, PKC activity was important for the potentiation caused by pep2-SVKI and in DD. Therefore, our data suggests that PKC is involved in the regulated insertion of AMPARs at synapses.

PKC-Dependent AMPAR Insertion Regulated by ct-GluR2 PDZ Interactions

A key feature of our model is that the binding of PDZ proteins to ct-GluR2/3 regulates the insertion of receptors. We hypothesize that during LTD, AMPARs are internalized (Carroll et al., 1999; Lüscher et al., 1999; Lüthi et al., 1999), and these internalized receptors bind to GRIP/ABP, which anchors them at the subsynaptic membrane (Dong et al., 1997; Srivastava et al., 1998), preventing their rapid reinsertion. However, a proportion of receptors can associate with PICK1 present in spines (Xia et al., 1999) and in this configuration may be mobile (Chung et al., 2000). The role of PICK1 is likely to be in targeting PKC to GluR2. During DD, AMPARs dissociate from PICK1 or GRIP/ABP and PKC phosphorylates serine 880. This prevents rebinding to GRIP/ABP (Chung et al., 2000) and allows insertion of receptors.

Although we have proposed that the phosphorylation state of serine 880 is the critical step for signaling AMPAR internalization/insertion, we do not, however, have any direct evidence that the PKC-dependent process is the phosphorylation of this site. It is possible that a different PKC-dependent phosphorylation step regulates the insertion of AMPARs; however, this too would have to be regulated by the ct-GluR2/3 PDZ interactions. Our model is consistent with the lack of effect of the peptide that selectively blocks the PICK1 interaction (pep2-EVKI) since GRIP/ABP functions to prevent unregulated reinsertion. The model is also consistent with the well-established role for phosphatases in the expression of hippocampal NMDAR-dependent LTD (Mulkey et al., 1993; Bear and Abraham, 1996). The lack of input specificity in the actions of pep2-SVKI may be due either to the mobility of the proportion of AMPARs associated with PICK or some other diffusible signal released from synapses that have undergone LTD. The input specificity of LTD and DD can be ascribed to the localized activation of phosphatases and kinases, respectively, provided by the NMDAR-mediated Ca^{2+} influx.

Our data are not consistent with the idea that GRIP/ABP function simply to anchor AMPARs once they are inserted in the postsynaptic membrane (Dong et al., 1997; Srivastava et al., 1998; Osten et al., 2000). If this were the case, pep2-SVKI would be expected to cause a reduction in AMPAR-mediated EPSCs, rather than the increase or no change that was observed. However, our results do not exclude a role for GRIP/ABP in the stabilization at the synaptic membrane of a more slowly recycling pool of AMPARs that would not be detected over the time course of the present experiments. Indeed, previous experiments from our and other groups, using peptides that block the NSF-GluR2 interaction, suggest the existence of just such a slowly recycling pool at these synapses (Nishimune et al., 1998; Song et al., 1998; Lüscher et al., 1999; Lüthi et al., 1999). An additional role, such as this for the GRIP/ABP-GluR2 interaction, could explain recent evidence in cultured neurons that this interaction is important for the surface accumulation of GluR2 (Osten et al., 2000). Furthermore, it should be noted that in this latter study, effects on the trafficking of GluR2 were observed when the extreme C terminus was removed or serine 880 was mutated. These are manipulations that, in addition to preventing GRIP/ABP binding, also prevent PKC phosphorylation of ct-GluR2, an event that may be critically important for the regulation of the surface expression of receptors as suggested by the data in the present study.

Our model can explain the finding in spinal cord neurons that the PKC-dependent insertion of AMPARs at silent synapses requires ct-GluR2 PDZ interactions (Li et al., 1999). This preparation is somewhat different from hippocampal slices in that there appears to be very little basal PKC activity since PKC had to be activated pharmacologically before effects were observed. One possibility, therefore, is that AMPARs ready for insertion are localized to silent synapses by being anchored to GRIP/ABP at the subsynaptic membrane. Blocking this anchoring with the introduction of a peptide could cause receptors to diffuse away from silent synapses before the PKC activator was applied, thus removing the substrate for PKC-dependent insertion. Thus, the peptide would prevent the PKC-driven insertion of new receptors. The lack of effect of the peptide on basal transmission observed in this study could reflect the lack of basal PKC activity.

Different mechanisms involving PKC and AMPA re-

ceptor movement clearly exist in other neurons, for example in the cerebellum (Matsuda et al., 2000), and may also coexist in hippocampal neurons (Chung et al., 2000). Our results are, however, consistent with the well-established role of kinases and phosphatases in NMDAR-dependent homosynaptic LTD and LTP in the hippocampus (Bliss and Collingridge, 1993; Bear and Abraham, 1996; Malenka and Nicoll, 1999).

Implications for Synaptic Plasticity and Development

One implication of the model presented in the present study is that GluR2/3 PDZ interactions are an essential component of the mechanism for the maintenance and expression of NMDAR-dependent LTD by preventing the constitutive reinsertion of AMPARs removed during LTD. This model does not exclude additional mechanisms for LTD involving, for example, the dephosphorylation of GluR1 (Kameyama et al., 1998; Lee et al., 1998) perhaps causing a reduction in P_{open} (Banke et al., 2000). A second implication is that PKC is involved in the insertion of AMPARs and that this process is important for the mechanism of DD. A recent study provided evidence that PKA was also involved in DD; however, it was not fully blocked by a PKA inhibitor suggesting an additional mechanism (Lee et al., 2000). In the present study, we found that DD was fully blocked by PKC inhibitors. One difference between the two studies is the age of the animals from which the slices were prepared; we used 12- to 16-day-old animals and Lee et al. (2000) used 21- to 30-day-old animals. This suggests that both PKC- and PKA-dependent mechanisms contribute to DD but that there is a developmental regulation of the relative contributions of the two mechanisms.

A number of other observations from the present and other studies suggest that this PKC mechanism may be regulated during development. We found an increase in EPSC amplitude with pep2-SVKI in a third of experiments, and this is very similar to the observed incidence of silent synapses in animals of this age (Isaac et al., 1995; Liao et al., 1995), which is highly developmentally regulated (Durand et al., 1996). Pep2-SVKI caused an increase in all experiments when NMDAR-dependent LTD was induced, and this form of LTD is prevalent in young animals (Bear and Abraham, 1996). Interestingly, at this age, LTP in approximately a third of experiments is due to an increase in the number of activated receptors, whereas it involves an increase in single channel conductance in the remainder (Benke et al., 1998). All this suggests that, in 2-week-old hippocampus, approximately a third of synapses, in primarily a cell- rather than pathway-specific manner, have AMPARs available for PKC-dependent insertion. Taken together with the results from the CV analysis, one possibility is therefore that AMPARs are preferentially inserted at silent synapses in response to the appropriate physiological stimulus. Induction of NMADR-dependent LTD produces silent synapses (Lüthi et al., 1999) and, therefore, primes synapses to enable them to utilize this mechanism. Development may regulate this mechanism so that it is less easy to access in older animals.

Conclusion

The interactions of PDZ proteins with ct-GluR2 are likely to have multiple roles and are much more dynamic in regulating synaptic transmission than was originally thought. They provide an extra level of complexity for interactions between the protein scaffold and the signal transduction pathways present at the synapse, and it is this interplay that is important for regulating AMPAR number at hippocampal synapses during basal transmission and synaptic plasticity.

Experimental Procedures

GST Pulldown Assays

GST was fused to ct-GluR2 by subcloning into pGEX-4T-1 (Pharmacia, Uppsala, Sweden). A flag-tagged PICK1 was prepared using PCR, with the tag introduced at the N terminus of PICK1 and subcloned into pCIneo (Promega, Madison, WI). COS-7 cells were transfected with flag-PICK1 using LipofectAMINE/PLUS (GIBCO Life Tech., Geithersburg, MD) and used 48 hr after transfection. *Escherichia coli* strain BL21 was transformed with GST-ct-GluR2 as described by manufacturer's protocol (Pharmacia). Sonicates of BL21 expressing GST-ct-GluR2 and lysates of COS-7 cells transiently expressing flag-PICK1 were prepared in PTxE buffer (PBS, 1% Triton X-100, 0.1 mM EDTA [pH 7.4]), and GST pulldown assays were performed essentially as described previously (Dev et al., 1999). Breifly, 1 ml GST-ct-GluR2 lysates (~1 mg/1 ml total protein) were coupled to 20 μl gluthathione Sepharose 4B (Pharmacia) in the presence of 1 mg/ml bovine serum albumin (BSA, Sigma) for 2 hr at 4°C. Meanwhile, 100 μl flag-PICK1 was incubated with or without peptide (100 or 133 μM final concentration) for 2 hr at 4°C. The coupled Sepharose was then washed with 3× 1 ml aliquots of PTx buffer (PBS, 0.1% Triton X-100 [pH 7.4]). The pellets were resuspended in 200 μl PTxE supplemented with 2 mg BSA and 300 μl flag-PICK1 ± peptide mixture (80 μM final) was added. After incubation for 2 hr at 4°C, all samples were washed, denatured, and then separated by electrophoresis on 9% SDS-polyacrylamide gels (SDS-PAGE). The primary and secondary antibodies used for Western blotting were the affinity-purified anti-PICK-rabbit antibody raised against peptide containing the residues 2–31 of rat PICK1 and the alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega), respectively.

Electrophysiology

Transverse hippocampal slices (400 μm thick) were prepared from rat pups (12–16 days old) using standard techniques (Nishimune et al., 1998; Lüthi et al., 1999). The extracellular solution was as follows (mM): 124 NaCl, 3 KCl, 1.25 $NaHPO_4$, 26 $NaHCO_3$, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 15 glucose, saturated with 95% O_2/5% CO_2. For extracellular recordings, field EPSPs were recorded at a temperature of 28°C–30°C with an electrode placed in the *stratum radiatum* of CA1 as previously described (Bortolotto and Collingridge, 2000). Two independent pathways were alternately stimulated at a frequency of 0.033–0.1 Hz. LTD was induced by 900 stimuli at 1 Hz (Dudek and Bear, 1992; Mulkey and Malenka, 1992), LTP and DD was induced by a single tetanus of 100 Hz, 1s at test intensity. Whole-cell patch-clamp recordings were made from CA1 pyramidal neurons using electrodes (2–6 MΩ) containing the following intracellular solution (mM): 135 $CsMeSO_4$, 8 NaCl, 10 HEPES, 0.5 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 5 QX-314 (pH 7.2), 285 mOsm. Whole-cell recordings were performed in the presence of picrotoxin (0.05 mM) in the extracellular solution. The majority of recordings were performed at room temperature except for the prior induction of LTD and associated controls in which 28°C–30°C was used. The intracellular solution also contained the protease inhibitors bestatin (100 μM), leupeptin (100 μM), and pepstatin A (100 μM). For the peptide experiments, peptides were dissolved in the intracellular solution at a concentration of 100 μM. All solutions were checked to ensure that the pH was 7.2 and osmolarity was 285 mOsm and were then aliquoted and stored at −80°C. On the day of the experiment, aliquots were thawed on ice and subsequently kept on ice. EPSCs, recorded at a holding potential of −70 mV, were evoked by electrical stimulation of Schaffer

collateral-commissural axons at a frequency of 0.1 Hz using bipolar stimulating electrodes placed in the *stratum radiatum*. For the majority of experiments, two pathways onto the same cell were alternately stimulated. For the induction of LTD using whole-cell recordings, EPSCs from two pathways were collected, and LTD was induced with a protocol of 300 stimuli at 1 Hz paired with a holding potential of −40 mV (Hjelmstad et al., 1997; Lüthi et al., 1999) applied to one pathway (test pathway). For these LTD experiments, recordings were maintained for at least 30 min before the induction protocol was applied to allow the peptide-containing intracellular solution to fully dialyze the cell. Data were recorded using an Axopatch 200-B amplifier, filtered at 5 kHz, digitized at 10 kHz and stored on computer. EPSC amplitude, series resistance, input resistance, and DC were analyzed and displayed online using the LTP program (http://www.ltp-program.com). Series resistance was calculated by estimating the peak of the unfiltered whole-cell capacitance transient in response to a 2 mV step (electrode capacitance was compensated for while in cell-attached mode). CV of EPSC amplitude was calculated as SD/mean, and a value obtained for the epoch at 0–5 min was compared to the value at 25–30 min. All numbers of observations reported represent number of pathways unless stated otherwise. All data are represented as percent of baseline (100% is no change) ± SEM. Statistical significance was assessed using the Student's t test.

Acknowledgments

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