Ca\(^{2+}\)–CALMODULIN SIGNALLING PATHWAY UP-REGULATES GABA SYNAPTIC TRANSMISSION THROUGH CYTOSKELETON-MEDIATED MECHANISMS

J. WEI, A, B M. ZHANG, A, B Y. ZHU AND J.-H. WANG A, B

\(^{a}\) Laboratory of Visual Information Processing, Center for Brain and Cognitive Sciences, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, PR China

\(^{b}\) The Department of Molecular Biosciences, University of Kansas, Lawrence, KS 66045, USA

Abstract—We investigated the role of calcium (Ca\(^{2+}\))–calmodulin (CaM) signalling pathways in modulating GABA synaptic transmission at CA1 pyramidal neurons in hippocampal slices. Whole-cell pipettes were used to record type A GABA receptor (GABA\(_{\text{A}}\))-gated inhibitory postsynaptic currents (IPSCs) and to perfuse intracellularly modulators in the presence of glutamate receptor antagonists. GABA\(_{\text{A}}\)-gated IPSCs were enhanced by the postsynaptic infusions of adenosphostin (1 \(\mu M\)), a potent agonist of inositol-1,4,5-triphosphate receptor (IP\(_{3}\)R) that induces Ca\(^{2+}\) release. The enhancement was blocked by co-infusing either 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (10 \(\mu M\)) or CaM-binding peptide (100 \(\mu M\)). Moreover, the postsynaptic infusion of Ca\(^{2+}\)–CaM (40/10 \(\mu M\)) enhanced both evoked and spontaneous GABA\(_{\text{A}}\)-gated IPSCs. The enhancement was attenuated by co-infusing 100 \(\mu M\) CaM-KII (281–301), an autoinhibitory peptide of CaM-dependent protein kinases. These results indicate that postsynaptic Ca\(^{2+}\)–CaM signalling pathways essentially enhance GABAergic synaptic transmission. In the investigation of synaptic targets for the enhancement, we found that IP\(_{3}\)R agonist-enhanced GABA\(_{\text{A}}\)-gated IPSCs were attenuated by co-infusing colchicine (30 \(\mu M\)), vincristine (3 \(\mu M\)) or cytochalasin D (1 \(\mu M\)) that inhibits tubulin or actin polymerization, implying that actin filament and microtubules are involved. We conclude that postsynaptic Ca\(^{2+}\)–CaM signalling pathways strengthen the function of GABAergic synapses via a cytoskeleton-mediated mechanism, probably the recruitment of receptors in the postsynaptic membrane. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Ca\(^{2+}\) release, calmodulin, cytoskeleton, GABA\(_{\text{A}}\) receptor anchoring, hippocampal CA1 pyramidal neurons.

Principal neurons synaptically connect with glutamatergic and GABAergic axons in the CNS. The activities of glutamatergic and GABAergic synapses on each neuron counterbalance in initiating action potentials, which maintains the functional stability of neurons. Fast inhibitory postsynaptic currents (IPSCs) are mediated by type-A GABA receptors (GABA\(_{\text{A}}\); Alger, 1991; Freund and Buzsaki, 1996). GABA\(_{\text{A}}\)Rs are believed to be pentameric heterooligomers constructed from \(\alpha\), \(\beta\), \(\gamma\) and \(\delta\) subunits, and different combinations display considerable variation in gating, ion selectivity and conductance (Kardos, 1999; McKernan and Whitting, 1996). Gephyrin and GABA\(_{\text{A}}\)-associated proteins anchor receptor subunits to the postsynaptic membrane and cytoskeleton (Essrich et al., 1998; Fallon, 2000; Kneussel and Betz, 2000; Wang et al., 1999). Therefore, the functional status of GABA\(_{\text{A}}\)Rs may rely on their intrinsic properties and anchoring machinery.

The activity of neurons and excitatory synapses may induce calcium influx and/or storage release, increasing the level of intracellular Ca\(^{2+}\) (Carafoli, 1987; Ehrlich, 1995; Fox et al., 1987; Jahr and Stevens, 1990; Mayer et al., 1989; Tsien et al., 1988). Ca\(^{2+}\)–CaM signalling cascades enhance the function of glutamatergic synapses (Barria et al., 1997; Malenka and Nicoll, 1999; Wang and Kelly, 1995): less is known about how such signaling pathways in the same neurons modulate GABA synaptic transmission to coordinate the activities of excitatory and inhibitory synapses. It was suggested that protein kinases phosphorylate the intracellular loop of GABA\(_{\text{A}}\) subunits (McKernan and Whitting, 1996); however, the results in studying the functional modulation of recombinant GABA\(_{\text{A}}\) subunits at cell lines are inconsistent (Angelotti et al., 1993; Krishkevich et al., 1994; Lin et al., 1994; Moss et al., 1992, 1995; Wang et al., 1997), implying that the function of GABA\(_{\text{A}}\)R may also be modulated by other mechanisms (e.g. receptor-anchoring machinery). In addition, extrasynaptic GABA\(_{\text{A}}\)Rs displayed different kinetics from synaptic receptors (Annet et al., 1999; Banks and Pearce, 2000; Brickley et al., 1999; Chen et al., 1999). It is necessary to investigate how intracellular signalling cascades modulate GABA\(_{\text{A}}\)Rs in natural synapses (synaptic GABA\(_{\text{A}}\)Rs). We have examined the role of Ca\(^{2+}\)–CaM signal in modulating GABA synaptic transmission.

IPSC enhancement was due to an increase in receptor number (Brooks-Kayal et al., 1998; Nusser et al., 1998), whereas its attenuation resulted from a decrease in receptor clustering (Crestani et al., 1999). The number of GABA\(_{\text{A}}\)Rs on cell surface was down-regulated by PKC (Connolly et al., 1999). Cytoskeleton and receptor-associated proteins involved in GABA\(_{\text{A}}\)R anchoring are the targets of protein kinases (Garner and Kindler, 1996; Jonshon et al., 1998; Langosch et al., 1992). Therefore, in addition to acting on...
GABA<sub>A</sub>Rs, intracellular signaling cascades may modulate the machinery of receptor recruitment to alter GABA synaptic function. We have examined this hypothesis by investigating the role of tubulin and actin polymerization in GABA synaptic potentiation induced by postsynaptic Ca<sup>2+</sup> /CaM signals.

**EXPERIMENTAL PROCEDURES**

**The preparation and perfusion of hippocampal slices**

The procedures for animal use were approved by IACUC in Beijing, China and the University of Kansas, USA. Efforts were made to minimize the number of animals used and their suffering. Hippocampal slices (400 μm) were prepared from Sprague–Dawley rats in postnatal day 17–24 (Wang and Kelly, 1995) when signaling molecules are well developed (Huang, 1989; Kelly, 1992). Rats were anesthetized with methoxyflurane and then decapitated. Hippocampus with partial cortex was quickly isolated in oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) ice-cold artificial cerebrospinal fluid (ACSF), in which 0.5 mM CaCl<sub>2</sub> and 4 mM MgSO<sub>4</sub> were used to reduce the neuronal excitation during procedures. Slices were cut with a Vibratome, and then held in the oxygenated ACSF (mM: 124 NaCl, 3 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 2.4 CaCl<sub>2</sub>, 1.3 MgSO<sub>4</sub>, 10 dextrose and 10 HEPES) at 25 °C for 1–2 h. A slice was transferred to a submerged recording chamber for experiments, which was perfused with oxygenated ACSF with a rate of 2 ml per minute at 31 °C.

**The isolation of GABA currents**

Bipolar tungsten electrodes (12 MΩ) were placed near CA1 pyramidal to stimulate interneurons and their axon arbors that release...
GABA. 6-Cyano-7-nitroquinoxaline-2,3-(1H,4H)-dione (CNQX; 10 μM) and D-amino-5-phosphonovanolic acid (D-AP5; 40 μM) were added in ACSF to block activities of glutamatergic synapses (Wang and Stelzer, 1996). These procedures allow GABAergic IPSCs to be isolated. At the end of experiments, bicuculline (10 μM) was applied to hippocampal slices to examine whether synaptic responses were purely mediated by GABAAR. Bicuculline did block synaptic currents recorded in our experiments.

**IPSC evoking and recording**

The intensity for stimulating GABA axons was constant during each experiment, and the frequency was 0.1 Hz. IPSCs were recorded by whole-cell patch clamp on the soma of CA1 pyramidal neurons that were visualized under DIC microscopy (Olympus, USA BX50WI or Nikon, USA E600FN). Standard pipette solution contained (mM) 135 K-gluconate, 20 KCl, 4 NaCl, 10 HEPES, 0.5 EGTA, 4 Mg-ATP, and 0.5 Tris–GTP, and was filtered with a 0.1 μm centrifuge filter before use. The osmolarity of pipette solutions was 295–310 mOsmol, and the resistance of filled pipettes was 5–7 MΩ. Based on Nernst equation, the concentration of chloride in our pipette solution makes reversal potential \(-43\) mV, which is consistent with values in our measurements. When the holding potential of membrane was \(-20\) mV in our experiments, GABAergic synapses were evoked and recorded.

**Fig. 3.** The postsynaptic infusion of \(\text{Ca}^{2+}\)-CaM enhances the response of GABAergic synapses on CA1 pyramidal neurons. (A) \(\text{Ca}^{2+}\)-CaM enhances IPSCs (dark-filled symbols) compared with a control (open symbols). IPSCs are blocked by 10 μM bicuculline. Inset shows IPSC waveforms at time points 1, 2, and 3 during \(\text{Ca}^{2+}\)-CaM infusion. (B) Averaged data show effects of \(\text{Ca}^{2+}\)-CaM infusions on IPSCs (dark-filled symbols, \(n=6\)) vs. controls (open symbols, \(n=6\)). Inset shows IPSC waveforms from a control (calibration, 150 pA and 30 ms).

**Fig. 4.** The postsynaptic perfusion of \(\text{Ca}^{2+}\)-CaM increased in the frequency and amplitude of sIPSCs on CA1 pyramidal neurons. (A) Cumulative probability is plotted as a function of sIPSC amplitudes. Perfusing \(\text{Ca}^{2+}\)-CaM into pyramidal neurons shifted IPSC amplitudes (open symbols) to bigger (filled symbols, \(n=5\)). (B) Cumulative probability is plotted as a function of sIPSC amplitudes. \(\text{Ca}^{2+}\)-CaM perfusion shifted inter-IPSC intervals (open symbols) to shorter (filled symbols, \(n=5\)). (C) sIPSC waveforms (up-fluctuation) show before (0−4 min, left) and after perfusing \(\text{Ca}^{2+}\)-CaM (15–18 min, right), and eight consecutive traces are superimposed in each condition. Calibration bars are 100 ms (horizontal) and 20 pA (vertical).
gic IPSCs were outward (up-fluctuation, see representative waveforms). We monitored the series and input resistance for all of the recording neurons by applying hyperpolarization pulses (−5 mV and 50 ms) throughout each experiment. Their values can be calculated by voltage pulses vs. instantaneous and steady-state currents (as shown the first part of waveforms in figures).

The infusion of modulators

Recording patch pipettes were also used to infuse reagents, which were dissolved in standard pipette solution, into neurons. Experiments to compare the effect of adenophostin and co-infusion of inhibitors with adenophostin were conducted on a daily basis, which reduces variation among rats and slices. The modulators were dissolved in distilled water for their stock solutions (100 times higher than the final concentration) that were diluted into the standard pipette solution before use. Ca²⁺-CaM was the mixture of Ca²⁺ and CaM at a ratio of 4:1, and after binding reaction it was dissolved in the standard pipette solution (Wang and Kelly, 1995). It should be emphasized that the concentration of inhibitors was above their respective IC₅₀ but did not affect basal synaptic transmission (see control experiments in Figs. 2 and 4–7). Therefore, that they attenuated synaptic potentiation during co-infusion experiments was unlikely due to their side effect. In addition, we could not see changes in the morphology of recording neurons under DIC microscopy while infusing colchicine, vincristine or cytochalasin D that inhibits tubulin and actin polymerization.

![Fig. 5](image1)

**Fig. 5.** The postsynaptic infusion of CaMKII(281–301) blocks the adenophostin-induced potentiation of GABA synaptic responses. (A) Individual example shows the effect of co-infusing CaMKII(281–301) with adenophostin (dark-filled symbols) on GABAergic IPSCs compared with adenophostin alone (open symbols). Inset a1 shows IPSC waveforms from adenophostin with CaMKII(281–301), and a2 CaMKII(281–301). (B) Averaged data show effects of co-infusing CaMKII(281–301) with adenophostin (dark-filled symbols, n=7) vs. adenophostin alone (open symbols, n=12) on GABAergic IPSCs. Inset shows waveforms of adenophostin-induced potentiation. Calibrations are 200 pA and 50 ms. CaMKII(281–301) (100 μM) did not significantly affect basal GABA synaptic transmission (gray-filled symbols in A and B, n=4).

![Fig. 6](image2)

**Fig. 6.** The postsynaptic infusion of colchicine blocks the adenophostin-induced potentiation of GABA synaptic responses. (A) Individual example shows the effect of co-infusing colchicine with adenophostin (dark-filled symbols) on GABAergic IPSCs compared with adenophostin alone (open symbols). Inset a1 shows IPSC waveforms from adenophostin with colchicine, and a2 colchicine. (B) Averaged data show effects of co-infusing colchicine with adenophostin (dark-filled symbols, n=7) vs. adenophostin alone (open symbols, n=12) on GABAergic IPSCs. Inset shows waveforms of adenophostin-induced potentiation. Calibrations are 200 pA and 50 ms. Colchicine at 30 μM did not significantly affect basal GABA synaptic transmission (gray-filled symbols in A and B, n=3).
While studying the effects of Ca$^{2+}$-CaM on spontaneous GABA$_{A}$-R-gated IPSCs, we used whole-cell pipette perfusion, in which pipette tips were filled with the standard solution and the back with Ca$^{2+}$-CaM-containing standard solution. This approach allows comparing the effect of modulators on synaptic transmission to control. The success of this method has been examined by perfusing neurobiotin and fluorescent that can be seen in recording neurons 2–4 min after the formation of whole-cell configuration.

**Equipment and data analysis**

The pulses of electrical stimulation were generated by either Master-8 (A.M.P.I.) or D/A output of pClamp-8. Patch pipettes (KG33; Garner Class, Inc.) were made by a puller (P-97; Sutter, Novato, CA, USA). Synaptic currents were recorded with an Axopatch-1D amplifier and then input to data acquisition system, Digipack-1200B and pClamp 8 (Axon Instrument, Inc., Foster, CA, USA). The pulses of electrical stimulation were generated by either Master-8 (A.M.P.I.) or D/A output of pClamp-8. Patch pipettes (KG33; Garner Class, Inc.) were made by a puller (P-97; Sutter, Novato, CA, USA). Synaptic currents were recorded with an Axopatch-1D amplifier and then input to data acquisition system, Digipack-1200B and pClamp 8 (Axon Instrument, Inc., Foster, CA, USA).

We set the following criteria for the acceptence of results to be analyzed. Neurons exhibited stable membrane potentials between −65 and approximately −70 mV, and no significant changes in series and input resistances throughout each experiment. We started recording IPSCs when whole-cell access was established, in which instantaneous currents were at least three-fold higher than steady-state currents (Marty and Neher, 1995; Wang and Zhang, 2004; Wang and Kelly, 2001). The amplitude and slope of IPSCs were measured and analyzed with Clampfit (Axon Instrument, Inc.). An average of the first three IPSCs was defined as baseline (100%) and used to normalize the remaining IPSCs. As adenophostin- and Ca$^{2+}$-CaM-induced synaptic potentiation developed quickly (see Results), the use of the first three IPSCs as the baseline should minimize the effects of modulators on baseline values. Spontaneous IPSCs were accounted only when the ratio of sIPSC to baseline noise was above three (Wang, 2003). Synaptic strength was represented as mean±S.E.M. The values of evoked IPSCs at 30 min between two groups were compared by ANOVA test. The comparison of spontaneous IPSCs was done between the initial 4 min and after 15 min recordings. Representative waves of evoked IPSCs were averaged from four consecutive responses, and spontaneous IPSCs were superimposed from eight traces.

**The source of reagents**

d-AP5, CNQX, bicuculline and 1,2-bis(2-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid (BAPTA) were purchased from Sigma-RBI. CaM, CaM-binding peptide, CaMKII(281–301), colchicine, vincristine and cytochalasin D were from CalBiochem. Other chemicals were from Fisher Scientific. Adenophostin was a gift from Dr. Masaaki Takahashi (Biological Research Laboratory, Sankyo Co., Ltd., Japan).

**RESULTS**

GABA synaptic potentiation induced by raising intracellular Ca$^{2+}$

GABA synaptic plasticity was blocked by the postsynaptic injections of BAPTA (Pitter and Alger, 1992; Wang and Stelzer, 1996), indicating a requirement of Ca$^{2+}$. If free calcium is essential to synaptic plasticity, raising postsynaptic Ca$^{2+}$ should induce a change in synaptic strength. We examined this possibility by evoking Ca$^{2+}$ release from intracellular stores in CA1 pyramidal neurons of hippocampal slices. Adenophostin [a potent agonist of inositol-1,4,5-triphosphate receptor (IP$_3$R); Delisle et al., 1997; Takahashi et al., 1994] was dissolved in the standard pipette solution at a final concentration of 1 μM, and infused into postsynaptic neurons to evoke Ca$^{2+}$ release. GABA$_{A}$-R-gated IPSCs were isolated by applying 10 μM CNQX and 40 μM d-AP5 to slices (see Experimental Procedures). Compared with a control (the standard pipette solution), the postsynaptic infusions of adenophostin enhanced GABA$_{A}$-R-gated IPSCs that were blocked by 10 μM bicuculline (Fig. 1A). Fig. 1B shows the averaged data from the effect of adenophostin on IPSCs (dark-filled symbols, 192±10%, n=12) and controls (open symbols, 97±4%,

![Fig. 7. The postsynaptic infusion of vincristine blocks the adenophostin-induced potentiation of GABA synaptic responses. (A) Individual example shows the effect of co-infusing vincristine with adenophostin (dark-filled symbols) on GABAergic IPSCs compared with adenophostin alone (open symbols). Inset a1 shows IPSC waveforms from adenophostin with vincristine, and a2 vincristine. (B) Averaged data show effects of co-infusing vincristine with adenophostin (dark-filled symbols, n=10) vs. adenophostin alone (open symbols, n=12) on GABAergic IPSCs. Inset shows waveforms of adenophostin-induced potentiation. Calibrations are 200 pA and 50 ms. Vincristine at 3 μM did not significantly affect basal GABA synaptic transmission (gray-filled symbols in A and B, n=3).](image-url)
n=6). Adenophostin strengthens GABA synaptic transmission.

To examine whether adenophostin increases intracellular Ca\(^{2+}\) levels and then enhances GABA synaptic function, we co-infused adenophostin (1 \(\mu\)M) with BAPTA (10 mM in pipette) that quickly chelates Ca\(^{2+}\) (Tsien, 1980). Compared with the infusions of adenophostin alone (open symbols, 192±10%, n=12), BAPTA blocked adenophostin-induced potentiation of GABA\(_{A}\)-R-gated IPSCs (dark-filled symbols, 99±4%, n=7; \(P<0.01\); Fig. 2A). It is noteworthy that infusing 10 mM BAPTA alone did not affect basal synaptic transmission (gray-filled symbols in Fig. 2A, n=6). In addition, adenophostin-enhanced GABA\(_{A}\)-R-gated IPSCs were not due to a change in series resistance, which has been a criterion for result acceptance (see Experimental Procedures). These results indicate that increases in postsynaptic Ca\(^{2+}\), which is released from intracellular stores through the activated IP\(_{3}\)Rs, sufficiently strengthen GABAergic synaptic function.

**Ca\(^{2+}\) release-induced potentiation requires CaM and CaM-KII**

As Ca\(^{2+}\) binds CaM with high affinity (Cohen, 1988; Klee and Cohen, 1988), the increased Ca\(^{2+}\) may activate Ca\(^{2+}\)-CaM signaling pathways to modulate neuronal and synaptic function. If Ca\(^{2+}\)-CaM signaling pathways are essential to GABA synaptic potentiation induced by an IP\(_{3}\)R agonist, the inactivation of CaM should block this potentiation and raising free Ca\(^{2+}\)-CaM should enhance GABA synaptic function. First, we co-infused adenophostin (1 \(\mu\)M) with a CaM-binding peptide (CBP, an antagonist of Ca\(^{2+}\)-CaM; Hanson et al., 1994; Ocorr and Schulman, 1991; 100 \(\mu\)M in the standard pipette solution) into postsynaptic neurons. Fig. 2B shows the comparison of the co-infusion and adenophostin alone. CBP attenuated the adenophostin-induced potentiation of GABA\(_{A}\)-R-gated IPSCs from 192±10% (open symbols, n=12) to 104±5% (dark-filled symbols, n=7; \(P<0.01\)). As 100 \(\mu\)M CBP did not affect basal GABA synaptic transmission (gray-filled symbols in Fig. 2B, n=5), these results indicate that CaM is required for adenophostin-induced potentiation.

Secondly, to examine whether Ca\(^{2+}\)-CaM sufficiently enhances GABA synaptic transmission, we infused free Ca\(^{2+}\)-CaM into postsynaptic neurons. Ca\(^{2+}\)-CaM was the mixture of Ca\(^{2+}\) and CaM at a ratio of 4:1 (Wang and Kelly, 1995; also see Experimental Procedures), and its final concentration was 40/10 \(\mu\)M in the standard pipette solution. Fig. 3 shows the effect of Ca\(^{2+}\)-CaM on evoked GABA\(_{A}\)-R-gated IPSCs. Compared with a control, Ca\(^{2+}\)/CaM enhanced IPSCs that were blocked by 10 \(\mu\)M bicusculine (Fig. 3A). Fig. 3B shows the averaged data from Ca\(^{2+}\)-CaM-enhanced IPSCs (dark-filled symbols, 178±8%, n=6) and controls (open symbols, 97±4%, n=6; \(P<0.01\)). We also examined the effect of Ca\(^{2+}\)-CaM on spontaneous GABA\(_{A}\)-R-gated IPSCs. Pipette tips were filled with standard solution and the back with Ca\(^{2+}\)-CaM (40/10 \(\mu\)M). sIPSCs were recorded for 4 min immediately after the formation of whole-cell configuration. After 15 min for Ca\(^{2+}\)-CaM to be diffused into neurons, we recorded siPSCs for another 4 min. Fig. 4 shows the relationship between cumulative probability and siPSC amplitudes (Fig. 4A) or inter-siPSC intervals (Fig. 4B). After infusing Ca\(^{2+}\)-CaM, siPSC amplitudes increased and inter-IPSC intervals shortened (n=5, filled symbols). Standard error bars show the variation of the cumulative probability in siPSC amplitude and intervals among neurons. These results indicate that Ca\(^{2+}\)-CaM signaling pathways essentially modulate GABAergic synaptic transmission.

CaM-KII, a major target of Ca\(^{2+}\)-CaM, is enriched in the postsynaptic density (Cohen, 1988; Kelly et al., 1984; Klee and Cohen, 1988) and required for rebound GABA potentiation in cerebellar Purkinje neurons (Kano et al., 1996). We examined whether CaM-KII was involved in GABA synaptic potentiation induced by Ca\(^{2+}\) signal. CaM-KII autoinhibitory peptide, CaMKII(281–301) (Hanson and Schulman, 1992), was co-infused with adenophostin into postsynaptic neurons. Compared with adenophostin alone (1 \(\mu\)M, open symbols in Fig. 5A), 100 \(\mu\)M CaMKII(281–301) attenuated adenophostin-induced potentiation of GABA\(_{A}\)-R-gated IPSCs (dark-filled symbols). Fig. 5B shows the averaged data from adenophostin-enhanced IPSCs (open symbols, 192±10%, n=12) and the effect of co-infusing CaMKII(281–301) (dark-filled symbols, 107±5%, n=7; \(P<0.01\)). Infusing 100 \(\mu\)M CaMKII(281–301) alone did not affect basal GABA synaptic transmission (gray-filled symbols in Fig. 5, n=4). Taking the results above together, postsynaptic Ca\(^{2+}\), Ca\(^{2+}\)/CaM and CaM-KII pathways enhance the function of GABAergic synapses.

**The involvement of machinery for receptor anchoring in Ca\(^{2+}\)-induced potentiation**

What postsynaptic elements modulated by Ca\(^{2+}\) signaling pathways contribute to GABA synaptic potentiation? Protein kinases phosphorylate not only GABA\(_{A}\)_Rs (Krishk et al., 1994; McKernan and Whiting, 1996; Moss et al., 1992, 1995), but also gephyrin, GABAR-associated proteins and cytoskeleton (Garner and Kindler, 1996; Jonhson et al., 1998; Langosch et al., 1992). Ca\(^{2+}\) signaling cascades may modulate these GABA\(_{A}\)_R-anchoring proteins (Essrich et al., 1998; Hirokawa, 1991; Kneussel and Betz, 2000; Wang et al., 1999) to recruit more GABA\(_{A}\)_R in the postsynaptic densities and strengthen GABA synaptic transmission. We examined the role of tubulin and actin polymerization in synaptic potentiation induced by postsynaptic Ca\(^{2+}\) signal.

If Ca\(^{2+}\) signaling cascades increase the polymerized microtubules to anchor more GABA\(_{A}\)_Rs in the postsynaptic membrane, the block of tubulin polymerization should attenuate Ca\(^{2+}\)-induced GABA synaptic potentiation. Colchicine and vincristine were used to inhibit the polymerization of tubulin (Aedreu and Timasheff, 1982; Lobert et al., 1999). As they act on tubulin polymerization and have different side effects, the use of two agents may strengthen the indication and reduce the possibility that results are due to their side effects. First, we co-infused colchicine (30 \(\mu\)M) with adenophostin (1 \(\mu\)M) into CA1 pyramidal neurons. Compared with adenophostin alone (open sym-
shows waveforms of adenophostin-induced potentiation. Calibrations are 200 pA and 50 ms. Cytochalasin D at 1/11006 inhibits the effect of co-infusing colchicine (dark-filled symbols, n=7;  P<0.01), implying an involvement of tubulin-polymerization in the potentiation. Next, we co-infused 3 μM vincristine with 1 μM adenophostin into CA1 pyramidal neurons. Similar to the effect of colchicine, vincristine attenuated adenophostin-induced potentiation of GABAAR-gated IPSCs (dark-filled symbols in Fig. 7A). Fig. 7B shows the averaged data from adenophostin-enhanced IPSCs (open symbols, 192±10%, n=12) and the effect of co-infusing vincristine (dark-filled symbols, 115±10%, n=10;  P<0.01). It is noteworthy that 30 μM colchicine or 3 μM vincristine did not affect basal GABA synaptic transmission (gray-filled symbols in Figs. 6, 7; n=3 for each group). Ca2+-signaling pathway may enhance the function of GABAergic synapses through increasing the polymerized microtubules that facilitate receptor anchoring in the postsynaptic membrane.

To examine the role of actin polymerization in Ca2+-induced potentiation, we co-infused cytochalasin D (an inhibitor of actin polymerization;  Sasaki et al., 1995 ) with adenophostin (1 μM). Compared with adenophostin alone (open symbols), 1 μM cytochalasin D attenuated adenophostin-induced potentiation of GABAAR-gated IPSCs (dark-filled symbols in Fig. 8A). Fig. 8B shows the averaged data from adenophostin-enhanced IPSCs (open symbols, 192±10%, n=12) and the effect of co-infusing cytochalasin D (dark-filled symbols, 118±10%, n=7;  P<0.01). 1 μM cytochalasin D did not affect basal GABAergic synaptic transmission (gray-filled symbols in Fig. 8, n=3). Thus, postsynaptic actin filament-mediated processes contribute to GABA synaptic potentiation induced by Ca2+-signaling cascades.

Fig. 8. The postsynaptic infusion of cytochalasin D blocks the adenophostin-induced potentiation of GABA synaptic responses. (A) Individual example shows the effect of co-infusing cytochalasin D with adenophostin (dark-filled symbols) on GABAergic IPSCs compared with adenophostin alone (open symbols). Inset a1 shows IPSC waveforms from adenophostin with cytochalasin D, and a2 cytochalasin D. (B) Averaged data show effects of co-infusing cytochalasin D with adenophostin (dark-filled symbols, n=7) vs. adenophostin alone (open symbols, n=12) on GABAergic IPSCs. Inset shows waveforms of adenophostin-induced potentiation. Calibrations are 200 pA and 50 ms. Cytochalasin D at 1 μM did not significantly affect basal GABA synaptic transmission (gray-filled symbols in A and B, n=5).

Ca2+-CaM signaling pathways differentially affect the activities of excitatory and inhibitory

Our results, together with previous studies, indicate that Ca2+-CaM signaling cascades enhance the function of both glutamatergic (Barria et al., 1997; Malenka and Nicoll, 1999; Wang and Kelly, 1995) and GABAergic synapses (Figs. 1–5). It is not known about how such signaling pathways activated in the neurons coordinately modulate the activities of excitatory and inhibitory synapses. We have addressed this question through infusing adenophostin and Ca2+-CaM into CA1 pyramidal neurons in hippocampal slices. Glutamatergic synaptic activities were isolated by adding 10 μM bicuculline; GABAergic synaptic transmission was isolated by applying 10 μM CNQX and 40 μM d-AP5 in ACSF. Fig. 9 shows the effects of adenophostin and Ca2+-CaM on the activities of GABAergic and glutamatergic synapses. Ca2+-CaM preferentially enhances EPSCs (dark-filled symbols, 346±32%, n=10) and IPSCs (open symbols, 178±8%, n=6) in Fig. 9A. Similarly, adenophostin increases EPSCs (dark-filled symbols, 339±27%, n=11) more than IPSCs (open symbols, 192±10%, n=12) in Fig. 9B. These results imply that Ca2+-CaM signaling pathways strengthen the excitatory activities of pyramidal neurons and protect them from over-excitation.

DISCUSSION

Our results show that raising postsynaptic Ca2+ or CaM strengthens GABAergic synaptic transmission, indicating that the activation of Ca2+-CaM signaling cascades during neuronal activity modulates the function of synaptic
GABA Rs besides enhancing glutamatergic synaptic transmission (Wang and Kelly, 1995). A preferential effect of Ca\(^{2+}\)-CaM signals on glutamatergic synapses grants a condition that they strengthen the excitatory activities of pyramidal neurons and protect them from the over-excitation. In addition, our studies can fit other physiological conditions. For example, as we raised postsynaptic Ca\(^{2+}\) by triggering IP\(_3\)Rs that are the targets of metabotropic glutamate receptor (mGluR)-G proteins (Schoepf and Conn, 1993), our experiments simulate the situation when mGluRs are activated. IP\(_3\)Rs are involved in Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Carafoli, 1987; Ehrlich, 1995), our results is also suitable for interpreting other conditions of intracellular Ca\(^{2+}\) increase. We did not apply depolarization pulses to evoke Ca\(^{2+}\) influx because the use of this protocol has caused inconsistent results (see below).

We observed that the postsynaptic infusions of CaM-KII autoinhibitory peptide (Fig. 5) attenuated IP\(_3\)R agonist-induced GABA synaptic potentiation. Therefore, postsynaptic Ca\(^{2+}\)-CaM signaling pathways including Ca\(^{2+}\), CaM and CaM-KII strengthen GABAergic synaptic transmission on CA1 pyramidal neurons in rat hippocampus. This indication is analogous to the conclusion that CaM-KII plays a role in rebound GABA potentiation on cerebellar Purkinje cells (Kano et al., 1996). As functional modulation of GABAergic synapses was examined in the neurons of brain slices, these results present a notion that Ca\(^{2+}\)-CaM signaling pathways modulate the function of synaptic GABA\(_A\)Rs. It extends the knowledge from studying the modulation of GABA\(_A\)Rs at the dissociated neurons or recombinant GABA\(_A\)Rs in cell lines (Chen and Wong, 1995; Jones and Westbrook, 1997; Krishek et al., 1994; Moss et al., 1992, 1995; Stelzer and Shi, 1994; Wang et al., 1995).

Results from studying the modulation of GABA\(_A\)R by intracellular signals were inconsistent First, PKA and PKC phosphorylated recombinant GABA\(_A\)R, decreasing (Krishek et al., 1994; Moss et al., 1992) or increasing GABA currents (Angelotti et al., 1993; Lin et al., 1994). Second, PKC and PKA differentially modulated spontaneous GABA synaptic currents in hippocampal CA1 and dentate areas (Poisebeau et al., 1999). Third, intracellular Ca\(^{2+}\) in frog sensory neurons (Inoue et al., 1986) and pyramidal neurons (Pitler and Alger, 1992) could suppress GABA currents. Increasing intracellular Ca\(^{2+}\) enhanced GABA currents transiently in cortical neurons (Tapia et al., 1997) or induced long-term potentiation of GABA synaptic transmission in hippocampal neurons (Caillard et al., 1999). Fourth, blocking Ca\(^{2+}\)-ATPase or activating ryanodine receptors to change intracellular Ca\(^{2+}\) led to variable effects on the amplitude and decay of GABA currents (De Koninck and Mody, 1996; Savi and Sciancalepore, 1998).

These inconsistencies may be caused by the followings. Different experimental conditions (e.g. preparations, animal age, GABA current isolation, receptor location and temperature) were used. Different combinations of GABA\(_A\)R subunits display variations in gating, conductance and functional modulation. The functional status of GABA\(_A\)R may be affected by local environments (e.g. the machinery of anchoring receptor subunits) since synaptic and extrasynaptic GABA\(_A\)Rs are different in kinetics (Annette et al., 1999; Banks and Pearce, 2000; Brickley et al., 1999; Chen et al., 1999). Therefore, it is necessary to examine the functional modulation of synaptic GABA\(_A\)Rs in different areas of the CNS to reveal their role in coding specific neuronal signals.

We observed that the inhibition of tubulin polymerization-attenuated Ca\(^{2+}\)-induced GABA synaptic potentiation, indicating that Ca\(^{2+}\) signaling pathways increase or stabilize polymerized microtubules. Microtubules under postsynaptic membrane constitute the deck for the anchoring of GABA\(_A\)R subunits such that an increase in
their size facilitates receptor anchoring. As inhibiting actin polymerization also attenuates GABA synaptic potentiation, receptor recycling by actin filament-dependent vesicle transport (Hirokawa, 1991) may be involved. Such increases in the number of postsynaptic GABA<sub>R</sub>s enhance synaptic responses. Our model emphasizes the role of cytoskeleton modulated by Ca<sup>2+</sup>-CaM signaling cascades in changing GABA synaptic function. It enriches the hypothesis that GABA<sub>R</sub>s are anchored on cytoskeleton by receptor-associated proteins (Essrich et al., 1998; Fallon, 2000; Kneussel and Betz, 2000; Wang et al., 1999). The cytoskeletal proteins are phosphorylated by protein kinases (Johnson et al., 1998; Langosch et al., 1992). The phosphorylation may promote their polymerization to facilitate the recruitment of GABA<sub>R</sub>s in the postsynaptic membrane and strengthen synaptic function. The interaction between GABA<sub>R</sub>s and cytoskeletal proteins may play an important role in synaptic plasticity, which has been proposed at glutamatergic synapses (Rossum and Hanish, 1999). To the reason why basal GABAergic responses are not significantly affected by microtubule depolymerization, we hypothesize that the reagents of the depolymerization mainly affect those microtubules and actin filaments phosphorylated by Ca<sup>2+</sup>-CaM signaling pathways, which will be examined in our future study.

Our results indicate the involvement of cytoskeleton in GABA synaptic potentiation induced by Ca<sup>2+</sup>-CaM signals. It remains to be studied how the recruitment machinery raises the number of GABA<sub>R</sub>s in postsynaptic membrane. Does the phosphorylation of gephyrin and GABAR-associated proteins enhance GABA synaptic transmission? This will be examined by a combination of functional study and phosphorylation assay. In addition, once reagents that affect these proteins are available, the further functional assay will be conducted.

In addition to modulating the machinery for receptor anchoring/recycling, Ca<sup>2+</sup>-CaM signaling cascades may phosphorylate GABA<sub>R</sub> channels and modulate their intrinsic properties (e.g. gating and conductance) to enhance the function of GABAergic synapse. It is not simple to judge which modulation is more important in natural synapses. It is predictable that Ca<sup>2+</sup>-CaM signaling cascades modulate properties of recombinant and extrasynaptic GABA<sub>R</sub>s. After receptors are inserted into postsynaptic membrane and cytoskeleton deck, certain exceptions should be considered. For example, do signaling molecules easily access to GABA<sub>R</sub>s imbedding in postsynaptic structures? Whether the modulation model of GABA<sub>R</sub>s switches to the alternation of receptor assembly from intrinsic properties needs to be examined.

In general, we describe the modulation of synaptic GABA<sub>R</sub>s at postsynaptic neurons, a straightforward interpretation by postsynaptic manipulations. Are retrograde messenger-initiated presynaptic mechanisms involved in the enhancement of GABA synaptic function (Caillard et al., 1999; Pitter and Alger, 1994)? It would appear unlikely that presynaptic mechanisms are involved because inhibiting the polymerization of postsynaptic tubulin and actin attenuated synaptic potentiation (Figs. 6–8).

Postsynaptic Ca<sup>2+</sup>-CaM signals enhance glutamatergic and GABAergic synaptic currents (Wang and Kelly, 1995; and present study). This is similar to observations that tetanus induced Ca<sup>2+</sup>-dependent increases in EPSCs and IPSCs during somatic recordings. However, the tetanus decreased IPSCs recorded at dendrite when EPSCs were intact (Stelzer et al., 1994; Wang and Stelzer, 1996). GABAergic synapses are mainly located at proximal dendrites and cell body (Freund and Buzsaki, 1996). As tetanus-enhanced EPSPs at dendrites counterbalance IPSCs propagated from soma and Ca<sup>2+</sup>-CaM signals preferentially increase EPSCs (data not shown), a decrease in IPSCs was observed during dendritic recordings. What is physiological significance of the modulation of Ca<sup>2+</sup>-CaM signals to these two groups of synapses? Pyramidal neurons synaptically connect with glutamatergic and GABAergic axons. A potentiation of glutamatergic synapses is essential to initiate action potentials for brain functions (e.g. learning/memory, cognition and behavior), whereas the potentiation at GABAergic synapses prevents hyperactivity in pyramidal neurons.

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