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Direct Presynaptic Regulation of GABA/Glycine Release by Kainate Receptors in the Dorsal Horn: An Ionotropic Mechanism

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Summary

In the spinal cord dorsal horn, excitatory sensory fibers terminate adjacent to interneuron terminals. Here, we show that kainate (KA) receptor activation triggered action potential-independent release of GABA and glycine from dorsal horn interneurons. This release was transient, because KA receptors desensitized, and it required Na^+ entry and Ca^{2+} channel activation. KA modulated evoked inhibitory transmission in a dose-dependent, biphasic manner, with suppression being more prominent. In recordings from isolated neuron pairs, this suppression required $GABA_B$ receptor activation, suggesting that KA-triggered GABA release activated presynaptic $GABA_B$ autoreceptors. Finally, glutamate released from sensory fibers caused a KA and $GABA_B$ receptor-dependent suppression of inhibitory transmission in spinal slices. Thus, we show how presynaptic KA receptors are linked to changes in GABA/glycine release and highlight a novel role for these receptors in regulating sensory transmission.

Introduction

Three types of ionotropic glutamate receptors (iGluRs) mediate fast excitatory transmission at synapses throughout the central nervous system. Of these, AMPA and NMDA receptors are best characterized for their roles in postsynaptic depolarization and activation of Ca^{2+}-dependent signaling cascades, respectively. More recent efforts to decipher the roles of kainate (KA) receptors have been facilitated by the introduction of selective pharmacology (Chittajallu et al., 1999) and KA receptor subunit knockout mice (Mulle et al., 1998, 2000). In addition to mediating a component of postsynaptic current at some excitatory synapses (reviewed by Frerking and Nicoll, 2000), KA receptors exert presynaptic control over transmitter release (Chittajallu et al., 1996). Interestingly, presynaptic KA receptors are not restricted to excitatory synapses, but have also been revealed at inhibitory synapses (Clarke et al., 1997; Rodriguez-Moreno et al., 1997). In both cases, KA was initially found to suppress synaptic transmission; however, subsequent work has demonstrated that KA can also act to facilitate transmitter release (Liu et al., 1999; Cossart et al., 2001; Contractor et al., 2001; Schmitz et al., 2001). There has been considerable effort to explain these presynaptic actions of KA (Rodriguez-Moreno and Lerma, 1998; Frerking et al., 1999; Rodriguez-Moreno et al., 2000; Kamiya and Ozawa, 2000; Schmitz et al., 2000; Frerking et al., 2001), but in most cases, the mechanisms remain unresolved.

In the spinal cord, KA receptors play an important role in sensory transmission. They are located on the postsynaptic membrane of dorsal horn neurons that respond to high-threshold primary afferent sensory fiber stimulation (Li et al., 1999), and they are present presynaptically on the sensory fibers themselves (Davies et al., 1979; Huettner, 1990), where they can regulate glutamate release (Kerchner et al., 2001). That spinal inhibitory neurons may also possess presynaptic glutamate receptors was suggested by experiments showing that glutamate, when applied focally in the neuritic field surrounding a cultured spinal neuron, sometimes triggered a barrage of inhibitory postsynaptic potentials (Ransom et al., 1977). Because glutamate-containing sensory fiber terminals come into close proximity with the GABA- and glycine-containing boutons of local interneurons at synaptic glomeruli (Ribeiro-da-Silva and Coimbra, 1982; Todd, 1996), and because inhibitory tone is so critical to the proper regulation of sensory transmission (Malcangio and Bowery, 1996), the possibility that the inhibitory terminals may respond directly to glutamate release is especially compelling. In the present study, we show that dorsal horn inhibitory neurons express presynaptic KA receptors that stimulate GABA and glycine release. In addition, we detail the mechanisms by which KA receptor activation can result in up- or downregulation of inhibitory transmission. Finally, we demonstrate that synaptic glutamate released from sensory fibers is sufficient to set these pathways in motion, culminating in a suppression of inhibitory transmission in the dorsal horn.

Results and Discussion

Activation of KA Receptors, but Not Other iGluRs, Enhances mIPSC Frequency

To test for the presence of presynaptic KA receptors at spinal dorsal horn inhibitory synapses, we examined the effect of KA receptor activation on spontaneous miniature inhibitory postsynaptic currents (mIPSCs). Recordings were obtained from dorsal horn neurons in dissociated culture to allow for more rapid and complete exchange of the extracellular medium than is possible during acute slice recordings. Speed of exchange is an important consideration because KA receptors exhibit prominent desensitization (Huettner, 1990). Recordings were made in the presence of 500 nM tetrodotoxin (TTX), which blocked all voltage-gated Na^+ current ($n = 5$; data not shown), as well as SYM2206 (100 μM) (Wilding and Huettner, 2001) and AP-5 (25 μM) to block AMPA and NMDA receptors, respectively. Neurons were voltage-clamped at 0 mV and perfused intracellularly with a

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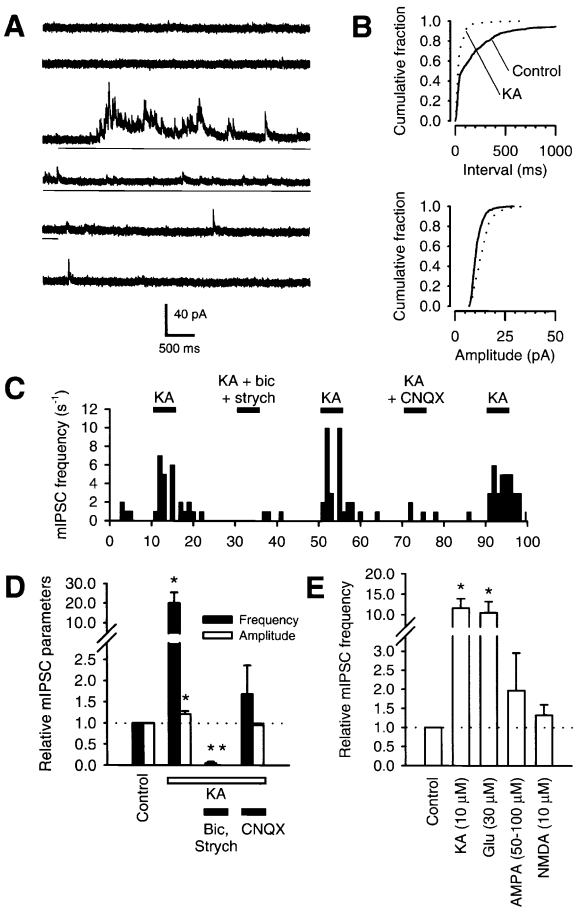

Figure 1. KA and Glutamate Trigger a Transient Increase in mIPSC Frequency

(A) A continuous recording of current is shown for a neuron before and during exposure to KA (10 μM). An underscore indicates the time of KA application. The burst of mIPSCs evident near the onset of KA application included overlapping, compound events and subsided after a few seconds (also see Figure 2). The neuron was held at 0 mV in the presence of TTX, SYM2206, and AP-5.

(B) Cumulative histograms of mIPSC interevent interval and amplitude in control conditions (solid line) and during the first four seconds of KA exposure (dotted line) are shown for a representative recording.

(C) mIPSC frequency (bin = 1 s) plotted over time for a representative experiment illustrates that KA triggered a burst of mIPSCs, that bicuculline (10 μM) and strychnine (1 μM) eliminated the events, and that the effect of KA was abolished upon coapplication of CNQX (100 μM).

(D) Pooled results are illustrated of experiments performed as in (C) (n = 10), with mIPSC frequency or amplitude normalized to the control value. (*) represents a significant difference from control.

(E) Pooled results compare the effects of KA, glutamate (with SYM2206, AP-5, and mGluR antagonists; see Results and Discussion), AMPA (with SYM2081), and NMDA on mIPSC frequency (n = 5–9 cells per condition). (*) represents a significant difference from control.

low-Cl^- pipette solution. In these conditions, mIPSCs appeared as outward currents at a background frequency of 3.8 ± 0.4 s^{-1} (n = 85). Upon application of KA (10 μM), this frequency increased to 850% ± 100% of the control value (n = 85; $p < 0.001$) during the first four seconds of the exposure (Figure 1A; and see below), suggesting that KA acted at a presynaptic locus. A small but significant increase in mIPSC amplitude was also observed during KA exposure (Figures 1B and 1D), but because of the prevalence of overlapping mIPSCs triggered by KA (Figure 1A), it is likely that this small change in amplitude was biased by coordinated, multiquantal release events. Application of bicuculline (10 μM) plus strychnine (1 μM) eliminated mIPSCs, confirming that the postsynaptic events resulted from release of GABA and/or glycine (Figures 1C and 1D; and see below). In addition, coapplication of KA with the nonselective AMPA/KA receptor antagonist CNQX (100 μM) resulted in no significant change in mIPSC frequency compared to control (Figures 1C and 1D).

KA receptors appeared to play a unique role among iGluRs in stimulating the release of inhibitory transmitters. AMPA receptor activation by AMPA (50–100 μM) (Figure 1E) or KA (10 μM) (Figure 3; and see below) had no significant effect on mIPSC frequency when applied in the presence of TTX, AP-5, and SYM2081 (1–10 μM), a selective KA receptor agonist that induces potent, complete receptor desensitization (Jones et al., 1997). Similarly, application of NMDA (10 μM) in the presence of TTX and SYM2206 also had no effect (Figure 1E). Although presynaptic AMPA (Bureau and Mulle, 1998; Satake et al., 2000) and NMDA receptors (Glitsch and Marty, 1999) have been reported to modulate inhibitory transmission in other systems, these receptors do not appear to play such a role in the dorsal horn.

KA and Glutamate, but Not ATPA, Reliably Increase mIPSC Frequency

KA was consistent in its ability to trigger GABA/glycine release. We tested whether this effect could be mimicked by the endogenous agonist glutamate. We found that 30 μM glutamate was sufficient to enhance mIPSC frequency to a similar extent as 10 μM KA (Figure 1E) when applied in the presence of TTX, SYM2206, AP-5, and metabotropic glutamate receptor (mGluR) antagonists, including the $mGluR_{1a}$-selective antagonist LY367385 (50 μM), the $mGluR_5$-selective antagonist MPEP (2 μM), and the group II/III mGluR antagonist CPPG (500 μM). Because we did not observe a subpopulation of cells in which KA or glutamate did not affect mIPSC frequency, and because dorsal horn neuronal cultures contain excitatory as well as inhibitory neurons (Kerchner et al., 2001), we conclude that these agonists stimulated inhibitory terminals that impinged on both types of postsynaptic cells (see Cossart et al., 2001).

We showed previously (Kerchner et al., 2001; Wilding and Huettner, 2001) that KA receptors in dorsal horn neurons were largely insensitive to the GluR5 subunit-preferring, KA receptor-selective agonist ATPA (2 μM) (Clarke et al., 1997). Consistent with that finding and with the low level of GluR5 mRNA expression observed in these cells (Tölle et al., 1993), we found that ATPA had varying effects on mIPSC frequency, inducing only minor changes in mIPSC frequency in six of ten recordings (95% ± 14% of the control value; p = 0.39) and a significant increase in the other four (860% ± 400%; p = 0.029). Because each recording sampled the effect of an agonist on axon terminals from many different presynaptic cells, we cannot be certain what

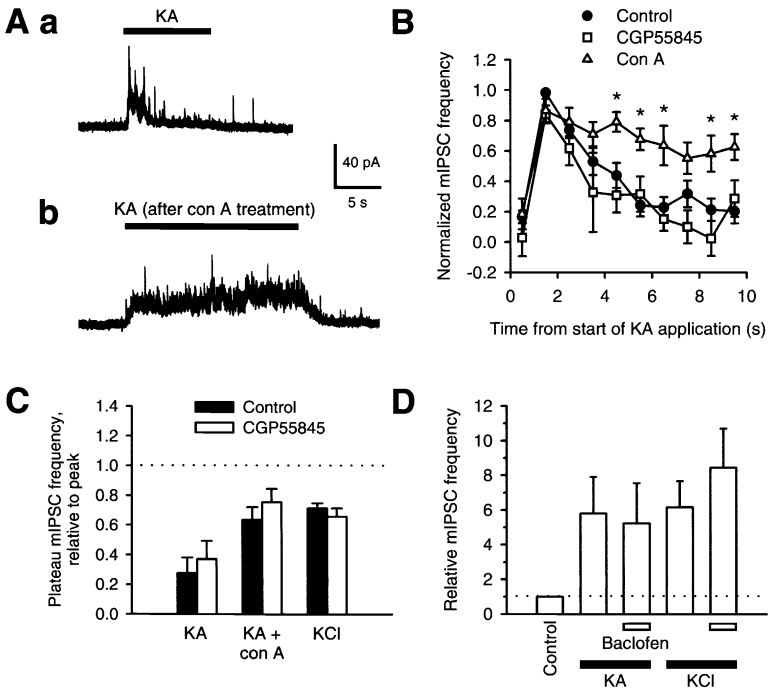

Figure 2. Receptor Desensitization Accounts for the Time Course of KA Action

(A) In control conditions, KA (10 μM) induced a burst of mIPSCs that subsided before the end of a 10 s application, as illustrated for a representative neuron (a). After a 25 min incubation with con A, another neuron in the same culture dish exhibited a burst of mIPSCs that persisted throughout a 20 s KA application (b).

(B) Variations in mIPSC frequency over time during a 10 s KA exposure are compared in control conditions (n = 7), in the presence of CGP55845 (10 μM) (n = 7; same cells as control), or after a 25 min exposure to con A (n = 6). For each experiment, mIPSC frequency was quantified at 1 s intervals and normalized on a scale from zero (background frequency in the absence of KA) to one (peak frequency attained during the 10 s KA exposure). (*) represents a significant difference from control.

(C) The ratio of mIPSC frequency during the last three seconds of a 10 s agonist exposure to the frequency during the first three seconds is plotted in the presence or absence of CGP55845 (10 μM) in experiments in which KA alone (n = 6), KA in con A-treated cultures (n = 13), or 20 mM KCl (n = 5) was used as the stimulus for vesicle release. CGP55845 had no significant effect in any condition.

(D) KA (10 μM; n = 4) or KCl (20 mM; n = 6) enhanced mIPSC frequency to a similar degree in the presence or absence of baclofen (5–10 μM).

fraction of the inhibitory interneurons was sensitive to ATPA. Our observations may reflect heterogeneity in the prevalence of the GluR5 subunit among dorsal horn neurons or nonselective actions on heteromeric receptors lacking GluR5 (Paternain et al., 2000).

KA Receptor Desensitization Dictates the Time Course of KA-Triggered Transmitter Release

KA typically triggered a burst of mIPSCs that subsided during a 10 s exposure (Figures 1A and 2A). This decline in mIPSC frequency over time might result from KA receptor desensitization, metabotropic inhibition of release through activation of presynaptic $GABA_B$ autoreceptors, or depletion of the readily releasable pool of synaptic vesicles (Rosenmund and Stevens, 1996). In cultures exposed to the lectin conconavalin A (con A), which selectively removes desensitization of KA receptors (Huettner 1990), mIPSC frequency remained high throughout a 20 s KA application (Figures 2A and 2B), suggesting that KA receptor desensitization played a large role in the waning of the KA-triggered mIPSC burst in control conditions.

By contrast, $GABA_B$ receptors did not contribute to the decay of KA-triggered mIPSC bursts. mIPSC frequency varied similarly over time whether KA was applied in control conditions or in the continual presence of the $GABA_B$ receptor antagonist CGP55845 (10 μM) (Deisz, 1999) (Figure 2B). Even in cultures treated with con A, CGP55845 had no effect on the time course of KA-triggered mIPSC bursts, a result mimicked when elevated extracellular [KCl] was used instead of KA as the trigger for vesicle release (Figure 2C). Further arguing against a role for $GABA_B$ receptors, KA and KCl each enhanced mIPSC frequency to a similar degree in the presence or absence of the $GABA_B$ receptor agonist baclofen (5–10 μM) (Figure 2D). Whereas it remains possible that GABA released consequent to KA receptor stimulation may activate $GABA_B$ receptors (see below), such activation clearly did not underlie the waning of mIPSC frequency during KA exposure.

The importance of KA receptor desensitization to the time course of a KA-induced increase in mIPSC frequency may explain why our findings differ from some previous reports showing that KA caused no change or even a decrease in the frequency of mIPSCs (Rodriguez-Moreno et al., 1997; Rodriguez-Moreno and Lerma, 1998; Cossart et al., 1998; Frerking et al., 1998, 1999; Bureau et al., 1999; Ali et al., 2001) or mEPSCs (Castillo et al., 1997; Frerking et al., 2001) in hippocampal slices. Even in studies reporting an increased mIPSC or mEPSC frequency (Liu et al., 1999; Mulle et al., 2000; Contractor et al., 2000; Cossart et al., 2001), the effects were significantly weaker (2- to 3-fold maximal enhancement) than the robust increases (more than 8-fold, on average, which itself is an underestimation of the full peak effect; see Experimental Procedures) observed with a similar KA dose in this study. Whereas there may be fundamental differences between slice and culture preparations, or presynaptic KA receptors may operate differently in the dorsal horn than in other brain regions, the notion that receptor desensitization may limit the effect of KA was not considered in previous studies. Our results do

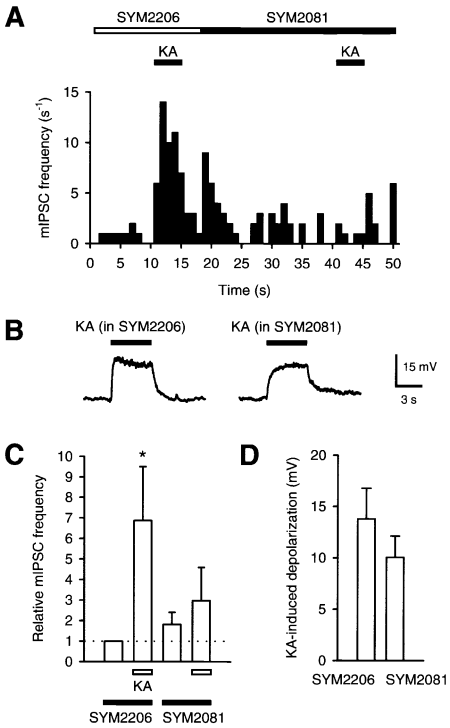

Figure 3. KA Enhances mIPSC Frequency by Activating Receptors Near Presynaptic Terminals

(A and C) As illustrated in a representative experiment (A) and pooled data (C) (n = 4), KA (10 μM) evoked a burst of mIPSCs in the presence of the AMPA receptor antagonist SYM2206 (100 μM) but not SYM2081 (10 μM), which desensitizes KA receptors. (*) represents a significant difference from SYM2206 alone.

(B and D) KA induced a similar degree of somatic depolarization whether applied with SYM2206 or SYM2081 (n = 11; p = 0.10). Whole-cell current clamp experiments were performed in the presence of bicuculline (10 μM), strychnine (1 μM), and TTX (0.5 μM), using a K^+-containing pipette solution. Resting membrane potential was −70 mV.

not make it clear how KA could decrease mIPSC frequency in some studies, but perhaps after many seconds or even minutes of continuous agonist exposure, either vesicle depletion or negative feedback pathways could limit the availability of releasable vesicles.

Somatodendritic Receptors Do Not Contribute to KA Action

Although an effect of KA to increase TTX-insensitive release of inhibitory neurotransmitters is consistent with the presence of KA receptors on axon terminals, it is conceivable that activation of receptors on presynaptic cell bodies or dendrites could induce sufficient passive depolarization of axons to trigger vesicle fusion. To test this possibility, we applied KA (10 μM) in the continuous presence of TTX, AP-5, and either SYM2206 (100 μM), to block AMPA receptors, or SYM2081 (10 μM), to desensitize KA receptors. In the former condition, KA receptors should be selectively activated, whereas in the presence of SYM2081, KA should induce a steady-state current through AMPA receptors (Wilding and Huettner, 1997) without activating KA receptors. KA caused an increase in mIPSC frequency in the presence of SYM2206 but not SYM2081 (Figures 3A and 3C), further supporting a specific role for KA receptors vis-à-vis AMPA receptors in regulating inhibitory transmitter release (see above). We also noted that SYM2081 by itself induced some transient enhancement of mIPSC frequency (Figure 3A), consistent with its action as a selective KA receptor agonist.

By contrast, the magnitude of somatic depolarization induced by KA was small and similar in both conditions (Figures 3B and 3D); importantly, within the region of membrane accessible to current clamp, no active depolarization (for instance, mediated by voltage-gated Ca^{2+} channels) was ever observed during KA application. Because the ability of KA to trigger bursts of mIPSCs did not correlate with its ability to induce somatic depolarization, we conclude that KA receptors responsible for influencing TTX-insensitive GABA/glycine release must reside somewhere electrically distant from the cell body but close to sites of vesicle release — likely on presynaptic terminals.

KA Triggers Release of Both GABA and Glycine

Glycine and GABA are probably copackaged in and coreleased from spinal interneurons (Burger et al., 1991; Christensen and Fonnum, 1991; Jonas et al., 1998). Bicuculline and strychnine each blocked a portion of IPSCs evoked by extracellular stimulation (eIPSCs), and a combination of the two antagonists blocked eIPSCs completely (Figures 4A and 4B), suggesting that both GABA and glycine were released from individual cultured dorsal horn neurons and contributed to postsynaptic currents. We predicted that GABAergic and glycinergic mIPSCs would be regulated similarly by presynaptic KA receptor activation. Indeed, in the presence of strychnine (500 nM), KA enhanced mIPSC frequency in all nine neurons tested (Figure 4C). Similarly, in the presence of bicuculline (5 μM), KA increased mIPSC frequency in six of eight experiments (Figure 4C); in two, no change in frequency was noted. Of side interest, whereas bicuculline and strychnine each depressed mIPSC frequency when applied alone, neither antagonist produced any significant effect on mIPSC amplitude (Figure 4C), suggesting that individual mIPSCs were typically mediated by GABA or glycine, but not both. Such a phenomenon has been attributed to differential clustering of the two receptor types at postsynaptic sites, not to differential release of GABA and glycine (Chéry and De Koninck, 1999).

KA Action Occurs by an Ionic Mechanism

We sought to determine how presynaptic KA receptor activation is linked to GABA/glycine release. Among various possible models, three include the following. First, Na^+ entry through KA receptors may depolarize nerve terminals, causing voltage-gated Ca^{2+} channel activation, Ca^{2+} entry, and vesicle fusion. Second, if axon terminals contain Ca^{2+}-permeable KA receptors (Kohler et al., 1993), Ca^{2+} entry and transmitter release could be achieved in the absence of membrane depolarization or Ca^{2+} channel activity, as has been proposed at hippocampal interneuron-interneuron synapses (Cossart et al., 2001). Third, KA receptors may be linked metabotropically to the transmitter release machinery (Rodriguez-Moreno and Lerma, 1998; Frerking et al., 2001).

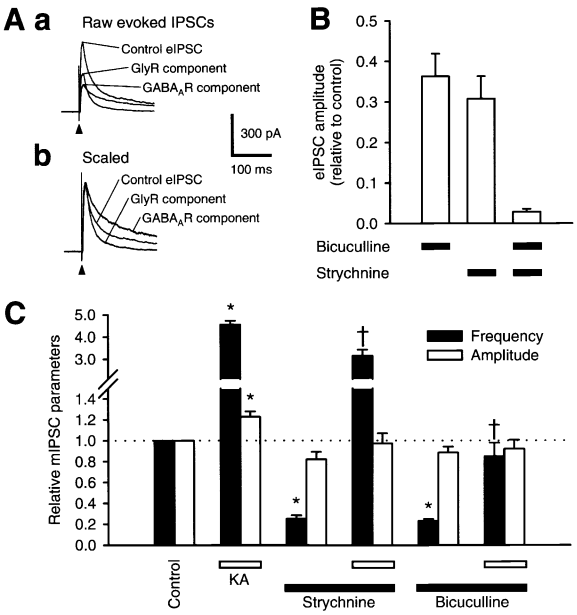

Figure 4. KA Triggers Release of Both GABA and Glycine

(A) eIPSCs, illustrated by averaged traces from a representative experiment (a), contained separate components mediated by glycine receptors (GlyR) (insensitive to 5 μM bicuculline) and by GABA$_A$ receptors (GABA$_A$R) (insensitive to 500 nM strychnine). The same traces, when normalized to their peak values (b), show distinct decay kinetics (Yoshimura and Nishi, 1995). Arrowheads indicate the time of stimulation.

(B) Pooled data for experiments performed as in (A) (n = 12) show the average proportion of an eIPSC blocked by bicuculline (5–10 μM), strychnine (0.5–1 μM), or both.

(C) In the presence of TTX, KA (10 μM) enhanced mIPSC frequency in the presence of either strychnine (500 nM) or bicuculline (5 μM) (n = 8–9 neurons per condition). (*) represents a significant difference from control; (†) represents a significant difference from strychnine or bicuculline alone.

To distinguish between these models, we first examined the ability of KA to enhance mIPSC frequency when extracellular Na^+ was replaced entirely by *N*-methyl-D-glucamine. Although background mIPSC frequency and amplitude were similar in this condition compared to control, KA no longer enhanced mIPSC frequency (Figures 5A and 5B). This observation suggests that Na^+ entry through activated KA receptors was essential, and that even if those receptors were Ca^{2+}-permeable, KA receptor-mediated Ca^{2+} entry was not sufficient to trigger transmitter release. Confirming that depolarization could indeed trigger GABA/glycine release and that removal of extracellular Na^+ per se did not interfere with this process, elevating extracellular [KCl] to 20 mM in Na^+-free medium induced a large, sustained enhancement of mIPSC frequency (Figure 5C). Although we do not formally exclude a more complex coupling between KA receptors and transmitter release (Rodriguez-Moreno and Lerma, 1998; Frerking et al., 2001), our findings indicate a requirement for an ionic component in KA action (Contractor et al., 2000; Cossart et al., 2001), most likely reflecting the ability of Na^+ entry to occur directly through ionotropic KA receptors (Huettner, 1990).

We next tested the Ca^{2+} dependence of KA-induced GABA/glycine release. The standard extracellular solution contained 2 mM Ca^{2+} and 2 mM Mg^{2+} (see Experimental Procedures). Lowering this Ca^{2+} to Mg^{2+} ratio from 2:2 to 1.5:2.5 resulted in a significant reduction in the ability of 10 μM KA to evoke mIPSC bursts while leaving background mIPSC frequency unaffected (Figures 5D and 5E). These data suggest that KA-triggered GABA/glycine release, but not spontaneous quantal release, was sensitive to changes in extracellular [Ca^{2+}]. To identify a role for voltage-gated Ca^{2+} channels in mediating the effects of KA, we first applied 50 μM $CdCl_2$, a concentration sufficient to block voltage-gated Ca^{2+} currents in cultured dorsal horn neurons (n = 7; data not shown) with little to no effect on the magnitude of KA-induced currents (Huettner et al., 1998). In this condition, KA was unable to trigger an increase in mIPSC frequency (Figure 5F). Whereas the L-type Ca^{2+} channel antagonist nimodipine had no effect on KA-induced GABA/glycine release, the selective N-type antagonist ω-conotoxin GVIA and the P/Q-type antagonist ω-conotoxin MVIIC (which also weakly blocks N-type channels) each partially blocked the phenomenon (Figure 5F). These data are consistent with the prevalence of N- and P/Q-type voltage-gated Ca^{2+} channels, but not L-type channels, on presynaptic axon terminals in the spinal cord (Westenbroek et al. 1998; see also Tsien et al., 1995). Because a combination of ω-conotoxin GVIA and ω-conotoxin MVIIC did not completely prevent KA action (n = 5; data not shown), it remains possible that other Ca^{2+} channel subtypes such as R-type channels may play some role.

Taken together, these data support a model in which GABA/glycine release is triggered by Na^+ entry through activated KA receptors and opening of presynaptic voltage-gated Ca^{2+} channels. Although we note that the magnitude of somatic depolarization induced by 10 μM KA was small (Figure 3)—too small, indeed, to cause detectable activation of voltage-gated Ca^{2+} channels in the current-clamped portion of the membrane—the magnitude of depolarization in axon terminals could be larger. At terminals, input resistance is greater than at the cell body, and KA receptors may be more densely expressed; such factors may underlie the ability of KA to cause sufficient terminal depolarization to activate presynaptic voltage-gated Ca^{2+} channels.

KA Can Modulate eIPSCs Biphasically

We next tested the effect of KA on action potential-evoked inhibitory transmission between dorsal horn neurons. Based on the ability of KA to enhance mIPSC frequency, we predicted that KA might facilitate eIPSCs. However, low doses of KA (50–200 nM) had no effect on eIPSCs, and higher doses (3–10 μM) caused only suppression (Figures 6B and 7). We considered that evoked inhibitory transmission may not be susceptible to facilitation by terminal depolarization in our standard conditions, so we raised divalent cation concentrations in the extracellular solution, a maneuver expected to reduce neuronal excitability, in part by shifting the voltage-dependence of Na^+ and Ca^{2+} channel gating (Hille, 1992). When Ca^{2+} and Mg^{2+} were each raised from 2 to 6 mM, a biphasic effect of KA on eIPSCs was revealed, with facilitation apparent at a low concentration (200

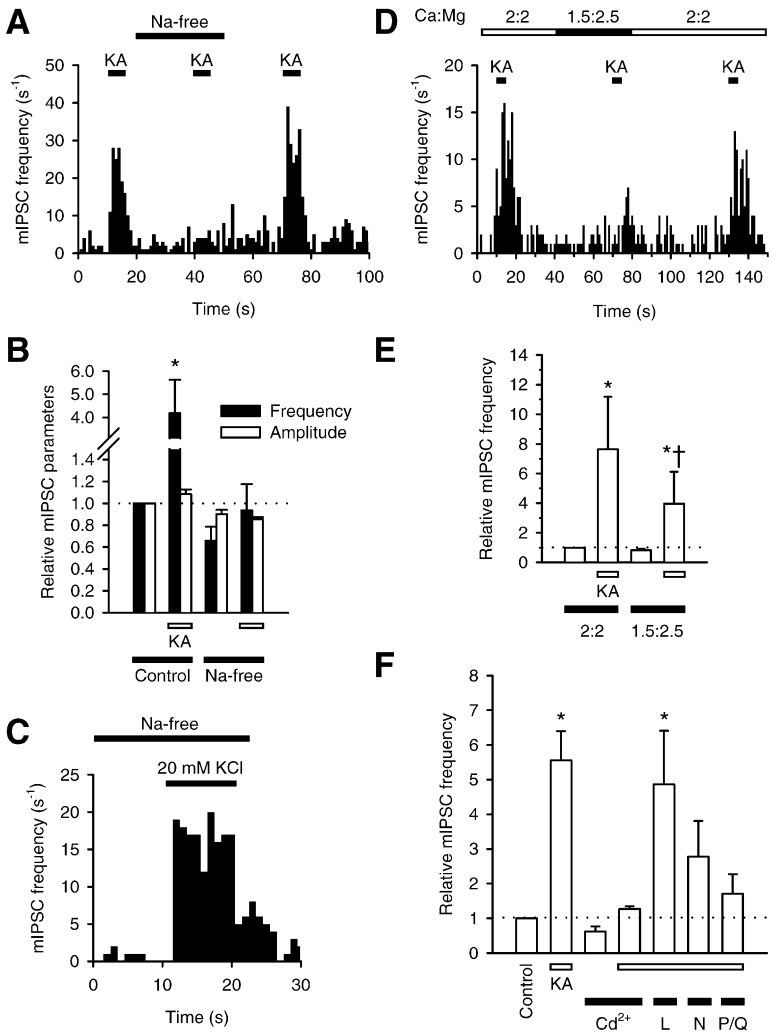

Figure 5. KA-Triggered GABA/Glycine Release Requires Membrane Depolarization and Voltage-Gated Ca^{2+} Channel Activation

(A and B) KA (10 μM) triggered a burst of mIPSCs in the presence, but not the absence, of extracellular Na^+, as illustrated by a representative experiment (A) and pooled data (B) (n = 8). (*) represents a significant difference from control (normal Na^+, no KA).

(C) Raising extracellular [K^+] in the absence of extracellular Na^+ induced a sustained increase in mIPSC frequency, as shown in a representative experiment.

(D and E). Lowering Ca^{2+}:Mg^{2+} from 2:2 to 1.5:2.5 reduced the ability of KA (10 μM) to trigger GABA/glycine release, as shown in a representative experiment (D) and by pooled data (E) (n = 7). (*) represents a significant difference from control (Ca^{2+}:Mg^{2+} = 2:2 alone); (†) represents a significant difference from the effect of KA in control conditions.

(F) Pooled data detail the effects of the nonselective Ca^{2+} channel antagonist Cd^{2+} (50 μM; n = 3), the L-type voltage-gated Ca^{2+} channel antagonist nimodipine (1 μM; n = 6), the selective N-type antagonist ω-conotoxin GVIA (0.5 μM; n = 5), and the potent P/Q-type and weak N-type antagonist ω-conotoxin MVIIC (0.5 μM; n = 4) on KA-triggered GABA/glycine release. (*) represents a significant difference from control.

nM) and suppression at a higher concentration (10 μM) (Figures 6A and 6B). This same elevation in divalent cation concentrations caused a small, statistically insignificant decrease in the ability of KA to enhance mIPSC frequency (mIPSC frequency triggered by 10 μM KA in elevated Ca^{2+} and Mg^{2+} was 80% ± 9% of the value in control conditions; n = 5; p = 0.24).

KA Suppresses Unitary IPSCs by $GABA_B$ Autoreceptor Activation

Whereas a facilitating effect of KA is consistent with its ability to excite nerve terminals, it is not clear how KA can suppress inhibitory transmission. It has been proposed that KA can affect synaptic transmission indirectly by inducing somatic depolarization and action potential firing, obscuring any effects on nerve terminals (Cossart et al., 1998; Frerking et al., 1998, 1999; Bureau et al., 1999; Chergui et al., 2000; Mulle et al., 2000). We avoided such problems by making simultaneous recordings from pairs of synaptically connected neurons isolated from other neurons in dissociated cultures either absolutely (by culturing neurons on glial microislands) or relatively (by plating neurons at a very low density and finding pairs of cells that did not appear to be contacted by neurites from any other cells). Both neurons in a pair were voltage-clamped to prevent KA-induced somatic depolarization, and unitary IPSCs (uIPSCs) were evoked using a paired-pulse protocol by delivering two brief (2 ms) depolarizing voltage steps at a 50 ms interval to the presynaptic cell, sufficient to trigger unclamped action potentials.

In these conditions, the inhibitory action of KA was preserved. A 10 s application of KA (3 μM) caused a reduction in the first uIPSC peak amplitude to 66% ± 9% of the control value (n = 7; p = 0.011). The amplitude of the second peak exhibited an insignificant change with KA (130% ± 30% of the control value; p = 0.36), but a significant increase occurred in the paired-pulse ratio (190% ± 30%; p = 0.04) (Figures 7A, 7C, and 7D). When both pre- and postsynaptic cells in an isolated pair were voltage-clamped, application of the same concentration of KA was able to trigger an increase in mIPSC frequency (Figure 7B). In some experiments, KA was delivered at a higher concentration (10 μM), and no difference was observed in the magnitudes of its effects (first peak amplitude, 77% ± 9% of control; paired-pulse ratio, 180% ± 20%; n = 5; see also Figure 6B). In other experiments, IPSCs were evoked between cell pairs in

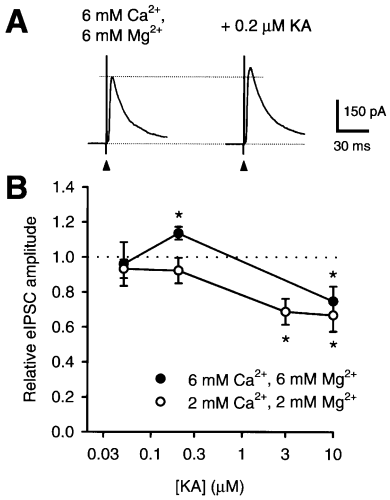

Figure 6. KA Can Exert Dose-Dependent, Biphasic Effects on Evoked Inhibitory Transmission

(A) Averaged eIPSCs are illustrated for a representative experiment before and during perfusion with 0.2 μM KA. Recordings were performed using elevated divalent cation concentrations. Arrowheads indicate time of stimulation.

(B) Pooled data illustrate a dose-dependent, biphasic effect of KA on eIPSC amplitude in the context of elevated Ca^{2+} and Mg^{2+}, but only a dose-dependent suppression at standard divalent cation concentrations (n = 3–7 cells per condition). (*) represents a significant difference from control (no KA).

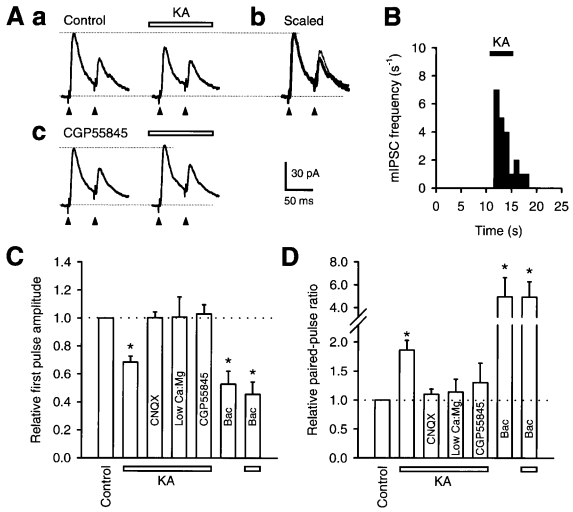

Figure 7. KA Suppresses Action Potential-Evoked Inhibitory Transmission by Causing Activation of $GABA_B$ Autoreceptors

(A) Averaged uIPSCs are illustrated for a representative experiment. Simultaneous recordings were made from two synaptically connected neurons in isolation, and the pre- and postsynaptic cells were voltage-clamped at −70 and 0 mV, respectively. Arrowheads indicate the times of stimulation. Brief (10 s) application of KA (3 μM) reduced the first pulse amplitude with little change in the second pulse (a). Traces in (a) from the control (heavy line) and KA (light line) conditions were peak-normalized and superimposed to illustrate an enhancement in the paired-pulse ratio, but no change in the kinetics of the responses (b). In the same neuron pair, application of KA in the presence of the $GABA_B$ receptor antagonist CGP55845 (10 μM) had no effect (c).

(B) While recording from a pair of isolated neurons, KA (3 μM) triggered a marked increase in spontaneous IPSC frequency. These spontaneous IPSCs were presumed to be identical to mIPSCs, as they had a small amplitude compared to uIPSCs in the same neuron pair, and spontaneous action potentials were prevented by holding both neurons in voltage-clamp. Similar results were obtained in two other recordings.

(C and D) Pooled data show the effects on first pulse amplitude (C) or the paired-pulse ratio (D) of KA applied alone (n = 21) or with CNQX (100 μM; n = 4), an extracellular solution containing 1.5 mM Ca^{2+} and 2.5 mM Mg^{2+} (n = 3; see Figure 5), or CGP55845 (10 μM; n = 6). Also illustrated are the effects of baclofen (5 μM) alone or with KA (n = 7). (*) represents a significant difference from control.

mass culture using extracellular stimulation; in these conditions, KA (3 μM) had similar effects as above (first peak amplitude, 65% ± 4% of control; paired-pulse ratio, 190% ± 30%; n = 9). Results from such experiments were thus pooled (Figures 7C and 7D). The effects of KA to reduce the first pulse amplitude and increase the paired-pulse ratio were blocked in the presence of CNQX (100 μM) (Figures 7C and 7D). KA likely acted presynaptically, as it increased the paired-pulse ratio and caused no significant change in input resistance in the postsynaptic cell (93% ± 13% of the control value; n = 17; p = 0.38) (see Frerking et al., 1999).

There are at least four possible models linking the abilities of KA to elicit action potential-independent GABA/glycine release and to reduce evoked inhibitory transmission. First, KA-induced GABA release may lead to activation of presynaptic $GABA_B$ autoreceptors, which are expressed on the terminals of dorsal horn inhibitory neurons (Malcangio and Bowery, 1996). Second, KA-induced depolarization of presynaptic terminals may cause voltage-gated Na^+ or Ca^{2+} channel inactivation. Third, by increasing membrane conductance in axon terminals, KA may cause sufficient electrical shunting to blunt the effect of an action potential. Fourth, KA-induced GABA/glycine release may lead to vesicle depletion.

In the presence of the $GABA_B$ receptor antagonist CGP55845 (10 μM), KA no longer affected evoked transmission (Figures 7A, 7C, and 7D). Also, baclofen (5 μM) could both mimic and occlude the effect of KA (Figures 7C and 7D); in the presence of baclofen, KA had little or no effect on the first peak amplitude (85% ± 6% compared to baclofen alone; n = 7; p = 0.10) or paired-pulse ratio (110% ± 10%; p = 0.95). Finally, KA was ineffective when the Ca^{2+} to Mg^{2+} ratio was lowered (Figures 7C and 7D). Neither $GABA_B$ receptor ligands nor alteration of the Ca^{2+} to Mg^{2+} ratio would be expected to change the ability of KA to cause voltage-gated channel inactivation or current shunting, arguing against these mechanisms. Reduced KA-triggered GABA/glycine release in the context of a lowered Ca^{2+} to Mg^{2+} ratio (see Figures 5D and 5E) may block the effect of KA on evoked transmission either by reducing $GABA_B$ receptor activation or preventing vesicle depletion; however, the latter explanation is unlikely, as vesicles were not depleted even after 20 s of KA exposure (Figure 2A). Taken together, these observations strongly implicate $GABA_B$ autoreceptor activation in KA-induced suppression of inhibitory transmission. Although we did not identify the downstream targets of $GABA_B$ autoreceptors, our data suggest that these targets include components that are essential for action potential-triggered vesicle release, but not for action potential-independent KA- or KCl-triggered release (see above).

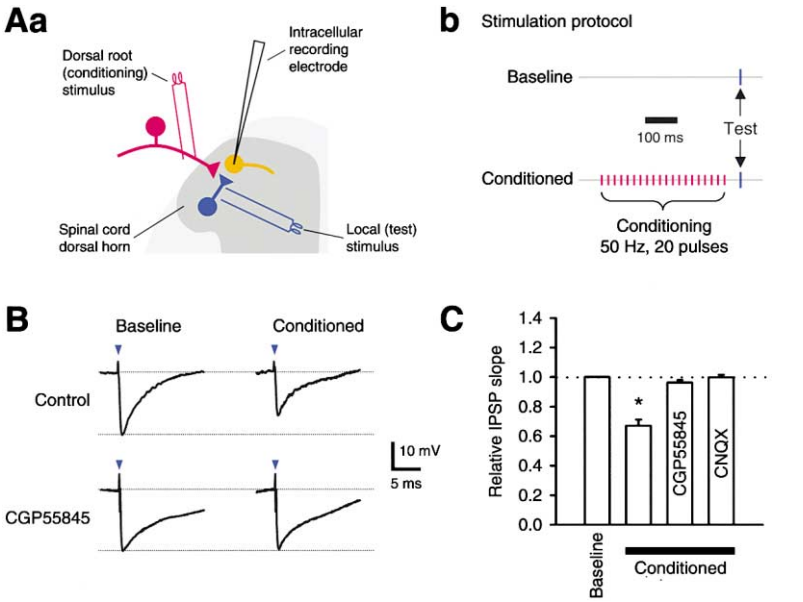

Figure 8. Synaptic Glutamate from Sensory Fibers Suppresses Inhibitory Transmission in the Dorsal Horn through KA and $GABA_B$ Receptor Activation

(A) A diagram illustrates the placement of recording and stimulating electrodes in a transverse spinal cord slice (a). Intracellular recordings were performed from dorsal horn neurons (dark yellow), and stimulating electrodes were placed in the dorsal root to activate sensory fibers (red) and in the dorsal horn to activate inhibitory interneurons (blue). The stimulation protocols are illustrated for baseline and conditioned responses (b).

(B) In representative recordings, averaged traces are shown of IPSPs evoked by local test stimulation before (Baseline) and immediately after a train of dorsal root stimulation (Conditioned). One cell was tested in the presence of SYM2206 and AP-5 alone (Control), and another was tested in the additional presence of CGP55845 (10 μM). Arrowheads indicate the time of test stimulation. During conditioning stimulation, no synaptic response was observed in the recorded neuron (data not shown).

(C) Pooled data illustrate the effects of conditioning on the slope of the rising phase of IPSPs evoked by test stimulation in control conditions (n = 6) or in the presence of CGP55845 (10 μM; n = 5) or CNQX (20 μM; n = 5). (*) represents a significant difference from baseline.

Because KA suppressed uIPSCs between pairs of isolated neurons, $GABA_B$ receptors were likely activated by GABA released consequent to presynaptic KA receptor activation at the same terminals. A similar $GABA_B$ autoreceptor-mediated negative feedback mechanism has been proposed to account for paired-pulse depression at inhibitory neocortical synapses at interpulse intervals of 150 ms or greater (paired-pulse depression at shorter intervals was $GABA_B$ receptor independent) (Deisz, 1999). In addition, $GABA_B$ autoreceptors have been shown to become activated at dorsal horn inhibitory synapses in conditions in which GABA release was insufficient to activate postsynaptic $GABA_A$ receptors (Chéry and De Koninck, 2000). Thus $GABA_B$ autoreceptors can respond quickly and sensitively to synaptic GABA. Importantly, our model differs from the one put forth by Frerking et al. (1999), who suggested that $GABA_B$ receptors on hippocampal nerve terminals respond to GABA released from "third party" neurons (i.e., not necessarily the presynaptic or postsynaptic cell being studied directly), which undergo depolarization and spontaneous action potential firing as a result of somatodendritic (not presynaptic) KA receptor activation. Although the autoreceptor pathway may be considered more compelling, as it does not invoke spillover of GABA from neighboring synapses, it is quite possible that in a preparation with many unclamped neurons, both pathways could occur in parallel. Finally, the importance of $GABA_B$ receptors for KA-induced inhibition of evoked transmission is consistent with the apparent role for a G protein-mediated pathway reported previously (Rodriguez-Moreno and Lerma, 1998).

Synaptic Glutamate Triggers a KA and $GABA_B$ Receptor-Mediated Suppression of Spinal Inhibitory Transmission

We next examined whether these effects of KA receptor activation could be reproduced in a more intact preparation using synaptic glutamate, instead of exogenously-applied agonists, as the trigger. Intracellular recordings were achieved from superficial dorsal horn neurons in spinal cord slices from adult rats, and two stimulating electrodes were introduced: one locally in the dorsal horn and one in the attached dorsal root (Figure 8A). In the presence of SYM2206 (100 μM) and AP-5 (50–100 μM), local stimulation produced a hyperpolarizing inhibitory postsynaptic potential (IPSP) that was sensitive to picrotoxin (20 μM) and strychnine (1 μM) (n = 4; data not shown), indicating that this stimulation activated local GABA- and glycinergic interneurons. By contrast, stimulation of excitatory sensory fibers in the dorsal root produced no response when the stimulus intensity was below the threshold needed to activate fibers eliciting EPSCs mediated by postsynaptic KA receptors (Li et al., 1999; G.-D.W. and M.Z., unpublished data).

Because primary afferent sensory fibers release glutamate in the vicinity of GABA- and glycine-containing boutons in the superficial dorsal horn (Ribeiro-da-Silva and Coimbra, 1982; Todd, 1996), we hypothesized that dorsal root stimulation may activate presynaptic KA receptors on spinal interneurons and thereby modulate inhibitory transmission. Indeed, when a conditioning stimulus train (50 Hz, 20 pulses) was delivered to the dorsal root (Figure 8A), both the amplitude and the slope of the rising phase of an IPSP generated 50 ms later by local stimulation were reduced relative to the baseline, unconditioned response (Figures 8B and 8C). The conditioning train itself triggered no EPSP or IPSP. In the presence of CNQX (20 μM) instead of SYM2206, conditioning did not affect IPSPs, suggesting that KA receptor activation was necessary (Figure 8B). Furthermore, conditioning produced no effect in the presence of CGP55845 (10 μM) (Figures 8B and 8C). Thus, in agreement with the effects of KA detailed above in cultured neurons, inhibitory transmission in spinal slices was

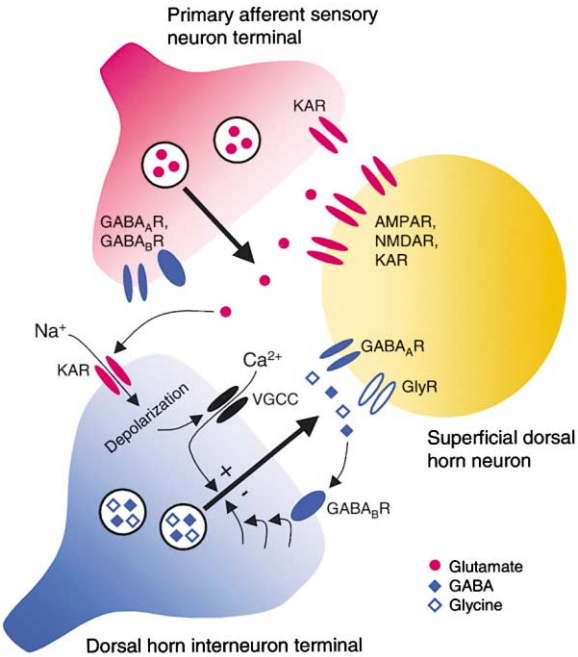

Figure 9. Presynaptic KA Receptors Regulate Spinal Inhibitory Transmission

A model of a synaptic glomerulus depicts the proposed function of presynaptic KA receptors at dorsal horn inhibitory synapses. These receptors, which can be activated by glutamate released from primary afferent sensory fibers, mediate Na^+ entry and terminal depolarization, triggering opening of voltage-gated Ca^{2+} channels (VGCC) and Ca^{2+}-dependent vesicle fusion. GABA released in this manner may activate presynaptic $GABA_B$ autoreceptors, reducing action potential-dependent transmitter release. Previous work has shown that sensory neuron terminals contain $GABA_A$ and $GABA_B$ receptors (Malcangio and Bowery, 1996), indicating that sensory neurons and dorsal horn interneurons engage in reciprocal heterosynaptic regulation of transmitter release. Other studies have documented additional roles for KA receptors in spinal sensory transmission. Along with AMPA and NMDA receptors, KA receptors mediate a component of the postsynaptic response of dorsal horn neurons to high-threshold sensory fiber stimulation (Li et al., 1999). In addition, sensory fibers themselves express presynaptic KA receptors that regulate glutamate release (Kerchner et al., 2001).

downregulated by synaptic glutamate through a mechanism involving KA receptors and $GABA_B$ receptors.

A Mechanism for Presynaptic KA Receptor-Mediated Regulation of Transmitter Release

We present unequivocal evidence that presynaptic KA receptors are present on GABA- and glycinergic interneurons in the spinal cord dorsal horn. In addition, we detail a mechanism linking presynaptic KA receptor activation to changes in transmitter release probability (Figure 9). This mechanism most likely involved an ionotropic action of KA receptors, which by permitting Na^+ entry, led to terminal depolarization, voltage-gated Ca^{2+} channel activation, and Ca^{2+}-dependent vesicle fusion. In this way, presynaptic KA receptor activation enhanced GABA/glycine release from dorsal horn neurons to an extent that could activate a negative feedback pathway, mediated by $GABA_B$ autoreceptors, inhibiting subsequent evoked release. Such a pathway appears to exist physiologically, as synaptically-released glutamate was able to suppress spinal inhibitory transmission in a KA and $GABA_B$ receptor-dependent fashion.

Biphasic effects of presynaptic KA receptor activation on excitatory transmission have also been demonstrated at hippocampal mossy fiber-CA3 synapses (Kamiya and Ozawa, 2000; Schmitz et al., 2000, 2001; Contractor et al., 2001). KA receptor activation caused a Cd^{2+}-sensitive increase in mEPSC frequency in CA3 neurons (Contractor et al., 2000) and enhanced the excitability of mossy fiber axons in a manner consistent with terminal depolarization (Kamiya and Ozawa, 2000; Schmitz et al., 2000). KA also suppressed mossy fiber-CA3 transmission and reduced action potential-evoked Ca^{2+} entry into mossy fiber axon terminals (Kamiya and Ozawa, 2000), possibly reflecting voltage-gated Na^+ or Ca^{2+} channel inactivation, current shunting, or stimulation of metabotropic glutamate autoreceptors, in analogy to the role of $GABA_B$ receptors in this study. Further work will be required to establish whether presynaptic KA receptors function similarly at other synapses in the central nervous system as they do at inhibitory synapses in the dorsal horn.

Significance

We demonstrate that glutamate released from primary afferent sensory fibers can regulate spinal inhibitory transmission by activating KA receptors. Taken together with the long known fact that sensory fibers themselves express $GABA_A$ receptors (Eccles et al., 1963), our data raise the novel possibility that heterosynaptic regulation of transmitter release by presynaptic ligand-gated ionic channels may be reciprocal between sensory fibers and dorsal horn interneurons. Because synaptically-released glutamate suppressed evoked inhibitory transmission, our findings suggest that with sufficiently high levels of sensory input, inhibitory tone may be reduced, possibly facilitating the relay of sensory information to higher brain centers, although other more complex scenarios cannot be excluded.

Regulation of inhibitory transmission in the dorsal horn is essential for proper processing of pain and other sensory information (Malcangio and Bowery, 1996); for instance, agonists of $GABA_A$ and $GABA_B$ receptors are antinociceptive, and spinal administration of antagonists induces chronic pain (Sawynok, 1987; Yaksh, 1989; Hammond and Washington, 1993). In their gate control theory of pain, Melzack and Wall (1965) proposed that pain transmission in the dorsal horn is enhanced by the activity of nociceptive sensory fibers, but suppressed by the activity of nonnociceptive fibers that may act in concert with local inhibitory neurons. The balance between enhancement and suppression determines the setting of a "gate" that permits or restricts the relay of nociceptive information from the spinal cord to higher brain centers. We suggest that the ability of glutamate released from sensory fibers to regulate GABA/glycine release via presynaptic KA receptors could represent one component of gate control.

Finally, our data broaden the known roles of KA receptors in spinal sensory pathways (Figure 9). We previously showed that KA receptors mediate a component of the postsynaptic response of dorsal horn neurons to high-

threshold primary afferent sensory fiber stimulation (Li et al., 1999). Antagonists that block these postsynaptic KA receptors and the presynaptic KA receptors on dorsal horn interneurons would be expected to have analgesic properties. We have also reported that sensory fibers themselves express presynaptic KA receptors, and that activation of those receptors with exogenous agonists suppressed sensory transmission (Kerchner et al., 2001). Because the KA receptors on peripheral sensory neurons could be pharmacologically separated from those expressed on spinal neurons (Kerchner et al., 2001; Wilding and Huettner, 2001), manipulation of spinal KA receptors with selective agonists and antagonists could represent a viable therapeutic strategy for the treatment of pain.

Experimental Procedures

Primary Neuronal Culture

Protocols for handling animals were approved by the Animal Studies Committee at Washington University. Dorsal horn neurons were taken from postnatal rats sacrificed by decapitation. The spinal cord was removed to a dish containing Earl's buffer, and the dorsal third of the cord was dissected and incubated for 30–90 min at 30°C–35°C in oxygenated Earl's buffer containing papain (Huettner and Baughman, 1986; Wilding and Huettner, 1997). Cells were dissociated mechanically in bovine serum albumin and ovomucoid, both at 1 mg/ml, and plated onto 35 mm culture dishes coated with matrigel (Becton & Dickinson, Bedford, MA). For microisland cultures (Mennerick et al., 1995), dishes were first coated with agar, sprayed with an aerosolized solution of collagen, and plated with rat spinal glial cells (prepared as above, with glutamate or NMDA added to the medium to eliminate neurons). Cultures were maintained at 37°C in a humidified, 5% CO_2 incubator in Eagle's minimal essential medium (supplemented with 20 mM glucose, 0.5 mM glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 4% rat serum), treated at 4 days in vitro with 10 μM cytosine β-D-arabinofuranoside, and used for experiments between days 7 and 35.

Electrophysiology in Cultures

On the stage of an Axiovert 25 inverted microscope (Carl Zeiss, Inc., Thornwood, NY), cultures were bath perfused with Tyrode's solution, containing (in mM): 150 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). During recordings, neurons were under constant local gravity-fed perfusion from a quartz glass pipette (inner diameter 300 μm) connected to a manifold with <1 μl dead space (ALA Scientific Instruments, Inc., Westbury, NY). Whole-cell recordings were established using heat-polished pipettes pulled from borosilicate capillary tubes (Warner Instrument Corp., Hamden, CT) with a tip resistance of 3–6 MΩ when filled with a solution containing (in mM): 140 $CsCH_3SO_3$, 5 CsCl, 5 $MgCl_2$, 10 EGTA, 10 HEPES, 5 Mg-ATP, and 1 Li-GTP (pH 7.4 with CsOH). (In current clamp recordings, K^+ replaced Cs^+; all other intracellular constituents were identical. Typical resting membrane potentials were between −70 and −75 mV, and a DC command current was applied to standardize this potential at −70 mV for all experiments.) Series resistance was not compensated but monitored throughout experiments. Recorded currents were filtered at 2 kHz, digitized at 10 kHz, and stored in a PC-compatible computer for display and analysis with an Axopatch 200B amplifier, Digidata 1320 interface, and the pCLAMP 8.1 software suite (Axon Instruments, Inc., Union City, CA). Extracellular stimulation of synaptic currents was achieved with the S48 single-channel stimulator and SIU5 stimulus isolation unit (Grass Instruments, W. Warwick, RI) connected to a bipolar stimulating electrode constructed with two Ag/AgCl wires immersed in Tyrode's solution within a theta glass electrode that was pulled and heat-polished to a final tip diameter of ~10–20 μm. This stimulus electrode was placed against the cell body of a neuron close to the recorded cell. We included only experiments in which evoked postsynaptic currents occurred at a fixed latency after stimulation. All compounds were obtained from Sigma Chemical Co. (St. Louis, MO), except ATPA ([*RS*]-2-α-amino-3-[3-hydroxy-5-tert-butylisoxazol-4-yl] propanoic acid), CGP55845 ([2*S*]-3-[{(15)-1-(3,4-dichlorophenyl)ethyl}amino-2-hydroxypropyl][phenylmethyl] phosphinic acid), CPPG ([*RS*]-α-cyclopropyl-4-phosphonophenylglycine), LY367385 ([*S*]-[+]-α-amino-4-carboxy-2-methylbenzeneacetic acid), MPEP (2-methyl-6-[phenylethynyl]pyridine), SYM2081 ([2*S*,4*R*]-4-methylglutamate), and SYM2206 ([*RS*]-4-[4-aminophenyl]-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphthalazine) (Tocris Cookson, Inc., Ellisville, MO).

Electrophysiology in Slices

Adult male rats were anaesthetized with urethane, and the spinal cord was removed to a dish containing chilled artificial cerebrospinal fluid (ACSF; in mM: 124 NaCl, 4 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2 $CaCl_2$, 1 $MgSO_4$, and 10 D-glucose) equilibrated with a 95% O_2, 5% CO_2 atmosphere. Transverse lumbar spinal cord slices, 450–500 μm thick with 7–12 mm of attached dorsal root, were isolated and maintained in oxygenated ACSF at 32°C. After 2–3 hr, slices were transferred to a recording chamber and perfused at 2–5 ml/min with oxygenated ACSF at 34°C. Intracellular recordings were made from superficial dorsal horn neurons (laminae I–II) using 3 M KCl-filled glass microelectrodes (DC impedance, 75–200 MΩ). Postsynaptic responses were evoked by electrical stimulation with a bipolar tungsten electrode, amplified with a high-input impedance bridge circuit amplifier (Axoclamp 2B, Axon Instruments, Inc.), and stored using pCLAMP software.

Data Analysis

Data records were analyzed with pCLAMP 8.1 and the Mini Analysis Program 5.2 (Synaptosoft, Inc., Decatur, GA). A portion of each record subjected to automated mIPSC detection was inspected visually in order to optimize algorithm parameters. Bin size for mIPSC frequency analysis was 1 s. Because of the transient nature of the KA-triggered mIPSC burst (Figures 1A and 2), mIPSC frequency was averaged during only the first four seconds of KA exposure. This method provided an appropriate standard of comparison, although it underestimated the full peak effect. Such underestimation was compounded by the presence of some multiquantal and complex, overlapping events near the peak of the mIPSC burst that could not be separated visually or with the event detection software (Figure 1A). Also, the magnitude of the KA-induced increase in mIPSC frequency varied between recordings, so the ability of an agent to reduce a KA-triggered increase in mIPSC frequency was always compared to the effect of KA alone in the same population of cells. Data are presented as mean ± standard error of the mean. To detect significant differences between two means, a paired t test or signed rank test was used. For comparison of multiple groups, Kruskall-Wallis ANOVA on ranks was performed with Dunn's or Student-Newman-Keuls test for post hoc comparison. In all cases, $p < 0.05$ was considered significant.

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