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Functional Expression of AMPA Receptors on Central Terminals of Rat Dorsal Root Ganglion Neurons and Presynaptic Inhibition of Glutamate Release

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Summary

No direct evidence has been found for expression of functional AMPA receptors by dorsal root ganglion neurons despite immunocytochemical evidence suggesting they are present. Here we report evidence for expression of functional AMPA receptors by a subpopulation of dorsal root ganglion neurons. The AMPA receptors are most prominently located near central terminals of primary afferent fibers. AMPA and kainate receptors were detected by recording receptor-mediated depolarization of the central terminals under selective pharmacological conditions. We demonstrate that activation of presynaptic AMPA receptors by exogenous agonists causes inhibition of glutamate release from the terminals, possibly via primary afferent depolarization (PAD). These results challenge the traditional view that GABA and $GABA_A$ receptors exclusively mediate PAD, and indicate that PAD is also mediated by glutamate acting on presynaptically localized AMPA and kainate receptors.

Introduction

The flow of information from peripheral sensory receptors to the central nervous system is under constant regulation by presynaptic inhibition via a mechanism known as primary afferent depolarization (PAD) (reviewed in Rudomin and Schmidt, 1999; Willis, 1999). Primary afferent terminals of thickly myelinated sensory fibers including muscle afferents receive axo-axonic contacts from GABAergic terminals of spinal interneurons. GABA released at these axo-axonic synapses is believed to be the primary transmitter responsible for presynaptic inhibitory control of transmitter release due to local depolarization at the terminals, or PAD, and accompanying depression of action potentials arriving at the terminals.

Induction of PAD in terminals of thinly myelinated Aδ and nonmyelinated C fibers in the mammalian spinal cord is controversial (Rudomin and Schmidt, 1999; Willis 1999). The only morphological evidence for GABAergic terminals synapsing on the afferent terminals is within complex structures called glomeruli that include both pre- and postsynaptic elements. Glomeruli including C and Aδ afferent fibers represent a small minority of central terminals in the superficial laminae of spinal cord. Nevertheless, Fitzgerald and Woolf (1981) showed a change in C fiber excitability following stimulation of dorsal gray matter near the C fiber terminals or stimulation of A fiber afferents to the same cord segment, suggesting synaptic modulation of C fiber central terminals. Furthermore, in a recent study using turtle spinal cord, dorsal root potentials, believed to reflect synaptically evoked PAD, were recorded in response to dorsal root stimulation (Russo et al., 2000). Because the recordings were made in low concentrations of tetrodotoxin (TTX), glutamate release was assumed to be restricted to C and Aδ fibers. A component of the dorsal root potential was sensitive to the $GABA_A$ receptor antagonist, bicuculline. The remaining component was fully blocked by APV and CNQX, antagonists of NMDA and non-NMDA receptors, respectively, indicating that PAD is found in C and Aδ fibers and that it is mediated in part by glutamate. The individual contribution of AMPA and kainate receptors to the non-NMDA receptor-mediated component of the response was not pharmacologically determined.

Physiological recordings have demonstrated that functional kainate receptors are expressed by small diameter dorsal root ganglion (DRG) neurons (Huettner, 1990). Immunocytochemical evidence indicates that AMPA receptor subunits are also expressed by subpopulations of DRG neurons, some of which are small and thus proposed to be nociceptors (Sato et al., 1993; Tachibana et al., 1994). Despite this indication of subunit expression, physiological tests have not detected direct evidence for functional AMPA receptor expression by DRG neurons (e.g., Huettner, 1990; Wong and Mayer, 1993), although functional AMPA receptor expression has been suggested in mesencephalic trigeminal neurons (Pelkey and Marshall, 1998). Furthermore, immunocytochemistry at the ultrastructural level using antibodies to AMPA receptors has provided evidence for localization in peripheral sensory axons (Coggeshall and Carlton, 1998).

Here we have tested the prediction that functional AMPA receptors are expressed by DRG neurons and found that while AMPA receptors are rarely detectable at the level of the soma, they are strongly expressed near the primary afferent central terminals. We also show that direct activation of these presynaptically localized AMPA receptors by exogenous agonist inhibits synaptic release of glutamate. In addition to presynaptic inhibition, these presynaptic AMPA receptors, as well as kainate receptors, are involved in the generation of PAD that may, in turn, drive dorsal root reflexes and an ac-

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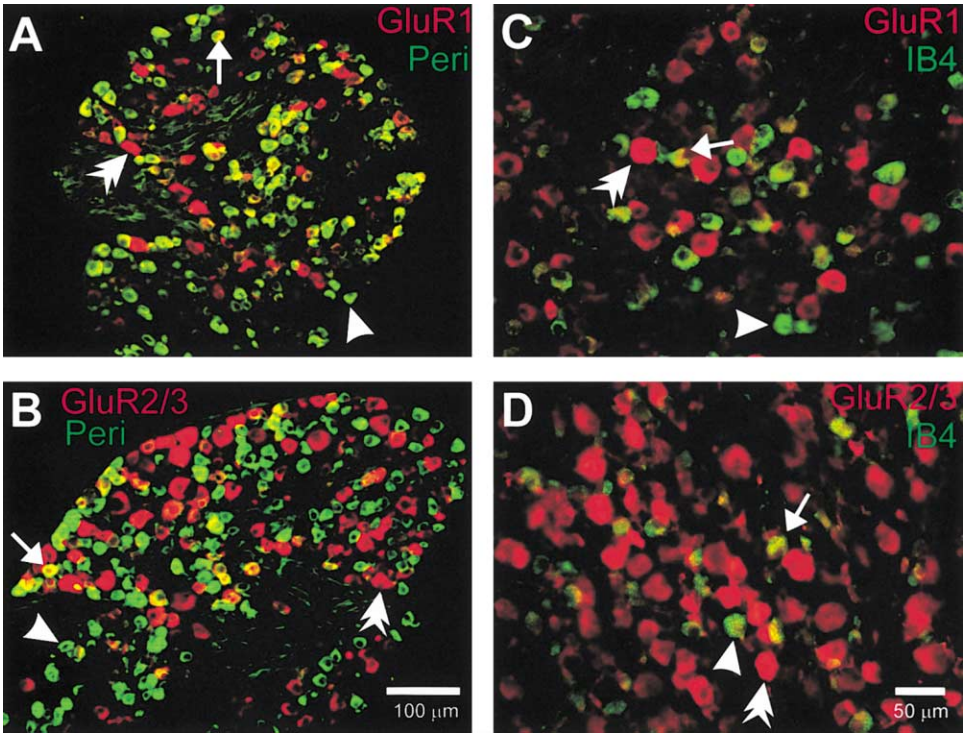

Figure 1. Immunocytochemistry with Antibodies to GluR1 and GluR2/3 plus Peripherin and IB4 Reveals Different Levels of Coexpression of AMPA Receptor Subunits with the Nociceptor Markers

(A) Section from a DRG (P7) was stained with antibodies to GluR1 (red) and peripherin (Peri, green). In this and the other three panels, double arrowheads indicate GluR-positive, peripherin/IB4-negative neurons; arrowheads indicate GluR-negative, peripherin/IB4-positive neurons; and arrows show double-labeled neurons. While 50% ± 4% of GluR1-positive neurons are peripherin positive, 24% ± 4% of peripherin-positive neurons are GluR1 positive.

(B) Section from a DRG (P7) was labeled with antibodies to GluR2/3 (red) and peripherin (green). 22% ± 4% of GluR2/3-positive neurons are peripherin-positive, while 20% ± 4% of peripherin-positive neurons are GluR2/3 positive.

(C) Section from a DRG (P8) labeled with antibodies to GluR1 (red) and with the lectin, IB4 (green). 9% ± 2% of GluR1-positive neurons are IB4 positive, while 13% ± 3% of IB4-positive neurons are GluR1 positive.

(D) Section from a DRG (P8) labeled with IB4 (green) and antibodies to GluR2/3 (red). 10% ± 1% of GluR2/3-positive neurons are IB4 positive, and 9% ± 1% of IB4-positive neurons are GluR2/3 positive.

companying neurogenic inflammation, as has been proposed for $GABA_A$ receptors (Willis, 1999).

Results

Immunocytochemical Evidence of AMPA Receptor Subunit Expression in Subpopulations of DRG Neurons

Histological sections were prepared from postnatal rat DRGs to test for expression of AMPA receptor subunits GluR1 and GluR2/3. There is evidence for a little expression of GluR4 in DRGs, and this may be mainly in satellite cells (Sato et al., 1993; Tachibana et al., 1994). Therefore we did not test for the presence of GluR4 in our studies. To correlate AMPA receptor subunit expression with nociceptor subpopulations, sections were double labeled with antibodies to GluR1 or GluR2/3 and peripherin or IB4. Peripherin is an intermediate filament protein that is expressed by small diameter DRG neurons, many of which express substance P and/or CGRP (Goldstein et al., 1991, 1996) and are therefore likely to be nociceptors. This subpopulation of DRG neurons is predominantly not overlapping with the IB4/LA4-positive, small diameter subpopulation of nociceptors as determined by IB4 and peripherin costaining (H.S.E, unpublished data), peptide and carbohydrate costaining (Dodd and Jessell, 1985; Molliver et al., 1997), and physiological characteristics (Stucky and Lewin, 1999).

Figure 1 shows examples of GluR and peripherin or IB4-positive staining on sections taken from DRG of postnatal day 7 (P7) or P8 rat pups. Figure 1A shows an example of GluR1 and peripherin staining. Examples of cells labeled for GluR1 (double arrowhead), peripherin (arrowhead), and for both (arrow) are indicated. As has been reported for adult DRG by Sato et al. (1993), some of the small neurons are immunopositive for GluR1 in the postnatal ganglion. Some of these are double labeled with peripherin. While staining for GluR2/3 is more widespread than for GluR1, a smaller percentage of neurons show costaining of GluR2/3 with peripherin (Figure 1B). Specifically, 50% ± 4% of the GluR1-positive DRG neurons were also positive for peripherin, whereas only 22% ± 4% of GluR2/3-positive neurons were costained for peripherin. Examples of double labeling of DRG with IB4 and GluR1 or GluR2/3 are shown in Figures 1C and 1D. Of the GluR1-positive neurons counted, 9% ± 2%

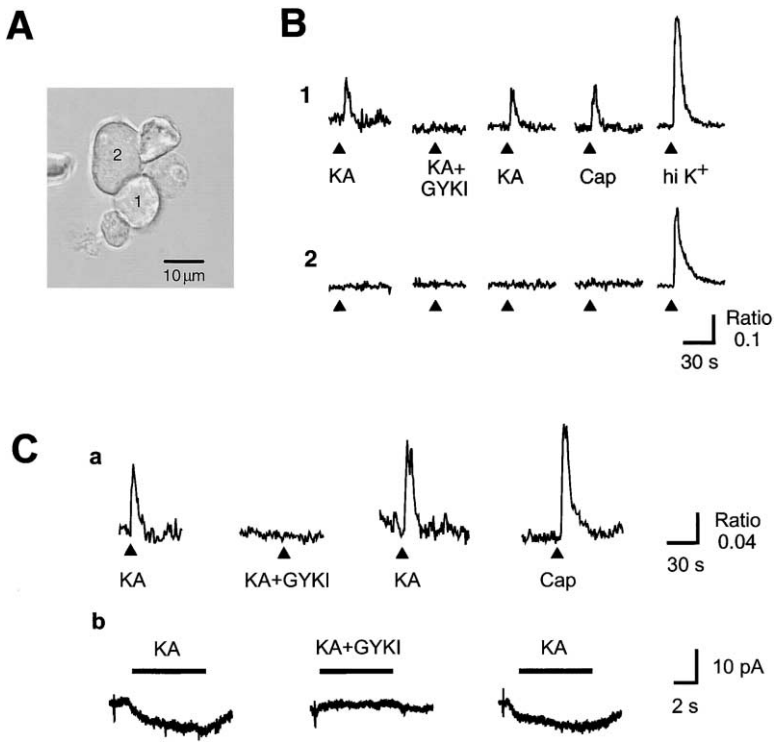

Figure 2. Few Acutely Dissociated DRG Cell Bodies Show Expression of Functional AMPA Receptors

(A) A bright-field image of acutely dissociated P4 DRG neurons. Cells were preloaded with fura-2 AM, and fluorescence ratios representing $[Ca^{2+}]_i$ were recorded in the presence of 3 μM SYM 2081. Results from DRG neuron #1 are shown in (B_1) and data from cell #2 in (B_2). (B_1) Neuron #1 showed expression of functional AMPA receptors activated by 100 μM kainate (KA), indicated by a transient increase in $[Ca^{2+}]_i$, which was completely blocked by 100 μM GYKI 52466, with full recovery after wash-out (n = 9). 1 μM capsaicin (Cap) and 50 mM K^+ (hi K^+) also induced a robust increase in $[Ca^{2+}]_i$. (B_2) Neuron #2 did not show kainate- or capsaicin-induced increase in $[Ca^{2+}]_i$. However, it displayed an increase in $[Ca^{2+}]_i$ with application of 50 mM K^+. All drugs were applied for 5 s. GYKI 52466 was applied 1 min prior to and during kainate application. (C_a) In another neuron, Ca^{2+} imaging was used to pre-identify the AMPA receptor-expressing cell. KA induced a robust elevation of $[Ca^{2+}]_i$, which was reversibly blocked by GYKI 52466. This cell showed sensitivity to capsaicin. (C_b) The same cell was patched under voltage clamp and was held at −70 mV. KA induced AMPA receptor-mediated currents that were blocked by GYKI 52466 and readily recovered upon washout (n = 2).

were IB4 positive, while 10% ± 1% of GluR2/3-positive DRG neurons were double labeled with IB4. Because peripherin and IB4 labeling reveal subpopulations of nociceptors in DRG, these colocalization studies indicate that some of the neurons immunopositive for GluR1 or GluR2/3 are nociceptive sensory neurons. Among nociceptors, GluR expression overlaps more with peripherin-positive neurons than with IB4-positive neurons. In addition, most neurons with the brightest staining for GluR subunit were larger and were not labeled with IB4 or peripherin, suggesting that these may be nonnociceptive sensory neurons. These results contrast with those of kainate receptors, which are expressed exclusively by nociceptors that are mostly IB4 positive (Lee et al., 2001).

Few DRG Cell Bodies Show Functional AMPA Receptor Expression

To test for expression of functional AMPA receptors by DRG neurons, we looked for AMPA receptor-mediated changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) from acutely dissociated postnatal DRG neurons. We have shown previously that by P1, 100% of acutely dissociated DRG neurons show expression of voltage-gated calcium channels (Lee et al., 2001). Sufficient depolarization following activation of AMPA receptors will open voltage-gated Ca^{2+} channels and produce detectable Ca^{2+} transients regardless of whether the AMPA receptors are Ca^{2+} permeable or impermeable. We used kainate as an agonist for AMPA receptors because kainate induces a mostly nondesensitizing current from AMPA receptors (Patneau et al., 1993). All the recordings were performed in the presence of 3 μM SYM 2081 to fully desensitize any kainate receptors present, eliminating their contribution to the response (Zhou et al., 1997; Donevan et al., 1998; Lee et al., 2001). Under these conditions, response to kainate application is mediated by AMPA receptors expressed on DRG cell bodies. Each neuron was also tested for sensitivity to capsaicin as a physiological assay of whether the neuron under study was a capsaicin-sensitive nociceptor.

Figure 2B shows the results recorded from two DRG neurons acutely dissociated from a P4 rat DRG. Figure 2B_1 shows the response of neuron #1 (seen in Figure 2A) to 100 μM kainate in the presence of 3 μM SYM 2081. Under these conditions, kainate caused an increase in $[Ca^{2+}]_i$ that was reversibly blocked by 100 μM GYKI 52466, an AMPA receptor antagonist. Capsaicin (1 μM) and K^+ (50 mM) were also able to elevate $[Ca^{2+}]_i$ in this neuron. In contrast, the larger neuron #2 (shown in Figure 2A) did not show a detectable change in $[Ca^{2+}]_i$ in response to kainate/SYM 2081 or capsaicin. The lack of response by neuron #2 was not due to the general health of the cell because of the robust elevation of $[Ca^{2+}]_i$ in response to 50 mM K^+ (Figure 2B_2). We were able to obtain AMPA receptor-mediated Ca^{2+} responses from a few cells although the total percentage of AMPA receptor-positive neurons was less than 1% (10 out of over 1000 DRG neurons tested). Nine out of ten neurons with AMPA receptor responses were sensitive to capsaicin with an average diameter of 19.5 ± 1.5 μm.

Whole-cell currents were recorded from two of the ten neurons with AMPA receptor-mediated $[Ca^{2+}]_i$ elevation. Figure 2C shows an example of a DRG neuron from a P3 rat pup preidentified as expressing AMPA receptors by its robust $[Ca^{2+}]_i$ elevation in response to kainate that was reversibly blocked by GYKI 52466 in the presence

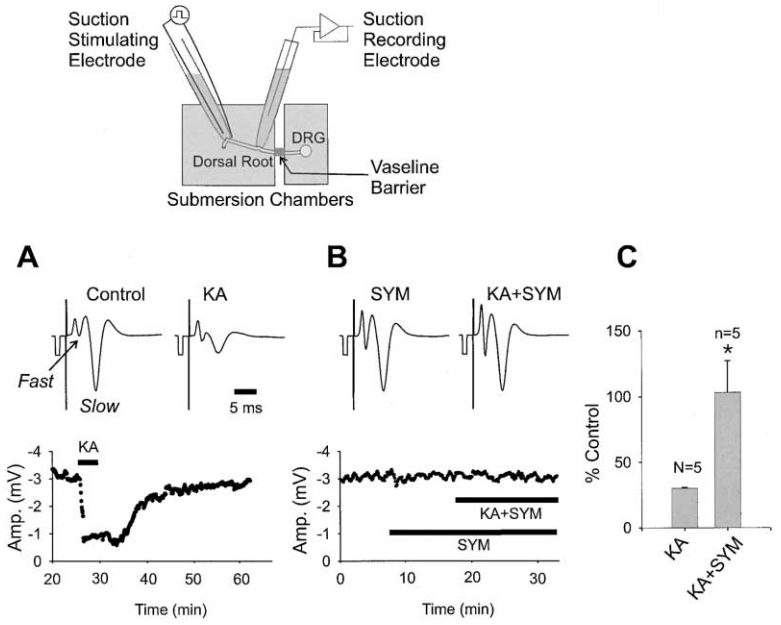

Figure 3. Dorsal Roots Show Kainate Receptor-Mediated Depression of Slow-Fiber Volley

The schematic drawing is of the experimental setup. Dorsal roots of S1–S4 with DRG attached were placed in a recording chamber. Two suction electrodes were for stimulation and recording. A Vaseline barrier was used to separate the chamber and isolate DRGs from drugs.

(A) Compound action potentials were recorded in the absence (control) and presence of 10 μM kainate (KA). The fast- and slow-fiber volleys are indicated on the control trace. The amplitude versus time plot (below) for slow-fiber volley shows that kainate rapidly depresses the slow-fiber volley and recovers upon washout.

(B) In the presence of 3 μM SYM 2081 (SYM), kainate-induced depression of slow-fiber volley was blocked (KA+SYM). Each trace is an average of ten raw traces.

(C) The bar graph shows the average percent control of the slow-fiber volley amplitude. The percent control is calculated for each dorsal root by taking a ratio of the peak amplitude of slow-fiber volley in KA over the control condition. KA+SYM showed no change in amplitudes. Numbers above each bar indicate the number of dorsal roots tested. The asterisk (*) indicates a significant difference based on unpaired Student's t test ($p < 0.05$).

of SYM 2081 (Figure 2C$_a$). It also responded to capsaicin. Electrophysiological responses were subsequently recorded from the same neuron. The current mediated by AMPA receptors was small but significant in both neurons tested (Figure 2C$_b$). We have also recorded AMPA receptor-mediated currents from embryonic DRG neurons cocultured with embryonic dorsal horn neurons and shown that about 50% of the cultured DRG neurons express functional AMPA receptors (our unpublished data). The fact that we were able to see functional AMPA receptor expression by embryonic cultured DRG neurons more often than by acutely dissociated postnatal DRG neurons could be explained by the observation that DRG neurons express AMPA receptors in vivo mostly at or near terminals (see Figure 4). These cultured DRG neurons extend their neurites and form synapses extensively (Gu and MacDermott, 1997).

Another possible explanation for the lack of functional AMPA receptors on acutely prepared DRG neurons is enzymatic disabling of the AMPA receptors. We tested if the trypsin used in the acute dissociation procedure was causing receptors to disappear from the surface of cell bodies by mechanically dissociating thin slices of DRGs (~75–100 μm thickness). When we looked for neurons showing AMPA receptor-mediated Ca^{2+} responses, we obtained a similar low percentage of less than 1% responsive neurons under these conditions (over 200 neurons tested in 3 preparations).

Kainate-Induced Depression of Compound Action Potentials Is Mediated by Kainate Receptors

An explanation for the low numbers of neurons expressing functional AMPA receptors on their cell bodies compared to those with immunocytochemical evidence of AMPA receptor subunit expression is that functional AMPA receptors may be highly localized to a cellular compartment separate from the cell body. Therefore, we tested if functional AMPA receptors are expressed along the dorsal roots. It has been shown previously that kainate depresses C fiber-mediated compound action potentials recorded from dorsal roots, probably by a shunting mechanism (Agrawal and Evans, 1986). Kainate receptors were proposed to be the mediators of this effect of kainate (Pook et al., 1993), although selective non-NMDA receptor antagonists were not available at the time of those studies.

Lower lumbar and upper sacral dorsal roots with attached DRGs were isolated from rat pups ages P6–P10 and used for recording. Using two glass suction electrodes, one for stimulation and the other for recording as schematized in the inset of Figure 3, we were able to record responses including fast- (0.42 ± 0.02 m/s, n = 94) and slow-conducting compound action potentials (0.18 ± 0.01 m/s, n = 101; Figure 3A). The slow-conducting compound action potentials were strongly depressed by capsaicin (data not shown) and assumed to be predominantly mediated by slower C and possibly Aδ fibers.

In five of five acutely prepared dorsal roots tested, 10 μM kainate caused an immediate and profound depression (67% ± 2%) of the slow-fiber compound action potential amplitude with minimal effect on the faster component (Figure 3A). In five of five separate dorsal roots tested, pretreatment with 3 μM SYM 2081, the rapidly desensitizing agonist for the kainate receptor, completely prevented the depressant action of kainate (Figure 3B). The action of kainate in the presence of SYM 2081 was significantly different from that of kainate alone ($p < 0.05$, Student's t test; Figure 3C).

We extended these studies using another pharmacological approach for receptor identification. Higher concentrations of SYM 2081 have been shown to activate AMPA receptors in a nondesensitizing response with an EC$_{50}$ of 325 μM (Zhou, et al., 1997). We tested 300 μM

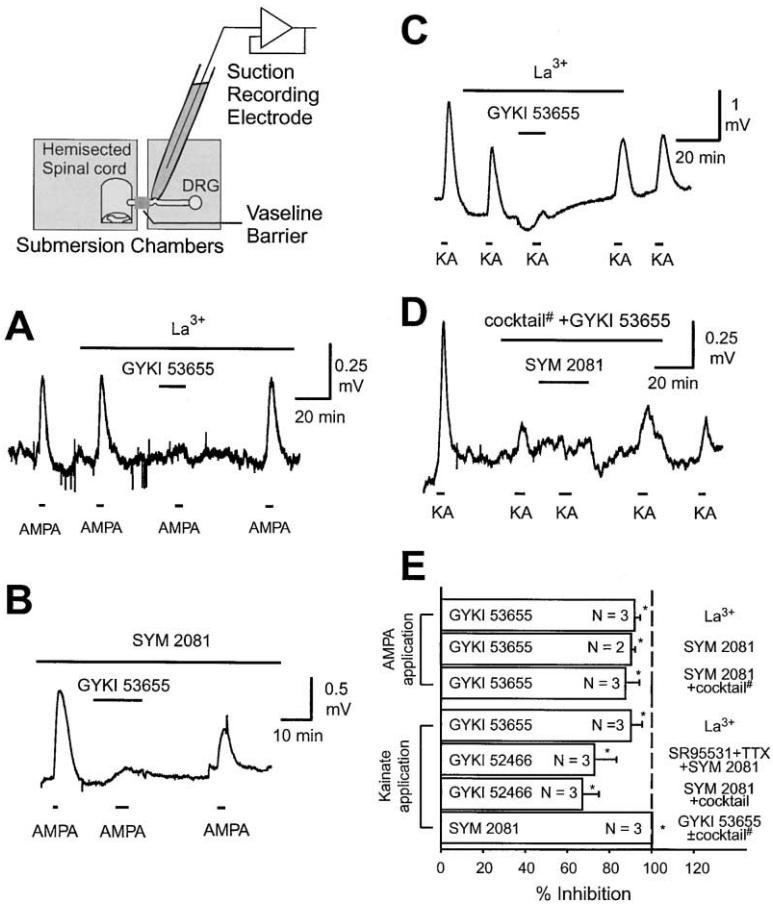

Figure 4. Functional AMPA and Kainate Receptors Are Expressed Near Primary Afferent Terminals

Schematic drawing of the recording setup is shown in inset. A glass suction electrode was placed on the dorsal root to record depolarizations from primary afferent terminals. Drugs were only applied to the spinal cord compartment, and the whole preparation was incubated in a low (20 μM) calcium medium. The time for drug to wash into the preparations began 45–60 s after the start of the horizontal bar in the figures. The voltage response from PAD was recorded from the root.

(A) In Krebs solution containing 30 μM La^{3+}, 20 μM AMPA was able to induce depolarization which was blocked by 50 μM GYKI 53655.

(B) In Krebs solution containing 3 μM SYM 2081, 20 μM AMPA caused a depolarization reversibly blocked by GYKI 53655.

(C) In La^{3+}, 100 μM kainate (KA) was able to induce depolarization that was reversibly blocked by GYKI 53655.

(D) Kainate could induce depolarization in a cocktail including GYKI 53655, and this depolarization was reversibly blocked by SYM 2081.

(E) The averaged percent inhibition by different antagonists was measured and plotted for each bath condition. Different additions to the bath are indicated on the right side of each bar graph. Each bar indicates percent inhibition by antagonists (GYKI 52466, GYKI 53655, or SYM 2081) of depolarization caused by kainate or AMPA application. Numbers inside each bar represent the number of preparations tested. Dashed line represents 100% inhibition. Cocktail# includes TTX+SR95531+SCH50911+PPADS+DPCPX. Cocktail indicates cocktail plus mGluR receptor antagonists MPPG and LY 341495. Significance (* = $p < 0.01$) was tested using paired t test to compare the depolarizations caused by KA or AMPA before and after antagonist wash in. In a few experiments, when very slow base line drifts (≤0.006 mV/min) were observed, a baseline calibration curve ($y = a + b \exp(-x/c)$) was used to fit the baseline, and this was subtracted from the original data.

SYM 2081, and it had no effect on slow-fiber compound action potential (0.4% ± 0.3% depression, n = 2). These data suggest that the action of 10 μM kainate on dorsal root fibers is mediated mostly by kainate receptors and not by AMPA receptors, consistent with the results of Pook et al. (1993).

Functional AMPA Receptors Are Expressed at or Near the Primary Afferent Terminals

We next tested for AMPA receptor expression at a cellular compartment different from the dorsal roots. Specifically, primary afferent axons near their central terminals were tested directly for the presence of functional AMPA receptors by recording depolarization of the terminals (PAD) from a suction electrode on the dorsal root. Kainate or AMPA were applied to the attached hemisected spinal cord isolated from the root by a Vaseline gap (Figure 4, inset). The hemisected spinal cord was continuously perfused in Krebs solution containing a low concentration (20 μM) of $CaCl_2$ in order to block any Ca^{2+}-dependent release of transmitters. This bath was sufficient to completely block dorsal root-evoked dorsal root potentials, which depend upon transmitter release, within 5 min (C.K.T., unpublished data). In order to distinguish the responses of AMPA receptors from kainate receptors, SYM 2081 (3 μM) or La^{3+} (30 μM) was used to block kainate receptors. La^{3+} was chosen because AMPA receptor-mediated current is enhanced by 30 μM La^{3+} (Reichling and MacDermott, 1991), while kainate receptor-mediated current is blocked 90%–100% (Huettner et al., 1998). GYKI 53655 (50 μM) or GYKI 52466 (100 μM) was used to block AMPA receptor-mediated responses. TTX (1 μM) was included to block TTX-sensitive sodium channels and SR 95531 (10 μM) (Uchida et al., 1996) was used to block $GABA_A$ receptors. In additional experiments, a broad cocktail of blockers was used in order to exclude indirect involvement of other receptors. The cocktail included 20 μM SCH50911 to block $GABA_B$ receptors, 25 μM PPADS to block P_{2x} receptors, 100 nM DPCPX to block adenosine receptors, and in some cases LY341495 (25 μM) and MPPG (100 μM) to block group I, II, III mGluR (mGluR 1–5, 7–8), and group III mGluR (mGluR4) receptors respectively.

AMPA (20 μM) and kainate (100 μM) routinely evoked PAD as illustrated in Figure 4. The PAD evoked by AMPA was maintained in the presence of La^{3+} or SYM 2081 and was reversibly blocked by the selective AMPA receptor antagonist, GYKI 53655 (Figures 4A and 4B) under a

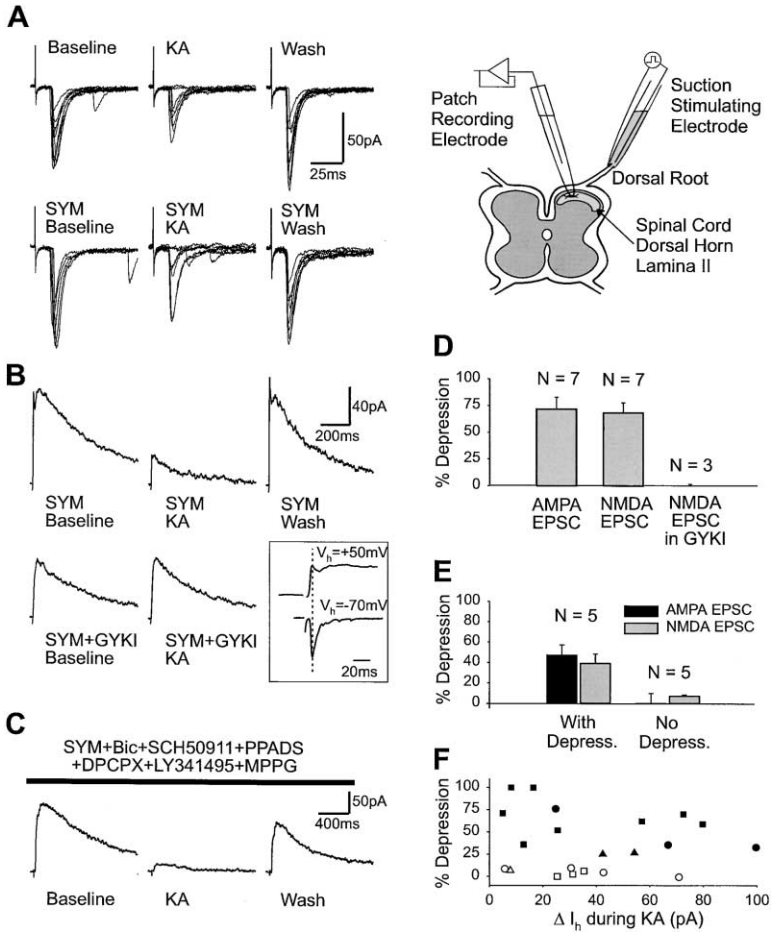

Figure 5. Activation of Presynaptic AMPA Receptor Decreases the Release of Glutamate from Primary Afferent Terminals

Schematic drawing of the recording setup with a transverse section of cervical spinal cord shown in inset.

(A) The release of glutamate was monitored by recording excitatory postsynaptic currents (EPSCs) from a lamina II neuron following dorsal root stimulation (0.03 Hz). The spinal cord slice was taken from a P9 rat pup, and the dorsal horn neuron was held at −70mV. The peak amplitudes of the EPSCs reversibly decreased in the presence of 5 μM kainate (KA; top panel). This also occurred in the continuous presence of 3 μM SYM 2081 (SYM) with an apparent increase in the number of synaptic failures (bottom panel).

(B) On a separate dorsal horn neuron, both fast AMPA and slow NMDA EPSCs were recorded while the cell was held at +50 mV in the presence of SYM. The inset demonstrates the alignment of the fast AMPA EPSC peaks at −70 mV and at +50 mV. The peak amplitude of AMPA EPSC is measured at this time point, whereas the peak amplitude of NMDA EPSC is measured at the same position of the peak in GYKI 52466. Kainate depressed the peak of both AMPA and NMDA EPSCs (top panel). GYKI blocked the depressant action of kainate (bottom panel). Each trace is an average of five raw traces.

(C) EPSCs were recorded at +50 mV in the presence of blocker cocktail containing 3 μM SYM 2081, 10 μM bicuculline, 20 μM SCH50911, 50 μM PPADS, 10 μM DPCPX, 25 μM LY341495, and 100 μM MPPG. The peak of the average EPSC reversibly decreased in the presence of 5 μM kainate (KA). Traces represent average trace from five raw traces during baseline, KA, and wash.

(D) The degree of depression induced by kainate in the presence of 10 μM bicuculline, 5 μM strychnine, and 3 μM SYM 2081 (example shown in [B]) is expressed as an average percentage of depression in amplitudes for AMPA EPSCs and NMDA EPSCs during kainate application compared to pre-kainate control EPSCs. Numbers above each bar indicate the number of recorded lamina II neurons.

(E) Depression of EPSC amplitudes induced by kainate in the presence of blocker cocktail (example shown in [C]), in most cases including mGluR antagonists, is summarized. Each cell is categorized based on unpaired t test between five peak NMDA EPSC amplitudes before and five during KA application. Error bars represent SEM.

(F) The relationship between the kainite-induced change in holding current and the percent depression is demonstrated in a scatter plot. Closed symbols represent the cells with depression, and open symbols represent the cells with no depression. Square symbols represent cells recorded in the presence of 10 μM bicuculline, 5 μM strychnine, and 3 μM SYM 2081 (as shown in [B]). Triangle symbols represent the cells recorded in the blocker cocktail containing 3 μM SYM 2081, 10 μM bicuculline, 20 μM SCH50911, 50 μM PPADS, and 10 μM DPCPX. Circle symbols represent the cells recorded in the blocker cocktail plus the mGluR antagonists, 25 μM LY341495, and 100 μM MPPG (as shown in [C]).

variety of bath conditions (Figure 4E). Likewise, kainate always induced a substantial PAD (n = 28; Figures 4C and 4D). In the presence of kainate receptor antagonists, kainate-induced depolarization was significantly blocked by both of the AMPA receptor antagonists, GYKI 53655 and GYKI 52466 (Figures 4C and 4E). Furthermore, in cocktail, kainate-induced depolarization was blocked by GYKI 52466 (Figure 4E). Taken together, our data indicate that the AMPA and kainite-induced PADs in Figures 4A–4C were mediated by AMPA receptors.

We were also able to detect PAD mediated by kainate receptors (Figure 4D). When AMPA receptors were blocked, the remaining PAD evoked by kainate was completely and reversibly blocked by SYM 2081 (Figures 4D and 4E), indicating that the remaining PAD was mediated by kainate receptors. Thus, both the kainate and AMPA receptor-mediated components of PAD were observed in the presence of cocktail when kainate was used as an agonist (Figure 4E). These data also indicate that SYM2081 is able to block all of the kainate receptor-mediated depolarization evoked by kainate and therefore is an efficient means to block functional kainate receptors expressed near primary afferent terminals.

Activation of Presynaptic AMPA Receptors Causes a Decrease in Glutamate Release from Primary Afferent Terminals in Acute Spinal Cord Slice Preparations

Because AMPA receptors are expressed near the central terminals of primary afferents as indicated by the measurements of PAD (Figure 4), they may modulate glutamate release from those terminals. To test this pos-

sibility, we used acutely prepared transverse spinal cord slices with attached dorsal roots. Glutamate release from primary afferent terminals was monitored by recording excitatory postsynaptic currents (EPSCs) from lamina II dorsal horn neurons while stimulating the dorsal roots as shown in the inset of Figure 5.

Figure 5A shows an experiment performed on a lamina II neuron from a P9 animal. The dorsal root was stimulated at 0.03 Hz, and EPSCs were recorded at a holding potential of −70 mV in the presence of 10 μM bicuculline and 5 μM strychnine to block $GABA_A$ and glycine receptors. Under these conditions, EPSCs were mediated mostly by postsynaptic AMPA receptors as they were readily blocked by 100 μM GYKI 52466. As shown in Figure 5A, AMPA EPSCs were recorded in the absence and presence of 5 μM kainate. The peak amplitudes decreased in the presence of kainate and recovered with washout (Figure 5A, top panel). This series was repeated following application of 3 μM SYM 2081 to the bath to block kainate receptors (Figure 5A, bottom panel). Under these conditions, there was still a decrease in the AMPA EPSC amplitude in the presence of kainate accompanied by an increase in synaptic failures. In eight of eight neurons, there was a significant depression of the EPSC in the presence of kainate, with three of these cells also showing an increase of synaptic failures in the presence of kainate.

When kainate is applied to neurons, it activates postsynaptic AMPA receptors and causes a small (~15%), immediately reversible decrease in miniature EPSC amplitude in the presence of 5 μM kainate (C.J.L, unpublished data). To clarify whether the effect of kainate on EPSC amplitude also had a presynaptic component, dorsal horn neurons were held at +50 mV to monitor both fast AMPA and slow NMDA EPSCs simultaneously (see Figure 5B, inset). SYM 2081 at 3 μM was present throughout the experiment to eliminate a contribution by presynaptic kainate receptors. When 5 μM kainate was applied, the amplitudes of both fast AMPA and slow NMDA EPSCs decreased significantly (Figure 5B) in 7 out of 11 cells. This kainate effect recovered during the washout (Figures 5B, top panel) and was blocked by the addition of 100 μM GYKI 52466 (Figure 5B, bottom panel).

Results from the 7 of 11 cells showing significant depression of EPSC amplitude are summarized in Figure 5D. Depression is calculated as the average percent depression of AMPA and NMDA EPSC amplitudes during kainate application relative to amplitudes before kainate application for each cell (see Experimental Procedures). Depression of NMDA EPSCs by kainate was blocked by GYKI 52466 (n = 3/3), indicating that presynaptic AMPA receptors are responsible for the depression. In 3 of the 11 cells, no depression of NMDA EPSCs by kainate was observed, although there was a depression of AMPA EPSCs (data not shown). This is consistent with the observation that not all DRG neurons express AMPA receptor subunits (see Figure 1). In 1 of the 11 cells, AMPA EPSCs could not be detected as a fast peak, although the NMDA EPSC showed a depression in response to kainate. These four cells were not included in Figure 5D.

During these slice recordings, kainate was applied to whole slices. Therefore, it is possible that kainate indirectly affected the glutamate release from primary afferent terminals by activating receptors on other cells, releasing unidentified modulators, and indirectly modulating glutamate release. In order to address this possibility, we performed additional slice recordings in the presence of a cocktail containing known blockers for various receptors. These blockers include 3 μM SYM 2081, 20 μM SCH50911, 50 μM PPADS, 10 μM DPCPX, and sometimes 25 μM LY341495 and 100 μM MPPG. As shown in Figure 5C, in the presence of blocker cocktail, kainate still induced a reduction in the amplitude of both fast AMPA and slow NMDA EPSCs, adding further support to our interpretation that the depressant effect of kainate is mediated by presynaptic AMPA receptors. Of the ten neurons tested in this way, five showed a robust and significant decrease in response to kainate with an average depression of 47% ± 10% for AMPA and 39% ± 9% for NMDA EPSCs (Figure 5E). The percent depression of NMDA EPSC amplitude is less when studied in the presence of cocktail, possibly suggesting that some of the depression observed in the absence of cocktail is due to indirect activation of one of the receptors blocked by cocktail. Nevertheless, this set of experiments further demonstrates that activation of presynaptic AMPA receptors causes inhibition of glutamate release from central terminals of primary afferents.

We were concerned that depression of EPSC amplitude may have been due to shunting of synaptic current by the change in holding current induced by kainate. To assess the possible impact of kainite-induced current on EPSC amplitude, we plotted percent depression of NMDA EPSC amplitude for all 21 neurons included in these studies as a function of the change in holding current following kainate application. As shown in Figure 5F, the kainite-induced holding current varied similarly among cells with and without significant depression of EPSC amplitude, indicating that shunting does not account for the synaptic depression.

AMPA Receptors Contribute to the Generation of Dorsal Root Potentials

Dorsal root potentials generated by stimulation of an adjacent dorsal root, or even a section of the same dorsal root, reflect the induction of PAD by endogenous agonists (Willis, 1999). GABA, acting on $GABA_A$ receptors, is the major mediator of PAD on primary afferents (Eccles et al., 1963; Rudomin and Schmidt, 1999). Even though non-NMDA receptor antagonists depress dorsal root potentials in rat spinal cord (Evans and Long, 1989), it was assumed in those studies that the non-NMDA receptors were mediating activation of inhibitory interneurons that release GABA. However, if AMPA receptors are expressed on primary afferents in the spinal cord and they are activated by synaptically released glutamate accompanying dorsal root stimulation, we would expect to see a component of the dorsal root potential that is sensitive to AMPA receptor antagonists even in the presence of $GABA_A$ receptor antagonists.

To test this possibility, we recorded dorsal root potentials while stimulating an adjacent dorsal root (Figure 6, inset). As shown in Figures 6A and 6B, the control dorsal root potential is partly blocked by the addition of 20 μM bicuculline, 3 μM SYM 2081, and 50 μM APV (Bic+

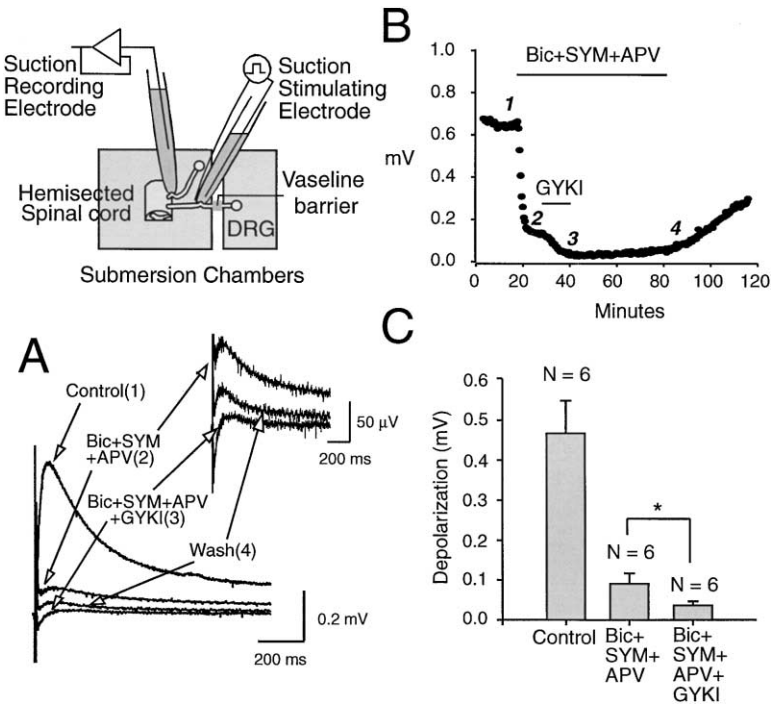

Figure 6. Endogenously Released Glutamate Contributes to the Generation of Dorsal Root Potentials

Dorsal root potentials were recorded from one of the attached dorsal roots while stimulating the adjacent dorsal root (inset) at ~0.03–0.1 Hz. Drugs were applied in the compartment with the spinal cord.

(A) The average traces for each condition are superimposed. The small inset shows an enlargement of the dorsal root potentials after Bic+SYM+APV and GYKI were superfused and washed. Each trace is an average of five traces.

(B) Peak amplitudes of dorsal root potentials plotted as a function of time under different conditions. Numbers 1, 2, 3, and 4 indicate the time that traces (1), (2), (3), and (4) in (A) were taken and averaged.

(C) Peak amplitudes of dorsal root potentials are pooled and plotted. The asterisk (*) indicates a significant difference between Bic+SYM+APV and Bic+SYM+APV+GYKI conditions (paired t test, $p < 0.05$, $n = 6$).

SYM+APV), selective antagonists for $GABA_A$, kainate, and NMDA receptors, respectively. The remaining component in the presence of Bic+SYM+APV was largely abolished with addition of 50 μM GYKI 53655 or 100 μM GYKI 52466, indicating that it requires activation of AMPA receptors. Results from six preparations are summarized in Figure 6C. Averages of five traces for each condition are used to calculate peak amplitudes of dorsal root potentials from each preparation. The peak (0.47 ± 0.08 mV, $n = 6$) was reduced to 19% ± 5% of control by addition of Bic+SYM+APV (0.09 ± 0.03 mV, $n = 6$). Peak responses remaining in Bic+SYM+APV were significantly different from dorsal root potentials in the presence of Bic+SYM+APV plus GYKI 53655 or GYKI 52466 (0.03 ± 0.01 mV, $p < 0.05$ for paired t test, $n = 6$). In four of six experiments, recovery following washout of the GYKI compounds averaged 60% ± 12% following 20–40 min wash. These results demonstrate that AMPA receptors contribute to the generation of the dorsal root potential. The GYKI 52466- or 53655-sensitive dorsal root potential cannot be simply due to block of AMPA receptors expressed by inhibitory interneurons that then release GABA onto the terminals, as suggested by Evans and Long (1989), because GYKI block was recorded in the presence of bicuculline.

Discussion

Subpopulations of DRG Neurons Express Kainate and AMPA Receptors

We have tested for colocalization of GluR1 and GluR2/3 subunit expression in DRG neurons with that of two markers for nociceptor subpopulations from P7–P8 animals: the intermediate filament, peripherin, and the lectin marker, IB4. Our data show that about 50% of GluR1-positive neurons and 23% of GluR2/3-positive neurons costained for peripherin. Interestingly, only ~10% of GluR1- and GluR2/3-positive DRG neurons were positive for IB4. This stands in contrast to our earlier observations showing that 90% of DRG neurons expressing functional kainate receptors also stain for IB4/LA4 markers (Lee et al., 2001). Because such a large proportion of the neurons expressing AMPA receptor subunit is not IB4/LA4 positive while a large proportion of neurons expressing kainate receptor is, we suggest that AMPA and kainate receptors are mostly expressed by different subpopulations of DRG neurons.

While all of the kainate receptor-expressing DRG neurons are nociceptors (Lee et al., 2001), this is not likely to be true of AMPA receptor-expressing neurons. Almost all of the acutely isolated DRG neurons that had physiological responses to AMPA receptor activation expressed capsaicin receptor, suggesting that some of these DRG neurons expressing functional AMPA receptors belong to a subset of capsaicin-sensitive nociceptors. However, many of the GluR1- or GluR2/3-expressing neurons were not positive for peripherin or IB4, suggesting that not all AMPA receptor-expressing DRG neurons are small diameter nociceptors. This is consistent with the observations of Coggeshall and Carlton (1998) who showed that AMPA receptor subunits were expressed on myelinated and unmyelinated peripheral sensory axons. Taken together, these data suggest that unlike kainate receptors, AMPA receptors may be expressed by nonnociceptive DRG neurons as well as nociceptors.

Functional AMPA and Kainate Receptor Expression by Sensory Neurons

Some years ago, kainate was shown to depress C fiber volleys in rat dorsal roots, suggesting a selective action of kainate on nociceptors (Agrawal and Evans, 1986).

This action of kainate was subsequently proposed to be mediated exclusively by kainate receptors because there was no physiological evidence for functional AMPA receptors on acutely dissociated DRG neurons (Huettner, 1990; Wong and Mayer, 1993) or on peripheral axons of nociceptors in the isolated rat spinal cord-tail preparation (Ault and Hildebrand, 1993). We directly tested the identity of the non-NMDA receptors involved with kainate-induced depression of compound action potentials and found that kainate receptors mediate the effect.

The best evidence that AMPA receptors are expressed near the primary afferent terminals is 2-fold. First, activation of AMPA receptors using exogenous agonists induces PAD in a low Ca^{2+} bath. This suggests that AMPA receptors are located on or near the terminals generating the depolarization rather than simply inducing release of other transmitters that activate PAD. Second, in the presence of SYM2081, kainate depressed NMDA receptor-mediated EPSCs, and this effect was blocked by GYKI 52466. Because kainate does not have any effect on NMDA receptor-mediated whole-cell currents in dorsal horn neurons (Kerchner et al., 2001b), we interpret the suppression of the NMDA EPSCs as evidence for a presynaptic action of kainate on AMPA receptors. An alternative interpretation is that kainate could cause changes in postsynaptic receptor levels by inducing postsynaptic receptor internalization. In this case, however, kainate should mainly depress AMPA EPSCs because in young animals, synaptic NMDA receptors do not internalize as readily as AMPA receptors (Grosshans et al., 2002; Luscher et al., 1999; Roche et al., 2001).

There is precedent for presynaptic localization of the ionotropic non-NMDA receptors, including both AMPA and kainate receptors, although the evidence has been more widespread for kainate receptors. A presynaptic role for kainate receptors has been described in the brain as well as in the spinal cord at both inhibitory and excitatory synapses (Lerma, et al., 2001; Kerchner et al., 2001a, 2001b). Kerchner et al., (2001b) recently presented evidence suggesting that exogenous activation of presynaptic kainate receptors is able to suppress glutamate release from primary afferent neurons. However, evidence for presynaptic AMPA receptors has been slower to develop. There are several reports indicating their role in regulation of inhibitory transmitter release. Activation of presynaptic AMPA receptors has been described recently in GABAergic interneurons of cerebellum to inhibit the release of GABA (Satake et al., 2000) and in cerebellar stellate cells to increase the release of GABA during development (Bureau and Mulle, 1998). Overcoming the difficulty of studying a presynaptic receptor at a synapse where it is also postsynaptically localized, Cochilla and Alford (1997) have demonstrated synaptic activation of presynaptic non-NMDA receptors in the lamprey spinal cord by recording directly from the axonal element. Another study has suggested that presynaptic non-NMDA receptors help to regulate transmitter release at sensory nerve terminals in the spinal cord based on their recording of dorsal root potentials but that work did not distinguish between AMPA and kainate receptors (Russo et al., 2000).

In our experiments, we have been able to show that presynaptic kainate and AMPA receptors are able to mediate PAD. We also show that presynaptic AMPA receptors mediate presynaptic inhibition and possibly dorsal root potentials. It is possible that these presynaptic AMPA receptors are developmentally restricted to early postnatal periods as in the experiments of Bureau and Mulle (1998). However, we have recently observed the presence of presynaptic AMPA receptor expression by inhibitory dorsal horn neurons during the 5th postnatal week (H.S.E., unpublished data), indicating that presynaptic AMPA receptors are not necessarily expressed only in the immature nervous system.

The apparent preferential expression of functional AMPA receptors near the primary afferent terminals is unusual for ionotropic receptors on DRG neurons. Most other families of ionotropic receptor are found both at the terminals and on the cell bodies. For example, $GABA_A$ receptors are found on primary afferent terminals (Sur et al., 1995), dorsal roots (Agrawal and Evans, 1986), and cell bodies (Desarmenien et al., 1984) of DRG neurons. Kainate receptors are also expressed on the cell bodies (Huettner, 1990), dorsal roots (Figure 3B) (Agrawal and Evans, 1986), and terminals (Figure 4) (Kerchner et al., 2001b; Hwang et al., 2001). Capsaicin-sensitive VR1 receptors are also found in all of these places (Guo et al., 1999). The importance of this specialized expression pattern for AMPA receptors is unknown. Interestingly, though, it is possible that NMDA receptors in DRG neurons follow an expression pattern similar to presynaptic AMPA receptors. This is suggested by the lack of functional responses in the somata (e.g., Huettner, 1990) but positive detection of NMDA receptor subunit mRNA and protein in the somata and dorsal roots (Sato et al., 1993; Liu et al., 1994).

Morphological and Physiological Basis for Action of AMPA Receptors

The local circuitry underlying GABA-mediated PAD in the dorsal horn is a matter of some debate (Rudomin and Schmidt, 1999; Willis, 1999), and for AMPA receptors, it is unknown. In the mammalian spinal cord, some primary afferent terminals form glomeruli where many axo-axonic, dendro-axonic, and axo-dendritic synapses are clustered in a giant synaptic complex (Ribeiro-Da-Silva et al., 1985). These glomeruli have been proposed as the structural basis for GABAergic PAD (Rudomin and Schmidt, 1999; Willis, 1999). The majority of our experiments on spinal cord preparations were performed using P6 –P10 animals. Even though glomeruli associated with C-type afferent terminals are present in the superficial dorsal horn toward the end of the first postnatal week (Pignatelli et al., 1989), glomeruli are believed to account for only a small proportion of the primary afferent terminals in both the developing and mature dorsal horn. Furthermore, the strongest sensory connections in lamina I and II at these ages are mediated by Aβ fibers that later withdraw to deeper lamina (Fitzgerald et al., 1994; Mirnics and Koerber, 1995). Immunocytochemical evidence from the present study suggests that both nociceptive and nonnociceptive sensory neurons express AMPA receptor subunits. Some portion of presynaptic inhibition of glutamate release in lamina II, of PAD, and of dorsal root potentials is mediated by AMPA re-

ceptors in the presence of $GABA_A$ and kainate receptor antagonists. Whether these effects are mediated by AMPA receptors expressed by the weakly connected Aδ and C fibers or by the transiently present Aβ fibers is not known.

Presynaptic AMPA receptor-mediated inhibition of GABA release onto Purkinje cells is believed to occur due to spillover of glutamate from the climbing fiber at its synapse with the Purkinje cell (Satake et al., 2000). Based on their dorsal root potential recordings in the presence of low concentrations of TTX, Russo et al. (2000) suggested that endogenously released glutamate can activate presynaptic AMPA receptors, possibly by a spill-over mechanism. In this case, dorsal root potentials may be generated due to glutamate release from neighboring primary afferent terminals and activation of AMPA receptors on adjacent primary afferent terminals. We were unable to record dorsal root potentials in low concentrations of TTX (C.K.T., unpublished data) as performed with the turtle spinal cord in Russo et al. (2000) and thus were unable to eliminate dorsal horn interneurons as a source of glutamate in our experiments. Nevertheless, spill-over from nearby primary afferent terminals could be a mechanism that explains our GYKI 52466- and GYKI 53655-sensitive dorsal root potentials. Another possibility is that the presynaptic AMPA receptors could serve as autoreceptors sensing glutamate release from their own terminals.

The mechanism driving AMPA receptor-mediated presynaptic inhibition at the primary afferent to dorsal horn neuron synapse is probably PAD (Rudomin and Schmidt, 1999), which in turn causes action potential shunting (Segev, 1990) or sodium channel inactivation at the sensory afferent central terminals (Graham and Redman, 1994). AMPA receptor-mediated depolarization may shunt the action potential propagation by creating a local area with elevated input conductance, which decreases the magnitude of depolarization during the action potential and slows action potential propagation. AMPA receptor-mediated depolarization may also inactivate voltage-gated sodium channels, thus making the sodium channels less available for action potential generation.

In the present study, presynaptic AMPA receptors, like kainate receptors (Kerchner et al., 2001b), are shown to have a strong inhibitory action on the amount of glutamate released from some primary afferent terminals in the superficial dorsal horn. The expression of AMPA and kainate receptors by nociceptors makes it plausible to predict that these receptors participate in activity-dependent generation of PAD in Aδ and C fibers. It is also possible that these receptors might participate in the generation of dorsal root reflexes. This is particularly significant as dorsal root reflexes have been implicated in generation of neurogenic inflammation (Willis, 1999). Along with the presynaptic kainate receptors, AMPA receptors should prove to be an important regulator of synaptic transmission in the pain pathway as well as an important modulator of nonnoxious sensory transmission.

Experimental Procedures

Immunocytochemisty of Postnatal DRG

Five P7–P8 pups were deeply anesthetized with isoflurane and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. Lumbar DRGs were removed and postfixed for 2 hr, rinsed in 0.1 M PB, and equilibrated in 30% sucrose in 0.1 M PB. DRGs were mounted in Tissue-Tek OCT compound. Cryostat sections of 15 μm thickness were prepared and transferred to Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were first incubated in a blocking solution of 10% normal goat serum in PBS with 0.1%–0.3% Triton X-100 (1% BSA was sometimes added). All antibodies were diluted in PBS with 0.1% Triton X-100 and 1% normal goat serum (PBS-TG) or in the blocking solution. Primary antibodies and lectins used were polyclonal rabbit anti-GluR1 or anti-GluR2/3 (1 μg/ml, Chemicon, Temecula, CA), and either monoclonal mouse anti-peripherin ascites fluid (1:100, Chemicon) or IB4-biotin (2 μg/ml, Sigma, St Louis, MO). For negative controls, DRG sections were incubated without primary antibodies or IB4. Secondary antibodies used were Cy3-conjugated goat anti-rabbit IgG (1:500, Jackson Immunoresearch, West Grove, PA) and Alexa488-conjugated goat anti-mouse IgG (1:500, Molecular Probes, Eugene, OR) for GluR/peripherin staining; Alexa488-conjugated goat anti-rabbit IgG and Alexa568-streptavidin (1:500, Molecular Probes) were used for GluR/IB4 staining. Sections were mounted in ProLong Antifade (Molecular Probes).

Digital images of GluR/peripherin staining (six sections per condition from three DRGs from two pups) and GluR/IB4 staining (four to seven sections from different DRGs from two pups) were acquired and analyzed with ScionImage and Corel Photo-Paint software. Pixel values for each image were stretched from 0 to 255 gray levels. As a criterion for counting cells, thresholds for positive staining were determined based on the highest measured intensity from a negative cell body (e.g., see Figure 1A, single arrowhead for GluR1 negative; or Figure 1B, double arrowhead for peripherin negative). As an additional criterion, cells with diffuse cytoplasmic staining for GluRs or peripherin, and punctate cytoplasmic staining for IB4 were counted as positive. Images were pseudo-colored red for GluR staining and green for peripherin or IB4 staining after thresholds were set. Each marker was independently counted and then checked for an overlap for double positives. All the average values in this study are expressed as mean ± SEM. More than 900 GluR1, 1300 GluR2/3, 1000 IB4, and 1700 peripherin-positive cells were counted.

Acute DRG Preparation and Ca^{2+} Imaging

P3–P9 rats were deeply anesthetized with isoflurane, decapitated, and then cervical, thoracic, and lumbar DRGs were dissected out. Isolated DRGs were exposed to 1 mg/ml trypsin (Type III, Sigma) dissolved in the external bath solution without $CaCl_2$ and $MgCl_2$ for 10 min at 37°C. The trypsin was subsequently inactivated by addition of an equal volume of 1 mg/ml trypsin inhibitor (Type II-O, Sigma), dissolved in the external bath solution with $CaCl_2$ and $MgCl_2$. The DRGs were mechanically dissociated with fire-polished glass pipettes. Cells were washed twice by centrifugation then plated on poly-D-lysine-coated glass coverslips. The coverslips were placed in an incubator for 30–40 min in order to allow cells to settle and attach. Cells were then loaded with 5 μM fura-2 AM for 15 min at room temperature and washed. Background-subtracted intensity images at two excitation wavelengths (340 and 380 nm) were acquired using an intensified CCD camera and Axon Imaging Workbench 2.1(Axon Instruments, Foster City, CA) or Video Probe (ETM Systems) image processing programs. For some experiments, ratios of the intensity images were further converted to Ca^{2+} concentration. Detailed methods are given elsewhere (Gu et al. 1996; Kyrozis et al., 1995). SYM 2081, a strongly desensitizing kainate receptor agonist, was present in the external bath solution at 3 μM all the time to fully desensitize and block kainate receptors. GYKI 52466 was preapplied by addition to the bath for at least 1 min before applying 100 μM kainate plus GYKI 52466 to ensure complete block. Drug solutions were applied local by perfusion through a capillary tube. The solution flow was driven by gravity and controlled by miniature solenoid valves (The Lee Company, Westbrook, CT).

Recording from Dorsal Roots and Primary Afferent Terminals

Lumbar and sacral spinal cords with roots attached were obtained from postnatal rats (P6–P12). The spinal cord with attached dorsal root was excised and placed in ice-cold oxygenated Krebs solution (95% O2/5% CO_2 saturated Krebs solution of 125 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM glucose, 1 mM

$MgCl_2$, and 2 mM $CaCl_2$, pH 7.4, 320 mOsm). After removal of the dura mater, all ventral roots were cut near the root entry zone. Several parallel cuts were made through the spinal cord between L1 and S4 roots and then the spinal cord sections were hemisected sagittally using a razor blade. After recovery in oxygenated Krebs for at least 1 hr at room temperature, the tissues or isolated roots were placed in a submersion-type recording chamber and superfused continuously with the Krebs solution at 23°C–25°C.

For compound action potential recording, dorsal roots of S1–S4 with DRG attached were transferred to a recording chamber (see Figure 3, inset). Two suction electrodes were used: one for electrical stimulation of the root and the other for recording compound action potentials. Electrical stimulation was performed at ~0.1–0.2 Hz for 100 μs. Experiments were performed at room temperature (23°C–25°C) in oxygenated Krebs solution. To rule out the possibility that the drug had some effect on the DRG itself, a Vaseline barrier was used to separate the compartments of the chamber and isolate the DRG so that the DRG was not in contact with any drug. Compound action potentials were filtered at 10 kHz and sampled at 20 kHz.

For spinal cord-dorsal root DC depolarization (PAD) recording, we modified the method of Hackman et al. (1997). The tissue was placed across a Vaseline seal between the hemisected cord and the dorsal root (see Figure 4B). The Vaseline seal made the drug containing Krebs solution flow exclusively through the cord without contacting the DRG and increased the output resistance of the dorsal root to magnify the amplitude of the voltage signals. A suction recording electrode was used with an indifferent electrode made of 3 M NaCl/3% agarose as the ground in the same chamber as the cord. DC potential was measured between the indifferent electrode and the root recording electrode. Potentials were filtered with a low pass 30 Hz or 3–10 kHz filter and sampled at 125–167 Hz. Reported peak amplitudes are averages calculated over a 0.2 s duration. For some experiments, kainate caused a slight hyperpolarization of the terminals in the presence of GYKI 53655 plus SYM 2081. In these cases, the blocking effect of the added drug on agonist induced depolarization was considered 100%.

For dorsal root-dorsal root potential (DR-DRP) recording, hemisected spinal cords with two roots attached were kept in a submersion style chamber. The DRG of the root being stimulated was kept in another compartment separated from the spinal cord with Vaseline (Figure 6, top). Electrical stimulation was performed at 0.03–0.1 Hz for 100 μs, and the DR-DRP was filtered at 10 kHz and recorded at 10 kHz for 120 ms and 833 Hz for 1 s. Experiments were performed at room temperature (23°C –25°C) in oxygenated Krebs solution.

Spinal Cord Slice Preparation, Stimulation of Dorsal Roots, and Recording

Recordings of excitatory postsynaptic currents were performed on lamina II neurons in spinal cord slices from postnatal rats (P2–P14). Postnatal rats were anaesthetized with halothane, decapitated, and the cervical or lumbar region of the spinal cord removed. Transverse slices (400–600 μm) with attached dorsal roots were obtained after gluing the spinal cord to an agarose block. Slices were incubated in oxygenated Kreb's at 35°C for 1 hr and used for recording. Intracellular recording solution contained 130 mM Cs-gluconate, 10 mM CsCl, 11 mM EGTA, 1 mM $CaCl_2$, 10 mM HEPES, and 2 mM Mg^{2+}-ATP, pH adjusted to 7.2 with NaOH, osmolarity adjusted to 305 mOsm with sucrose. 10 μM bicuculline and 5 μM strychnine were included in the control bath.

Dorsal roots were stimulated using a glass suction electrode. The stimuli had intensities ranging from 5 to 500 μA (constant current SIU output) and durations of 0.05–0.5 ms at a frequency of ~0.05–0.03 Hz. Threshold was found by adjusting the intensity from low to high. Once the threshold was identified, the stimulation intensity was adjusted slightly higher than threshold. Patch-clamp recording in whole-cell configuration was performed on visually identified lamina II neurons at room temperature. Data were recorded and acquired using an Axopatch-1D amplifier and pClamp6 software. The EPSC amplitudes were detected with Clampfit 6 (Axon Instruments) and Minianalysis (Synaptosoft). At +50 mV, peaks of AMPA EPSCs were measured 3–4 ms after the start of the rise and peak NMDA EPSCs were measured 15–16 ms after the start of the rise. Significance of depression of EPSC amplitude was determined for each cell by comparing the five NMDA EPSCs recorded before kainate to the last five NMDA EPSCs evoked in kainate using an unpaired t test with a two-tailed p value. $p < 0.05$ was considered significantly depressed. The average traces were obtained by averaging five consecutive events. The percent depression was calculated using average traces and was defined as following:

$$\%\ \text{depression} = (EPSC_{peak,control} - EPSC_{peak,kainate}) \times 100/EPSC_{peak,control}.$$

All of the averaged data in this paper are expressed as mean ± SE.

Materials

GYKI 52466 and cyclothiazide were purchased from RBI (Natick, MA). GYKI 53655 was a gift from EGIS Pharmaceutical Ltd. Kainate, SYM 2081, SR95531, and SCH50911 were purchased from Tocris Cookson, (Ballwin, MO). Fura-2 AM was from Molecular Probes. Other drugs were obtained from Sigma.

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