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Subpopulations of GABAergic and non-GABAergic rat dorsal horn neurons express Ca^{2+}-permeable AMPA receptors

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Abstract

Subpopulations of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors that are either permeable or impermeable to Ca^{2+} are expressed on dorsal horn neurons in culture. While both mediate synaptic transmission, the Ca^{2+}-permeable AMPA receptors provide a Ca^{2+} signal that may result in a transient change in synaptic strength [Gu, J.G., Albuquerque, C., Lee, C.J. & MacDermott, A.B. (1996) *Nature*, **381**, 793]. To appreciate the relevance of these receptors to dorsal horn physiology, we have investigated whether they show selective expression in identified subpopulations of dorsal horn neurons. Expression of Ca^{2+}-permeable AMPA receptors was assayed using the kainate-induced cobalt loading technique first developed by Pruss *et al.* [Pruss, R.M., Akeson, R.L., Racke, M.M. & Wilburn, J.L. (1991) *Neuron*, **7**, 509]. Subpopulations of dorsal horn neurons were identified using immunocytochemistry for γ-aminobutyric acid (GABA), glycine, substance P receptor (NK1 receptor) and the Ca^{2+}-binding proteins, calretinin and calbindin D28K. We demonstrate that, in dorsal horn neurons in culture, kainate-induced cobalt uptake is selectively mediated by Ca^{2+}-permeable AMPA receptors, and that a majority of GABA and NK1 receptor-expressing neurons express Ca^{2+}-permeable AMPA receptors. GABAergic dorsal horn neurons are important in local inhibition as well as in the regulation of transmitter release from primary afferent terminals. NK1 receptor-expressing dorsal horn neurons include many of the projection neurons in the nociceptive spino-thalamic pathway. Thus, we have identified two populations of dorsal horn neurons representing important components of dorsal horn function that express Ca^{2+}-permeable AMPA receptors. Furthermore, we show that several subpopulations of putative excitatory interneurons defined by calretinin and calbindin expression do not express Ca^{2+}-permeable AMPA receptors.

Introduction

Ionotropic glutamate receptors that respond to the glutamate analogue α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) mediate most fast synaptic excitation in the central nervous system. The majority of natively assembled AMPA receptors have low permeability to Ca^{2+}. At synapses with these AMPA receptors, another ionotropic glutamate receptor, the *N*-methyl D-aspartate (NMDA) receptor, is often the mediator of activity-dependent postsynaptic Ca^{2+} increases that can initiate changes in synaptic strength (Bliss & Collingridge, 1993). A subpopulation of AMPA receptors, however, has substantial Ca^{2+} permeability. This Ca^{2+} permeability is regulated by the inclusion of one or more GluR2 (GluRB) subunits into the heteromeric protein structure, which dramatically reduces Ca^{2+} flux through the AMPA receptor channels (Burnashev *et al.*, 1992). Our studies have shown that individual dorsal horn neurons can express both Ca^{2+}-permeable and Ca^{2+}-impermeable AMPA receptors (Goldstein *et al.*, 1995; Gu *et al.*, 1996). The implication is that the same neurons can express AMPA receptors including GluR2 and others excluding GluR2, indicating that GluR2 expression cannot be taken as evidence for the absence of Ca^{2+}-permeable AMPA receptors.

Understanding the role of Ca^{2+}-permeable AMPA receptors in the transmission and modulation of synaptic signals requires characterization of their distribution in specific neuronal types. In the superficial dorsal horn of the spinal cord, a region where nociceptive input is relayed and modulated, expression of Ca^{2+}-permeable AMPA receptors has been demonstrated using kainate-induced cobalt loading (Nagy *et al.*, 1994; Engelman *et al.*, 1997), which takes advantage of the known cobalt permeability of Ca^{2+}-permeable AMPA receptors (see Pruss *et al.*, 1991). This assay for functional Ca^{2+}-permeable AMPA receptors is essential because immunostaining for GluR2 does not accurately identify neurons excluding Ca^{2+}-permeable AMPA receptors. Previously, Ca^{2+}-permeable AMPA receptors were detected in ≈60% of embryonic dorsal horn neurons grown in culture by measuring Ca^{2+} fluxes (Reichling & MacDermott, 1993). Later, it was demonstrated that Ca^{2+}-permeable AMPA receptors are expressed at synapses on cultured dorsal horn neurons and can trigger synaptic potentiation there (Gu *et al.*, 1996). However, the distribution of Ca^{2+}-permeable AMPA receptors by dorsal horn neuronal type is still largely unknown. One exception is the identification of Ca^{2+}-permeable AMPA receptor expression by lamina I neurons expressing the NK1 receptor, the receptor for the neuromodulator, substance P (Engelman *et al.*, 1999). These neurons are likely to be spino-thalamic projection neurons and thus important in the transmission of nociceptive information to higher brain centres

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(Littlewood *et al*., 1995; Marshall *et al*., 1996). Here we use a combination of immunocytochemical markers for cell type in combination with kainate-induced cobalt loading to further investigate dorsal horn neuronal cell populations that do or do not express Ca^{2+}-permeable AMPA receptors. Parts of this work were published previously in abstract form (Albuquerque *et al*., 1996, Albuquerque & MacDermott, 1998).

Materials and methods

Cell culture

Dorsal horn neurons and dorsal root ganglion (DRG) neurons were isolated from rat embryos aged 15–16 days and were cultured on glass coverslips coated with a monolayer of rat cortical astrocytes (modified from Reichling & MacDermott, 1993). Briefly, pregnant rats were killed by CO_2 asphyxiation followed by cervical dislocation, and the embryos were removed and transferred to ice-cold Leibowitz-15 medium (Gibco, Grand Island, NY, USA). The embryos were then removed from the uterus and the spinal cord dissected out. The dorsal third of the spinal cord was removed and incubated for 20 min with trypsin in S-MEM (Gibco) at 37 °C in a 5% CO_2, humidity-saturated atmosphere, then centrifuged and resuspended in culture medium (either MEM + vitamins + glucose + 10% horse serum, or neurobasal + glutamine + B-27 supplement, all from Gibco). The DRGs were plucked from the spinal cord and treated in the same way as the dorsal horns. For cultures containing DRG neurons, 50 ng/mL nerve growth factor (NGF; Boehringer Mannheim, Indianapolis, IN, USA) was added to each dish. For all cultures, 10 μM each of uridine and 5′-fluoro-2′-deoxyuridine (Sigma, St Louis, MO, USA) were added to the cultures to inhibit cell division (next day for dorsal horn neuron-only cultures, same day for DRG neuron-containing cultures). Cultures were maintained for up to 5 weeks at 37 °C, in a 5% CO_2, humidity-saturated atmosphere, with half of the medium being replaced with fresh medium each week.

Cobalt loading

Dorsal horn neuron cultures or dorsal horn/DRG neuron co-cultures were washed once with uptake buffer (in mM: sucrose, 139; NaCl, 57; KCl, 5; $MgCl_2$, 2; $CaCl_2$, 1; glucose, 12; HEPES, 10; pH 7.6 with NaOH) and then incubated at room temperature with 250 μM kainate and 5 mM $CoCl_2$ in uptake buffer for 20 min. This was followed by washing with uptake buffer [sometimes two washes with uptake buffer plus 5 mM ethylenediaminetetraacetic acid (EDTA) were included to reduce extracellular silver deposition], then incubation in a 0.12% solution of ammonium sulphide in uptake buffer for 5 min. The cells were then washed and fixed for 10–30 min in 4% paraformaldehyde in phosphate-buffered saline (PBS, usually 4% sucrose was added to the fixative to help preserve morphology). Finally, the cobalt sulphide formed inside the cobalt-permeable cells by the ammonium sulphide incubation was silver-intensified using the method described by Davis (1982). The coverslips with cells were mounted in Aquamount (Lerner Laboratories, Pittsburgh, PA, USA) plus 50% glycerol and sealed with nail polish.

To test for permeation of cobalt through voltage-gated Ca^{2+} channels, a high-potassium uptake buffer, consisting of (in mM): KCl, 140; $MgCl_2$, 2; $CaCl_2$, 1; $CoCl_2$, 5; glucose, 12; HEPES, 10; pH 7.61 with NaOH, was applied to the cells for 20 min. Controls included normal uptake buffer plus kainate, kainate plus CNQX (5-[3H]6-cyano-7-nitro-quinoxaline-2,3-dione, from Tocris, Ballwin, MO, USA), kainate plus GYKI-53655 (a generous gift from Lilly Research Laboratories) and high-potassium uptake buffer plus 30 μM $LaCl_3$ (to block voltage-gated Ca^{2+} channels).

To test for cobalt permeation through NMDA receptors, we added 100 μM NMDA, 5 mM $CoCl_2$ and 5 μM glycine to uptake buffer without $MgCl_2$.

To control for the possibility of a negative interaction between cobalt staining and the presence of calretinin or calbindin due to buffering of the cobalt by the Ca^{2+}-binding proteins, we compared kainate-stimulated cultures with sister cultures treated for the same time with the ionophore lasalocid (X537A; 40 μM; Sigma).

JsTx block

Joro spider toxin (JsTx, Calbiochem, La Jolla, CA, USA) is a slow, open-channel blocker of Ca^{2+}-permeable non-NMDA receptors. In order to assess its ability to block kainate-induced cobalt uptake, the cultures were pre-incubated with 5–10 μM JsTx plus 100–250 μM kainate for 4–5 min, immediately followed by the regular cobalt-loading incubation. In one experiment, both incubations were performed in uptake buffer; in the others, the pre-incubation was performed in a 0Na/0Ca/20Cs buffer (in mM: NMDG, 155; HCl, 144; CsCl, 20; $CaCl_2$, 0.02; HEPES, 10; glucose, 5.5; pH 7.3, 317 mOsm, adjusted with sucrose; Gu *et al*., 1996). This was done to prevent the neuritic blebbing that was observed in Ca^{2+}-permeable AMPA receptor-positive cells subjected to this protocol. While using low-Ca^{2+} solutions did preserve the cells from neuritic blebbing, it also caused retraction of the underlying astrocytic layer.

Immunocytochemistry

All antibodies used in this study have been previously characterized and found to be highly selective. Rabbit anti-GABA antiserum (INCStar/DiaSorin, Stillwater, MN, USA, Maley & Newton, 1985) was used at 1 : 1000; the monoclonal antibody anti-GABA MAB316 (Szabat *et al*. 1992) and rabbit anti-glycine antiserum (both from Chemicon, Temecula, CA, USA) were used at 1 : 100; rabbit anti-calretinin and mouse monoclonal anti-calbindin D28K (both from SWant, Bellinzona, Switzerland, Celio *et al*., 1990; Schwaller *et al*., 1993) were used at 1 : 1000 to 1 : 5000; and the rabbit anti-NK1 receptor (a generous gift from S.R. Vigna, Duke University, Durham, NC, USA; Vigna *et al*., 1994) was used at 1 : 1000.

Dorsal horn or dorsal horn–DRG cultures were fixed for 15 min with 4% paraformaldehyde in PBS plus 4% sucrose, except for the GABA/glycine co-staining, where the GABA monoclonal antibody required that 0.005% glutaraldehyde be added to the fixative. The cultures were then incubated for 30 min to 2 h with the appropriate concentration of primary antibody, in PBS plus 0.1% (or 0.5%) Triton X-100, at room temperature. After careful washing, the cells were treated either with the LSAB kit (DAKO, Carpinteria, CA, USA) or fluorophore-coupled secondary antibodies. The LSAB kit was used according to the instructions of the manufacturer (a 20-min development time was usually required for optimum staining), except that the hydrogen peroxide incubation was eliminated because it interfered with the cobalt staining. No increased background was observed in the absence of primary antibody because of this modification of the original protocol. Fluorophore-coupled secondary antisera were added for 30 min at room temperature, at the following concentrations: 1 : 500 for Cy3-coupled antisera; 1 : 200 for Cy2-coupled antisera (both from Jackson Immunoresearch, West Grove, PA, USA), 1 : 500 and 1 : 100 for Alexa 488- and AMCA-S-coupled antisera, respectively (both from Molecular Probes, Eugene, OR, USA).

Combined cobalt and immunocytochemistry

In those experiments where cobalt staining and immunostaining were sequentially performed, the cells were subjected to the cobalt uptake protocol prior to the fixation step. After that, cells were processed according to the appropriate immunostaining technique, and the coverslips were then mounted in a perfusion chamber to maintain a constant orientation. Images of sequentially located fields were acquired with a CCD-coupled frame grabber or standard photographic techniques, using a water-immersion objective. The coordinates of each field were recorded. The cobalt staining was then silver-enhanced as described, and pictures of the same fields were taken again. Cell counts were then performed from the pairs of pictures taken of each field.

In the Results, *n* means the number of different individual culture dishes counted from the same or different experiments, except for the time-course experiment', where *n* represents different sets of culture dishes, originating from different dissections. All results are percentages (± SEM).

Results

Specificity of cobalt uptake

Dorsal horn neurons maintained for 14 days *in vitro* (DIV) or longer were exposed to 250 μM kainate and 5 mM $CoCl_2$. Some neurons demonstrated kainate-induced cobalt uptake, which could be readily detected after silver development (Fig. 1A) as an amber to black staining. Even in these relatively mature cultures, different levels of staining were always present, with some cells showing strong staining while others showed a staining that was weaker but still made them clearly distinguishable from cobalt-negative cells. This variability reflects the previously reported variability in the proportion of Ca^{2+}-permeable AMPA receptors relative to the total AMPA receptor population on individual dorsal horn neurons in culture (Goldstein *et al*., 1995; Gu *et al*., 1996).

The non-NMDA receptor competitive antagonist, CNQX (50 μM), completely prevented kainate-induced cobalt uptake (Fig. 1B), indicating that cobalt-permeable AMPA or kainate receptors were mediating cobalt uptake. As dorsal horn neurons have been reported to express both AMPA and kainate receptor subunits (Tachibana *et al*., 1994; Petralia *et al*., 1994), we tested whether the AMPA receptor-selective, non-competitive antagonist, GYKI 53655, was also able to prevent kainate-induced cobalt uptake. As shown in Fig. 1C and D, complete block of cobalt staining was obtained with 50 μM GYKI 53655, indicating that cobalt entry occurred predominantly through Ca^{2+}-permeable AMPA receptors. As a further test, we applied the selective antagonist of Ca^{2+}-permeable non-NMDA receptors, JsTx. JsTx had to be pre-applied with kainate in order to block cobalt uptake because it is a use-dependent, open-channel blocker (Blaschke *et al*., 1993; Iino *et al*., 1996). JsTx (10 μM) produced a strong but incomplete block of cobalt loading as seen by comparing a blocked culture with its paired, unblocked control (Fig. 1E and F; see Materials and methods). The JsTx block was never as complete as with the other, fast-acting antagonists, presumably because of the more complex mechanism of this block.

We tested whether kainate-induced cobalt influx could occur through NMDA receptors indirectly activated by kainate-stimulated glutamate release or through voltage-gated Ca^{2+} channels activated by kainate-induced depolarization. These possibilities were assessed by determining whether cobalt uptake was induced when mature dorsal horn neuron cultures were treated with a modified uptake buffer containing 140 mM KCl to activate voltage-gated Ca^{2+} channels. In this case, no cobalt uptake was observed (Fig. 1G). The high-KCl experiments also included a paired control using kainate as the agonist which always resulted in strong staining in a subpopulation of dorsal horn neurons (data not shown). Similarly, NMDA (250 μM), together with 5 μM glycine in uptake buffer with no added Mg^{2+}, did not induce cobalt uptake (Fig. 1H), even though strong staining was detected in a sister culture treated with kainate (not shown). Thus, based on these experiments under the conditions we have defined, kainate-induced cobalt uptake is a direct indication of expression of Ca^{2+}-permeable AMPA receptors.

Developmental expression of Ca^{2+}-permeable AMPA receptors

The percentage of cells expressing Ca^{2+}-permeable AMPA receptors varied considerably from experiment to experiment (average, 46.9 ± 3.7%, $n = 13$; range, 23–69%). Many factors could be responsible for this variation, including slight variations in dissection, density of cultures and time in culture. We specifically investigated the change in percentage of cells showing kainate-induced cobalt permeability over time in culture by counting the number of cobalt-positive cells grown from 1 to 32 DIV ($n = 2$). There was a steep increase in the percentage of cobalt-positive cells over the first week in culture, and a slower increase over the second week, until it stabilized at ≈60% between 16 and 32 DIV (Fig. 2). These data indicate a developmental regulation of Ca^{2+}-permeable AMPA receptor expression with the most rapid increase in expression happening at a time of intense synaptogenesis in the cultures.

GABA and glycine immunoreactivities

One striking aspect of the distribution of Ca^{2+}-permeable AMPA receptors in cortical structures is their restriction to subpopulations of GABAergic interneurons (Yin *et al*., 1994; Kharazia *et al*., 1996; Racca *et al*., 1996). A correlation is thus suggested between expression of Ca^{2+}-permeable AMPA receptors and inhibitory function in a neuron. We tested whether Ca^{2+}-permeable AMPA receptor expression in dorsal horn neurons correlates with GABA expression, and examples of those results are shown in Fig. 3. In cultures between 9 and 22 DIV, we found 32.5 ± 2.9% of the total neuronal population to express GABA-like immunoreactivity (GABA-LI; $n = 13$; 2176 neurons counted; Table 1). Sequential staining for GABA and kainate-induced cobalt uptake was performed, and the majority of the GABAergic neurons was found to express Ca^{2+}-permeable AMPA receptors. Based on counts of 661 neurons in five dishes, we found that 58.6 ± 4.2% of the GABA-LI neurons were cobalt-positive.

In the dorsal horn, glycine is also used as a fast, inhibitory neurotransmitter and is often, but not always, co-localized with GABA (Todd & Sullivan, 1990). To determine whether co-expression of glycine and GABA occurs in culture, we performed double-staining experiments with antibodies against GABA and glycine. We found that 83.2 ± 3.6% of the cells expressing glycine-LI in our cultures also expressed GABA-LI ($n = 3$; 404 neurons counted). This is likely to be an underestimate as the glutaraldehyde fixation required for these experiments enhanced detection of glycine-LI, making it harder to distinguish cells with metabolic levels of glycine from those that actually concentrate the amino acid to use as a neurotransmitter. Nevertheless, because 83% of the glycine-LI neurons are GABA-LI, our results with kainate-induced cobalt uptake and GABA-LI neurons may be representative of the glycine-LI population of neurons.

Cobalt-positive GABA-LI neurons comprise only 38.8 ± 5.9% of the total population of cobalt-positive neurons in our dorsal horn

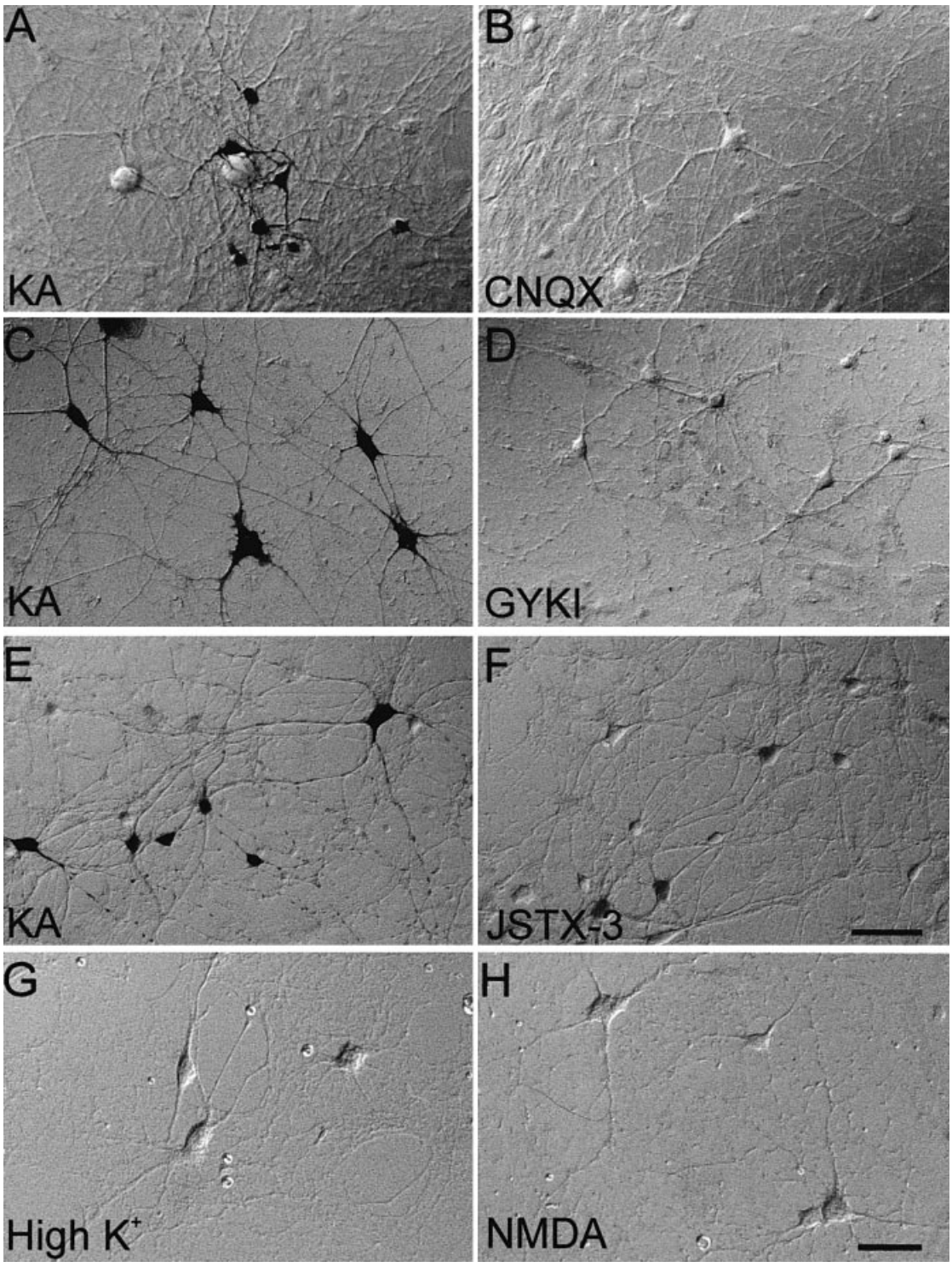

FIG. 1. Kainate-induced cobalt uptake specifically reveals neurons expressing Ca^{2+}-permeable AMPA receptors. Neuronal cultures were exposed for 20 min to agonist or agonist plus antagonist in the presence of 5 mM $CoCl_2$, then washed and the cobalt uptake revealed with silver enhancement (see Materials and methods). (A, C and E) Positive controls for conditions depicted to their right: cells were exposed to 250 μM kainate. (B) Inhibition of cobalt uptake by 50 μM CNQX. (D) Inhibition of cobalt uptake by 50 μM GYKI 53655. (F) Inhibition of cobalt uptake by 5 μM joro spider toxin (JsTx). Because JsTx is an open-channel blocker, in E and F cells were pre-incubated for 5 min with 100 μM kainate with or without JsTx, in the absence of $CoCl_2$. (G) Failure of high-potassium solution to cause cobalt uptake. (H) Failure of 100 μM NMDA with 5 μM glycine to cause cobalt uptake. In G and H the procedure was similar to the experiments with blockers, except that the stimulation solution was changed as appropriate (see Materials and methods). Controls with kainate-stimulated cobalt uptake for G and H were similar to A, C and E (data not shown). Scale bars, 50 μm (A–F); 25 μm (G and H).

cultures, indicating that the majority of the neurons expressing Ca^{2+}-permeable AMPA receptors are not GABAergic. We thus attempted to identify other, presumably excitatory, neuronal subpopulations that are cobalt-positive. Results are summarized in Table 1.

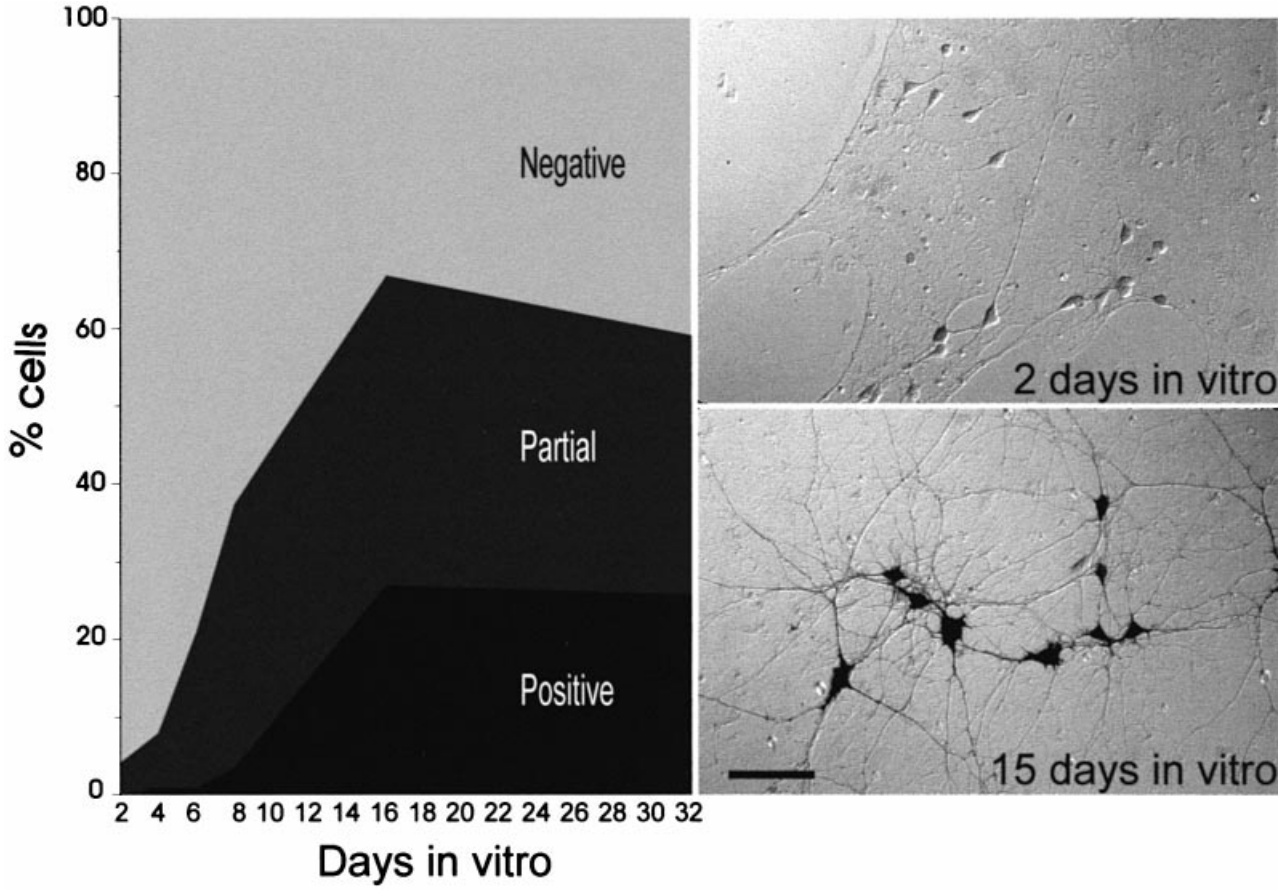

FIG. 2. Time course of expression of Ca^{2+}-permeable AMPA receptors in embryonic dorsal horn neuron cultures. Left, the area graph depicts the number of 'positive', 'partial' and 'negative' cells according to the level of cobalt uptake (respectively, high, intermediate and none). Data were obtained from two culture dishes plated from different dissections and sampled at 2, 4, 6, 8 or 9, 15 or 16 and 32 days after plating. Right, the top picture shows a representative field from a culture stained for kainate-induced cobalt uptake 2 days after plating. The bottom picture shows a field from a culture stained at 15 days after plating. Scale bar, 50 μm.

Neurokinin-1 receptor immunoreactivity

The NK1 receptor is expressed in the adult dorsal horn in neurons that lack GABA- and glycine-LI (Littlewood *et al*., 1995), and therefore is a good marker for a subpopulation of excitatory neurons. We examined whether NK1 receptor-like immunoreactivity (NK1-LI) and kainate-induced cobalt uptake co-localize in our cultures. Using DRG/dorsal horn co-cultures, we found that 4.6 ± 0.3% of the dorsal horn neurons ($n = 3$; 1165 neurons counted) were positive for NK1-LI. Of these NK1-LI neurons, 77 ± 11.9% expressed kainate-induced cobalt permeability. Figure 4 shows examples of NK1-LI neurons that are both cobalt-positive and -negative. The high correlation of NK1-LI with kainate-induced cobalt uptake indicates that we have identified a population of excitatory neurons in the dorsal horn cultures, the majority of which expresses Ca^{2+}-permeable AMPA receptors.

Ca^{2+}-binding proteins

Calbindin and calretinin are Ca^{2+}-binding proteins expressed in neurons throughout the nervous system. Their expression patterns in different neuronal populations have been found to partially overlap. In adult spinal cord sections, both calretinin-LI and calbindin-LI neurons are present, especially in the superficial dorsal horn (Ren & Ruda, 1994). In our cultures, calretinin-LI was found in 20.8 ± 1.4% of the neurons ($n = 17$; 4670 neurons counted), and calbindin-LI was present in 17.5 ± 1.4% of the total neuronal population ($n = 13$; 2507 neurons counted). The two markers overlapped in ≈38% of each subpopulation, and therefore, calbindin-LI and/or calretinin-LI were present in a third of the dorsal horn neurons (see Table 1).

Throughout the mammalian central nervous system (CNS), GABA and glycine are the only well-established fast inhibitory transmitters that have been unambiguously identified. Thus, we have used GABA-LI and glycine-LI as an operational definition of inhibitory neurons in our study, and their absence as an indication that a neuron is excitatory, possibly glutamatergic. While these definitions are not rigorously proved, they are reasonable working definitions. To determine whether calbindin-LI and calretinin-LI identify inhibitory or excitatory neurons, double-labelling experiments were performed with antibodies against calretinin versus GABA and glycine as well as calbindin versus GABA and glycine, using GABA and glycine to identify inhibitory neuronal populations. Figure 5 shows fields of dorsal horn neurons double-labelled with the different marker combinations. The results indicate that ≈90% or more of the cells immunoreactive for each of the two Ca^{2+}-binding proteins were not immunoreactive for either GABA or glycine (Table 1). These observations suggest that calbindin-LI and calretinin-LI dorsal horn neurons in culture use excitatory amino acids as fast neurotransmitters.

To determine whether these two populations of putative excitatory dorsal horn neurons express Ca^{2+}-permeable AMPA receptors, staining was performed for calretinin, calbindin and kainate-induced

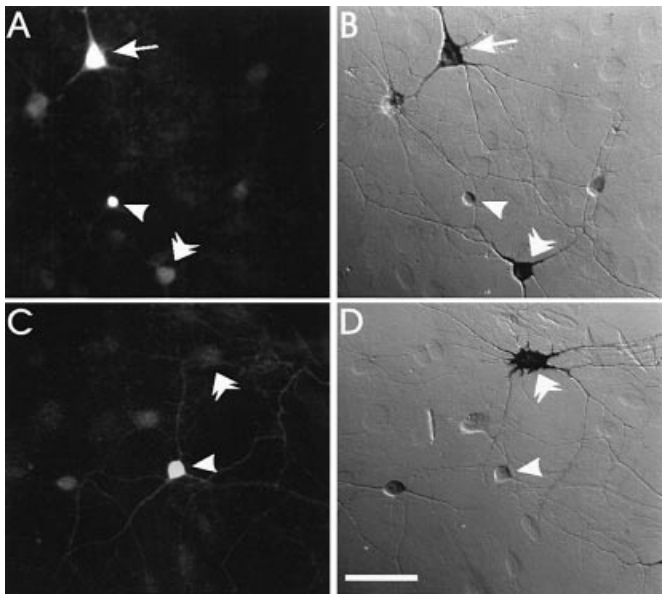

FIG. 3. Ca^{2+}-permeable AMPA receptors are expressed in, but not restricted to, most GABAergic dorsal horn interneurons. (A and C) Two different fields from a dorsal horn neuron culture showing cells expressing GABA-LI. (B and D) DIC pictures of the same fields as A and C, respectively, after silver enhancement of cobalt uptake. Arrows, cells positive for both GABA-LI and cobalt; single arrowheads, cell positive for GABA-LI but negative for cobalt; double arrowheads, cells negative for GABA-LI but positive for cobalt. Scale bar, 50 μm.

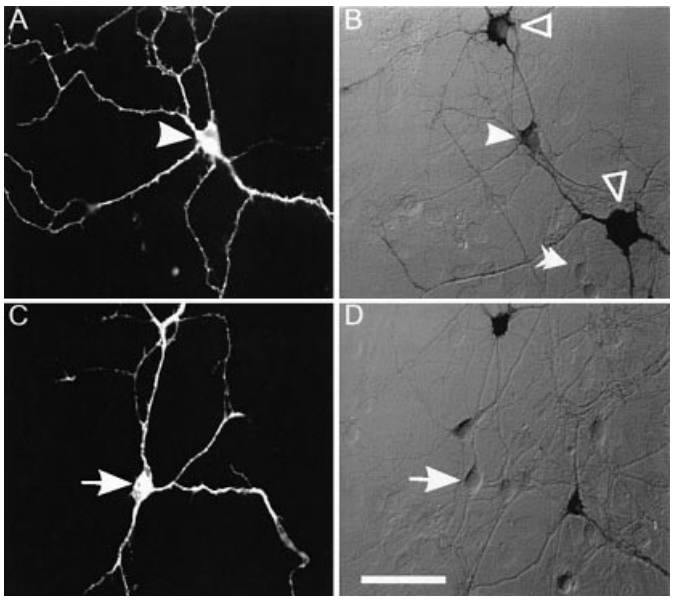

FIG. 4. Ca^{2+}-permeable AMPA receptors are co-expressed with NK1 receptor in some neurons. (A and C) Two different fields from a dorsal horn culture showing cells expressing NK1-LI. (B and D) DIC pictures of the same fields as A and C, respectively, after silver enhancement of cobalt uptake. Single arrowheads, cell positive for both NK1-LI and cobalt (this cell is an example of a 'partial' cell; double arrowheads point to a negative cell in the same field, and empty arrowheads to fully positive cells, for comparison). Arrows, cell positive for NK1-LI but negative for cobalt. Scale bar, 50 μm.

TABLE 1. Summary of cobalt-staining and immunocytochemistry results

	Positive for marker × 100	Cobalt-staining × 100	GABA-positive × 100	Glycine-positive × 100
Marker	Total	Positive for marker	Positive for marker	Positive for marker
Cobalt	46.9 ± 3.7 (13)	100	38.8 ± 5.9 (5)	ND
GABA-LI	32.5 ± 2.9 (13)	58.6 ± 4.2 (5)	100	88.6 ± 3.1 (3)
Glycine-LI	29.8 ± 3.4 (10*)	ND	83.2 ± 3.6 (3)	100
NK1-LI	4.6 ± 0.3 (3)	77 ± 11.9 (5)	ND	ND
CB-LI	17.5 ± 1.4 (13)	10 ± 0.5 (2)	7.2 ± 1.1 (2)	10.5 ± 2.8 (2)
CR-LI	20.8 ± 1.4 (17)	4.4 ± 2.3 (3)	12.3 ± 3.1 (3)	7 ± 1.2 (2)

Data are percentages ± SEM. Numbers in parentheses are the number of experiments (typically > 100 cells counted per experiment). *Data pooled from experiments with and without 0.005% glutaraldehyde in the fixative. ND, not determined.

cobalt uptake. Figure 6 shows results from a triple label experiment with a field of dorsal horn neurons with both calretinin-LI neurons and a calbindin-LI neuron. It is apparent that the cobalt-positive neurons do not include either the calretinin-LI or calbindin-LI neurons. Indeed, very little overlap was found between calretinin-LI and calbindin-LI and cobalt staining, and then only in cells that stained weakly for cobalt. Specifically, 4.4 ± 2.3% of calretinin-LI neurons were cobalt-positive ($n = 3$; 567 neurons counted) and 10 ± 0.5% of calbindin-LI neurons were cobalt-positive ($n = 2$; 335 neurons counted).

These results contrast with those from the sequential staining for cobalt and GABA-LI, or cobalt and NK1-LI shown before, where the majority of both of these two neuronal populations were cobalt-positive. Although those previous results make it clear that immunocytochemistry and cobalt staining are not incompatible techniques, it is possible that cobalt specifically interferes with antibody binding to calbindin and/or calretinin. If true, this would create an artificially low correlation between marker expression and cobalt uptake. In control experiments, we compared the number of calretinin- or calbindin-LI cells in sister cultures that were not exposed to cobalt, that were treated with cobalt, or that were loaded with cobalt in the presence of kainate, then treated with $(NH_4)_2S$ (a condition similar to that in the sequential staining). No significant difference in the percentage of cells expressing immunoreactivity to calbindin and calretinin was observed among these three groups. This makes it unlikely that the low correlation between cobalt staining and Ca^{2+}-binding proteins arises from an interference of the cobalt loading with the immunoreactivity of the relevant epitopes. We also examined the possibility that calretinin or calbindin sequestered cobalt, thus making it undetectable with the silver enhancement technique. We compared sister cultures treated with cobalt plus kainate or with cobalt plus the cobalt (and calcium) ionophore lasalocid (X537A). As expected, all cells, including underlying astrocytes, took up cobalt when exposed to lasalocid. Even so, it was possible to detect neurons strongly immunoreactive to calretinin and calbindin in those cultures. Although present in all cells, the staining caused by lasalocid treatment was less strong than that seen in the Ca^{2+}-permeable AMPA receptor-positive cells revealed by cobalt

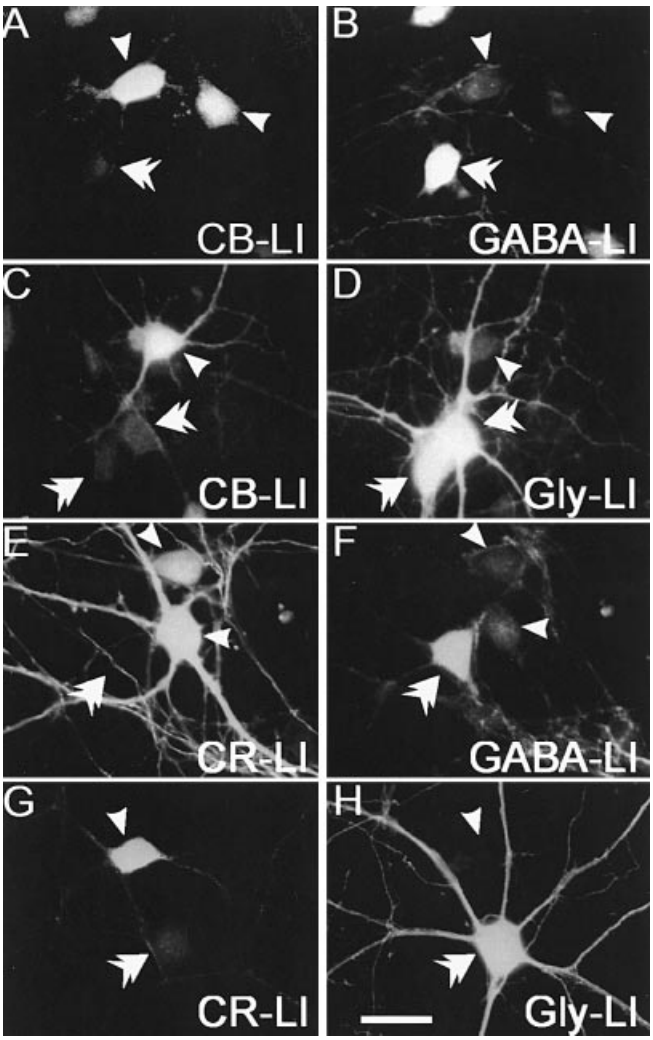

FIG. 5. Calbindin and calretinin are not expressed by GABAergic or glycinergic interneurons. (A and B) Same field stained for calbindin (A, simple arrowheads) and GABA (B, double arrowheads). (C and D) Culture stained for calbindin (C, simple arrowheads) and glycine (D, double arrowheads). (E and F) Culture stained for calretinin (E, simple arrowheads) and GABA (F, double arrowheads). (G and H) Culture stained for calretinin (G, simple arrowheads) and glycine (H, double arrowheads). Scale bar, 25 μm.

plus kainate treatment (data not shown). Thus, it is unlikely that, in the presence of lasalocid, the cobalt influx overwhelmed the putative cobalt-buffering capacity of calretinin or calbindin.

Discussion

We have used antibodies to a variety of functional cell-type markers to extend our previous studies and identify the kinds of neurons expressing Ca^{2+}-permeable AMPA receptors in dorsal horn cultures. Kainate-induced cobalt staining provided a functional assay for the presence of Ca^{2+}-permeable AMPA receptors. This allowed us to account for both cells that express GluR2 subunit in only part of their receptors, as well as for those that express no GluR2 subunit. As a result, we found that 59% of GABA-LI neurons and 77% of NK1-LI neurons express Ca^{2+}-permeable AMPA receptors. Ten per cent or less of calbindin-LI and calretinin-LI neurons express Ca^{2+}-permeable AMPA receptors. Because these results were obtained from dorsal horn neurons in culture, the possibility exists that the patterns we found significantly diverge from those found *in vivo*. Nonetheless, some of our results are consistent with data obtained from spinal cord sections. Examples of that include the correlations between NK1-LI and cobalt staining, and between GABA-LI and cobalt staining, which are similar to those reported in spinal cord sections (see below).

Developmental changes in Ca^{2+}-permeable AMPA receptor expression

In an early study on developmental changes in Ca^{2+}-permeable AMPA receptor expression, it was suggested that receptor expression was highest in the immature brain. This was based on changes in the ratio of GluR1 and GluR3 versus GluR2 mRNA expression viewed within brain regions (Pellegrini-Giampietro *et al*., 1992). When tested at the single-cell level in retinal explants, the percentage of cells expressing Ca^{2+}-permeable AMPA receptors, as detected by kainate-induced cobalt loading, increased sharply and then decreased over the first 8 DIV (Allcorn *et al*., 1996). Recently, Gleason & Spitzer (1998) have shown that spinal cord neurons removed from the neural plate of *Xenopus* embryos express Ca^{2+}-permeable AMPA receptors when tested within only a few hours of plating, at a time when there is no detectable NMDA receptor expression. Using embryonic rat dorsal horn neurons grown in culture, we show here that the number of neurons expressing Ca^{2+}-permeable AMPA receptors increases over the first few weeks in culture (Fig. 2). The increase in receptor expression occurs at a time of tremendous neurite outgrowth and synapse formation. We do not know whether these receptors contribute to this maturation process or are a consequence of it. However, once the receptors are expressed, they appear to be sustained over the subsequent few weeks in culture. Thus, our data are consistent with a possible role for Ca^{2+}-permeable AMPA receptors at mature synapses as well as during development.

GABA staining

In our dorsal horn cultures, Ca^{2+}-permeable AMPA receptors were present in almost two-thirds of the GABA-LI neurons. These results raise the possibility that Ca^{2+}-permeable AMPA receptors may play an important role in the regulation of inhibition in the spinal dorsal horn and thus, in the modulation of the flow of nociceptive information through the dorsal horn. This possibility is supported by our previous studies showing that Ca^{2+}-permeable AMPA receptors can mediate potentiation of synaptic transmission in dorsal horn neurons (Gu *et al*., 1996), and by a recent report of Ca^{2+}-permeable AMPA receptor-mediated modulation of synaptic inputs onto GABAergic neurons in the amygdala (Mahanty & Sah, 1998). Interestingly, our data indicate that the majority of dorsal horn neurons expressing Ca^{2+}-permeable AMPA receptors in culture are not inhibitory, but rather, use an excitatory substance, e.g. glutamate as their fast neurotransmitter. Spike *et al*. (1998) demonstrated that the vast majority of GluR2/3-LI neurons in the rat adult dorsal horn express no GABA- or glycine-LI. Taken together with our work, this would suggest that a good proportion of the cobalt-positive excitatory dorsal horn neurons express a mixed population of Ca^{2+}-permeable and Ca^{2+}-impermeable AMPA receptors.

NK1 immunoreactivity

While little is known about how excitatory dorsal horn interneurons modulate pain perception, synaptic modulation of excitatory synapses onto projection neurons should clearly influence the effectiveness of nociceptive transmission. With that in mind, we specifically assessed the expression of Ca^{2+}-permeable AMPA receptors and NK1-like immunoreactivity (NK1-LI), a marker of excitatory, largely projection, neurons in the superficial dorsal horn (Littlewood *et al*., 1995;

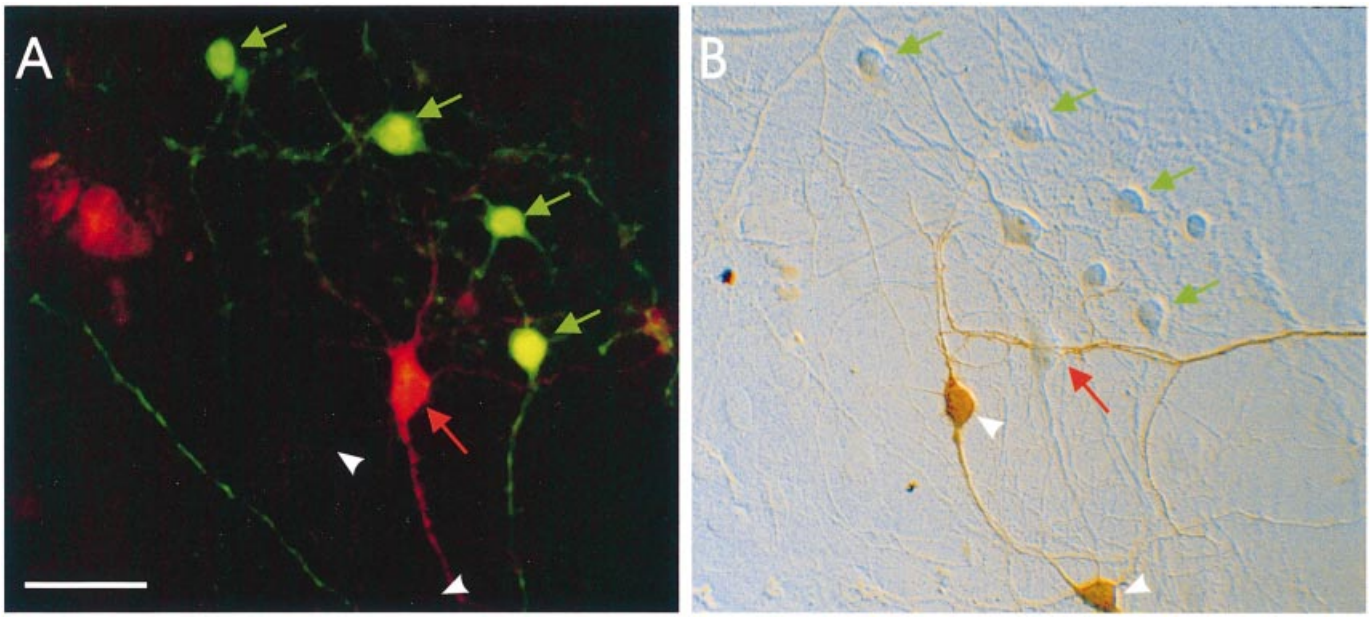

FIG. 6. Calbindin and calretinin are not co-expressed with Ca^{2+}-permeable AMPA receptors. (A) Representative field from a dorsal horn neuron culture showing cells expressing calretinin-LI (CR-LI, green) and calbindin-LI (CB-LI, red). (B) Same field after silver enhancement of cobalt stain. Green arrows, CR-LI-positive cells; red arrow, CB-LI-positive cell; arrowheads, cobalt-positive cells. Scale bar, 50 μm.

Marshall *et al*., 1996). Although only a small fraction (4.6%) of the dorsal horn neurons in co-cultures with DRG neurons expressed NK1-LI, most of those expressed kainate-induced cobalt uptake. This is consistent with sequential staining experiments performed in dorsal horn slices that detected the presence of Ca^{2+}-permeable AMPA receptors in almost half of the NK1-LI cells in lamina I (Engelman *et al*., 1999). While the role of NK1 receptors in pain transmission is not yet established, selective ablation of NK1-LI neurons in lamina I prevents hyperalgesia (Mantyh *et al*., 1997). Our results thus raise the possibility that Ca^{2+}-permeable AMPA receptors might have a direct effect in modulating the synaptic input to the nociceptive projection neurons, with possible bearing on longer-term changes in pain perception, e.g. hyperalgesia.

Ca^{2+}-binding proteins

Calretinin- and calbindin-LI were altogether present in about one-third of the total population of neurons in our cultures, and for the most part, did not co-localize with GABA- or glycine-LI or kainate-induced cobalt permeability. Thus, calretinin- and calbindin-LI neurons encompass the large majority of putative excitatory dorsal horn neurons devoid of Ca^{2+}-permeable AMPA receptors, and are useful markers for that population in culture.

The physiological role of calretinin and calbindin is still not entirely clear. Calbindin has been more extensively studied, and was shown to be able to act as a fast buffer, affecting the amplitude of postsynaptic Ca^{2+} transients (Airaksinen *et al*., 1997), as well as determining firing patterns in some neurons (Li *et al*., 1995). The absence of either protein has been shown to affect long-term plasticity in the hippocampus, although apparently by different mechanisms (Molinari *et al*., 1996; Schurmans *et al*., 1997). Considering that parvalbumin is reportedly restricted to laminae II–III GABAergic/glycinergic neurons (Laing *et al*., 1994; Yamamoto *et al*., 1989), and that calmodulin is all but absent in the dorsal horn (Ren & Ruda, 1994), Ca^{2+}-permeable AMPA receptor-expressing excitatory neurons in the dorsal horn may have a low level of cytoplasmic Ca^{2+}-binding proteins that could act as fast Ca^{2+} buffers. This could make those neurons especially sensitive to excitotoxic injury. From a physiological point of view, one might speculate that the expression of a low level of fast Ca^{2+} buffering is beneficial for the function of the Ca^{2+}-permeable AMPA receptors, which have two- to fourfold lower Ca^{2+} permeability than NMDA receptors (Jonas & Burnashev, 1995).

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Abbreviations

AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CNQX, 5-[3H]6-cyano-7-nitro-quinoxaline-2,3-dione; CNS, central nerve system; DIV, days *in vitro*; DRG, dorsal root ganglion; EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; -LI, -like immunoreactivity; NGF, nerve growth factor; NK1, neurokinin-1; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline.

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