Electrophysiological Properties of Cultured Neonatal Rat Dorsal Horn Neurons Containing GABA and Met-Enkephalin-Like Immunoreactivity

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Jo, Y. H., M. E. Stoeckel, and R. Schlichter. Electrophysiological properties of cultured neonatal rat dorsal horn neurons containing GABA and met-enkephalin-like immunoreactivity. J. Neurophysiol. 79: 1583–1586, 1998. We have developed a culture of neurons dissociated from the superficial laminae of the neonatal rat spinal cord dorsal horn (DH). By using the perforated patch-clamp technique, we distinguished four types of neurons based on their firing properties in response to intracellular injection of 900 ms lasting current pulses. Type 1 neurons were characterized by a tonic firing. Type 2 neurons displayed marked spike accommodation and lasting current pulses. Type 1 neurons were characterized by a tonic firing. Type 2 neurons displayed marked spike accommodation and lasting current pulses. Type 4 neurons exhibited different types of firing patterns, but all of them possessed a time-dependent inwardly rectifying current activated by membrane hyperpolarization. Met-enkephalin-like immunoreactivity (met-ENK-LI) and glutamic acid decarboxylase-like immunoreactivity (GAD-LI) were colocalized in 42% of the neurons (n = 59), which were previously identified electrophysiologically. Type 1–4 neurons represented respectively 4, 64, 20, and 12% of the population of neurons colocalizing met-ENK-LI and GAD-LI. We conclude that the electrophysiological properties of DH neurons present in our cultures are similar to those described in acute slice or hemisected spinal cord preparations and that met-ENK-LI and GABA-LI are preferentially colocalized in type 2 neurons.

INTRODUCTION

The dorsal horn (DH) of the spinal cord gray matter is involved in the transmission and in the modulation of peripheral nociceptive signals (Besson and Chaouch 1987; Willis et al. 1995). GABAergic neurons represent ~30% of the neurons in laminae I-II and ~45% of the neuronal population in lamina III (Todd and Sullivan 1990). In addition, γ-aminobutyric acid (GABA)-like immunoreactivity is present in ~70% of met-enkephalin-positive neurons located in laminae II and III (Todd et al. 1992). Therefore the neurons which colocalize GABA and met-enkephalin are likely to represent local inhibitory DH interneurons involved in the modulation of pain transmission. Several recent studies have characterized in more detail the membrane properties of DH neurons (Thomson et al. 1989; Yoshimura and Jessel 1989; Lopez-Garcia and King 1994) but none of them has aimed at correlating these properties with the neurotransmitter/neuropetide phenotype of the neurons. We have developed a culture of neonatal DH neurons to address this question. The electrophysiological properties of the cultured DH neurons were determined with the perforated patch-clamp technique, which preserves the peptide content of the cells and the presence of met-enkephalin (met-ENK) and/or glutamic acid decarboxylase (GAD)-like immunoreactivities was revealed in the electrophysiologically identified neurons by using a double immunofluorescence labeling technique.

METHODS

Cell culture

Primary cultures of spinal DH neurons were prepared from 3- to 4-day-old Wistar rats. After decapitation under deep diethyl ether anesthesia, a laminectomy was performed and the dorsal third of the spinal cord was cut with razor blade and incubated at 37°C in oxygenated divalent-free Earle’s balanced salt solution (EBSS, Gibco) containing papain (20 U/ml, Sigma). After 45 min, the enzymatic digestion was stopped by adding 3 ml EBSS containing bovine serum albumin (BSA, 1 mg/ml, Sigma), trypsin inhibitor (10 mg/ml, Sigma), and DNase (0.01%, Sigma). After trituration with a 1-ml plastic pipette, the homogenate was deposited on top of 4 ml of a solution of similar composition to that described above, except that the concentration of BSA was increased to 10 mg/ml. After centrifugation (5 min at 500 rpm), the supernatant was removed and replaced with culture medium composed of: MEM-alpha (Gibco), fetal calf serum (5% vol/vol, Sigma), heat-inactivated horse serum (5% vol/vol, Gibco), penicillin and streptomycin (50 IU/ml each, Gibco), transferrin (10 mg/ml, Sigma), insulin (5 mg/ml, Sigma), putrescine (100 mM, Sigma), and progesterone (20 mM, Sigma). Cells were dissociated by trituration with a fire-polished Pasteur pipette and plated onto 35-mm collagen-coated culture dishes, which were modified by heating in a press (BB-form2, Mecanex, Switzerland) to print an alphabetical grid on the bottom of the dish, thus allowing to localize the recorded neuron for immunolabeling. Two days after seeding, cytosine arabinoside (10 μM) was added to the culture medium for 24 h to reduce glial proliferation.

Electrophysiological recording

Perforated patch-clamp recordings using amphotericin B as the pore-forming agent (Rae et al. 1991) were performed at room temperature (20–22°C) after 6–10 days in culture with an Axopatch 200 A amplifier (Axon instruments) and low resistance (3–4 MΩ) electrodes. The external solution contained (in mM) 135 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgCl₂, 5 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and glucose 10, pH 7.3. The tip of the electrode was first dipped into a solution containing (in mM) 125 KCl, 5 CaCl₂, 2 MgCl₂, 10 HEPES, and 10 ethylene glycol-bis(β-aminoethyl ether)-N,N’,N’’,N’’-tetraacetic acid (EGTA), pH 7.3, and the pipette was then backfilled with the same solution...
Electrophysiological properties of cultured DH neurons.

A1: Type 1 neurons were characterized by a tonic firing pattern in response to depolarizing current pulses; $V_m = -60$ mV. A2: Type 2 neurons fired a brief burst of action potentials; $V_m = -66$ mV. A3: Type 3 discharged a single spike. $V_m = 59$ mV. A4: Type 4 neurons displayed tonic firing for moderate depolarization (middle), spike accommodation for stronger depolarizing current pulses (top) and possessed a time-dependent inwardly rectifying current revealed as an initial sag in voltage response during hyperpolarization (bottom); $V_m = -62$ mV. B1–4: Current-to-voltage relationships of type 1–4 neurons illustrated in A1–4.

B4: ●, peak amplitude of hyperpolarization; □, amplitude of hyperpolarization measured just before termination of current injection.

Immunofluorescence

After the recording session, the cultures were fixed (paraformaldehyde 4%, 30 min), permeabilized (Triton $\times 100, 0.5%$) and incubated overnight at room temperature with a goat polyclonal antibody against glutamic acid decarboxylase (GAD, 1/750, gift of Dr. Tappaz, Lyon, France) and a rabbit polyclonal antibody against met-ENK (1/2,000, TEBU, France). The following day, cultures were incubated for 1 h at room temperature with a fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG (1/200, Biosys, France) to reveal GAD-like immunoreactivity and an indocarbocyanine (Cy3)-conjugated antirabbit IgG (1/400, Jackson Immunoresearch Laboratories) to reveal met-ENK-like immunoreactivity.

RESULTS

Electrophysiological properties of cultured neonatal DH neurons

Four different types of neurons were identified on the basis of their spike discharge pattern in response to 900 ms lasting intracellular current pulses. Type 1 neurons (Fig. 1, A1 and B1) discharged a continuous train of action potentials during suprathreshold depolarizing current pulses. There was no obvious sign of spike accommodation or attenuation. Type 2 neurons (Fig. 1, A2 and B2) displayed marked spike accommodation including a progressive increase in interspike intervals and attenuation of spike amplitude. This resulted in a discharge pattern consisting of an initial burst of action potentials not exceeding a total duration of 500 ms. Type 3 neurons (Fig. 1, A3 and B3) fired a single action potential upon depolarization regardless of the intensity of the current pulse applied. Finally, type 4 neurons (Fig. 1D) were characterized by tonic firing during moderate depolarization and marked spike accommodation for larger depolarizing current pulses. Upon hyperpolarization the voltage trace of all type 4 neurons displayed an initial sag indicating the presence of an inwardly rectifying current that was not further characterized. Type 1–4 neurons represented respectively, 15.5, 36.7, 32.2, and 15.5% of the total population ($n = 90$). Their membrane properties and action potential firing characteristics are summarized in Table 1. Among all neurons from which we recorded, only three cells (all classified as type 4 neurons) displayed spontaneous action potential activity with a mean discharge frequency of 9.9 ± 3.9 Hz.

Immunofluorescence

Fifty-nine neurons previously identified electrophysiologicallly were processed for immunofluorescent detection of met-enkephalin-like (met-ENK-LI) and GAD-like (GAD-LI) immunoreactivities (Table 2 and Fig. 2). Forty-two and four-tenths percent of the neurons (25/59) co-localized met-ENK-LI and GAD-LI; 6.8% (4/59) expressed only met-ENK-LI; 5% (3/59) displayed only GAD-LI; and the remainder, i.e., 45.8% (27/59) possessed neither met-ENK-LI nor GAD-LI. When considering the distribution of the 25 neurons that colocalized met-ENK-LI and GAD-LI (Fig. 3 and Table 2), it appeared that colocalization of both markers was most frequently encountered in type 2 neurons (64%, 16/25) and less frequently observed in type 3 (20%, 5/25), type 4 (12%, 3/25), and type 1 (4%, 1/25) neurons (Fig. 3B). A $\chi^2$ test on the contingency table (Table 2) showed that there was a significant relation between cell type and immunoreactivity, i.e., there was a clear tendency for type 2 neurons to colocalize preferentially GAD-LI and met-ENK-LI and for type 3 neurons to express none of the two markers.
PROPERTIES OF DH NEURONS CONTAINING MET-ENK AND GABA

TABLE 1. Membrane properties of the four types of dorsal horn neurons

<table>
<thead>
<tr>
<th>Cell type</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>33</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>RMP (mV)</td>
<td>−60.2 ± 2.4</td>
<td>−57.1 ± 1.2</td>
<td>−56.8 ± 1.3</td>
<td>−58.1 ± 1.9</td>
</tr>
<tr>
<td>$R_h$ (MΩ)</td>
<td>556.2 ± 69.3</td>
<td>691.8 ± 42.5</td>
<td>358.4 ± 40.3*</td>
<td>804.6 ± 42.4</td>
</tr>
<tr>
<td>$\tau$ (ms)</td>
<td>23.5 ± 1.8</td>
<td>30.2 ± 3.7</td>
<td>16.2 ± 1.6</td>
<td>44.5 ± 4.5</td>
</tr>
<tr>
<td>FF (Hz)</td>
<td>12.6 ± 0.9</td>
<td>14.4 ± 0.8</td>
<td>44.5 ± 4.5</td>
<td>12.3 ± 1.1</td>
</tr>
</tbody>
</table>

Data (mean ± SE) concern resting membrane potential (RMP), input resistance ($R_h$), and time constant ($\tau$). The action potential firing frequency (FF) was determined for just suprathreshold current pulses (+20 to +40 pA). * a statistically significant difference with all other groups ($P < 0.05$, Student-Newman-Keuls method).

DISCUSSION

The aim of this work was to develop a culture of neonatal dorsal horn (DH) neurons and to characterize their membrane properties in relation with their neurotransmitter/neuropeptide content.

A major finding of the present study was that in our culture preparation the electrophysiological properties of DH neurons were similar to those of laminae I and II DH neurons recorded in adult spinal cord slices (Thomson et al. 1989) or superficial and deeper DH neurons recorded in a hemisected spinal cord-hindlimb preparation from 10- to 14-day-old rats (Lopez-Garcia and King 1994), the major differences observed in our study being markedly higher input resistances of all categories of neurons (probably because of the fact that we used the patch-clamp technique and not conventional intracellular recordings) and a 30% lower firing frequency of the different types of neurons in our system. This difference may be explained in part by the fact that we determined the discharge frequency for just suprathreshold current pulses (+20 to +40 pA) and this value does not represent the maximal firing frequency that can be achieved by the neuron. It must be noticed however, that the overall discharge properties of our type 1–4 neurons compare well to those of type A, A-B, B, and C neurons described by Lopez-Garcia and King (1994). Similar properties were also observed in the study by Thomson et al. (1989), the only difference being that their type 2 neurons correspond to a pool of our type 2 and 4 neurons or of Lopez-Garcia and King’s type A-B and C neurons. Moreover, we found that about half of the cultured neurons displayed GAD and/or met-ENK like immunoreactivity, which is typical of DH neurons found in laminae II and III (Todd et al. 1992).

This suggested that our culture system contained essentially neurons from the most superficial laminae of the DH.

Colocalization of GAD-LI and met-ENK-LI was preferentially associated with type 2 neurons. Interestingly and with the exception of type 1 neurons, the proportion of neurons colocalizing GAD-LI and met-ENK-LI within type 2–4 neurons in our study (64, 20, and 12%, respectively) is very close to the proportion of neurons having the properties of type 2–4 neurons within the population of nociceptive specific neurons described by Lopez-Garcia and King (1994) (i.e., 53, 15, and 8%, respectively). Moreover, a recent in vivo study in the cat has shown that most nociceptive neurons express enkephalin immunoreactivity, whereas

![FIG. 2. Double labeling of glutamic acid decarboxylase (GAD) (A1 and B1) and met-ENK (A2 and B2) immunoreactivities. A: neuron indicated by an arrow in A1 was electrophysiologically identified as a type 2 neuron and colocalized GAD and met-ENK. B: cluster of 3 GAD immunoreactive neurons (B1), one of which was also met-ENK positive (B2). Bars, 25 µm.]

TABLE 2. Distribution of GAD-LI and/or met-ENK-LI among the four types of dorsal horn neurons

<table>
<thead>
<tr>
<th>Cell type</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>26</td>
<td>18</td>
<td>8</td>
<td>59</td>
</tr>
<tr>
<td>GAD+/ENK+</td>
<td>1 (3)</td>
<td>16 (11)</td>
<td>5 (8)</td>
<td>3 (3)</td>
<td>25</td>
</tr>
<tr>
<td>GAD+/ENK−</td>
<td>2 (0)</td>
<td>0 (1)</td>
<td>0 (1)</td>
<td>1 (0)</td>
<td>3</td>
</tr>
<tr>
<td>GAD−/ENK+</td>
<td>2 (0)</td>
<td>2 (2)</td>
<td>0 (1)</td>
<td>0 (1)</td>
<td>4</td>
</tr>
<tr>
<td>GAD−/ENK−</td>
<td>2 (3)</td>
<td>8 (12)</td>
<td>13 (8)</td>
<td>4 (4)</td>
<td>27</td>
</tr>
</tbody>
</table>

Numbers in parentheses are those expected for a random distribution of the immunoreactivity among the different cell types. The signs + and − stand for presence or absence, respectively, of a given type of immunoreactivity.
speculate that a substantial fraction of nociceptive specific neurons would be inhibitory interneurons. Activation of these neurons by nociceptive stimuli could therefore lead to the corelease of GABA and met-enkephalin, which could have synergistic inhibitory effects on the postsynaptic target neuron. Our culture model will be useful to address the question of such a corelease because ~50% of the neurons appear to contain both neurotransmitters and because in culture it is possible to study the synaptic transmission between pairs of neurons.

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REFERENCES


