Membrane Dysfunction Induced by In Vitro Ischemia in Immature Rat Hippocampal CA1 Neurons

T. ISAGAI,1 N. FUJIMURA,2 E. TANAKA,1 S. YAMAMOTO,1 AND H. HIGASHI1
1Department of Physiology and 2Department of Neurosurgery, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan

Isagai, T., N. Fujimura, E. Tanaka, S. Yamamoto, and H. Higashi. Membrane dysfunction induced by in vitro ischemia in immature rat hippocampal CA1 neurons. J. Neurophysiol. 81: 1866–1871, 1999. We investigated differences between immature and mature hippocampal neurons in their response to deprivation of oxygen and glucose (in vitro ischemia), using intracellular recording techniques from CA1 pyramidal neurons in rat brain slices. The membrane was more depolarized in immature hippocampal CA1 neurons (postnatal day 7, P7) compared with the adult neurons (P140), and the apparent input resistance in immature neurons was higher than that in adult neurons. In immature neurons, the threshold for action potential generation was high, and the peak amplitude of the action potential was low in comparison with adult neurons. A time-dependent inward rectification, at potentials negative than the resting potential, was prominent in neurons of P14 and P21. After P21, the resting membrane potential, the apparent input resistance, and the threshold and the peak amplitude of the action potential did not significantly change with increasing age. In adult neurons, application of ischemia-simulating medium caused irreversible changes in membrane potential consisting of an initial hyperpolarization followed by a slow depolarization and a rapid depolarization. Once the rapid depolarization occurred, reinduction of oxygen and glucose failed to restore the membrane potential, a state referred to as irreversible membrane dysfunction. In neurons of ages P7 or P14, the initial hyperpolarization was not apparent, whereas a slow depolarization followed by a rapid depolarization was observed. With development of the neurons, the latency for onset of the rapid depolarization became shorter and its maximal slope increased. Moreover, neurons of ages P14 or P21 showed a partial or complete recovery after reintroduction of oxygen and glucose, unlike mature neurons. In summary, the present study has demonstrated that the initial hyperpolarization and rapid depolarization induced by in vitro ischemia is age dependent. The rapid depolarization is not readily produced in the neurons in age less than P21 during ischemic exposure.

INTRODUCTION

A considerable number of experimental studies have demonstrated that the immature brain, including brain stem (Balkanyi et al. 1992), hippocampus (Cherubini et al. 1989; Friedman and Haddad 1993), and cerebral cortex (Bickler et al. 1993; Hansen 1977; Luhmann and Krä 1997; Luhmann et al. 1993), is markedly resistant to oxygen deprivation. There have been some reports that the neural activity in immature rat hippocampal neurons is also resistant to oxygen and glucose deprivation (in vitro ischemia) (Kawai et al. 1989; Nabetani and Okada 1994a,b; Nabetani et al. 1995, 1997). This insensitivity of the immature brain to lack of oxygen or in vitro ischemia probably results from developmental differences in brain energy production and energy consumption (for review, see Ben-Ari 1992; Hansen 1985; Luhmann 1996).

We previously have reported that adult hippocampal CA1 neurons show an initial hyperpolarization followed by a slow depolarization, which leads to a rapid depolarization after ~6 min of exposure to oxygen and glucose-deprived medium (Tanaka et al. 1997). When oxygen and glucose are reintroduced immediately after generating the rapid depolarization, the membrane potential depolarizes further and approaches 0 mV (the persistent depolarization) (Rader and Lanthorn 1989). Thus the neuron shows no functional recovery (Higashi 1990; Higashi et al. 1990; Kudo et al. 1989; Rader and Lanthorn 1989; Tanaka et al. 1997; also see Martin et al. 1994). Moreover, simultaneous recordings of changes in intracellular Ca2+ concentration ([Ca2+]i) and membrane potential recorded in Fura-2/AM-loaded slices revealed a rapid increase in [Ca2+]i, corresponding to the rapid depolarization in all CA1 layers (Tanaka et al. 1997; also see Hansen and Zeuthen 1981; Silver and Erecinska 1990; Uematsu et al. 1988). Moreover, pretreatment with a N-methyl-D-aspartic acid (NMDA) receptor antagonist or a non-NMDA receptor antagonist inhibits the persistent depolarization and restores the membrane potential to preexposure levels when oxygen and glucose have been reintroduced (Rader and Lanthorn 1989; Tanaka et al. 1997; Yamamoto et al. 1997b). Thus the activation of non-NMDA and NMDA receptors and the accumulation of [Ca2+]i, have important roles in the membrane dysfunction induced by in vitro ischemia. Nevertheless, the potential responses induced by in vitro ischemia in immature CA1 hippocampal neurons are still unclear.

The rapid depolarization induced by in vitro ischemia (Tanaka et al. 1997) corresponds to the terminal depolarization (phase II depolarization) produced by in situ ischemia or asphyxia (Hansen 1985). The differences in the mechanisms underlying the generation of the rapid depolarization after in vitro ischemia between immature and adult rats are of interest because the rapid depolarization is crucial for the irreversible membrane change that leads to neuronal death (Tanaka et al. 1997). Thus the present study addresses processes involved in the membrane dysfunction induced by in vitro ischemia in hippocampal CA1 neurons in slice preparations from rats of different ages. We have examined whether or not in vitro ischemia produces the rapid depolarization in immature hippocampal CA1 neurons and whether the rapid depolarization...
IN VITRO ISCHEMIA IN IMMATURE HIPPOCAMPAL NEURONS

TABLE 1. Parameters of resting and active membrane properties in CA1 neurons of different ages

<table>
<thead>
<tr>
<th>Postnatal Age</th>
<th>Resting Membrane Potential, mV</th>
<th>Apparent Input Resistance, MΩ</th>
<th>Peak of the Action Potential, mV</th>
<th>Duration of the Action Potential, ms</th>
<th>Threshold of the Action Potential, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>P7</td>
<td>−66 ± 4∗ (10)</td>
<td>69 ± 37† (10)</td>
<td>−16 ± 14‡ (10)</td>
<td>1.9 ± 0.8 (10)</td>
<td>−47 ± 4‡ (10)</td>
</tr>
<tr>
<td>P14</td>
<td>−62 ± 5† (13)</td>
<td>51 ± 23 (13)</td>
<td>8 ± 6 (13)</td>
<td>1.7 ± 0.4 (13)</td>
<td>−53 ± 6 (13)</td>
</tr>
<tr>
<td>P21</td>
<td>−68 ± 2 (10)</td>
<td>36 ± 10 (10)</td>
<td>16 ± 4 (10)</td>
<td>1.4 ± 0.2 (10)</td>
<td>−59 ± 2 (10)</td>
</tr>
<tr>
<td>P28</td>
<td>−69 ± 3 (4)</td>
<td>28 ± 10 (4)</td>
<td>15 ± 2 (4)</td>
<td>1.3 ± 0.2 (4)</td>
<td>−59 ± 3 (4)</td>
</tr>
<tr>
<td>P35</td>
<td>−71 ± 3 (7)</td>
<td>40 ± 24 (7)</td>
<td>16 ± 5 (7)</td>
<td>1.2 ± 0.1 (7)</td>
<td>−57 ± 3 (7)</td>
</tr>
<tr>
<td>P42</td>
<td>−72 ± 4 (6)</td>
<td>35 ± 14 (6)</td>
<td>16 ± 6 (6)</td>
<td>1.2 ± 0.2 (6)</td>
<td>−57 ± 2 (6)</td>
</tr>
<tr>
<td>P140 (Adult)</td>
<td>−73 ± 2 (10)</td>
<td>31 ± 8 (10)</td>
<td>17 ± 3 (10)</td>
<td>1.2 ± 0.2 (10)</td>
<td>−60 ± 1 (10)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD with values of n shown in parentheses. ∗ P < 0.02, † P < 0.05, and ‡ P < 0.001 the one-way ANOVA with Scheffé post hoc comparisons.

METHODS

The preparation and recording techniques employed were similar to those described in the previous paper (Tanaka et al. 1997). Briefly, the forebrain of immature (postnatal day (P7, P14, P21, P28, P35, and P42) and adult (P140) male Wistar rats was removed quickly under ether anesthesia and placed in chilled (4–6°C) Krebs solution aerated with 95% O2-5% CO2. The composition of the solution was (in mM) 117 NaCl, 3.6 KCl, 2.5 CaCl2, 1.2 MgCl2, 1.2 NaH2PO4, 25 NaHCO3, and 11 glucose. The hippocampus was dissected and then sliced with a Vibratome (Oxford) at a thickness of ~400 μm. The slice preparation was submerged completely in superfusing solution (preheated to 36.5 ± 0.5°C). Intracellular recordings from CA1 pyramidal neurons were made with glass micropipettes filled with K acetate (2 M). The electrode resistance ranged between 40 and 80 MΩ.

Slices were made “ischemic” by superfusing them with medium equilibrated with 95% N2-5% CO2 and deprived of glucose, which was replaced with NaCl isosmotically (ischemia-simulating medium). When switching the superfusing media, there was a delay of 15–20 s before the new medium reached the chamber due to the volume of the connecting tubing. Thus there was an ~30-s delay in saturating the bath with test solution after initial switching to test solution.

The response to deprivation of oxygen and glucose mainly consists of an initial hyperpolarization, a slow depolarization, a rapid depolarization and a persistent depolarization (Tanaka et al. 1997). The onset of the rapid depolarization produced by ischemia-simulating medium was estimated by extrapolating the slope of the slow depolarization to the rapid depolarization. The latency of the rapid depolarization was measured from onset of superfusion to onset of the rapid depolarization (Tanaka et al. 1997). Recovery after reintroduction of oxygen and glucose was defined as follows: no recovery, 30–60 min after reintroduction the membrane potential lay between 0 and −19 mV; complete recovery, the membrane potential was more negative than −60 mV; partial recovery, membrane potential repolarized to values between −20 and −59 mV (Yamamoto et al. 1997b).

In most neurons with complete recovery, action potentials and fast excitatory postsynaptic potentials elicited by direct and focal stimulation, respectively, were similar to those observed during the pre-exposure period, i.e., in normal medium. All quantitative results were expressed as means ± SD. The number of neurons examined is given in parentheses. The one-way ANOVA with Scheffé post hoc comparisons was used to compare data, with P < 0.05 considered significant, unless specified otherwise.

RESULTS

This study was based on intracellular recordings from 60 CA1 pyramidal neurons of immature (P7, n = 10; P14, n = 13; P21, n = 10; P28, n = 4; P35, n = 7; P42, n = 6) and adult (P140, n = 10) rats with stable resting membrane potentials.

Membrane properties of immature and adult neurons

Table 1 summarizes resting and active membrane properties of hippocampal CA1 neurons of different ages. The membrane was more depolarized in immature (P7 and P14) CA1 neurons compared with adult CA1 neurons (P < 0.02 and P < 0.001, respectively). The apparent input resistance was significantly higher in immature CA1 neurons (P7) (P < 0.05). Figure 1 shows typical action potentials elicited by brief depolarizing current pulses through the recording electrode at different ages. The action potential was abolished reversibly by tetrodotoxin (TTX 0.3 μM) in immature (P7, n = 6 and P14, n = 6) and adult CA1 neurons (P140, n = 6; not shown). The threshold for action potential generation in immature (P7 and P14) CA1 neurons was depolarized significantly compared with that in adult CA1 neurons (P < 0.001 and P < 0.05, respectively; Table 1). The peak amplitude of

FIG. 1. Action potentials of hippocampal CA1 pyramidal neurons of different ages. TTX-sensitive action potentials were elicited by brief depolarizing current pulses (from left to right 0.2, 0.4, 0.9, and 1.2 nA for 5 ms). Note that with development of the neurons, the peak of action potential was shifted in the depolarizing direction, whereas the threshold for generation was shifted in the hyperpolarizing direction. Resting membrane potentials of the neurons at ages P7, P14, and P21 and in adult were −61, −65, −69, and −75 mV, respectively.
the action potential was significantly lower in immature CA1 neurons (P7; \( P < 0.001 \)) compared with adult neurons. The duration of the action potential was not significantly different between immature and adult CA1 neurons.

Figure 2A illustrates typical potential responses to injection of hyperpolarizing and depolarizing current pulses in CA1 neurons at different ages. The resting membrane was more hyperpolarized in adult neurons (P140) compared with immature neurons (P7–P21). The apparent input resistance decreased as the age increased. In addition, a time-dependent inward rectification elicited at potentials negative to the resting potential was prominent in neurons of P14 and P21. The \( V-I \) relationship of these neurons also showed the presence of the time-dependent inward rectification in neurons of P14 and P21 but not in neurons of P7 and P140 (Fig. 2B).

Responses to perfusion with ischemia-simulating medium in immature and adult CA1 neurons

As described previously (Tanaka et al. 1997), deprivation of oxygen and glucose produced a sequence of potential changes consisting of an initial hyperpolarization, a slow depolarization followed by a rapid depolarization in adult neurons (at age more than P56). All responses were accompanied by decreases in the apparent input resistance. Reintroduction of oxygen and glucose after the rapid depolarization failed to restore the membrane potential to control levels. The membrane continued to depolarize progressively to 0 mV (a persistent depolarization), and this was accompanied by a further decline in the apparent input resistance (Fig. 3D). These changes in membrane potential and input resistance were never restored to control levels even when the slice was perfused with normal medium for \( >60 \) min (Onitsuka et al. 1998; Tanaka et al. 1999). At ages older than P21, the membrane potential changes induced by in vitro ischemia were essentially the same as those described in adult neurons. In neurons of P21, the potential changes were similar, but reintroduction of oxygen and glucose restored the membrane potential partially or completely; 1 of 10 neurons showed complete recovery, 4 neurons showed partial recovery (Fig. 3C), and the remaining 5 neurons showed no recovery.
At ages P7 and P14, the initial hyperpolarization was, however, absent and the slow depolarization occurred after superfusion of ischemia-simulating medium (Fig. 3, A and B). In all neurons at age P14, the slow depolarization was followed by the rapid depolarization (Fig. 3B). Reintroduction of oxygen and glucose restored the membrane potential partially or completely; 1 of 13 neurons showed complete recovery, 3 neurons showed partial recovery, and the remaining 9 neurons showed no recovery. At age P7, the rapid depolarization occurred after 43.3 min superfusion with ischemia-simulating medium in only one of seven neurons (Fig. 3A). When oxygen and glucose were reintroduced, the membrane potential did not recover but further depolarized to 0 mV. In the remaining six neurons, intracellular recording was suddenly lost from impaled neurons after 40–55 min superfusion without obvious tissue movement.

Table 2 summarizes the latency of onset and the maximal slope of the rapid depolarization in different age groups. The onset of the rapid depolarization was earlier and its maximal slope increased as the age was increased. The onset was significantly later in neurons at age P14 (P < 0.002) and P21 (P < 0.05) compared with adult (P140) neurons. The maximal slope was significantly reduced in neurons at age P14 and P21 (P < 0.002 and P < 0.02, respectively) compared with adult neurons. These results suggest that the rapid depolarization is not readily produced in the immature neurons.

### DISCUSSION

Resting and active membrane properties of immature and adult neurons

The present study demonstrates that the membrane was more depolarized in immature hippocampal CA1 pyramidal neurons compared with adult neurons, and the apparent input resistance in immature neurons was higher than that of adult neurons. In the immature neurons, the peak amplitude of the action potential was low in comparison with that of adult neurons. These experiments were compared with previous reports of developmental changes in the electrophysiological properties of hippocampal CA1 neurons (Cherubini et al. 1989; Mueller et al. 1981; Schwartzkroin 1982; Schwartzkroin and Altschuler 1977; Zhang et al. 1991), neocortical pyramidal neurons (McCormick and Prince 1987), and trigeminal neurons (Guido et al. 1998). It is possible that a positive shift of the resting potential in the immature neurons is due to a lower activity of Na, K-ATPase because the rate of energy metabolism in the immature brain is 5–20% of the adult (Duffy et al. 1975; Hansen and Nordstrom 1979; Thurston and McDougal 1969). Moreover, there is a previous report that in the first postnatal week the Na, K-ATPase density on the membrane of hippocampal CA1 neurons is low and insufficient to allow substantial activity of Na, K-ATPase (Fukuda and Prince 1992). The high apparent input resistance in the immature neurons may be the result of the presence of fewer active voltage-dependent ion channels compared with adult CA1 neurons (Costa et al. 1994; Spigelman et al. 1992) or relatively small cell soma and less branched dendrites (Pokorny and Yamamoto 1981). A time-dependent inward rectification elicited at potentials negative to the resting potential was prominent in neurons of P14 and P21. This result is also comparable with previous reports in hippocampal CA1 neurons (Krnevitch et al. 1989; Schwartzkroin 1982), neocortical pyramidal neurons (McCormick and Prince 1987), and trigeminal neurons (Guido et al. 1998). The present study demonstrates that the threshold for generating the TTX-sensitive action potential was high in immature neurons. It is likely that Na\(^+\) channels in immature neurons are activated at more positive potentials than those in adult neurons because the activation curve for the Na\(^+\) current and the potential corresponding to half-activation in immature neurons is shifted in the depolarizing direction (Costa 1996). After the age P21,
values of the resting membrane potential, the apparent input resistance, and the threshold and the peak amplitude of the action potential were not different between immature and adult neurons. These results suggest that the resting and the active membrane properties reached maturity between P14 and P21.

Responses to in vitro ischemia in immature and adult neurons

Deprivation of oxygen and glucose produced a sequence of potential changes consisting of an initial hyperpolarization, a slow depolarization, a rapid depolarization, and a persistent depolarization in adult neurons. When oxygen and glucose were reintroduced immediately after generating the rapid depolarization, the neuron did not repolarize and the membrane potential finally became 0 mV after ~5 min. At ages P7 and P14, the initial hyperpolarization was, however, absent, and the slow depolarization was followed by the rapid depolarization. Previously, we have reported that the initial hyperpolarization is mediated mainly by activation of ATP-sensitive K⁺ channels (Fujimura et al. 1997b; Fujiwara et al. 1987; Yamamoto et al. 1997a). The binding densities of the sulfonylurea receptor antagonist, glibenclamide in rat brain are lower within 3 wk of birth compared with adults (Xia et al. 1993). It is, therefore, possible that in neurons of ages P7 and P14 the lack of the initial hyperpolarization is the result of an absence of sulfonylurea receptors.

We already have reported that the rapid depolarization is Na, K-ATPase dependent and is due to a nons elective increase in permeability to all participating ions in the adult neurons (Tanaka et al. 1997). In addition, the latency of the rapid depolarization is prolonged and the maximal slope is decreased at low temperatures (27–33°C). The temperature coefficient (Q₁₀) of the latency and the maximal slope is 2.5 and 2.9, respectively (Onitsuka et al. 1998). Okada (1988) reported that in adult hippocampal slice preparations of the rat, the level of ATP and creatine phosphate (CrP) is reduced to 30 and 10% of the preexposure level respectively, after 5 min of in vitro ischemia, and the reduction of the high energy phosphates is suppressed markedly at low temperatures (28 and 21°C); Q₁₀ of the high energy phosphates is ~2. Energy metabolism in immature animals is much lower than that in adult animals, as described previously (Duffy et al. 1972; Kawai et al. 1989; Lowry et al. 1964; Thurston and McDougal 1969), and anaerobic glycolysis is sufficient for the relatively small energy requirements of the immature brain (Duffy et al. 1975; Kawai et al. 1989; Samson et al. 1960). The present study showed that in immature neurons, the latency of the rapid depolarization was prolonged and the maximal slope was reduced. It is therefore possible that the prolonged latency of the rapid depolarization in immature neurons is due to a relatively slow depletion of ATP (Lowry et al. 1964).

When oxygen and glucose were reintroduced to the slices immediately after the rapid depolarization, the neurons at age P14 and P21 showed partial or complete recovery. We previously have reported that in adult neurons, at temperatures <33°C, the persistent depolarization is fully or partially reversible after re introduction of oxygen and glucose (Onitsuka et al. 1998). Taken together, these results suggest that a low-energy metabolism in immature neurons may have a central role in recovery after in vitro ischemia. In adult rat neurons, the membrane potential is well restored when the slices are pre treated with inorganic Ca²⁺ antagonists, low Ca²⁺ medium, or antagonists for Ca²⁺-induced Ca²⁺ release from intracellular stores (Yamamoto et al. 1997b). Moreover, an increase in intracellular Ca²⁺ concentration ([Ca²⁺]i) caused by in vitro ischemia in immature neurons shows relatively slow onset and the slope of raised [Ca²⁺]i is much reduced in comparison with those of adult neurons (Nabetani et al. 1997). It is therefore possible that the recovery of the membrane potential in immature neurons is partly the result of a decrease in the accumulated [Ca²⁺], during in vitro ischemia. However, most of the neurons at age P7 did not show the rapid depolarization, but the intracellular recording of the impaled neurons was suddenly and abruptly lost. It is likely, although not proven, that swelling of the cell following a prolonged application of ischemia-simulating medium is the cause of the displacement of the neuron relative to the recording electrode.

In summary, the present study has demonstrated that the initial hyperpolarization and the rapid depolarization produced by ischemic exposure are age dependent. The resistance of immature neurons against ischemic exposure is probably due to a low-energy metabolic rate.

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Address for reprint requests: E. Tanaka, Dept. of Physiology, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan.

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