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CHANGES OF *N*-METHYL-D-ASPARTATE ACTIVATED CHANNELS OF CEREBELLAR GRANULE CELLS WITH DAYS IN CULTURE

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N-Methyl-D-Aspartate (NMDA) activated channels were studied in enzymatically dissociated cerebellar granule cells primary cultures. Measurements of single channel currents were made on different days in culture. Changes in the electrophysiological behaviour of NMDA-activated channels, which were dependent on the time in culture, were found. The variations of single channel maximum conductance during the development of the cells in culture were detected. Three different characteristic periods could be distinguished: the first period (1-3 days) in which the conductance assumed a value of 15.5 pS; the second one (5-8 days) characterized by a value of 35.7 pS and the last one (9-11 days) in which the conductance reached values of 46.8 pS. Moreover mean open time of NMDA-activated channels was less than 1 msec during the first two days in culture and stabilized at 3 to 6 msec around the fifth day.

Dicarboxylic amino acids are known to act as excitatory synaptic transmitters. One of the most common excitatory neurotransmitter in the vertebrate central nervous system is the *L*-glutamate which activates an ionic channel [19]. Glutamate receptors are grouped in, at least, three different types, according to their sensitivity to different agonists as *N*-Methyl-D-aspartic acid (NMDA), kainic acid and quisqualic acid receptors [19]. NMDA is the most potent and selective agonist of the glutamate receptor. The electrophysiological properties of NMDA-activated channels have been investigated in different central nervous system cell preparations [1, 6, 13]. These channels can be characterized as voltage independent with cationic selectivity and maximum conductance states of 30 to 50 pS and several subconductance states.

Rat cerebellar granule cells grown in "high" potassium medium (25 mM) are very homogeneous and long-term cell cultures [17]. This preparation has been widely used for biochemical investigation of glutamate receptors [see for example 7, 8, 9, 15, 16, 17, 18]. Some electrophysiological [4, 11] and biochemical properties [8, 9, 11] were changed through days in culture (DIC). We report here the changes of electrophysiological properties of NMDA-activated channels with DIC (from 1 to 11 days; seeding: day 0).

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METHODS

Cerebellar granule cells were obtained from 8 days newborn Wistar rats, dissociated with trypsin (0.02%) and cultured as previously described [17]. For patch–clamp experiments, intracellular solution (pipette) contained (in mM): 140 CsCl, 1 $MgCl_2$ 1 EGTA, 10 HEPES–KOH, 22 *D*–glucose. The extracellular (bath) solution contained (in mM): 144 NaCl, 3 KCl, 1.5 $CaCl_2$, 10 HEPES-NaOH, 6 *D*-glucose. The pH of both solutions was adjusted at 7.4. Tetrodotoxin 1μM (Sigma, St. Louis, USA) was added to the extracellular solution to block sodium channels. Culture dishes were intensely washed with external solution before experiments. NMDA (Sigma) was applied changing the bath solution by gravity perfusion.

Single–channel currents were measured using the outside–out configuration of the patch clamp technique [10]. Borosilicate patch pipettes were coated with Sylgard (Corning) and fire polished to obtain tip resistences of 8-10 MΩ. Single NMDA-activated channel currents were recorded with an EPC-7 amplifier (List Medical Instruments) and stored in video recorder tapes for later analysis. Experiments were performed at room temperature (22–24 °C). Data stored in magnetic tapes were filtered with a 4–pole Bessel filter (4302, Ithaco) at a cut–off frequency of 1 to 5 KHz, and transfered to an Atari (1040ST) microcomputer, using a 12 bit analog to digital converter (M2 LAB Instrutech) at a sampling interval of 200 to 500 μsec.

RESULTS

An application of 20 μM NMDA in the bath solution, evoked, in outside–out configuration, single channel currents. These currents usually did not constitute a homogeneous population. For each patch it was possible to identify, unambiguously maximum single channel conductances with the same size (fig. 1A). Channel currents were studied in their steady–state conditions (usually 10 minutes after the beginning of NMDA perfusion). Only patches without background channels, that open prior to agonist application, were considered for further analysis.

The rate of success to find channels that were activated by NMDA application was higher in cells with more than 5 DIC (90% patches). The probability to have more than one channel in a patch also increased with time in culture using the same pipette size. This could be explained as an increase of NMDA receptor–channel complex density.

The amplitude of NMDA–activated single channel currents was linearly dependent on the applied membrane potential and the channels showed a reversal potential of about 0 mV, as shown in fig. 1A. Single channel maximum current-voltage relationship (I–V) at different DIC showed that the linearity of this function was mantained at different DIC (see below). In fig. 1B I–V fits, obtained at three different DIC displayed as example.

NMDA-activated channels showed different maximum current amplitudes in patches obtained from cells at different DIC (fig. 2). At 1 DIC, for most of the neurons examined, no NMDA–activated channel currents were detected. We measured the NMDA-activated channel currents only in one patch out of 10. For this case we found a maximum conductance of 4 pS. At 2 to 3 DIC we found a maximum conductance of 15.5$\pm$3 pS (mean$\pm$s.d.; n= 6). Then, the maximum NMDA-activated channel conductance increased to reach

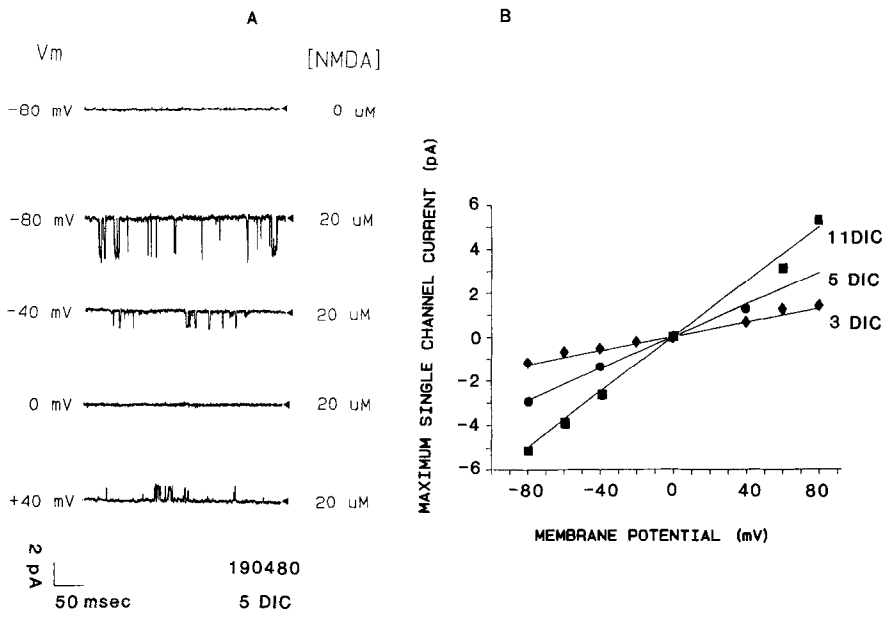

Figure 1 (**A**): NMDA evoked single–channel currents in an outside–out patch obtained from a 5 DIC cell. Control trace (0 μM NMDA) is shown at the top of records. 20 μM NMDA-activated a channel population at different membrane potentials (V_m). (**B**): NMDA-activated single channel maximum current amplitude (I_m) as a function of membrane potential, measured in ouside-out patches obtained from cells of 3 DIC (diamonts), 5 DIC (circles) and 11 DIC (squares).

35.7±6.6 pS (n=9) between 5 to 8 DIC. Between 9 to 11 DIC NMDA–activated channels had a maximum conductance of 46.8±7.7 pS (n=6).

NMDA-activated channels revealed different subconductance states, as was also found in other neuron preparations [1, 5, 13]. Up to 5 different conductance states could be easly detected in most of the experiments. In fig. 3B an example of two conductance states observed in a patch obtained from a 5 DIC cell is depicted. At least two different values of current amplitude can be clearly detected from the data (see amplitude histogram in fig. 3C). Subconductance states have been observed at all potentials and at all times in culture. For cells at more than 5 DIC, besides the maximum conductance and subconductance values described above, some brief events were easly distinguished from the maximum conductance channel openings, and had a conductance of about 15 pS. (fig. 3A). This is the value of conductance of NMDA–activated channels observed for younger cells. If the observed big channels are an addition of small channels (15 pS) events, the probability to find such superimposition would be much smaller than the probability to find a single channel. As this is not the case, we can conclude that small conductance and high conductance channels are independent units.

Open time histograms of NMDA–activated channels were analized in different DIC. They showed always a single exponential distribution [1, 12], so the open time constant was not different from the mean open time, $< t_o >$. The mean open time is clearly shorter

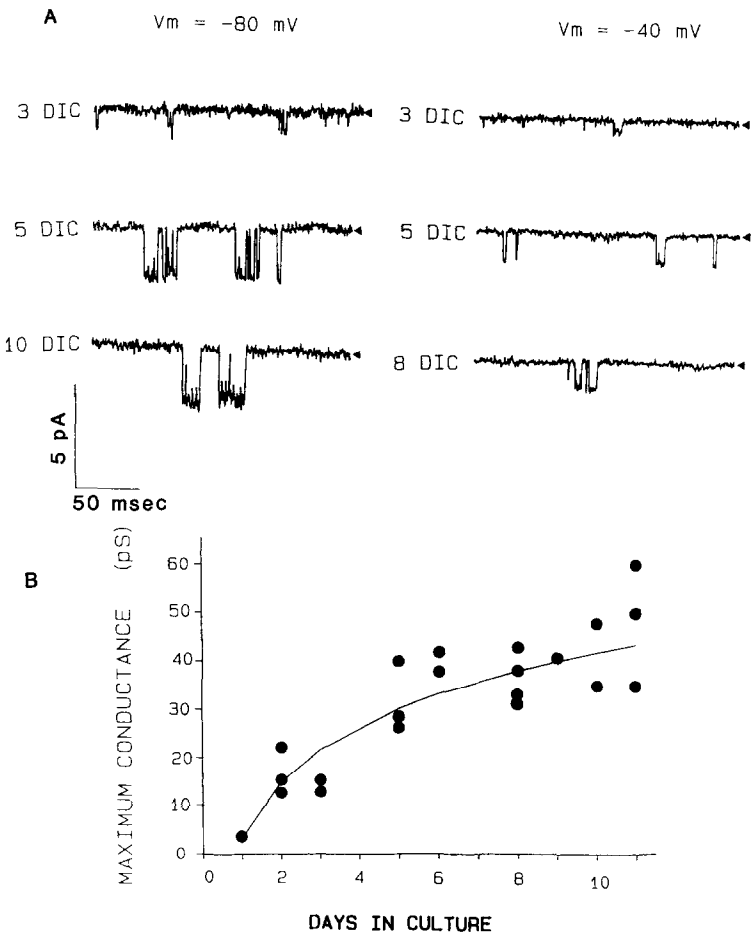

Figure 2 (**A**): Single channels activated by 20 μM NMDA at different DIC. Membrane potential (V_m) is indicated at the top of each column. (**B**): NMDA-activated maximum conductance as function of time in culture, from 1 to 11 DIC.

when the cells are younger. On cells of 1 to 3 DIC $< t_o >$ was shorter than 1 msec and never longer than 2.5 msec, while in cells with more than 3 DIC $< t_o >$ was 5.9±3.7 msec (n=11). Open time distribution in NMDA-activated currents did not appear to change significatively in cells older than 3 DIC.

DISCUSSION

The primary culture of enzymatically dispersed cerebellar granule cells that we studied consisted in immature cells, that were subjected to a developing process *in vitro*. The development of granule cells in this period was indicated by changes in the metabolic pathways [8, 9, 15], in the neurocytochemical and morphological characteristics of the cells [8, 11], and in voltage–dependent channel expression [4, 11]. Here we report that there is also a change in the electrophysiological properties of NMDA-activated channels during differentiation *in vitro*.

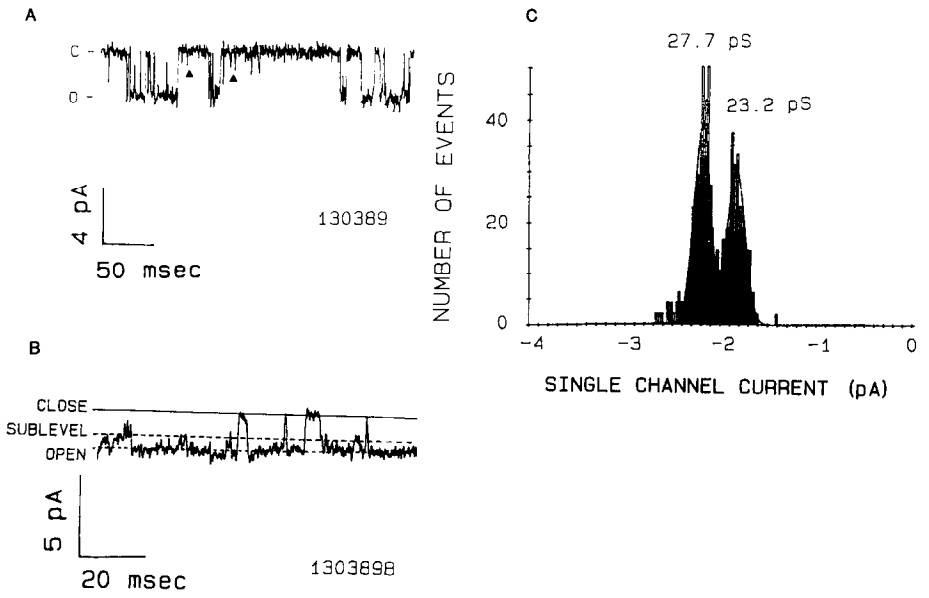

Figure 3 (**A**): Record of NMDA–activated channels of an outside–out patch obtained from an 8 DIC cell at a membrane potential (V_m) of –80 mV. Maximum single channel conductance of 38 pS is accompained by some brief events (arrows) of 15 pS. (**B**): Single channel currents activated by 20 μM NMDA at a V_m of –80 mV, where a subconductance level is observed is presented as an example. (**C**): The amplitude histogram corresponding to the record shown in (**B**) where sub–states of 27.7 pS and 23.2 pS conductance are resolved.

The first important observation that we report here is that the NMDA–activated single channel maximum conductance increases with days in culture. Ascher and Nowak [2] observed an increase of the whole-cell response to NMDA after 7 DIC in cortical neurons. They attributed this effect to an increase of glycine concentration in the media surrounding the cells. Under our conditions, if glycine modulation was involved in the change of the response of NMDA–receptors, it would not be expected an increment neither in single channel conductance nor in $< t_o >$ [14]. On the other hand, our experiments were performed after an intensive washing of the culture dishes , that should remove the glycine present in the culture media, and using a fast perfusion that should improve the washing of glycine [1].

We can tentatively propose that small conductance events observed under NMDA stimulation during the first 3 DIC can be attributed to the response of other receptors, as quisqualic acid and kainic acid receptors, which are known to have an unspecific response to NMDA [3, 5, 6]. It may be probable that NMDA specific receptors during this period are absent because of a natural immature state of the cells or the damage produced on the NMDA receptor–channel complexes by trypsin digestion. This could be supported by the fact that it is possible to find isolated events similar to those found during the first days also in cells with more than 6 DIC (see fig. 3B). Nevertheless, we can not exclude the possibility that the NMDA different responses are due to changes of the NMDA-receptor activated channels themselves, either by modification of the NMDA channel-receptor complex or by

ex novo syntesis of NMDA-activated channels, expressing channels that are different from those present in the first days in culture.

It would be interesting also to notice that all the changes that we observed during the development of granule cells *in vitro* occurred in artificial conditions, with the lack of their natural synaptic target (Purkinje cells), and in the absence of glial cells, factors normally partecipating in the neuronal differentiation process [7].

We can conclude that enzymatically dissociated granule cells responses to NMDA change with days in culture. This different responses can be related to development of the cells *in vitro* as result of a natural process or by the resynthesis of NMDA receptor-channel complexes damaged by the trypsin digestion. Our results show the necessity to define the developmental stage of these cell preparation for studies of glutamate receptor–channel complex.

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