Patch-clamp recording of amino acid-activated responses in "organotypic" slice cultures

cerebellum/hippocampus/γ-aminobutyric acid-activated channels/glutamate-activated channels

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ABSTRACT

Patch-clamp recording techniques were used to study the properties of amino acid-activated channels in cultured "organotypic" slices from rat cerebellum and hippocampus. Hippocampal pyramidal cells responded to the three main glutamatergic agonists, N-methyl-D-aspartate (N-Me-D-Asp), quisqualate, and kainate, whereas Purkinje cells responded only to quisqualate and kainate. Analysis of single-channel events recorded in outside-out patches from hippocampal neurons showed large conductance events (50 pS), which occurred more frequently in the presence of glycine. These events could be produced by N-Me-D-Asp and also, at low frequency, by quisqualate. On the other hand, 50-pS events were never observed in Purkinje neurons. This supports the hypothesis that N-Me-D-Asp and "non-N-Me-D-Asp" receptors are distinct molecular entities. Comparison of whole-cell and outside-out patch recordings from Purkinje cells revealed a clear spatial segregation of γ-aminobutyric acid (GABA) and glutamate receptors: although GABA receptors are found at high density in somatic membrane, quisqualate and kainate receptors are mostly extrasomatic. The results show that organotypic slice cultures are amenable to patch-clamp methods. They also show that, in these cultures, amino acids receptors have specific distribution patterns according to cell type and to region within a cell.

Our understanding of the basic membrane conductances operating in vertebrate central neurons rests largely on studies using two types of in vitro preparations: primary cultures prepared from dissociated neurons and brain slices. Both preparations have specific advantages. One of the key advantages of primary cultures is that the neuronal surface is well exposed, allowing the use of patch-clamp techniques (1). On the other hand, classical slices permit the study of identified neurons connected by normal synapses. A third type of preparation, the "organotypic" culture of brain slices, stands as an attractive intermediate between dissociated neurons and classical slices. In these cultures (2–4) slices are placed for a few weeks in roller tubes, where they undergo a progressive thinning. When most successful, this process yields one or two layers of cells that retain the general organization of the original slice and the main morphological and physiological properties of the various neurons present, including specific synaptic connections (refs. 2–4; see also refs. 5 and 6). Furthermore, organotypic cultures can be used to characterize synaptic interactions between anatomically remote brain areas, since functional synaptic connections can be established between cocultured slices derived from various brain regions (7).

In the present work, we show that patch-clamp recording methods can be applied to organotypic cultures of brain slices. To explore some of the potentials of this approach, we have studied the responses of hippocampal and cerebellar neurons to neuroactive amino acids. We have specifically addressed three questions related to the spatial organization and developmental stage of neurons in organotypic cultures. (i) We have examined whether Purkinje cells contained N-methyl-D-aspartate (N-Me-D-Asp) receptors, since these receptors, which are absent in in vitro slices of adult rats (8, 9), have been reported to appear transiently during development (10, 11) and to be present in neurons presumed to be Purkinje cells from dissociated cultures (12). (ii) We have studied responses to glutamatergic agonists in hippocampal pyramidal cells to compare the results with those obtained in organotypic cerebellar cultures, hippocampal cultures (13), and hippocampal slices (14). (iii) We have investigated if functional amino acid receptors in Purkinje cells are differentially distributed in somatic vs. dendritic membranes.

METHODS

Organotypic cultures of rat cerebellar and hippocampal slices were prepared from 1-day-old rats and 3- to 7-day-old rats, respectively, and maintained in culture as described (2). To reduce the number of nonneuronal cells, some of the cultures were subjected to moderate doses of ionizing irradiation [x-ramp, 250 kV, 1200 rads (1 rad = 0.01 gray)] at the time of explantation. In some cases, the antimitotic agents uridine, 5-fluorodeoxyuridine, and cytosine β-D-arabinofuranoside were added to the culture tubes at equal concentrations (1–0.1 μM) starting on day 3 or 4 of culture and were removed after 20 hr. Exposure to ionizing radiation and application of antimitotic agents can be expected not only to reduce the number of nonneuronal cells but also to reduce the number of neuroblasts still undergoing division and/or migration. However, Bodian silver staining indicated that intermediate-size neurons (presumed interneurons) and small neurons (tentatively identified as granule cells) were still numerous in cultures subjected to these manipulations (B.H.G., unpublished observations). The slices were used within 2–5 wk after explantation.

During experimental recordings, the slices were maintained at room temperature while being perfused with a solution containing (in mM) 140 NaCl, 2.5 KCl, 1 CaCl2, and 10 Heps (sodium) (pH 7.2). During most experiments 200 nM tetrodotoxin was added and in some 5.5 mM glucose was added. Variations in the composition of this solution are indicated in the figure legends. The whole-cell configuration and the outside-out patch configuration of the patch-clamp technique (1) were used. The composition of the internal solutions was as follows (in mM): (a) KCl: 120 KCl/10 EGTA (potassium)/5 Heps (potassium); (b) CsCl: 120 CsCl/10 EGTA (cesium)/5 Heps (cesium); (c) CsF: 120 CsF/10 CsCl/10 EGTA (potassium)/10 Heps (potassium)

Abbreviations: GABA, γ-aminobutyric acid; N-Me-D-Asp, N-methyl-d-aspartate.
(internal pH, 7.2). Electrode resistance was 1.5–5 MΩ. During the recording of large whole-cell currents, the series resistance led to errors in the imposed membrane potential of up to 10 mV. In addition, some of our results indicate lack of voltage control of neurites (see Results). These errors were left uncorrected.

Purkinje cells were recognized in the living state by their large size and their characteristic dark cytoplasm as visualized with phase-contrast microscopy. They were always localized in peripheral parts of the culture and could, therefore, easily be distinguished from neurons derived from the deep cerebellar nuclei, which were localized in the center of the cerebellar culture. Observation of dendritic processes in 20 Purkinje cells from cultures treated with antimitotics and x-rays and intrasomatically injected either with horseradish peroxidase or Lucifer yellow (B.H.G., unpublished observations) showed that their dendritic trees were very similar to those described in control cultures (4). The identification of the Purkinje cells was confirmed (J. Mariani, personal communication) by the fact that the cells identified as such (in all cases; data from unidentified hippocampal neurons are also included). In all cases the cell under study was identified as a neuronal cell by the presence of large currents activated by depolarization.

In some cultures sealed and the progression to whole-cell recording proceeded smoothly with the majority of neurons chosen for study. In other slices, however, a high-resistance seal was formed but following patch breakage, the response to voltage steps resembled the response of a partly obstructed pipette. Voltage- and time-dependent currents were not elicited by depolarizing voltage commands. A negative resting potential (−60 to −80 mV) was typically recorded in these cases, even with Cs+ containing pipette solutions. A possible explanation of these observations may be that seals were made on nerve or glial cell processes that ran on the exposed surface of neuronal cell bodies. This problem arose more frequently in cultures that had been treated with antimitotics and x-rays and intrasomatically injected with horseradish peroxidase or Lucifer yellow (B.H.G., unpublished observations) showed that their dendritic trees were very similar to those described in control cultures (4). The identification of the Purkinje cells was confirmed (J. Mariani, personal communication) by the fact that the cells identified as described above were all selectively stained by an antibody to calbindin (28-kDa calcium-binding protein) (15).

In hippocampus, pyramidal cells were recognized by their large size and location in or near cell layer CA1 or CA3. Only in those cultures with a clearly visible pyramidal cell layer was identification considered possible (data from pyramidal cells are specified as such; in other cases, data from unidentified hippocampal neurons are also included). In all cases the cell under study was identified as a neuronal cell by the presence of large currents activated by depolarization.

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Fast microperfusion of agonists was achieved either with a U tube (16) or with a double-barreled tube (17).

Noise analysis and single-channel analysis were performed as described by Ascher et al. (18).

RESULTS

Purkinje Cells and Pyramidal Hippocampal Cells Respond Differently to Excitatory Amino Acids. At least three different types of glutamate receptors can be distinguished in the vertebrate nervous system (reviewed in ref. 19). One receptor type is selectively activated by N-Me-D-Asp; the corresponding ion channel is blocked by Mg2+ ions (20). The other two receptor types are selectively activated by quisqualate and kainate, respectively. Adult Purkinje cells are known to respond with an increase in cationic conductance to quisqualate and kainate but to have a low sensitivity to N-Me-D-Asp (8, 9). In contrast, adult hippocampal pyramidal cells and, in particular, those of the CA1 region respond to all three compounds (14, 19).

Under whole-cell voltage-clamp, all Purkinje cells tested responded to applications of quisqualate and kainate with a large conductance increase. At a holding potential of −60 mV, 2 μM quisqualate elicited inward currents that ranged in amplitude from 0.6 to 4.2 nA (mean = 1.32; n = 8), whereas application of 10 μM kainate led to currents ranging from 0.5 to 1.9 nA (mean = 1.07; n = 7). In contrast, none of the cells tested (n = 6) responded to N-Me-D-Asp (100 μM), even when glycine, known to potentiate the response to N-Me-D-Asp, was added at concentrations (1–10 μM) that saturate the potentiating effect in those cultures. The selective sensitivity to "non-N-Me-D-Asp" agonists is illustrated in Fig. 1, which shows current records obtained from a Purkinje cell upon application of each of the three compounds. In the experiment shown in Fig. 1, Cd2+ was included in the bath solution to block synaptic transmission. The same result was obtained in the absence of Cd2+, which was previously shown not to block N-Me-D-Asp receptors (19). As illustrated in Fig. 1, when pipettes filled with the KCl internal solution were used, the initial inward current elicited by quisqualate or kainate was often followed by an outward current. No attempt was made to identify the ionic mechanism of this component of the response. A similarly activated outward current in other neuronal types has been attributed by some authors to activation of the Na/K pump and by others to the activation of Ca-dependent K currents (reviewed in ref. 19).

In contrast to the N-Me-D-Asp insensitivity of cerebellar Purkinje cells, hippocampal cells responded to N-Me-D-Asp as well as to quisqualate and kainate (Fig. 2). The responses usually had a much slower time course than those recorded in dissociated cultures from mouse brain with a similar perfusion system: the half-time of the onset and the offset was in the range of tens of seconds in organotypically cultured hippocampal neurons instead of hundreds of milliseconds in dissociated cultures. The response kinetics did not depend on drug perfusion pipette position but did vary from agonist to agonist (Fig. 2) and cell to cell. A possible explanation of these observations is that many of the receptors are on distant dendritic branches, partially buried within the slice. The existence of accessible somatic receptors and more distant dendritic receptors is expected to give rise to rather complicated current kinetics. A second difference with respect to the responses obtained in dissociated neuronal cultures was the absence of a large effect of glycine on the N-Me-D-Asp response recorded in the whole-cell mode. The response to 10 μM N-Me-D-Asp was barely affected by 1 μM glycine in five of six whole-cell recordings from hippocampal neurons of cultured slices (mean augmentation, 1.1; range, 0.97–1.2). In one cell, however, faster response

![FIGURE 1](https://example.com/figure1.png)  
**FIG. 1.** Whole-cell current responses of a Purkinje cell to glutamatergic agonists. Holding potential, −60 mV. Agonists were applied during the time indicated by the bars above each record. The slice was bathed in Mg2+-free external medium, which was supplemented with 5.5 mM glucose, 200 mM tetrodotoxin, and 250 μM Cd2+. Internal solution, KCl. Quis, quisqualate; Kai, kainate; NMDA, N-Me-D-Asp.
kinetics and a significant augmentation by glycine (2.4-fold) were observed (see Discussion).

Single-Channel Analysis of Excitatory Amino Acid-Induced Currents. Fig. 3A (upper trace) illustrates the response of an outside-out patch from a hippocampal pyramidal cell to 10 μM N-Me-D-Asp with 1 μM glycine. The mean amplitude of the most commonly observed single-channel event at −50 mV was 2.3 pA in this patch. With a reversal potential of 0 mV, the corresponding value of the single-channel conductance is 46 pS. The mean conductance (±SD) estimated in nine different patches was 48 ± 4 pS. The influence of 1 μM glycine on the response to 5 or 10 μM N-Me-D-Asp was measured in seven patches. In five of these patches, the total channel open time was increased by glycine by a factor of 4.9 ± 3.3 (mean ± SD). This was due predominantly to an increase in the frequency of channel opening. There was only a slight increase in channel mean open time from a value of 7.5 ± 1.8 ms (mean ± SD; n = 5) with N-Me-D-Asp alone to a value of 9.1 ± 1.2 ms (mean ± SD; n = 7) in the presence of glycine. In the remaining two patches glycine clearly augmented the response to N-Me-D-Asp, but the effect could not be quantified because too few channel openings were observed with N-Me-D-Asp alone.

The lower trace of Fig. 3A presents some of the single-channel events recorded during application of quisqualate (2 μM) in the same patch as the upper trace. In this case the main event was a 12-pS channel, but many 6-pS events (of which only one is illustrated) were also recorded. In addition, there were a few large openings having the same conductance as the main event observed after addition of N-Me-D-Asp. These large-conductance events were observed, albeit at low frequency, in all three patches studied. They carried 5% of the quisqualate-induced current.

The responses to kainate (data not shown) were associated with current fluctuations in which individual events could not be resolved. The ratio of the noise variance to the total current yielded a "mean" single-channel conductance of 2.1 pS. When fitted with a single Lorentzian, the noise spectra yielded time constants of about 1–3 ms, values comparable to those found in dissociated cultures (21).

The single-channel records obtained from Purkinje cells differed markedly from those obtained in hippocampal neurons by the (expected) absence of responses to N-Me-D-Asp. As discussed below, none of the patches studied showed a detectable response to 2 μM quisqualate (n = 5). With a higher concentration (10 μM), responses to quisqualate were obtained in four of nine patches tested. These responses consisted of an initial inward current of 3–4 pA, which desensitized within 30–50 ms to a level of activity where single-channel events could be clearly resolved. Over the entire period of recording analyzed (13 min), transitions corresponding to the N-Me-D-Asp-induced conductance

![Fig. 2](image_url) Whole-cell current responses of a hippocampal pyramidal cell to glutamatergic agonists. Holding potential, −50 mV. Agonists were applied during the times indicated by the bars above each record. The slice was bathed in Mg²⁺-free external medium with 200 nM tetrodotoxin. Internal solution, CsF. See Fig. 1 legend for abbreviations.

![Fig. 3](image_url) (A) Selected records obtained from an outside-out patch of a hippocampal pyramidal cell during application of the indicated agonists. Holding potential, −50 mV; Mg²⁺-free external medium; pipette solution, CsF. The arrows mark the current level corresponding to the mean single-channel current of N-Me-D-Asp-activated channels in this patch at −50 mV. (B) Selected records from an outside-out patch of a cerebellar Purkinje cell during application of the indicated agonists. Holding potential, −60 mV. The dotted lines indicate the expected current level for an N-Me-D-Asp-activated channel at this potential. In the case of kainate, the beginning of the dotted line marks the time of agonist application. Mg²⁺-free external medium, 5.5 mM glucose; pipette solution, CsCl. All records were filtered with an 8-pole Bessel filter at a cutoff frequency of 1 kHz. See Fig. 1 legend for abbreviations.
(45–50 pS) were never observed. To examine more rigorously the possibility that 45- to 50-pS channels may nevertheless have contributed to the response, 3 min of recordings at low mean current level (<0.3 pA; e.g., Fig. 3B, upper trace), where overlaps were relatively infrequent, were further analyzed. A cumulative transition histogram obtained on such recordings showed a predominant step amplitude of 0.86 pA at −60 mV, corresponding to an elementary conductance of 14 pS. Smaller step sizes were also apparent. Direct transitions corresponding to a 45- to 50-pS conductance were not found, but there were a few cases (seven) of multistep transitions that reached the 45- to 50-pS level. The total duration of these events was 7.5 ms. Even if this time was considered as reflecting the opening of N-Me-D-Asp-type channels, such events would represent only 0.1% of the total charge transfer during the entire recording period. If, in addition, an average N-Me-D-Asp-like opening (of 7.5-ms duration) was artificially added to the record to allow for the statistical nature of channel opening, the total charge contribution of N-Me-D-Asp-like channels would still be <0.2%.

The current fluctuations induced by 50 μM kainate in Purkinje cell patches are exemplified by the lower trace of Fig. 3B, obtained from the same outside-out patch as Fig. 3B, upper trace. The spectrum of this current noise increase could be described by a single Lorentzian with a time constant of 1 ms. Analysis of the ratio between the mean current and the variance yielded an estimate of 2.5 pS for the single-channel conductance. An analysis of this patch by using the same method as that described for quisqualate-induced currents indicated that N-Me-D-Asp-like channels, if present, would contribute <0.5% of the total current.

Dendritic vs. Somatic Localization of Amino Acid Receptors. Although the classical concept that inhibitory synaptic contacts are generally placed on the soma whereas excitatory synapses are most often found on dendrites (22) has not been found to apply universally, in the case of Purkinje cells it is well established that γ-aminobutyric acid (GABA) receptors are mostly somatic (23) whereas glutamate receptors are mostly dendritic (24). Electrophysiological studies indicating a dendritic localization for the excitatory responses to glutamatergic agonists have provided further support for this notion (8).

To determine if this segregation is also present in the Purkinje cells of cultured slices, we compared the responses of these neurons to GABA and to excitatory amino acids, in the whole-cell recording configuration and in outside-out patches excised from the cell soma. Pipettes with relatively large tip diameters (resistances in KCl solutions, 2–2.5 MΩ) were used to obtain large outside-out patches. At a holding potential of −60 mV and with symmetrical Cl− concentrations, whole-cell inward currents induced by 2 μM GABA varied between 0.6 and 1.8 nA (mean = 1.39; n = 6), which is within the range of the response magnitudes obtained with 2 μM quisqualate and 10 μM kainate (see above). In contrast, when the same compounds were applied to outside-out patches, it was observed that the response to GABA was always much larger than the response to excitatory amino acids (Fig. 4). All patches studied responded to 2 μM GABA, the size of the induced current ranging from 10 to 150 pA at −60 mV (mean = 50 pA; n = 6). However, none of the patches showed detectable responses to 2 μM quisqualate and the response to 10 μM kainate was either small or undetectable (range, 0–3 pA; mean = 1.23 pA; n = 5). These findings strongly suggest that functional GABA receptors are found at high density on the soma of Purkinje cells, whereas most of the excitatory amino acid receptors are extrasyomatic. An alternative interpretation of the results would be that whole-cell responses to glutamatergic agonists were caused by the activation of excitatory interneurons rather than by a direct action on Purkinje cells. This seemed unlikely since most experiments were performed in the presence of tetrodotoxin. Nevertheless, additional experiments were performed in the presence of 250 μM Cd2+ to prevent synaptic transmission (e.g., Fig. 1). The whole-cell responses to kainate and quisqualate were as large as in the normal external solution. These experiments exclude the possibility that an indirect activation of Purkinje cells by kainate and quisqualate accounts for the present results.

A dendritic localization of excitatory receptors was also suggested by comparisons of the reversal potential for the responses to quisqualate and kainate in whole-cell and in outside-out patches. In outside-out patches, these responses reversed at 0 mV, as described (12, 13, 21). However, the excitatory amino acid-induced currents recorded in the whole-cell mode remained inward with applied membrane potential values as large as 20–40 mV. This difference is most likely observed because most functional receptors are on the cells’ dendrites, which are not accurately voltage-clamped. On the other hand, responses to GABA in whole-cell recording and outside-out patches were found to reverse near 0 mV, the Cl− equilibrium potential under our ionic conditions. This is in agreement with the hypothesis that GABA receptors are mostly somatic.

The GABA responses studied with patch-clamp techniques on Purkinje cells from organotypic cultures resembled those of cultured spinal neurons (25, 26) and chromaffin cells (27). The currents desensitized quite strongly in response to GABA concentrations between 2 and 10 μM (see Fig. 4, upper traces) and were blocked by bicuculline meth-
ochloride (2.5–10 μM) in a partially reversible manner \((n = 6)\). Analysis of single-channel records, obtained in smaller outside-out patches, indicated that there are multiple conductance states. The most frequent state had a conductance of 28–30 pS, which compares well with the value of 30 pS reported by Bormann et al. (26) for dissociated cultures of spinal neurons. A less frequent state with a conductance of 19–20 pS was also observed, but the 40-pS events described by these authors were not found in the patches studied.

**DISCUSSION**

N-Me-D-Asp induced responses in hippocampal neurons but not in cerebellar Purkinje cells. The sensitivity of hippocampal neurons was expected since all previous studies except one (28) have indicated that pyramidal cells bear N-Me-D-Asp receptors and are excited by N-Me-D-Asp (reviewed in ref. 19). The pattern of sensitivity to excitatory amino acids and, in particular, the absence of N-Me-D-Asp sensitivity in Purkinje cells suggest at first sight that the neurons present in organotypic slices have the properties of adult neurons. This should be qualified, however, since it has been suggested that the sensitivity of rat Purkinje cells to N-Me-D-Asp is also absent at birth, that it develops in the days after birth, and that it then disappears again about 1 month later (10, 11).

The properties of N-Me-D-Asp responses recorded in outside-out patches from hippocampal cells are similar to those reported in cultures of dissociated neurons from mammalian brain (12, 13, 18, 20). The observation here of an augmentation by glycine suggests that glycine sensitivity (17) is a general property of N-Me-D-Asp channels, although the effect is not of uniform magnitude.

The whole-cell responses of the hippocampal neurons to N-Me-D-Asp, however, differ from those of dissociated cultures in their slow time course and in their insensitivity to glycine. A link between these properties is suggested by the fact that the only whole-cell recording exhibiting a significant augmentation of the N-Me-D-Asp response by glycine (2.4-fold) also had the most rapid responses to application and removal of agonists. The results could be explained by the presence of a diffusion barrier between the bathing solution and neuronal receptors. Such a barrier could delay the access of exogenous agonists to their receptors and permit the accumulation of glycine in the intercellular space up to a concentration sufficient to mask any potentiating effect of exogenous glycine (see ref. 17).

In outside-out patches from cerebellar and hippocampal neurons, quisqualate opened channels of several different conductances. The main conductance state, defined as the conductance state observed most frequently among single-channel events, had a value of 12–14 pS. In the case of kainate, analysis of the ratio of the current noise variance to the total agonist-induced current yielded a value of 2.1–2.3 pS for the main conductance state. Both of these values are in agreement with what has been found in dissociated cultures from various brain regions (12, 13, 21).

The study of the "minor" states elicited by non-N-Me-D-Asp agonists, however, revealed a marked difference between Purkinje cells and hippocampal neurons. In the case of hippocampal neurons, the minor states included some large single-channel events with a conductance very similar to that of the main event activated by N-Me-D-Asp agonists. The activation of these large conductance events by non-N-Me-D-Asp agonists, which have also been observed in dissociated cultures from hippocampus (13), cerebellum (12), and cortex (21), has been interpreted by two types of theories: one in which the 50-pS openings represent a "substate" of a channel that can be activated through separate receptors by N-Me-D-Asp and non-N-Me-D-Asp agonists (12, 13) and one in which the large conductance openings observed with non-N-Me-D-Asp agonists indicate that these compounds are weak agonists for the N-Me-D-Asp receptor (21). The fact that the non-N-Me-D-Asp agonists did not induce any 50-pS openings in Purkinje cells supports the second type of interpretation, in which the 50-pS conductance is exclusively linked with the N-Me-D-Asp receptor.

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