Optical Mapping of Early Embryonic Expressions of Mg^{2+} -/APV-Sensitive Components of Vagal Glutaminergic EPSPs in the Chick Brainstem

Yoko Momose-Sato, Tetsuro Sakai, Akihiko Hirota, Katsushige Sato, and Kohtaro Kamino

Department of Physiology, Tokyo Medical and Dental University School of Medicine, Bunkyo-ku, Tokyo 113, Japan

Multiple-site optical recording of transmembrane voltage activity, using a voltage-sensitive dye (NK2761) and a 12 × 12 element photodiode array, was employed to monitor neural responses in the slice preparations that were dissected from 6–9-d-old embryonic brainstems. Transmembrane voltage-related optical responses evoked by a brief square current pulse applied with a microsuction electrode to the vagus nerve were recorded simultaneously from many sites in the preparation. In preparations from 6-d-old embryos, only action potential-related spike-like optical signals were often detected in the normal bathing solution containing 0.5 mM Mg^{2+} . However, in several loci of some preparations, excitatory postsynaptic potential-related slow signals were elicited in a Mg^{2+} -free bathing medium, and these elicited slow signals were blocked by DL-2-amino-5-phosphonovaleric acid (2-APV). These results show a possible embryonic expression of NMDA receptor-mediated excitatory postsynaptic potentials. The slow signals were usually detected from tested preparations from 7–9-d-old embryos in normal bathing solution. The later phase of the slow signals was enhanced in a Mg^{2+} -free bathing medium, and the enhanced component was also sensitive to 2-APV and insensitive to 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). There were regional differences in the degree of the enhancement of the slow signals in the Mg^{2+} -free bathing medium, and of the 2-APV-induced reduction of the slow signals. We have constructed early developmental maps of the spatial patterns of the slow signals related to Mg^{2+} - and 2-APV-sensitive postsynaptic potentials. These maps reflect the spatial distribution of functional expression of the NMDA receptors during early development of the chick brainstem. The present findings are new data that have not been reported previously, and they have been obtained for the first time using the multiple-site optical recording technique with a voltage-sensitive dye.

[Key words: glutaminergic EPSP, Mg^{2+} sensitivity, APV sensitivity, embryonic brainstem, nucleus tractus solitarius, optical recording]

In the experimental analysis of the early embryonic CNS, microelectrode examination of the electrophysiological behavior of neurons, which provides the most direct test of their function, is often extremely difficult or impossible because of the small size and fragility of the cells. In the last few years, using optical techniques for monitoring of cellular transmembrane voltage (Cohen and Salzberg, 1978; Freedman and Laris, 1981; Salzberg, 1983), our laboratory has shown that it is possible to record directly from embryonic nerve fibers (Sakai et al., 1991), ganglia (Sakai et al., 1985; Momose-Sato et al., 1991a), and brainstems (Kamino et al., 1989b, 1990; Komuro et al., 1991, 1993; Momose-Sato et al., 1991b; for a review, see Kamino, 1990) at early stages of their development.

In addition, optical techniques using a multiple-element photodiode array have facilitated simultaneous recordings of electrical activity from many regions in one preparation (for reviews, see Cohen and Leshner, 1986; Grinvald et al., 1988; Kamino, 1991). Using such methods, we have already investigated the appearance of excitatory postsynaptic potentials evoked by vagal stimulation in the embryonic brainstem at early stages of its development, and we reported that distinct postsynaptic potentials appear in 7-d-old embryonic brainstem, and that it is glutamate mediated (Komuro et al., 1991). Furthermore, in experiments using two antagonists such as DL-2-amino-5-phosphonovaleric acid (2-APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), we have previously identified NMDA and non-NMDA receptor-related components of postsynaptic potential-related slow optical signals in response to vagal stimulation in the 8-d-old embryonic chick brainstem (Komuro et al., 1991; Momose-Sato et al., 1991b; Kamino et al., 1992). On the other hand, it is well known that the NMDA receptor currents are inhibited by external Mg^{2+} (Evans et al., 1977; Nowak et al., 1984; Collingridge et al., 1988; for a review, see Collingridge and Lester, 1989). These data and ideas have suggested two questions to be resolved with the optical recording technique, at the earliest stage of the generation of the glutaminergic excitatory postsynaptic potential. One is in what manner the embryonic onset of the functional expression of the NMDA receptors is effected by external Mg^{2+} , and the other is in what spatial pattern the NMDA receptor function is generated.

Thus, we have examined the effects of external Mg^{2+} and 2-APV on the onset and early development of the glutamate-mediated excitatory postsynaptic potentials in 6–9-d-old embryonic chick brainstems. Subsequently, we have constructed maps of their spatial patterning, and we attempted to identify the onset of the physiological expression of the NMDA receptor.

A preliminary account of some of these experiments has been presented (Momose-Sato et al., 1992; Sakai et al., 1992).

Received Apr. 4, 1994; revised May 26, 1994; accepted June 2, 1994.

We are grateful to Drs. Larry Cohen and Brian Salzberg for their reading of the manuscript and comments. We also thank the journal reviewers for helpful suggestions concerning the manuscript. This work was supported by grants from the Ministry of Education, Science and Culture of Japan, the Mitsubishi Foundation, and the Uehara Memorial Life Science Foundation.

Correspondence should be addressed to Kohtaro Kamino, Department of Physiology, Tokyo Medical and Dental University School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan.

Copyright © 1994 Society for Neuroscience 0270-6474/94/147572-13\$05.00/0

Materials and Methods

The methods that we have used for multiple-site optical recording of electrical activity in embryonic brainstem preparations have been described in detail elsewhere (Komuro et al., 1991; Momose-Sato et al., 1991b).

Preparations. In the present experiments, we used embryonic chick brainstem slice preparations. Fertilized eggs of white Leghorn chickens were incubated for 6–9 d in a forced-draft incubator (type P-03, Showa Incubator Lab., Urawa, Japan) at a temperature of 37°C and 60% humidity, and were turned once each hour. The brainstems, with vagus nerve fibers attached, were dissected from the embryos. The isolated brainstem preparation was attached to the silicone (KE 106LTV; Shinetsu Chemical Co., Tokyo, Japan) bottom of a simple chamber by pinning it with tungsten wires. The preparation was kept in a bathing solution with the following composition (in mM): NaCl, 138; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 0.5; glucose, 10; and Tris HCl buffer (pH 7.2), 10. The solution was equilibrated with oxygen. The pia mater attached to the brainstem was carefully removed in the bathing solution under a dissecting microscope. Slices were then prepared, with the right and/or left vagus nerve fibers attached, by sectioning the embryonic brainstem transversely at the level of the root of the vagus nerve. The thickness of the slice was about 1 mm.

Dye staining. The isolated slice preparation was stained by incubating it for 15–25 min in a Ringer's solution containing 0.1–0.2 mg/ml of the voltage-sensitive merocyanine-rhodanine dye NK2761 (Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan; Kamino et al., 1981, 1989a; Salzberg et al., 1983; Kamino, 1991), and the excess (unbound) dye was washed away with dye-free Ringer's solution before recording.

Electrical stimulation. For preparations in which the vagus nerve was stimulated, the cut end of the nerve was drawn into a microaspiration electrode fabricated from TERUMO-hematocrit tubing (VC-H075P, TERUMO Co., Tokyo, Japan), which had been hand pulled to a fine tip (about 100 μ m internal diameter) over a low-temperature flame.

Optical recording. Light from a 300 W tungsten-halogen lamp (type JC-24V/300W, Kondo Sylvania Ltd., Tokyo, Japan) was collimated, rendered quasi-monochromatic with a heat filter (32.5B-76, Olympus Optical Co., Tokyo, Japan), and an interference filter having a transmission maximum at 703 ± 15 nm (Asahi Spectra Co., Tokyo, Japan), and focused on the preparation by means of a bright-field condenser with a numerical aperture (NA) matched to that of the microscope objective (S plan Apo, 10 $\times$, 0.4 NA). The objective and photographic eyepiece (2.5 $\times$) projected a real image of the preparation onto a 12 $\times$ 12 element silicon photodiode matrix array (MD-144-4PV, Centronic Ltd., Croydon, UK) mounted on an Olympus Vanox microscope (type AHB-L-1, Olympus Optical Co., Tokyo, Japan). The magnification of the image was usually 25 $\times$. Each pixel (element) of the array detected light transmitted by a square region ($56 \times 56 \mu\text{m}^2$) of the preparation. The output of each detector in the diode array was passed to an amplifier (time constant of AC-coupling = 3 sec) via a current-to-voltage converter. The amplified outputs from 127 elements of the detector were first recorded simultaneously on a 128-channel recording system (RP-890 series, NF Electronic Instruments, Yokohama, Japan), and then were passed to a computer (LSI-11/73 system, Digital Equipment Co., Tewksbury, MA). The 128-channel data recording system is composed of a main processor (RP-891), eight I/O processors (RP-893), a 64K word wave-memory (RP-892), and a videotape recorder. The program for the computer was written in assembly language (Macro-11) called by Fortran, under the RT-11 operating system (version 5.0). The optical recording was carried out in a still chamber without continuous perfusion with Ringer's solution, at room temperature, 26–28°C. The incident light was turned off except during the measuring period. Under these conditions, in 6–9-d-old embryonic brainstem preparations, the evoked optical signals can often be detected continuously for 60–150 min.

Results

The initial appearance of Mg²⁺-sensitive component

We have previously reported that in 7–8-d-old embryonic chick brainstems, voltage-sensitive dye-related absorption signals, composed of two components (a fast spike-like signal and a slow signal), were evoked by vagal stimulation, and the slow signal was inhibited by kynurenic acid, reflecting a glutamate-mediated excitatory postsynaptic potential (Komuro et al., 1991;

Momose-Sato et al., 1991b). We also showed that the postsynaptic potential reflects the activity of both the NMDA and non-NMDA subtype receptors. In the 6-d-old embryonic brainstem, the appearance of slow signals has usually been ambiguous in normal Ringer's solution containing 0.5 mM Mg²⁺, so we have tentatively determined the embryonic onset of the generation of the glutamate-mediated postsynaptic function to occur in 7-d-old embryonic chick brainstems. In the present experiments, we have first searched for the appearance of the slow signals in 6-d-old embryonic brainstem preparations, in more detail.

Figure 1 shows two optical recordings of neural activity in the slice preparation dissected from a 6-d-old embryo (J988). The optical signals were evoked by a brief square current pulse (7 μ A/5 msec) applied to the right vagus nerve, and recorded simultaneously from 127 contiguous loci of the preparation. The left panel illustrates the recording made in normal Ringer's solution containing 0.5 mM Mg²⁺, and the recording in the right panel was made in a Mg²⁺-free bathing solution. These optical signals were wavelength dependent, as expected for the action spectra of merocyanine-rhodanine dyes, and no signals were detected in unstained preparations; the recordings shown in Figure 1 were made using an interference filter of 703 ± 15 nm. Therefore, these optical signals are indeed the light absorption changes originating only from transmembrane voltage changes.

In normal Ringer's solution containing 0.5 mM Mg²⁺, apparently, only fast spike-like signals were observed. Whereas, when Mg²⁺ was removed from the Ringer's solution, small slow signals, which followed the fast spike-like signals, were evident, and they were elicited in a restricted area (indicated by arrowheads in Fig. 1). To see the time course of the signals in more detail, we show examples of enlarged traces in Figure 2A. These traces were obtained from six positions (F-3, G-3, F-4, J-7, I-8, and J-8) of a different preparation (J1058). In these traces, the slow signals were not elicited in normal Ringer's solution. Nevertheless, when Mg²⁺ was removed from the Ringer's solution, the slow signals, which were very small (fractional change, $\Delta I/I < 2 \times 10^{-4}$), appeared to be elicited in four traces (G-3, J-7, I-8, and J-8). These small slow signals disappeared again when the Mg²⁺-free solution was replaced by the normal Ringer's solution. Thus, it is likely that the detected slow signals were revealed by removal of Mg²⁺ from the bathing solution. The traces in Figure 2B were obtained from positions H-4, I-4, and J-4 of the same preparation. In these traces, slow signals with extremely small amplitudes can be seen in normal Ringer's solution, and their amplitudes were enhanced twofold in the Mg²⁺-free bathing solution.

The slow signals that appeared in the Mg²⁺-free solution were completely blocked in the presence of kynurenic acid (e.g., Collingridge and Lester, 1989) or 2-APV (Davies et al., 1981; Evans et al., 1982), as shown on the bottom traces in Figure 2B, and they were not affected by CNQX (data not shown). These results strongly suggest that in the 6-d-old embryonic brainstem, the slow signals in normal Ringer's solution, and the slow signals that were enhanced in Mg²⁺-free bathing medium are mostly mediated by the NMDA subtype glutamate receptor.

The slow signals detected from the 6-d-old embryonic preparations appeared to be concentrated in a restricted narrow area. One example of the spatial distributions of the slow signals is illustrated in Figure 3, where the positions of the slow signals appearing in normal Ringer's solution are indicated by black grid, and the extended regions that exhibited slow signals in Mg²⁺-free solution are indicated by shadowed grids. Similar

6-day

Figure 1. Multiple-site optical recording of the electrical activity from a 6-d-old embryonic brainstem slice preparation. The electrical activity was evoked by a depolarizing square current pulse ($7 \mu\text{A}$, 5 msec) applied to the right vagus nerve using a microsuction electrode. This stimulating condition was adequate for eliciting the maximum responses. The *left recording* was obtained in normal Ringer's solution and the *right recording* was obtained in Mg^{2+} -free solution. The preparation was fixed to the bottom of the chamber with the cephalic side down. In this figure and Figures 4 and 6, the traces are arranged so that their relative positions in the figures correspond to the relative positions of the sites of the preparation imaged onto the detector. The recordings were made in a single sweep. The *direction of the arrow at the lower right corner of the recording* indicates a decrease in transmission (an increase in absorption) and the *length of the arrow* indicates the stated value of the fractional change. *Arrowheads* indicate the slow signals that were elicited in the Mg^{2+} -free solution.

patterns were obtained in four other preparations from the 6-d-old brainstems. Therefore, it is suggested that, by the 6-d-old embryonic stage, the NMDA-subtype receptor-mediated post-synaptic function has been generated potentially within the medulla oblongata, and that, in normal bathing solution, it is mostly suppressed by external Mg^{2+} .

Early development

Similar experiments have been carried out on 7–9-d-old embryonic brainstem preparations. Two examples of the original recordings are shown in Figure 4; one was obtained from a 7-d-old preparation (*A*), and the other from an 8-d-old preparation (*B*). In the preparations from these older embryonic brainstems, the slow signals were usually recorded in the normal Ringer's solution and they were enhanced by removal of Mg^{2+} from the bathing medium. This effect depended upon the concentration of Mg^{2+} in the bathing solution (data not shown). In the bottom of each recording, the enlargements of typical signals are illustrated.

In previous experiments (Komuro et al., 1991), we have shown that the initial phase of the slow signal is reduced by CNQX

and the later phase is reduced by 2-APV. We have thus examined effects of 2-APV and CNQX on the enhanced fraction of the slow signal in the Mg^{2+} -free solution. The enlargements of the signals obtained from three conditions are compared in Figure 5. These signals were obtained from two different 8-d-old embryonic brainstems. As shown in Figure 5*A*, the later phase of the slow signal was reduced by 2-APV (200 μM ; in the early embryonic preparations used in the present experiments, the maximal effects of 2-APV were obtained at around 200 μM) and in a Mg^{2+} -free solution containing 2-APV: in the presence of 2-APV, no effects of removal of Mg^{2+} from the bathing solution were observed. On the other hand, as shown in Figure 5*B*, the initial phase of the slow signal was reduced by CNQX (5 μM), and the later phase of the slow signal was enhanced in the Mg^{2+} -free solution containing CNQX. Similar effects were observed in the 7- and 9-d-old embryonic preparations. Furthermore, when the concentration of the external Mg^{2+} ($[\text{Mg}^{2+}]_o$) was raised, the later phase of the slow signal was reduced in a manner similar to the effect of 2-APV (data not shown). These results strongly suggest that the enhanced component of the slow signal in Mg^{2+} -free bathing solution is the postsynaptic potential

Figure 2. *A*, Enlargements of six optical signals from six sites (*F-3*, *G-3*, *F-4*, *J-7*, *I-8*, and *J-8*) of a 6-d-old preparation (*J1058*) in normal and Mg^{2+} -free solutions. Note that the very small slow optical signals elicited in the Mg^{2+} -free solution were recorded from four sites (indicated by asterisks: *G-3*, *J-7*, *I-8*, and *J-8*). *B*, Optical signals recorded simultaneously from three sites (*H-4*, *I-4*, and *J-4*) of the 6-d-old preparation (*J1058*) in normal Ringer's (top), Mg^{2+} -free (middle), and 2-APV-containing Mg^{2+} -free (bottom) solutions. In these positions, very small slow signals were detected in the normal Ringer's solution, and they were enhanced in the Mg^{2+} -free solution. The slow signals, which appeared in the normal solution and enhanced in the Mg^{2+} -free solution, were eliminated in the 2-APV-containing Mg^{2+} -free solution. The experimental conditions were the same as in Figure 1.

mediated by the NMDA receptor function. Therefore, it is likely that in the 7–9-d-old embryonic preparations, NMDA receptor function is markedly restrained by extracellular 0.5 mM Mg^{2+} .

2-APV-induced reduction of slow optical signals

In the next step, we examined the 2-APV-sensitive component. Figure 6 shows two examples of the experiments that were carried out to examine the regional distribution of the suppressive effects of 2-APV on postsynaptic potential-related slow optical signals that appeared in the brainstem slice preparations dissected from an 8-d-old (*A*) and a 9-d-old (*B*) chick embryo.

In Figure 6, the left recordings are the control signals recorded in a normal Ringer's solution and the right ones are the signals recorded in a bathing solution to which 2-APV (200 μ M) was added. In every preparation, the later phase of all the slow signals was markedly reduced when 2-APV (200 μ M) was added to the bathing solution.

Distribution mapping

Mg^{2+} sensitivity. As can be seen in the original recordings shown in Figure 4, there were regional differences in the amplitudes both of the slow signals in normal Ringer's solution and the slow signals enhanced in the Mg^{2+} -free bathing solution. We have measured the amplitudes of all of the slow signals and constructed the contour maps of the slow signal magnitudes in normal Ringer's solution and in Mg^{2+} -free bathing solution (Fig. 7). The data were obtained from the 7-d-old (*J971*), 8-d-old (*J981*), and 9-d-old (*J1102*) embryonic preparations; the 7- and 8-d-old preparations were the same as those shown in Figure 4.

In Figure 7, the differences in amplitude between the control and the Mg^{2+} -free bathing solution records are also shown. The amplitudes of the signal were distributed in a layered pattern surrounding the site in which the signal exhibited the maximal amplitude (the peak amplitude site). In these maps, we are able

Figure 3. An example of the areas in which slow optical signals appeared in a 6-d-old embryonic brainstem preparation (*J984*). The positions indicated by black is the area in which small slow signals were detected in normal Ringer's solution; the positions indicated by gray were the area in which slow signals were elicited in Mg^{2+} -free solution.

A 7-day

(J971)

B 8-day

(J981)

Table 1. Parameters associated with the maximum area of optical signals under the normal and Mg^{2+} -free conditions

	Preparation	Control (S_{max})	Difference (ΔS_{max})	$\Delta S_{max}/S_{max}$ (%)
7 day	J967	5.8	3.4	59
	J971(R)	8.6	9.4	109
	J971(L)	8.7	7.6	87
	J973	4.9	9.4	192
8 day	J957	10.5	13.5	129
	J960	6.5	12.6	194
	J981	15.3	16.2	106
	J824	11.4	4.5	39
9 day	J1097	15.7	13.8	88
	J1098	10.2	10.2	100
	J1102(R)	14.0	8.9	64
	J1102(L)	11.6	6.4	55

R and L indicate the right and left sides of the preparations.

to see the developmental changes in the amplitude and the distribution pattern of the slow signals.

In each of the preparations shown in Figure 7, the patterns of the contour lines obtained in the control and in the Mg^{2+} -free bathing solution are similar. Also the peak amplitude sites are located approximately at the same position in the control and in the Mg^{2+} -free solution. On the other hand, there were differences in the positions of the peak amplitude site among the three preparations. In the 7-d-old preparation, the peak amplitude site of the slow signal was positioned in the lateral region, and in the 8- and 9-d-old preparations, it translocated toward the medial region. The results obtained from 60 preparations are summarized in Figure 8. The peak amplitude site of the difference was also located in the lateral side in the 7-d-old preparation; in contrast, the peak amplitude site in the 8- and 9-d-old preparations appeared to be located in the medial side. The relative locations of the peak amplitude site of the differences in each preparation are illustrated with the black grids on the photodiode array elements over the image of the preparations, on the bottom of the maps in Figure 7.

In the Mg^{2+} -free solution, not only the amplitude of the slow signal but also the duration increased. We have also measured the enhanced area [ΔS = (area estimated in the Mg^{2+} -free solution) – (control area)] under the slow signals (illustrated in Fig. 9), with time integration using a computer. Figure 9A illustrates three examples of the contour line maps of ΔS . The data were obtained from the 7-d-old (J971), 8-d-old (J981), and 9-d-old (J1102) slice preparations used in Figure 7. The patterns of the contour lines of ΔS were similar to those of the contour lines of the differences in the amplitude shown on the third row in Figure 7.

Next, in order to examine the regional differences in the relative sensitivity of the slow signal to external Mg^{2+} , we estimated the ratio of the ΔS to the control area (S) of the slow signal, in normal Ringer's solution, $\Delta S/S$. The ratios were ob-

Figure 5. Evoked optical signals typical of those that appeared in 2-APV-containing, and 2-APV-containing Mg^{2+} -free solutions (*A*), and CNQX-containing, and CNQX-containing Mg^{2+} -free solutions (*B*). Optical recordings were made in two different 8-d-old preparations (J1077 and J1076). They were evoked by depolarizing square current pulses of 8 μA , 5 msec.

tained from all areas in which signals were detected. In Figure 9B, the values of the ratios are represented by the relative sizes of the solid circles: the size was divided into six grades. The data were obtained from the three preparations shown in Figure 9A. In these maps, interestingly, the relative sensitivity to external Mg^{2+} is greatest in the 8-d-old preparation (see also Table 1), and the ventral side of the response area is relatively more effected by external Mg^{2+} than is the dorsal side. These results show that developmental changes in the relative sensitivity to $[Mg^{2+}]_o$ do not occur in a simple fashion.

2-APV sensitivity. We have also evaluated the areas under the slow signal curves (signal area) obtained in the normal and 2-APV-containing bathing solution, and then constructed maps of the contour lines of the signal areas.

The maps shown in Figure 10A were constructed using the recordings from the 7-d-old preparation (J1084) together with the 8-d-old preparation (J673) and the 9-d-old preparation

Figure 4. Multiple-site optical recordings of neural voltage responses from a 7-d-old (*A*) and an 8-d-old (*B*) preparation in normal Ringer's (*left*) and Mg^{2+} -free (*right*) solution. The neural responses were evoked by depolarizing square current pulses of 7 μA , 5 msec (for the 7 d embryo) and 8 μA , 5 msec (for the 8 d embryo). Enlargements of the optical signals characteristic of one position (J-6 for *A*, H-7 for *B*; indicated by asterisks) in each multiple-site recording are shown in the *bottom*. Note that the slow optical signals were enhanced in the Mg^{2+} -free solution. Other experimental conditions were the same as in Figure 1.

Figure 6. Multiple-site optical recordings of neural voltage responses from an 8-d-old preparation (*A*, J673) and a 9-d-old preparation (*B*, J1112) in normal Ringer's solution (*left traces*) and in 2-APV (200 μM)–containing solution (*right traces*). The optical signals were evoked by a depolarizing square current of 8 μA , 5 msec.

Figure 7. Contour line maps of the amplitude of the slow optical signals in 7-d-old (*A*), 8-d-old (*B*), and 9-d-old (*C*) preparations. The *top* row shows the control, the *second* row is for the signals in the Mg^{2+} -free solution, and the *third* row is the difference = (the amplitude in Mg^{2+} -free solution) – (the control). Data used for the 7- and 8-d-old preparations were obtained from the recordings shown in Figure 4. The numerals on the contour lines represent the fractional change ($\times 10^{-4}$) of the signal. The black grids indicate the relative locations of the peak amplitude site of the differences. See Results for additional details.

(J1112) used in Figure 6. The top row illustrates the control maps for each preparation, the second row was made with the data obtained in the 2-APV-containing bathing solution, and the third row with the differences [$\Delta S = (\text{control area}) - (\text{area estimated in the 2-APV-containing solution})$]. The value of ΔS reached saturation level with the 2-APV concentration used in the present study.

The sizes of the slow signal areas were regionally distributed in a layered pattern surrounding the site in which the signal area exhibited the maximal size (peak area site). Under the control condition, the peak size increased from 6 (arbitrary units) to 16 (arbitrary units) as development proceeded from 7 to 9 d. On the other hand, similarly to the case observed in the Mg^{2+} experiment, there were differences in the regional distribution among the three preparations: the position of the peak area site was located in the lateral region in the 7-d-old preparation, and it translocated to the medial region in the 8- and 9-d-old pre-

parations. These patterns were observed in all the tested preparations.

Here, we note once again that the slow signal areas were markedly decreased in the 2-APV-containing bathing solution. In these maps, an additional feature is that in the 7- and 9-d-old preparations, the relative positions of the peak area site in the control (top) and in the 2-APV-containing solution (second row) coincided with each other, but in the 8-d-old preparation, the position of the peak area site of the signal in the presence of 2-APV was deviated laterally with respect to the control signal.

As can be seen in the third row in Figure 10*A*, in every preparation, the distribution pattern of ΔS is very similar to that of the control signal area (*S*), and the relative position of the peak area site of ΔS corresponded to that of the control signal area (*S*). Furthermore, in comparison with the data obtained from the Mg^{2+} experiment, although the position of the

Figure 8. Variations in the relative location of the peak amplitude site of the fast and slow signals obtained from the 7-, 8-, and 9-d-old preparations: $n = 16$ for the 7-d-old preparations, $n = 22$ for the 8-d-old preparations, and $n = 22$ for the 9-d-old preparations. Open circles indicate the center of the locations of the peak amplitude site of the fast signals, and solid circles indicate the center of the locations of the peak amplitude site of the slow signals. These illustrations were drawn by superimposing traces from different preparations. One circle corresponds to one preparation, but the data from some preparations were overlapped. Y-axes correspond to the midline of the preparations, and the dashed lines indicate the averaged anatomical outlines of the preparations at each stated stage. The scales on the abscissae (x-axes) indicate the distance (in μm) from the midline (y-axes), and the scales on ordinates (y-axes) indicate the distance (in μm) from the x-axes to the

peak area site in the Mg^{2+} -free solution deviated in the ventral direction, as a whole, the distribution patterns shown in the third row in Figure 10A appeared to correspond to those obtained in a Mg^{2+} -free bathing solution. This is consistent with the suggestion that the ΔS corresponds to NMDA receptor-mediated component of the glutaminergic excitatory postsynaptic function. The relative locations of the peak area site in the 2-APV-containing solution (indicated by triangles) and those of the difference (ΔS) (indicated by black grids) are illustrated on the photodiode array elements over the image of the preparations, on the bottom of the maps in Figure 10A.

Finally, to examine the relative sensitivity to 2-APV, we have estimated the ratios of the 2-APV-induced reduction area (ΔS) to the control area (S), and we have made the distribution maps of the ratios ($\Delta S/S$). Figure 10B shows the distribution of the ratio for the signal component reduced by 2-APV. The data were obtained from the maps shown in Figure 10A. In these maps, the values of the ratio ($\Delta S/S$) are indicated by the relative size of the solid circles: the ratios were divided into six sizes. There were differences in the regional distribution pattern of the ratio ($\Delta S/S$) between the three preparations. In the 7-d-old preparation, the ratio was larger in the lateral/dorsal region; in the 8-d-old preparation, the ratio was larger in the medial region; and in the 9-d-old preparation, the ratio increased over the whole response area and was largest in the marginal zone. These features are considered to correspond to the regional distribution of the sensitivity to 2-APV and of the ratio of functionally expressed NMDA receptor to total glutaminergic receptor function.

Discussion

In the present experiments, we have examined changes in the regional distribution of the Mg^{2+} - and 2-APV-sensitive components of excitatory postsynaptic potentials evoked by stimulation of the vagus nerve in the early embryonic chick brainstem. Recordings from brainstem slice preparations were carried out using multiple-site optical recording with a 12×12 element photodiode array and a voltage-sensitive merocyanine-rhodanine dye. We discuss the results obtained in connection with the regional distribution of electrophysiological expression of NMDA subtype receptors during early development of the chick brainstem.

The earliest embryonic Mg^{2+} -sensitive component

The first results described in this article provide new information about the extracellular Mg^{2+} dependence of postsynaptic potentials in response to vagal stimulation in the brainstem during early embryonic development. In particular, we have detected the Mg^{2+} -sensitive component of glutaminergic excitatory postsynaptic potential in the 6-d-old embryonic brainstem, and we have demonstrated the developmental changes in location of the area in which the Mg^{2+} -sensitive component is generated in the 6- to 9-d-old embryonic chick brainstems.

In our early experiments, we tentatively suggested that the embryonic onset of the glutaminergic excitatory postsynaptic potential in response to vagal stimulus is first observed in the 7-d-old embryonic brainstem (Komuro et al., 1991; Momose-

←

dorsal direction. Thus, the location of the peak-amplitude site is represented on the orthogonal coordinate.

Figure 9. *A*, Contour line maps of the difference of the slow signal areas evaluated in normal Ringer's solution and in Mg²⁺-free solution, (Mg²⁺-free signal area) – (control signal area), for the 7-d-old (J971), 8-d-old (J981), and 9-d-old preparations (J1102). Data were obtained from the preparations shown in Figure 7. The areas were evaluated using a computer (PC9801VX, NEC, Tokyo, Japan). Numerals on the contour lines are in arbitrary units. The program is available from Y. Momose-Sato. *B*, Regional distributions of the ratio of the signal area enhanced in the Mg²⁺-free solution to the control area, in the 7-d-, 8-d-, and 9-d-old preparations. The ratios are displayed by black circles, which were ranked into six classes according to the values of the ratios. Data were obtained from the preparations shown in *A*.

Sato et al., 1991b). Indeed, in the recording shown in Figure 1 of the present paper, it is very difficult to identify postsynaptic potential-related slow signals in normal Ringer's solution with any assurance. However, in Mg²⁺-free solution, slow signals are clearly recognizable, as shown in Figures 1 and 2. This evidence clearly indicates that postsynaptic potentials appear initially in some embryonic brainstems as early as the 6-d-old stage, and some of them appear to be suppressed by external Mg²⁺.

In addition, Figure 2*B* leads us to conclude that postsynaptic potential-related slow signals detected from the 6-d-old embryonic brainstem, including the signals enhanced in Mg²⁺-free solution, are mediated by NMDA receptor. Thus, we suggest that the NMDA receptor function is expressed prior to the functional expression of the non-NMDA receptor in the process of embryonic organization of glutamatergic postsynaptic function. Expansion of the region where the slow signals are observed in Mg²⁺-free solution reflects the fact that physiological expression of the NMDA receptor, during the early stage of the brainstem organization, is closely dependent on external Mg²⁺.

Concerning the postsynaptic response area, we considered the following basic points.

(1) The vagus nerve bundle contains both motor and sensory nerve fibers. Thus, in the present experiments, the applied stimulation was simultaneously orthodromic for the sensory nerve fibers, and antidromic for the motor nerve fibers. Thus, it is likely that the vagal response area within the early embryonic brainstem includes many motoneurons and sensory nerve terminals.

(2) The size of the slow optical signals was decreased by continuous stimulation, reduced by low external calcium ion concentrations and eliminated in the presence of manganese or cadmium ions (Komuro et al., 1991). Furthermore, the slow optical signals were eliminated by kynurenic acid (Komuro et al., 1991). Therefore, we concluded that the slow optical signals correspond to glutamate-mediated postsynaptic potentials. It is technically difficult or impossible to make intracellular recording of the early embryonic EPSPs, because the neurons are too small to be impaled with microelectrodes.

Figure 10. *A*, Contour line maps of the time-integrated area of the slow optical signals in 7-d-old (J1084), 8-d-old (J673), and 9-d-old (J1112) preparations. The top row shows the control, the second row is for the signals in the Ringer's solution containing 2-APV, and the third row is the difference = (the control area) – (the area in the Ringer's solution containing 2-APV). Data used in the 8- and 9-d-old preparation were obtained from the recordings shown in Figure 6. The numerals on the contour lines represent the areas in arbitrary units. Triangles indicate the relative positions of the peak area site in the 2-APV-containing solution, and black grids indicate those of the difference (ΔS). *B*, Regional distributions of the fraction of the slow optical signal area reduced by 2-APV to the control area. The fractions are displayed by black circles that were ranked into six classes according to the size of the ratio (%). Data were obtained from the same preparations shown in *A*. See Results for additional details.

(3) Anatomically, it is known that the sensory nerve fibers contained within the vagus nerve project into the nucleus tractus solitarius (Breazile, 1979). In the nucleus tractus solitarius of adult vertebrates, L-glutamate has been shown to be a chemical transmitter (Perrone, 1981; Talman et al., 1984). Thus, it is reasonably assumed that postsynaptic optical response area detected in the present experiment corresponds to the nucleus tractus solitarius. Our morphological investigations, using the fluorescent carbocyanine dyes, support this idea (Momose-Sato et al., unpublished observations).

Accordingly, the present results suggest that in the nucleus tractus solitarius, the sensitivity to Mg^{2+} of the NMDA receptor is originally generated during early embryonic development.

Thus, we may speculate that the Mg^{2+} sensitivity of the NMDA receptor is related to the early development of synaptic transfer efficacy within the nucleus tractus solitarius. On the other hand, considering that the inhibitory effect of Mg^{2+} on NMDA receptors is voltage dependent (Mayer et al., 1984; Nowak et al., 1984), it is possible that during the developmental changes in the Mg^{2+} sensitivity of NMDA receptor, developmental changes in resting membrane potential may play a role. Furthermore, in comparison with adult neurons (Mayer et al., 1984) and cultured mammalian neurons (Forsythe and Westbrook, 1988), in the present data, higher concentrations of external Mg^{2+} are required to suppress the postsynaptic potential, suggesting that the affinity of Mg^{2+} to the receptor is weak in the early embryonic

neurons, and/or that the resting membrane potential is relatively depolarized in early embryonic neurons.

2-APV-sensitive component

According to the conventional notion that glutamate receptors can be grouped into several distinct subtypes, namely, NMDA, and non-NMDA including kainate, AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), and L-AP4 (L-2-amino-4-phosphonobutanoate) (Lodge and Johnson, 1990; Watkins et al., 1990), and that 2-APV is a specific antagonist to the NMDA subtype of glutaminergic receptor (Davies et al., 1981; for reviews, see Watkins and Evans, 1981; Collingridge and Lester, 1989; MacDonald and Nowak, 1990), we have used 2-APV as a pharmacological tool to separate the NMDA and non-NMDA components of the slow optical signal that is related to glutaminergic excitatory postsynaptic potentials. It seems reasonable to assume that the signal area diminished at the saturation level of 2-APV (difference, ΔS , the third row in Fig. 10A) may be considered as a measure of the component mediated by the NMDA receptor, and that the signal area unaffected by 2-APV ($S - \Delta S$, the second row in Fig. 10A) corresponds to the component mediated by the non-NMDA receptor, at least as a first approximation.

Possible interpretations

Generally, the linear relationship of the optical signal with changes in intracellularly recorded membrane potential has been established in other preparations (Cohen and Salzberg, 1978). We further assume that each detector received light from many neurons and processes; signals therefore are likely to represent the summed responses from simultaneously active neurons. Thus, in the present experiments, it seems likely that the amplitude of the slow signal is a function of some kind of average of the potential changes in the postsynaptic neurons and the number of synapses within the receptive field of one detector. Accordingly, developmental changes in the amplitude of the slow signal reflect the developmental increase of the postsynaptic potential and/or the developmental increase in the number of active synapses. Similarly, the regional differences of the amplitude of the slow signals correspond to the regional distribution of the postsynaptic potential and the regional differences in the number of active synapses.

In the maps shown in Figures 7 and 10A, (1) the control contour lines (illustrated in the top rows) reveal the distribution of activity of the glutaminergic EPSPs: the activity expands in a layered pattern, and the core area, in which the activity is highest, shifts with development, from the lateral region (in the 7 d embryo) to the medial region (in the 8 and 9 d embryo); (2) the regional distribution patterns of Mg^{2+} - and 2-APV-sensitive components related to the NMDA receptor activity (illustrated in the third row) is similar to the control patterns in each embryonic stage; and, furthermore, (3) in Figure 10A, the distribution of the non-NMDA receptor activity (illustrated in the second row) is somewhat different from that of the NMDA receptor activity: in the 7- and 9-d-old preparations, the relative positions of the peak regions are similar to those of the NMDA receptor activity, but in the 8-d-old preparation, the position of the peak region of the non-NMDA receptor activity deviates markedly toward the lateral region from that of the NMDA receptor activity.

The results shown in Figure 9B and Table 1 are of special interest for the following reasons. The ratio of external Mg^{2+}

sensitivity of the NMDA receptor, which is indicated by the relative change in the area of the slow signal, markedly increased in the 8-d-old embryo, and decreased again in the 9-d-old embryo. This developmental change of the ΔS -to- S ratio may argue that the fraction of the NMDA receptor component decreases in the 9-d-old preparation. Another possibility is that this change reflects the process by which NMDA receptor function is elicited during early development.

References

- Breazile JE (1979) Systema nervosum centrale. In: Nomina anatomica avium (Baumel JJ, King AS, Lucas AM, Breazile JE, Evans HE, eds), pp 417–472. New York: Academic.
- Cohen LB, Leshner S (1986) Optical monitoring of membrane potential: methods of multisite optical measurement. In: Optical methods in cell physiology (De Weer P, Salzberg BM, eds), pp 71–99. New York: Wiley.
- Cohen LB, Salzberg BM (1978) Optical measurement of membrane potential. *Rev Physiol Biochem Pharmacol* 83:35–88.
- Collingridge GL, Lester RAJ (1989) Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol Rev* 40:143–210.
- Collingridge GL, Herron CE, Lester RAJ (1988) Synaptic activation of *N*-methyl-D-aspartate receptors in the Schaffer collateral-commissural pathway of rat hippocampus. *J Physiol (Lond)* 399:283–300.
- Davies J, Francis AA, Jones AW, Watkins JC (1981) 2-Amino-5-phosphonovalerate (2APV), a potent and selective antagonist of amino acid-induced and synaptic excitation. *Neurosci Lett* 21:77–81.
- Evans RH, Francis AA, Watkins JC (1977) Selective antagonism by Mg^{2+} of amino acid-induced depolarizations of spinal neurons. *Experientia* 33:489–491.
- Evans RH, Francis AA, Jones AW, Smith DAS, Watkins JC (1982) The effects of a series of ψ -phosphonic α -carboxylic amino acids on electrically evoked and excitant amino acid-induced responses in isolated spinal cord preparation. *Br J Pharmacol* 75:65–75.
- Forsythe ID, Westbrook GL (1988) Slow excitatory postsynaptic currents mediated by *N*-methyl-D-aspartate receptors on cultured mouse central neurons. *J Physiol (Lond)* 396:515–534.
- Freedman JC, Laris PC (1981) Electrophysiology cells and organelles: studies with optical potentiometric indicators. *Int Rev Cytol [Suppl]* 12:177–246.
- Grinvald A, Frostig RD, Lieke E, Hildesheim R (1988) Optical imaging of neuronal activity. *Physiol Rev* 68:1285–1366.
- Kamino K (1990) Optical studies of early developing cardiac and neural activities using voltage-sensitive dyes. *Jpn J Physiol* 40:443–461.
- Kamino K (1991) Optical approaches to ontogeny of electrical activity and related functional organization during early heart development. *Physiol Rev* 71:53–91.
- Kamino K, Hirota A, Fujii S (1981) Localization of pacemaking activity in early embryonic heart monitored using voltage-sensitive dye. *Nature* 290:595–597.
- Kamino K, Hirota A, Komuro H (1989a) Optical indications of electrical activity and excitation-contraction coupling in the early embryonic heart. *Adv Biophys* 25:45–93.
- Kamino K, Katoh Y, Komuro H, Sato K (1989b) Multiple-site optical monitoring of neural activity evoked by vagus nerve stimulation in the embryonic chick brain stem. *J Physiol (Lond)* 409:263–283.
- Kamino K, Komuro H, Sakai T, Sato K (1990) Optical assessment of spatially ordered patterns of neural response to vagal stimulation in the early embryonic chick brainstem. *Neurosci Res* 8:255–271.
- Kamino K, Sakai T, Momose-Sato Y, Komuro H, Hirota A, Sato K (1992) Optical monitoring of postsynaptic potential in the early embryonic avian brain stem using a voltage-sensitive dye. In: *Fluorescence spectroscopy, new methods and applications* (Wolfbeis OS, ed), pp 289–301. Berlin: Springer.
- Komuro H, Sakai T, Momose-Sato Y, Hirota A, Kamino K (1991) Optical detection of postsynaptic potentials evoked by vagal stimulation in the early embryonic chick brain stem slice. *J Physiol (Lond)* 442:631–648.
- Komuro H, Momose-Sato Y, Sakai T, Hirota A, Kamino K (1993) Optical monitoring of early appearance of spontaneous membrane potential changes in the embryonic chick medulla oblongata using a voltage-sensitive dye. *Neuroscience* 52:55–62.

- Lodge D, Johnson KM (1990) Non-competitive excitatory amino acid receptor antagonists. *Trends Pharmacol Sci* 11:81–86.
- MacDonald JF, Nowak LM (1990) Mechanisms of blockade of excitatory amino acid receptor channels. *Trends Pharmacol Sci* 11:167–172.
- Mayer ML, Westbrook GL, Guthrie PB (1984) Voltage-dependent block by Mg^{2+} of NMDA responses in spinal cord neurons. *Nature* 309:261–263.
- Momose-Sato Y, Komuro H, Sakai T, Hirota A, Kamino K (1991a) Optical monitoring of cholinergic postsynaptic potential in the embryonic chick ciliary ganglion using a voltage-sensitive dye. *Biomed Res* 12[Suppl 2]:139–140.
- Momose-Sato Y, Sakai T, Komuro H, Hirota A, Kamino K (1991b) Optical mapping of the early development of the response pattern to vagal stimulation in embryonic chick brain stem. *J Physiol (Lond)* 442:649–668.
- Momose-Sato Y, Sakai T, Hirota A, Sato K, Kamino K (1992) Optical mapping of regional patterning of Mg^{2+} -sensitive postsynaptic potentials in the embryonic chick brain stem. *Neurosci Res [Suppl]* 17:S80.
- Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A (1984) Magnesium gates glutamate-activated channels in mouse central neurons. *Nature* 307:462–465.
- Perrone MH (1981) Biochemical evidence that L-glutamate is a neurotransmitter of primary vagal afferent nerve fibers. *Brain Res* 230:283–293.
- Sakai T, Hirota A, Komuro H, Fujii S, Kamino K (1985) Optical recording of membrane potential responses from early embryonic chick ganglia using voltage-sensitive dyes. *Dev Brain Res* 17:39–51.
- Sakai T, Komuro H, Katoh Y, Sasaki H, Momose-Sato Y, Kamino K (1991) Optical determination of impulse conduction velocity during development of embryonic chick cervical vagus nerve bundles. *J Physiol (Lond)* 439:361–381.
- Sakai T, Momose-Sato Y, Hirota A, Sato K, Kamino K (1992) Optical demonstrations of NMDA-/non-NMDA-subtype synaptic responses in the embryonic chick brain stem. *Neurosci Res [Suppl]* 17:S80.
- Salzberg BM (1983) Optical recording of electrical activity in neurons using molecular probes. In: *Current methods in cellular neurobiology*, Vol 3, Electrophysiological techniques (Barker JL, McKelvy JF, eds), pp 139–187. New York: Wiley.
- Salzberg BM, Obaid AL, Senseman DM, Gainer H (1983) Optical recording of action potentials from vertebrate nerve terminals using potentiometric probes provides evidence for sodium and calcium components. *Nature* 306:36–40.
- Talman WT, Granata AR, Reis DJ (1984) Glutamatergic mechanisms in the nucleus tractus solitarius in blood pressure control. *Fed Proc* 43:39–44.
- Watkins JC, Evans RH (1981) Excitatory amino acid transmitters. *Annu Rev Pharmacol Toxicol* 21:165–204.
- Watkins JC, Krogsgaardarsen P, Honore T (1990) Structure activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonist. *Trends Pharmacol Sci* 11:25–33.