A Modified Coaxial Compound Micropipette for Extracellular Iontophoresis and Intracellular Recording: Fabrication, Performance and Theory

John E. Remmers[1], Stanley A. Schultz[1], Jacqueline Wallace[1], Ryuji Takeda[2] and Akira Haji[2,*]

[1]*Respiratory Research Group, University of Calgary, Calgary, Alberta T2N 4N1, Canada*
[2]*Department of Pharmacology, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930–01, Japan*

ABSTRACT—Investigation of the identity and modes of action of neurotransmitters in the mammalian central nervous system can be facilitated by simultaneous intracellular recording of membrane potential and extracellular iontophoresis of agonists and antagonists. We describe here techniques for conveniently constructing a compound microelectrode, originally described by Sonnhof (Pflugers Arch **341**, 351–358, 1973), suitable for such studies. The Sonnhof electrode consists of two components, a centraxial micropipette for recording membrane potential surrounded by a cylindrical array of 6 pipettes for iontophoresis. The cylindrical array tapers coaxially and terminates in 6 contiguous, crescent-shaped orifices surrounding the terminal portion of the central pipette, 25–50 μm from the tip. Pipettes were constructed from borosilicate glass tubing of 1-mm wall thickness having a 10-mm or 16-mm outer diameter. The resistances, flux and transport numbers for iontophoresis of glycine were measured for pipettes constructed from both sizes of glass. Flux increased with increasing levels of current, and transport number decreased with increasing micropipette resistance. A spherical diffusion model points out the steep dependence of steady state concentration on diffusional distance, stressing the importance of diminishing the distance between the iontophoresis source and the recording site. This is particularly true when brief pulses of current are used.

Keywords: Transport number, Brief pulse application, Diffusional distance, Membrane potential, Iontophoresis

Combining intracellular recording of membrane potential with local, extracellular application of drugs provides a useful experimental method for investigating the identity and modes of action of putative trans-synaptic mediators. In well-visualized, in vitro preparations, this can be accomplished using separate movable recording and iontophoresis pipettes. However, neurophysiological or pharmacological investigation of a vertebrate central nervous system entailed blind recordings, precluding the use of two independent pipettes. In such situations, compound electrodes having a fixed spatial relationship between iontophoresis and recording pipettes allow simultaneous recording of membrane potential and iontophoresis of drugs in the vicinity of the neuron. Two types of compound electrodes have been used: para-axial and coaxial. In the para-axial electrode, the shafts of the recording and iontophoresis pipettes are glued with the

tips separated 100–200 μm (1–6). Because of its asymmetry and its relatively large cross-sectional area at the level of the micropipette's shank, the para-axial electrode is not well-suited for studying neurons located deep below the surface of the brain. In addition, the tips of the pipettes cannot be routinely brought closer together than 100 μm. Lastly, these tips may be dislocated as the compound electrode advances in the brain.

A coaxial electrode, described by Sonnhof (7), consists of a recording pipette encircled by an array of iontophoresis pipettes. This Sonnhof compound micropipette possesses a number of advantages. Its long, slender shank allows deep penetrations, and the fused walls of adjacent iontophoresis pipettes obviate spatial dislocation of the barrels of the pipette. A final advantage of the coaxial electrode is that the distance between the tips of the recording and the iontophoresis pipettes can be easily varied. The smaller distance between the tips of iontophoresis and recording pipettes reduces the diffusion distance

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*To whom correspondence should be addressed.

between the iontophoretic source and receptors near to the point of cellular impalement. As is described in the present communication, this diffusion distance influences the drug concentration at the point of recording, as well as the dynamics of change in this concentration, when iontophoretic pulses are applied.

Despite the desirable features of the coaxial compound electrodes system, it has been used sparingly; only Engberg et al. (8), Durand and Gueritaud (9) and ten Bruggencate and Sonnhof (10) have reported successful use of the Sonnhof micropipette. The elaborateness of construction and the reported large capacitative coupling between control recording and peripheral iontophoresis barrels (7) may have discouraged use. Our technique has largely circumvented both problems. The present report describes reliable and convenient methods for constructing a Sonnhof micropipette that does not require shielding of the central barrel for capacitance neutralization and that has tips of recording and iontophoresis pipettes in close proximity, approximately $20-50~\mu m$. Electrodes have been constructed from two sizes of glass, and the in vitro performance characteristics of the two have been compared.

MATERIALS AND METHODS

Construction

The cylindrical array of 6 micropipettes was constructed from 6 borosilicate glass tubes, each 8-cm-long. Two sizes were used: one 10-mm outer diameter with a 1-mm-thick wall and the other, 16-mm outer diameter with a 1.2-mm-thick wall (Corning #7740 "Pyrex"; Corning,

NY, USA). The 6 tubes were positioned circularly around a seventh of equal size. At each end of this array, adjacent tubes were tacked together with melted glass. The central tube was removed, and axial tubes (40-cm-long) were fused to the center of each end of the cylinder. These tubes served as handles during the initial pulling stage.

The initial pulling of the cylinder was found to critically influence the subsequent construction of the micropipette. The central 30% to 50% of the cylindrical array was heated in a glass blower's blast burner until red and slightly soft. At this time, the array was twisted somewhat (45–90 degrees) to ensure that the separate tubes in the array would touch while soft and slightly adhesive, and therefore, would adhere to each other during subsequent manipulations. The cylindrical array was then stretched axially in a vertical orientation and simultaneously twisted an additional 45–90 degrees. The linear stretch served to reduce the collective outer diameter of the array from 30 mm (or 48 mm) to 4–6 mm, while the vertical orientation maintained axiality during the stretch. The desired cross-sectional configuration was achieved by varying the amount of twist. The rotational twist at this stage counteracted the tendency of the peripheral bores to collapse radially during the stretch. Best results were obtained when the peripheral lumina were nearly circular and diameters of the center and peripheral lumina were approximately equal.

The resulting stock of 6-barrel tubing was cut into 4-cm-long segments. Each segment was divided into two 2-cm segments by heating, thereby producing a multibarrel array fused at one end and open at the other (Fig. 1A). A 2-mm diameter glass rod was tacked to the side of the
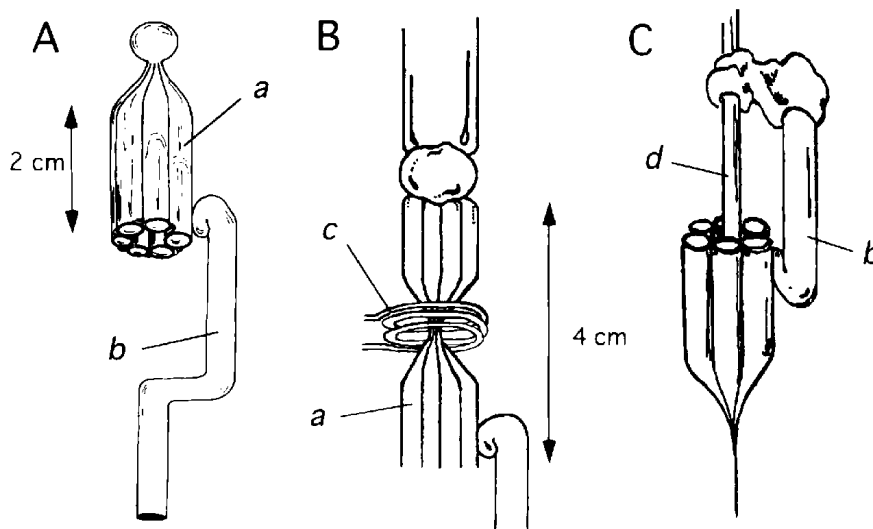

Fig. 1. Construction of the compound micropipette. Panel A: Six tubes (a) fused together with the axial handle attached (b). Panel B: Pulling the fused tubes in a micropipette puller with a spiral heating coil (c). Panel C: A coaxial micropipette with the center electrode (d) glued to the external handle (b) attached to the iontophoresis array.
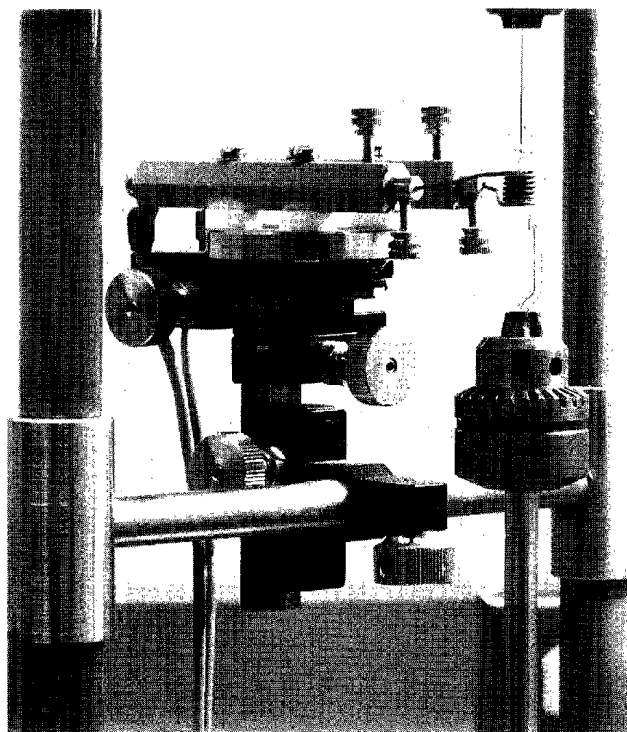
Fig. 2. A photograph showing a 6-barrel pipette mounted on the modified vertical micropipette puller.

segment, 0.5 cm from the open end. The rod was heated and bent to axiality to produce a convenient handle (Fig. 1A). The free end of this rod was mounted in the inferior chuck of a vertical micropipette puller (Narishige PE2, Tokyo). This puller was modified by mounting its heating coil on a micromanipulator (Narishige 3 M), allowing three-dimensional movement of the coil so that it could be conveniently centered coaxially around the glass cylinder (Fig. 2). A 4-mm glass rod, mounted in the superior chuck of the puller, was fused to the inferior, fused end of the multibarrel array with the heating coil surrounding the array (Fig. 2).

A tapered micropipette was pulled in two stages, the heat intensity and pull force being adjusted to produce a 5- to 7-mm shank length and a 1- to 2-μm tip diameter (Fig. 1B). This tip was broken under microscope visualization or beveled to achieve a diameter of 4–6 μm. This tip diameter was selected to correspond to the diameter of the shaft of the center pipette at the distance from the tip corresponding to the length of exposed recording electrode (see below). The axial portion of the rod was removed (Fig. 1C), and the multibarrel array was mounted on a three dimensional micromanipulator (Narishige 3M).

The central recording pipette was pulled (Flaming /Brown micropipette puller P-87; Sutter Instrument Co., Novato, CA, USA), and its tip was broken manually to
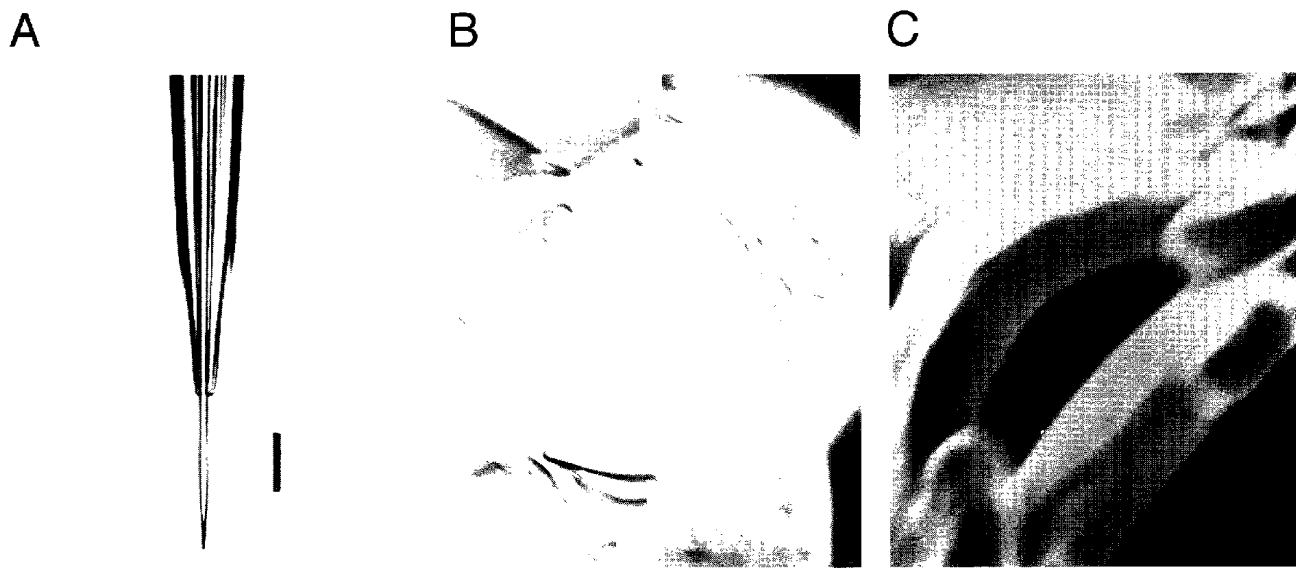
A   B   C

Fig. 3. Photographs of multibarrelled electrodes. Panel A: a light photomicrograph of the tip of the coaxial compound micropipette. The vertical bar indicates 10 μm. The profile reveals a shoulder 30 μm from the tip of the center electrode. This shoulder is comprised of the tips of the iontophoresis pipettes. Panel B: a scanning electronmicroscopic photograph of the end of the iontophoresis pipettes. End-on view of an array of iontophoresis pipette fabricated from 10-mm glass showing crescent shape mouths of the iontophoresis barrels encircling a center lumen through which the recording pipette can be inserted. Panel C: a scanning electronmicroscopic photograph of the mouth of a single iontophoresis barrel fabricated from 10-mm glass. Bars in B and C indicate 5 μm.

0.5-μm diameter with a 5-mm shank. This micropipette was mounted on a three dimensional micromanipulator system capable of angular movement (Narishige ME-7). The conventional micropipette and multibarrel array were axially aligned, and the former was then threaded into the center lumen of the latter under microscope visualization (Wild M5A; Heerbrugg, Switzerland or Olympus SZ, Tokyo). The central pipette was advanced until its shaft filled the lumen of the multibarrel array, as shown in Fig. 3A, and the shaft of the center pipette was then attached to the rod on the multibarrel array using an automotive body filler as an adhesive (Fig. 1C).

The morphology of the multibarrel array was assessed with an inverted microscope (Nikon TMD, Tokyo) equipped with Hoffman optics (Hoffman Modulation) and with a scanning electron microscope (Hitachi 450, Tokyo).

In vitro evaluation

The performance of the compound micropipette was evaluated by measuring the iontophoretic flux and transport number of glycine using a six channel iontophoresis current source (Dagan 6400; Minneapolis, MN, USA). To measure iontophoretic flux and transport number for glycine, the pipettes were filled with 1 M ^{3}H-glycine (pH 4.5), and cathodic currents (50–200 nA) were applied through silver-silver chloride electrodes with the tips of the array in 2 ml of normal saline. To measure diffusional egress of glycine from the pipette, no current was applied in some cases. This solution was analyzed for ^{3}H-glycine by a scintillation counter.

In vivo evaluation

In vivo performance was evaluated in decerebrate cats with the axial shaft of the compound electrode fixed to a stepping-motor-driven micromanipulator (Nanostepper; List-Electronic, Darmstadt, Germany or Burleigh 6000; Fishers, NY, USA). The recording electrode was filled with 2 M potassium citrate and coupled through a silver-silver chloride electrode to the head of a high impedance electrometer with a capacitance compensator (Dagan 8100). The iontophoresis barrels were coupled through silver-silver chloride electrodes to a current source (WPI S7100A; New Haven, CT, USA or Medical System IP-2; Great Neck, NY, USA). This instrument allowed application of brief large voltages to the iontophoresis electrodes for dislodging proteinaceous plugs at their tips. The electrodes were tested by iontophoresing GABA (1 M, pH 4.5), glycine (1 M, pH 4) and glutamate (0.5 M, pH 8) onto impaled bulbar respiratory neurons. One barrel filled with normal saline served as a "summing" channel carrying a current equal to the sum of currents passed through all other channels but opposite in sign.

To theoretically evaluate the importance of distance between the tips of the iontophoresis and recording electrodes, distance-concentration relationships were calculated using the following equation described by Nicholson and Phillips (11):

$$C(r,t) = Q\lambda^2 / 4\pi Dar \times \mathrm{erfc}(r\lambda / 2\sqrt{(Dt)})$$

where $C(r,t)$ is the concentration of glycine at the distance r and the time t, and erfc is the complementary error function. Volume fraction ($\alpha = 0.21$) and path tortuosity ($\lambda = 1.55$) were assigned to describe spherical diffusion from a point iontophoretic source, as reported by Nicholson and Phillips (11) for the cerebellum. The diffusivity D of glycine was assumed to be $1.055 \times 10^{-5}/cm^2$/sec and a transport number n of 0.05 was used.

$$Q = In / F$$

where Q is the source strength in mol/sec, I is the current and F is Faraday's electrochemical equivalent. The uptake of glycine by neural elements in the diffusion sphere was ignored.

RESULTS

Morphology

The tips of the multibarrelled-pipettes constructed from 10-mm glass, shown in Fig. 3B, formed a circular array of crescents shaped mouths, similar to that described by Sonnhof (7), although the overall dimensions were somewhat smaller. No consistent morphological difference was noted between arrays constructed from 10-mm and from 16-mm glass tubing. A view of an individual mouth of iontophoretic pipettes is shown in Fig. 3C.

In vitro studies

The average resistance was 17.4 ± 4.8 MΩ (n = 9) and 9.8 ± 4.5 MΩ (n = 10) for pipettes filled with 1 M glycine, which were constructed from 10-mm and 16-mm glass, respectively. Mean transport numbers at all levels of current tested were 0.042 ± 0.011 (n = 9) and 0.066 ± 0.043 (n = 10) for pipettes constructed from 10-mm and 16-mm glass, respectively. These values were not significantly different. The dependence of glycine flux on current intensity for each array of pipettes studied is shown in Fig. 4A. As expected, flux increased with increasing current for both types of pipettes. The larger variability in values for pipettes from 16-mm glass is apparent from the S.D. bars in this figure. Similarly, this figure illustrates the tendency for glycine flux from pipettes constructed from 16-mm glass to exceed that from pipettes constructed from 10-mm glass. This difference was statistically significant only for values at 50 nA (P < 0.05, Student's
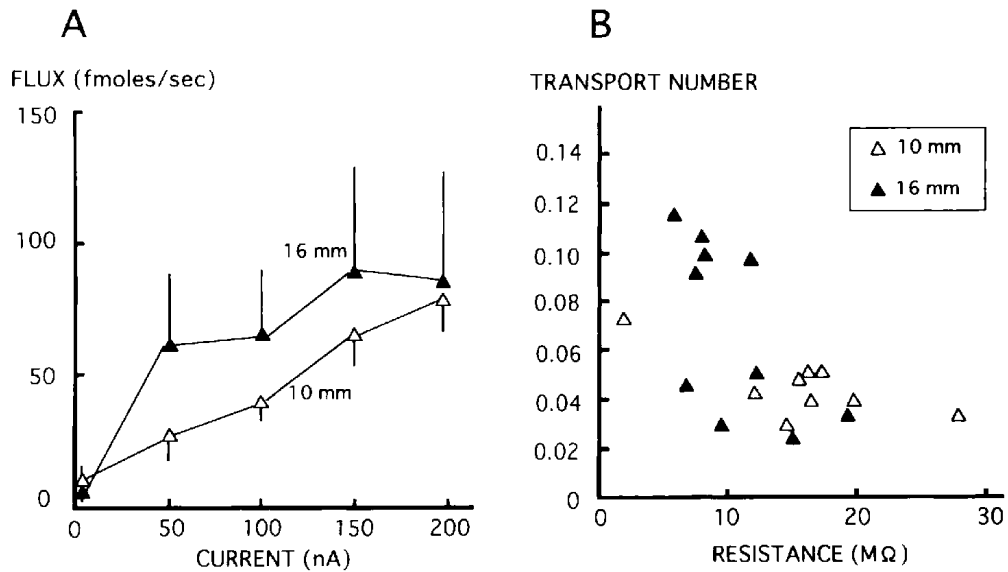
Fig. 4. Performance of the coaxial multibarrelled electrode. Panel A: theoretical calculations of the relationship between the amount of glycine fluxed from the iontophoretic pipette (ordinate) and current intensities (abscissa). Panel B: relationship between the measured transport numbers of iontophoresis pipettes constructed from 10-mm (open triangles) and from 16-mm (filled triangles) glass and measured resistances of the pipettes.
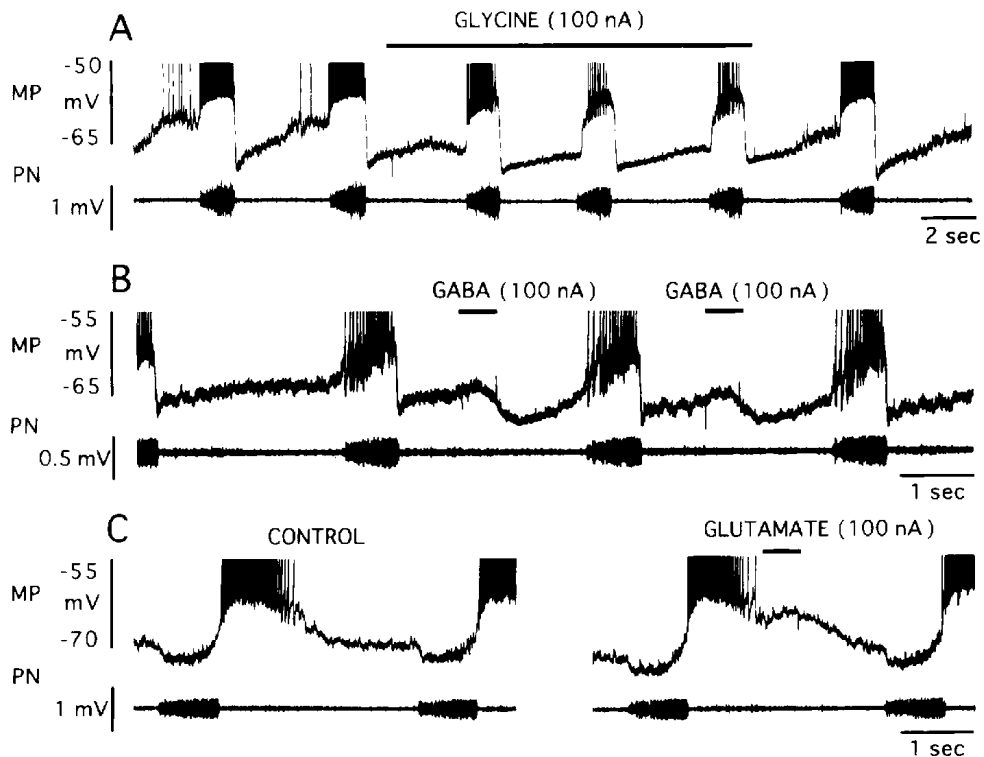

Fig. 5. Effects of iontophoresed glycine (A), GABA (B) and glutamate (C) on respiratory neurons recorded in the medulla oblongata. Panel A: a long pulse iontophoresis of glycine on an inspiratory neuron. Panel B: a 0.5-sec pulse iontophoresis of GABA on an inspiratory neuron. Panel C: a 0.5-sec pulse iontophoresis of glutamate on a postinspiratory neuron. Drugs were applied during the bars. MP, membrane potential; PN, phrenic nerve discharge.

unpaired *t*-test). The transport numbers decreased with increasing resistance as shown in Fig. 4B. The values for pipettes constructed from 16-mm glass exceeded those for pipettes from 10-mm glass (P<0.05, Student's unpaired *t*-test) when 50 nA current was used.

In vivo studies

Respiratory neurons located 2.0–4.0 mm below the dorsal surface of the medulla oblongata were impaled with the center pipette, and stable recordings of membrane potential were obtained. In virtually all cases, stray capacitance in the center pipette was compensated completely, as indicated by a square wave output from the electrometer in response to a 1-nA sqaure wave applied to the center electrode. The d.c. resistance of the center pipette ranged from 20 to 40 MΩ. Electronically induced shifts in membrane potentials caused by iontophoretic current were always less than 0.5 mV. Steady iontophoresis with currents ranging from 25–100 nA of inhibitory amino acids routinely decreased input resistance and synaptic noise and generally hyperpolarized the membrane (see refs. 12–14). A typical example of effects of glycine (100 nA) on an inspiratory neuron is shown in Fig. 5A. Membrane hyperpolarization was observed during both the active and inactive phases of the respiratory cycle. Application of brief pulses (0.5 sec) of 100 nA of GABA produced similar but transient effects (Fig. 5B). In this case, membrane hyperpolarization started 0.3 sec after the onset of iontophoresis. Iontophoresis of an excitatory amino acid, glutamate, with a brief pulse (0.5 sec) produced membrane depolarization 0.1 sec after the pulse started (Fig. 5C). The tips of the center pipettes used in Fig. 5, B and C, extended 25 μm beyond the tips of the peripheral array. The rapid response of membrane potential to a brief pulse of iontophoretic current demonstrates that our multibarrel, compound micropipette allowed ejection of one or more drugs at preselected times into the neural respiratory cycle such that their discrete effects occurred after a relatively short delay. Repeated large current pulses (300–500 nA, 0.3 sec, 2 Hz) were applied to all iontophoresis barrels to counteract the tendency of these pipettes to plug as evidenced by a rise in resistance and by a loss of effects of the amino acids. Pipettes constructed from 16-mm glass appeared to be more fragile than those from 10-mm glass so that for routine iontophoresis experiments, the latter seemed preferable.

Calculated concentration profiles

Figure 6 illustrates the calculated dependence of concentration on distance from the iontophoretic source during steady state iontophoresis. Concentration decreases in a curvilinear fashion as distance increased, and the concentration-distance relationship shifts upward
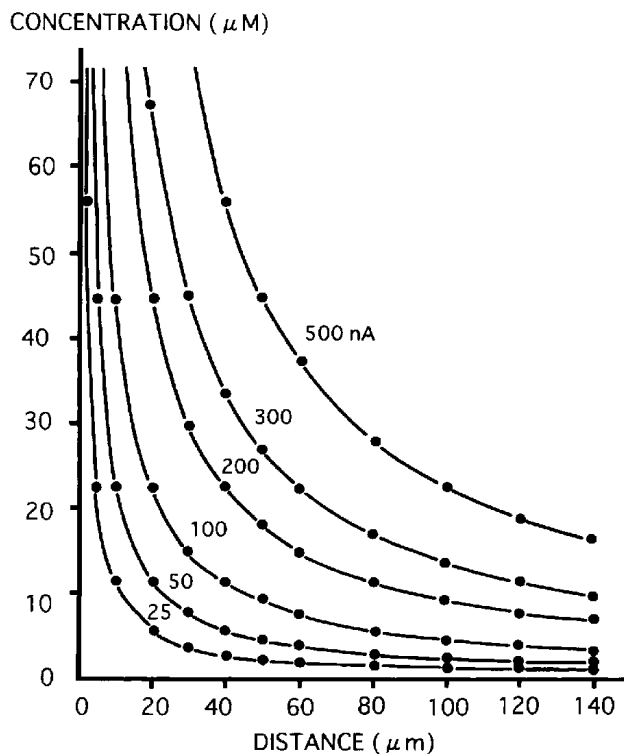

Fig. 6. Theoretical calculations of the relationship between concentration of glycine ejected from a point source (ordinate) and distance from the source (abscissa). Each curvilinear relationship applies to constant current steady state iontophoresis of various currents ranging from 25 to 500 nA.

and to the right with increasing levels of current. The calculated temporal variation in glycine concentration with a 100-nA pulse lasting 3 sec is shown in Fig. 7. Near the iontophoretic source (distance <50 μm), the concentration rose along a trajectory concave to the time axis, reaching a peak at the end of the current pulse. Farther from the iontophoretic source, e.g., 50–100 μm, the concentration reached a peak after termination of the current pulse. The dependence of 90% rise time on distance is shown in Fig. 8 for a 100-nA pulse (dashed line). Comparison of 25 μm with 150 μm revealed that the rise time is 1000-fold greater for the longer distance. Also illustrated in Fig. 8 is a comparison of concentrations achieved at the end of a 3-sec pulse and in the steady state (continuous lines). A concentration in the range of 5 μM can be achieved either using a 3-sec pulse at a diffusion distance of 30–50 μm or a 600-sec iontophoresis period and a distance of 125–150 μm. In other words, the shorter diffusing distance decreases by a factor of 200 the time required to achieve this concentration.
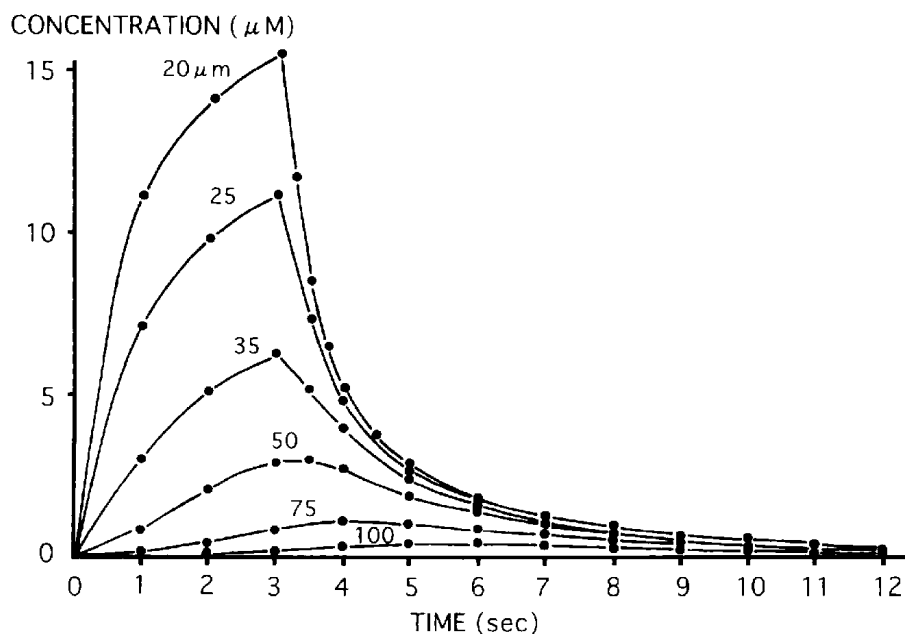
CONCENTRATION (μ M)

Fig. 7. Theoretical calculation showing a time course of the concentration change of glycine ejected by a 3-sec pulse of current. The concentration was calculated for various distance from the iontophoretic point source (20–100 μm).
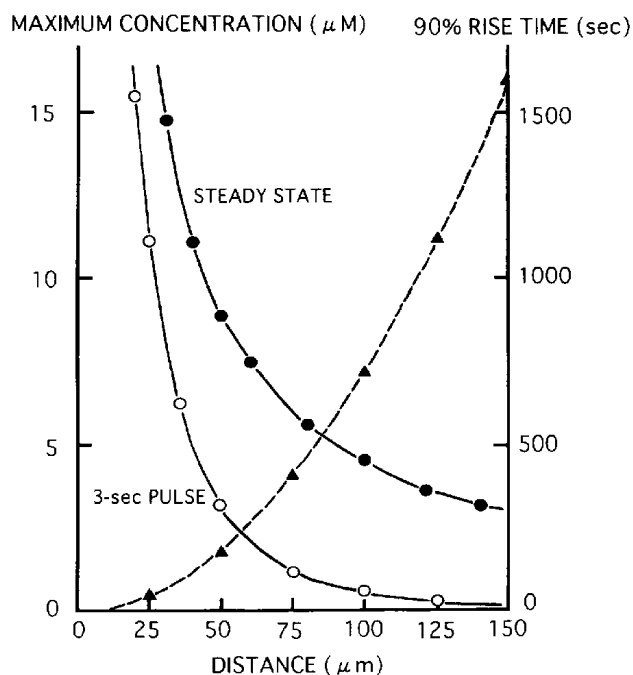

Fig. 8. Peak concentration (left-hand ordinate) associated with a 3-sec pulse (open circles) and a steady state (filled circles) of 100-nA iontophoretic current plotted against distance from the source. The right-hand ordinate shows the relationship between 90% rise time and distance (filled triangles).

DISCUSSION

The construction methods described here allow convenient fabrication of a Sonnhof style compound micropipette. In our multibarrelled electrode, fitting the shaft of the center pipette in the lumen of the peripheral array can minimize inflow of fluid into the interspace of the center and peripheral pipettes. This may result in a small distributed capacitance between them. Because our recording electrode could be capacity compensated without shielding of the central micropipette, the construction of the compound micropipette was greatly simplified. The electrode's electrical and mechanical characteristics provide intracellular recording of membrane potential from bulbar respiratory neurons comparable to that obtained with a single micropipette. Its iontophoretic capability allows application of up to 200 nA of current with negligible electrotonically induced shifts in membrane potential. Finally, by using electrodes with small distances between the tip of the recording pipette and the tip of the iontophoresis pipette, we obtained excellent dynamic responses to brief pulses of iontophoretic current.

The iontophoretically induced flux of glycine from the tips of pipettes constructed from 16-mm glass exceeded that from pipettes derived from 10-mm glass when low currents (50 nA) were used. Similarly, the transport number was greater for pipettes derived from 16-mm than 10-mm glass when the electrode resistance was low (<10 MΩ). This superior performance of pipettes derived from

the larger glass tubing presumably results from larger orifices of the iontophoresis pipettes made from 16-mm glass. On the other hand, compound micropipettes are more easily constructed from 10-mm glass, and proved to be more durable. Because the advantages associated with pipettes constructed from the larger glass appear only in restricted circumstances, pipettes from 10-mm glass appear to be preferable for most applications. Nonetheless, when low current and low pipette resistance are required, pipettes constructed from 16-mm glass will probably prove more effective.

The magnitude and dynamics of the postsynaptic responses to a brief pulse of the inhibitory amino acids are comparable to those observed by Diamond and Roper (15) and Mathews and Winckelgren (16) using visually controlled, independently manipulated iontophoresis pipettes in vitro. One performance benchmark of our compound micropipette is the observed 50% decrease in input resistance typically caused by a 50–100 nA, 1–2 sec pulse of glycine or GABA (12, 13). We postulated that the high dynamic responsiveness of respiratory neurons to brief pulse iontophoresis derives principally from the close approximation of the tips of the recording and iontophoresis electrodes. To explore this, we expressed mathematically the dependence of glycine concentration on diffusion distance, current and time of application. According to the spherical diffusion model described by Nicholson and Phillips (11), the calculations predict the inverse, curvilinear relationship between concentration and distance from the iontophoretic source (Fig. 6). For currents of 50–100 nA, concentrations change little when diffusion distance varies between 100 and 140 μm. However, with smaller distances, the steady state concentration increases dramatically as diffusion distance is shortened.

An additional consideration applies in non-steady state conditions because the rate of change in concentration varies with distance. The temporal concentration profiles produced by a 3-sec, 100-nA pulse are shown in Fig. 7 for a distance between 20–100 μm. The shape differs amongst the various curves, being more curvilinear for the shorter diffusion distances. In addition, the peak concentration lags behind the termination of the pulse for distances of 50 μm or greater. The 90% rise time for concentration is predicted to increase curvilinearly with distances up to 100 μm, having a slope of 11/sec μm. Accordingly, the peak concentration associated with a pulse varies inversely with distance because of the steady state dependence and because of the dynamic dependence. While these considerations ignore consumption of the iontophoresed compound, uptake of this compound within the diffusion sphere will only accentuate the fall off in concentration with distance and, hence, will in-

crease the need for short diffusion distances.

Our results with the improved Sonnhof style coaxial compound micropipette system reveal recording and iontophoretic characteristics that are suitable for the investigation of membrane responses to extracellularly applied drugs in a variety of neurons in the mammalian central nervous system (see our recent papers: 12–14, 17–19). In this style of compound electrode, the tips of the recording and iontophoresis barrels can be closely approximated, thereby allowing the use of brief pulses.

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