

## Potential Clamp of Isolated Dialyzed Neuron: Minimalization of the Effect of Series Resistance

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**Abstract.** A modification of the technique of intracellular dialysis of isolated single excitable cells, such as rat spinal ganglion neuron, suitable for potential clamping of its somatic membrane is described. The advantage of the new modification is the substantial reduction of the effect of inherent resistance in series ( $R_S$ ) to the membrane resistance ( $R_M$ ) on precision of potential clamping. This is attained by reversal of cell position in the perfusion pipette resulting in an approximately tenfold reduction in the area of active membrane. The resistance of this area proportionally increased while  $R_S$  remained unchanged. Hence the error in potential fixation, which is inversely proportional to the ratio  $R_M/R_S$ , is by approximately one order smaller with the new technique than with the original one. An essential step in the new technique is the osmotic expansion of the cell to improve the contact of the cell with the perfusion pipette in the pore and to facilitate disruption of the appropriate part of the membrane. All features and advantages of the technique of intracellular dialysis, such as simplicity, the possibility to easily change ionic composition of media, and/or to apply drugs to any side of the membrane in the same cell, etc., have been maintained.

**Key words:** Potential clamp — Intracellular perfusion method modification — Series resistance — Isolated single neuron

### Introduction

The technique of potential clamping of intracellularly dialyzed single neuron introduced by Krishtal and Pidoplichko (1975) was used for the first time in giant neurons isolated from the periesophageal ganglion of the snail *Helix pomatia*. It was routinely used later for these cells (Kostyuk et al. 1976; Krishtal

and Pidoplichko 1977; Kostyuk and Krishtal 1981) as well as for much smaller cells, such as neuroblastoma cells (Veselovsky et al. 1977; 1984; Kostyuk et al. 1978), neurons from rat dorsal root ganglia (Veselovsky et al. 1979; Kostyuk and Krishtal 1981; Kostyuk et al. 1981a, b, c), rat cardiomyocytes (Undrovinas et al. 1980; Undrovinas 1984; Pidoplichko 1986), and in canine Purkinje cells (Fozzard et al. 1984). The development and the use of the intracellular perfusion technique of isolated cells was concisely described by Kostyuk et al. (1984).

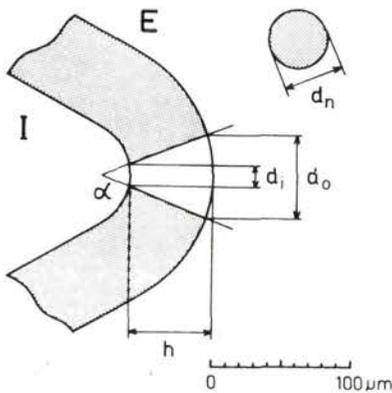
Compared to other techniques used for potential clamp studies in isolated cells, such as whole cell patch clamp using glass suction pipette (Neher and Lux 1969; Lee et al. 1980; Reuter and Stevens 1980) or the two microelectrode system (Kostyuk et al. 1974; Selyanko et al. 1979; Smith et al. 1980), the intracellular dialysis technique provides some advantages. Of these the most remarkable are the easy preparation of the perfusing pipettes of plastic material, the possibility to readily change the composition of perfusing media on any side of the membrane of the cell studied, the yield of complex information about a population of ion channels in the membrane, and the use of macroelectrodes only.

As this technique employs the two electrode system, the effect of resistance in series to the neuronal membrane inherent to such electrical schemes is a serious limiting factor. This resistance can remarkably impair the precision of potential clamping of the membrane (Poindessault et al. 1976; Kostyuk et al. 1981a; Moore et al. 1984; Pidoplichko 1986). As outlined by Kostyuk et al. (1981a) essentially there are three possible ways how to eliminate the effect of series resistance ( $R_s$ ): by electrical compensation of  $R_s$ , by decrease in  $R_s$  by increasing the diameter of the perfusion tool pore in which the cell is fixed, and by diminishing the size of active membrane area. The first frequently used approach (Lee et al. 1980; Reuter and Stevens 1980; Smith et al. 1980; Kostyuk et al. 1981a; Moore et al. 1984) is, however, rather demanding as it requires comparatively complicated schemes of the compensation circuitry. Moreover, the insufficient stability of these systems revealed by high frequency oscillations as well as difficulties in proper adjustment of the feedback system present further disadvantages. The second approach, a decrease in  $R_s$  by enlarging the pipette pore, is limited by the size of the cell itself. Although the third way to diminish the effect of  $R_s$  by increasing the membrane resistance was considered technically difficult (Kostyuk et al. 1981a), a procedure of reducing the effective area of the cell membrane by approximately one order is presented in our paper. Thus, by substantially increasing membrane resistance, with  $R_s$  remaining unchanged, the effect of  $R_s$  can easily be reduced to insignificant values. The described modification keeps all the other features of the technique of intracellular dialysis of isolated cells unchanged.

## Materials and Methods

### Pipette Preparation

Perfusion pipettes were prepared from polyethylene tubing (VEB MLW Polyplast Halberstadt, GDR) with external and internal diameters of 1.5 and 1.1 mm, respectively. The procedure was essentially the same as that described by Kostyuk and Krishtal (1981). Briefly, a piece of the tubing approximately 12 cm long was bent in the middle of its length in a stream of hot air to an angle of approximately 45°. The tip of the bend was slightly covered with smear containing liquid mineral oil, lanolin, and Parafilm\* (American Can Co.) mixed in an approximate ratio of 3:2:1. Then the tip of the pipette was heated again and pushed from inside by a metal rod to diminish the wall thickness to 50–70  $\mu\text{m}$ . Using a precisely shaped, electrolytically sharpened and polished steel needle fixed to a micromanipulator a conical hole was made in the pipette tip under visual control. To facilitate its penetration across the tubing wall, the needle was slightly heated during the procedure. A minute amount of the smear pushed by the needle onto the hole surface was essential for successful sealing of the cell to the pipette. The dimensions of the pore are shown in Fig. 1.



**Fig. 1.** Geometry of the tip of the polyethylene perfusion pipette with precise conical hole creating communication between intraluminal (*I*) and extraluminal (*E*) compartments. For comparison a sphere with a diameter similar to that of rat dorsal root ganglion neuron is shown. Typical dimensions are  $d_i = 14 \mu\text{m}$ ,  $d_o = 60\text{--}70 \mu\text{m}$ ,  $\alpha = 43.5^\circ$ ,  $d_n = 35\text{--}50 \mu\text{m}$ .

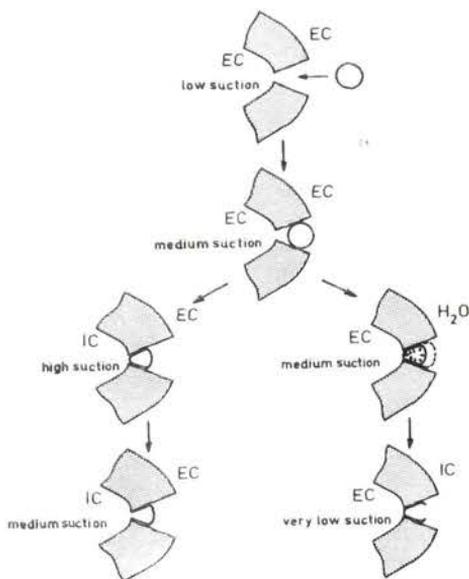
### Cell Isolation

The cells used in our study were neurons of dorsal root ganglia from 7 to 10 day old rats. The ganglia were enzymatically treated at 32°C for 15 min in Eagle's Minimal Essential Medium (MEM) supplemented with 0.65 % trypsin and 0.25 % collagenase. The enzymes were washed out of the tissue during a subsequent soaking in plain MEM at 22°C for an additional 30 min. The vials were gently agitated during the incubations. The ganglia were then stored at 14°C in MEM until final mechanical microdissection of single neurons was carried out under a stereomicroscope using fine needles. The diameter of the cells ranged from 35 to 50  $\mu\text{m}$ .

### Intracellular Dialysis

The cells isolated by the above procedure were transferred into a glass experimental chamber in which the tip of a V-shaped perfusion pipette was placed. The pipette and the chamber were filled by "normal" extracellular medium containing NaCl 140, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 2, and TRIS 5 (concentra-

tions in mmol/l). The pH was adjusted with hydrochloric acid to 7.3. The temperature of the chamber was maintained at 18°C. By application of low negative pressure inside the pipette ( $-1.47$  to  $-1.96$  kPa, i.e.  $-15$  to  $-20$  cm of  $H_2O$ ) one of the cells closest to the pipette tip was sucked into the hole and got stuck there. This was indicated by a sudden increase in the resistance of the pipette which resistance in the "normal" extracellular medium ranged from  $0.3$  to  $0.5$  M $\Omega$ . By slowly increasing the suction pressure up to  $-4.90$  kPa ( $-50$  cm of  $H_2O$ ) the cell was allowed to settle in the hole (Fig. 2).



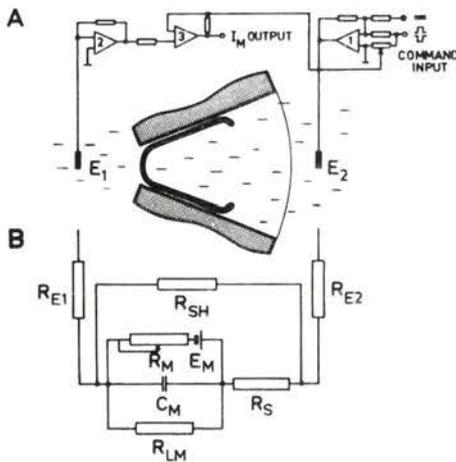
**Fig. 2.** Sequence of steps in neuron fixation in the conical hole of the perfusion pipette, rupture of the appropriate part of the membrane with the technique according to Kostyuk et al. (1981a) (left side) and with the new version of the technique (right side). EC — extracellular solution, IC — intracellular solution,  $H_2O$  — distilled water used to osmotically expand the cell. For details see text.

In the original arrangement used by Krishtal and Pidoplichko (1977), Veselovsky et al. (1977; 1979), Kostyuk and Krishtal (1981), and Kostyuk et al. (1981a) (Fig. 2, left side) the extracellular medium in the pipette is switched to a low calcium intracellular solution which can consist of e.g. KF 70, TRIS 50, glucose 20, and EDTA 1 (concentrations in mmol/l), the pH being adjusted with hydrofluoric acid to 7.3. Then, by high negative pressure (up to  $-9.79$  kPa, i.e.  $-100$  cm of  $H_2O$ ) and by vibrating the water column in the perfusing system by gentle tapping on the outflow tube the part of the membrane adjacent to the perfusion system is broken. This creates a communication

between intracellular and intraluminal compartments permitting easy access of ions and/or drugs from the solution to the inner side of the membrane. The cross section area of the inner aperture of the pore is one factor which limits this diffusion and determines a substantial portion of the resistance in series to the active part of the neuronal membrane (Fig. 3B). Due to the conical shape of the pore, the diameter of this active part is definitely larger than the diameter of the inner aperture of the pore.

The new modification and its procedure is illustrated in Fig. 2 (right). Essentially, it is the reversed configuration of the original one. An adequate wedging of the cell into the pore, its breaking at the appropriate side of the pore, and keeping the membrane in the pipette were crucial problems to be solved in this modification. After the cell had been sucked into the pore at low negative pressure ( $-1.47$  to  $-1.96$  kPa, i.e.  $-15$  to  $-20$  cm of  $H_2O$ ) it was allowed to settle at

medium suction ( $-3.92$  to  $-4.90$  kPa, i.e.  $-40$  to  $-50$  cm of  $H_2O$ ). To improve its pressing to the pipette wall the cell was osmotically expanded. The expansion was achieved by replacing the extracellular medium in the chamber by distilled water for approximately 1 min. The decrease in osmotic pressure resulted in an increase in the cell volume and was essential for satisfactory clinging of the cell to the pipette as well as for breaking the cell membrane in the wider part of the pore. This usually occurred after replacing the distilled water for low calcium intracellular medium, and could be facilitated by sudden changes in voltage applied across the pore. During the above procedure a potential of  $100$ – $120$  mV, positive inside the perfusion pipette was applied. The breaking of the membrane was indicated by a decrease in the input resistance and by appearance of typical inward and outward currents during depolarizing pulses. The parameters of the currents were the same as those observed in the former configuration except for by approximately one order smaller amplitude. This is consistent with an approximately 10 times smaller area of active membrane obtained with the new technique compared to the former one. Immediately following disruption of the membrane the suction pressure was reduced to  $-0.98$  kPa ( $-10$  cm of  $H_2O$ ) and maintained at this level throughout the experiment.

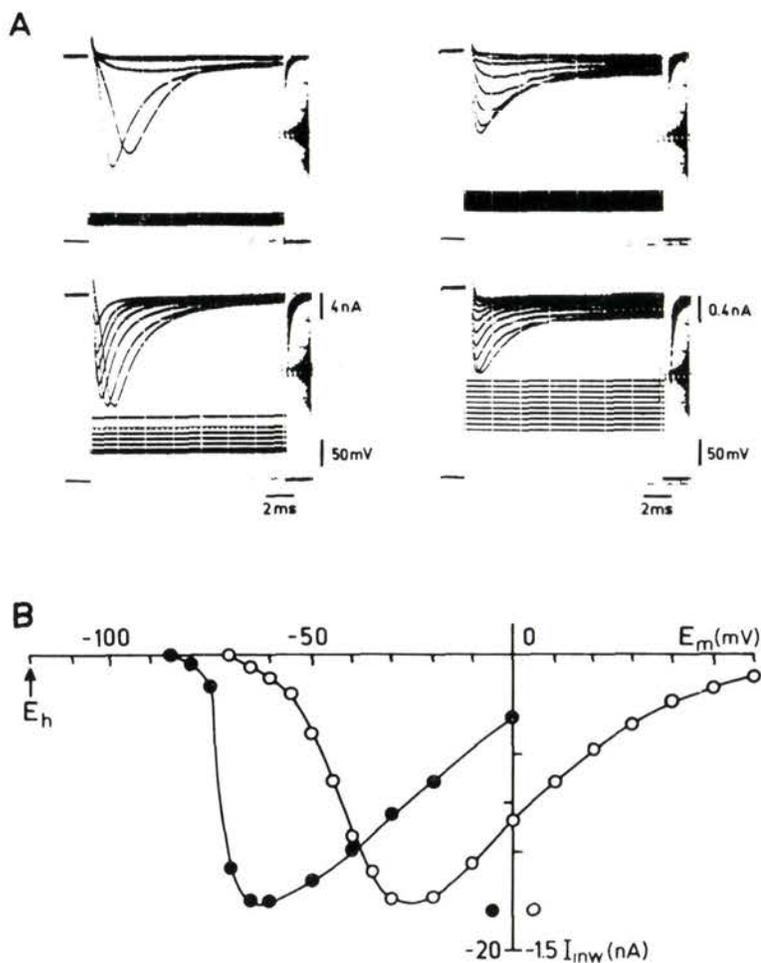


**Fig. 3.** *A* — Somatic membrane fixed in the pore and block scheme of the potential clamping system. Amplifiers 1, 2, and 3 are command former, current to voltage converter, and differential amplifier, respectively.  $E_1$  and  $E_2$  represent extracellular and intracellular macroelectrodes immersed in perfusing media. *B* — Simplified electrical scheme of the pore with the membrane.  $R_{E1}$  and  $R_{E2}$  are resistances of the macroelectrodes,  $R_{SH}$  is the resistance shunting the membrane in space between cell and pipette walls,  $R_M$  is the variable membrane resistance,  $E_M$  is the driving force depending on ion diffusion potentials,  $C_M$  is the membrane capacitance,  $R_{LM}$  is the membrane leakage resistance, and  $R_S$  is the series resistance created by fragments of the membrane, the neuronal soma, and the pore itself.

The electrical system used for potential clamping was essentially the same as that used by Kostyuk et al. (1978; 1981a). It consisted (Fig. 3A) of an injecting amplifier with low impedance output used to sum up the desired holding potential and the command voltage pulses. The resulting potential from this amplifier was applied through one of the macroelectrodes into the cell interior. The potential of the other macroelectrode immersed in extracellular medium was maintained using the current to voltage converter on a potential close to zero. The voltage output of this converter was proportional to the transmembrane current. A fraction of command voltage was fed into the output of the current to voltage converter to compensate shunting and leakage resistances ( $R_{SH}$  and  $R_{LM}$ ) in parallel to that of membrane resistance ( $R_M$ ).

#### Chemicals Used

Trypsin (TRYPsin® Spofa), collagenase crude (ÚSOL Bohumile, ČSSR), Eagle's Minimal Essential Medium (MEM® Sevac, ČSSR), tris(hydroxymethyl)aminomethane puriss. p.a. (TRIS, Fluka),



**Fig. 4.** *A* — Typical current responses (upper traces) evoked by depolarizing voltage pulses (lower traces) in a neuron dialyzed in the original (left records) and the new arrangement (right records). The upper and lower records show the descending and ascending phases of current — voltage characteristics; the calibrations apply to both records. Note different current calibrations for both neurons. Holding potential was 120 mV negative inside in both neurons. *B* — Current-voltage characteristics for the neurons shown in part *A*. The cell dialyzed by the modified technique (O) reveals much flatter descending phase of the curve comparing to the cell dialyzed by the former technique (●). This undoubtedly points to much improved potential clamping of the neuronal membrane in the suggested version.  $E_m$ ,  $E_h$ , and  $I_{inw}$  are membrane potential, holding potential, and peak amplitude of the inward current, respectively.

ethylenediaminetetracetic acid disodium salt dihydrate puriss. p.a. (EDTA, Fluka). All other chemicals were of analytical grade.

## Results and Discussion

Resistance in series ( $R_S$ ) to the membrane resistance ( $R_M$ ) (Fig. 3B) is inherent to any potential clamping system. It can be responsible for a comparatively large error in membrane potential fixation. This error is inversely proportional to the ratio  $R_M/R_S$ . The effect of  $R_S$  can be remarkable especially with the two electrode systems, with the exception of systems using the electrodes alternately for current injection and membrane potential sampling on time sharing basis (Merickel 1980; Sigworth 1983). The series resistance in the potential clamp technique of intracellularly dialyzed neurons is a complex resistance. Essentially, it consists of resistances of both macroelectrodes, of resistances of the extra- and intracellular perfusing media, of that of fragments and residues of disintegrated cell membrane, and of the resistance of the cell soma. The most important limiting factor in determining  $R_S$  is, however, geometry of the pore.

Kostyuk et al. (1981a) considered three possible ways how to diminish the effect of  $R_S$ . Changes in pore geometry by increasing its diameter are limited by the dimensions of the cells. In experiments with cells similar to those used in our experiments the inner diameter of the pore cannot exceed 15–20  $\mu\text{m}$ ; thus the typical pipette resistance is 0.5–0.3  $\text{M}\Omega$ . The second possibility is the compensation for  $R_S$  by an appropriate control of clamping voltage derived from changes in transmembrane current, hence from changes in  $R_M$ . The systems described in the literature (Lee et al. 1980; Reuter and Stevens 1980; Smith et al. 1980; Sigworth 1983; Moore et al. 1984) are, however prone to be unstable during the compensation. The one proposed by Romanyuk (Kostyuk et al. 1981a) which is devoid of this instability requires comparatively complicated electronics. Another serious problem common to all these systems is, however, the proper adjustment of the feedback impeded by the uncertainty in  $R_M/R_S$  determination. The third way of diminishing the effect of  $R_S$ , namely by increasing  $R_M$  using a smaller area of active membrane was considered in this technique to be technically difficult (Kostyuk et al. 1981a).

If the diameter of a typical rat spinal ganglion is 40  $\mu\text{m}$ , the volume of this spherically shaped cell is 33510  $\mu\text{m}^3$ . If such a cell is pushed into a conical pore with dimensions indicated in Fig. 1 deep enough to obturate its narrower aperture, the ratio between both areas of the membrane exposed outside of the pore would be approximately 1:11.3. No concomittant change in the cell volume, as well as planar shape of the exposed membrane areas have been assumed with this calculation. Consequently, a mere reversion of the experi-

mental arrangement results in a reduction in the active membrane area by approximately one order. Indeed, the maximal inward current measured with the new modification in potassium free intracellular medium was  $1.19 \pm 0.24$  nA ( $\bar{x} \pm \text{SEM}$ ;  $n = 13$ ), while in the former version it was  $14.42 \pm 3.41$  nA ( $n = 5$ ). This yields a ratio of 1 : 12.1 which agrees fairly well with the above geometrical considerations. The total resistance of the pipette, after penetrating both membrane patches with fragments of the cell left in the pipette, was  $1.21 \pm 0.15$  M $\Omega$  ( $n = 5$ ) with the net pipette pore resistance being  $0.39 \pm 0.03$  M $\Omega$ . Let us consider sodium equilibrium potential ( $E_{\text{Na}}$ ) to be +40 mV and maximal sodium inward current ( $I_{\text{max}}$ ) 1 nA at a testing potential ( $E_{\text{max}}$ ) -40 mV. The net membrane resistance ( $R_{\text{M}}$ ) at the moment of maximal current should be about 80 M $\Omega$  ( $R_{\text{M}} = I_{\text{max}} / (E_{\text{max}} - E_{\text{Na}})$ ). If  $R_{\text{S}} = 1$  M $\Omega$ , the error of potential to which the membrane should be clamped would be only -1.2%. This is an approximately 10 times smaller error than at  $I_{\text{max}} \doteq 10$  nA, as typical of the original arrangement (Kostyuk et al. 1981a).

The representative records of current responses to depolarizing voltage pulses in two neurons perfused in the original and the new arrangements (left and right side of Fig. 2, respectively) are shown in Fig. 4A. The corresponding current-voltage characteristics are shown in Fig. 4B. An approximately 10 times lower peak inward current can be seen in the neuron dialyzed by the new technique comparing to that dialyzed by the original one. The difference in the shapes of the current-voltage curves demonstrates that the suggested modification of the dialysis technique indeed remarkably improves the quality of the cell membrane potential clamping.

A comparison of input resistance measured at various steps of cell fixation in the pipette with the original and the modified procedures did not reveal any corresponding increase in the input resistance in the latter one. This obviously can be attributed to a less complete sealing of the gap between the cell and pipette walls since high negative pressure cannot be used for cell wedging with the new arrangement.

The technique described in this paper has been routinely used in our laboratory. It has greatly improved the reliability of potential fixation, it completely eliminated the necessity of discarding cells with too high currents, hence with unsatisfactory potential clamping, and the necessity of using rather complicated compensation electronics. All the other features and advantages of the technique described by Kostyuk et al. (1981a) have been fully preserved.

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