A Triple-barreled Microelectrode for Simultaneous Measurements of Intracellular Na^+ and K^+ Activities and Membrane Potential in Biological Cells

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Abstract A triple-barreled Na^+ , K^+ -selective microelectrode was constructed with liquid ion exchangers for Na^+ (monensin) and K^+ (Corning #477317) to measure the intracellular Na^+ and K^+ activities ($(\text{Na})_i$ and $(\text{K})_i$) of a single cell and its membrane potential (E_M), simultaneously. The tip of the triple-barreled assembly was made less than $0.6 \mu\text{m}$ in outside diameter.

Prior to *in vivo* measurements, some physicochemical properties of microelectrodes were examined *in vitro* for the slope constant, selectivity coefficient, electrical resistance, and pH effect, as well as measurements of the activity coefficient of ions in blood serum and Ringer solution.

Carrying out direct micropunctures on single cells of the sartorius muscle and renal proximal tubule of bullfrogs *in vivo*, we obtained the following results: (1) In sartorius muscle, the average $(\text{Na})_i$ was 14.8 mEq/liter, the $(\text{K})_i$ 64.5 mEq/liter, and the E_M -68.2 mV. (2) In proximal tubule cells, the average $(\text{Na})_i$, $(\text{K})_i$ and E_M were 16.8, 63.0 mEq/liter and -65.9 mV, respectively. (3) There were significant correlations in the proximal tubule between $(\text{K})_i$ and E_M , and inversely between $(\text{Na})_i$ and E_M , and between $(\text{Na})_i$ and $(\text{K})_i$. These facts may somehow be related to both the activity of Na^+ - K^+ exchange pump and the osmotic equilibrium of water across the membrane.

Further, several problems inherent in the multibarreled microelectrode were discussed from the practical point of view.

The development of the K^+ -selective liquid ion exchanger microelectrode by WALKER (1971) and its subsequent improvement by KHURI and his associates (1972) and VYSKOCIL and KRIZ (1972) permit the direct observation of electrochemical events for K^+ or Cl^- of a single cell *in situ* in multicellular tissues under approximately normal conditions. This technique has been extended to simultaneous measurement of intracellular Na^+ activity and the membrane potential

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in the proximal tubule of amphibian kidneys (KOTERA *et al.*, 1979) along with extension of other kinds of double-barreled microelectrodes (FUJIMOTO *et al.*, 1980a). Recent advances in the microelectrode technique have made possible the manufacture of multibarreled microelectrodes within a tip size of $1.0\ \mu\text{m}$ (SILVER, 1976; KESSLER *et al.*, 1976). However, many difficulties still remain not only in techniques but also in practical applications to biological fluids.

Extending our earlier techniques (FUJIMOTO and KUBOTA, 1976), we have manufactured a triple-barreled microelectrode which is capable of measuring the ionic activities of both Na^+ and K^+ and the membrane potential difference (PD) and have applied this technique to intact sartorius muscle and proximal tubule *in situ* of bullfrog kidneys.

This paper characterizes the physicochemical properties of this microelectrode and discusses its applicability to both *in vitro* and *in vivo* measurements.

METHODS

Microelectrode techniques. The triple-barreled Na^+ , K^+ -selective microelectrodes were fabricated in the following manner. Borosilicate glass-capillaries having 0.9 mm outer diameter (OD), 0.7 mm inner diameter (ID) and 15 cm in length were used. These were made of Pyrex glass tubing (Corning medium wall #10) with a capillary puller (Narishige PY-6). All the capillaries were cleaned by immersion in dichromate-sulfuric acid solution, rinsed repeatedly with redistilled water and kept dry in a desiccator. Three capillaries were mounted in a pyramidal form on a horizontal micropipette-puller (Narishige PD-5), gently rotated 360° along their longitudinal axes upon heating with a platinum ribbon, and drawn carefully in a two-step pull: *i.e.*, initially pulled manually by approximately 1 mm, and then pulled automatically by machine to a fine tip of less than $1\ \mu\text{m}$.

Figure 1 presents photographs showing a tip of a triple-barreled micropipette taken by a scanning electron microscope (Hitachi S-500A). To facilitate contrast, a gold spattering has been made in 8 mA for 2 min. To the lower in Fig. 1 is a picture in low magnification taken obliquely downward, whereas the upper picture represents the top view at higher magnification. Without metal spattering, the size of the tip diameter is estimated to be approximately $0.5\ \mu\text{m}$.

For membrane potential measurements, one of three barrels (referred to as PD-barrel) was filled with 1.0 M NaCl solution from the stem end of the capillary. To determine intracellular activities of Na^+ and K^+ , the remaining two barrels (referred to as ionic barrels) of the electrode were filled individually with Na^+ - and K^+ -selective liquid ion-exchangers. For these procedures to perform successfully, the internal surfaces of the two ionic barrels had to be made hydrophobic at the very tips (approximately $250\ \mu\text{m}$ in length), while the remainder (the PD barrel) was kept hydrophilic. The technique for siliconization in general has been described by FUJIMOTO and KUBOTA (1976). For triple-barreled microelectrodes, the following procedures should be carried out. First, after the tip of

Fig. 1. Scanning electron micrographs of the tip of a triple-barreled Na^+ , K^+ -selective microelectrode. The tip of the microelectrode was visualized in aid of ion sputtering of Au by 8 mA for 2 min. The magnification: the lower, $\times 40$ the bar shown at the right indicates $500\text{ }\mu\text{m}$; the upper, $\times 20,000$ (the bar, $0.5\text{ }\mu\text{m}$).

the PD-barrel had been completely filled with pure acetone from the stem-end of the capillary, the tip assembly of all three barrels was dipped into a diluted silicone solution (0.15% silicone oil (KF-96, Shinetsu Chemical Industry Co., Tokyo)

diluted with the purest trichlorethylene ("Trichlene" for electronics use, Nakarai Chemicals, Kyoto)) for several seconds to make the other two tips of ionic barrels silicone-filled. Second, the silicone- and acetone-holding triple-barreled micro-pipette thus produced was baked on a hot plate at 300°C for 15–30 min to permit evaporation of the solvents. Liquid ion-exchangers were introduced into the tips by a "back-fill" technique: *i.e.*, injected from the stem-end of an individual glass-capillary by means of a long needle (10 cm long, 0.2 mm OD) attached to a syringe.

The exchangers used were Corning #477317 for K⁺-selective barrel, and 10% (w/w) H⁺-monensin (a Na⁺-selective antibiotic ionophore) dissolved in Corning #477317 (KOTERA *et al.*, 1979) for Na⁺-selective barrel. The internal solutions of individual ionic barrels were 0.5 M KCl solution for K⁺-selective barrel and 0.49 M NaCl plus 0.01 M KCl solution for Na⁺-barrel. The pH of the internal solution for Na⁺-barrel should be adjusted to 3 with 0.1 M citrate buffer (consisting of disodium citrate and HCl) to lower the pH sensitivity.

Methods of calibrations, slope constants and selectivity coefficients of the ratios of the ionic barrels are similar to those described by FUJIMOTO and KUBOTA (1976). Solving simultaneously two different kinds of Eisenman-Walker's equations (WALKER, 1971) obtained from the Na⁺- and K⁺-selective barrels, intracellular activity of Na⁺ or K⁺ was calculated from the following equation, as:

$$a_i^{\text{ICF}} = \frac{(a_i^{\text{ECF}} + K_{ij} a_j^{\text{ECF}}) 10^{\Delta E_i / \alpha_i} - K_{ij} (a_j^{\text{ECF}} + K'_{ji} a_i^{\text{ECF}}) 10^{\Delta E_j' / \alpha_j'}}{1 - K_{ij} K'_{ji}}, \quad (1)$$

where a stands for the ionic activity; the subscripts i and j refer to the test and interfering ion, respectively; the superscripts ICF and ECF represent the intra- and extra-cellular fluid; ΔE denotes the difference of the electromotive force (EMF) measured by each ionic barrel between ICF and ECF; K_{ij} represents the selectivity coefficient (the reciprocal of selectivity ratio, as shown later in Table 1) to the i th ion over the j th ion, and α_i the slope constant in the ionic barrel selective to the i th ion. All the measured parameters, which are derived from the ionic barrel selective to the j th ion and used for correcting the effect of the i th ion, are apostrophized, such as $\Delta E_j'$, K'_{ji} and α_j' . This has been done to avoid confusion with the data (ΔE_j , K_{ji} and α_j) obtained from the i th ion-selective barrel, which are to be used for correction of the i th ion.

In practice, the product of K_{ij} and K'_{ji} is usually as small as 0.003, therefore, neglecting this term, the above Eq. (1) is simplified as:

$$a_i^{\text{ICF}} = (a_i^{\text{ECF}} + K_{ij} a_j^{\text{ECF}}) 10^{\Delta E_i / \alpha_i} - K_{ij} (a_j^{\text{ECF}} + K'_{ji} a_i^{\text{ECF}}) 10^{\Delta E_j' / \alpha_j'}. \quad (2)$$

Animal experiments. Male bullfrogs (*Rana catesbeiana*), weighing from 100 to 150 g, were used for animal study. Each animal was anesthetized by immersion in 3% urethane solution for 15 to 20 min. For the kidney experiment, the left kidney was exposed through a wide abdominal incision, while for the muscle experiment, the sartorius muscle was exposed by opening the skin and fascia covering

the muscle. Throughout the experiment, a continuous infusion was performed through a fine catheter inserted caudally into the pre-abdominal vein in the hypogastric region. The infusate was frog Ringer solution having the following composition (in mmol): NaCl 110, NaHCO₃ 15, KCl 3.0, KH₂PO₄ 0.5, CaCl₂ 1.0, MgSO₄ 0.5, gassed with a mixture of 1.5% CO₂ and 98.5% O₂, the ranging from 7.4 to 7.6 and the osmolality from 208 to 213 mOsm.

The experimental design for renal micropuncture is depicted in Fig. 2. A double-barreled micropipette with a beveled tip was inserted into a Bowman's capsule of a superficial glomerulus to perfuse the tubular lumen with a marker dye (200 mg % Lissamine Green dissolved in Ringer solution) or with normal Ringer solution. Meanwhile, a triple-barreled ion-selective microelectrode was advanced into the intracellular space at the desired spot downstream.

The procedure for micropuncture was similar to that described previously (KUBOTA and FUJIMOTO, 1978). Single cells of sartorius muscles were also punctured in a similar fashion. All these experiments were carried out under stereo-

Fig. 2. Block diagram of the electrical measurements and experimental design of micropuncture study using a triple-barreled Na⁺-, K⁺-selective microelectrode. A single cell of the proximal tubule in bullfrog kidney is impaled with a triple-barreled microelectrode to measure the membrane PD and chemical potentials (ionic activities) of K⁺ and Na⁺, simultaneously. The differential output between K⁺-barrel and PD barrel and that between Na⁺-barrel and PD barrel are both connected with cables to the input stages of individual differential amplifiers (A and B). Those outputs and the output of PD barrel are displayed in both digital and analog notations.

microscope (Leitz Model TS) with the illumination provided by a glass-fiber illuminator (Narishige MEI-2). The micromanipulators used were a step-motor-driven hydraulic manipulator (Narishige MO-81) in combination with a three-dimensional hydraulic manipulator and a metal-screw type manipulator (Narishige MO-103 and MM-3).

The differential outputs of the triple-barreled microelectrode were connected with high impedance differential amplifiers (Keithley 604) and the output of the reference barrel (the PD barrel) with an electrometer (Keithley 616) for the membrane PD. The method of cable connections is also shown diagrammatically in Fig. 2. The digital and analog voltages were recorded with digital multimeters (Keithley 160), in combination with a data-logger (Fluke 2240-A) and a 3-channel pen-recorder (Rikadenki B-381, Tokyo).

Sample analyses. For analysis of electrolytes in biological fluids, such as human and frog serum, the total concentration of Na^+ and K^+ were calculated with a flame-photometer (Corning 450, New York) and activity coefficients (γ) of Na^+ and K^+ were calculated as: $\gamma = (\text{ionic activity})/(\text{total ionic concentration})$, using the measured data with microelectrodes and flame-photometer.

Blood samples were collected from the cubital vein for human subjects and from the trunk vessel for bullfrogs. For the determinations of pH and P_{CO_2} of perfusates and blood samples a digital pH meter (HM-15A, Toa Electronics, Ltd., Tokyo) and P_{CO_2} meter (Corning 161, New York) were employed.

Statistical analyses were performed by Student's t test, and the regression lines were calculated by the least squares method. The numerical data were expressed as the mean ($\bar{M}$) $\pm$ SD (σ), unless otherwise noted in the text, and the coefficient of variation (C) was calculated as: $C = 100\sigma/\bar{M}$ (SNEDECOR, 1956).

RESULTS

For a preliminary test, one of the microelectrodes was examined for its sensitivities to the various cations, such as Na^+ , K^+ and Mg^{2+} , at 25°C. Figure 3 shows an example of the EMFs of the ionic barrels in solutions with various cationic concentrations. Open marks represent the data of the Na^+ -barrel, whereas solid marks represent those of the K^+ -barrel. An individual barrel can respond to all the cationic species in order of their selectivities. It is confirmed that the K^+ - and the Na^+ -barrels respond most sensitively to K^+ (solid circles) and Na^+ ions (open triangles), respectively. Their slope constants to Mg^{2+} ion (squares) are lower than those to K^+ (circles) or Na^+ ions (triangles). The calibration curve for the Na^+ -barrel exhibits a non-linear behavior in the concentration range of Na^+ lower than 10 mEq/liter, representing a detection limit of Na^+ based on the criteria of I.U.P.A.C. (1976) at approximately 3 mEq/liter for this particular microelectrode.

Electrochemical properties of 12 arbitrarily chosen triple-barreled microelectrodes are summarized in Table 1. The average electrical resistances of the PD

Fig. 3. EMF vs. ionic activities plots for various cations obtained with a triple-barreled Na^+ -, K^+ -selective microelectrode. The EMFs of the Na^+ -barrel are presented by the open marks, whereas those of the K^+ -barrel by the closed marks. The marks of triangles, circles and squares are, respectively, Na^+ , K^+ and Mg^{2+} ions in a series of calibration solutions with Cl^- as counter ions.

and ionic barrels were of the order of 10^7 and $10^{10}\Omega$, respectively. It is shown that the electrical resistance of the Na^+ -selective barrels is about 4 times larger than that of the K^+ -selective barrels. The tip potential of the PD barrels determined *in vitro* as a difference of the EMFs (in mV) between Ringer and 0.1 M KCl solutions is 3.2 ± 2.0 mV on average. The mean values for the slope constants in the Na^+ - and K^+ -selective barrels are 56.0 ± 11.0 and 53.4 ± 6.0 mV per 10-fold change of respective ionic activities.

The K^+ -selective barrel (K^+ -barrel) had a selectivity coefficient for Na^+ over K^+ of approximately 0.016 ($\text{K}^+/\text{Na}^+ = 1/61.5$) in the higher concentration range of test solutions (0.05–0.25 M NaCl and KCl), and approximately 0.40 ($=1/25.3$) in the lower concentration range around 0.01 M. In contrast, the Na^+ -selective barrel (Na^+ -barrel) had a selectivity only 6 times more sensitive to Na^+ than K^+ in the lower concentration range (0.025–0.01 M), although it showed a better selectivity ($\text{Na}^+/\text{K}^+ = 12.5$) in the higher concentration range around 0.1 M. As noticed in Table 1, selectivity coefficients of the ionic barrels depend on the concentration of test solutions. For the calculation of intracellular activities of K^+ and Na^+ , we have used those selectivity coefficients of the higher concentra-

Table 1. Electrochemical properties of triple-barreled Na^+ -, K^+ -selective microelectrodes.

No.	PD barrel			K ⁺ -barrel			Na ⁺ -barrel					
	Resist- ance (10 ⁷ Ω)	Tip potential (mV)	Resist- ance (10 ¹⁰ Ω)	Slope (mV)		Selectivity K ⁺ /Na ⁺ 0.01 M 0.1 M	Slope (mV)		Selectivity Na ⁺ /K ⁺ 0.01 M 0.1 M			
				α _K	α' _{Na}		α' _{Na}	α' _K				
1	2.7	3.2	0.4	56.5	33.9	16.3	45.1	6.5	56.5	54.0	4.9	6.8
2	4.7	0.3	1.3	54.9	35.0	37.0	78.1	4.4	67.6	46.8	3.7	8.7
3	1.8	4.5	2.1	54.0	35.3	15.4	39.8	8.9	69.5	67.0	7.4	8.0
4	2.3	3.8	2.6	50.4	39.4	33.7	66.6	1.3	35.1	17.2	2.2	8.1
5	4.8	7.8	3.5	57.3	56.9	29.5	61.0	6.3	65.3	63.4	2.9	17.0
6	3.2	2.0	1.2	58.3	52.8	21.0	75.1	8.2	49.1	25.8	3.8	15.0
7	2.0	4.0	3.5	60.6	61.0	17.2	71.9	5.7	58.0	62.8	0.4	8.6
8	2.4	1.0	2.5	44.0	18.1	9.3	40.2	7.3	69.2	57.8	5.6	12.0
9	4.5	5.2	0.4	55.5	48.0	32.9	68.5	5.3	46.2	42.1	0.1	12.3
10	2.2	2.0	0.1	53.9	28.4	29.0	94.5	4.3	50.0	43.8	12.4	32.1
11	0.2	2.0	0.1	40.0	44.2	46.8	47.3	0.1	60.2	53.8	3.8	6.1
12	1.8	2.3	0.1	56.2	31.1	15.9	50.3	0.6	45.8	35.8	2.1	14.9
Mean	2.7	3.2	1.5	53.4	40.0	25.3	61.5	5.2	56.0	47.5	5.8	12.5
±SD	±1.4	±2.0	±1.3	± 6.0	±12.6	±11.1	±17.2	±3.1	±11.0	±15.4	± 3.5	± 7.1

Individual barrels of 12 different triple-barreled microelectrodes were examined for the electrochemical properties at 25°C. The symbols, α and α'_K , are the slopes in mV for the K^+ - and Na^+ -barrel, respectively, per 10-fold change in K^+ activities, whereas α_{Na} and α'_{Na} those for the Na^+ - and K^+ -barrel, respectively, per 10-fold change in Na^+ activities.

tion range for K^+ -barrels and those of the lower concentration range for Na^+ -barrels because the intracellular fluid under investigation contains high K^+ and low Na^+ .

Figure 4 shows the effect of pH changes ranging from 6.3 to 8.8 on the estimation of ionic activities of Na^+ and K^+ with triple-barreled microelectrodes in test solutions containing the ionic concentration of 115 mM Na^+ and 4 mM K^+ . No pH effect is found on K^+ measurements, whereas a minor but significant effect is induced on Na^+ measurements. The gentle slope observed in the Na^+ -barrel would be due to a pH sensitivity of monensin-based microelectrodes (KOTERA *et al.*, 1979). It is considered, however, that under experimental conditions in which the pH changes are small the pH effects on Na^+ are not so serious in practice.

Fig. 4. Effects of pH on triple-barreled Na^+ -, K^+ -selective microelectrodes. The vertical bar through open mark gives the mean $\pm$ SD obtained with 12 different microelectrodes.

Activity coefficients of Na^+ and K^+ in some biological fluids (human serum, frog serum and frog Ringer solution) were determined with triple-barreled microelectrodes, the results being summarized in Table 2. Human sera were collected from 11 healthy male subjects, whereas frog sera were from 8 normal male bullfrogs. The values of activity coefficients for Na^+ and K^+ ions ranged from 0.6 to 0.8 in both species. It is found that the data of mammalian sera (0.716 for Na^+ and 0.725 for K^+) were lower than those of amphibian sera (0.749 for Na^+ and 0.742 for K^+), being in agreement with our previous observation by double-barreled K^+ - or Cl^- -selective microelectrodes (FUJIMOTO and KUBOTA, 1976).

The coefficient of variation (C) as a measure of variation in the values obtained by electrometry is larger than that by flame photometry: *i.e.*, about 7 times for Na^+ and 3 times for K^+ . In view of the electrochemical behavior of ions in complex solutions and of the tip size of less than 1 μm in the microelectrode, the above results are not necessarily inaccurate.

Table 2. Determination of the ionic activities of Na^+ and K^+ , and activity coefficients in biological samples with triple-barreled Na^+ -, K^+ -selective microelectrodes and flame-photometer.

Samples	Number of samples	Electrometry		Flame photometry		Activity coefficient	
		Na^+ activity (mEq/liter H_2O)	K^+ activity (mEq/liter H_2O)	Na^+ concentration (mEq/liter H_2O)	K^+ concentration (mEq/liter H_2O)	Na^+	K^+
Human serum	11	106.5 ± 9.5 (8.9)	2.9 ± 0.2	148.8 ± 2.4 1.6	4.0 ± 0.2 (5.0)	0.716 ± 0.050	0.725 ± 0.060
(C)							
Frog serum	8	89.2 ± 8.0 (9.0)	2.3 ± 0.2 (8.7)	119.1 ± 0.1 (1.4)	3.1 ± 0.1 (3.2)	0.749 ± 0.080	0.742 ± 0.060
(C)							
Frog ringer	18	76.1 ± 8.1 (10.6)	2.5 ± 0.2 (8.0)	100.9 ± 1.3 (1.3)	3.4 ± 0.1 (2.9)	0.761 ± 0.160	0.735 ± 0.100
(C)							

The values are presented as mean $\pm$ SD. C is the coefficient of variation. Protein concentrations of human and frog sera were assumed to be 6 and 4%, respectively.

An actual trace of the EMFs in an intracellular measurement of bullfrog proximal tubule is presented in Fig. 5, showing changes in the membrane potential (E_M), the observed chemical (ionic) potentials for K^+ (E_K) and Na^+ (E_{Na}). The E_M registered -58.1 mV with respect to the outer cell surface 2 min after impalement, the observed E_K and E_{Na} were approximately 64 and -23 mV, corresponding to 54.7 mEq/liter for $(\text{K})_i$ and 25.7 mEq/liter for $(\text{Na})_i$, respectively.

Table 3 summarized the data of the E_M , the intracellular activities of Na^+ $(\text{Na})_i$ and K^+ $(\text{K})_i$ in proximal tubules and sartorius muscles *in vivo*. In both tissues, most values of the E_M ranged between -60 and -73 mV, the $(\text{Na})_i$ between 7 and 25 mEq/liter, the $(\text{K})_i$ between 55 and 70 mEq/liter. There were significant correlations between E_M and $(\text{K})_i$ in both kinds of cells: *i.e.*, the correlation coefficient (r) was 0.815 ($n=24$, $p<0.001$) for proximal tubule and 0.802 ($n=8$, $p<0.05$) for sartorius muscle. In sartorius muscle, no significant correlation was observed either between E_M and $(\text{Na})_i$ ($r=-0.723$, $p>0.05$) or between $(\text{Na})_i$ and $(\text{K})_i$ ($r=-0.380$, $p>0.1$), whereas in proximal tubule, significant inverse proportions were recognized between E_M and $(\text{Na})_i$ ($r=-0.585$, $p<0.01$) and between $(\text{Na})_i$ and $(\text{K})_i$ ($r=-0.755$, $p<0.01$).

In Fig. 6 are plotted the individual values of E_M in 24 different proximal tubules against $(\text{Na})_i$ and $(\text{K})_i$ which have been measured simultaneously with triple-barreled microelectrodes, indicating that the peritubular membrane potential directly correlates with the concentration of K^+ and inversely with Na^+ in the cell. The finding that the $(\text{K})_i$ in the proximal tubule cell correlates reciprocally with the $(\text{Na})_i$ agrees well with the data of double-barreled Na^+ -selective microelectrode (KOTERA *et al.*, 1979). This may somehow be related to the activity of

Fig. 5. Actual trace in a cellular impalement of the proximal tubule of bullfrog kidney with a triple-barreled Na^+ -, K^+ -selective microelectrode. A single cell was impaled (I) with the microelectrode, and the E_M , E_K and E_{Na} were directly observed for 3 min, then the electrode was manually withdrawn (W).

Table 3. Summarized data of intracellular activities of Na^+ and K^+ in proximal tubules and sartorius muscles measured *in vivo* at 25°C .

	<i>n</i>	E_M (-mV)	$(\text{Na})_i$ (mEq/liter)	$(\text{K})_i$ (mEq/liter)
Proximal tubule	24	65.9 ± 5.9	16.8 ± 8.2	63.0 ± 5.3
Sartorius muscle	8	68.2 ± 2.3	14.0 ± 5.6	64.5 ± 5.6

n is number of samples.

Na^+ - K^+ exchange across the cell membrane. Further, it also relates to the fact that, unlike muscle cells, in the renal proximal tubule cell whose membrane is one of the most permeable to water, the total ionic concentration, hence the osmolarity, is kept relatively constant across the cell membrane.

DISCUSSION

The measurement of intracellular Na^+ activity by means of a Na^+ -selective microelectrode has been attempted by a number of researchers (PALMER and CIVAN, 1977; KOTERA *et al.*, 1979; ARMSTRONG *et al.*, 1979; KIMURA and SPRING,

Fig. 6. Relationships among E_M , $(Na)_i$ and $(K)_i$ in the proximal tubule cell of 24 bullfrog kidneys. The solid lines represent their respective regression lines. r is the correlation coefficient. The broken line in the middle figure indicates Nernstian curve, whose potential is higher than the observed data. * is significant at 1% level, whereas ** is significant at 0.1% level.

1979). However, no success has been attained so far in simultaneous measurement of Na^+ and K^+ within a common cell. The triple-barreled Na^+ -, K^+ -selective microelectrode reported here has been designed for accomplishing this purpose with additional data of the electrical potential difference (PD) across the cell membrane. In the electrometry of ionic activities within single cells, the multibarreled electrode which simultaneously monitors change of the membrane PD offers many advantages, such as (1) the measurement of intracellular ionic activity *per se* becomes accurate and reliable, (2) tip position of the microelectrode can be exactly located within the tissue, (3) analysis of relationship between the ionic activities and the membrane PD is instantaneously possible, and (4) electrochemical potential gradient for the test ion across the cell membrane can be monitored continuously in various experimental conditions. Thus, the use of multibarreled electrodes in the study of an epithelial cell is one of the most powerful tools for analysing various phenomena, such as electrogenic activities of

Na⁺, sugar-evoked depolarization and others (FUJIMOTO and HONDA, 1980).

Although single-barreled ion-selective microelectrodes are relatively easy to fabricate, their use in experiments for monitoring changes in the ionic activity of the cell is often inappropriate, because the EMFs of such electrodes do not represent the ionic potential (E_{ionic}) reflecting the intracellular ionic activity, but represent a total potential (E_{total}) consisting of the E_{ionic} and the electrical potential across the membrane (E_{M}) as:

$$E_{\text{total}} = E_{\text{ionic}} + E_{\text{M}} \quad (3)$$

To obtain E_{ionic} , therefore, the EMF of the electrode must be subtracted by the E_{M} of the same cell. The results by other workers in the past were calculated from the difference between the mean values of E_{total} in one group of cells and those of E_{M} in another group of similar cells. Thus, the data obtained by this method are not of a single cell but of the cell normalized on the assumption that all the cells have a common membrane potential. The use of a multibarreled microelectrode is advantageous because the net change in E_{ionic} can be recorded by a differential output between the PD and ionic barrels.

There are, however, a number of problems in the use of the multibarreled electrode. The size of the tip of the electrode, in general, becomes larger with increasing number of barrels. However, in our triple-barreled microelectrodes, the tips have been made less than 1 μm in OD (see Fig. 1), which are small enough to impale most animal cells. Further, mechanical strength of the tip is increased in the triple-barreled rather than the single- or double-barreled microelectrode. An increase in the electrical resistance associated with a smaller tip of the individual barrel is to such an extent as could be compensated for by improvements of input-impedance of electrometers and prevented by beveling the tip of the electrode (KUBOTA *et al.*, 1980).

Another problem inherent in a high-resistance multibarreled electrode is relevant to the cross-coupling among individual barrels. By connections of plural numbers of input terminals of electrometers with a multibarreled microelectrode simultaneously, the chance of partial shunting among individual limbs would increase. The use of high input-impedance electrometers with complete isolations of the individual terminals is essential for recordings of not only the ionic potentials but also the simple membrane potential.

A possible electrical coupling among three barrels of an electrode is diagrammed in Fig. 7. In an equivalent circuit model shown here, if wall resistances (R_1 , R_2 and R_3) among three barrels are infinite, then it is possible to measure the ionic potential (E_{K} or E_{Na}) and the membrane potential (E_{M}), independently. However, a cross-coupling would occur when the electrical resistance across the glass wall becomes close to that of the electrical resistance of the ionic barrel (R_{Na} or R_{K}) or of the PD barrel (R_{T}). By analysing the equivalent circuit shown in Fig. 7, one can formulate a change of the tip potential (ΔE_{T}) due to cross-

Fig. 7. Equivalent circuit diagram for the triple-barreled microelectrode. Left: a possible circuit which contains multiple RC-coupling among 3 limbs. E'_M is the observed membrane potential after cellular impalement. Right: a simplified circuit which contains only wall- and fluids-resistance of the microelectrode.

coupling as:

$$\Delta E_T(R_1 + R_2) = \Delta i_K R_2(R_K + R_1) + \Delta i_{Na} R_1(R_{Na} + R_2), \quad (4)$$

where Δi denotes the change in the current flowing through the ion-exchanger. If we assume a parallel resistance (R) of R_1 and R_2 , Eq. (4) is rewritten in the form,

$$\Delta E_T = \Delta E'_K \left(1 + \frac{R_K}{R}\right) + \Delta E'_{Na} \left(1 + \frac{R_{Na}}{R}\right), \quad (5)$$

where

$$\frac{1}{R} \equiv \frac{1}{R_1} + \frac{1}{R_2}$$

and

$$\Delta E'_K \equiv \Delta i_K R \quad \text{and} \quad \Delta E'_{Na} \equiv \Delta i_{Na} R.$$

Thus, the potential change generated at the tip of the PD barrel is associated with the change in ionic potentials. When the values of R_1 and R_2 are relatively large as compared to R_K and R_{Na} , ΔE_T is minimum, and Eq. (5) is expressed as:

$$\Delta E_T \simeq \Delta E'_K + \Delta E'_{Na}. \quad (6)$$

In this study, the change in EMF of PD barrel was measured *in vitro* in the calibration stage prior to biological measurement, the values ranging from 0 to 20 mV in response to a 10-fold change of ionic activities. We selected those electrodes within 5 mV in the calibration stage in biological measurements. For comparison of electrode properties, the tip potentials of PD barrel measured be-

tween Ringer and 0.1 M KCl solution *in vitro* have been represented in Table 1 as 3.2 ± 2.0 mV ($n=12$). Even in intracellular recording with such electrodes, the possibility of a large change in the tip potential might not be expected, because the effect of a decrement of ionic potential for Na^+ (*i.e.*, $\Delta E'_{\text{Na}} < 0$) and an increment of that for K^+ ($\Delta E'_{\text{K}} > 0$) would cancel each other, as is predicted by Eq. 6. However, since the response time of the Na^+ -barrel is more sluggish than the PD barrel, an anomalous deflection in the EMF of the Na^+ -barrel would appear momentarily at the insertion of the electrode tip into or withdrawal from the cell (see arrows I and W in Fig. 5). These abnormalities disappear when two ionic barrels are stabilized. At any rate, in the practice of PD measurement across the cell membrane, the tip potential determined for an individual microelectrode at the stage of electrode calibration has to be subtracted from the observed potential difference.

The electrode resistances of the ionic barrels are relatively high as compared to the PD barrel: *i.e.*, the K^+ -barrel had an average of $1.5 \pm 3.1 \times 10^{10} \Omega$, and the Na^+ -barrel $5.2 \pm 3.1 \times 10^{10} \Omega$. The data suggest high input-impedance electrometers are necessary for the use of a triple-barreled microelectrode.

On the other hand, one difficulty in a Na^+ -selective single- or double-barreled microelectrode is relevant to its poor selectivity to the test ion with respect to the other interfering ions. Especially, when it is applied to intracellular measurement, the problem often becomes apparent, because the test ion (Na^+) is relatively low and the interfering ions, such as K^+ and proteins, are high in the cell. The triple-barreled microelectrode capable of measuring not only the test ion (Na^+) but also one of the major interfering ions (K^+) could solve this problem. It permits us to calculate exactly the ionic activity of the test ion by knowing the extent of interference of the other ion.

In earlier studies in our laboratory, in which double-barreled Na^+ -selective microelectrodes were used for the proximal tubule cell, the correction for K^+ -interference was made on the EMF of Na^+ -barrel indirectly on the basis of a known relationship between $(\text{K})_i$ and E_M (KOTERA *et al.*, 1979; FUJIMOTO *et al.*, 1980b). However, the measured values for $(\text{Na})_i$ and E_M in the proximal tubule cell by means of the double-barreled microelectrode (20.7 ± 6.0 mEq/liter, 68.7 ± 3.4 mV) are virtually in agreement with those measured by triple-barreled microelectrodes in this study (Table 3). Furthermore, the values for $(\text{K})_i$ measured by the double-barreled K^+ -selective microelectrode (62.6 ± 12.2 mEq/liter) are also identical with the present data (63.0 ± 5.3 mEq/liter, see Table 3). Thus, it is obvious that both electrodes can yield a fair result with respect to the intracellular ionic activity of Na^+ .

Recently, O'DOHERTY *et al.* (1979) and STEINER *et al.* (1979) reported a Na^+ -selective microelectrode with a high selectivity (Na^+/K^+ ratio) of more than 100. The Na^+ -ligand, a synthetic neutral carrier consisting of 1, 1, 1-tris[1-(2-oxa-4-oxo-5-aza 5-methyl)-dodecanyl]propane, was first developed by Simon's group

(see AMMANN *et al.*, 1976), and it has just come into the stage of testing. Since this material has an extremely high electrical resistance of $10^{11} \Omega$ or so, further developmental work on Na^+ -ion-specific ligands and their practical application needs to be done.

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REFERENCES

- AMMANN, D., BISSIG, R., CIMERMAN, Z., FIEDLER, U., GÜGGI, M., MORF, W. E., OEHME, M., OSSWALD, H., PRETSCH, E., and SIMON, W. (1976) Synthetic neutral carriers for cations. *In: Ion and Enzyme Electrodes in Biology and Medicine*, ed. by KESSLER, M. *et al.* Urban & Schwarzenberg, München, pp. 22–37.
- ARMSTRONG, W. McD., BIXEMAN, W. R., FREY, K. F., GRACIA-DIAZ, J. F., O'REAGAN, M. G., and OWENS, J. L. (1979) Energetics of coupled Na^+ and Cl^- entry into epithelial cells of bullfrog small intestine. *Biochim. Biophys. Acta*, **551**: 207–219.
- FUJIMOTO, M. and KUBOTA, T. (1976) Physicochemical properties of a liquid ion exchanger microelectrode and its application to biological fluids. *Jpn. J. Physiol.*, **26**: 631–650.
- FUJIMOTO, M., KOTERA, K., and MATSUMURA, Y. (1980a) The direct measurement of K, Cl, Na and H ions in bullfrog tubule cells. *In: Current Topics in Membranes and Transport*, ed. by BOULPAEP, E. Academic Press, New York, pp. 49–61.
- FUJIMOTO, M., NAITO, K., and KUBOTA, T. (1980b) Electrochemical profile for ion transport across the membrane of proximal tubule cells. *Membr. Biochem.*, **3**: 67–97.
- FUJIMOTO, M. and HONDA, M. (1980) Direct measurement of intracellular Na and K activities in the renal tubular cells with triple-barreled micro-electrodes. Symposium "Cell Ionic Activity and Element Analysis," Proc. 28th Int. Congr. Physiol. Sci. Budapest, p. 119.
- I.U.P.A.C. (1976) Recommendations for nomenclature of ion-selective electrodes. *Pure Appl. Chem.*, **48**: 127–132.
- KESSLER, M., HÁJEK, K., and SIMON, W. (1976) Four-barreled microelectrode for the measurement of potassium-, sodium- and calcium-ion activities. *In: Ion and Enzyme Electrodes in Biology and Medicine*. ed. by KESSLER, M. *et al.* Urban & Schwarzenberg, München, pp. 136–140.
- KHURI, R. N., AGULIAN, S. K., and KALLOGHLIAN, A. (1972) Intracellular potassium in cells of the distal tubule. *Pflügers Arch.*, **335**: 297–308.
- KIMURA, G. and SPRING, K. R. (1979) Luminal Na^+ entry into *Necturus* proximal tubule cells. *Am. J. Physiol.*, **236**: F295–F301.
- KOTERA, K., SATAKE, N., HONDA, M., and FUJIMOTO, M. (1979) The measurement of intracellular sodium activities in the bullfrog by means of double-barreled sodium liquid ion-exchanger microelectrodes. *Membr. Biochem.*, **2**: 323–338.
- KUBOTA, T. and FUJIMOTO, M. (1978) Seasonal changes of membrane potential across the proximal tubular epithelium in bullfrog kidneys. *Jpn. J. Physiol.*, **28**: 181–196.
- KUBOTA, T., HONDA, M., KOTERA, K., and FUJIMOTO, M. (1980) The effect of diffusible ions on the peritubular membrane potential of proximal tubular cells in perfused bullfrog kidneys. *Jpn. J. Physiol.*, **30**: 775–790.
- O'DOHERTY, J., GRACIA-DIAZ, J. F., and ARMSTRONG, W. McD. (1979) Sodium-selective

- liquid ion-exchanger microelectrodes for intracellular measurements. *Science*, **203**: 1349–1351.
- PALMER, L. G. and CIVAN, M. M. (1977) Distribution of Na^+ , K^+ and Cl^- between nucleus and cytoplasm in *Chironomus* salivary gland cells. *J. Membr. Biol.*, **33**: 41–61.
- SILVER, I. A. (1976) Multi-parameter electrodes. In: Ion and Enzyme Electrodes in Biology and Medicine, ed. by KESSLER, M., CLARK, L. C., Jr., LÜBBERS, D. W., SILVER, I. A., and SIMON, W. Urban & Schwarzenberg, München, pp. 119–122.
- SNEDECOR, G. W. (1956) Statistical Methods, 5th ed., Iowa State Univ. Press, Ames, 478 pp.
- STEINER, R. A., OEHME, M., AMMANN, D., and SIMON, W. (1979) Neutral carrier sodium ion-selective microelectrode for intracellular studies. *Anal. Chem.*, **51**: 351–353.
- VYSKOCIL, F. and KRIZ, N. (1972) Modification of single and double-barreled potassium specific microelectrodes for physiological experiments. *Pflügers Arch.*, **337**: 265–276.
- WALKER, J. L. (1971) Ionic specific liquid ion exchanger microelectrodes. *Anal. Chem.*, **43**: 89A–93A.