NEUTRAL CARRIER Na^+- AND Ca^{2+}-SELECTIVE MICROELECTRODES FOR INTRACELLULAR APPLICATION

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ABSTRACT Na^+- and Ca^{2+}-selective microelectrodes were made with Simon's neutral carrier ETH 227 and ETH 1001, respectively, and their properties were studied for intracellular application. The k_{NaK} (selectivity coefficient for Na^+ with respect to K^+) values of the Na^+-selective microelectrodes were in the range of 0.01–0.02, which is comparable to those of recessed-tip Na^+-selective glass microelectrodes. The k_{NaMg} values of the microelectrodes were ~0.005 so that the interference by intracellular Mg^{2+} levels could be negligible. The k_{NaCa} values were ~2 and the Na^+-selective microelectrodes were more selective to Ca^{2+} than Na^+. This indicates that their intracellular application requires special care to handle Ca^{2+} interference under certain conditions. The k_{NaK}, k_{NaMg}, and k_{NaCa} values did not depend significantly on the methods used for their determination or on the ion activity levels tested. The Nicolsky equation described well the microelectrode potentials in the mixed solutions of NaCl (1–100 mM) and KCl. Potential and resistance of the microelectrodes were stable for a long period and their response time was fast. The results indicate that the Na^+-selective microlectrodes are suitable for measurements of intracellular Na ion activities. Ca^{2+}-selective microelectrode potentials at Ca^{2+} concentrations lower than 10^{-4} M changed significantly for the first 2–3 h and then became fairly stable. The rate of the potential change was dependent on the column length of the Ca^{2+}-selective liquid filled. Potentials of the microelectrodes varied from 10–20 mV for Ca^{2+} between 10^{-7} and 10^{-6} M concentrations, which may be the cytosolic free-Ca^{2+} range. With the Ca^{2+} concentrations $>10^{-6}$ M, the microelectrodes had potential changes of ~30 mV or greater for a tenfold change in Ca^{2+} concentration. The k_{CaK} and k_{CaNa} values were in the ranges of 10^{-5}–10^{-6} and 10^{-4}–10^{-5}, respectively. The k_{CaMg} values were ~10^{-7}. The results show that the Ca^{2+}-selective microelectrodes can be used for measurements of cytosolic Ca ion activities.

INTRODUCTION

Measurements of intracellular Na and Ca ion activities are important for the study of cell membrane transport of these ions, and cellular functions related to the ion activities. Intracellular ion activities have been measured directly with ion-selective microelectrodes. When new types of ion-selective microelectrodes are developed, their properties must be studied for application. Intracellular Na ion activities have been measured with recessed-tip Na^+-selective glass microelectrodes because selectivity of the glass microelectrodes for Na^+ over K^+ is better than that of the Na^+-selective microelectrodes made with available Na^+-selective liquid ion exchangers (Lee, 1981). However, the major disadvantages of the recessed-tip Na^+-selective glass microelectrodes are difficulty in the microelectrode construction and slow response time. The response times of the recessed-tip microelectrodes are inevitably slower than those of liquid ion-exchanger microelectrodes because ions must diffuse into the recessed space for an equilibrium between the space and a test solution (Thomas, 1976). The blockade of the microelectrode tip is also a problem, particularly in cases of impalement for a long period. It is obvious that the ion-selective microelectrodes made with a liquid ion-exchanger overcome the disadvantages of the recessed-tip glass microelectrodes. Recently Steiner et al. (1979) have reported Na^+-selective microelectrodes made with a Na^+-selective neutral carrier, ETH 227 (Ammann et al., 1974). ETH 227 represents a Na^+-selective ligand and its structure has been shown by Ammann et al. (1974). Their study indicated that the Na^+-selective microelectrodes with tip diameters of ~1 μm have adequate selectivities for Na^+ over K^+ and Mg^{2+} for measurements of intracellular Na ion activities. However, the Na^+-selective microelectrodes are more selective to Ca^{2+} than to Na^+ so that they must be used carefully under certain conditions. The present study is concerned with the properties and intracellular application of the neutral carrier (ETH 227) Na^+-selective microelectrodes with tip diameters <1 or ~1 μm. The properties include selectivity, resistance, stability, and response time. We describe how the problems of Ca^{2+}

interference can be managed. Construction of the neutral carrier Na^+-selective microelectrodes is much easier than that of recessed-tip Na^+-selective glass microelectrodes. The results of this study indicate that the neutral carrier Na^+-selective microelectrodes are suitable for measurements of intracellular Na ion activities in small cells.

Recently Ca^{2+}-selective microelectrodes were made by using a Ca^{2+}-selective neutral carrier, ETH 1001 (Ammann et al., 1975), and have been used for measurements of intracellular Ca ion activities (Rink et al., 1980; O'Doherty et al., 1980; Lee et al., 1980*a*; Marban et al., 1980; Lee et al., 1980*b*; Sheu and Fozzard, 1981; Alvarez-Leefmans et al., 1981; Lux et al., 1981). ETH 1001 represents a Ca^{2+}-selective ligand and its structure has been shown by Ammann et al. (1975). The Ca^{2+}-selective microelectrodes have some problems and their intracellular application is not easy (Tsien and Rink, 1980; Lee, 1981). The major problems may be changes of the microelectrode potentials with time (potential drift) and super-Nernstian slopes over a certain range of Ca^{2+} concentration. Despite such problems, the microelectrodes can be used to measure intracellular Ca ion activities under certain conditions. In the present study, we report some properties of the neutral carrier Ca^{2+}-selective microelectrodes that may be useful for their intracellular application. Tsien and Rink (1980) have modified the original Ca^{2+}-selective liquid provided by W. Simon (Simon et al., 1978). They reported that such a modification improved the microelectrode stability and reduced hysteresis greatly in potential responses of the microelectrodes.

METHODS

Construction of Na^+- and Ca^{2+}-selective Microelectrodes

To prepare glass micropipettes, glass tubing was cleaned by the following procedures. Borosilicate glass tubing (ID, 1.1 mm; OD, 1.8 mm; length, 7 cm; Corning Glass Works, Corning, NY, code 7740) was soaked in ethanol for ~30 min, boiled in distilled water for ~20 min, and then dried by heating at ~200°C for ~1 h. Glass micropipettes with tip diameters <1 μm were pulled from the cleaned glass tubing using a micropipette puller (David Kopf Instruments, Inc., Tujunga, CA, model 700 C). The surface of the micropipettes was silanized by exposure to dichlorodimethylsilane gas (Eastman Kodak Co., Rochester, NY). 10–15 micropipettes were placed vertically (tip up) in a glass staining jar with a ground-glass cover. This glass jar was transferred to an oven, preheated to ~200°C. After heating at this temperature for ~30 min, a small amount (0.03 ml) of dichlorodimethysilane was dropped quickly into the glass jar and covered. The glass jar was transferred to the oven and heated further for ~30 min. Then the jar was cooled slowly. For a Na^+-selective microelectrode, the shank and shoulder of the silanized micropipette were filled with a 100-mM NaCl solution by a syringe and then the solution was pushed down to the tip by applying pressure. Na^+-selective liquid was drawn up to 100–400 μm from the tip by applying back pressure. For a Ca^{2+}-selective microelectrode, the silanized micropipette was filled with a 5-mM $CaCl_2$ solution or a buffer solution of pCa 6.6 as described for a Na^+-selective microelectrode. Ca^{2+}-selective liquid then was drawn up to 500–900 μm from the tip. Both ion-selective liquids (ETH 227 and ETH 1001) were provided by W. Simon of the Eidgenössische Technische Hochschule, Zürich, Switzerland.

Solutions and Measurement of Microelectrode Potential

Single (pure) and mixed electrolyte solutions were used for investigating properties of neutral carrier Na^+-selective microelectrodes. The single electrolyte solutions were 1, 2, 5, 10, 50, 100, and 150 mM NaCl; 50, 100, and 150 mM KCl; 1.8 mM $CaCl_2$; 1 mM $MgCl_2$. The mixed electrolyte solutions consisted of 1 mM NaCl + 149 mM KCl, 2 mM NaCl + 148 mM KCl, 5 mM NaCl + 145 mM KCl, 10 mM NaCl + 140 mM KCl, 50 mM NaCl + 100 mM KCl, 100 mM NaCl + 50 mM KCl, and 150 mM NaCl + 1.8 mM $CaCl_2$. The mixed solutions of NaCl and KCl had a constant ionic strength of 0.15 M. Ionic activities of the electrolyte solutions were calculated using the activity coefficient of each solution. Mean ionic activity coefficients of single electrolyte solutions are known or can be calculated by the extended Debye-Hückel equation (Robinson and Stokes, 1965). The known activity coefficients (determined experimentally) agree well with the calculated activity coefficients. Activity coefficients of the mixed electrolyte solutions also were calculated by the extended Debye-Hückel equation.

The solutions used for studying neutral carrier Ca^{2+}-selective microelectrodes had Ca ion concentrations of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M; they also contained 150 mM K^+, and 1 mM Mg^{2+}, and had a pH of 7.0. Also used were solutions containing 10^{-7} or 10^{-8} M Ca^{2+} and 140 mM K^+ plus 10 mM Na^+. The single electrolyte solutions used were 150 mM KCl, 10 mM NaCl, and 2 mM $MgCl_2$. These solutions contained 0.1 mM EGTA so that the solutions were Ca^{2+} free. The solutions containing 10^{-3}, 10^{-4}, and 10^{-5} M Ca^{2+} were made by dilution of 100 mM $CaCl_2$ solution. The solutions of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M Ca^{2+} were made from a Ca^{2+} buffer containing EGTA. Two sets of these solutions were made using the apparent stability constants of 4.90×10^6 M^{-1} (Schwarzenbach constant) and 2.51×10^6 M^{-1} (Allen and Blinks constant) for Ca-EGTA complex at a pH of 7.0 (Fabiato and Fabiato, 1978). Note that the solutions containing 10^{-5} M Ca^{2+} were made by EGTA buffer as well as simple dilution. The mixed electrolyte solutions had virtually the same ionic strength and were assumed to have the same activity coefficient for Ca^{2+}. The Ca^{2+} activity coefficient of 0.32 was used to calculate Ca^{2+} activities in the solutions (Lee, 1981).

Potential and resistance of the microelectrodes were measured as described previously (Lee and Fozzard, 1974).

RESULTS

Selectivity of Na^+-selective Microelectrodes

The evaluation of ion-selective electrodes focuses on their selectivity coefficients (k_{AB}), i.e., mathematical expression of their ability to distinguish between the primary ion A and the interfering ion B. In determination of the selectivity coefficients, two major problems have emerged (Moody and Thomas, 1971; Simon et al, 1978; Lee, 1981). First, the selectivity coefficient of an ion-selective microelectrode varies depending on the methods used. Second, the selectivity coefficient varies with the levels of the primary ion activity and the interfering ion activity. In the present study we have determined selectivity coefficients of neutral carrier Na^+-selective microelectrodes with the different methods and at different ion activity levels. The behavior of ion-selective microelectrodes is described by the following empirical equation (extended Nicolsky equation):

$$E = E_0 + RT/z_AF \ln (a_A + k_{AB}(a_B)^{z_A/z_B}). \quad (1)$$

E is the potential measured with an ion-selective microelectrode. E_0 is a constant potential of a given ion-selective microelectrode. RT/z_AF is the Nernstian factor. z_A and z_B are the valences of A and B ions, respectively. a_A and a_B are the activities of A and B ions, respectively. k_{AB} is the selectivity coefficient of an ion-selective microelectrode. Several different methods for determining the selectivity coefficient have been described (Moody and Thomas, 1971; Lee, 1981). Selectivity coefficients of the Na^+-selective microelectrodes were determined by the following equations (methods):

$$k_{AB} = \frac{a_A}{(a_B)^{z_A/z_B}} \times 10^{(E_B - E_A)/S} \quad (2)$$

$$k_{AB} = \frac{1}{(a_B)^{z_A/z_B}} \times 10^{(E_B - E_0)/S} \quad (3)$$

$$k_{AB} = \frac{a_A}{(a_B)^{z_A/z_B}} \quad (4)$$

$$k_{AB} = [10^{(E-E_0)/S} - a_A]/(a_B)^{z_A/z_B}. \quad (5)$$

E_A and E_B are the potentials measured with single electrolyte solutions containing the ion A and the ion B, respectively. S is Nernstian factor (slope) determined experimentally. Eqs. 2–5 were derived or modified from Eq. 1.

Fig. 1 shows the potentials measured with a typical neutral carrier Na^+-selective microelectrode. The number(s) above each potential recording represent the concentration (mM) of the primary ion (Na^+) and interfering ions (K^+, Ca^{2+}, and Mg^{2+}) of the electrolyte solutions. Note that single and mixed electrolyte solutions were used. The selectivity coefficients of the Na^+-selective microelectrodes were calculated by the Eqs. 2–5, using the measured potentials as shown in Fig. 1 and ionic activities of the electrolyte solutions. Table I shows the selectivity coefficients of the Na^+-selective microelectrode used in the experiment shown in Fig. 1. The k_{NaK} values were in the range of 0.011 to 0.014 regardless of the methods (equations) used. The values of 0.011 to 0.014 correspond that the Na^+-selective microelectrode is 90–71 times more selective to Na^+ than to K^+. The results indicate that any methods (equations) shown above can be used for determination of k_{NaK} of the microelectrodes. The results (Table I) also indicate that the k_{NaK} values determined by each method do not vary significantly with the ion activity levels tested.

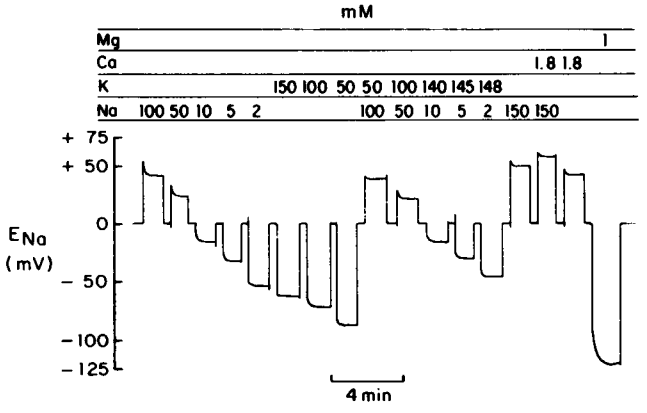

FIGURE 1 Potential recording of a typical neutral carrier Na^+-selective microelectrode. The potentials were measured with single and mixed electrolyte solutions of NaCl, KCl, $CaCl_2$, and $MgCl_2$ as shown above the potential recording. The solutions of Na^+ concentration ≤10 mM contained 0.1 mM EGTA. The zero potential represent a grounded reference potential.

TABLE I
SELECTIVITY COEFFICIENTS (k_{NaK}, k_{NaCa} AND k_{NaMg}) OF A NEUTRAL CARRIER Na^+-SELECTIVE MICROELECTRODE

Method	Solutions	k_{NaK}	k_{NaMg}	k_{NaCa}
	(*mM*)			
Eq. 2	100 NaCl and 150 KCl	0.013		
	10 NaCl and 150 KCl	0.013		
	5 NaCl and 150 KCl	0.013		
	2 NaCl and 150 KCl	0.012		
	100 NaCl and 100 KCl	0.013		
	10 NaCl and 100 KCl	0.013		
	5 NaCl and 100 KCl	0.013		
	2 NaCl and 100 KCl	0.012		
	100 NaCl and 50 KCl	0.014		
	10 NaCl and 50 KCl	0.014		
	5 NaCl and 50 KCl	0.014		
	2 NaCl and 50 KCl	0.013		
	100 NaCl and 1 $MgCl_2$		0.005	
	10 NaCl and 1.8 $CaCl_2$			1.92
Eq. 3	150 KCl	0.013		
	100 KCl	0.013		
	50 KCl	0.014		
	1 $MgCl_2$		0.005	
	1.8 $CaCl_2$			1.85
Eq. 4	150 KCl	0.013		
	100 KCl	0.013		
	50 KCl	0.013		
Eq. 5	10 NaCl + 140 KCl	0.011		
	5 NaCl + 145 KCl	0.012		
	2 NaCl + 148 KCl	0.011		
	150 NaCl + 1.8 $CaCl_2$			1.93

The average k_{NaK} value obtained from 40 Na^+-selective microelectrodes was 0.017 ± 0.008 (mean ± SD). The k_{NaMg} values of the Na^+-selective microelectrodes were ~0.005 (Table I). This indicates that the interference by Mg^{2+} activities in cells is negligible. The k_{NaMg} value obtained from five Na^+-selective microelectrodes was 0.0052 ± 0.0014 (mean ± SD). The k_{NaCa} values of the microelectrodes were ~2 as shown in Table I, indicating that the Na^+-selective microelectrodes are more selective to Ca^{2+} than to Na^+. The k_{NaCa} value obtained from eight Na^+-selective microelectrodes was 1.97 ± 0.25 (mean ± SD). Under certain conditions, special handling of Ca^{2+} interference is required for measuring intracellular Na^+

activities with the Na^+-selective microelectrodes. This will be described.

Intracellular Application of Na^+-selective Microelectrodes

Generally there are two methods to determine intracellular ion activities with ion-selective microelectrodes (Lee, 1981). One method is to use the equation (Nicolsky equation) describing the ion-selective microelectrode potentials. The other is to determine intracellular ion activities using a calibration curve. The use of the Nicolsky equation for a new type of ion-selective microelectrode must be justified (Lee, 1981). For this justification, the microelectrode potentials observed in various mixed electrolyte solutions are compared with those calculated by the Nicolsky equation using a selectivity coefficient determined. In the present study, a k_{NaK} value (0.016) of a Na^+-selective microelectrode was determined by a simple method (Eq. 3) using 150 mM KCl solution. In Fig. 2, the calculated and measured potentials were plotted against $-\log a_{Na}$. The continuous curve (—) represents the potentials calculated by the Nicolsky equation. The filled circles (●) are the microelectrode potentials measured with the mixed solutions of NaCl and KCl. The calculated potentials agree well with the measured potentials. This indicates that the Nicolsky equation can be used to determine intracellular Na ion activities with neutral carrier Na^+-selective microelectrodes. The broken line (---) represents the microelectrode response with a slope of 59.8 mV in single solutions of NaCl.

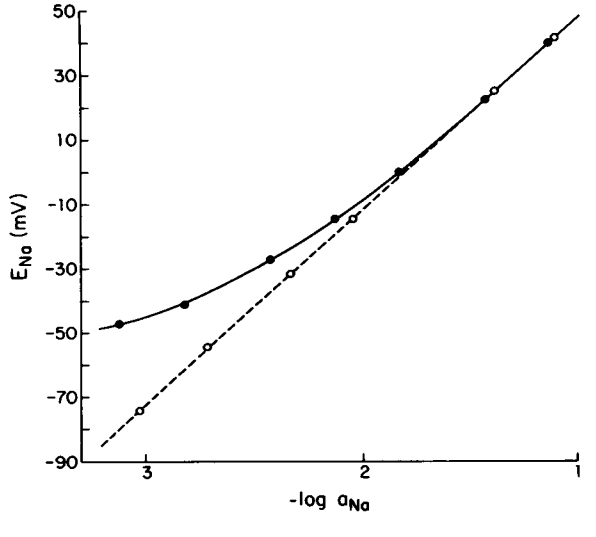

FIGURE 2 Comparison of calculated potentials solid curve (——) with measured potentials (●) in various mixed solutions of NaCl and KCl for a typical Na^+-selective microelectrode. The open circles (○) represent the microelectrode potentials measured with single electrolyte solutions of NaCl. The mixed solutions used were (1 mM NaCl + 149 mM KCl), (2 mM NACl + 148 mM KCl), (5 mM NaCl + 145 mM KCl), (10 mM NaCl + 140 mM KCl), (20 mM NaCl + 130 mM KCl), (50 mM NaCl + 100 mM KCl), and (100 mM NaCl + 50 mM KCl). The solutions of Na^+ concentrations ≤10 mM contained 0.1 mM EGTA.

One can use the Nicolsky equation (Eq. 1) to determine intracellular Na^+ activity (a^i_{Na}). Appropriate corrections should be made for interference by $k_{AB}(a_B)^{z_A/z_B}$ in the equation. For an example, the value of k_{NaK} (a_K) is 1.5 mM for 100 mM a^i_K (intracellular K^+ activity) and 0.015 of k_{NaK}. The value of k_{NaCa} $(a_{Ca})^{1/2}$ is 0.6 mM for 100 nM (intracellular Ca^{2+} activity) and 2.0 of k_{NaCa}. Thus, selectivity coefficients (k_{NaK} and k_{NaCa}) of each electrode should be determined. The interference of 1.5 mM or 0.6 mM is smaller than the intracellular Na^+ activity range of 6 to 11 mM in resting cardiac muscle. The interferences due to changes in a^i_K and a^i_{Ca} may be insignificant when the changes in a^i_K and a^i_{Ca} are less than 30 mM and 50 nM, respectively.

In the other method, intracellular Na ion activities are determined using calibration curves. In this method, a calibration curve for a Na^+-selective microelectrode is constructed with the potentials measured in a series of mixed solutions such as 1 mM NaCl + 149 mM KCl, 5 mM NaCl + 145 mM KCl, 10 mM NaCl + 140 mM KCl, 20 mM NaCl + 130 mM KCl, 30 mM NaCl + 120 mM KCl, and 149 mM NaCl + 1 mM KCl. Note that all solutions have the same ionic strength. The measured potentials are plotted against Na^+ activities (Ellis, 1977; Thomas, 1978; Lee, 1981). The calibration solutions must include an electrolyte solution similar to the extracellular bathing fluid (Edelman et al., 1978) because the potential measured in the bathing fluid is used in the method. The solution of 149 mM NaCl and 1 mM KCl is nearly the same as the extracellular fluid containing 150 mM Na^+. The microelectrode potential measured in a solution containing 149 mM NaCl and 1 mM KCl must be the same as the potential measured in the extracellular fluid. Then the former potential is set arbitrarily as zero in the calibration curve. The 1 mM K^+ is different from the normal K^+ concentrations (3–5 mM) in the extracellular fluid, but the effect of the difference on the microelectrode potential is not significant because of high selectivity of the microelectrodes for Na^+ over K^+. It was found, however, that the method using a calibration curve for neutral carrier Na^+-selective microelectrodes is quite complicated. With these microelectrodes there is significant interference by Ca^{2+}. It has been shown that the k_{NaCa} values are ~2 (Table I). As shown in Fig. 1, the difference between the potential measured with 150 mM NaCl and that measured with the mixed solution of 150 mM NaCl and 1.8 mM $CaCl_2$ was ~8 mV. We observed that the potential differences were in the range of 7 to 12 mV, depending on the k_{NaCa} value of the individual microelectrode. Therefore, the calibration solution equivalent to the extracellular fluid must contain 1.8 mM Ca^{2+} in addition to K^+, but other calibration solutions must contain a Ca^{2+} concentration equivalent to intracellular free Ca^{2+}. Then microelectrode potentials are not linear in the range of high Na^+ activities. If the

calibration solution (150 mM NaCl) without Ca^{2+} is used and the potential is set as zero in the calibration curve, this potential differs from that measured in the extracellular fluid and significant errors are produced in the determination of intracellular Na^+ activities. A convenient method is to use the Nicolsky equation, which does not require potential measured in the bathing fluid but contains appropriate corrections for intracellular interfering ions.

Stability of Na^+-selective Microelectrodes

Ion-selective microelectrodes must be stable for reliable and wide application. The tips of ion-selective microelectrodes are chemically and electrically complicated because of the small tip diameter, the possibility of poor silanization, and possible shunt through the glass wall. The selectivity coefficients and resistances of some ion-selective microelectrodes change with aging (Lee, 1979; Lee, 1981). Table II shows slopes (S), selectivity coefficients (k_{NaK}), and resistances (R) of a neutral carrier Na^+-selective microelectrode for ~120 h aging after construction. The microelectrode had ~300 μm column of the Na^+-selective liquid from the tip (the tip diameter <1 μm). The microelectrode had been kept in air except for the periods of potential measurements. The slopes were measured with 10 and 100 mM NaCl solutions. The selectivity coefficients were determined by Eq. 3 using the potential measured with 150 mM KCl solution. As seen in Table II, the slope of the microelectrode was stable and very close to the theoretical slope (59.8 mV). The selectivity coefficient and resistance of the microelectrode did not change substantially during this period. Four additional Na^+-selective microelectrodes showed results similar to those in Table II. We also measured the slope, selectivity coefficient, and resistance of Na^+-selective microelectrodes whose tips were immersed continuously in 100 mM NaCl solution for ~10 h. The experiments done with three microelectrodes showed that the slope, selectivity coefficient and resistance were stable. The results indicate that the properties of neutral carrier Na^+-selective microelectrodes are quite stable for a long period. The data also suggest that the Na^+-selective microelectrodes did not have an electrical shunt by poor silanization or hydration of the glass wall.

Response times of neutral carrier Na^+-selective microelectrodes were in the range of 10 to 20 s for more than 95% response of the final stable potential. Such response times may be due to the high resistance and capacitance of the microelectrodes (Lee, 1981). A slow response time can be improved by silver conductive paint of ion-selective microelectrodes as described by Tsien and Rink (1980) and Lewis and Wills (1980). Two Na^+-selective microelectrodes were painted with conductive silver. These microelectrodes showed a response time of <5 s for a full response.

TABLE III
SELECTIVITY COEFFICIENTS (k_{CaK}, k_{CaNa}, AND k_{CaMg}) OF Ca^{2+}-SELECTIVE MICROELECTRODES (n = 6)

Solutions used	k_{CaK}	k_{CaNa}	k_{CaMg}
150 mM KCl	$(4.6 \pm 2.1) \times 10^{-6}$		
10 mM NaCl		$(5.2 \pm 2.2) \times 10^{-5}$	
2 mM $MgCl_2$			$(2.9 \pm 2.4) \times 10^{-7}$

Change of Ca^{2+}-selective Microelectrode Potential with Time

The major problems of neutral carrier Ca^{2+}-selective microelectrodes are potential drift and super-Nernstian slope (Tsien and Rink, 1980; Lee, 1981). Fig. 3 shows potential changes of two Ca^{2+}-selective microelectrodes with time after they were constructed. The Ca^{2+}-selective microelectrode used in the upper panel was filled with 5 mM $CaCl_2$ solution and had ~550 μm column length of Ca^{2+}-selective liquid, while the Ca^{2+}-selective microelectrode used in the lower panel was filled with a buffered solution of pCa 6.6 and had ~600 μm column length of Ca^{2+}-selective liquid. The potentials of both electrodes changed considerably for ~2 h before becoming stable. For ~2 h after construction the slopes in the range of pCa 4 to 6 were ~50 mV/pCa, a value greater than the theoretical one of 30 mV. The slopes reached the theoretical value with time. The potential differences between pCa 6 and 7 remained constant or sometimes increased slightly, which is important for intracellular application of the microelectrodes because cytosolic free Ca concentrations were reported to be in the range of pCa 6 to 7 (Lee, 1981). It was also observed that the rate of the microelectrode potential

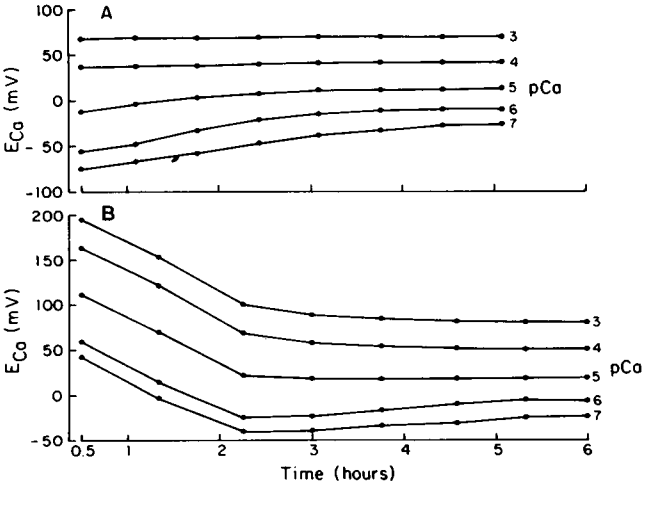

FIGURE 3 Change in Ca^{2+}-selective microelectrode potential (E_{Ca}) with time. The potentials were measured with the calibration solutions containing pCa 3, 4, 5, 6, and 7. *A*, microelectrode filled with 5-mM $CaCl_2$ solution. *B*, microelectrode filled with buffered pCa 6.6 solution.

change depended on the column length of the Ca^{2+}-selective liquid. The longer the column length, the slower the rate of the microelectrode potential change. The microelectrodes with a column length of 1 to 2 mm often had super-Nernstian slopes for a long period. Such microelectrodes may still be useful as long as the potential drift is not significant.

Selectivity of Ca^{2+}-selective Microelectrodes

Selectivity coefficients of ion-selective microelectrodes can be determined by several methods as described for Na^+-selective microelectrodes. The method using Eq. 3 is suitable to determine selectivity coefficients of Ca^{2+}-selective microelectrodes (Lee, 1981). Ca^{2+}-selective microelectrodes were aged for 3 to 4 h until their slopes in the range of pCa 6 to 3 were linear and close to the theoretical value (see Fig. 3). Then the potentials of these microelectrodes were measured with the single electrolyte solutions (Table III) containing the interfering ions K^+, Na^+, and Mg^{2+}. The potential (E_B of Eq. 3) measured with each solution was used for calculating k_{CaK}, k_{CaNa}, and k_{CaMg} values. Table III shows the calculated selectivity coefficients. It appears that the values of k_{CaK} influence significantly the Ca^{2+}-selective microelectrode potential at pCa 7. One can estimate the influence (interference) by calculating the $k_{AB}\ (a_B)^{z_A/z_B}$ value of Eq. 1. The value of $4.6 \times 10^{-6}(10^{-1})^2$ M for 0.1 M K^+ (intracellular K^+ activity) affects significantly the Ca^{2+}-selective microelectrode potential measured with pCa 7 solution. The value of $5.2 \times 10^{-5}\ (10^{-2})^2$ M for 0.01 M Na^+ (intracellular Na^+ activity) is about one order lower than the value $4.6 \times 10^{-6}\ (10^{-1})^2$ M for intracellular K^+ activity. It was observed that the selectivity and slope of the Ca^{2+}-selective microelectrodes were considerably improved by breaking the tips to 1–2 μm. The neutral carrier Ca^{2+}-selective microelectrodes have excellent selectivity for Ca^{2+} over Mg^{2+}. The interference by Mg^{2+} in cells is almost negligible if cytosolic Ca^{2+} and Mg^{2+} activities are 10^{-7} and 10^{-3}–10^{-2} M, respectively.

To test whether Eq. 1 describes the Ca^{2+}-selective microelectrode potentials, the microelectrode potentials measured with the solutions of pCa 3–8 were compared with the potentials calculated by Eq. 1 using a k_{CaK} value. The solutions contained 150 mM K^+. The measured values agreed with the calculated values.

TABLE II
EFFECTS OF AGING ON SLOPE (S), k_{NaK}, AND RESISTANCE (R) OF A NEUTRAL CARRIER Na^+-SELECTIVE MICROELECTRODE

Aging	S	k_{Nak}	R
hours	*mV*		Ω
0.5	59.5	0.013	7.3×10^{10}
19	59.6	0.014	7.4×10^{10}
43	59.4	0.012	8.1×10^{10}
68	59.5	0.013	8.1×10^{10}
92	59.3	0.014	6.8×10^{10}
118	59.7	0.012	7.0×10^{10}

Intracellular Application of Ca^{2+}-selective Microelectrodes

Recently, cytosolic Ca^{2+} activities have been measured with neutral carrier Ca^{2+}-selective microelectrodes (Lee, 1981). In most measurements, the method using calibration curves was chosen. This is because the neutral carrier Ca^{2+}-selective microelectrodes often showed super-Nernstian slope, drift, and hysteresis in their potential responses. In the method, standard calibration solutions containing Ca^{2+} and interfering ions are prepared and a calibration curve for each microelectrode is constructed. A problem is the uncertainty of the apparent stability constant for the Ca-EGTA complex in preparation of the calibration solutions containing low Ca^{2+} concentrations (Fabiato and Fabiato, 1978; Lee, 1981). The Ca^{2+} concentrations in the solutions depend on the apparent stability constant chosen. In the present study, we have chosen the stability constants (K) of $2.51 \times 10^6\ M^{-1}$ (Allen and Blinks constant) and $4.90 \times 10^6\ M^{-1}$ (Schwarzenbach constant) that have been frequently used. Marban et al. (1980) used the stability constant of $2.63 \times 10^6\ M^{-1}$. Fig. 4 shows calibration curves constructed with the solutions made using the two stability constants in the range of pCa 8 to 5 and the solutions made by simple dilution in the range of pCa 5 to 3. The potentials were measured with a Ca^{2+}-selective microelectrode aged for ~4 h. The potential changes are nonlinear in the Ca^{2+} concentrations lower than 10^{-6} M. This is due to the interference by K^+ and Na^+ in the calibration solutions. At

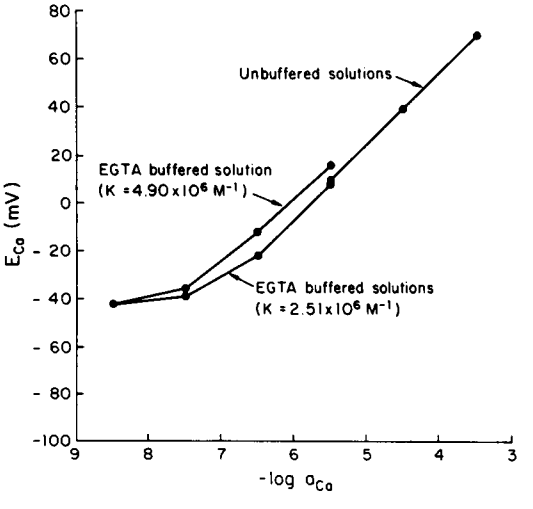

FIGURE 4 Calibration curves constructed for a Ca^{2+}-selective microelectrode using apparent stability constants (K) of $4.90 \times 10^6\ M^{-1}$ and $2.51 \times 10^6\ M^{-1}$ for Ca-EGTA complex at pH of 7.0. The solutions of pCa 7 and 8 contained 140 mM K^+ and 10 mM Na^+. The solutions of pCa 3, 4, 5, and 6 contained 150 mM K^+.

the pCa of 7, 6, and 5, the potentials measured with the solutions made using the two stability constants were different as expected. At the pCa of 8, the potentials were similar because of relatively large interference by the K^+. At the pCa of 5, the potential measured with the unbuffered solution is closer to the potential measured with the solution made using the value of $2.51 \times 10^6\ M^{-1}$ than to the potential measured with the solution made using the value of $4.90 \times 10^6\ M^{-1}$. This suggests that the apparent stability constant of $2.51 \times 10^6\ M^{-1}$ is more likely to be closer to the true value. As shown in Fig. 4, two calibration curves were constructed using different stability constants and intracellular Ca^{2+} activities (a^i_{Ca}) were determined (Lee and Dagostino, 1981). The a^i_{Ca} value determined using $2.51 \times 10^6\ M^{-1}$ was about twice the value determined using $4.90 \times 10^6\ M^{-1}$. This reflects the difference of the two stability constants, indicating that the a^i_{Ca} value determined using a stability constant can be converted to that corresponding to another stability constant.

DISCUSSION

Selectivity coefficients (k_{NaK}) of neutral carrier Na^+-selective microelectrodes have been determined by various methods at different levels of primary and interfering ion activities (Table I). It was observed that the k_{NaK} values do not depend significantly on the methods used and the ionic activity levels in the physiological ranges. The k_{NaK} values obtained in the study are similar to the k_{NaK} of 0.02 (log $k_{NaK} = -1.7$) reported by Steiner et al. (1979) and those (mean $k_{NaK} = 0.008$; k_{NaK} range of 0.005 to 0.01) reported initially by O'Doherty et al. (1979). However, the k_{NaK} values in the present study are substantially lower than the k_{NaK} of 0.038 (0.071 for unshielded microelectrodes) reported by Lewis and Wills (1980) and those (k_{NaK} range of 0.031 to 0.055) reported by Garcia-Diaz and Armstrong (1980). It should be pointed out that the Na^+-selective liquids used by Lewis and Willis (1980) and Garcia-Diaz and Armstrong (1980) were the mixtures of K^+-selective exchanger resin and neutral carrier Na^+-selective resin. Eaton (1981) reported that the selectivity coefficients (k_{NaK} range of 0.04 to 0.06) of Na^+-selective microelectrodes made with the monensin exchanger were not significantly different from those of Na^+-selective microelectrodes made neutral carrier Na^+-selective resin. It is apparent that the reported k_{NaK} values vary from one investigator to another and the k_{NaK} values differ somewhat between individual microelectrodes. Such variations of selectivity coefficients may be also true in Ca^{2+}-selective microelectrodes. Although the reason for such variations is not completely clear, there are a few possible explanations. First, poor silanization of the glass surface has been suggested (Armstrong and Garcia-Diaz, 1980). Second, an electrical shunt through the glass wall may affect ion-selective microelectrode potentials (Lewis and Wills, 1980; Armstrong and Garcia-Diaz, 1980; Baumgarten, 1981). Third, poor contact (phase boundary) between test solution and ion-selective resin at the end of the tip is also possible. These possibilities are examined with a schematic diagram of the tip of ion-selective microelectrodes as shown in Fig. 5.

Poor silanization produces a shunt resistance (R_s in Fig. 5), attenuating the potential E_L of an ion-selective microelectrode. This attenuation depends on the relative magnitude of R_s to the total resistance of an ion-selective microelectrode. A long column of ion-exchanger liquid may reduce the possibility of such a shunt resistance. The poor silanization is a technical problem.

The shunt resistance (R_g in Fig. 5) through glass wall attenuates the potential of E_L of an ion-selective microelectrode, depending on its relative magnitude to the total microelectrode resistance. The resistance of the glass wall of a micropipette was estimated to be $\sim 10^{13}$ ohms (Lewis and Wills, 1980; Lee, 1981). This value is at least ~100 times greater than the resistances (10^{10}–10^{11} ohms) of ion-selective microelectrodes. This indicates no significant attenuation of the potentials of ion-selective microelectrodes. However, it was postulated that glass hydration may cause a reduction of the resistance through the glass wall (Lewis and Wills, 1980; Armstrong and Garcia-Diaz, 1980; Baumgarten, 1981). The resistances of glass wall of the microelectrodes have not been directly measured during hydration except those of microelectrodes made from Eisenman and co-workers (Eisenman et al., 1957; Eisenman, 1962) $NAS_{27\text{-}4}$ and $NAS_{11\text{-}18}$ glass (Lee and Fozzard, 1974; Lee, 1979). However, Eisenman (1969) has demonstrated the existence of different thicknesses of hydrated layers in $NAS_{27\text{-}4}$ and $NAS_{11\text{-}18}$ glasses. He concludes that the $NAS_{11\text{-}18}$ glasses hydrate for >10 Å whereas the

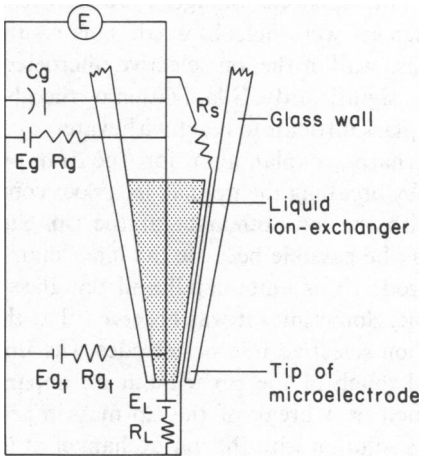

FIGURE 5 Schematic diagram of a portion of ion-selective microelectrode and equivalent circuit. C_g represents distributed capacitance across microelectrode glass wall. R_s is shunt resistance along glass surface of poorly silanized microelectrodes. Electrical potential and resistance across the glass wall are represented by E_g and R_g for the shank and E_{gt} and R_{gt} near the tip. E_L and R_L are potential and resistance across ion-selective liquid column.

NAS_{27-4} glasses hydrate uniformly with time up to depths exceeding 100,000 Å. Although NAS_{27-4} glass is subject to hydration, it took about one month for about a tenfold decrease in the resistance of the glass microelectrodes with sealed tips. The resistance of the microelectrodes made with NAS_{11-18} glass increased with aging in electrolyte solutions (Lee, 1979). Therefore hydration of glass depends on its composition, and so hydration of chemically durable glasses such as Corning 7740 may be very slow. Fig. 5 shows two lumped resistances (R_{gt} and R_g) through a glass wall of an ion-selective microelectrode. R_{gt} and R_g represent the resistance of the glass wall within 1–2 μm from the tip and that of the rest of glass wall, respectively. The glass wall is assumed to have no hydration layer initially because the micropipettes are pulled by heat and heated at a high temperature (~200°C) during silanization. It is unlikely that R_g decreases significantly by hydration within several hours, and this affects the microelectrode potential (E_L). This is because the glass wall is relatively thick. If hydration occurs, it starts from the tip because of the thinner glass wall. Hydration of the glass wall near the tip is often supported by an improvement of selectivity when the tip of an ion-selective microelectrode is broken by ~1 μm or less. Such an improvement of selectivity by breaking the tip was observed in this study. Walker (1976) has shown an increase in selectivity of K^+-selective microelectrodes when the tips are broken to ~2 μm diameter. However, even if the glass wall near the tip is hydrated, the resistance of R_{gt} may be high because of the small surface area of the glass wall near the tip (Lewis and Wills, 1980). In the present study, possible hydration of the glass wall has been tested by measuring resistance and selectivity coefficient of Na^+-selective microelectrodes immersed in an electrolyte solution for ~10 h and in air for ~1 wk (Table II). Any apparent changes in resistance and selectivity coefficients were not observed. This result indicates that the glass wall of the ion-selective microelectrodes did not hydrate significantly. Silanization of the glass surface makes the glass difficult to react with water.

An alternative explanation for the improvement of selectivity by breaking the tip may be a poor contact of test solution with the ion-exchanger at the tip. Such a poor contact may be possible because the inner diameter of the microelectrode tip is quite small and the glass surface is hydrophobic. Sometimes it was observed that the selectivity of the ion-selective microelectrodes was improved by mechanical touch of the tip without an apparent break. Such a touch or a break of the tip may improve a poor contact of a solution with the ion-exchanger at the tip.

Selectivity coefficients (k_{NaK}) of neutral carrier Na^+-selective microelectrodes are comparable to those of recessed-tip Na^+-selective glass microelectrodes (Lee, 1979) and the microelectrode potentials are quite stable. The response time of neutral carrier Na^+-selective microelectrodes are faster than those of recessed-tip Na^+-selective glass microelectrodes. Furthermore, the construction of neutral carrier Na^+-selective microelectrodes is much easier. These offer wide application of the microelectrodes for measuring intracellular Na ion activities. However, neutral carrier Ca^{2+}-selective microelectrodes have considerable problems, although they can be used for measuring cytosolic Ca ion activities. Recently Tsien and Rink (1980) and Rink and Tsien (1980) reported that the improved Ca^{2+}-selective microelectrodes made with a modified Ca^{2+}-selective liquid had quite stable potentials. Such improvements are needed for convenient and wide use of the Ca^{2+}-selective microelectrodes.

Finally, it should be mentioned that the Na^+- and Ca^{2+}-selective microelectrodes may be interferred with by certain anions and drug molecules. This has not been investigated in the present study. However, Dani et al. (1982) have reported that the Ca^{2+}-selective liquid membrane electrode (Orion Research Inc., Cambridge, MA) was interfered with by lyotropic anions. Therefore, possible interferences by anions and drugs for the cation-selective microelectrodes should be examined in certain relevant experiments.

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REFERENCES

Alvarez-Leefman, F. J., T. J. Rink, and R. Y. Tsien. 1981. Free calcium ions in neurones of Helix aspersa measured with ion-selective microelectrodes. *J. Physiol. (Lond.)*. 315:531–548.

Ammann, D., M. Güggi, E. Pretsch, and W. Simon. 1975. Improved calcium ion-selective electrode based on a neutral carrier. *Anal. Lett.* 8:709–720.

Ammann, D., E. Pretsch, and W. Simon. 1974. A sodium ion-selective electrode based on a neutral carrier. *Anal. Lett.* 7:23–32.

Armstrong, W. M., and J. F. Garcia-Diaz. 1980. Ion-selective microelectrodes: theory and technique. *Fed. Proc.* 39:2851–2859.

Baumgarten, C. M. 1981. An improved liquid ion exchanger for chloride ion-selective microelectrodes. *Am. J. Physiol.* 241:C258–C263.

Dani, J., J. Sanchez, and B. Hille. 1982. Lyotropic anions affect a Ca-selective electrode. *Biophys. J. (Abstr.)*. 37:321*a*.

Eaton, D. C. 1981. Intracellular sodium activity and sodium transport in rabbit urinary bladder. *J. Physiol. (Lond.)*. 316:527–544.

Edelman, A., S. Curci, I. Samarzija, and E. Fromter. 1978. Determination of intracellular K activity in rat kidney proximal tubular cells. *Pflugers Arch. Eur. J. Physiol.* 378:37–45.

Eisenman, G. 1962. Cation selective glass electrodes and their mode for operation. *Biophys. J. (Suppl.)*. 2:259–323.

Eisenman, G. 1969. The ion exchange characteristics of the hydrated surface Na^+ selective glass electrodes. *In* Glass Microelectrodes. M. Lavallee, O. F. Schanne, and N. C. Hebert, editors. John Wiley & Sons, Inc., New York. 32–61.

Eisenman, G., D. O. Rudin, and J. U. Casby. 1957. Glass electrode for measuring for sodium ion. *Science (Wash. D.C.)* 126:831–834.

Ellis, D. 1977. The effects of external cations and ouabain on the intracellular sodium activity of sheep heart Purkinje fibers. *J. Physiol. (Lond.)*. 273:211–240.

Fabiato, A., and F. Fabiato. 1978. Effect of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscle. *J. Physiol. (Lond.).* 276:233–255.

Garcia-Diaz, J. F., and W. M. Armstrong. 1980. The steady-state relationship between sodium and chloride transmembrane electrochemical potential differences in Necturus gallbladder. *J. Membr. Biol.* 55:213–222.

Lee, C. O. 1979. Electrochemical properties of Na^+- and K^+-selective glass microelectrodes. *Biophys. J.* 27:209–220.

Lee, C. O. 1981. Ionic activities in cardiac muscle cells and application of ion-selective microelectrodes. *Am. J. Physiol.,* 241:H459–H478.

Lee, C. O., and M. R. Dagostino. 1981. Sarcoplasmic Ca ion activity in canine cardiac Purkinje fibers measured with Ca^{2+}-selective microelectrodes. *Biophys. J. (Abstr.)* 33:284.

Lee, C. O., and H. A. Fozzard. 1974. Electrochemical properties of hydrated cation-selective glass membrane: a model of K^+ and Na^+ transport. *Biophys. J.* 14:46–68.

Lee, C. O., A. Taylor, and E. E. Windhager. 1980*a*. Cytosolic calcium ion activity in epithelial cells of Necturus kidney. *Nature (Lond.).* 287:859–861.

Lee, C. O., D. Y. Uhm, and K. Dresdner. 1980*b*. Sodium-Calcium exchange in rabbit heart muscle cells: direct measurement of sarcoplasmic Ca activity. *Science (Wash. D.C.)* 209:699–701.

Lewis, S. A., and N. K. Wills. 1980. Resistive artifacts in liquid-ion exchanger microelectrodes estimates of Na^+ activity in epithelial cells. *Biophys. J.* 31:127–138.

Lux, H. D., G. Hofmeier, and J. B. Aldenhoff. 1981. Intracellular free calcium affects electrical membrane properties. A study with calcium-selective microelectrodes and with Arsenazo III in Helix neurons. *In* Ion-selective Microelectrodes and Their Use in Excitable Tissues. E. Sykova, P. Hnik, and L. Vyklicky, editors. Plenum Publishing Corp., New York. 99–117.

Marban, E., T. J. Rink, R. W. Tsien, and R. Y. Tsien. 1980. Free calcium in heart muscle at rest and during contraction measured with Ca-selective microelectrodes. *Nature (Lond.).* 286:845–850.

Moody, G. J., and J. D. R. Thomas. 1971. Selective Ion Sensitive Electrodes. Merrow Publishing Co. Ltd., Watford, U.K. 4–32.

O'Doherty, J., J. F. Garcia-Diaz, and W. M. Armstrong. 1979. Sodium-selective liquid ion-exchange microelectrodes for intracellular measurements. *Science (Wash. D.C.).* 203:1349–1351.

O'Doherty, J., S. J. Youmans, W. M. Armstrong, and R. J. Stark. 1980. Calcium regulation during stimulus-secretion coupling: continuous measurement of intracellular calcium activities. *Science (Wash. D.C.).* 209:510–513.

Rink, T. J., and R. Y. Tsien. 1980. Calcium-selective microelectrodes with bevelled, sub-micron tips containing poly(vinylchloride)-gelled neutral-ligand sensor. *J. Physiol. (Lond.) (Abstr.).* 308:5P.

Rink, T. J., R. Y. Tsien, and A. E. Warner. 1980. Free calcium in Xenopus embryos measured with ion-selective microelectrodes. *Nature (Lond.).* 283:658–660.

Robinson, R. A., and R. H. Stokes. 1965. Electrolyte solutions. Butterworth & Co. (Publishers) Ltd., London. 223–252.

Sheu, S-S., and H. A. Fozzard. 1981. The stoichiometry of Na/Ca exchange in the mammalian myocardium. Biophys. J. 33:11*a*.

Simon, W., D. Ammann, and W. E. Morf. 1978. Calcium-selective electrodes. *Ann. N.Y. Acad. Sci.* 307:52–70.

Steiner, R. A., M. Oehme, D. Ammann, and W. Simon. 1979. Neutral carrier sodium- ion-selective microelectrode for intracellular studies. *Anal. Chem.* 51:351–353.

Thomas, R. C. 1976. Construction and properties of recessed-tip microelectrodes for sodium and chloride ions and pH. *In* Ion and Enzyme Electrodes in Biology and Medicine. M. Kessler, L. C. Clark, D. W. Lubbers, I. A. Silver, and W. Simon, editors. Urban and Schwarzenberg, Munich. 141–148.

Thomas, R. C. 1978. Ion-Sensitive Intracellular Micro-Electrodes. Academic Press, Inc., London. 89–94.

Tsien, R. Y., and T. J. Rink. 1980. Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. *Biochim. Biophys. Acta.* 599:623–638.

Walker, J. L. 1976. Ion-selective liquid ion exchanger microelectrodes. *In* Ion and Enzyme Electrodes in Biology and Medicine. M. Kessler, L. C. Clark, E. W. Lubbers, I. A. Silver, and W. Simon, editors. Urban and Schwarzenberg, Munich. 116–118.