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CHARACTERIZATION OF $^{42}K^+$ AND $^{86}Rb^+$ TRANSPORT AND ELECTRICAL MEMBRANE PROPERTIES IN EXPONENTIALLY GROWING NEUROBLASTOMA CELLS

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Summary

For measuring K^+ efflux from exponentially growing neuroblastoma cells (clone Neuro-2A), two methods were used, a sampling method and a washing method. Both methods indicated that K^+ efflux kinetics were as from a two-compartment system, but the two compartments could only be resolved completely using the washing method. A fast compartment, containing 143 ± 16 nmol $K^+/10^6$ cells, was found to be associated to the cell surface, and a slow compartment, containing 151 ± 7 nmol $K^+/10^6$ cells, was found to represent the intracellular K^+. The rate constant of the slow compartment was 0.0164 ± 0.0005 min^{-1}, and the K^+ efflux rate was 2.46 ± 0.14 nmol $K^+/10^6$ cells per min. Using the appropriate conditions to measure K^+ influx, the kinetics of influx were equal to the kinetics of efflux, indicating steady-state conditions.

In addition a comparison was made between $^{42}K^+$ and $^{86}Rb^+$ as radioactive tracers for K^+ flux. It was found that $^{86}Rb^+$ was specifically bound on both the inside and the outside of the cells, and for this reason was not a suitable tracer for studying K^+ flux kinetics in Neuro-2A cells.

A membrane potential of −42.9 ± 1.3 mV and intracellular K^+ activity of 108.1 ± 3.0 mM were measured using conventional and ion-selective microelectrodes. A correlation was made between the K^+ flux and electrophysiological data, using the equations of electrodiffusion theory. Thus, the permeabilities of K^+ and Na^+ were calculated as $(3.9 \pm 0.4) \cdot 10^{-8}$ cm/s and $(0.6 \pm 0.1) \cdot 10^{-8}$ cm/s respectively, together with K^+ conductance of $(2.8 \pm 0.3) \cdot 10^{-6}$ Ω^{-1}/cm^2.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

Introduction

Considerable evidence has accrued implicating a variety of plasma membrane properties in the control of cell growth [1—3]. Several findings have been particularly pertinent on the role of the plasma membrane in regulating proliferation of neuroblastoma cells [4,5].

The properties of the cation transport systems and related membrane potentials have been shown to be involved in the regulation of cell proliferation [6—11]. A particular role of the K^+ transport system in these processes has been suggested. Under conditions where a decrease in intracellular K^+ occurs, inhibition of cell proliferation is observed in many cell lines [12—15]. Furthermore, elevated K^+ transport and enhanced $(Na^+ + K^+)$-ATPase activity have been found for virally transformed cells as compared to their normal counterparts [16]. The role of cation transport and the $(Na^+ + K^+)$-ATPase in the regulation of cell proliferation has been demonstrated by the stimulation of growth by serum or growth factors in quiescent cells. In both cases an immediate rise in K^+ flux was observed [17—20].

In the study of K^+ transport in mammalian cells, $^{86}Rb^+$ is frequently used as an analogue for K^+ instead of $^{42}K^+$, in view of its longer half-life. Small differences in the behaviour of Rb^+ compared with K^+ may result in misleading results [21,22], so for each cell line studied, it should be established whether $^{86}Rb^+$ is indeed an analogue for K^+ in the cell. For this reason we have compared the use of $^{86}Rb^+$ and $^{42}K^+$ as radioactive tracers for determining K^+ flux in neuroblastoma cells. Further, the study of the role of cations in living cells requires an accurate method of determining net fluxes and of measuring the total cellular and intracellular contents of an ion [23,24]. We have developed such a method, involving rapid and frequent washing of cells and are now able to characterize compartmentalization and flux kinetics of K^+ in cells grown in monolayer. We have applied the method both in exponentially growing neuroblastoma cells and to synchronized cells during the cell cycle [25]. Furthermore, we describe the measurement of membrane potential and intracellular K^+ activity using conventional and ion-selective microelectrodes and the correlation made between the results obtained by tracer flux studies and electrophysiological studies.

Materials and Methods

Cell culture

C1300 mouse neuroblastoma cells, clone Neuro-2A, were obtained from the American Type Culture Collection, Rockville MD, U.S.A. The cells were grown in Dulbecco's modified Eagle's medium without bicarbonate, buffered with 25 mM Hepes at pH 7.5 and supplemented with 10% fetal calf serum (Flow), at 37°C in a humidified atmosphere.

For tracer flux experiments, cells were plated at a density of $1.5 \cdot 10^4$ cells/cm^2 in culture dishes (3.5 cm diameter, Costar, Cambridge MD, U.S.A.) and grown overnight to a final density of $4.5 \cdot 10^4$ cells/cm^2. The cell doubling time is approximately 10 h, 60—70% of the cells being in S phase during exponential growth [4]. For electrophysiological measurements, cells were plated on plastic

tissue culture cover slips (3.2 cm^2, Lux Scientific Corp., Thousand Oaks, CA, U.S.A.) and grown as described above.

Determination of membrane potential and intracellular K^+ activity

Electrophysiological measurements were made in growth medium or phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/6.5 mM $Na_2HPO_4 \cdot 2H_2O$/1.5 mM KH_2PO_4/0.9 mM $CaCl_2 \cdot 2H_2O$/0.5 mM $MgCl_2 \cdot 6H_2O$, pH 7.3) at room temperature on exponentially growing cells as described previously [25].

Efflux measurements

Sampling efflux method. Medium from cells grown in 3.5 cm diameter culture dishes was replaced by 1 ml growth medium at 37°C. The cells were labelled with ^{42}KCl (Interuniversity Reactor Institute, Delft, The Netherlands) or $^{86}RbCl$ (Radiochemical Centre, Amersham, U.K.), added to a final concentration of approx. 2—5 nM (spec. act. 1 Ci/mol K^+) and incubated for at least 3 h at 37°C. At the end of the incubation period, the radioactively labelled medium was removed, the cells washed five times in 1 ml phosphate-buffered saline at room temperature and, finally, 1 ml of phosphate-buffered saline or growth medium at room temperature added to the culture, the whole procedure being completed within 20 s. Samples of 0.05 ml were taken at various time intervals and assayed for radioactivity by measuring Cerenkov radiation in a liquid scintillation counter (Packard, Tricarb 2450). After the experiment, cells were lysed in ice-cold 10% trichloroacetic acid. The lysate was transferred to a scintillation vial and isotope content determined as described above. The initial intracellular content of the isotope at time zero was determined from the sum of the counts leaving the cells during the time of the experiment and those remaining at the end of the experiment obtained from the trichloroacetic acid lysate.

Washing efflux method. Cells were equilibrated with isotope as above. Labelled medium was removed from the cells and the cells washed with 1 ml phosphate-buffered saline at room temperature at various frequent intervals over a 10 min period, the washing fluid removed each time being reserved for counting. The radioactivity remaining in the cells at the end of the experiment was determined from the trichloroacetic acid lysate. In order to distinguish between radioactivity in the washing fluid due to efflux from the cells and due to that remaining after removal of the high specific activity incubation medium, a correction procedure was developed as described in Results section. A check for possible cell loss during the washing procedure was made by measuring the loss of protein-incorporated [^3H]alanine (New England Nuclear, Boston MA, U.S.A.; specific activity 5.5 mCi/mmol) during the procedure from cells previously incubated with the amino acid (1.8 μM final concentration) for 24 h.

Influx measurements

Medium from cells grown in 3.5 cm diameter culture dishes was replaced by 1 ml fresh growth medium at 37°C. At zero time a trace of ^{42}KCl was added as described for efflux measurements, and incubated at 37°C. At various time intervals the labelled medium was removed and the cells washed 5-times in 1 ml

phosphate-buffered saline (pH 7.3) at room temperature. Washing was completed within 20 s. The isotope content was determined from a trichloroacetic acid lysate as described above.

Determination of total K^+ content

Cells were grown in culture dishes (14 cm diameter, Sterilin, Teddington, U.K.) as described above, washed gently 5-times in 10 ml of ice-cold 0.3 M sorbitol. Cells were then lysed in 15 ml of distilled H_2O, the lysate lyophilized and subsequently resuspended in 1.0 ml H_2O. This dilute solution was analyzed for K^+ activity using the K^+-selective microelectrode, with an activity coefficient of 1.

Cell surface area

Two methods were used to estimate the cell surface area.

(1) Trypsinized cells were suspended in medium made progressively hypotonic by adding H_2O. The maximum diameter attained by the cells was measured using a calibrated eyepiece micrometer. The mean cell surface area was then estimated assuming the maximally swollen cell to be spherical.

(2) The majority of cells growing exponentially in monolayer are in S phase and are extremely flattened, with height of negligible dimensions relative to the cross-section parallel to the substratum. The surface area was estimated as twice that obtained by tracing around the periphery of these cells in projected time-lapse films, using a Wang digitizer connected to a Wang 2200 minicomputer.

Results

Membrane potential and intracellular K^+ activity

Electrophysiological measurements were made on cells selected on basis of size and shape to be predominantly in S phase. The membrane potential (E_m) of these cells was -42.9 ± 1.3 mV (mean ± S.E.; $N = 45$; range: -31 — -50 mV), and the intracellular K^+ activity (a_K^i) 108.1 ± 3.0 mM ($N = 57$; range: 85—130 mM).

In order to establish the accuracy of a_K^i measurements in these small cells, the a_K^i was calculated from the modified Goldman-Hodgkin-Katz [26] equation. With K^+ and Na^+ as the permeable ions and Cl^- in thermodynamic equilibrium with E_m, E_m can be described by:

$$E_m = \frac{RT}{F} \ln \frac{a_K^o + \alpha a_{Na}^o}{a_K^i + \alpha a_{Na}^i} \quad (1)$$

in which R, T and F have their usual meanings, α represents the permeability ratio of Na^+ to K^+ ($\alpha = P_{Na}/P_K$), a_K^o and a_{Na}^o represent the extracellular activities of K^+ and Na^+, respectively, and a_K^i and a_{Na}^i the corresponding intracellular activities. The a_K^o and a_{Na}^o were obtained from the external concentrations of the ions using an experimentally determined activity coefficient of 0.755.

Usually $\alpha a_{Na}^i << a_K^i$, so Eqn. 1 can be written as

$$e^{E_m F/RT} = \frac{(a_{Na}^o + a_K^o)}{a_K^i} + \frac{a_K^o(1-\alpha)}{a_K^i} \quad (2)$$

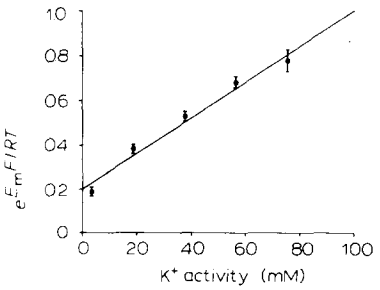


Fig. 1. Relationship between membrane potential and external K^+ activity in Neuro-2A cells. Relationship between $e^{E_m F/RT}$ and a_K^o; each point represents the mean of 11 measurements. The linear regression line was significant for $P < 0.001$.

This equation shows clearly that plotting $e^{E_m F/RT}$ versus a_K^o should yield a straight line under conditions of equimolar replacement of Na^+ by K^+. The regression line through the experimentally determined points was significant for $P < 0.001$, as shown in Fig. 1. The linearity of this relationship demonstrates the validity of applying Eqn. 2 to E_m measurements in Neuro-2A cells. α and a_K^i were then calculated from the slope of this line and at the point $E_m = 0$, i.e. $a_K^o = 98$ mM, yielding values of 0.18 and 101 mM, respectively. This calculated value of a_K^i obtained using independent experimental data is, within error, equal to that obtained directly from the K^+-selective microelectrode. The α calculated above was also close to α calculated from Eqn. 1 using the measured data, being 0.15.

K^+ efflux measurements

The K^+ gradient established across the plasma membrane is determined by the membrane permeability properties and the activity of K^+ translocation systems, such as the $(Na^+ + K^+)$ATPase. Due to the asymmetric K^+ distribution, internal K^+ being high relative to external K^+, K^+ efflux measurements provide a simple method for obtaining data to calculate K^+ permeability.

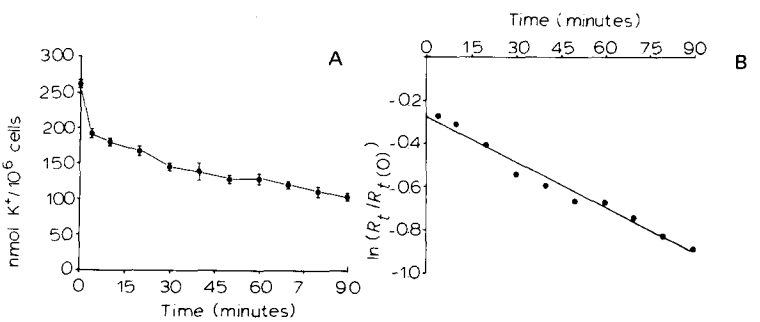


Fig. 2. K^+ efflux from exponentially growing Neuro-2A cells. A: K^+ efflux from with $^{42}K^+$ preloaded cells as measured by the sampling method (for details see Materials and Methods). The efflux was into growth medium at room temperature at a cell density of 45 000 cells/cm^2. The data are presented as mean ± S.E., $N = 6$. B: Plot to obtain the unidirectional K^+ efflux rate from data of Fig. 2A. The slope of the linear regression line represents the rate constant k, equal to 0.0071 ± 0.0004 min^{-1} ($N = 10$). The regression line was significant for $P < 0.001$. See also text.

K^+ efflux from preloaded, exponentially growing cells using $^{42}K^+$ as a radioactive tracer and the sampling method described in Materials and Methods is shown in Fig. 2A. The total K^+ content of the cells was calculated as 261 ± 5 nmol $K^+/10^6$ cells (N = 5). K^+ efflux can usually be considered as from a one-compartment system [27,28] under steady-state conditions, and thus is described by:

$$R_t/R_{t(0)} = e^{-kt} \quad (3)$$

where R_t and $R_{t(0)}$ represent the counts in the cells at times t and zero, respectively, and k the rate constant for efflux (min^{-1}). Plotting ln ($R_t/R_{t(0)}$) against time should give a straight line with slope k. The data of Fig. 2A are thus plotted in Fig. 2B. A straight line is obtained which does not, however, pass through the origin, suggesting a second compartment for K^+, from which efflux to the medium is very rapid. Efflux experiments using shorter time intervals between samples did not resolve the fast efflux compartment in more detail (unpublished results). Therefore, a method was developed for studying K^+ efflux as described in Materials and Methods, the so-called washing method. This involved rapid and repeated rinsing of the cells, the efflux from the cells being determined from the radioactivity present in the consecutive wash-fractions. The radioactivity in these fractions had to be corrected for: (a) the remainder of the initial radioactivity and (b) possible cell loss. Correction for (a) was made by adding a high concentration of gramicidin (15 μM) to the cells at 37°C at least 15 min prior to efflux measurement. Gramicidin increases the membrane permeability to various ions, including K^+ and Na^+ [29], thus the intra- and extracellular K^+ concentrations become equal in its presence. Fig. 3 shows the amount of radioactivity, expressed as nmol K^+/wash, measured in consecutive washes from cells in the presence of gramicidin. That this curve indeed represents removal of the remainder of the initial radioactivity was con-

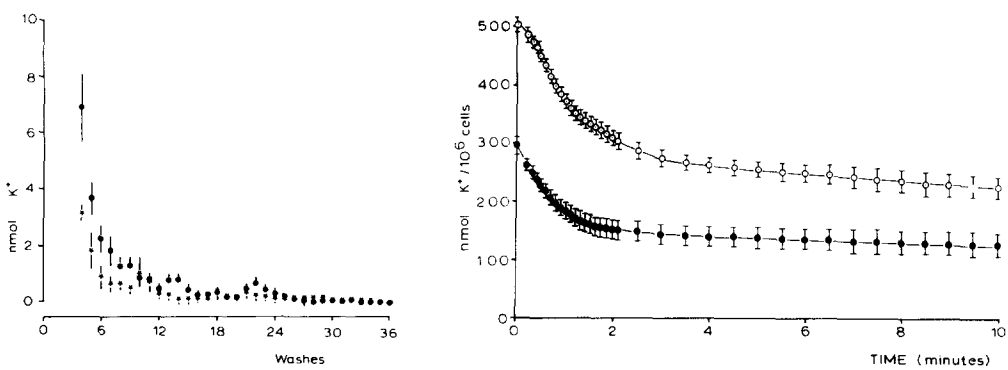


Fig. 3. Removal of extracellular radioactivity. •, carry-over of ^{42}K from gramicidin-treated cells (N = 13). Cells were treated with 15 μM gramicidin as described in the text; ★, carry-over of [^{3}H]inulin (Radiochemical Centre, Amersham, U.K.; spec. act., 530 Ci/mol) used as an extracellular marker from untreated cells (N = 3). Each point represents one wash.

Fig. 4. K^+ efflux from exponentially growing Neuro-2A cells. $^{42}K^+$ (•) and $^{86}Rb^+$ (○) efflux from preloaded cells measured by the washing method as described in Materials and Methods. The radioactivity in the washes was corrected for initial extracellular activity using the data presented in Fig. 3. Each point represents one wash. Data are presented as mean ± S.E. (N = 6 for $^{42}K^+$; N = 13 for $^{86}Rb^+$).

firmed by experiments in which inulin was used as an extracellular marker (Fig. 3) or in which K^+ carry-over was determined from dishes without cells (unpublished data). In all cases the curves obtained were not significantly different.

In order to determine whether repeated washes during the experiment caused cell loss, cells were labelled with [^{3}H]alanine for 24 h. The subsequent washes contained the same fraction of the initial amount of extracellular amino acid as was found for ^{42}K (Fig. 3), indicating that no significant cell loss occurred during the experiment. This observation was supported by counting the cells after various numbers of washes (data not shown).

K^+ efflux from preloaded cells using the washing method is shown in Fig. 4, with both $^{42}K^+$ and $^{86}Rb^+$ as tracers, and corrected for initial extracellular radioactivity according to Fig. 3. There is a clear difference between curves obtained using $^{42}K^+$ and $^{86}Rb^+$ as tracer; with ^{42}K the total K^+ content of the cells was determined to be 294 ± 15 nmol $K^+/10^6$ cells ($N = 6$), whereas with $^{86}Rb^+$ it was 505 ± 11 nmol $K^+/10^6$ cells. The total K^+ content was also determined by washing the cells very gently in isotonic sorbitol with subsequent lysis in H_2O, as described in Materials and Methods. The K^+ content was measured to be 276 ± 9 nmol $K^+/10^6$ cells ($N = 9$), which is in a good agreement with the results presented above using $^{42}K^+$ as radioactive tracer. This suggests a specific association of Rb^+ with the cells compared with K^+, and that $^{86}Rb^+$ does not behave as an analogue for K^+ in neuroblastoma cells. Plotting the data of Fig. 4 as ln $(R_t/R_{t(0)})$ versus time results in a curve resolvable into two components, suggesting a system of two compartments (Fig. 5). The compartment from which efflux is slow will from now on be referred to as the slow compartment and the fast compartment is similarly defined. Since the efflux rates from the compartments are sufficiently different, the contents of the compartments can be obtained from extrapolation of the line representing the slow compartment to $t = 0$ [30]. This represents the K^+ content of the slow compartment, while the difference between the total content and the content of the slow compartment gives the K^+ content of the fast compartment. The slopes of the lines represent the rate constants for efflux. A line obtained by regression analysis on the points, from 4 min onwards, was considered to characterize the slow compartment. The slope of this line yielded the rate constant k as 0.0165 ± 0.0005 min^{-1} ($N = 13$) using $^{42}K^+$ and 0.0272 ± 0.0005 min^{-1} ($N = 13$) using $^{86}Rb^+$ as a tracer (lines significant for $P < 0.001$). The contents of the slow compartment were 151 ± 7 and 291 ± 6 nmol $K^+/10^6$ cells with $^{42}K^+$ and $^{86}Rb^+$, respectively.

Increasing the extracellular K^+ concentration to 16.4 mM resulted in an increase of the total K^+ content to 399 ± 38 nmol $K^+/10^6$ cells and a further increase was observed upon an increase of extracellular K^+ concentration to 27.4 mM, while no increase was observed of a_K^i (Table I). At both concentrations the content of the slow compartment was virtually unchanged at 150 nmol $K^+/10^6$ cells (Table I). These results demonstrate that the slow compartment represents the intracellular K^+ and that the fast compartment probably represents extracellularly associated K^+, as has similarly been demonstrated for other cell types [24,31]. These results clearly demonstrate that only the washing method leads to accurate determination of the parameters of K^+ efflux.

TABLE I

DEPENDENCE OF K^+ CONTENT AND INTRACELLULAR K^+ ACTIVITY ON EXTERNAL K^+ CONCENTRATION IN NEURO-2A CELLS

K^+ content was calculated from efflux experiments using the washing method as described in the text, and intracellular K^+ activity using a K^+-selective microelectrode as described in Materials and Methods. Data presented as mean ± S.E. The number of observations is shown in parentheses. n.d., not determined.

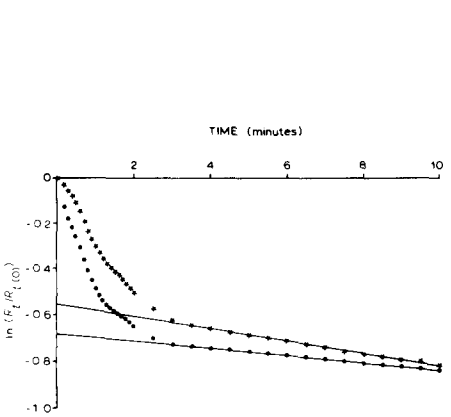
Extracellular K^+ concentration (mM)	K^+ content (nmol $K^+/10^6$ cells)		a^i_K (mM)
	Total	Slow compartment	
5.4	294 ± 15 (6)	151 ± 7 (6)	108.1 ± 3.9 (57)
16.4	399 ± 38 (3)	143 ± 3 (3)	n.d.
27.4	450 ± 15 (3)	150 ± 8 (3)	109.3 ± 4.7 (19)

K^+ influx measurements

The finding that a large fraction of K^+ is associated with the cell surface is, inevitably, important for the interpretation of K^+ influx data. A curve for K^+ influx with time, washing the cells routinely 5 times in phosphate-buffered saline, is shown in Fig. 6, using $^{42}K^+$ as tracer. From the rate constant and intracellular K^+ content obtained from the efflux studies (Fig. 5), a prediction could be made for the K^+ influx in the cells, according to

$$R_t = R_\infty (1 - e^{-kt}) \quad (4)$$

In which R_∞ represents the intracellular radioactivity at equilibrium of the slow compartment (Fig. 6). There is clearly a large difference between the experi-



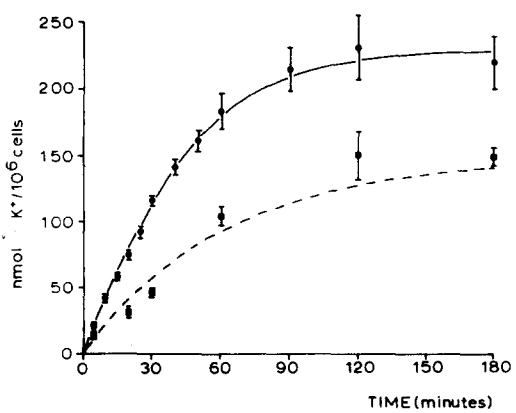


Fig. 5. Determination of the unidirectional K^+ efflux rate. Data of Fig. 4 were plotted according to Eqn. 3. The slope of the regression line through the points taken after 4 min was considered as the rate constant of the slow compartment of efflux. Using $^{42}K^+$ (•), $k = 0.0164 \pm 0.005$ min^{-1}, while using $^{86}Rb^+$ (⋆), $k = 0.0272 \pm 0.0005$ min^{-1}. Both lines were significant for $P < 0.001$.

Fig. 6. K^+ influx in exponentially growing Neuro-2A cells. K^+ influx was estimated using $^{42}K^+$ as a radioactive tracer as described in Materials and Methods, at a cell density of 45 000 cells/cm^2. Data presented as mean ± S.E. ($N = 6$) (•). The dotted line represents a hypothetical influx curve according to $R_t = R_\infty (1 - e^{-kt})$ and using a rate constant and K^+ content from K^+ efflux studies from the washing method, as described in the text. ■, K^+ influx estimated from a plot of $\ln(R_t/R_{t(0)})$ versus time, using the washing method after various labelling times.

mental influx curve and the predicted influx. Since it was shown that five washes were not sufficient to remove the K^+ from the fast compartment completely (Fig. 5), the difference between experimental and predicted influx is due to a fraction of K^+ remaining in the fast compartment which can only be entirely removed by increasing the number of washes. Therefore, the K^+ uptake was estimated from logarithmically transformed K^+ efflux curves, obtained after various labelling times, in the same way as for Fig. 5. The K^+ uptake was then very close to the predicted influx, indicating again that the real influx could only be determined from data if the cells were washed sufficiently to remove the extracellularly associated K^+.

K^+ and Na^+ permeability

Using the data presented above, the K^+ and Na^+ permeability may be calculated using the equations of the conventional electrodiffusion theory [26], provided that the cell surface area and intracellular H_2O volume are known. The cell surface area was 13.42 ± 1.66 cm^2/10^6 cells ($N = 32$), using the methods described in Materials and Methods.

Due to the lack of reliable alternative methods, the intracellular H_2O volume was calculated from the intracellular K^+ content and a_K^i, using an experimentally determined intracellular activity coefficient of 0.52 [25]. The value thus obtained was 0.74 μl/10^6 cells.

The unidirectional efflux rate (J_{eff}) may be calculated according to:

$$J_{eff} = C_i \cdot V/S \cdot k \tag{5}$$

in which C_i is the intracellular K^+ concentration (pmol K^+/cm^3), J_{eff} the unidirectional efflux rate (pmol K^+/cm^2 per s), V the intracellular H_2O volume (cm^3/10^6 cells), S the cell surface area (cm^2/10^6 cells) and k the rate constant (sec^{-1}). J_{eff} was thus calculated as 3.04 ± 0.40 pmol K^+/cm^2 per s.

The K^+ permeability is given by

$$P_K = \frac{J_{eff}}{C_i} \frac{1 - e^{-E_m F/RT}}{E_m F/RT} \tag{6}$$

in which P_K is the K^+ permeability (cm/s). For exponentially growing cells P_K was $(3.9 \pm 0.4) \cdot 10^{-8}$ cm/s. Using the value of α of 0.152, the P_{Na} was estimated to be $(0.6 \pm 0.1) \cdot 10^{-8}$ cm/s. The K^+ conductance can then be calculated according to:

$$G_K = P_K \frac{F^2}{RT} \frac{E_m}{E_m - E_K} \frac{a_K^i e^{E_m F/RT} - a_K^o}{e^{E_m F/RT} - 1} \tag{7}$$

in which G_K is the K^+ conductance (Ω^{-1}/cm^2) and E_K is the K^+ potential, obtained from the Nernst equation:

$$E_K = \frac{RT}{F} \ln(a_K^o/a_K^i) \tag{8}$$

E_K was -89.4 ± 2.4 mV, hence G_K was $(2.8 \pm 0.3) \cdot 10^{-6}$ Ω^{-1}/cm^2. The K^+ resistance ($1/G_K$) was thus 0.36 ± 0.04 M$\Omega \cdot$ cm^2. The total membrane resistance was measured as described previously [25] and found to be 7.2 ± 0.8 MΩ. From these data the specific resistance was calculated to be 96.6 ± 16.1 $\Omega \cdot$

cm^2. These results clearly indicate that the K^+ resistance is much higher than the total specific resistance, and thus K^+ contributes to only a small extent to the total membrane conductance.

Discussion

In connection with studies of the properties of the plasma membrane in the regulation of growth in neuroblastoma cells [4,5] we have measured the K^+ distribution and membrane potential in the cell cycle of neuroblastoma cells [25]. For interpretation of such results, a knowledge of the K^+ distribution and of possible K^+ binding is pertinent. For this reason we have characterized the kinetics of K^+ transport in exponentially growing cells.

Measurements of K^+ efflux using a sampling method indicated that this flux in Neuro-2A cells could not be considered as being from one compartment under steady-state conditions since plotting ln $(R_t/R_{t(0)})$ against time resulted in a straight line which did not pass through the origin (Fig. 2B). The results indicated the presence of a compartment from which efflux is rapid, within the first minutes of the assay. The two compartments could be more clearly resolved using a washing method which involved rapid rinses of the cells and a correction for radioactivity remaining after removal of the labelled incubation medium, as shown in Fig. 3. This method requires that the cells are firmly attached to the substratum to avoid cell loss and our system of Neuro-2A cells was demonstrated as suitable. The fast compartment of efflux was shown to be associated with the cell surface, while the slow compartment represented the intracellular K^+, as inferred from experiments where increased extracellular K^+ concentrations resulted in an increase in the K^+ content of the fast compartment but not of the slow compartment, the intracellular K^+ activity remaining constant.

The finding of relatively high amounts of K^+ associated with the cell surface has also been observed in other cell lines. Recently, binding of Na^+, K^+, Mg^{2+} and Ca^{2+} to the cell surface of chicken embryo fibroblasts was described using a method similar to the washing method described in this paper [24], the amount of K^+ binding being similar to the amount found in Neuro-2A cells. However, in contrast to these cells, in Neuro-2A cells the externally bound K^+ could not be displaced by protons. Cellular K^+ concentrations of about 300 mM have been measured is SV40-3T3 cells [15], which is comparable with the sum of the amounts of K^+ in the fast and slow compartments in our cells.

Using the efflux rate constant k and the intracellular K^+ content we calculated a hypothetical influx curve (Fig. 6) but found that experimental data obtained for K^+ influx with time was not identical with this curve when only five washes were used. The difference is due to insufficient removal of the K^+ from the fast compartment after different incubation times. In most circumstances a curve such as that given in Fig. 4 would indicate the number of washes necessary for accurate influx measurements and for correct interpretation of results. With increased washing, a close fit was found between the predicted curve and the experimental influx data. These results clearly show the necessity of characterizing all aspects of K^+ flux, since pulse labelling techniques are frequently used to measure cellular response in terms of ion flux changes induced by external factors.

In many studies of K^+ transport through the plasma membrane $^{86}Rb^+$ is used as a radioactive analogue for K^+ in view of its relatively long half-life and has indeed been reported to behave more like K^+ than the other alkali ions [17,32, 33]. However, some studies have indicated a selective uptake or accumulation of Rb^+ over K^+ in various organs and cell lines [21,22]. In cultured glioma and neuroblastoma cells $^{86}Rb^+$ resembles $^{42}K^+$ in its ouabain-sensitive uptake [13]. Our results clearly demonstrate a difference between the use of $^{86}Rb^+$ and $^{42}K^+$ in determining K^+ content of the cells. Using $^{86}Rb^+$, a higher content was calculated in both the fast and slow compartments, indicating specific intra- and extracellular binding of Rb^+. In addition, the K^+ influx rate was different using data obtained with $^{86}Rb^+$ compared with $^{42}K^+$ and indicated that Rb^+ is accumulated selectively by the cells. The apparent difference in total cell K^+ of 184 nmol $K^+/10^6$ cells between using $^{86}Rb^+$ and $^{42}K^+$, however, actually represents only 0.08 pmol/10^6 cells of specifically bound Rb^+. Therefore, only a very small amount of specific binding can produce anomalous results in K^+ flux kinetics if $^{86}Rb^+$ is used as an analogue for K^+.

The K^+ flux studies were correlated with measurements of membrane potential, intracellular K^+ activity and membrane resistance. Correlation required an estimate of the cell surface area and of the intracellular H_2O volume. Unfortunately, technical difficulties prevented reliable determination of the intracellular H_2O volume of cultured cells in monolayer despite using various combinations of fully permeable and non-permeable compounds (e.g. ^3H_2O, [^{14}C]urea with [^{14}C]inulin and [^{3}H]inulin, respectively), all of which appeared to bind to some extent. A frequently used method to measure the intracellular H_2O volume of cells grown in monolayer is to measure the diameter of trypsinized cells [15,34,35]. However, the intracellular volume obtained in this way is likely to be slightly overestimated since trypsinization of flattened cells results in an increase in the H_2O volume [36]. Therefore, we have calculated the intracellular H_2O volume from the intracellular K^+ content and activity, using an experimentally determined activity coefficient of 0.52 [25].

Since trypsinized cells have a considerably undulated membrane, calculation of the surface area from their diameter would give an underestimated value. We have, therefore, used the maximum diameter attained by cells suspended in hypotonic solution to calculate the surface area. The data obtained were similar to those obtained from scanning electron micrographs and photographs of cells in S phase on monolayer.

The membrane potential, E_m, and intracellular K^+ activity, a_K^i, were measured using conventional and ion-selective microelectrodes. The E_m value obtained is in agreement with measurements reported in other neuroblastoma cell lines [37—39] using microelectrodes and also with data using lipophilic cations [38,40]. The observation that a_K^i is not dependent on a_K^o (Fig. 1) is in agreement with the K^+ flux experiments in which no increase of the intracellular K^+ content was measured by increasing the extracellular K^+ concentration.

The K^+ conductance and K^+ permeability, using the equations of conventional electrodiffusion theory [26], have values which are low compared with reports from other authors [33]. These parameters are, however, inversely dependent on cell surface area which may be underestimated using the method of measuring the diameter of trypsinized cells. Furthermore, the E_m of these

cells is low compared with excitable cells, which indicates a lower P_K.

In summary, we have demonstrated that a washing-method is required to resolve K^+ flux kinetics, in order to correlate tracer flux studies with electrophysiological measurements, and, furthermore, the $^{86}Rb^+$ is not a suitable tracer to study K^+ fluxes in Neuro-2A cells.

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