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Membrane Regulation of the Na^+, K^+-ATPase During the Neuroblastoma Cell Cycle: Correlation With Protein Lateral Mobility

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The pumping activity of the plasma membrane-bound Na^+,K^+-ATPase shows considerable variation during the cell cycle of mouse neuroblastoma Neuro-2A cells. Addition of external ATP at millimolar concentrations, which selectively enhances the plasma membrane permeability of Neuro-2A cells for sodium ions, stimulates the Na^+,K^+-ATPase pumping activity at all phases of the cell cycle from a factor of 1.05 in mitosis up to 2.2 in G_1 phase. Determination of the number of Na^+,K^+-ATPase copies per cell by direct ^{3}H-ouabain binding studies in the presence of external ATP shows a gradual increase in the number of pump sites on passing from mitosis to the late S/G_2-phase by approximately a factor of 2. From these data the pumping activity per copy of Na^+,K^+-ATPase, optimally stimulated with respect to its various substrate ions, has been determined during the various phases of the cell cycle. This optimally stimulated pumping activity per enzyme copy, which is a reflection of the physicochemical state of the plasma membrane, is high in mitosis, almost twofold lower in early G_1 phase, and increases gradually again during the other phases of the cell cycle. This shows that the observed regulation of Na^+,K^+-ATPase activity during the cell cycle is caused by a combination of three independent factors—namely variation in intracellular substrate availability (Na^+), changes in number of enzyme copies per cell, and modulation of the plasma membrane environment of the protein molecules. The modulation of the optimal pumping activity per enzyme copy shows a good correlation ($\rho = 0.96$) with the known modulation of protein lateral mobility during the cell cycle, such that a high protein lateral mobility correlates with a low enzyme activity. It is concluded that changes in plasma membrane properties take place during the Neuro-2A cell cycle that result in changes in the rate of protein lateral diffusion and Na^+,K^+-ATPase activity in a directly correlated way.

Key words: Na^+,K^+-ATPase, cell cycle, protein lateral mobility, regulation, neuroblastoma cells, ouabain binding

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The Na^+,K^+-ATPase is the enzyme responsible for maintaining gradients of sodium and potassium ions across cellular plasma membranes [for recent reviews 1, 2]. A number of recent studies have revealed the relationship of this enzyme to processes of cellular transformation [3, 4], differentiation [5], and regulation of cellular growth [6–10]. In considering the role of the plasma membrane in the control of cellular growth and differentiation, we are studying the regulation of the Na^+,K^+-ATPase during the cell cycle of mouse neuroblastoma Neuro-2A cells [11]. It has been demonstrated that in sparse Neuro-2A cultures the functional pumping activity of the Na^+,K^+-ATPase, defined as the rate of ouabain-sensitive K^+ uptake by these cells under conditions of growth, is high in mitosis, drops approximately threefold in G_1 phase, and gradually rises again during S phase, with a transient increase near the G_1/S phase transition [9]. The hydrolysis activity of the enzyme, defined as the rate of ATP hydrolysis per cell in cell homogenates, shows qualitatively similar modulations during the cell cycle, lacking, however, the transient increase near the G_1/S phase transition [9]. Evidence has recently been presented for the involvement of intracellular sodium ions in the transient stimulation of the Na^+,K^+-ATPase pumping activity near the G_1/S phase transition [12].

In the present study we have investigated to what extent regulation of the Na^+,K^+-ATPase activity during the Neuro-2A cell cycle is caused by variation in availability of intracellular sodium ions, by changes in the number of enzyme copies per cell, and by modulation of the plasma membrane environment of the Na^+,K^+-ATPase molecules. Use has been made of the recent observation [13] that ATP, externally added to Neuro-2A cells in the millimolar range, selectively enhances the plasma membrane permeability of these cells for sodium ions. This permits determination of the Na^+,K^+-ATPase pumping activity under conditions of optimal stimulation with respect to its various substrate ions.

As typical for rodent cells, the Neuro-2A Na^+,K^+-ATPase has a relatively low affinity toward ouabain. External ATP, however, enhances the affinity of the Neuro-2A Na^+,K^+-ATPase for inhibition by ouabain such that it permits determination of the number of Na^+,K^+-ATPase copies per cell by direct ouabain-binding studies [13]. Taken together, we have determined the optimally stimulated pumping activity per copy of Na^+,K^+-ATPase during the Neuro-2A cell cycle. Our results show that the optimally stimulated pumping activity per enzyme copy is high in mitosis, almost twofold lower in early G_1 phase, while gradually increasing again during interphase. This demonstrates that changes in membrane environment of the Na^+,K^+-ATPase occur during the Neuro-2A cell cycle. The changes observed in optimally stimulated pumping activity per enzyme copy show a good correlation with the changes described in the lateral mobility of membrane proteins during the Neuro-2A cell cycle as measured by means of the fluorescence photobleaching recovery technique [14], such that a high Na^+,K^+-ATPase activity is correlated with a low membrane protein mobility. This is the first demonstration of a direct correlation between membrane protein lateral mobility and the activity of a membrane-associated enzyme.

MATERIALS AND METHODS

Cell Culture

C1300 mouse neuroblastoma cells, clone Neuro-2A, were grown in a 1:1 mixture of Ham's F_{12} medium and Dulbecco's modified Eagle's medium without

bicarbonate, but with 25 mM N-2-hydroxy-ethyl-piperazine-N'-2-ethane sulphonic acid (HEPES), pH 7.6, and supplemented with 7.5% fetal calf serum (Flow Laboratories, Irvine, Scotland) at 37°C in a humidified atmosphere. Synchronized cells were obtained by selective detachment of mitotic cells from exponentially growing cultures at a density of approximately 2.10^5 cells/cm^2 as described previously [15].

$^{86}Rb^+$ Uptake Measurements in Monolayer Cells

Synchronized mitotic cells were plated in 35-mm diameter culture dishes (Costar, Cambridge, Massachusetts) in growth medium at a density of 2.0×10^4 cells/cm^2. After the required time cells were washed three times with 1 ml 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and subsequently incubated in 0.65 ml of a medium containing 150 mM NaCl, 4.5 mM KCl, 3.5 mM ATP, 4 mM $MgSO_4$, and 10 mM Tris-HCl, pH 7.4 (cation uptake medium), unless stated otherwise. After a preincubation period of 15 min at room temperature with gentle shaking, a trace amount of $^{86}RbCl$ (Radiochemical Centre, Amersham, United Kingdom) was added up to a final concentration of 2–5 nM (specific activity 1 Ci/mole K^+). After a 5-min pulse, during which uptake is linear with time [16], cells were rapidly washed five times with 1 ml ice-cold phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4), and then lysed in 1 ml of an ice-cold 10% trichloroacetic acid solution. The lysate was transferred to a scintillation vial, and radioactivity was counted by measuring Cerenkov radiation in a liquid scintillation counter. Active Rb^+ uptake, mediated by the Na^+,K^+-ATPase, was determined by labeling cells in parallel experiments in the presence and absence of 3.5 mM ouabain in the cation uptake medium. K^+ influx was calculated on the basis of equal affinity and transport rates of the pump for K^+ and Rb^+.

$^{86}Rb^+$ Uptake Measurements in Mitotic Cells

Mitotic cells were plated in growth medium in 35-mm diameter culture dishes, precoated overnight in a polylysine solution (50 μg/ml, MW 60,000). After 5 min at room temperature without shaking, the medium was exchanged for phosphate-buffered saline. After another 5 min the cells were washed, incubated in the cation uptake medium, and treated as described above. The trichloroacetic acid precipitate of the cells was solubilized in 0.5 N NaOH, and used for protein determination [17] in order to determine the number of cells on the dish, using a value of 188 μg protein/10^6 mitotic cells [9]. Uptake measurements were also performed on mitotic cells in suspension according to the method described by Mummery et al [9], with similar results.

Ouabain Sensitivity of Active K^+ Influx

The inhibition constant of ouabain (K_i), defined as the concentration of ouabain required to give 50% inhibition of active K^+ influx, was determined by measuring $^{86}Rb^+$ uptake in the presence of four different ouabain concentrations in the cation uptake medium. K_i values were determined from the linear double reciprocal plot of percent inhibition of total K^+ influx versus ouabain concentration, as described in detail by van Zoelen et al [13].

^3H-Ouabain Binding

Synchronized mitotic cells were plated in 60-mm diameter culture dishes (Costar, Cambridge, Massachusetts) in growth medium at a density of 2.0×10^4 cells/

cm^2. After the required time, cells were washed three times with 2 ml 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and incubated in 1.0 ml of a medium containing 150 mM NaCl, 3.5 mM ATP, 4 mM $MgSO_4$, 5 μM ^{3}H-ouabain (17 Ci/mmol; Radiochemical Centre, Amersham, United Kingdom), 5 μM unlabeled ouabain, and 10 mM Tris-HCl, pH 7.4. After incubation for 15 min at room temperature with gentle shaking, which is sufficient to obtain binding equilibrium, the cells were washed five times with an ice-cold solution of 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, containing 2% ethanol [13]. Cells were solubilized in 0.5 N NaOH, and radioactivity was assayed according to standard procedures. In order to correct for binding of ouabain at nonspecific sites, duplicate experiments were performed using a medium containing 150 mM KCl instead of 150 mM NaCl. Specific binding was assayed by subtracting the binding in 150 mM KCl from that in 150 mM NaCl [13, 18]. ^{3}H-Ouabain binding on mitotic cells was performed in a similar way, after attachment of the cells on polylysine-coated dishes as described above.

Chemicals

ATP was obtained as a disodium salt from Sigma Chemical Co (St. Louis) and added to the incubation media from 50 mM Tris-buffered solution, pH 7.4. Polylysine and ouabain were both obtained from Sigma Chemical Co and used from a water solution.

RESULTS

Active K^+ Influx During the Neuro-2A Cell Cycle

Mouse neuroblastoma Neuro-2A cells can be synchronized simply by mitotic shakeoff. When plated on a suitable substrate, the mitotic cells proceed through the various phases of the cell cycle as indicated in the upper part of Figure 1 [15]. The variation in intermitotic time between individual cells resulting from a random transition in the cell cycle, as well as other nonprobabilistic variation in intermitotic time, is relatively small for this cell line [19], and these cultures therefore maintain a high degree of synchronization throughout the first cell cycle. Eight hours after shakeoff the first mitotic cells of the next generation appear. This relatively short generation time, combined with the other properties above, makes this cell line very suitable for studying variation in cellular properties during the cell cycle.

Figure 1 shows the rate of active potassium influx during the Neuro-2A cell cycle at various external potassium and ATP concentrations. Recent studies have demonstrated that external ATP in the millimolar range is able to enhance specifically the plasma membrane permeability of Neuro-2A cells for sodium ions, such that the Na^+,K^+-ATPase pumping activity is optimally stimulated with respect to internal sodium ions under these conditions [13]. By comparing the rate of active potassium influx during the Neuro-2A cell cycle at physiological potassium concentration (4.5 mM) in the presence and absence of 3.5 mM external ATP (Fig. 1, upper and middle curve), it is shown that active potassium influx can be stimulated by external ATP in all phases of the cell cycle. However, the extent of stimulation of the Na^+,K^+-ATPase pumping activity by external ATP varies significantly during the cell cycle, from a factor of 1.05 in mitosis up to 2.2 in G_1 phase (Fig. 2). Mummery et al [12] have shown that the internal sodium concentation of Neuro-2A cells is high in mitosis, low in G_1 phase, increases during S phase until 6 hr after mitosis, and then decreases

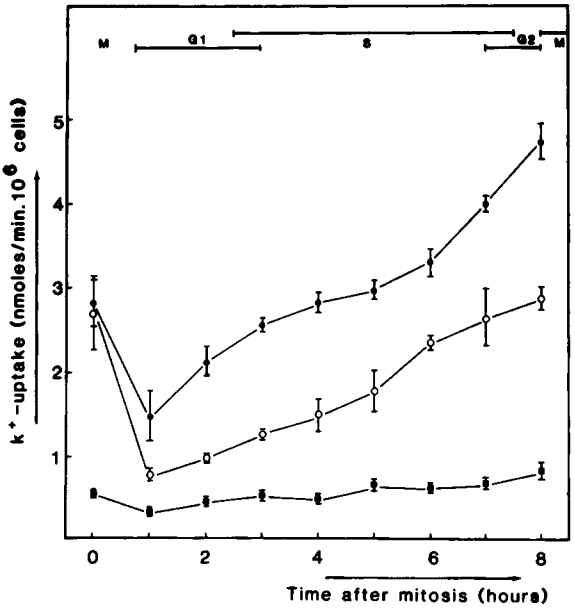

Fig. 1. Rate of active K^+ influx in synchronized Neuro-2A cells during the cell cycle. Measurements were performed in cation uptake medium containing (●——●) 4.58 mM K^+, 3.5 mM ATP (n = 4; optimal pumping activity); (○—○) 4.58 mM K^+, no ATP (n = 2); and (■——■) 0.23 mM K^+, 3.5 mM ATP (n = 3). K^+ influx was assayed from ouabain-sensitive $^{86}Rb^+$ uptake. n denotes the number of independent cell cycles analyzed. The upper part of this graph shows the relation between the time after mitosis and the various phases of the cell cycle.

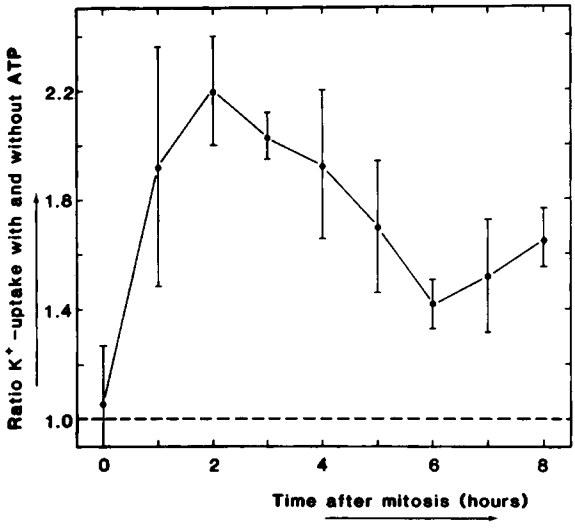

Fig. 2. Stimulation of active K^+ influx by external ATP (3.5 mM) during the Neuro-2A cell cycle. The ratio of ouabain-sensitive $^{86}Rb^+$ uptake with and without ATP was obtained by combining the data from the two upper curves of Figure 1.

again. It therefore follows from Figure 2 that the lower the internal sodium concentration of the cells, the more the Na^+,K^+-ATPase pumping activity can be stimulated by external ATP. This demonstrates that during the cell cycle the internal sodium concentration is suboptimal for stimulation of the Na^+,K^+-ATPase, but that in addition the extent to which the pump can be stimulated by internal sodium ions varies during the cell cycle. Moreover, it follows that in the presence of external ATP and a sufficiently high external potassium concentration [13], the pumping activity of the Na^+,K^+-ATPase can be measured while optimally stimulated with respect to its substrate ions.

Calculations by Van Zoelen et al [13] on exponentially growing Neuro-2A cells have shown that the value of the pumping activity of the Na^+,K^+-ATPase in the presence of extracellular ATP is very similar to that of the hydrolysis activity of the Na^+,K^+-ATPase in cell homogenates, which is measured under optimal conditions for all substrate molecules, including Na^+, K^+, Mg^{++}, and ATP. In addition, the modulation of the Na^+,K^+-ATPase pumping activity in the presence of external ATP (see Fig. 1) shows a close resemblance with the modulation of the hydrolysis activity of the Na^+,K^+-ATPase during the Neuro-2A cell cycle as measured by Mummery et al [9]. The actual values cannot be compared directly in this case because of temperature differences between the two experiments, but in both situations a decrease in activity is observed on going from mitosis to G_1 phase followed by a continuous threefold increase during the interphase of the cell cycle. The good correlation between the Na^+,K^+-ATPase hydrolysis activity and the pumping activity in the presence of external ATP indicates that the availability of intracellular substrate molecules other than Na^+ is not limited, or at least does not change during the cell cycle to an extent that significantly modulates the Na^+,K^+-ATPase activity.

Ouabain Sensitivity of the Na^+,K^+-ATPase During the Neuro-2A Cell Cycle

The extent to which the number of Na^+,K^+-ATPase copies per cell changes during the Neuro-2A cell cycle necessitates measurement of the number of ouabain binding sites per cell in the various phases of the cell cycle. To find suitable experimental conditions for such binding studies, the affinity constant for binding of ouabain to the Na^+,K^+-ATPase is required. This affinity constant can be measured either directly from ouabain binding studies in the presence of various external ouabain concentrations, or indirectly, by measuring the ouabain concentration required for 50% inhibition of the Na^+,K^+-ATPase mediated Rb^+ uptake. Measurements on exponentially growing Neuro-2A cells have shown that the two methods give similar results [13]. We have used the indirect method, which is experimentally more convenient, by measuring the inhibition constant of ouabain toward active $^{86}Rb^+$ uptake during the Neuro-2A cell cycle in the presence of 3.5 mM external ATP, in the presence of both a high (4.5 mM) and a low (0.23 mM) external potassium concentration. External ATP is known to enhance the affinity of the Neuro-2A Na^+,K^+-ATPase for ouabain [13], whereas external potassium ions competitively inhibit the binding of ouabain [1]. Therefore, at least two different potassium concentrations are required to determine, by extrapolation, the inhibition constant of ouabain obtained in the absence of potassium ions [13].

Figure 1 shows the rate of active K^+ influx during the Neuro-2A cell cycle in the presence of 3.5 mM ATP in a medium containing either 0.23 or 4.5 mM K^+ (lower and upper curve, respectively). The higher rate of influx at the latter potassium

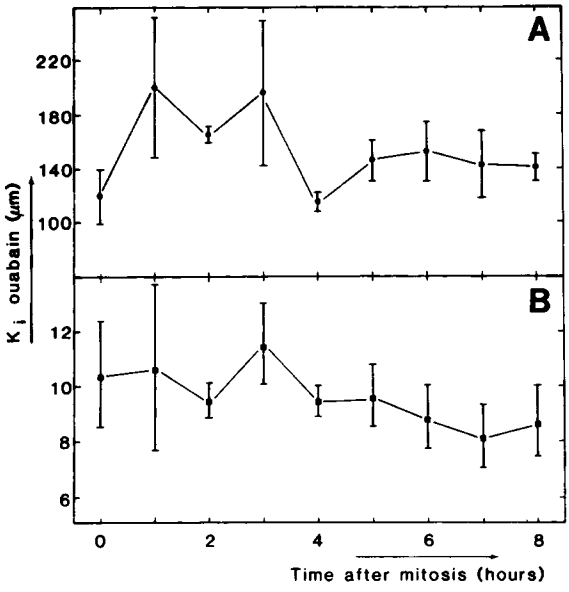

Fig. 3. Sensitivity of the Neuro-2A Na^+,K^+-ATPase for inhibition by ouabain. The ouabain inhibition constant K_i, defined as the concentration of ouabain required to inhibit 50% of active K^+ influx, has been determined in media containing 4.58 mM K^+, 3.5 mM ATP (A; n = 4) and 0.23 mM K^+, 3.5 mM ATP (B; n = 3). In each experiment four different ouabain concentrations were used, and K_i values were determined from the linear double-reciprocal plot of percent inhibition of active K^+ influx versus ouabain concentration [see van Zoelen et al 13].

concentration illustrates the stimulation of the Na^+,K^+-ATPase pumping activity by external potassium ions. Figure 3 shows the values of the ouabain dissociation constant, defined as the concentration of ouabain required for 50% inhibition of active K^+ influx, under the above two conditions. The difference in ouabain sensitivity between the two potassium concentrations reflects the inhibition of ouabain binding by external potassium ions. It is shown in Figure 3 that the affinity of the Na^+,K^+-ATPase for ouabain varies considerably during the cell cycle, especially at the higher potassium concentration (Fig. 3A). An almost twofold decrease in ouabain affinity is observed on passing from mitosis to G_1 phase, which might be illustrative of conformational changes of the Na^+,K^+-ATPase, possibly as a result of changes in membrane properties, during the cell cycle. The large standard error of the ouabain inhibition constant in G_1 phase (1 hr after mitosis) might well result from the very low pumping activity and relatively large contribution of ouabain insensitive K^+ uptake at this time point [9], but it is striking that a relatively large standard error is also observed 3 hr after mitosis, close to the G_1/S phase transition, where the functional pumping activity of the enzyme shows its transient increase [9]. This could indicate that different protein conformations are present at this time point. The ouabain inhibition constant at low potassium concentration (Fig. 3B) shows little significant variation during the cell cycle, indicating that changes in the ouabain inhibition constant during the cell cycle result more from changes in the potassium inhibition constant for ouabain binding than in the affinity constant of the enzyme for ouabain as such.

Calculation of the ouabain inhibition constant in the absence of external potassium ions by linear extrapolation of the data presented in Figure 3 results in values between 1 and 3 μM throughout the cell cycle, which is in good agreement with the value of 2.6 $\pm$ 0.4 μM observed for exponentially growing Neuro-2A cells [13]. Since no error analysis can be performed on data extrapolated from two points, these values have not been listed explicitly. The above data demonstrate that ouabain concentrations of at least 10 μM are required in a direct ouabain binding assay to obtain relevant information on the number of ouabain binding sites available.

Ouabain Binding During the Neuro-2A Cell Cycle

Figure 4 shows the number of ouabain binding sites per cell during the Neuro-2A cell cycle, as measured in a medium containing 10 μM ouabain, 3.5 mM external ATP, but no external potassium ions. In order to correct for binding of ouabain at non-specific sites, parallel experiments have been performed in a medium with a high potassium concentration, which results in a strong inhibition of specific ouabain binding to the Na^+,K^+-ATPase [13]. The number of ouabain binding sites per cell increases on going from mitosis to mid G_1 phase, remains almost constant during late G_1 and early S phase, and then increases rapidly in late S and G_2 phase (Fig. 4). The relatively large standard error in the number of ouabain binding sites 6 hr after mitosis is caused by the fact that in some of the cell cycle experiments the rapid increase in number of binding sites occurred later in the cycle. The reason for the large standard error in the case of mitotic cells is not clear.

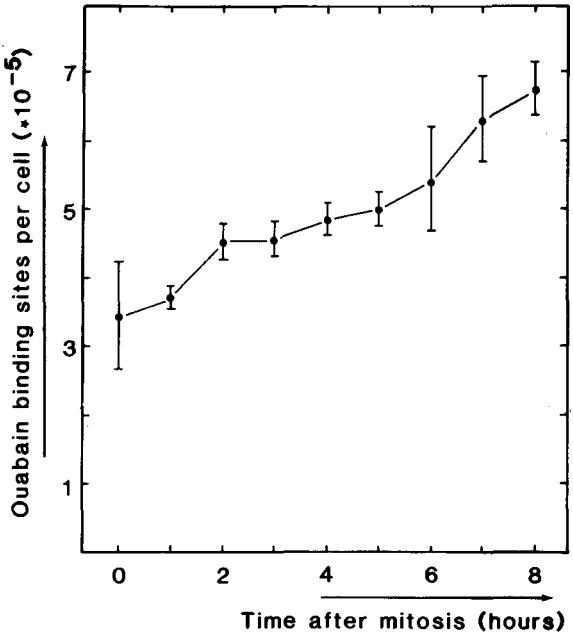

Fig. 4. Number of ouabain binding sites per cell during the Neuro-2A cell cycle. Ouabain binding was performed in media containing 10 μM ouabain and 3.5 mM ATP, either in the presence or absence of 150 mM K^+ (n = 4).

It follows from Figure 4 that the number of ouabain binding sites almost doubles on passing from mitosis to the next G_2 phase. The cells thus attain the required number of Na^+,K^+-ATPase molecules to divide between their daughter cells at the next mitosis. The increase in number of ouabain binding sites per cell parallels to a large extent the increase in cell surface area through the cell cycle [20]. This remains almost constant during the first part of the interphase, but increases rapidly at the late S/G_2 phase. This suggests that, at a first approximation, the number of Na^+,K^+-ATPase molecules per surface area remains constant during the cell cycle. This agrees with freeze-fracture electron microscopy data [45] that the density of intramembraneous particles with a diameter larger than 90 Å remains almost constant during the interphase of the Neuro-2A cell cycle. From reconstitution work of purified Na^+,K^+-ATPases it is known that this protein gives rise to intramembraneous particles with a diameter of 90–100 Å [21]. The above data show that variation in the number of Na^+,K^+-ATPase molecules per cell is one of the factors that contribute to the modulation of active K^+ influx during the cell cycle.

Membrane Regulation of the Na^+,K^+-ATPase During the Neuro-2A Cell Cycle

When comparing the data of Figures 1 and 4, it follows that during the interphase of the Neuro-2A cell cycle the optimally stimulated pumping activity of the Na^+,K^+-ATPase per cell increases by more than a factor of 3, whereas the number of enzyme copies per cell increases by less than a factor of 2. In addition, on passing from mitosis to early G_1 phase, the number of enzyme copies hardly changes, but the optimally stimulated pumping activity decreases by almost a factor of 2. This demonstrates that the optimally stimulated pumping activity per Na^+,K^+-ATPase copy is modulated during the cell cycle. Figure 5, which has been obtained from a combination of the data in Figures 1 and 4, shows that the optimally stimulated pumping activity per Na^+,K^+-ATPase copy is high in mitosis, almost twofold lower in early G_1 phase, while gradually increasing again during interphase following an S-shaped curve. It is important to note that a similar result is obtained when this method is used to calculate the modulation of the Na^+,K^+-ATPase hydrolysis activity (as measured by Mummery et al [9]) per enzyme copy during the cell cycle (not shown). This demonstrates that variation in the optimally stimulated pumping activity of each enzyme molecule also contributes to the regulation of the active K^+ influx during the Neuro-2A cell cycle.

The above observations imply that properties of the plasma membrane in the immediate environment of the Na^+,K^+-ATPase molecules have to change during the Neuro-2A cell cycle, such that they permit a high enzyme activity in mitosis and late S/G_2 phase, compared to a low activity in early G_1 phase. From other studies it is known that various physicochemical properties of the Neuro-2A plasma membrane,in particular the microviscosity [15] and rates of lateral diffusion of lipids and membrane proteins [14], change during the cell cycle. The rate of membrane protein lateral diffusion, as measured by means of the fluorescence photobleaching recovery technique, using an aspecific antibody against mouse cell surface determinants, is low in mitosis, threefold higher in early G_1 phase, while gradually decreasing again during the other phases of the cell cycle following an S-shaped curve [14]. This behavior parallels to a large extent the variations in optimally stimulated Na^+,K^+-ATPase pumping activity per copy during the cell cycle observed in this study, such that a

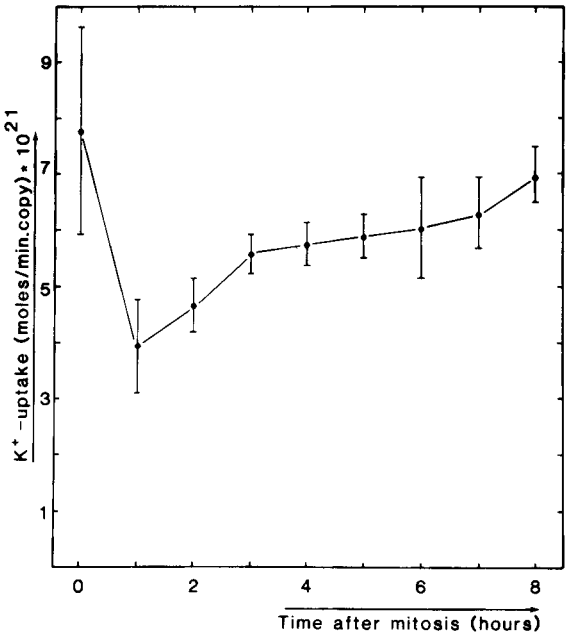

Fig. 5. Optimally stimulated Na^+,K^+-ATPase activity per ouabain binding site during the Neuro-2A cell cycle. This graph was obtained by combining the data of Figure 1 (upper curve) with those of Figure 4.

high rate of protein lateral diffusion is accompanied by a low Na^+,K^+-ATPase activity. Figure 6 shows that a very good correlation exists between these two parameters during the Neuro-2A cell cycle ($\rho = 0.96$). The correlation between Na^+,K^+-ATPase activity and changes in microviscosity [15] or rate of lipid lateral diffusion [14] during the cell cycle is much less pronounced (data not shown). In conclusion, we have shown that changes in plasma membrane properties take place during the Neuro-2A cell cycle that result in changes in the rate of protein lateral diffusion and the Na^+,K^+-ATPase activity in a directly correlated way. Finally, it is important to realize that the data in Figures 2, 5, and 6 have been obtained by combination of individually determined, independent parameters, and that the relatively large standard errors in the values therefore result from accumulation of the standard errors in the individual parameters.

DISCUSSION

The activity of the plasma membrane-bound Na^+,K^+-ATPase shows considerable variation during the cell cycle [9, 22]. In this study it has been shown that in the case of Neuro-2A cells this regulation of enzyme activity is caused by a combination of three factors. First, the extent of stimulation of the Na^+,K^+-ATPase by internal sodium ions varies; second, the number of enzyme copies per cell changes; and third, the optimally stimulated activity of each enzyme copy is modulated during the cell cycle. The regulation of this latter parameter correlates well with modulations in the rate of lateral diffusion of plasma membrane proteins during the Neuro-2A cell cycle.

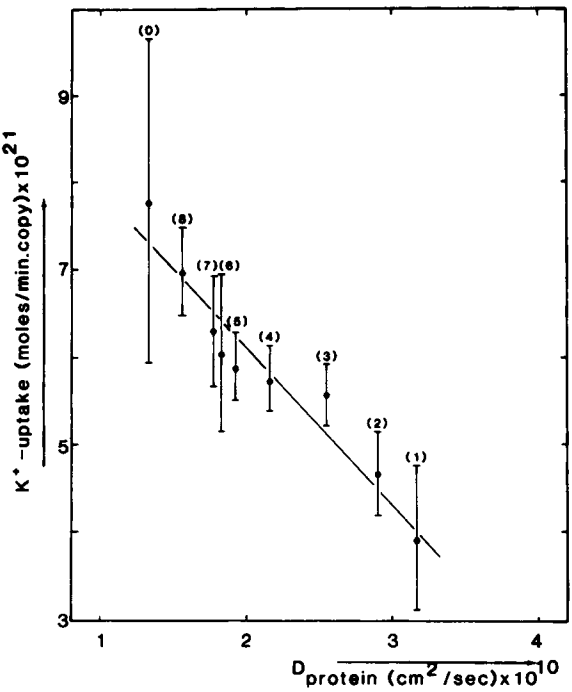

Fig. 6. Relationship between the membrane protein lateral diffusion coefficient (D_{prot}) and the optimally stimulated Na^+,K^+-ATPase pumping activity per ouabain binding site during the Neuro-2A cell cycle. Values for D_{prot} were taken from de Laat et al [14]; the values in the graph denote the corresponding time points (in hours) during the cell cycle. The observed relation has a correlation coefficient of 0.96. The values of the standard error of the mean for D_{prot} are relatively small and have not been indicated in the figure.

We have made use of the observation [13] that ATP, added externally to Neuro-2A cells at millimolar concentrations, enhances the affinity of the cell for binding of ouabain. Since rodent cells are relatively insensitive to ouabain [23], addition of ATP is a necessity for detemining the number of Na^+,K^+-ATPase cells by direct ouabain binding studies. In addition, external ATP enhances the plasma membrane permeability of these cells for sodium ions, which permits determination of the Na^+,K^+-ATPase pumping activity optimally stimulated with respect to its substrate ions. In so doing, we have assumed that the concentrations of other intracellular substrates for the Na^+,K^+-ATPase, such as Mg^{2+}, internal ATP, and inorganic phosphate, do not vary to the extent that they modulate the enzyme activity during the cell cycle. Evidence for this statement comes from a numerical comparison of the optimally stimulated pumping activity and the hydrolysis activity of the Neuro-2A Na^+,K^+-ATPase [13]. In this latter study it has been established that in addition to the effects described above, external ATP may also stimulate the Na^+,K^+-ATPase pumping activity directly, either because it brings the enzyme into a more active conformation or because it unmasks additional Na^+,K^+-ATPase copies in the plasma membrane. This aspect should be considered when interpreting the stimulating effect of external ATP on the Na^+,K^+-ATPase pumping activity, as shown in Figure 2. The number of Na^+,K^+-ATPase molecules per cell, which equals the number of ouabain binding

sites [24], increases during the Neuro-2A cell cycle in a way parallel to the changes in cell surface area. This is in contrast to the data of Rabito and Tchao [18], who observed a decrease in the number of ouabain binding sites on passing from early S phase to mitosis in Madin-Darby canine kidney (MDCK) cells. A direct comparison with studies on such epithelial cell lines is hampered, however, by the fact that these cells show pronounced polarization, with the Na^+,K^+-ATPase preferentially localized at the basolateral side [25]. Whether any polarization occurs in the sparse Neuro-2A cultures used in this study is at present unknown. Since our experiments are based on the rate of ouabain-sensitive $^{86}Rb^+$ uptake and number of ouabain binding sites, our results refer only to those Na^+,K^+-ATPase copies that can be reached externally by ouabain. This should be kept in mind particularly when considering the results obtained on mitotic cells bound to polylysine-coated surfaces.

Functional Aspects of the Na^+,K^+-ATPase Activity Regulation During the Neuro-2A Cell Cycle

When comparing the data of Figure 1 with the known modulation of the functional pumping activity of the Na^+,K^+-ATPase during the Neuro-2A cell cycle [9], it is striking that the transient, Na^+-dependent increase in the rate of active K^+ influx near the G_1/S phase transition is not observed in the present study, even in the absence of external ATP. This may be due to the fact that the present studies were carried out at room temperature, whereas the previous studies were performed at 37°C. In addition, the presence of fetal calf serum may be required for observing this transient increase in pumping activity. Evidence has been presented that under the tested conditions of growth the occurrence of this transient increase in Na^+,K^+-ATPase pumping activity is a prerequisite for progression of the cells from the G_1 phase to S phase [12]. Studies on the effect of ouabain on the growth characteristics of Neuro-2A cells have shown that a proper functioning of the Na^+,K^+-ATPase is required both for transition of the cells from the G_1 to S phase, and for progression through the other phases of the cell cycle [9].

From previous studies it is also known that the membrane potential of sparsely cultured Neuro-2A cells show significant modulation during the cell cycle, being high in mitosis, low in G_1 phase, and gradually increasing during S phase [20]. Preliminary results from the growth kinetics of Neuro-2A cells in media with a high potassium concentration have shown that experimental reduction of the membrane potential retards the progression of these cells through the cell cycle, but enhances the so-called transition probability, the probability that cells in G_1 phase enter the S phase (van Zoelen and Defize, unpublished). This would suggest that a low membrane potential in G_1 phase, as observed under normal culture conditions, facilitates the entry of the cells into S phase, whereas a high membrane potential is required for the cells to proceed optimally through the S phase. The activity of the Na^+,K^+-ATPase largely contributes to the membrane potential, and therefore a low enzyme activity in G_1 phase and a high one in S phase will contribute to an optimal progression of the cells through the cell cycle. On the other hand, it can be argued that under conditions of growth the functional activity of the Na^+,K^+-ATPase will never reach the optimally stimulated level observed under the present experimental conditions. It can be argued, however, that the modulation of optimal pumping activity per enzyme copy reflects a conformational change of the enzyme, which directly affects the affinity of the enzyme for its substrate molecules. Work is in progress to investigate this

possibility by measuring the affinity constant of the Na^+,K^+-ATPase for stimulation by internal sodium ions during the cell cycle in cell homogenates. Since the internal sodium ion concentration is generally suboptimal for stimulation of the Na^+,K^+-ATPase activity, modulation of this affinity constant can directly affect the functional pumping activity of the enzyme.

It is important to note that the hydrolysis activity per copy of Na^+,K^+-ATPase shows qualitatively similar modulation during the Neuro-2A cell cycle as the optimally stimulated pumping activity per enzyme copy. This observation excludes the possibility that the observed modulation in enzyme activity results, for example, from the concomitant variation in membrane potential or from changes in the ion-pumping stoichiometry. The modulation in optimally stimulated pumping activity per enzyme copy can therefore only result from changes in plasma membrane properties during the cell cycle.

Activity Regulation of the Na^+,K^+-ATPase During the Cell Cycle in Relation to Membrane Fluidity

Cell cycle–dependent changes in plasma membrane properties provide a good system for studying membrane regulation phenomena. The modulations observed during the cell cycle remain within narrow limits, but they can be studied without any external disturbances, simply by following processes as a function of time. Previous studies have shown that the microviscosity [15] and the rate of plasma membrane lipid and protein lateral diffusion [14] change during the Neuro-2A cell cycle. Such changes in membrane fluidity might be due to variations in the plasma membrane lipid composition, the cholesterol-to-lipid ratio, and protein-to-lipid ratio [see review by Quinn, 26], or by variations in the cytoskeleton arrangement, or even in the amount of bound water [27]. In addition, the variation in cell membrane potential during the cell cycle [20] can affect the lateral mobility of membrane components [28]. The present study shows that the regulation of Na^+,K^+-ATPase activity is inversely related to that of the protein lateral mobility. It is known that the activity of the Na^+,K^+-ATPase depends on the presence and/or absence of specific lipid molecules [29–31] and divalent cations [32–34]. Moreover, it has been established for the Na^+,K^+-ATPases from various sources that an increase in membrane fluidity results in a stimulation of enzyme activity [35, 36], although one situation has been described in which the hydrolysis activity but not the steady-state pumping activity of the enzyme was affected [37, 38]. None of the above considerations, however, seem appropriate to explain the observed relation between increased Na^+,K^+-ATPase and reduced membrane protein lateral mobility. We can explain our data in terms of the bilayer thickness concept [39], which is basically similar to the optimal fluidity concept [40], and the so-called lipid dimpling model [41] for lipid-protein interactions. According to the bilayer thickness concept, the nearer the thickness of the hydrophobic region of an enzymatic membrane protein matches that of the surrounding lipid molecules, the higher the activity will be. If it is assumed that the thickness of the lipid bilayer in Neuro-2A cells is smaller than required for optimal Na^+,K^+-ATPase activity, but increases during the cell cycle, this will result in an enhanced Na^+,K^+-ATPase activity together with an increased packing of the lipid bilayer when proceeding through the cell cycle, giving rise to a reduced rate of protein lateral mobility [42–44]. In this way, the conformation of a membrane enzyme can manifest itself in the rate of protein lateral diffusion. However, it remains unclear why the rate of lipid lateral diffusion is much less affected during this process.

In this study we have related the activity of Na^+,K^+-ATPase to the lateral mobility of plasma membrane proteins. It is important to realize that the external conditions used to determine these two parameters were not identical, since external ATP were not present in the photobleaching experiments. The effects of external ATP on protein lateral mobility are under current investigation, in particular because external ATP is known to affect the membrane potential of Neuro-2A [13]. The fluorescence photobleaching recovery technique permits direct determination of the lateral mobility characteristics of the Na^+,K^+-ATPase molecules themselves, if a specific fluoresecent label can be attached to this enzyme. Work is in progress to determine the lateral mobility of the Na^+,K^+-ATPase during the Neuro-2A cell cycle after labeling with a specific antibody, in order to investigate, more directly, the relation between activity and mobility of the Na^+,K^+-ATPase. Since the activity regulation of this enzyme is known, such measurements can elucidate the functional aspects of the generally observed "immobile fraction" in fluorescence photobleaching recovery measurements of membrane proteins [42–44]. In addition, work is in progress to modify the plasma membrane composition and plasma membrane–cytoskeleton interactions in Neuro-2A cells, and to measure the effects of such modifications on the Na^+,K^+-ATPase activity and protein lateral mobility. Such measurements will give information as to whether a causal relationship exists between membrane enzyme activity and membrane protein lateral mobility.

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