SODIUM-DEPENDENT EFFLUX OF K^+ AND Rb^+ THROUGH THE ACTIVATED SODIUM CHANNEL OF NEUROBLASTOMA CELLS

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Summary: Passive efflux of ^{42}K or ^{86}Rb from differentiated mouse neuroblastoma cells in culture was stimulated up to 8-fold by 10^{-4} M veratridine. The increased efflux could be blocked by low concentrations of tetrodotoxin (K_i = 4×10^{-9} g/ml), and did not occur with other cell types lacking an excitable membrane. The temperature sensitivity of the activated component was much higher than that of the normal passive outflow. It is suggested that the veratridine-dependent, tetrodotoxin-sensitive efflux represents passage of ions through the excitable Na^+ channel. Replacement of extracellular Na^+ by $Tris^+$ abolished the activation by veratridine. Titration of the Na^+ requirement resulted in a hyperbolic relationship between external Na^+ concentration and efflux rate, with an apparent K_m of 66.7 mM for Na^+. This phenomenon may reflect an interaction between extracellular ions and a regulatory site on the Na^+ channel.

Introduction

Under physiological conditions, the fast inward current through the voltage-dependent ionic channels of many neuronal membranes is carried by Na^+ ions (1). Voltage-clamp experiments reveal that these channels are not entirely specific as similar currents of varying magnitude can be recorded following substitution of extracellular Na^+ by other metal cations such as Li^+ or K^+ (2, 3). In addition, it is known that in the squid giant axon, cations can flow out through the Na^+ channel under certain conditions. Two groups have demonstrated the existence of an early outward current carried by K^+ when this ion is the only internal cation present (4, 5). Kinetic considerations indicate that this current is mediated by the Na^+ channel itself, being easily distinguished from the late outward current component which passes through the excitable K^+ channel. For both inward and outward currents the measured selectivity of the channel for Na^+ over K^+ is on the order of 12-25:1 (4, 5). Using a recently developed method for following fluxes through the Na^+ channel by means of tracers (6, 7) we have now found that in addition to the expected inward Na^+ flux, both K^+ and Rb^+ are capable of effluxing via the activated channel of

differentiated neuroblastoma cells. This activated efflux can be blocked by tetrodotoxin (TTX) confirming that is is truly mediated by the Na^+ channel. Furthermore, it is totally dependent on the presence of Na^+ ions in the external medium.

Materials and Methods: Cells of clone N1E-115 (see ref. 8 for details) were maintained in confluency for 1 week in Dulbecco's modified Eagle's medium (DMEM+$NaHCO_3$, 1.2 g/l) plus 7.5% fetal calf serum (FCS) plus penicillin (50 u/ml) and streptomycin (10 μg/ml). They were then replated into 60 mm Falcon tissue culture dishes at a density of $8x10^5$ cells/dish in a medium consisting of DMEM + 7.5% FCS+ 1% dimethylsulfoxide (8). After 4 days, when morphological and electrical differentiation was maximal (8), the cells were loaded on a thermostated table at 35° for 75 min., with a medium consisting of DMEM buffered with 20 mM HEPES (pH 7.3) plus 10-20 μCi/ml ^{86}Rb (Radiochemical Centre). Each plate was washed three times with 5 ml HEPES-buffered DMEM at room temperature, then efflux was allowed to proceed at 35° into 3.5 ml of the same medium. Aliquots of 100 μl were withdrawn at 2 minute intervals over a period of 40 min, and counted in a Packard Auto-gamma spectrometer. In treated cultures 6 μl of a 50 mM stock solution of veratridine (K & K Labs) in ethanol was added to the medium 15 min following the commencement of efflux to make a final concentration of 100 μM veratridine and 0.2% ethanol. The latter alone had no effect on the rate of ^{86}Rb efflux. In experiments using tetrodotoxin (Calbiochem, 10^{-6} - 10^{-9} g/ml) this agent was present from the beginning of the efflux. When efflux was conducted in solutions of differing Na^+ concentrations, isotonicity and ionic strength were maintained using Tris-Cl buffer (pH 7.3).

Results and Discussion

The efflux of ^{42}K or ^{86}Rb from preloaded neuroblastoma cells in culture is an energy-independent, ouabain-insensitive process with a $T_{1/2}$ of 30-40 min. (^{42}K) or 45-55 min (^{86}Rb). As demonstrated in a variety of other transport systems, ^{86}Rb serves as an effective replacement for ^{42}K, with the advantage of being a more stable isotope. Further analysis of passive Rb^+ or K^+ diffusion indicated the presence of both a simple electrodiffusion component and a varying fraction exhibiting the characteristics of exchange (K^+ for K^+) diffusion (unpublished results). The method for measurement of fluxes through the Na^+ channel depends on the capacity of certain neurotoxic drugs, such as veratridine, batrochotoxin and scorpion venom to maintain the channel in an open conformation for extended periods of time (see refs. 6 and 7 and refs. therein for details). Fig. 1a shows that addition of veratridine (100 μM) during the early phase of ^{86}Rb efflux resulted in an immediate stimulation of outflow, the degree of which depended on the growth state of the cells. Thus, logarithmically growing cells, which are known to be relatively electrically inexcitable, showed an approximately 1.5 - 2 fold stimulation by veratridine, whereas cells differentiated to a high level of excitability in the presence of dimethylsulfoxide (8), exhibited increases of up to 8-fold in the efflux rate constant. Moreover, inexcitable cells such

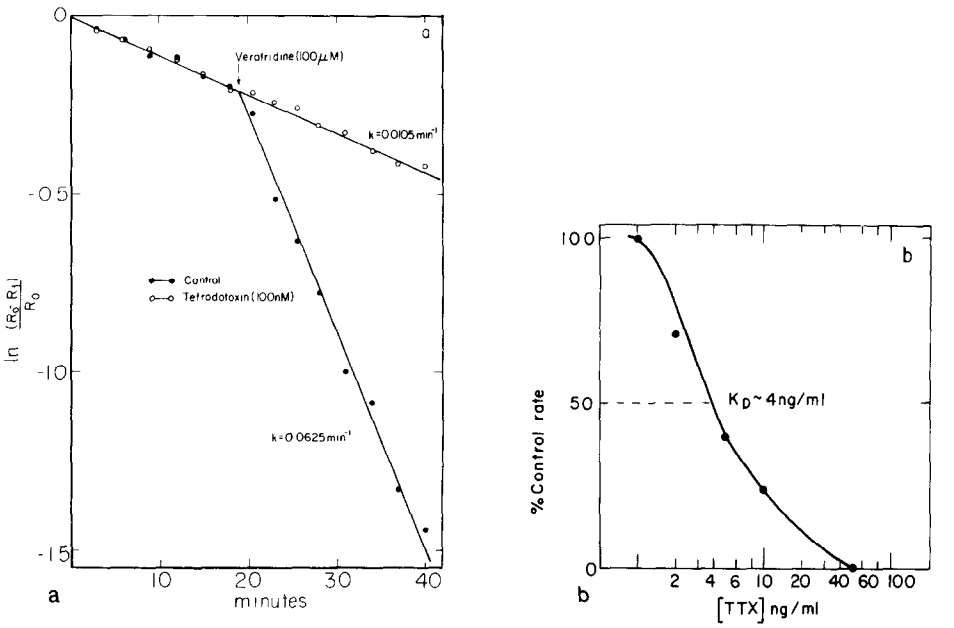

Fig. 1: (a) ^{86}Rb efflux through the Na^+ channel of differentiated neuroblastoma cells. ●—● control, 0—0 + TTX (10^{-7} g/ml). Ordinate: fraction of isotope remaining in cells; abscissa: time. Veratridine was added to both cultures at the time indicated by the arrow.
(b) Titration of TTX sensitivity of veratridine-activated ^{86}Rb efflux. The percentage of the maximal activated efflux rate in the presence of veratridine alone, is plotted against the particular concentration of TTX included in the efflux medium.

as cultured fibroblasts and rat glioma (C 6) cells were totally unresponsive to the addition of the drug.

The increased efflux was completely blocked by low concentrations of TTX, an agent known to interact specifically with an external site on the Na^+ channel of many excitable membranes, including that of neuroblastoma (9, 10). Fig. 1b shows that the K_i for TTX inhibition was about 4 ng/ml, a value in good agreement with that found for inhibition of veratridine-dependent Na^+ influx in these cells (11). TTX had no influence on the normal rate of passive efflux of either ^{42}K or ^{86}Rb, and was effective in blocking the induced flux when added either before, or several minutes after, the addition of veratridine. This latter finding militates against the possibility that Na^+ influx itself, in some way, could be responsible for the observed change in K^+ or Rb^+ permeability. A second indirect mechanism considered was that of a reduction in membrane potential upon veratridine administration, which would be expected

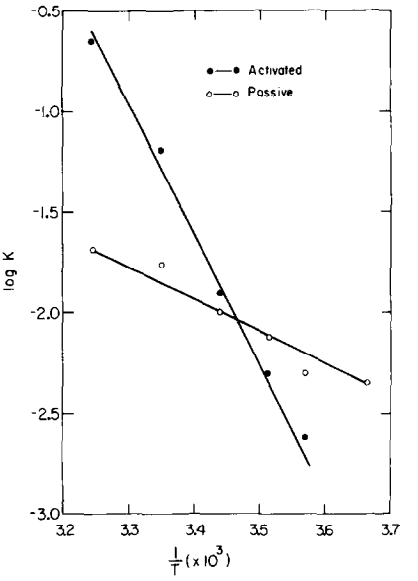

Fig. 2: Aarhenius plot of temperature dependence of ^{42}K efflux rates in the presence (activated) and absence (passive) of veratridine (100 μM).

to lead to an increase in the electrochemical gradient driving passive cation efflux. This possibility was eliminated by a continuous electrophysiological monitoring of the membrane potential during application of veratridine. Excitable neuroblastoma cells having membrane potentials of between -40 mv to -50 mv showed no detectable change in this parameter upon addition of the alkaloid (100 μM).

When efflux was performed at different temperatures between 0° and $37^{\circ}C$, the veratridine stimulated rate was found to be much more affected than the normal passive rate. Fig. 2 shows an Aarhenius plot of these data, the two lines yielding activation energies of 24.2 Kc/mole (Q_{10} = 2.6) and 7 Kc/mole (Q_{10} = 1.5) for the stimulated and normal rates respectively. This difference is compatible with the hypothesis that two independent transport processes are involved.

Taken together, these results strongly suggest that the veratridine-induced, TTX-sensitive cation efflux can be considered as occurring via the Na^+ channel itself. Preliminary experiments with both batrachotoxin (1 μM) and scorpion (Leiurus quinquestriatus) venom (5 μg/ml), indicate that these agents can replace veratridine in the activation of efflux. This method thus offers a useful and convenient alternative to Na^+ influx measurements for developmental studies of Na^+

channel density in excitable cells, a major advantage being that complete information on both passive and activated fluxes can be obtained from a single preparation or culture plate. We are currently using ^{86}Rb efflux to estimate the effects of various culture conditions on the appearance of the Na^+ channel during differentiation.

Recent models of Na^+ channel function derived from voltage-clamp experiments on frog nodes of Ranvier and squid axons (12, 13), have suggested that the independence principle first enunciated by Hodgkin and Huxley (16) is not strictly adhered to in the case of Na^+ influx. This principle implies that the passage of any ion through the membrane is not influenced by the presence of other ions on either side of the membrane, apart from the contribution of their electrochemical gradients to the transmembrane potential. Deviations from this ideal behaviour are observed with a variety of external ions which can interfere with the passage of Na^+ through the channel (2, 12, 13). To test the validity of this principle for K^+ or Rb^+ outflow via the neuroblastoma channel, efflux was performed in solutions of differing ionic composition. It was found that removal of external Na^+ (isosmotically replaced by Tris-Cl, pH 7.3) resulted in a complete elimination of veratridine-stimulated ion efflux. As shown in Fig. 3a, titration of the Na^+ requirement for ^{86}Rb efflux yielded a hyperbolic relationship, which showed signs of saturation within the physiological range (130 mM). A double reciprocal plot of the same data (Fig. 3b) was linear, and an apparent K_m of 66.7 mM and V_{max} of 0.0953 min^{-1} could be calculated from the curve. Values for passive efflux, which were obtained from the same culture plate prior to veratridine addition, were only slightly (<20%) reduced in a solution devoid of Na^+. Lithium ions efficiently substituted for Na^+ in the activation process (data not shown), and a study of the specificity of the reaction for a series of metal cations is in progress.

These results could be explained either by a direct effect of Na^+ on the conductance mechanism itself, or by a dependence of veratridine activation of the channel on extracellular Na^+. Although the latter cannot as yet be eliminated, it is pertinent to note that recent results on extra ^{22}Na efflux from electrically-stimulated perfused squid axons, have also revealed that removal of external Na^+ causes a dramatic reduction in Na^+ channel flux (17). Such findings appear to have an important bearing on the models currently being constructed from kinetic data for the mode of operation of the channel (12, 13). Several lines of evidence suggest that an external acidic "coordination" site associated with the channel plays a central role in controlling transport through the pore itself (2, 12, 13). This site has

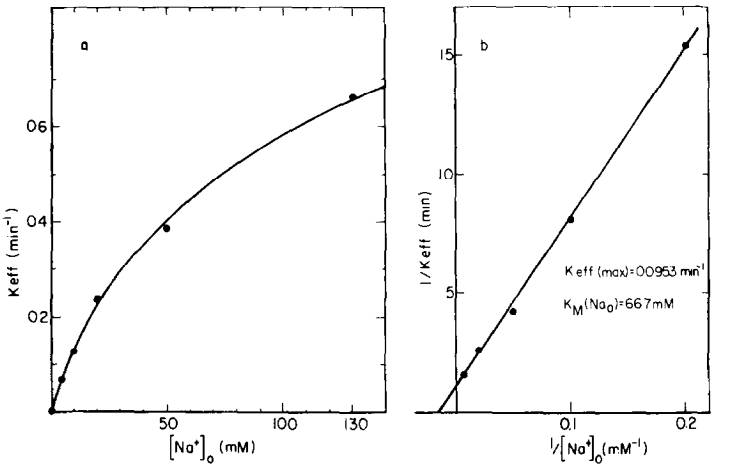

Fig. 3: Sensitivity of veratridine-activated ^{86}Rb efflux to external Na^+, (a) k (rate constant of ^{86}Rb efflux in min^{-1}) plotted against external $[Na^+]$. (b) double-reciprocal (Lineweaver-Burk) plot of the same data as in (a). Cells were loaded as described in Materials and Methods and allowed to efflux into solutions of differing Na^+ concentrations.

the capacity to bind Na^+, other metal cations, TTX and saxitoxin (2, 12-15). The relationship of this site to the transport site or pore is not yet clear, but binding at this point could explain the abovementioned deviations from ion independence. Occupation of the site by an appropriate ion may be a prerequisite event for, and may actually regulate, transport from the second site through the membrane. The dependence of K^+ or Rb^+ efflux on extracellular Na^+ reported here could be interpreted in the light of this hypothesis, in which case the interaction of Na^+ with the first site seems to control the degree of opening of the pore in an apparently linear manner. Although reports on the affinity of Na^+ for this site vary (12-15), a recent study (15) of the inhibition of ^3H-TTX binding to electroplax membrane fragments by Na^+ yielded a K_i value of 71 mM, reasonably close to that found here for activation of ^{86}Rb efflux. Further work utilizing combinations of toxins which act synergistically in promoting fluxes through the channel (7) may help in the precise elucidation of these phenomena.

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