Biochimica et Biophysica Acta, 649 (1981) 625–632
Elsevier/North-Holland Biomedical Press

BBA 79501

POTASSIUM FLUXES IN THE RAT RETICULOCYTE

OUABAIN SENSITIVITY AND CHANGES DURING MATURATION

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(Received June 1st, 1981)

Key words: K^+ transport; Ouabain sensitivity; Maturation; (Rat erythrocyte)

K^+ turnover is markedly enhanced in the rat reticulocyte, both influx and efflux rates being increased by factors of approximately 3 over the corresponding rates in adult cells. These accelerated fluxes are observed despite the absence of any appreciable change in intracellular K^+ concentration during the course of maturation. Qualitative characteristics of the active transport process for K^+ influx appear to be identical in reticulocytes and mature erythrocytes with regard both to K^+ sensitivity, and to ouabain sensitivity as a function of external K^+ concentration. The number of ouabain binding sites per unit volume of cells, however, is increased by a factor of approximately three in the reticulocyte and thus correlates well with the observed degree of enhancement of active K^+ influx in these cells. Half-maximal rates of ouabain-sensitive K^+ influx are observed at external K^+ concentrations well below 1 mM for both reticulocytes and mature erythrocytes. It is concluded that the enhanced rate of K^+ accumulation in the reticulocyte can be quantitatively attributed to an increased number of pump units which are qualitatively identical to those in the mature cell, and which function at a near-maximal rate at the ambient K^+ concentration present in normal rat plasma.

Introduction

Despite considerable interest in processes underlying erythrocyte maturation, and numerous indications that such processes are accompanied by changes in univalent cation transport, no study to date has addressed itself to a detailed comparison of the characteristics of active ion accumulation in reticulocytes and mature erythrocytes in species in which the interpretation of such changes is uncomplicated by accompanying changes in intracellular ionic composition. It is known, for example, that reticulocytes induced by hemorrhage in sheep with low-potassium erythrocytes show a marked increase in both active K^+ accumulation [1–3] and ouabain binding capacity [2] over what is exhibited by mature cells, but the former cells are characterized by a high intracellular K^+ concentration that falls strikingly during the final stages of maturation.

In contrast, the rat erythrocyte, like erythrocytes from the majority of other mammals, is a 'high-potassium' cell whose K^+ concentration changes very little during the process of reticulocyte maturation. In the studies to be described it will be shown that, despite the constancy of its internal ionic composition, maturation of the rat reticulocyte is accompanied by marked changes in the rate of active K^+ influx, passive K^+ efflux and the number of (Na^+,K^+)-ATPase units per cell. Enhanced influx in the reticulocyte appears to be effected by a proportionate increase in the number of pump units per cell, with each pump unit functioning at near-maximal rate and being indistinguishable both qualitatively and in its rate of function from those present in the mature erythrocyte.

Materials and Methods

Materials. Experiments were performed on erythrocytes obtained from male Sprague-Dawley rats

weighing 220–260 g. The animals were maintained on standard laboratory chow (Ralston Purina Co., St. Louis, MO) and were provided with water ad libitum. Ouabain was obtained from the Sigma Chemical Co. (St. Louis, MO) and phenylhydrazine hydrochloride from Matheson, Coleman & Bell (Norwood, OH). [^{3}H]Ouabain (19.5 Ci/mmol) and ^{42}KCl (0.12–0.17 Ci/g K^+) were purchased from the New England Nuclear Corp. (Boston, MA).

Induction of reticulocytosis. Reticulocytosis was induced by the subcutaneous injection of phenylhydrazine hydrochloride according to the protocol described by Bilezikian et al. [4]. Reticulocytes, identified by methylene blue staining, consistently accounted for over 90% of the total erythrocytes in any given preparation.

Preparation of washed erythrocytes. Approx. 7 ml blood were obtained from each animal by exsanguination into a heparinized vessel. After centrifugation at 400 × *g* for 10 min, the plasma and buffy coat were removed and the erythrocytes resuspended in incubation buffer containing 150 mM NaCl, 11.1 mM glucose and 10 mM Tris at pH 7.4. After resedimentation of the erythrocytes and two further washes, the cells were resuspended in incubation buffer and kept on ice unless otherwise indicated.

Hemoglobin and hematocrit determinations. Hematocrits (per cent packed red blood cell volumes) were measured in capillary tubes by means of an Adams microhematocrit centrifuge (Clay-Adams, Inc., New York, NY). Hemoglobin was measured by the cyanmethemoglobin method [5]. Erythrocyte counts were performed under direct observation in a hemacytometer counting chamber.

Measurement of Na^+ and K^+ concentrations. K^+ concentrations in incubation media were checked at the end of each incubation in an Instrumentation Laboratory Model 143 flame photometer (Boston, MA). Intracellular concentrations of K^+ and Na^+ were determined according to the method of Sachs and Welt [6].

Measurement of K^+ influx. K^+ influx was determined by a minor modification of the method of Gardner et al. [7] for turkey erythrocytes. Aliquots of washed, packed erythrocytes were transferred to capped scintillation vials containing prewarmed incubation medium with added stock ^{42}KCl (0.12–0.17 Ci/g K^+) at the indicated total K^+ concentrations in a shaking water bath at 37°C. Final hematocrits in the resulting incubation mixtures ranged between 5 and 10%. At specified times, 100-μl aliquots of the incubation mixtures were transferred to plastic tubes containing 300 μl chilled non-radioactive incubation medium and the erythrocytes were immediately centrifuged down in a Beckman Microfuge (Beckman Instruments, Inc., Palo Alto, CA). After aspiration of the supernatant fluid, the erythrocytes were resuspended in 300 μl fresh cold medium and were re-sedimented. After two additional identical washings, each plastic tube containing the final washed erythrocyte pellet was placed in a 12 × 75 mm glass tube and the radioactivity determined directly in a Packard Auto-Gamma Model 5266 scintillation spectrometer (Packard Instrument Co., Downers Grove, IL). The range of variation between triplicate samples by this method is extremely narrow, maximal variations rarely exceeding 2%.

Measurement of K^+ efflux. K^+ efflux was also determined by a minor modification of the method of Gardner et al. [7]. Washed cell suspensions were incubated for 2 h at 37°C at a hematocrit of approx. 10% in incubation buffer in which the K^+ concentration had been brought to 10 mM by the addition of stock ^{42}KCl. The cells were then chilled, spun down, washed three times with $^{42}K^+$-free incubation buffer containing 10 mM KCl, and resuspended at 0°C in the latter buffer at a hematocrit of approx. 30%. K^+ efflux rates were observed by adding samples of these suspensions to pre-warmed (37°C) aliquots of incubation buffer with 10 mM KCl to a final hematocrit ranging between 5 and 10% in a shaking water bath, and thereafter periodically removing samples to determine the radioactivity present in the external medium. In order to accomplish the latter, 400 μl aliquots of mixture were withdrawn and the red cells immediately centrifuged down in a Beckman Microfuge. 100 μl of the supernatant phase were then withdrawn and placed in a 12 × 75 mm glass tube and the radioactivity determined in a Packard Auto-Gamma Model 5266 scintillation spectrometer. Total initial radioactivity in the incubation mixtures was determined by directly counting 100-μl aliquots, and that initially present in the red cells themselves was determined by subtracting from this value the radioactivity observed in the supernatant samples at zero-time. As

in the influx experiments, the range of variation between triplicate samples by this method is extremely narrow, maximal variations rarely exceeding 2%.

Binding of [^{3}H]ouabain. Suspensions of washed erythrocytes were added to glass scintillation vials containing incubation medium and [^{3}H] ouabain either in the presence or absence of an additional displacing concentration (1 mM) of non-radioactive ouabain as indicated below. Stock [^{3}H]ouabain (19.5 Ci/mmol) in ethanol : benzene (9 : 1) was evaporated to dryness under nitrogen and redissolved in buffer prior to use, and a known concentration of non-radioactive ouabain added to reduce the specific activity of the radioligand to a range of 1.2 to 2.2 Ci/mmol. The vials were then capped, and the resulting suspensions, at a final hematocrit ranging between 3 and 5%, were incubated in a shaking water bath at 37°C. Ouabain binding to erythrocyte membranes was determined by transferring duplicate 100-μl aliquots of incubation mixture to 5 ml distilled water at 0°C, vortex mixing, and trapping the hemolyzed erythrocyte membranes on Gelman A/E glass filters (Gelman Instrument Co., Ann Arbor, MI). The filters were washed four times with additional 5-ml aliquots of iced distilled water and transferred to glass scintillation vials, and radioactivity was determined in a Packard Model 3003 liquid scintillation spectrometer after the addition of 10 ml Bray's solution [8].

Results

Fig. 1 shows K^+ influx as a function of time for both mature erythrocytes and reticulocytes incubated in the presence of $^{42}K^+$. Influx in the mature cells proceeds linearly with time throughout the 45-min period of observation, whereas the rate of influx in the reticulocytes shows a gradual decrease with time. Comparison of the initial rates of K^+ influx in normal mature cells and reticulocytes in multiple experiments showed a consistent 3–4-fold greater rate of influx in the immature cells, yielding mean influx rates of 5.7 ± 0.3 and 21.1 ± 0.9 mequiv./l cells per h, respectively (means ± 1 S.E.).

K^+ efflux from reticulocytes, studied in parallel experiments, was similarly found to be enhanced, as shown in Fig. 2. Fractional efflux of $^{42}K^+$ from cells pre-loaded with radioisotope is constant with time for both mature cells and reticulocytes, and, as is the

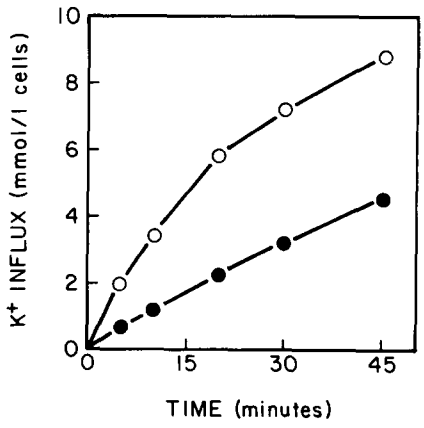

Fig. 1. K^+ influx as a function of time in rat reticulocytes and mature erythrocytes. External K^+ concentration 10 mM. Open circles, reticulocytes; closed circles, mature erythrocytes. Points indicate total influx; at 10 mM external K^+, 75% to 80% of total influx is inhibitable by ouabain (see legend to Fig. 4).

case for influx, efflux is considerably more rapid in reticulocytes. Multiple determinations of the rate constants for K^+ efflux for normal cells as compared to reticulocytes yielded values of 0.094 ± 0.006 and 0.262 ± 0.014 h^{-1}, respectively (means ± 1 S.E.). In contrast to the substantial increment in bi-directional K^+ fluxes observed in the reticulocyte, intracellular K^+ and Na^+ concentrations in reticulocytes (101 ± 5 and 3.7 ± 0.1 mmol/l cells, respectively) were found

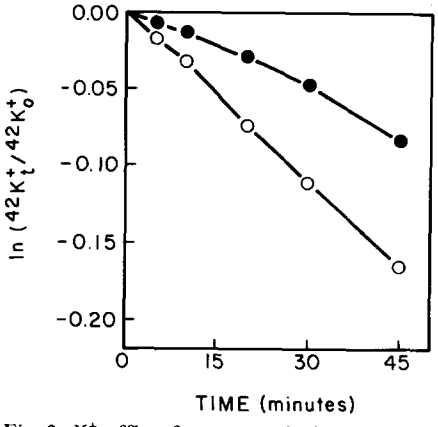

Fig. 2. K^+ efflux from rat reticulocytes and mature erythrocytes. External K^+ concentration 10 mM. The logarithm of the fraction of $^{42}K^+$ initially present in the cells at $t = 0$ is plotted as a function of time. Open circles, reticulocytes; closed circles, mature erythrocytes.

to be substantially the same as those in mature erythrocytes (98 ± 1 and 4.3 ± 0.3). The latter values agree well with those reported earlier for rat erythrocytes by Beaugé and Ortíz [9].

Although both influx and efflux rates for K^+ were shown to be enhanced in reticulocytes, the quantitative dependence of ouabain-sensitive K^+ influx rate upon external K^+ concentration was found to be virtually identical to that for mature erythrocytes. Fig. 3 shows that the form of the K^+ concentration-response curve for both cells is identical, the ratio of the ordinates of the two curves at any given external K^+ concentration being constant, and that when influx is expressed as per cent of maximum the two curves are in fact superimposable. Hill plots of the relation between ouabain-sensitive influx rate and external K^+ concentration show consistent slopes of two, corresponding to earlier observations on erythrocytes from other species that simultaneous occupancy of two potassium-binding sites is required for active K^+ translocation (see, for example, Ref. 4).

The similarity of the dependence of K^+ influx upon external K^+ concentration for both reticulocytes and mature erythrocytes observed in the preceding experiments suggested qualitative similarity between the effector units responsible for active K^+ accumulation in the two types of cell. Further evidence of similarity was provided in another series of experiments in which the sensitivity of active K^+ accumulation to inhibition by ouabain was examined at a variety of different external K^+ concentrations. Fig. 4 shows that, at each external K^+ concentration examined, the concentration-response curves to varying concentrations of ouabain were indistinguishable in reticulocytes and mature erythrocytes, fractional inhibition of active K^+ accumulation in all instances being identical for both cells at any given concentration of ouabain.

Several additional aspects of the relationships shown in Fig. 4 are of interest. First, the concentra-

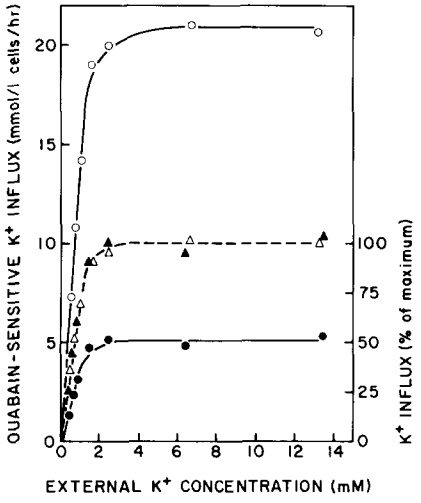

Fig. 3. Ouabain-sensitive K^+ influx in reticulocytes and mature erythrocytes as a function of external K^+ concentration. The difference in K^+ influx in the absence and presence of a maximally inhibitory concentration of ouabain (5 mM) was determined at each external K^+ concentration. Left-hand ordinate (solid lines), influx expressed as mmol K^+ per l cells per h (open circles, reticulocytes; closed circles, mature erythrocytes); right-hand ordinate (dashed line), influx expressed as per cent of maximum (open triangles, reticulocytes; closed triangles, mature erythrocytes).

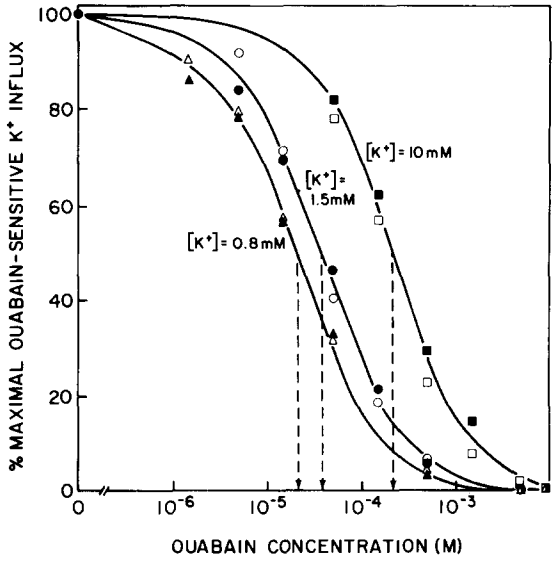

Fig. 4. Per cent of maximally inhibitable K^+ influx as a function of ouabain concentration at three different external K^+ concentrations. Open symbols, reticulocytes; closed symbols, mature erythrocytes. Triangles, $[K^+] = 0.8$ mM; circles, $[K^+] = 1.5$ mM; squares, $[K^+] = 10$ mM. Half-maximal inhibitory concentrations of ouabain at these three external K^+ concentrations are $2.1 \cdot 10^{-5}$ M, $3.8 \cdot 10^{-5}$ M and $2.1 \cdot 10^{-4}$ M, respectively. Ouabain-inhibitable influx, expressed as per cent of total influx at these three external K^+ concentrations, is 95%, 94% and 81%, respectively.

tions of ouabain required for half-maximal inhibition of influx are far higher than the corresponding concentrations which have previously been shown to inhibit influx at comparable external K^+ concentrations in erythrocytes from other species [10,11]. Second, at external K^+ concns. substantially exceeding the estimated dissociation constant for the potassium ion-ATPase complex for erythrocytes from other species [11] and that required for half-maximal ouabain-sensitive K^+ influx in the rat cells themselves (less than 1 mM, see Fig. 3), the concentration of ouabain required for half-maximal inhibition of K^+ influx is closely proportional to the external K^+ concentration itself (see theoretical discussion in Ref. 9). Although the form of the relationship between ouabain sensitivity and external K^+ concentration follows that observed for erythrocytes from other species, the preceding results confirm in a quantitative fashion the striking resistance of rat red cells to the inhibitory effects of ouabain noted in earlier experiments [12, 13]. Table I presents a comparison of our quantitative observations in rat reticulocytes and mature erythrocytes with previous observations on mature human and turkey erythrocytes. Rat cells are seen to be less sensitive to ouabain than turkey cells by two orders of magnitude, and less sensitive than human cells by between three and four orders of magnitude.

Despite their decreased sensitivity to ouabain, mature rat erythrocytes and reticulocytes were found to respond to changing external ouabain concentrations in a perfectly classical manner. Fig. 5 presents an analysis of the results shown in Fig. 4 by a method analogous to that of Scatchard [14]. The linearity of the plots is consistent with the interaction of a single ouabain molecule per pump site,

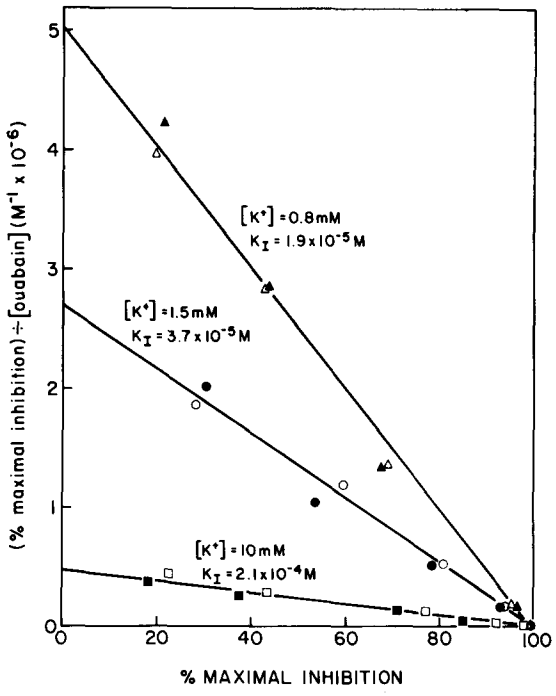

Fig. 5. Plot of (% maximal inhibition ÷ ouabain concentration) vs. (% maximal inhibition) as a function of external K^+ concentration. The linearity of this relationship indicates that, at all concentrations of K^+ examined, inhibition of ion transport is strictly proportional to ouabain binding, and hence that membrane (Na^+,K^+)-ATPase sites function independently of each other (see text). Open symbols, reticulocytes; closed symbols, mature erythrocytes.

TABLE I

COMPARISON OF OUABAIN SENSITIVITY IN ERYTHROCYTES FROM DIFFERENT SPECIES

External $[Na^+]$ = 150 mM in all instances.

Species; cell type	Concentration of ouabain required for 1/2-maximal effect		
	$[K^+]$=0.8 mM	$[K^+]$ = 1.5 mM	$[K^+]$ = 10 mM
Human erythrocyte [a]	$1.2 \cdot 10^{-8}$ M	$1.9 \cdot 10^{-8}$ M	$7.9 \cdot 10^{-8}$ M
Turkey erythrocyte [b]	$3.6 \cdot 10^{-7}$ M	$5.7 \cdot 10^{-7}$ M	$3.1 \cdot 10^{-6}$ M
Rat erythrocyte (mature)	$2.1 \cdot 10^{-5}$ M	$4.2 \cdot 10^{-5}$ M	$2.3 \cdot 10^{-4}$ M
Rat reticulocyte	$2.1 \cdot 10^{-5}$ M	$3.5 \cdot 10^{-5}$ M	$1.9 \cdot 10^{-4}$ M

[a] Calculated from Ref. 10.
[b] Calculated from Ref. 11.

and moreover indicates that each pump unit functions independently of its neighbors; i.e., that inhibition of any given number of sites by ouabain does not affect the rate of K^+ transport at unoccupied sites. The slope of the lines in each instance provides a measure of the association constant for ouabain with the pump site, and its negative reciprocal indicates the concentration of ouabain required for half-maximal inhibition of transport at the three different external K^+ concentrations.

The preceding observations are all consistent with the following conclusions: (i) both mature rat red cells and reticulocytes possess an ouabain-sensitive ATPase with qualitative characteristics similar to those described for erythrocytes from other species but exhibiting a markedly reduced affinity for the glycoside; (ii) the qualitative characteristics of the ATPase (including its rate of pumping as a function of external K^+ concentration, as well as its ouabain sensitivity and the dependence of the latter upon external K^+ concentration) are similar in mature cells and reticulocytes; and (iii) the number of active pump sites is markedly increased in the latter cells. Direct confirmation of these conclusions should in principle be provided by an examination of radioactive ouabain binding, which additionally should provide a direct measure of the absolute number of binding sites (and hence of the number of pump units) per cell. While such studies can be readily performed on erythrocytes from other species (see Refs. 8 and 9), the low affinity of the rat cells for the radioligand requires such high free radioligand concentrations to result in satisfactory levels of receptor saturation that nonspecific binding contributes substantially to total binding, and hence background 'noise' is greater than for other analogous systems. Nevertheless, by performing experiments at sufficiently low external K^+ concentrations (so that binding affinity is maximized), data can be obtained which result in satisfactory Scatchard [14] plots. Fig. 6 shows the results of such an experiment comparing the binding of ouabain by reticulocytes and mature erythrocytes, in this instance carried out at an external K^+ concentration of 0.4 mM. The slopes of the two lines are identical, indicating a dissociation constant for the ouabain-ATPase complex at this K^+ concentration that is the same for reticulocytes and mature erythrocytes and of the order of $9 \cdot 10^{-6}$ M. The different intersections

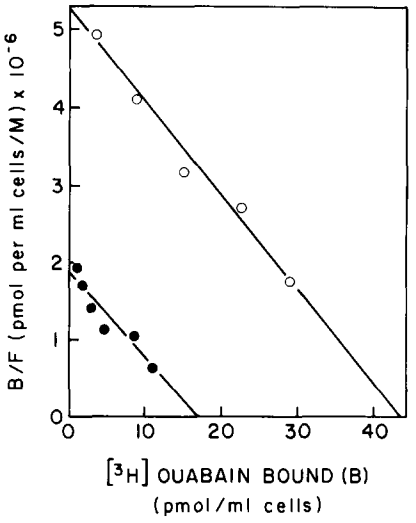

Fig. 6. Scatchard [14] analysis of ouabain binding by rat reticulocytes (open circles) and mature rat erythrocytes (closed circles) in the presence of 0.4 mM external K^+. Both lines have identical slopes corresponding to a dissociation constant for the ouabain-ATPase complex, at this external K^+ concentration, of $8 \cdot 10^{-6}$ M. The different intersections on the abscissa indicate an ouabain binding capacity that is about 3-times as great per unit vol. cells for reticulocytes as it is for mature erythrocytes.

of the two lines on the abscissa indicate that the number of binding sites per unit vol. cells is approx. 3-times as great for reticulocytes (44 pmol/ml cells) as it is for mature erythrocytes (16 pmol/ml cells). The linearity of these plots correlates with that of the analogous plots for inhibition of active ion transport shown in Fig. 5, and further confirms that inhibition of active K^+ influx is directly proportional to occupancy of membrane (Na^+,K^+)-ATPase sites by ouabain with a stoichiometry of one molecule of glycoside per site.

Discussion

The preceding observations indicate that K^+ fluxes in the rat reticulocyte are enhanced by a factor of 3 to 4 over those in the mature erythrocyte. In contrast to earlier observations in other species in which mature red cells have a low intracellular K^+ concentration, and where enhanced K^+ transport in reticulocytes has been shown to be associated with a strikingly higher K^+ concentration in the immature cell

[1–3], the enhanced transport of K^+ in the immature rat cell is observed in the absence of any appreciable change in intracellular K^+ concentration during the process of cell maturation. The enhancement of K^+ fluxes becomes even more striking when calculated per cell or on the basis of estimated surface area. Mean cell volume for the reticulocytes in these studies was increased by a factor of 1.69 ± 0.12 (mean ± 1 S.E.) over that for the mature cells, leading to a calculated 6-fold increase in K^+ transport per cell, and an estimated 4- to 5-fold increase in ion flux per unit of surface area, based on an assumption of a comparable geometry of the two cell types.

As would be expected under ordinary steady-state conditions, the independently measured rate of passive K^+ efflux was found in each of the present experiments to be enhanced in strict proportion to the rate of K^+ influx. Bernstein [15] first described enhanced rates of K^+ accumulation and Na^+ extrusion in reticulocytes in 1959, and Wiley and Schaller [16] have recently reported an increase in both ouabain binding and active K^+ influx in reticulocyte-rich human blood. Strikingly enhanced influx rates in reticulocytes for Ca^{2+} [16], Na^+ [16] and amino acids [17], as well as an increased rate of glucose utilization [15], have additionally been demonstrated in previous studies. As has been shown to be the case for erythrocytes from other species [18,19], the ouabain concentration-dependence of the inhibition of active K^+ influx in these rat cells displays a form consistent with simple hyperbolic binding of the glycoside and linearity between binding and inhibition of transport. Despite the markedly enhanced rate of active K^+ accumulation observed in the rat reticulocyte, the calculated affinity constants for ouabain and the ATPase in both reticulocytes and mature cells are identical, suggesting marked similarity, if not actual identity, of the ATPase units in the mature and immature cells.

The strong dependence of ouabain sensitivity upon external K^+ concentration was found to be identical for both reticulocytes and mature erythrocytes, and moreover to be of a form indistinguishable from that previously observed for ouabain binding and for ouabain-induced inhibition of active K^+ transport in detailed studies on both human [10,18] and turkey [11,19] erythrocytes. The magnitude of the affinity constant for ouabain at any given external K^+ concentration, however, shows enormous variation between these three species, being lower for the rat cell than for turkey or human cells by factors of approximately 100 and 2000, respectively (Table I). Direct estimation of rat-cell ouabain affinities under conditions of varying external K^+ concentration in the present experiments confirms earlier qualitative observations of others and explains the well-known resistance of rat tissues to concentrations of cardiac glycosides that are maximally inhibitory in other species (see review in Ref. 20). In contrast to the profoundly reduced binding affinity, which was shown to be identical in mature and immature cells, the number of binding sites per unit vol. reticulocytes was shown to be triple that present in mature cells. This number correlates well with the magnitude of the increase in reticulocyte (Na^+,K^+)-ATPase activity previously demonstrated for cell membrane preparations by classical enzymatic techniques in vitro [12].

A priori, the observed 3–4-fold enhancement of K^+ influx in reticulocytes over that observed in mature red blood cells might be conceived of as occurring by one of three possible mechanisms: (1) the increased rate of influx might represent a 3–4-fold increase in the rate of ion transport per ATPase pump site; (2) the rate of pumping per site might instead remain constant but additional pumps become activated; or (3) the total number of pump sites itself might be increased. The first possibility would appear unlikely in view of the shape of the concentration-response curve for active K^+ influx as a function of external K^+ concentration: Fig. 3 showed that active K^+ influx rate rises rapidly with external K^+ concentration and is already maximal at ambient K^+ concentrations corresponding to those present in normal rat plasma (6.2 ± 0.3 mM [21]). While the second possibility cannot be formally excluded, the radioactive ouabain binding data provide direct evidence in support of the third possibility by showing that radioactive ouabain binding is enhanced in reticulocytes by almost exactly the same proportion as that by which the rate of active K^+ transport is increased. This being the case, it would appear that the enhanced rate of K^+ accumulation in the reticulocyte can be quantitatively attributed to an increase in pump units qualitatively identical to those in the mature cell, and functioning, as they do in the latter cell, at near-maximal rate. The mechanism by which the presumed decrease in K^+-leakiness,

resulting in a decreased requirement for active K^+ influx, leads to a proportionate loss of (Na^+,K^+)-ATPase membrane pump units during the course of cell maturation poses an interesting problem for future study.

Acknowledgements

This work was supported in part by United States Public Health Service Grants HD-05506, HL-20859, and AM-07271. J.P.B. is the recipient of Research Career Development Award 5 K04 HL-00383.

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