Biochimica et Biophysica Acta 859 (1986) 56–60
Elsevier

BBA 73138

Analysis of the all or nothing behaviour of Ca-dependent K channels in one-step inside-out vesicles from human red cell membranes

Javier Alvarez, Javier García-Sancho * and Benito Herreros

Departamento de Fisiología y Bioquímica, Facultad de Medicina, Universidad de Valladolid, 47005-Valladolid (Spain)

(Received January 23rd, 1986)

Key words K^+ transport, K^+ channel, Ca^{2+} dependence, Redox state, Inside-out vesicle, (Erythrocyte membrane)

The all or nothing behaviour of Ca^{2+}-dependent K^+ channels has been analyzed in one-step inside-out vesicles. There is a threshold for Ca^{2+} below which the K^+ channels remain silent, and which ranges between the 10^{-6} and 10^{-8} M for different vesicles under the experimental conditions tested, in the absence of Mg^{2+}. The increase of Ca^{2+} concentration within this range recruits a larger fraction of the vesicles to the active (permeable to $^{86}Rb^+$) state. The apparent rate of $^{86}Rb^+$ transport through each individual channel was found to increase, however, with Ca^{2+} concentration This finding is not an artefact due to size heterogeneity of the vesicle population, and it is consistent with the variations of the mean open time of the channels with Ca^{2+} concentration reported previously in patch-clamp experiments. The electron donor system ascorbate + phenazine-methosulphate increases the rate of $^{86}Rb^+$ transport through the channels whereas oxidized cytochrome *c* has the opposite effect.

Introduction

It has been reported before that Ca^{2+} accelerates the uptake of $^{86}Rb^+$ by one-step inside-out vesicles derived from the human erythrocyte membrane by activation of the Ca^{2+}-dependent K^+ channel [1,2] The rate of $^{86}Rb^+$ uptake remains increased during the first few minutes after Ca^{2+} addition, returning afterwards to about the same value observed without Ca^{2+} [2] The amount of $^{86}Rb^+$ taken up during the rapid uptake phase, henceforth called $^{86}Rb^+$ level at the 'steady-state', increases with the Ca^{2+} concentration within the 10^{-8} to 10^{-6} M range This has been interpreted to reflect an all or nothing behaviour of the individual vesicles vesicles with activated channels equilibrate with ^{86}Rb during the first few minutes of incubation, after that, the rate of uptake depends on the influx of ^{86}Rb into vesicles whose channels were not activated at all, declining for this reason to about the same value observed in the Ca^{2+}-free situation The value of the Ca^{2+}-dependent $^{86}Rb^+$ uptake reached at the steady-state reflects then the fraction of vesicles whose channels were activated by a particular Ca^{2+} concentration

Since the mean number of channels per vesicle is close to one [1], the all or nothing behaviour of the vesicles has been proposed to reflect the behaviour of the individual channels [1,2] This view assumes that only two states of the channel, either active or inactive, are possible and that the shift from one to the other requires a change of Ca^{2+} concentration Models assuming the existence of at least two Ca^{2+}-binding sites, one of which can

* To whom correspondence should be addressed

Abbreviations EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid, Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, asc-PMS 1 mM potassium ascorbate + 0 01 mM phenazine methosulphate

be occluded on occupation of the other, have been proposed to explain this behaviour [1,3] A pure all or nothing response seems, however, difficult to reconcile with observations from patch-clamp experiments, where a progressive increase of the mean open time of the K^+ channels with the increase of the Ca^{2+} concentration has been reported [4,5]

If intermediate activation states of the K^+ channels do exist, a decrease of the relative rate of $^{86}Rb^+$ transport on going from maximally- to submaximally-activating Ca^{2+} concentrations should be expected The time-course of Ca^{2+}-dependent $^{86}Rb^+$ transport by inside-out vesicles does not follow single exponentials, suggesting size heterogeneity of the vesicles [2] Since the rate of equilibration of the vesicles with the external $^{86}Rb^+$ depends on its volume/surface ratio, differences among inside-out vesicles subpopulations, which may differ in vesicular size, should be taken into account on interpreting the results

In this work we have investigated the effects of Ca^{2+} on the rate of $^{86}Rb^+$ uptake by inside-out vesicles The possible contribution of vesicular size heterogeneity has been evaluated Finally, the effects of redox agents, which have been shown before to modify the sensitivity (threshold) to Ca^{2+} of the K^+ channels [6], have also been studied

Materials and Methods

Methods for preparation of one-step inside-out vesicles from human erythrocyte membranes and for measurements of $^{86}Rb^+$ transport were as described before [2,6] $^{86}Rb^+$ behaves similarly to K^+ for transport through the Ca^{2+}-dependent channels in red cells [7] The composition of the final incubation medium used for the flux measurements was (in mM) KCl, 18, K-Hepes (pH 7 5), 16 5, Tris-EGTA, 0 4, and $CaCl_2$ to give the Ca^{2+} concentrations shown in the figures, which were calculated using a value of $6\,5\ \ 10^{-8}$ for the apparent dissociation constant of EGTA The concentrations of K^+, Cl^- and Hepes inside the vesicles were the same as in the medium This simplifies the interpretation of the results, especially in view of the 'single-file diffusion' behaviour proposed recently for the Ca^{2+}-dependent K^+ channels of human red cells [8] Experimental details for the use of redox agents were as described previously [6]

$^{86}Rb^+$ chloride was purchased from Amersham International, p l c Chemicals were obtained either from Sigma London, Chem Co Ltd , Boehringer Mannheim or E Merck, Darmstadt

Results and Discussion

Fig 1 shows the kinetics of Ca^{2+}-dependent $^{86}Rb^+$ uptake by inside-out vesicles at different Ca^{2+} concentrations The effects of redox agents, shown also in Fig 1 (central and right-hand side panels), will be discussed later The $^{86}Rb^+$ uptake at the steady state (20 min) is also shown in the figure The time-course of the uptake of $^{86}Rb^+$ during the first 5 min could not be described by single exponentials For this reason the time needed to take up one half of the amount of $^{86}Rb^+$ taken up at the steady state ($T_{1/2}$) was used as an operational parameter to compare the rate of uptake at different Ca^{2+} concentrations

Fig 2 shows the relation of $T_{1/2}$ to the fraction of activated vesicles reached at the steady state, expressed as per cent of maximal activation $T_{1/2}$ decreased about 5 times in passing from 40% activation to 100% activation The changes of $T_{1/2}$ with variations of the fraction of activated vesicles seem inconsistent with a pure all or nothing response of the channels The simplest interpretation is that, even though a threshold for Ca^{2+} below which the K^+ channel is permanently closed seems to exist, submaximal Ca^{2+} concentrations are not associated with an 'always-open' state of the active channels, but intermediate states exist This view is consistent, on the other hand, with the changes of the mean open time of the channels observed with variations of the Ca^{2+} concentration in patch-clamp experiments [4,5]

The correct interpretation of our results must include, however, an evaluation of the possible effects of the heterogeneity of vesicles' size on the estimates of the $T_{1/2}$ values In fact, even if pure all or nothing behaviour were applicable, size heterogeneity would tend to displace the results towards the direction observed On purely statistical considerations the larger vesicles would be more likely to be activated at the lower Ca^{2+} concentra-

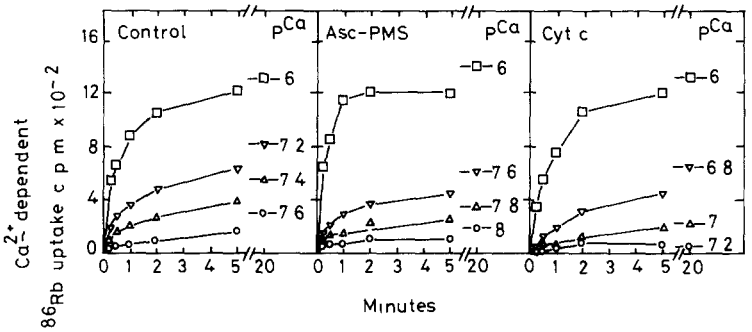

Fig 1 Time-course of Ca^{2+}-dependent ^{86}Rb uptake by one-step inside-out vesicles The uptake of ^{86}Rb in the absence of Ca^{2+} (0 4 mM EGTA) was not modified by the addition of either asc-PMS or cytochrome *c*, and it has been substracted from all the values Asc-PMS 1 mM potassium ascorbate + 0 01 mM phenazine methosulphate, catalase (800 U/ml) was also added to the medium to avoid oxidative damage of the vesicles [6] Cyt *c* 0 03 mM oxidized cytochrome *c* The experiments were performed at 20 °C The values of p*Ca* are indicated at the right-hand side for each curve Note that the values of p*Ca* for each symbol differ from panel to panel

tions, since they should contain a larger number of channels per vesicle Owing to the larger volume/surface ratio in these vesicles and also to the fact that only one or a few among many channels would be active in each vesicle, the $T_{1/2}$ would be longer than in the smaller vesicles

The previous observation that fractions of activated vesicles as low as 5–10% can be obtained at the lower Ca^{2+} concentrations [2] argues against the presence of a large fraction of the $^{86}Rb^+$ space contained within a few large vesicles with many channels per vesicle It seems critical, however, to obtain a more direct indication of the size distribution within our population of inside-out vesicles in order to interpret correctly the results Morphologic measurements are meaningless since only the everted vesicles, which include only 20–40% of the total membrane surface, are relevant to our experiments

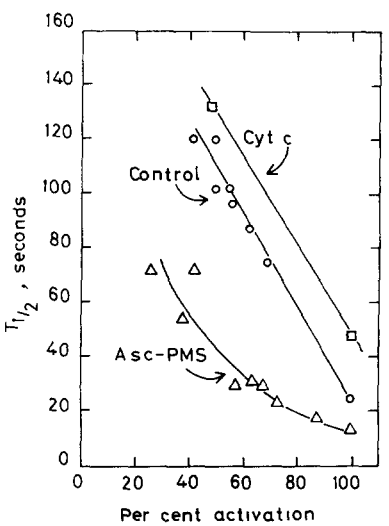

Fig 2 Half-equilibration times for Ca^{2+}-dependent ^{86}Rb uptake by one-step inside-out vesicles at different fractions of activated vesicles Values were calculated from experiments similar to those shown in Fig 1 The fraction of activated vesicles was estimated by extrapolation of the slow rate phase of ^{86}Rb uptake (20–50 min of incubation) to zero-time [2]

The approach chosen to test the effects of size heterogeneity of the measured $T_{1/2}$ values was as follows inside-out vesicles were incubated during 30 min with $^{86}Rb^+$ at a submaximal Ca^{2+} concentration, which activated only about 50% of the vesicles The fraction of vesicles equilibrated with $^{86}Rb^+$ by the end of this incubation period should include the larger ones according to the above reasoning Then the vesicles were transfered to Ca^{2+}-free medium Under this conditions no measurable loss of $^{86}Rb^+$ from the vesicles took place The vesicles' suspension was then divided into two aliquots which were transferred either to medium containing $^{86}Rb^+$ at the same specific activity as that used during the first incubation or to medium containing no radioactivity Finally, the Ca^{2+} concentration was increased to 1 μM, a concentration which gives maximal activation of the K^+ channels, and the changes of the $^{86}Rb^+$ contents of the

vesicles with time were followed The results of one of such experiments are shown in Fig 3 In the vesicles incubated with $^{86}Rb^+$ the intravesicular radioactivity increased to reach about the same level as in the maximally activated vesicles On the contrary, the vesicles incubated in medium containing no radioactivity lost $^{86}Rb^+$ to attain about the same level as in the vesicles incubated with $^{86}Rb^+$ in Ca^{2+}-free medium In the first case we are observing the influx of $^{86}Rb^+$ in the fraction of vesicles which were not activated during the first 30-min incubation with a submaximal Ca^{2+} concentration In the second case we are observing the efflux of $^{86}Rb^+$ from the fraction of vesicles which were activated during the first 30-min incubation with a submaximal Ca^{2+} concentration We can then compare directly the kinetics of $^{86}Rb^+$ transport by both subpopulations of inside-out vesicles The $T_{1/2}$ values estimated from Fig 3 for the influx and the efflux of $^{86}Rb^+$ were, 0 37 and 0 25 min, respectively Contrary to our expectations the uptake (supposedly by the smaller vesicles) was somewhat slower than the loss (supposedly from the larger vesicles) In any case the differences were small indicating that size heterogeneity does not have a large influence in determining differences in kinetics of $^{86}Rb^+$ transport by fractions of vesicles which have a different threshold for Ca^{2+}

It we now reexamine Fig 2, we must conclude that a pure all or nothing behaviour is incompatible with our results Even though each channel has a threshold for Ca^{2+}, below which no measurable activation occurs, at submaximal Ca^{2+} concentrations a gradual activation of the individual channels can take place, with an increase of the apparent permeability on increasing Ca^{2+} concentrations This conclusion is, on the other hand, consistent with the observations performed with isolated membrane patches, where changes of the mean open time with changes of the Ca^{2+} concentration have been reported [4,5]

A second aspect which deserves discussion is the effect of redox agents on the kinetics of $^{86}Rb^+$ transport through the Ca^{2+}-dependent K^+ channel The electron donor agent asc-PMS increased the fraction of activated vesicles at a given Ca^{2+} concentration (compare the 20-min $^{86}Rb^+$ uptake in left-hand side and central panels of Fig 1), and

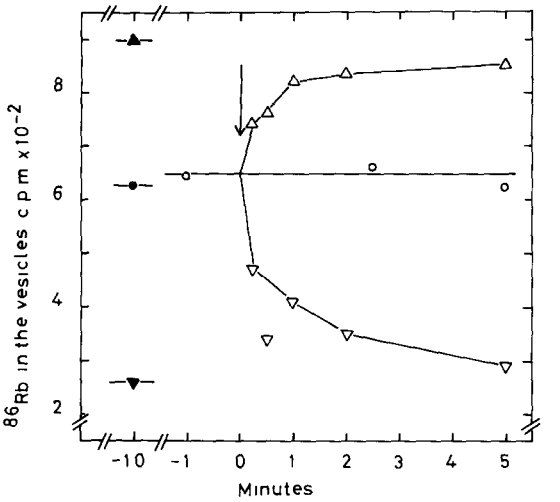

Fig 3 Time-course of ^{86}Rb transport by vesicle subpopulations with different Ca^{2+} thresholds One-step inside-out vesicles were first incubated during 30 min with ^{86}Rb at three different pCa values ≤ 9 (0 4 mM EGTA, ▼), 7 2 (●) and 6 (▲) The ^{86}Rb uptake by the end of this period is shown at $t = -10$ min in the figure The vesicles were then sedimented by centrifugation (5 min at $12000 \times g$) and resuspended at about 100% equivalent haematocrit [1] in Ca^{2+}-free (0 4 mM EGTA) medium Only the data obtained with the vesicles first loaded at pCa 7 2 are shown in the figure for clarity After 10 min incubation in Ca^{2+}-free medium ($t = 0$ in the figure) the vesicles' supension was divided in three aliquots which were diluted with 10 volumes of medium containing EGTA and Ca as required to give a pCa of either ≤ 9 (○) or 6 (△ and ▽) In one case (▽) the diluting medium contained no radioactivity whereas in the other two (○) and (△) ^{86}Rb was added to give the same specific activity as in the first loading incubation The $T_{1/2}$ value for the loss of ^{86}Rb from vesicles first loaded at pCa 6 (▲) on dilution with ^{86}Rb-free medium at pCa 6 did not differ significantly from the $T_{1/2}$ for the uptake of ^{86}Rb at pCa 6 by vesicles first loaded in Ca^{2+}-free medium (▼) These results have not been represented in the figure for clarity

cytochrome c had the reverse effect (right-hand side panel of Fig 1) This result confirms previous observations [6] The rate of equilibration with ^{86}Rb was also affected by the redox agents (Fig 2) Asc-PMS decreased $T_{1/2}$ to 1/2–1/3 of the control values at all the Ca^{2+} concentrations tested, including the one which activates 100% of the vesicles On the contrary, cytochrome c increased $T_1/_2$ to about twice the control value at the highest Ca^{2+} concentration (Fig 2) The accelerating effect of asc-PMS on the uptake of $^{86}Rb^+$ could be thought to arise from the reactivation of channels

otherwise insensitive to Ca^{2+} This effect has been reported in ghosts with inactivated K^+ channels [9] If that were the case, asc-PMS would increase the vesicular space which equilibrates with $^{86}Rb^+$ at the steady-state under conditions of maximal stimulation by Ca^{2+} However, this has not been observed (Ref 6, see also Fig 1) On the other hand the opposite effect should be expected of cytochrome *c*, that is a decrease of the vesicular space which equilibrates with $^{86}Rb^+$ under conditions of maximal stimulation by Ca^{2+}, and this was not observed either [6]

An alternative explanation would be to postulate two different states of the K^+ channel, one oxidized and the other reduced, the last one having a 3–4-times larger conductance The control situation in our experiments would be an intermediate one with some channels in the reduced state and some in the oxidized one The hypothesis is consistent with the observation of low-conductance states of this channel in patch clamp experiments [4] In this way the redox state would modulate not only the Ca^{2+} affinity (threshold) of the K^+ channel, as reported before [6], but also the conductance of the channels The importance of this double modulation is unknown at the moment

Acknowledgement

Financial support from the spanish Fondo Nacional para el Desarrollo de la Investigación Científica (Proyecto no 2873/83) is gratefully acknowledged

References

1 Lew, V L , Muallem S and Seymour, C A (1982) Nature 296, 742–744
2 García-Sancho, J , Sanchez, A and Herreros B (1982) Nature 296, 744–746
3 Lew, V L Muallem, S and Seymour C A (1983) Cell Calcium 4, 511–517
4 Grygorczyk, R and Schwarz W (1983) Cell Calcium 4, 499–510
5 Grygorczyk, R Schwarz, W and Passow H (1984) Biophys J 45, 693–698
6 Alvarez, J , García-Sancho, J and Herreros B (1984) Biochim Biophys Acta 771, 23–27
7 Simons T J B (1975) J Physiol 256, 209–225
8 Vestergaard-Bogind, B , Stampe P and Christophersen P (1985) J Membrane Biol 88, 67–75
9 Alvarez, J Garcia-Sancho J and Herreros, B (1985) J Physiol 369, 113P