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An estimate of the number of Ca^{2+}-dependent K^+ channels in the human red cell

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An original approach has been designed to count Ca^{2+}-dependent K^+ channels in the human red cell using a preparation of inside-out vesicles. The relative frequency of vesicles having no K^+ channels is estimated from the fraction of $^{42}K^+$ (or $^{86}Rb^+$) which is not released from loaded vesicles on maximal stimulation with Ca^{2+}. The mean number of channels per vesicle is then calculated from this figure assuming a Poisson distribution for the K^+ channels. From this value and the mean vesicular radius, computed from the volume/surface ratio, the mean number of channels per cell can be estimated. A value of 142 ± 27 (mean $\pm$ S.E.) was obtained, which is well above that estimated by comparison of unitary conductance and tracer equilibration rate measurements (about 10 channels/cell, Grygorczyk, R. Schwarz, W. and Passow, H. (1984) Biophys. J. 45, 693–698), but compares favourably with the channel density inferred from comparison with the number of Na^+ pumps in a similar preparation of inside-out vesicles (100–200/cell, Lew, V.L., Muallem, S. and Seymour, C.A. (1982) Nature 296, 742–744). The procedure described here can be considered for general application as an alternative to other known procedures.

Usual procedures to count channels include the use of specific high-affinity ligands or measurements of gating current or single channel conductance [1]. High-affinity ligands for Ca^{2+}-dependent K^+ channels of red cells are not available at present. On the basis of comparison of measurements of unitary conductance in membrane patches and tracer equilibration rate in intact cells a mean number of about 10 Ca^{2+}-dependent K^+ channels per cell has been estimated [2]. In another study using inside-out vesicles a mean number of 100–200 channels per cell has been proposed on the basis of comparison of the fraction of intravesicular $^{86}Rb^+$ which was released on maximal activation of either Na^+ pumps or Ca^{2+}-dependent K^+ channels. Since both fractions were similar, it was concluded that the numbers of channels and pumps were also similar [3]. In the present paper we propose a procedure of application to inside-out vesicles based on the simultaneous estimate of the mean number of channels per vesicle and the mean vesicular radium.

One-step inside-out vesicles [3] were prepared as described elsewhere [4]. Vesicles were loaded with the tracer, either $^{86}Rb^+$ (Amersham International) or $^{42}K^+$ (produced from ^{42}Ar in a generator provided by Professor H. Morinaga, Physik-Department, Technische Universität, München, F.R.G.), added before the vesiculation step. Flux measurements were performed under equilibrium exchange conditions for K^+ and Cl^- as described previously [4,5].

Fig. 1 shows the results of a typical experiment

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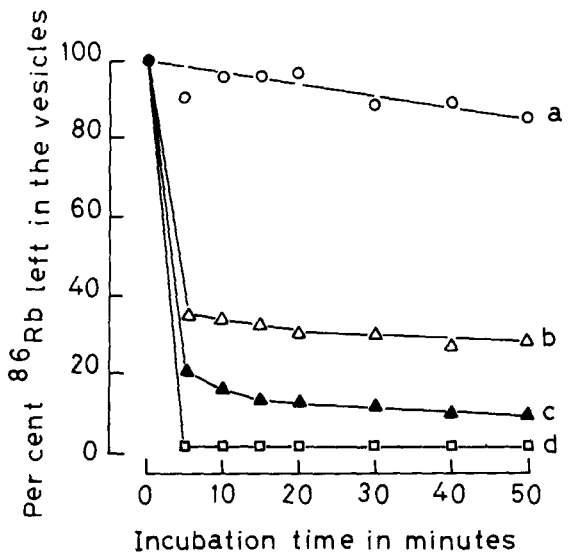

Fig. 1. Exit of $^{86}Rb^+$ from one-step inside-out vesicles. The incubation medium contained 18 mM KCl and 16.5 mM K^+-Hepes, pH 7.5, and the following additions: (a) 0.4 mM EGTA; (b) 0.1 mM Ca^{2+}; (c) 0.1 mM Ca^{2+} and 2 μM A23187; (d) 1 μM valinomycin.

designed to measure both the mean vesicular size and the mean number of channels per vesicle. After a brief period of incubation in Ca^{2+}-free medium to allow the release of $^{86}Rb^+$ from leaky vesicles and ghosts (not shown in the figure), the exit of the tracer became extremely slow ($t_{1/2}$ = 161 min). The resealed vesicular space [2] was at this stage 7.6% of that of the original erythrocytes, and the everted membrane surface, estimated from acetylcholine sterase accessibility [6], was 32%. The addition of 0.1 mM Ca^{2+}, a concentration documented to give maximal response of the K^+ channels [4], produced rapid release of 69% of the tracer. The emptied space would correspond to inside-out vesicles containing one or more Ca^{2+}-dependent K^+ channels. The joint addition of excess Ca^{2+} and the divalent cation ionophore A23187 extended the rapid $^{86}Rb^+$ release to 86% of the total. This suggests that 86 − 69 = 17% of the total $^{86}Rb^+$ was contained in right-side-out vesicles whose K^+ channels had access to Ca^{2+} only after selective permeabilization of their membrane by A23187. Valinomycin produced rapid release of 97% of the total $^{86}Rb^+$, indicating that 97 − 86 = 11% of the tracer was contained within resealed vesicles, either right-side-out vesicles or inside-out vesicles, which did not possess K^+ channels. If the relative proportion of inside-out to right-side-out vesicles within this fraction were similar to that observed in the channel-possessing vesicles (0.69/0.17) then about 9% of $^{86}Rb^+$ should be contained in K^+ channel-free inside-out vesicles.

If channels distribute at random among vesicles, the probability (P_x) of a vesicle having a given number (x) of channels would be; $P_x = (m^x/x!)e^{-m}$, where m is the mean frequency of channels per vesicle. The fraction of vesicles having no channels (P_0) would then be: $P_0 = e^{-m}$. From the data of the experiment of Fig. 1 the fraction of everted vesicles having no K^+ channels can be estimated: $P_0 = 9/(69 + 9) = 0.115$. The mean frequency of channels per vesicle would then be: $m = -\ln 0.115 = 2.16$. From this value the fraction of vesicles with different numbers of channels can be easily estimated: $P_1 = 0.25$; $P_2 = 0.27$; $P_3 = 0.19$; $P_4 = 0.10$; $P_5 = 0.05$, etc.

The mean radius of the everted vesicles (r) can also be estimated from the data of the experiment of Fig. 1. The resealed vesicular space contained in inside-out vesicles would be 7.6 × 0.78 = 5.92% of that of the original erythrocytes, and the everted membrane surface was 32% of the total. If we assume spherical shape, and taking into account the volume and surface of the original cells (86 μm^3 and 140 μm^2, respectively) we can write: volume/surface = $r/3$ = (86 × 5.92)/(140 × 32) = 0.112; then r = 0.34 μm.

The mean number of channels per μm^2 would then be $2.16/(4 \times 3.14 \times 0.34^2) = 1.49$ and the number of channels per original cell = 1.49 × 140 = 209. Table I summarizes the results of four similar experiments using either $^{86}Rb^+$ or $^{42}K^+$ as the tracer. The estimates of the mean number of channels per cell oscillated between 77 and 209.

The size heterogeneity of the vesicles could affect the estimates of the mean radius and the number of vesicles per cell. Table II shows the discrepancies between the true and the estimated values for gaussian distributions of vesicles with different sizes and dispersions. The estimated radius is always above the true value, ant it increases with increasing dispersion degrees. The estimated number of channels per cell is first

TABLE I

MEAN NUMBER OF CHANNELS PER CELL AND RELATED PARAMETERS ESTIMATED IN FOUR INDEPENDENT EXPERIMENTS

Experimental details are as in Fig. 1, except for the use of $^{42}K^+$ instead of $^{86}Rb^+$ as the tracer in 3 and 4. Resealed vesicular space (RVS) is expressed as ml vesicular space/100 ml original cells. Percent everted membrane surface (% inside-out vesicles (IOV)) was estimated from acetylcholinesterase accessibility [6]. Mean radius is given in μm. See text for details on calculations.

Exp. No.	RVS	Percent of total vesicular space released by:			%IOV	Mean radius	Channels/ vesicle	Channels/ cell
		Ca^{2+}	Ca^{2+} + A23187	valino-mycin				
1	7.6	69	86	97	32	0.34	2.18	209
2	9.0	78	83	99	30	0.51	1.82	77
3	10.1	62	84	97	34	0.39	2.01	146
4	4.2	70	77	97	19	0.36	1.58	136
Mean	7.7	70	83	98	29	0.40	1.90	142
S.E.	1.3	3	2	1	3	0.04	0.13	27

increased and then decreased with regard to the true value with increasing dispersion degrees. Figures associated with an estimated mean radius of about 0.4 μm should apply to our case. In the worst case, true radius = 0.1 and S.D. = 0.2, an overestimation of near 3-times the real value would apply.

We have reported elsewhere that kinetics of Ca^{2+}-dependent $^{86}Rb^+$ uptake do not follow single exponentials [7]. That could certainly reflect size heterogeneity of the vesicle population but it would also be expected from the random distribution of channels in a vesicle population of uniform size, since the number of channels varies from vesicle to vesicle, specially if the mean number approaches 1, as is the case. We have shown elsewhere that the rate constants for $^{86}Rb^+$ fluxes are very similar for subpopulations of vesicles with lower (which should include larger vesicles) and higher threshold sensitivities to Ca^{2+} [7]. This result argues against gross size heterogeneity of the population of vesicles.

Attempts were made to separate fractions of larger and smaller vesicles without much success. Centrifugation at 2500 × g for 30 min sedimented a fraction which seemed enriched in unvesiculated ghosts when observed under the phase contrast microscope. This fraction contained about 10% of both the total acetylcholinesterase activity (membrane surface) and the total resealed vesicular space, and was composed mainly of unsealed membranes. Estimated mean radius computed

TABLE II

EFFECT OF SIZE HETEROGENEITY OF VESICLES ON THE ESTIMATED VALUES OF THE MEAN RADIUS AND THE MEAN NUMBER OF CHANNELS PER CELL

Vesicle populations with different mean radii were modeled to conform normal distributions with different standard deviations (S.D.). Values assumed for original cells were: volume, 86 μm^3; surface, 140 μm^2; number of channels, 200 per cell. Values for radii are given in μm.

True mean radius	Estimated mean radius/mean number of channels per cell				
S.D.:	0.05	0.1	0.2	0.3	0.4
0.1	0.14/382	0.21/550	0.37/576	0.53/483	0.69/390
0.2	0.22/248	0.28/325	0.43/377	0.57/346	0.74/297
0.3	0.32/214	0.36/242	0.49/272	0.63/260	0.79/233
0.4	0.41/201	0.45/205	0.56/212	0.70/204	0.85/188

from the above values was about 1.5 μm. The fraction of the vesicles which did not sediment had an estimated mean radius of about 0.2 μm. The kinetics of $^{86}Rb^+$ uptake by this 'purified' fraction was, however, very similar to that obtained with the crude preparation.

The accuracy of our estimates of the mean radius and the number of channels per cell can be checked by comparing the rate constants obtained at maximal activation in vesicles and in intact cells. In one-step inside-out vesicles half-equilibration of $^{86}Rb^+$ is obtained within 30–45 s [7]. This corresponds to a first-order rate constant of 55–83 h^{-1}. Since the surface/volume ratio is about 4.6-times larger in intact cells than in vesicles (from mean radius = 0.4 μm), then we should expect rate constants of 12–18 h^{-1} in intact cells. These values are not far from those reported in the literature (6–10, Refs. 8–10). All the above evidence supports the fact that the figures reported here for the mean number of channels per cell are reasonably accurate. The values agree closely with those reported by Lew et al. [3] and differ significantly from those estimated by Grygorczyk et al. [2] by comparison of measurements of unitary conductance in patch-clamp experiments and tracer exchange fluxes in intact cells. As discussed elsewhere [11], technical reasons could contribute to explain these discrepancies. The recent discovery of a high-affinity toxin against Ca^{2+}-dependent K^+ channels of red cells [12] will hopefully soon allow alternative estimates to be made.

The procedure described here could find general application in the counting of channels when specific high-affinity ligands are not available and gating current or single channel conductance measurements cannot be conveniently performed or interpreted. since a mean number of channels per vesicle close to 1 is required to obtain reliable values with this procedure, it should be most appropriate to count channels which are distributed at low densities in the cell membrane.

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