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The Dual Effect of Rubidium Ions on Potassium Efflux in Depolarized Frog Skeletal Muscle

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Summary. The effects of external Rb^+ on the efflux of $^{42}K^+$ from whole frog sartorius muscles loaded with 305 mM K^+ and 120 mM Cl^- were studied. K^+ efflux is activated by $[Rb^+]_o$ less than about 40 mM according to a sigmoid relation similar to that for activation by $[K^+]_o$. At $[Rb^+]_o$ greater than 40 mM, K^+ efflux declines, although at $[Rb^+]_o = 300$ mM it is still greater than at $[Rb^+]_o = 0$ mM. For low concentrations, the increment in K^+ efflux over that in K^+- and Rb^+-free solution, Δk, is described by the relation $\Delta k = a[X^+]_o^n$, for both K^+ and Rb^+. The value of a is larger for Rb^+ than for K^+, while the values of n are similar; the activation produced by a given $[Rb^+]_o$ is larger than that by an equal $[K^+]_o$ for concentrations less than about 40 mM. Adding a small amount of Rb^+ to a K^+-containing solution has effects on K^+ efflux which depend on $[K^+]_o$. At low $[K^+]_o$, adding Rb^+ increases K^+ efflux, the effect being greatest near $[K^+]_o = 30$ mM and declining at higher $[K^+]_o$; at $[K^+]_o$ above 40 mM, addition of Rb^+ decreases K^+ efflux. At $[K^+]_o$ above 75 mM, where K^+ efflux is largely activated, Rb^+ reduces K^+ efflux by a factor b, described by the relation $b = 1/(1 + c[Rb^+]_o)$. Activation is discussed in terms of binding to at least two sites in the membrane, and the reduction in K^+ efflux by Rb^+ at high $[K^+]_o$ in terms of association with an additional inhibitory site.

Key words anomalous rectifier · inward rectifier · potassium efflux · rubidium · muscle membrane

Introduction

Considerable differences exist among the effects of Rb^+ on potassium permeability systems in vertebrate excitable membranes. For example, in the delayed rectifier of frog myelinated nerve, Rb^+ is an excellent K^+ substitute: P_{Rb}/P_K is near 1, and voltage-clamp currents in Rb^+ solution are only slightly smaller than in K^+ (Hille, 1973). In contrast, the inward rectifier of frog muscle is at most only slightly permeable to Rb^+, and furthermore Rb^+ interferes with the movement of K^+ through this system (Adrian, 1964; Standen & Stanfield, 1980).

In this paper, we examine the effects of external Rb^+ on the efflux of K^+ through the inward rectifier of depolarized frog skeletal muscle. At high $[K^+]_o$, Rb^+ reduces K^+ efflux, an effect probably not unlike the interference with K^+ movement referred to above. In addition, Rb^+ itself partially activates K^+ efflux, as first reported by Adrian (1962). The purpose of the experiments described in this report was to quantitate both the activating and inhibiting effects of external Rb^+ on K^+ efflux in the absence and presence of varying amounts of external K^+.

Materials and Methods

The efflux of $^{42}K^+$ from sartorius muscles from the frog *Rana pipiens* was measured in these experiments. In many experiments, both muscles from the same frog were used, one muscle serving as a control for the other. Muscles were loaded with K^+ by a 1-hr soak in a solution containing 305 mM K^+ and 120 mM Cl^- ("305-K^+" solution below), preceded by a 30-min soak in an isotonic K_2SO_4 solution. After the transient volume changes were over, the muscles were placed in 305-K^+ solution prepared from ^{42}KCl (New England Nuclear) for another hour. The muscles were then attached to stainless steel frames, and efflux was measured by suspending the muscles in a series of tubes containing various (inactive) solutions. Muscles were transferred to new tubes after timed intervals (generally 5 min). Washout of the extracellular space was facilitated by continuously rotating the tubes. The radioactivity in each of the collection tubes and that remaining in the muscles were measured in a gamma counter. After correction for isotope decay and background, K^+ efflux was calculated as the fraction of counts lost from the muscle and expressed as an apparent efflux rate coefficient, k. Throughout the paper, this efflux rate coefficient is referred to simply as "K^+ efflux" and has the units of min^{-1}. "Relative K^+ efflux" is the efflux rate coefficient in a given solution normalized to that in the equilibrating (305-K^+) solution measured near the beginning of each experiment. In general, efflux rate coefficients in a given solution were taken as the average of the values for the last three or four intervals (15–20 min) in that solution.

The solutions used in this study were prepared from three stock solutions of the following composition (in mM): alkali cation (*see* below), 305; Ca^{2+}, 4; Mg^{2+}, 2; Cl^-, 120; SO_4^{2-}, 96; and 5 meq phosphate buffer at pH 7.2. The alkali cation composition of the three solutions was 305 mM K^+ ("305-K^+"), 305 mM Na^+ ("305-Na^+") and 300 mM Rb^+ and 5 mM Na^+ ("300-Rb^+"). "300-Li^+" solution, used only in Fig. 1, was of similar composition but with 300 mM Li^+ and 5 mM Na^+.

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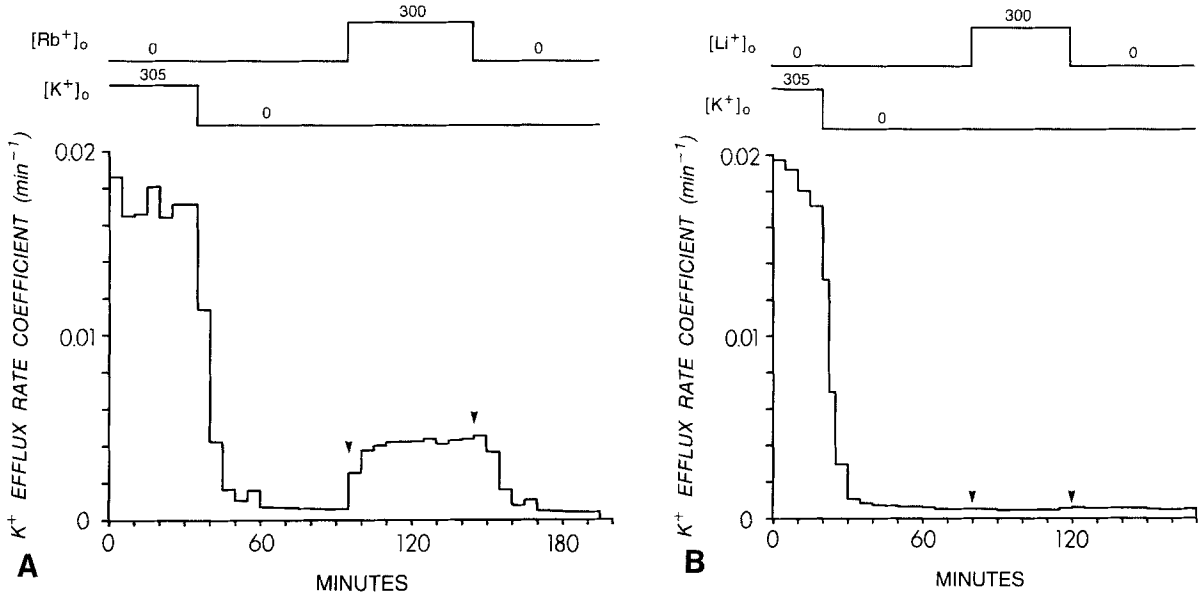

Fig. 1. K^+ efflux in 300-Rb^+ and 300-Li^+ solutions. K^+ efflux rate coefficients in each collection period in solutions with the K^+, Rb^+ and Li^+ concentrations (in mM) indicated. Arrows indicate solution changes. (*A*): Muscle 175; (*B*): muscle 28

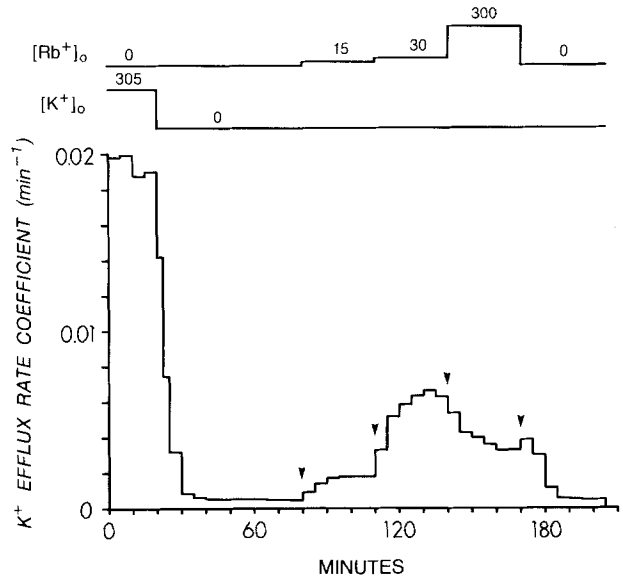

Fig. 2. K^+ efflux in various $[Rb^+]_o$. K^+ efflux rate coefficients during each collection period in solutions with the K^+ or Rb^+ concentrations (in mM) indicated. Arrows indicate solution changes. (Muscle 26)

The soaking solution ("305-K^+") was designed with the idea that after equilibration the membrane chloride conductance would be greatly increased by the high internal Cl^- concentration so that the membrane potential was determined by the chloride concentration gradient when $[K^+]_o$ was lowered (Hodgkin & Horowicz, 1959; Spalding, Senyk, Swift & Horowicz, 1981). In a series of duplicate experiments in which muscles were soaked in inactive 305-K^+ solution, the membrane potential measured with microelectrodes at the end of the soaking period was $+0.1 \pm 1.5$ mV (mean $\pm$SD, 39 muscles). In muscles as depolarized as the ones in this paper, inactivation of the delayed rectifier potassium channel is virtually complete (Adrian, Chandler & Hodgkin, 1970). The issue of the constancy of membrane potential in these experiments is taken up more fully later in this paper. All experiments were performed at room temperature (near 22 °C).

Results

Activation of K^+ Efflux by External Rb^+

The activation by Rb^+ of K^+ efflux from a KCl-loaded, depolarized muscle is most plainly seen in an experiment of the type shown in Fig. 1. The large K^+ efflux in 305-K^+ solution is greatly reduced by switching to 305-Na^+, as has been described previously (Spalding et al., 1981). Changing to 300-Rb^+ solution (Fig. 1*A*) results in a significant stimulation of K^+ efflux over the level in 305-Na^+; in this experiment the K^+ efflux in 300-Rb^+ is about one-fourth that in 305-K^+. That the K^+ efflux increase in 300-Rb^+ is not due simply to the removal of Na^+ is apparent from Fig. 1*B*. The K^+ efflux in 300-Li^+ solution is indistinguishable from that in 305-Na^+, even though Standen and Stanfield (1979) found in experiments at large negative membrane potentials that Na^+ and Li^+ have quantitatively different effects on the inward rectifier system.

The dependence of K^+ efflux on $[Rb^+]_o$ was investigated in a series of experiments like that in Fig. 2. It is apparent from this figure that K^+ efflux depends strongly on $[Rb^+]_o$ at low concentrations, K^+ efflux in 30-Rb^+ being much larger than in 15-

Rb^+. Interestingly, the K^+ efflux-$[Rb^+]_o$ relation goes through a maximum, and the K^+ efflux in 300-Rb^+ is less than in 30-Rb^+, although still larger than in Rb^+-free (305-Na^+) solution. The maximum in this relation at an intermediate Rb^+ concentration is responsible for the transient increase in efflux when the muscle is returned from 300-Rb^+ to 300-Na^+. Apparently, as the extracellular space is washed out, the muscle is transiently exposed to a low $[Rb^+]_o$ at which the level of K^+ efflux activation is greater than in 300-Rb^+. The stimulation of K^+ efflux by Rb^+ is reversible, as demonstrated by the return of K^+ efflux in 305-Na^+ at the end of the experiment to the same level as before exposure to Rb^+.

The results of several efflux experiments at various $[Rb^+]_o$ are summarized in Table 1 and plotted in Fig. 3. At low $[Rb^+]_o$, K^+ efflux increases with $[Rb^+]_o$ according to a sigmoid relation similar to the K^+ efflux-$[K^+]_o$ relation described elsewhere (Adrian, 1962; Horowicz, Gage & Eisenberg, 1968; Spalding et al., 1981). At $[Rb^+]_o$ in the range 30–40 mM, K^+ efflux reaches a maximum at a value approximately one-third the K^+ efflux in 305-K^+. On further increasing $[Rb^+]_o$, K^+ efflux gradually declines, reaching a value which appears to be independent of $[Rb^+]_o$ but still greater than the K^+ efflux in 305-Na^+.

Comparison of K^+ Efflux Activation by K^+ and Rb^+ at Low Concentrations

Although the activation of K^+ efflux by external Rb^+ resembles that by external K^+, there are quantitative differences, as shown in Fig. 4. The activation of K^+ efflux in 20-Rb^+ is significantly larger than that in 20-K^+ (Fig. 4*A*), indicating that Rb^+ is actually a more potent activator than K^+. This result is shown in another way in Fig. 4*B*, where the activation in 20-K^+ is no greater than that in 12-Rb^+. In this experiment, the muscle was returned to 305-Na^+ after the 20-K^+ exposure to demonstrate that the larger activation in Rb^+ was also observed when the Rb^+ exposure was not immediately preceded by exposure to K^+ and therefore was not due to the presence of K^+ retained in the extracellular space during the first few collection periods. The effects of solutions containing mixtures of K^+ and Rb^+ are considered later in the paper. In several experiments similar to those in Fig. 4, the activation of K^+ efflux by a given concentration of Rb^+ was consistently greater than that by an equal concentration of K^+, for concentrations less than 40 mM.

For quantitative comparison of the activating effects of K^+ and Rb^+, the K^+ efflux in each solution was expressed as the "relative increment," Δk, the difference between the relative K^+ efflux in each solution and that in 305-Na^+, which is a measure of the net stimulation due to the presence of K^+ or Rb^+. Relative increments for various $[Rb^+]_o$ and $[K^+]_o$ are presented in Tables 1 and 2, respectively. Next, it was noted that the dependence of K^+ efflux on $[Rb^+]_o$ (Fig. 3), like the dependence on $[K^+]_o$ (Spalding et al., 1981) follows a markedly sigmoid relation, with slope near zero at the lowest concentrations, suggesting a power-law relation of the form

$$\Delta k = a[X^+]_o^n \qquad (1)$$

Table 1. Dependence of K^+ efflux on external Rb^+

$[Rb^+]_o$ (mM)	$[Rb^+]'_o$ (mM)	n	Relative efflux	Relative increment
0		59	0.054 ± 0.003	–
5	5.0	3	0.066 ± 0.016	0.011 ± 0.005
9	9.0	6	0.095 ± 0.008	0.042 ± 0.007
10	10.0	3	0.072 ± 0.008	0.044 ± 0.010
12	12.3	5	0.126 ± 0.011	0.068 ± 0.005
15	15.8	4	0.129 ± 0.012	0.080 ± 0.007
18	19.7	4	0.229 ± 0.024	0.178 ± 0.022
20	22.4	3	0.239 ± 0.043	0.164 ± 0.052
24	28.6	3	0.328 ± 0.027	0.290 ± 0.021
30	39.3	3	0.338 ± 0.011	0.292 ± 0.031
45	60.0	1	0.328	0.281
75	97.4	4	0.300 ± 0.007	0.262 ± 0.005
150	165.4	42	0.223 ± 0.004	0.163 ± 0.005
225		12	0.205 ± 0.008	0.140 ± 0.009
300		24	0.221 ± 0.006	0.174 ± 0.005

Relative efflux is the K^+ efflux rate coefficient in the indicated solution divided by that in 305-K^+ solution. Relative increment is this value minus the relative efflux in 305-Na^+ solution. Mean ± SE; n = number of muscles.

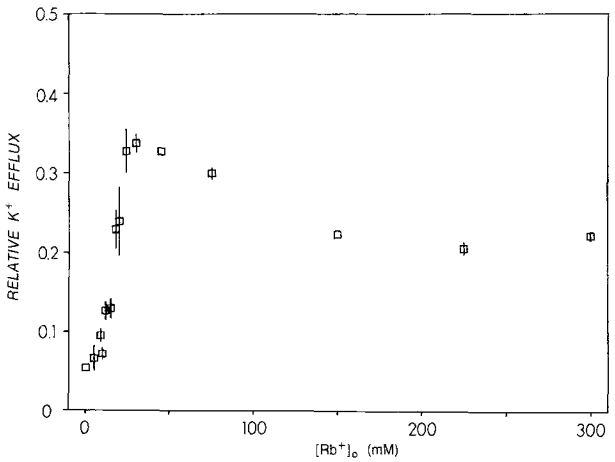

Fig. 3. Relative K^+ efflux as a function of $[Rb^+]_o$. Mean ± SE plotted from Table 1

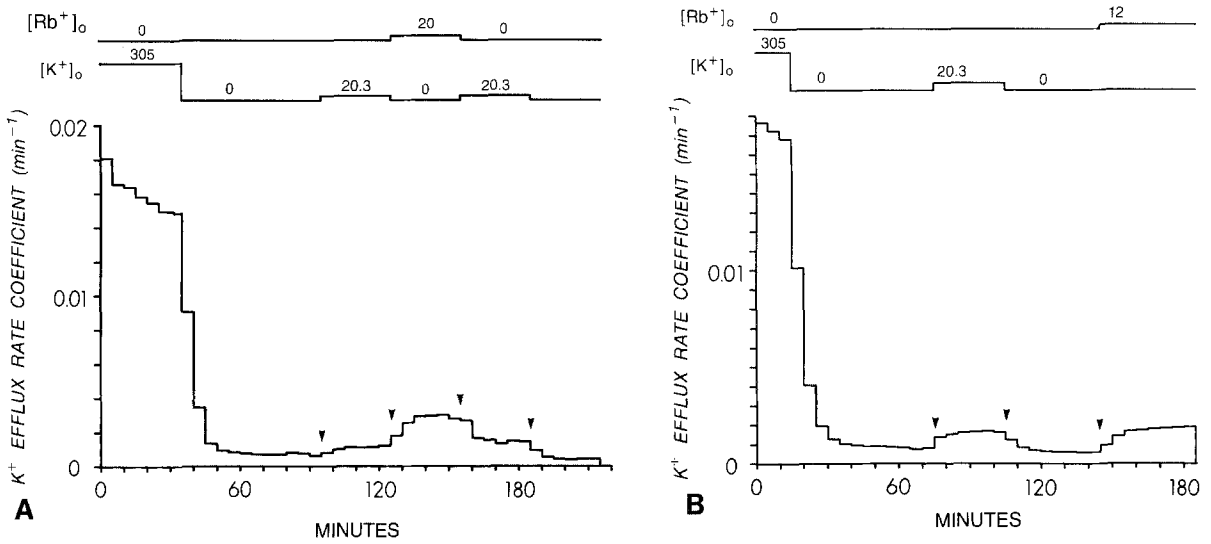

Fig. 4. Comparison of K^+ efflux in low concentrations of either K^+ or Rb^+. K^+ efflux rate coefficients during each collection period in solutions with the K^+ or Rb^+ concentrations (in mM) indicated. Arrows indicate solution changes. (*A*): Muscle 44; (*B*): muscle 302

Table 2. Dependence of K^+ efflux increment on external K^+

$[K^+]_o$ (mM)	$[K^+]'_o$ (mM)	n	Relative efflux	Relative increment
15.2	17.0	16	0.084 ± 0.004	0.030 ± 0.004
20.3	24.2	18	0.103 ± 0.006	0.049 ± 0.006
30.5	41.4	13	0.213 ± 0.015	0.159 ± 0.015
40.7	63.0	5	0.435 ± 0.064	0.381 ± 0.064
45.8	78.0	6	0.486 ± 0.063	
61.1	119.6	12	0.711 ± 0.026	
76	178.0	55	0.860 ± 0.018	
152	234.4	21	0.931 ± 0.019	
219	262.6	37	0.973 ± 0.015	

Relative increment is the difference between the efflux rate coefficient in the indicated solution and that in 305-Na^+ solution (0.054 ± 0.003, $n=59$), divided by the efflux rate coefficient in 305-K^+ solution. Mean $\pm$ SE; n = number of muscles.

where X^+ is K^+ or Rb^+. To explore this relation, the relative increments have been plotted against $[K^+]_o$ or $[Rb^+]_o$ in a log-log plot in Fig. 5*A*. It can be seen that Eq. (1) adequately describes the results; the values of n for K^+ and Rb^+ from a linear regression analysis were 2.64 and 2.02, respectively. These values are subject to a correction to be described below and may therefore be taken as upper bounds for n.

Estimate of Membrane Potential Effects

One interpretation of the activating effects of K^+ and Rb^+ is to associate activation with binding of these ions to sites in the membrane. If binding is influenced by membrane potential, it is not unreasonable to suppose that K^+ efflux could be influenced by small differences in membrane potential from solution to solution. We now describe an analysis to estimate the magnitude of membrane potential effects on K^+ efflux activation.

At a given membrane potential, V_1, the local concentration of a monovalent cation X^+ at equilibrium with a site in the membrane, $[X^+]_s(V_1)$, can be shown to be

$$[X^+]_s(V_1) = [X^+]_o \xi \exp -(\alpha V_1 F/RT) \tag{2}$$

where ξ is a factor which depends on the membrane surface potentials and other intrinsic factors; F, R and T have their usual meaning; $[X^+]_o$ is the external concentration of X^+; and α is an empirical factor which can be interpreted, for example, as the fraction of the membrane field influencing binding in the absence of surface potentials. In general, ξ includes a multiplicative term which takes account of that portion of the membrane field produced by surface potentials. If $[X^+]_o$ remains constant but membrane potential is changed to V_2, then from Eq. (2)

$$\frac{[X^+]_s(V_2)}{[X^+]_s(V_1)} = \frac{[X^+]_o \xi \exp -(\alpha V_2 F/RT)}{[X^+]_o \xi \exp -(\alpha V_1 F/RT)}$$
$$= \exp -(\alpha (V_2 - V_1) F/RT) \tag{3}$$

assuming ξ is unchanged.

Applying this result to the present experiments, we estimate the effect of membrane potential on K^+ efflux by defining an equivalent or "corrected" activator concentration, $[X^+]'_o$, by

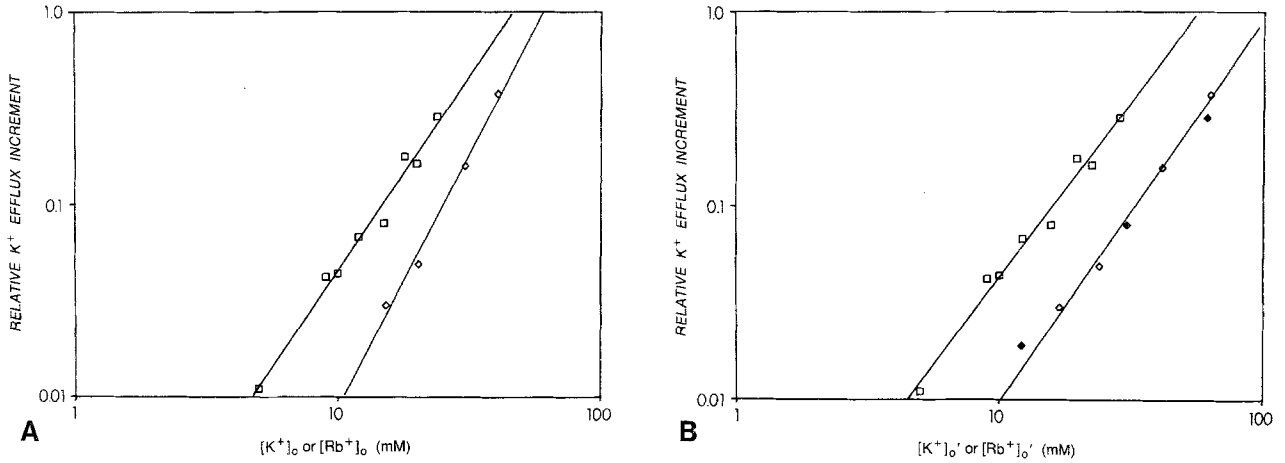

Fig. 5. Comparison of activation of K^+ efflux by K^+ and by Rb^+. (*A*): The relative K^+ efflux increments in Rb^+-containing solutions (squares) or K^+-containing solutions (diamonds) from Tables 1 and 2 are plotted against $[K^+]_o$ or $[Rb^+]_o$. Note logarithmic scales. (*B*): The same data from part *A* are plotted against $[K^+]'_o$ or $[Rb^+]'_o$ as calculated from Eq. (4) and presented in Tables 1 and 2. The filled diamonds are relative K^+ efflux increments from experiments on small bundles (Spalding et al., 1981) plotted against $[K^+]_o$. The lines in both parts of the figure are drawn according to Eq. (1) with the following parameters (from linear regression analysis) for concentrations in mM: (*A*) $a=4.4\times10^{-4}$ and $n=2.02$ for Rb^+, $a=2.0\times10^{-5}$ and $n=2.64$ for K^+; (*B*) $a=6.5\times10^{-4}$ and $n=1.82$ for Rb^+, $a=1.0\times10^{-4}$ and $n=1.98$ for K^+

$$[X^+]'_o=[X^+]_o \exp-(\alpha(V_2-V_1)F/RT) \quad (4)$$

and noting from Eq. (2) that $[X^+]_s$ is the same for membrane potential V_2 and activator concentration $[X^+]'_o$, defined in this way, as for membrane potential V_1 and activator concentration $[X^+]_o$. This issue is considered further in Appendix A, where the case of multiple binding sites is treated.

In practice, we calculated $[X^+]'_o$ from $[X^+]_o$ taking $V_2=-0.8$ mV, i.e., the membrane potential in 305-Na^+ (Table 3), and V_1, the membrane potential in $[X^+]_o$, from microelectrode measurements. Membrane potentials were measured in a series of experiments where muscles were loaded in 305-K^+ and placed in various experimental solutions according to the same protocols as used in the efflux experiments summarized in Tables 1 and 2. Membrane potentials in each muscle were measured with KCl-filled microelectrodes in several fibers in each solution; generally, at least 20 impalements were made in each solution. Table 3 presents the results of measurements on 39 muscles, and the tabulated membrane potentials were taken as V_1 for use in Eq. (4). For solutions in which membrane potential measurements were not made, V_1 was estimated by linear interpolation. To estimate the value of α, it was noted that in a previous study doubling $[K^+]_o$ resulted in an increase in K^+ efflux roughly equivalent to that from doubling $[Cl^-]_o$, which alters the membrane potential by about 17 mV in the negative direction (Fig. 6 of Spalding et al., 1981). From the relation given in Eq. (4), equal effluxes would give $\alpha = 1$, and we have used this value in our calculations of $[X^+]'_o$. The calculated values of $[X^+]'_o$ are listed in Tables 1 and 2.

Table 3. Membrane potentials in various solutions used in this study

Solution	n	V_1(mV)
305-K^+	39	+0.1
305-Na^+	39	−0.8
$[K^+]_o$(mM)		
15.2	6	−3.5
30.5	6	−8.6
76	4	−21.7[a]
152	4	−11.0[a]
219	4	−4.7
$[Rb^+]_o$(mM)		
10	4	−0.9
15	4	−2.1
20	4	−3.7
30	5	−7.7
75	2	−6.7[b]
150	2	−2.5[b]

Potentials in each muscle were estimated as the mean of several (usually 20–30) separate measurements; the values in the table are the means of these means (n=number of muscles).

[a] In 76 and 152 mM K^+, repolarization was slow so the mean presented includes only measurements taken after 40 min in the K^+ solution.

[b] Muscles placed in 75 or 150 mM Rb^+ slowly repolarized over the next 2–3 hr; the values presented were taken at times between 43 and 49 min after exposure to Rb^+ solution.

The activating effects of K^+ and Rb^+ corrected for the effects of membrane potential are presented

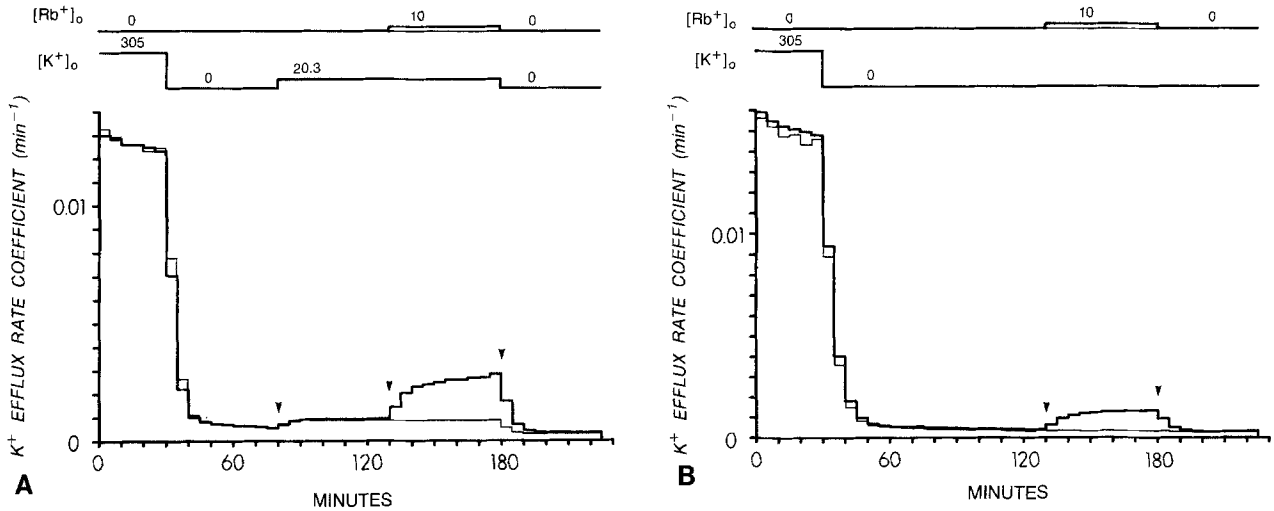

Fig. 6. Activation of K^+ efflux by low $[Rb^+]_o$ in the presence and absence of external K^+. K^+ efflux rate coefficients during each collection period in solutions with the K^+ and Rb^+ concentrations (in mM) indicated. Arrows indicate solution changes. (*A*): Muscles 379 (thick line) and 380 (thin line), both from the same frog. (*B*): Muscles 381 (thick line) and 382 (thin line), both from the same frog

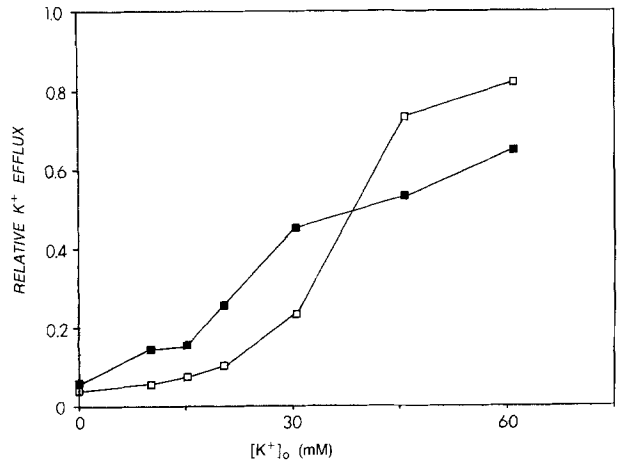

Fig. 7. Relative K^+ efflux in solutions of various $[K^+]_o$ and in the same solutions to which 10 mM Rb^+ was added. Protocol was similar to that for muscle 397 in Fig. 6*A*. Seven muscles (47–53) were used, one for each $[K^+]_o$

in Fig. 5*B*, which is a log-log plot of relative increments against $[K^+]'_o$ or $[Rb^+]'_o$, calculated as just described. As an internal check, relative increments from an earlier paper (Spalding et al., 1981), in which K^+ efflux measurements were made on small bundles of fibers, are plotted without correction in Fig. 5*B*. Although similar solutions were used in the earlier study, we consider that the effects of membrane potential changes were relatively minor, largely because of the short exposures to test solutions (usually 10 min) used. Indeed, the potential changes in Table 3 occurred slowly, particularly the large changes in high $[K^+]_o$, and brief exposure produced only small potential changes. The excellent agreement between the small bundle data (filled diamonds in Fig. 5*B*) and the whole muscle data after correction for potential changes (open diamonds) is evidence that the correction procedure is adequate.

Having assessed the importance of membrane potential changes, it is apparent that the results in Fig. 5*B* are again adequately described by Eq. (1). The values of n from a linear regression analysis are 1.98 and 1.82 for K^+ and Rb^+, respectively. It is clear that n is about the same for Rb^+ as for K^+, while the value of a is greater, reflecting the larger increment, at a given concentration, produced by Rb^+ than by K^+ in this range. Further, if activating effects are attributed to binding to membrane sites, the value of n indicates the presence of at least two activation sites per channel (Spalding et al., 1981). These issues are taken up further in the discussion.

Effect of Mixtures of K^+ and Rb^+ at Low Concentrations

Figure 6 presents an experiment to investigate the effect on K^+ efflux of applying low concentrations of K^+ and Rb^+ simultaneously. Comparison of the two parts of the figure reveals that K^+ and Rb^+ act synergistically in this concentration range. 10 mM Rb^+ produced a larger increase in K^+ efflux when added to 20-K^+ solution than when added to 305-Na^+. Further, the K^+ efflux increment in 20-K^+, 10-Rb^+ was larger than the sum of the increments in 20-K^+ and 10-Rb^+ alone. These changes were reversible on returning to 305-Na^+ solution.

Figure 7 summarizes a series of experiments showing the K^+ efflux change produced by adding

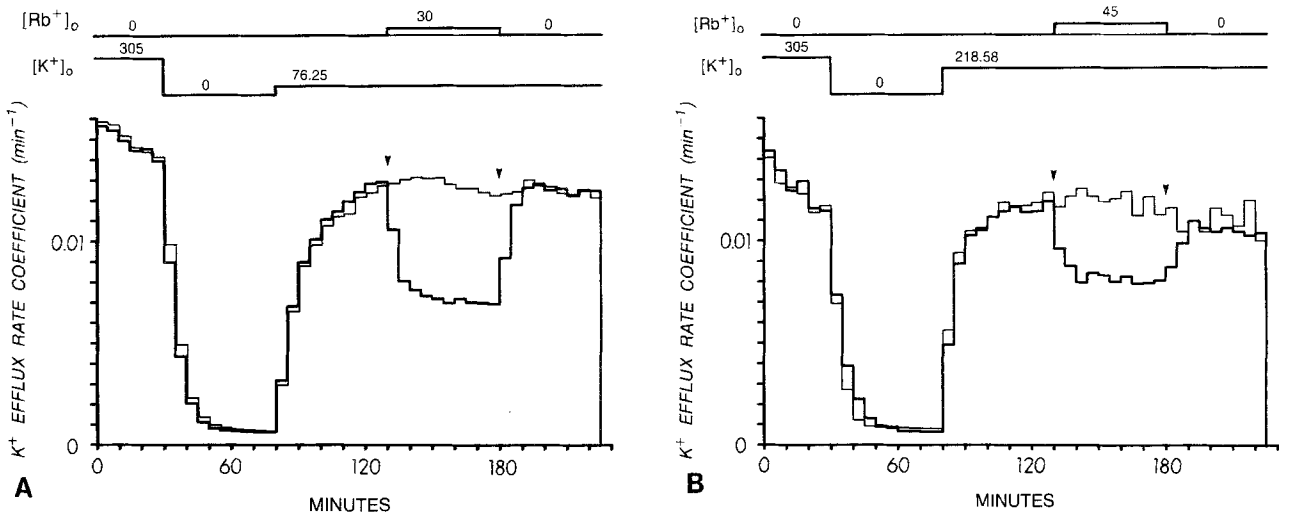

Fig. 8. Inhibition of K^+ efflux by Rb^+ at high $[K^+]_o$. K^+ efflux rate coefficients during each collection period in solutions with the K^+ and Rb^+ concentrations (in mM) indicated. Arrows indicate solution changes. (*A*): Muscles 107 (thick line) and 108 (thin line), both from the same frog. (*B*): Muscles 105 (thick line) and 106 (thin line), both from the same frog

10 mM Rb^+ to solutions containing various $[K^+]_o$, using protocols similar to that for muscle 379 in Fig. 6*A*. At the lower $[K^+]_o$, adding 10 mM Rb^+ increased K^+ efflux, the largest increase occurring when $[K^+]_o = 30$ mM. At higher $[K^+]_o$, adding 10 mM Rb^+ decreased K^+ efflux. Similar effects of smaller magnitude were observed in experiments using 5 mM Rb^+. These results may be compared with those of Fig. 3, where adding Rb^+ to a Rb^+ solution also increased K^+ efflux at low concentrations, but decreased K^+ efflux at higher concentrations. The decrease in K^+ efflux from adding Rb^+ to solutions of high $[K^+]_o$ is taken up in the next section.

Effect of Rb^+ on K^+ Efflux in Solutions of High $[K^+]_o$

Figure 8 presents two experiments from a series designed to investigate the decrease in K^+ efflux when Rb^+ is added to solutions of $[K^+]_o \geqq 76$ mM. At these $[K^+]_o$, the K^+ efflux is largely activated as is apparent from Fig. 8 where the K^+ efflux in 76-K^+ and in 219-K^+ was nearly as large as in 305-K^+ (*see also* Spalding et al., 1981). Changing the solution to one with the same $[K^+]_o$ but containing Rb^+ caused a prompt reduction in K^+ efflux which was reversible on return to Rb^+-free solution. Note that the reduction in K^+ efflux was greater when 30 mM Rb^+ was added to 76-K^+ solution than when a larger amount of Rb^+ (45 mM) was added to 219-K^+.

For analysis, the K^+ efflux in pairs of muscles like those in Fig. 8 was treated as follows. The K^+ efflux in the Rb^+-containing solution was divided by that in the other muscle of the pair at the same time (in Rb^+-free solutions of the same $[K^+]_o$). This ratio was then normalized by dividing by the ratio (always near one) of the K^+ effluxes in the two muscles in the high-K^+ solution before exposure to Rb^+, to correct for small muscle-to-muscle differences. The resulting normalized ratios, b, are summarized in Table 4 and plotted in Fig. 9.

It is apparent from Fig. 9*A* that the dependence of b on $[Rb^+]_o$ is well described by the empirical relation

$$b = \frac{1}{1 + c[Rb^+]_o} \tag{5}$$

where c is a parameter which is constant for a given $[K^+]_o$. The curves in Fig. 9*A* are drawn according to Eq. (5) using the value of c from a linear regression analysis of the results for each $[K^+]_o$ plotted in the linearized form of Eq. (5):

$$b^{-1} - 1 = c[Rb^+]_o. \tag{6}$$

Examined in this way, at each $[Rb^+]_o$ the fraction of K^+ efflux inhibited appears to increase as $[K^+]_o$ is decreased.

Before any conclusion can be drawn, however, about the dependence on $[K^+]_o$ of the Rb^+-induced inhibition, allowance has to be made for the fact that as $[K^+]_o$ is lowered, from 219 to 76 mM, the internal potential becomes more negative, as shown in Table 3. For this purpose, use can be made of the observations of Standen and Stanfield (1980) that the fractional inhibition of inward current by exter-

nal Rb^+ at constant $[K^+]_o$ increases as the internal potential is made more negative in the range of potentials between 0 and −25 mV. As is shown in Appendix B, the fraction of K^+ efflux remaining when $V=0$, b^*, can be calculated from the measured fraction of K^+ efflux remaining at potential V_1, b, from the relation

$$b^* = \frac{1}{1+(b^{-1}-1)\,e^{\delta V_1 F/RT}} \tag{7}$$

where $\delta = 1.4$ (*see* Standen & Stanfield, 1980); and F, R, and T have their usual significance. Microelectrode measurements on muscles exposed to experimental solutions similar to those in which efflux was determined did not show significant changes in membrane potential upon addition of external Rb^+ when $[K^+]_o$ was either 219 or 152 mM. For these concentrations of external K^+, the average value of V_1 shown in Table 3 was used in Eq. (7). When $[K^+]_o$ was 76 mM, addition of external Rb^+ up to 225 mM produced significant depolarizations. The potentials in these solutions were estimated by the equation, $V_1 = -21.7\,\text{mV} + 0.04133\,[Rb^+]_o\ \text{mV mM}^{-1}$, obtained by linear regression from potential measurements in 76 mM $[K^+]_o$ and varying $[Rb^+]_o$.

Table 4. Effect of Rb^+ on K^+ efflux at high $[K^+]_o$

$[Rb^+]_o$(mM)	n	b	V_1(mV)	b^*
$[K^+]_o$=76 mM				
10	3	0.837	−21.29	0.942
20	3	0.708	−20.87	0.883
25	3	0.612	−20.67	0.831
30	3	0.599	−20.46	0.820
45	2	0.493	−19.84	0.739
50	1	0.456	−19.63	0.712
75	2	0.394	−18.60	0.644
112	2	0.296	−17.05	0.518
150	2	0.229	−15.50	0.394
188	2	0.160	−13.95	0.290
225	2	0.184	−12.40	0.304
$[K^+]_o$=152 mM				
10	2	0.852	−11.0	0.913
20	2	0.784	−11.0	0.869
30	2	0.704	−11.0	0.814
45	2	0.647	−11.0	0.771
75	2	0.483	−11.0	0.631
150	1	0.341	−11.0	0.487
$[K^+]_o$=219 mM				
10	3	0.937	−4.7	0.951
20	2	0.838	−4.7	0.870
30	2	0.766	−4.7	0.808
45	3	0.683	−4.7	0.735
65	2	0.627	−4.7	0.682
85	3	0.556	−4.7	0.618

b is the normalized ratio of K efflux in paired muscles in the indicated $[K^+]_o$ with or without Rb^+. b^* was calculated from Eq. (7) using values for V_1 obtained as described in the text. n = number of muscle pairs.

Figure 9*B* shows the fraction of K^+ efflux remaining after correction for potential plotted as a function of $[Rb^+]_o$. It is clear that all the points now follow a common curve independent of $[K^+]_o$. For the points where $[Rb^+]_o < 100$ mM, the relation

$$b^* = \frac{1}{1+c^*[Rb^+]_o} \tag{8}$$

with $c^* = 7.49\ \text{M}^{-1}$ (95% confidence interval: 7.06 to 7.91 M^{-1}) fits the results adequadely. It is clear, therefore, that external K^+ does not measurably influence the fractional inhibition produced by external Rb^+ under conditions of constant membrane potential. The apparent effect of $[K^+]_o$ in Fig. 9*A*

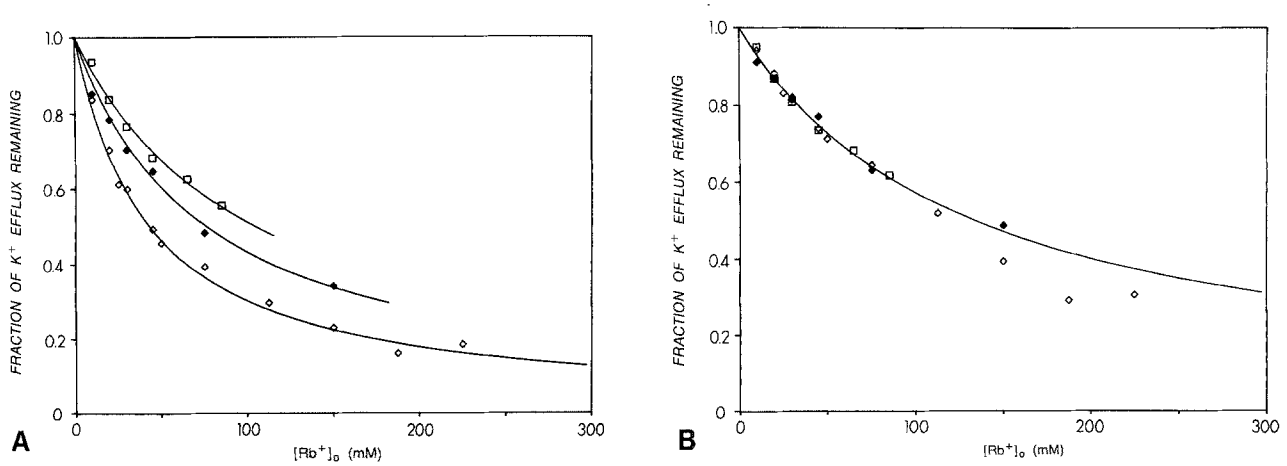

Fig. 9. Inhibition of K^+ efflux by Rb^+ at high $[K^+]_o$. Values of the normalized K^+ efflux ratios, b, from Table 4 are plotted against $[Rb^+]_o$ in *A*, and the corrected ratios, b^*, are plotted in *B*. The symbols refer to different $[K^+]_o$ as follows: open diamonds, 76-K^+; filled diamonds, 152-K^+; squares, 219-K^+. The curves are drawn according to Eqs. (6) and (8) with the following values of c or c^* (in mM^{-1}): (*A*) $c = 2.3\times10^{-2}$ for 76-K^+, $c = 1.3\times10^{-2}$ for 152-K^+, and $c = 9.6\times10^{-3}$ for 219-K^+; (*B*) $c^* = 7.5\times10^{-3}$

can be ascribed to differences in membrane potential in the various solutions.

It can be noted that the simple relation given above does not adequately describe the data over the entire range of Rb^+ concentrations used. For $[Rb^+]_o > 100$ mM, the data points fall below the curve when extended into this region. Analysis of the data for $[Rb^+]_o > 100$ mM with Eq. (8), gives a best fit of 10.2 M^{-1} for c^*, which is outside the 95% confidence interval determined from the data for $[Rb^+]_o < 100$ mM. Similarly, analysis of the data over the entire range of $[Rb^+]_o$ gives a best fit of 9.68 M^{-1} for c^*, and the 95% confidence interval (8.26 to 11.1 M^{-1}) does not overlap with the interval determined from the data for $[Rb^+]_o < 100$ mM. This suggests that at higher Rb^+ concentrations, Rb^+ becomes a more effective inhibitor of K^+ efflux than at lower concentrations, at least as judged by Eq. (8). This point is further explored in the discussion.

Discussion

From the results of the experiments in this study it is clear that in depolarized fibers addition of Rb^+ to the external solution has two distinct effects on K^+ efflux through the inward rectifier system. One effect is to stimulate K^+ exit when K^+ efflux is reduced to low rates at low $[K^+]_o$. The other effect is to inhibit K^+ exit when K^+ efflux is at intermediate or high rates produced by intermediate concentrations of either external K^+ or external Rb^+.

The stimulation of K^+ efflux produced by the addition of external Rb^+ to K^+-free solutions parallels the stimulation produced by the addition of external K^+; for relatively low concentrations the increment in K^+ efflux varies approximately as the square of the external alkali metal ion concentration with Rb^+ being more effective than K^+ when compared on a molar scale. Previous studies on the behavior of the ratio of unidirectional K^+ fluxes as a function of external K^+ have shown that increasing external K^+ converts the inward rectifier system from a state with low K^+ flux rates obeying the independence principle to a state with high K^+ flux rates no longer obeying the independence principle (Spalding et al., 1981). In the high flux state the K^+ flux ratio is given by the relation influx/efflux $= \exp[-n(V-V_K)F/RT]$ with $n=2$. It was suggested that these results can be rationalized on the notion that external K^+ has access to at least two distinct sites within the membrane which, when associated with K^+, convert the inward K^+ rectifier channel from a low to a high flux state. The stimulatory effects on K^+ exit produced by external Rb^+ at low concentrations when compared to the similar effects of external K^+ also support this notion.

The additional finding that the stimulatory action of external Rb^+ or K^+ is potentiated by the presence of low concentrations of the other ion (*see* Figs. 6 and 7) provides further strong support for this notion. The simplest explanation for the mutual potentiation of these ions at low concentration is that activation is produced whenever both sites are occupied by K^+ or Rb^+ either alone or in combination. If association with two membrane sites is required for K^+ exit to increase and both K^+ and Rb^+ derived from the external solution can reside at these sites, then any one of a number of simple reaction schemes will generate a relation in which, at low concentrations, the increment in K^+ efflux will be proportional to the sum $a_1[K^+]_o^2 + a_2[K^+]_o[Rb^+]_o + a_3[Rb^+]_o^2$, where a_1, a_2, and a_3 are independent of ion concentrations but are dependent on reaction rate constants which in turn depend on membrane potential. The middle term contributes to the increment of K^+ efflux only when both ions are present in the external solution, and this accounts for the mutual potentiation observed.

Since external Rb^+ has an additional inhibitory effect which becomes evident once K^+ efflux is stimulated beyond a certain level, whether the activating ion is K^+ or Rb^+, it seems certain that at least one additional membrane site, also accessible to external alkali metal ions, is involved in the control of K^+ movements through the inward rectifier. The fact that in completely depolarized fibers the fractional inhibition of K^+ efflux at high $[K^+]_o$ is a function of $[Rb^+]_o$ independent of $[K^+]_o$ (*see* Fig. 9*B*) suggests strongly that, for the low membrane potentials and net outward K^+ flow in the present experiments, K^+ derived from external solutions does not interact with the inhibitory site to which Rb^+ binds. Standen and Stanfield (1980) have shown for membrane potentials in the range -40 to 0 mV under conditions of net inward current flow that Rb^+ produces a fractional inhibition of the current which is insensitive to variations of $[K^+]_o$ between 80 and 160 mM. Further evidence indicating that external K^+ does not interact measurably with a membrane site to produce inhibition of K^+ efflux is the fact that K^+ efflux rises monotonically to a plateau as external K^+ concentration is increased in depolarized fibers (*see* Fig. 8 of this paper; Fig. 8 of Adrian, 1962; Fig. 1 of Horowicz et al., 1968; and Fig. 2 of Spalding et al., 1981).

Although the inhibition of K^+ efflux produced by Rb^+ seems simple, the data available suggest that, in fact, it is more complex than the association of Rb^+ with a membrane site having a unique equilibrium constant at a given membrane potential resulting in block of K^+ exit. First, it has already been noted in the results section that, for solutions

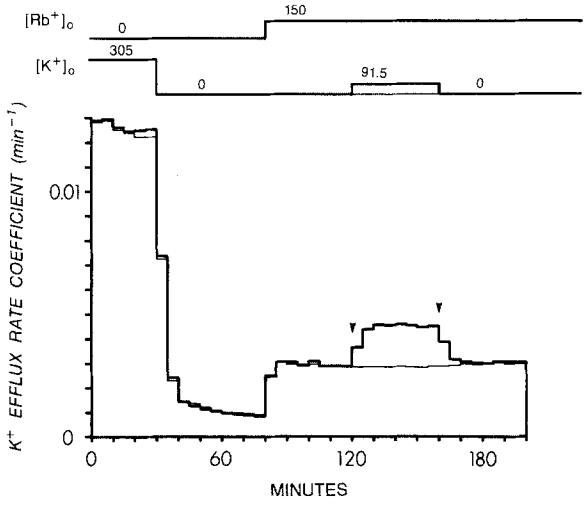

Fig. 10. Disinhibition of K^+ efflux by K^+ at high $[Rb^+]_o$. K^+ efflux rate coefficients during each collection period in solutions with K^+ and Rb^+ concentrations (in mM) indicated. Arrows indicate solutions changes. Muscles 491 (thick line) and 492 (thin line) both from the same frog

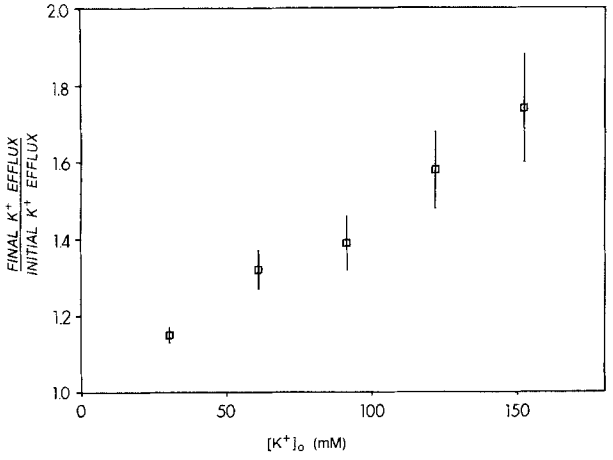

Fig. 11. Disinhibition of K^+ efflux by K^+ at high $[Rb^+]_o$. From a series of experiments like that in Fig. 10, the squares are the ratio of the K^+ efflux rate coefficient in a mixture containing 150-Rb^+ and the indicated external K^+ concentration to that in 150-Rb^+ alone before exposure to the mixture, normalized to the ratio taken at the same time in the control muscle from the same frog. Mean ± SE

with $76\,\text{mM} \leqq [K^+]_o \leqq 219\,\text{mM}$, the fraction of K^+ efflux remaining after addition of external Rb^+, b^*, can be approximated by the relation $b^* = 1/(1+c^*[Rb^+]_o)$ with $c^* = 7.49 \times 10^{-3}\,\text{mM}^{-1}$ for $[Rb^+]_o \leqq 85\,\text{mM}$. This kind of relation can be derived from a model in which Rb^+ coming from the external solution and associating with a single membrane site blocks K^+ exit; e.g., *see* Woodhull (1973). Provided $[Rb^+]_i = 0$, the general form of the above relation holds whether or not the Rb^+ associated with the site can leave the site for the internal solution as well as returning to the external solution. Yet when $[K^+]_o = 76\,\text{mM}$ and $[Rb^+]_o > 100\,\text{mM}$, the fraction of K^+ efflux remaining is less than that estimated by the above relation (*see* Fig. 9*B*); i.e., the inhibition produced by Rb^+ at high concentrations is greater than that expected based on extrapolating inhibition measured at low Rb^+ concentrations.

A second difficulty with the view that Rb^+ 'blocks' K^+ exit by reacting with a single membrane site is that one would expect K^+ efflux to decline to zero as one gradually increases $[Rb^+]_o$. Yet as Table 1 and Fig. 3 show, K^+ efflux between 150 and 300 mM is not measurably dependent on $[Rb^+]_o$ and is well above its value when both external K^+ and Rb^+ are absent. This behavior strongly suggests that the membrane sites with which Rb^+ combines to produce inhibition of K^+ efflux are essentially saturated when $[Rb^+]_o \geqq 150\,\text{mM}$, at least when the external solutions are free of K^+, and yet at these Rb^+ concentrations the probability of K^+ leaving the fiber is substantial.

The implications of these findings can be pursued further. The fact that K^+ efflux is independent of $[Rb^+]_o$ between 150 and 300 mM means that externally derived Rb^+ has occupied not only all the sites leading to inhibition but also those sites leading to activation of K^+ efflux. This is consistent with the finding that at low concentrations Rb^+ has about a two- to sixfold greater affinity for the activation sites than does K^+ (*see* Fig. 5) and the result that the K^+ efflux when $[K^+]_o = 150\,\text{mM}$ is within 17% of its value when $[K^+]_o = 305\,\text{mM}$ (Spalding et al., 1981). Since, as reviewed above, the available evidence shows that K^+ does not interact directly to any appreciable extent with the sites producing inhibition, any effects produced by adding K^+ to the external solution when $[Rb^+]_o = 150\,\text{mM}$ must be produced by replacing Rb^+ with K^+ at the sites producing activation. Figure 10 shows the result of an experiment comparing the K^+ efflux in 150-Rb^+ solution with that in a solution containing 150 mM Rb^+ plus 92 mM K^+. It is apparent that on addition of K^+, the K^+ efflux increased substantially, returning to its original value when the muscle was returned to 150-Rb^+, while K^+ efflux for the control muscle remained stable in 150-Rb^+ throughout. Figure 11 summarizes a series of such experiments in which the amount of external K^+ added was varied, from which it is clear that when $[Rb^+]_o$ is 150 mM, K^+ efflux increases approximately in proportion to $[K^+]_o$.

In the absence of additional sites or effects controlling K^+ exit, these results show that replacing Rb^+ by K^+ at the activation sites alters the probability of K^+ leaving the fibers in the presence of a constant, high $[Rb^+]_o$. In other words, mere occup-

ancy of these sites does not uniquely specify the behavior of the inward rectifier, at least in the presence of external Rb^+. There are two different classes of mechanisms which could account for the observations. In one, replacement of Rb^+ by K^+ at an activation site could markedly lower the association constant of the inhibitory site for external Rb^+. In this case, external Rb^+ at a concentration of 150 mM would no longer be sufficient to fully saturate the inhibitory sites. In the other mechanism, replacement of Rb^+ by K^+ at an activation site could increase the probability of K^+ exit without altering the association constant of the inhibitory site for external Rb^+. That is, the probability of K^+ exit would be larger in the states in which the two activation sites are occupied both by K^+ or by K^+ and Rb^+ than when both are occupied by Rb^+. In this mechanism, since there is no interaction between the activation sites and the inhibitory sites, occupancy of the inhibitory sites by Rb^+ would reduce the probability of K^+ exit by a constant fraction regardless of the ligands occupying the activation sites. These two mechanisms are not mutually exclusive and both can be present simultaneously. With the data presently available, however, it is not possible to quantitate accurately the relative contribution of each mechanism.

The conclusions we draw from the experimental results of this and an earlier report (Spalding et al., 1981) can be summarized as follows. Each inward rectifier channel has associated with it at least three membrane sites which control the movements of ions through it. When K^+ is the only alkali metal ion present other than Na^+ in the external fluid, the inward rectifier can exist in either of two conductance states. One is a low conductance state in which the unidirectional K^+ fluxes are linear functions of the source compartment K^+ concentrations, and the other is a high conductance state in which the unidirectional fluxes are proportional to the square of the source compartment concentration. The conversion from the low conductance state to the high conductance state ordinarily requires the presence of external K^+ and is a function of the membrane potential, so that K^+ is more effective as an activator when the internal potential is made more negative and is less effective when the internal potential is made more positive. Leech and Stanfield (1981) have also shown that inward rectification depends upon membrane potential and external K^+. A minimum of two membrane sites have to be occupied by K^+ for the conversion or activation to take place. Rb^+ can also function as an activator when associated with these sites and is more effective in this regard than external K^+ at relatively low concentrations when the membranes are fully depolarized. An additional membrane site controlling the fluxes through each inward rectifier channel is required to account for the inhibitory action of external Rb^+ on K^+ efflux. When this site binds Rb^+, K^+ efflux is reduced. There is no evidence to indicate that externally derived K^+ binds to this site to produce inhibition of K^+ efflux. Finally, the extent to which external Rb^+ inhibits K^+ efflux depends on which ions are occupying the activation sites.

Although the exact mechanisms by which these effects are produced are as yet unclear, there are reports in the literature that bear on them. Standen and Stanfield (1978) have shown that Ba^{2+} blocks the inward rectifier, and recently Ohmori, Yoshida and Hagiwara (1981) have detected unitary, step currents in cultured rat myotubes caused by the blocking and unblocking by Ba^{2+} of K^+ channels, which they identify with the inward rectifier. In addition, it can be noted that in modelling the inward rectifier channels as multi-ion, single-file pores Hille and Schwarz (1978) were able to reproduce many features of the inward rectifier using a three-site model. Further experiments are required for a broad range of experimental conditions to determine the detailed applicability of such models.

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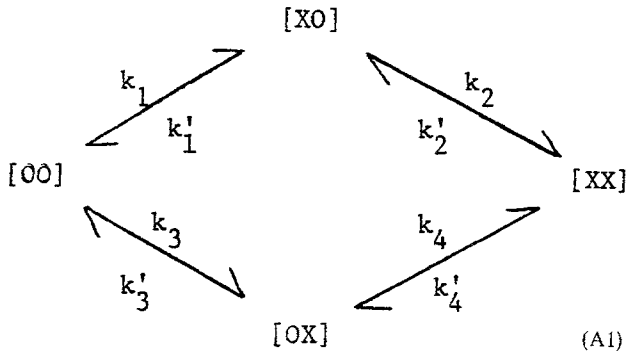
Appendix A

Potential Transformation for Activation Sites

The purpose of this section is to derive expressions which make explicit the membrane potential dependence of K^+ efflux activation by external monovalent cations. For simplicity, we shall assume that only two membrane sites associated with each rectifier channel are related to activation. Our conclusions, however, can be generalized to any integral number of sites, but the mathematical development becomes more complex. The reactions governing the occupancy is given by the following scheme:



(A1)

As written, $[OO]$, $[OX]$, $[XO]$, and $[XX]$ denote the number of channels with completely vacant, partially vacant, and fully occupied activation sites. The k_i and k'_i denote the pseudo first-order rate constant for the individually labeled reactions. Assuming that the number of channels is constant, one finds

$$[OO]+[XO]+[OX]+[XX]=T \tag{A2}$$

where T is constant. In addition, at equilibrium the following relations hold:

$$[XO]=(k_1/k'_1)[OO] \tag{A3}$$

$$[OX]=(k_3/k'_3)[OO] \tag{A4}$$

$$[XX]=(k_2/k'_2)[XO]=(k_2/k'_2)(k_1/k'_1)[OO] \tag{A5}$$

and

$$[XX]=(k_4/k'_4)[OX]=(k_4/k'_4)(k_3/k'_3)[OO]. \tag{A6}$$

Substituting Eqs. (A3), (A4), and (A5) into (A2), one obtains

$$[OO]\cdot Q=T \tag{A7}$$

where

$$Q=1+(k_1/k'_1)+(k_3/k'_3)+(k_2/k'_2)(k_1/k'_1). \tag{A8}$$

If one assumes that the increment in K^+ efflux, Δ, is proportional to the fraction of channels with completely occupied activation sites, then from Eqs. (A5) and (A7)

$$\Delta \propto [XX]/T=(k_2/k'_2)(k_1/k'_1)/Q. \tag{A9}$$

Using Eyring rate theory, assuming only one potential energy barrier between the external solution and the location of the individual activation sites, the expressions for the pseudo first-order rate constants are given by the following relations:

$$k_i=[X]_o a_i e^{-\theta_i VF/RT} \tag{A10}$$

and

$$k'_i=a'_i e^{+\theta'_i VF/RT} \tag{A11}$$

where i takes on the values 1 through 4. Here $[X]_o$ represents the concentration of the external cation; a_i and a'_i are constants independent of potential; and θ_i and θ'_i give the fraction of membrane potential felt in jumping the barrier in forward and reverse directions, respectively. Note that since k_1, k'_1, k_4, and k'_4 refer to occupation of the first site and k_2, k'_2, k_3 and k'_3 to occupation of the second site, $\theta_1=\theta_4$, $\theta'_1=\theta'_4$, $\theta_2=\theta_3$ and $\theta'_2=\theta'_3$. Substituting Eqs. (A8), (A10), and (A11) into (A9) one obtains:

$$\Delta \propto \frac{[XX]}{T} = \frac{r_1 r_2\{[X]_o e^{-\langle\eta\rangle VF/RT}\}^2}{1+\{r_1 e^{-\eta_1 VF/RT}+r_3 e^{-\eta_3 VF/RT}\}[X]_o+r_1 r_2\{[X]_o e^{-\langle\eta\rangle VF/RT}\}^2}, \tag{A12}$$

where $r_i=(a_i/a'_i)$; $\eta_i=\theta_i+\theta'_i$; and $\langle\eta\rangle=(\eta_1+\eta_2)/2$. At low $[X]_o$ where the system is mainly in the $[OO]$ state, Eq. (A12) reduces to

$$\Delta \propto [XX]/T=r_1 r_2\{[X]_o e^{-\langle\eta\rangle VF/RT}\}^2. \tag{A13}$$

That is, the potential dependence of the increment in K^+ efflux expresses itself as a simple factor multiplying the external concentration.

If the two activating sites span the same fraction of the membrane potential, as might happen if each channel is composed of two subunits, each of which contains one activation site, then $\eta_1=\eta_2=\eta_3=\eta_4=\eta$. In this case Eq. (A12) can be rearranged to give

$$\Delta \propto \frac{[XX]}{T} = \frac{r_1 r_2\{[X]_o e^{-\eta VF/RT}\}^2}{1+(r_1+r_3)\{[X]_o e^{-\eta VF/RT}\}+r_1 r_2\{[X]_o e^{-\eta VF/RT}\}^2}. \tag{A14}$$

In Eq. (A14), $[X]_o$ appears everywhere in the relation with $e^{-\eta VF/RT}$ present as a multiplicative factor and may be written

$$\Delta \propto \frac{[XX]}{T}=\frac{r_1 r_2([X]'_o)^2}{1+(r_1+r_3)[X]'_o+r_1 r_2([X]'_o)^2} \tag{A15}$$

where $[X]'_o=[X]_o e^{-\eta VF/RT}$. In other words, for this situation an alteration of V simply expands or compresses the $[X]_o$ axis by a constant factor in the function relating Δ to $[X]_o$. Equations (A14) and (A15) are valid for all values of $[X]_o$; that is, they are not restricted to the low concentration range where the system is mainly in the $[OO]$ state.

Appendix B

Membrane Potential Correction Factor for Inhibitory Site

Our aim in this section is to correct the fractional inhibition produced by external Rb^+ for measurements made at different membrane potentials. We assume that inhibition of K^+ efflux is produced when a specific membrane site associated with each channel is occupied by a monovalent cation coming from the external solution. Translated into the nomenclature adopted above in the results section, Standen and Stanfield (1980) have shown that for membrane potentials between 0 and -25 mV the fractional inhibition produced by external Rb^+ of inward current through the inward rectifier can be approximated by the relation

$$b=1/(1+c^* e^{-\delta VF/RT}[Rb^+]_o). \tag{B1}$$

In this equation c^* is a constant and $\delta=1.4$.

Our aim is to correct the measured fractional inhibition determined at a potential V, b, to the fractional inhibition at $V=0$, b^*. When $V=0$, Eq. (1) yields

$$b(V=0)=b^*=1/(1+c^*[Rb^+]_o). \tag{B2}$$

Rearranging Eqs. (B1) and (B2), one has

$$(b^{-1}-1)e^{\delta VF/RT}=c^*[Rb^+]_o=(b^*)^{-1}-1. \tag{B3}$$

b^* can then be calculated from the following form of Eq. (B3):

$$b^*=1/(1+(b^{-1}-1)e^{\delta VF/RT}). \tag{B4}$$

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