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# EFFECTS OF INTERNAL AND EXTERNAL $Na^+$ IONS ON INWARDLY RECTIFYING $K^+$ CHANNELS IN GUINEA-PIG VENTRICULAR CELLS

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SUMMARY

1. The effects of internal and external $Na^+$ ions on the inwardly rectifying $K^+$ channel were studied in guinea-pig ventricular cells.

2. Single-channel currents through the inwardly rectifying $K^+$ channel were recorded in the open cell-attached or inside-out configuration at 150 mM internal $K^+$ and either 150 or 25 mM external $K^+$. Internal $Na^+$, at a concentration of 5–40 mM, reduced the unitary amplitude of the outward current. No increase in open-channel current noise was detected with the filter cut-off frequency of 3 kHz. Substate behaviour seen with internal $Mg^{2+}$ at a micromolar level was not observed. The inward currents were little affected by internal $Na^+$.

3. The unitary current–voltage relation rectified inwardly in the presence of internal $Na^+$ in a concentration-dependent manner.

4. Outward unitary currents were normalized to those measured in the absence of $Na^+$. The normalized current–voltage relation was shifted in the negative direction by 20–25 mV by decreasing external $K^+$ from 150 to 25 mM, indicating that the blocking effect increases with low external $K^+$ when compared at a fixed voltage.

5. The normalized current–$Na^+$ concentration curve was fitted by a one-to-one binding curve at each voltage. In a semi-logarithmic plot of dissociation constant *versus* membrane potential, data points for 150 and 25 mM external $K^+$ were fitted by straight lines with nearly the same slope. The dissociation constant at 0 mV is 154 mM in 150 mM external $K^+$ and 89 mM in 25 mM external $K^+$. The voltage dependence of dissociation constants gives a value for the effective valency of the $Na^+$ ion of around 0·5.

6. To study effects of external $Na^+$, single-channel currents were recorded with pipette solutions containing 125 mM $Na^+$, 125 mM choline or 125 mM *N*-methyl-D-glucamine (NMDG) in addition to 25 mM $K^+$. Current amplitude was smaller with choline than with $Na^+$ or NMDG. The reduction in current amplitude with choline was more evident in the inward current, resulting in a stronger outward rectification of the current–voltage relation. This finding and prolonged mean open time (see Summary point 7) was interpreted by assuming that choline is an open-channel blocker.

7. The lifetimes of the openings in the inward currents were distributed according to a single exponential. The mean open time with $Na^+$ was similar to that with

NMDG, which decreased with hyperpolarization. The mean open time with choline was much longer and less voltage dependent. The closed times were fitted by a single exponential function with $Na^+$, while at least two exponentials were required to fit the frequency histogram of the closed times with NMDG or choline.

8. It is concluded that internal $Na^+$ acts as an open-channel blocker and that external $K^+$ relieves the block as in the block by internal $Mg^{2+}$. On the other hand, external $Na^+$ does not block the open channel, but may modify transitions from a short-lived closed state ($C_1$) to other states (O, open; $C_2$, long-lived closed) if we assume a $C_2$-$C_1$-O) model.

## INTRODUCTION

Most cardiac $K^+$ channels show inward rectification, permitting a greater entry of $K^+$ under hyperpolarization than exit under depolarization. This behaviour plays an important role in maintaining the long-lasting action potential characteristic of cardiac cells. The mechanism by which such rectification might occur has remained a puzzle for close to 40 years since it was first reported in the resting $K^+$ conductance of skeletal muscle (Katz, 1949). Recently, however, evidence has accumulated from several cardiac $K^+$ channels that voltage-dependent block by internal $Mg^{2+}$ causes the inward rectification: in the inwardly rectifying $K^+$ channel (Matsuda, Saigusa & Irisawa, 1987; Vandenberg, 1987; Matsuda, 1988); in the adenosine 5′ triphosphate (ATP)-regulated $K^+$ channel (Horie, Irisawa & Noma, 1987; Findlay, 1987); in the muscarinic receptor-operated $K^+$ channel (Horie & Irisawa, 1987, 1989); and in the $Na^+$-activated $K^+$ channel (Wang, Kimitsuki & Noma, 1991).

It is also reported that $Na^+$, which exists physiologically inside cells as well as $Mg^{2+}$, can produce inward rectification in the ATP-regulated $K^+$ channel (Kakei, Noma & Shibasaki, 1985; Horie *et al.* 1987) and the $Na^+$-activated $K^+$ channel (Wang *et al.* 1991). Thus the effects of internal $Na^+$ were studied, to determine whether $Na^+$ blocks the inwardly rectifying $K^+$ channel in a voltage-dependent manner or not and, if so, whether $Mg^{2+}$ or $Na^+$ is more important for producing rectification.

Another question addressed here is how external $Na^+$ acts on the cardiac inwardly rectifying $K^+$ channel. In skeletal muscle (Standen & Stanfield, 1979), egg cells (Ohmori, 1978, 1980) and cardiac cells (Biermans, Vereecke & Carmeliet, 1987; Harvey & Ten Eick, 1989*a*) external $Na^+$ is reported to produce a voltage-dependent block of inward $K^+$ currents. However there are few reports on the effect of $Na^+$ at the single-channel level (Fukushima, 1982; Payet, Rousseau & Sauvé, 1985; Matsuda & Stanfield, 1989).

Present results show that internal $Na^+$ acts as an open-channel blocker but that the blocking effect of $Na^+$ is much weaker than that of $Mg^{2+}$. It is suggested that external $Na^+$ does not block the channel but may modify its gating kinetics.

## METHODS

*Preparations.* Guinea-pigs were anaesthetized with intraperitoneal injections of sodium pentobarbitone (30 mg $kg^{-1}$) and the chest was opened under artificial respiration. The ascending aorta was cannulated *in situ* and the heart was dissected out. The blood was washed out by coronary perfusion with Tyrode solution equilibrated with 100% $O_2$. The composition of the main solutions is listed in Table 1. After the heart was perfused with about 50 ml of $Ca^{2+}$-free Tyrode

solution, the perfusate was switched to a $Ca^{2+}$-free Tyrode solution containing 0·4 mg $ml^{-1}$ collagenase (Sigma, Type I) and 0·8 mg $ml^{-1}$ bovine albumin (Sigma), which was recirculated with a peristaltic pump for about 30 min. In later experiments $Ca^{2+}$-free Tyrode solution containing only collagenase (Yakult, Tokyo, Japan) at a concentration of 0·1–0·15 mg $ml^{-1}$ was perfused for about 10 min. Thereafter collagenase was washed out with 100 ml of a high-$K^+$, low-$Cl^-$ solution

TABLE 1. Composition of solutions (mM)

Bathing solution

| | NaCl | $NaH_2PO_4$ | KCl | $CaCl_2$ | $MgCl_2$ | HEPES | Glucose | $V_j$ (mV) |
|---|---|---|---|---|---|---|---|---|
| Tyrode solution | 140 | 0·33 | 5·4 | 1·8 | 0·5 | 5 | 5·5 | 0 |

| | Potassium aspartate | KCl | $KH_2PO_4$ | NaCl | EDTA | $K_2ATP$ | HEPES | $V_j$ (mV) |
|---|---|---|---|---|---|---|---|---|
| 150 mM $K^+$–$Na^+$ free | 60 | 65 | 1 | 0 | 5 | 3 | 5 | −9·0 |
| 150 mM $K^+$–$Na^+$ test | 60 | 65 | 1 | 5–40 | 5 | 3 | 5 | −7·9 to −8·8 |

| Pipette solution | KCl | NaCl | Choline chloride | NMDG-Cl | $CaCl_2$ | HEPES | $V_j$ (mV) |
|---|---|---|---|---|---|---|---|
| 150 mM $K^+$ | 150 | 0 | 0 | 0 | 1 | 5 | −2·0 |
| 25 mM $K^+$–125 mM $Na^+$ | 25 | 125 | 0 | 0 | 1 | 5 | 0 |
| 25 mM $K^+$–125 mM choline | 25 | 0 | 125 | 0 | 1 | 5 | +1·0 |
| 25 mM $K^+$–125 mM NMDG | 25 | 0 | 0 | 125 | 1 | 5 | +2·6 |

The pH of solutions was adjusted to 7·4 with KOH or NaOH (Tyrode solution, 25 mM $K^+$–125 mM $Na^+$ pipette solution) or HCl (25 mM $K^+$–125 mM NMDG solution). The $K^+$ concentration after titration was approximately 155 mM in internal solution, 152 mM in 150 mM $K^+$ pipette solution and 27 mM in 25 mM $K^+$–125 mM choline pipette solution. Dipotassium salt of EDTA was used. $V_j$ was the liquid junction potential between the solution and the Tyrode solution (the sign was positive when the Tyrode solution side was negative).

containing (mM): KCl, 30; glutamic acid, 70; $KH_2PO_4$, 10; $MgCl_2$, 1; taurine, 20; HEPES, 10; glucose, 10 and ethyleneglycol-bis-(β-aminoethylether)*N*,*N*′-tetraacetic acid, 0·3; pH was adjusted to 7·3 with KOH. The temperature of all perfusates was kept at 36–37 °C during coronary perfusion, and the hydrostatic pressure for perfusion was approximately 65 $cmH_2O$. Finally, the ventricles were cut and chopped with scissors. Gentle agitation of the chunks released the cells into the high-$K^+$, low-$Cl^-$ solution. The cells were filtered through a 150 μm mesh net and centrifuged at 800 r.p.m. for 4 min. The cell pellet was resuspended in modified Eagle's medium (Flow Laboratories, Irvine, Scotland) and was kept at room temperature.

*Recording techniques*

Recordings of single-channel currents were performed using a heat-polished patch electrode (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Pipettes were made from capillaries of hard borosilicate glass (Pyrex) and were coated near their tips with silicone to reduce electrical capacitance. The electrode resistance ranged between 10 and 15 MΩ when filled with 150 mM KCl pipette solution.

Current records illustrated in this paper were obtained from open cell-attached (Horie *et al.* 1987) or inside-out patches. After the gigaseal was attained in Tyrode solution, nominally $Ca^{2+}$-free Tyrode solution and then 150 mM $K^+$–$Na^+$-free, divalent cation-free solution were perfused. To make the open cell-attached patch, the cell membrane was ruptured in the last solution by crushing the tip of another patch pipette (filled with the same solution as the bathing solution) against the cell on the glass bottom of the recording chamber. The intracellular milieu was equilibrated with the bathing solution through the hole made in the membrane. ATP (dipotassium salt) at a concentration of 3 mM suppressed the ATP-regulated $K^+$ channel. Thus the channel observed

constantly under this condition was the inwardly rectifying $K^+$ channel responsible for the resting conductance. The channel activity was maintained for as long as 30–60 min in a total absence of $Mg^{2+}$ (internal solution contained 5 mM ethylenediamine tetraacetic acid, EDTA; but see Takano, Qin & Noma, 1990). NaCl (5–40 mM) was added to 150 mM $K^+$, divalent cation-free solution without compensation for changes in osmolarity and ionic strength. The temperature of the solution in the chamber was kept at 24–26 °C.

Recordings were made with an EPC-7 patch clamp amplifier (List electronics). A steady potential was applied to the inside of the electrode to set the holding potential of the membrane patch, and outward currents were elicited by depolarizing steps of 130 ms every 1 s. Membrane potentials are expressed in the conventional way, inside relative to outside, and outward currents are ascribed a positive sign. The membrane potentials were corrected for the liquid junction potential at the tip of the patch pipette in Tyrode solution and also that at the tip of the indifferent reference electrode filled with Tyrode solution in the bathing solution.

*Data analysis.* Data were recorded on a video cassette (Victor, BR-6400) using a PCM converter system (NF, RP-880) and stored for subsequent computer analysis (NEC, PC-98 XL). Currents were filtered using a four-pole low-pass Bessel filter (NF, FV-665) with a -3 dB corner frequency of 1·2 kHz (outward currents) or 1 kHz (inward currents) and sampled every 0·2 ms, unless otherwise indicated. For outward currents, capacitive and leakage currents were removed by the transient cancellation facility of the amplifier and by subtracting from each trace the average of current traces without events. Unitary current amplitudes were measured by forming histograms of baseline and open-level data points and fitting these histograms with Gaussian curves using a least-squares algorithm to find the area under each curve and its mean and variance. Open and closed times were measured in patches where only one channel was active, using a cursor set midway between open and closed levels. Open- and closed-time histograms were formed and then fitted with exponential(s) using a least-squares algorithm. Events of very short duration, too rapid to properly resolve, were excluded from the fitting by omitting the first bin. Results throughout this paper are given as means ± S.D.

## RESULTS

### *Reduction of the unit amplitude of outward currents by internal $Na^+$*

Outward single-channel currents through the inwardly rectifying $K^+$ channel are not recorded in the cell-attached configuration (Sakmann & Trube, 1984*a*). Outward currents, however, appear in response to voltage steps or voltage ramps to levels more positive than the equilibrium potential for $K^+$ when the internal surface of the cell is exposed to divalent cation-free, $K^+$ solution (Matsuda *et al.* 1987; Vandenberg, 1987; Matsuda, 1988, 1991).

Figure 1*A* shows outward currents induced by voltage steps to +30 mV from a holding potential of −89 mV with 25 mM $K^+$–125 mM $Na^+$ pipette solution. The zero-current potential was around −40 mV at 25 mM external $K^+$. Millimolar quantities of internal $Na^+$ decreased the unitary amplitude of the outward current. The reduction increased with increasing $Na^+$ concentration. With $Mg^{2+}$ at a micromolar level, outward open-channel currents show sublevels with one-third and two-thirds of the unit amplitude and fluctuate between sublevels (Matsuda, 1988, 1991). $Na^+$ did not produce such sublevels nor large fluctuations (flicker) in the open-channel currents such as those observed in $Na^+$ block in the $Ca^{2+}$-activated $K^+$ channel of bovine chromaffin cells (Marty, 1983; Yellen, 1984*a*, *b*). Note that the standard deviation of the open-channel current was not increased in the presence of internal $Na^+$ (Fig. 1*B*). Flickering was not seen even when the filter cut-off frequency was increased to 3 kHz.

The reduction in current amplitude is enhanced by positive membrane potentials as shown in Fig. 2*A*, where outward currents at different voltages in the presence of

20 mM internal $Na^+$ are illustrated. Arrow-heads indicate the unit amplitude of the outward current in the absence of internal $Na^+$.

Thus the current–voltage (*I–V*) relation rectified inwardly in a $Na^+$-concentration-dependent manner. Figure 2*B* shows the *I–V* relations at different $Na^+$ concentrations

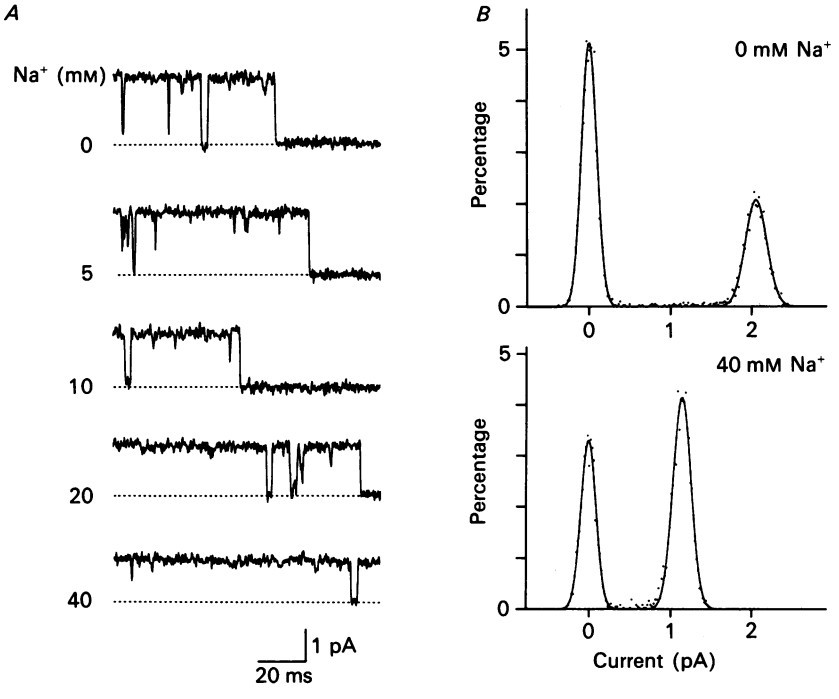


Fig. 1. *A*, effect of internal $Na^+$ on the outward single-channel current through an inwardly rectifying $K^+$ channel. After recording the control trace while perfusing the open-cell patch membrane with $Na^+$-free solution, the $Na^+$ concentration of the perfusing solution was increased progressively. Then $Na^+$ was washed out and recording at 0 mM was repeated. A trace for illustration at 0 mM was obtained during the second control run. The dotted line in this and subsequent figures indicates zero-current level; 25 mM $K^+$–125 mM $Na^+$ pipette solution; depolarizing step to +30 mV from −89 mV. *B*, histograms of current amplitude with 0 and 40 mM $Na^+$ from the same patch as in *A*. The curve is the sum of two Gaussian distributions fitted by a least-squares method. The parameters (area, mean, standard deviation) were; 0·624, 0, 0·09; 0·357, 2·05, 0·13 at 0 mM: 0·392, 0, 0·09; 0·591, 1·15, 0·11 at 40 mM. The standard deviation of the open-channel current was not increased in the presence of 40 mM $Na^+$.

(0–40 mM) measured in the same patch shown in Figs 1 and 2*A*. In this case a slight inward rectification was seen even in the absence of internal $Na^+$, which was rather exceptional (Matsuda, 1988, 1991). Inward currents were little affected by internal $Na^+$ and are omitted in the figure.

In a simple model for channel blocking, the voltage dependence arises because the blocking ion binds to a site inside the channel within the transmembrane electric field (Woodhull, 1973). Internal blocking ions should be driven into the channel by depolarized voltages to produce a more pronounced block. The voltage-dependent reduction of the unitary amplitude by internal $Na^+$ suggests that $Na^+$ moves into the

inner mouth of the channel and binds to a site inside the channel to prevent $K^+$ efflux. It is considered that blocking by $Na^+$ is much too fast to observe the discrete open and blocked states of the channel and rather we see the time-averaged value of the current through the channel.

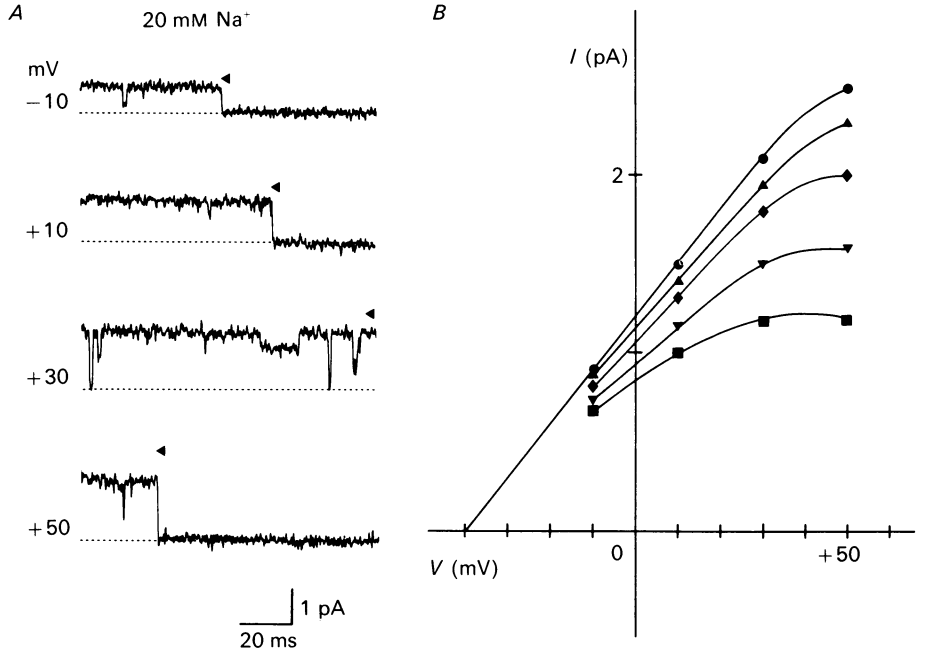


Fig. 2. *A*, outward currents at different voltages in the presence of 20 mM internal $Na^+$. Arrow-heads indicate the unit amplitude of the outward current in the absence of $Na^+$. The same patch as in Fig. 1. *B*, unitary current–voltage relationship obtained from the same patch at a $Na^+$ concentration of 0 (●), 5 (▲), 10 (◆), 20 (▼) and 40 (■) mM.

If we assume that internal $Na^+$ acts as an open-channel blocker and that the only route between the blocked state and the closed state is via the open state, it is predicted that the mean burst length will increase with the concentration of the blocking ion (Neher & Steinbach, 1978). A burst is the time that the channel spends in a sequence of open and blocked states, and is terminated by a transition to a closed state. The transitions between the open and blocked state cannot be resolved in $Na^+$ block and the open times measured in the presence of internal $Na^+$ represent burst durations. As described previously (Matsuda, 1988), there is a tendency for open times of the channel in the outward direction to be prolonged during perfusion of the artificial internal solution. The washing away of a soluble gating component by the perfusion may hinder the channel from closing. Although such prolongation of the open time with time even in the absence of blocking ions made systematic analysis difficult, the open time with 40 mM $Na^+$ was compared with that after washing out $Na^+$. In the experiment shown in Fig. 1*A*, the mean open time decreased from 20 to 12 ms after wash-out of 40 mM $Na^+$. A similar result was obtained in three other experiments, supporting the theory that a voltage-dependent block by internal $Na^+$ with fast kinetics causes the reduction of the outward current.

In terms of a voltage-dependent block by internal $Na^+$, the time-averaged channel current at a membrane potential of $V$, $i(V)$, will be:

$$i(V) = i_0(V)\{1 + ([Na^+]_i/K_D(V))^n\}^{-1},$$

where $i_0(V)$ is the current through the unblocked channel, $[Na^+]_i$ the intracellular $Na^+$ concentration, $K_D(V)$ a voltage-dependent dissociation constant, and $n$ a Hill

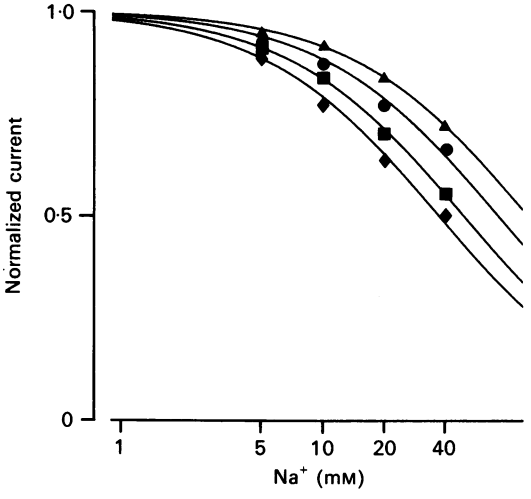


Fig. 3. Normalized current–concentration curves obtained from the patch shown in Figs 1 and 2. Each curve was fitted by assuming one-to-one binding of $Na^+$ to a site. The dissociation constant was 101·8 mM at −10 mV (▲), 72·6 mM at +10 mV (●), 49·1 mM at +30 mV (■) and 37·1 mM at +50 mV (◆), respectively.

coefficient. Figure 3 shows a plot of the unit amplitude of the outward current normalized to that in the absence of $Na^+$ ($i(V)/i_0(V)$) against the $Na^+$ concentration at four different voltages. The effect of internal $Na^+$ was studied at concentrations lower than 40 mM so that the $Na^+$-activated $K^+$ channel was not activated (Kameyama, Kakei, Sato, Shibasaki, Matsuda & Irisawa, 1984). Results give, at each voltage, a reasonable fit to concentration–effect curves predicted by the above equation with $n$ of 1, or by assuming one-to-one binding of $Na^+$ to a site.

*Relief of the block by external $K^+$*

Outward currents were also recorded with 150 mM $K^+$ pipette solution and compared to those with 25 mM $K^+$ pipette solution to study the effect of external $K^+$ on the block by internal $Na^+$. Figure 4*A* shows current traces in the presence and absence of 40 mM $Na^+$ at different external $K^+$ concentrations. The current amplitude was decreased to about half of the control value with 25 mM $K^+$, while it was decreased to only two-thirds with 150 mM $K^+$. The normalized current averaged in seven experiments at each $K^+$ concentration was plotted against the membrane potential in Fig. 4*B*. No differences were found in the extent of the block between experiments with 25 mM $K^+$–125 mM NMDG and with 25 mM $K^+$–125 mM $Na^+$ pipette solutions or between experiments in the open cell-attached and inside-out patch configuration. Thus average values shown in Figs 4*B* and 5 were calculated

neglecting differences in the substitutes for $K^+$ or in the methods of exposure of the patch membrane to the artificial solution. The relation is shifted in the positive direction by 20–25 mV by increasing external $K^+$ from 25 to 150 mM, i.e. the blocking effect decreases with high external $K^+$ when compared at a fixed voltage. The shift of 20–25 mV is smaller than that of the zero-current potential (about 40 mV).

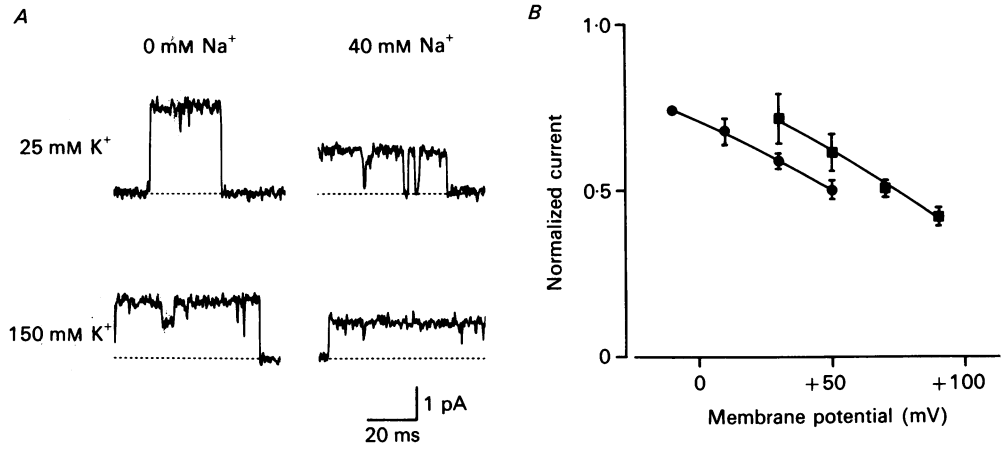


Fig. 4. *A*, effect of external $K^+$ on the block by internal $Na^+$. Outward current at the same voltage (+50 mV) is depressed more by 40 mM $Na^+$ with 25 mM $K^+$–125 mM NMDG pipette solution (upper panel) than with 150 mM $K^+$ pipette solution (lower panel). *B*, normalized current–voltage relationships in the presence of 40 mM $Na^+$. ●, 25 mM $K^+$; ■, 150 mM $K^+$. Each data point represents the mean of seven experiments. Bars give ±S.D. of means; where no standard deviation is shown it was smaller than the symbol size.

A similar shift was observed in a semilogarithmic plot of dissociation constant *versus* membrane potential (Fig. 5). Data points at each pipette $K^+$ concentration, averaged in seven experiments, can be fitted by a straight regression line, indicating that the dissociation constant decreases exponentially as the membrane potential is increased. The dissociation constant at a membrane potential of $V$, $K_D(V)$ is described as:

$$K_D(V) = K_D(0)\exp(-z'VF/RT),$$

where $z'$ is the so-called effective valency of the blocking ion, equal to the actual valency multiplied by the fractional electrical distance between the internal mouth of the aqueous pore and the $Na^+$ binding site (Woodhull, 1973). $F$, $R$ and $T$ have their usual meanings. The slope of the regression lines gave a value for $z'$ of 0·45 with 25 mM $K^+$ and 0·51 with 150 mM $K^+$. This difference probably results from experimental errors, considering that external $K^+$ did not change the slope in $Mg^{2+}$ block (Matsuda, 1991). The value of $K_D$ at 0 mV, $K_D(0)$, is dependent on the external $K^+$ (154 mM in 150 mM $K^+$ and 89 mM in 25 mM $K^+$).

*Effects of external $Na^+$ on inward currents through the channel*

As described so far, the effect of internal $Na^+$ on the outward current can be interpreted as a voltage-dependent block of the channel with very fast kinetics. $Na^+$ present in the external solution is also supposed to block the inward current in a

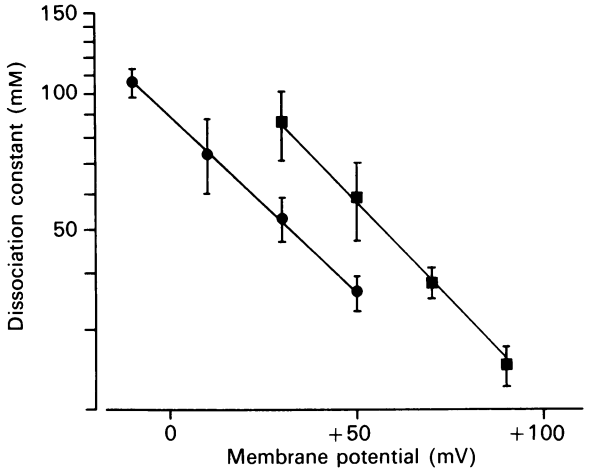


Fig. 5. Dependence of the dissociation constant on membrane potential. ●, 25 mM $K^+$; ■, 150 mM $K^+$. The bars give ±S.D. of means. Dissociation constants ($n = 7$) were 106·3±8·4 mM at −10 mV, 74·1±14·6 mM at +10 mV, 53·3±6·1 mM at +30 mV and 36·4±3·4 mM at +50 mV with 25 mM $K^+$, and 86·9±15·5 mM at +30 mV, 58·5±12·0 mM at +50 mV, 37·9±3·5 mM at +70 mV and 24·9±2·4 mM at +90 mV with 150 mM $K^+$. In a semilogarithmic plot, data points at each external $K^+$ are fitted by a straight regression line. The slope of the line gave the fractional electrical distance of the $Na^+$ binding site of 0·45 with 25 mM $K^+$ and 0·51 with 150 mM $K^+$.

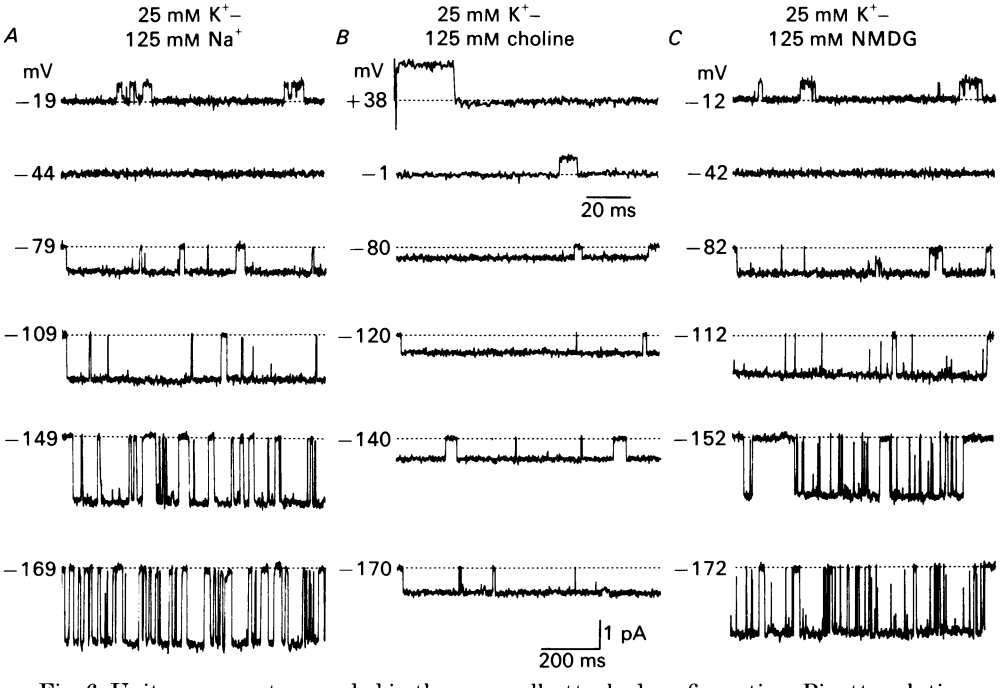


Fig. 6. Unitary currents recorded in the open cell-attached configuration. Pipette solution contains: *A*, 125 mM $Na^+$; *B*, 125 mM choline; and *C*, 125 mM NMDG in addition to 25 mM $K^+$. Numbers to the left of each current trace refer to the holding potential or the potential levels during the depolarizing steps from −97 mV (upper two panels of *B*). Steady-state currents for display were filtered at 1 kHz and sampled every 1 ms.

voltage-dependent manner, mainly based on the observation that the macroscopic inward current inactivates during hyperpolarization in the presence of $Na^+$ (see Introduction for references).

To examine voltage-dependent block by external $Na^+$, I recorded single-channel currents with pipette solutions of several kinds. Figure 6 shows records obtained

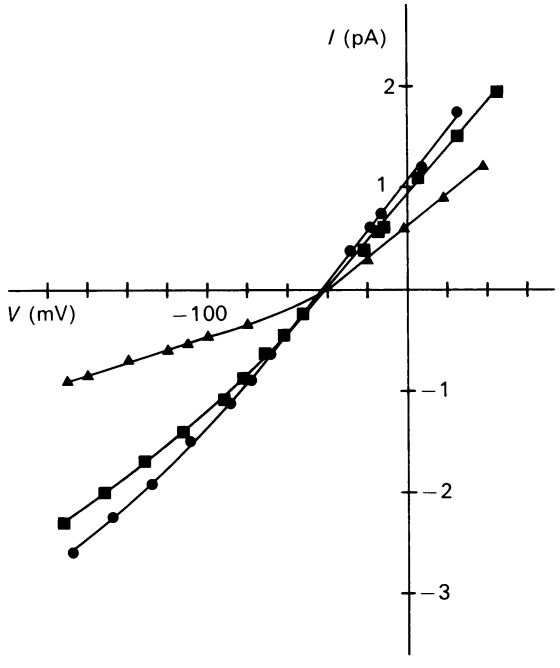


Fig. 7. Unitary current–voltage relationships obtained from the same patches as in Fig. 6. ●, 125 mM $Na^+$; ▲, 125 mM choline; ■, 125 mM NMDG.

from different patches using 25 mM $K^+$–125 mM $Na^+$ solution (*A*), 25 mM $K^+$–125 mM choline solution (*B*) and 25 mM $K^+$–125 mM NMDG solution (*C*). As in egg cells (Fukushima, 1982) and skeletal muscle (Matsuda & Stanfield, 1989), the open time of the inward current decreased remarkably in the presence of external $Na^+$ as voltage was made increasingly negative. With choline, the open time was longer and much less dependent on voltage. Choline reduced the amplitude of unitary currents, especially in the inward direction. The open time decreased with hyperpolarization in the presence of NMDG as steeply as with $Na^+$ present. Short closed states were frequently observed with NMDG.

The *I–V* relation showed a slight outward rectification with $Na^+$ or NMDG (Fig. 7). The average slope conductance measured in the voltage range between −130 and −70 mV was $20{\cdot}2 \pm 2{\cdot}1$ pS ($n = 6$) with $Na^+$, $19{\cdot}2 \pm 1{\cdot}4$ pS ($n = 5$) with NMDG and $6{\cdot}8 \pm 1{\cdot}5$ pS ($n = 7$) with choline. Choline present in the pipette solution decreased the amplitude of the inward current more markedly than that of the outward current, resulting in a noticeable outward rectification. The voltage-dependent reduction of the unit amplitude with choline in addition to the prolongation of the open time

suggests that choline may not be a simple substitute for $Na^+$. The same effects as choline were observed in two experiments with tetramethylammonium (TMA); the slope conductance measured in the negative voltage range was 8·4 and 9·2 pS, and the mean open time at −140 mV was 171 and 183 ms.

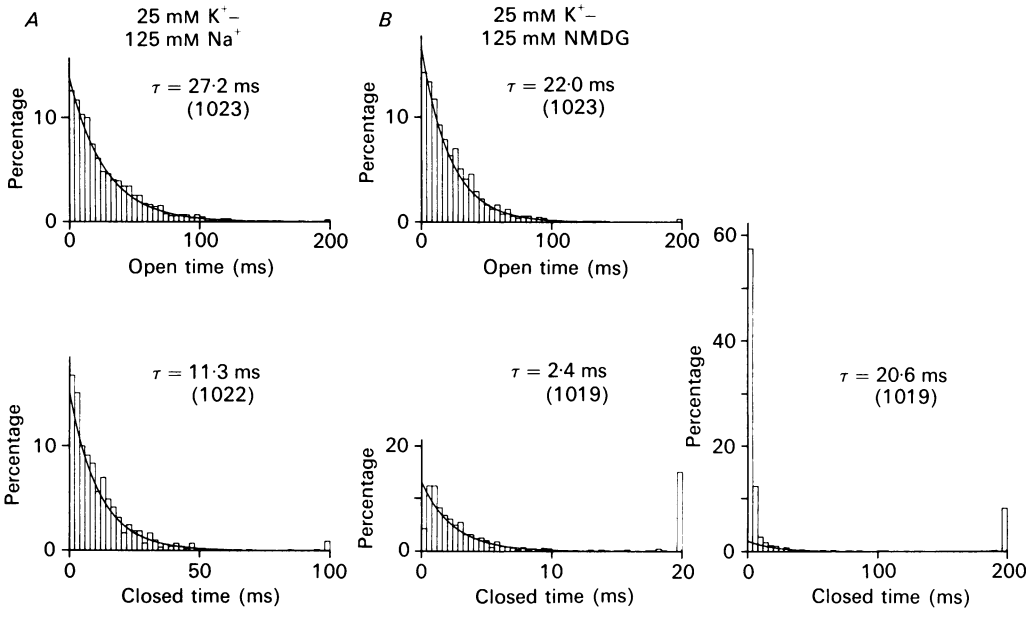


Fig. 8. Histograms of open and closed times with 25 mM $K^+$–125 mM $Na^+$ (*A*) and 25 mM $K^+$–125 mM NMDG pipette solution (*B*). Holding potential, −149 mV (*A*) and −152 mV (*B*). The open-time histograms and the closed-time histogram with 125 mM $Na^+$ were fitted with a single-exponential function with the time constant ($\tau$) indicated. The closed-time histogram with 125 mM NMDG were fitted with two exponentials; the fitted probability density function is $f(t) = 0{\cdot}366 \exp(-t/2{\cdot}41) + 0{\cdot}00568 \exp(-t/20{\cdot}6)$; $t$ = time (ms). The numbers of events are shown in parentheses.

Figure 8 shows open- and closed-time histograms recorded at around −150 mV with 25 mM $K^+$–125 mM $Na^+$ (*A*) or with 25 mM $K^+$–125 mM NMDG (*B*). The lifetimes of the openings were distributed according to a single exponential. The mean open time with $Na^+$ is similar to that with NMDG. The distribution of closed times with $Na^+$ was different from that with NMDG or choline. Most of the closed times were fitted by a single exponential function having a time constant of about 10 ms in the case of $Na^+$, while at least two exponentials were required to fit the closed-time histogram in the case of NMDG or choline.

Figure 9 summarizes the mean open and closed times with each pipette solution. The open time showed a strong voltage dependence in the presence of $Na^+$ and NMDG. The open time with $Na^+$ was not shorter than that with NMDG. The distribution of closed times with $Na^+$ was fitted by a single exponential function, whose time constant is weakly voltage dependent. In the case of NMDG or choline at least two exponentials were needed to fit the closed-time histogram and both fast and slow time constants increased with hyperpolarization. One possible explanation

of these results in a linear $C_2$-$C_1$-O model is that external $Na^+$ modifies transitions from a closed state of short lifetime ($C_1$) to other states (O, open; $C_2$, long-lived closed) (see Discussion).

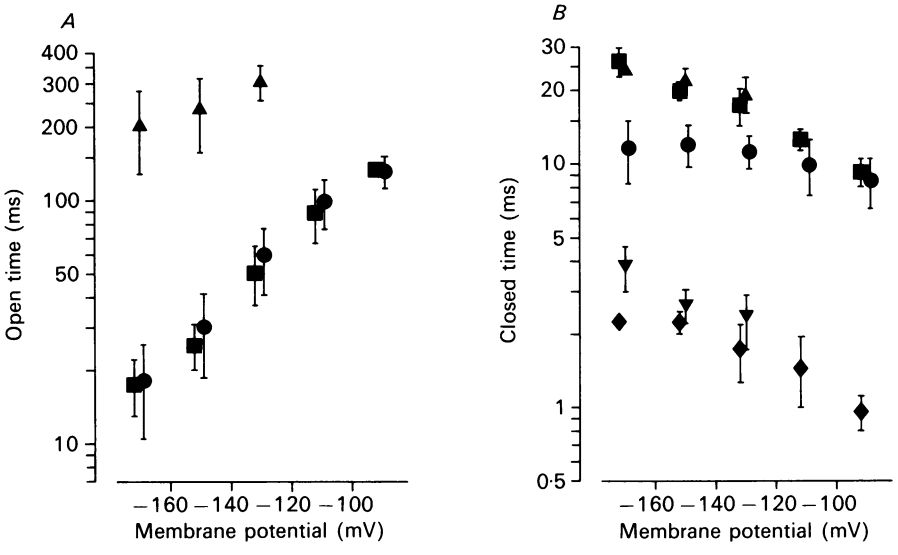


Fig. 9. Dependence of mean open time (*A*) and mean closed time (*B*) on membrane potential. ●, 125 mM $Na^+$ ($n = 5$); ▼, ▲, 125 mM choline ($n = 4$); ◆, ■, 125 mM NMDG ($n = 4$). The bars give ±S.D. of means; when no bars are shown S.D. is smaller than the symbol size.

## DISCUSSION

In this work, the effects of internal and external $Na^+$ ions on the inwardly rectifying $K^+$ channel were studied. Internal $Na^+$ reduced the unit amplitude of the outward current in a voltage-dependent manner and the depressive effect of $Na^+$ was increased by reducing the external $K^+$ concentration at a given voltage.

A number of $K^+$ channels are known to be the subject of voltage-dependent block by internal $Na^+$: the $Ca^{2+}$-activated $K^+$ channel of bovine chromaffin cells (Marty, 1983; Yellen, 1984*a*, *b*), the ATP-regulated $K^+$ channel of guinea-pig ventricular cells (Kakei *et al.* 1985; Horie *et al.* 1987), of insulin-secreting cell line RINm5F (Ciani & Ribalet, 1988), and of frog skeletal muscle (Quayle & Stanfield, 1989), and the $Na^+$-activated $K^+$ channel of guinea-pig ventricular cells (Wang *et al.* 1991). In the $Ca^{2+}$-activated $K^+$ channel $Na^+$ produces flicker in the open level (Marty, 1983; Yellen, 1984*a*, *b*). In other channels $Na^+$ reduces single-channel amplitude without increasing noise in the open level, suggesting $Na^+$ blockage is very rapid. As has been described here, this is the case in $Na^+$ block of the inwardly rectifying $K^+$ channel.

In the presence of internal $Mg^{2+}$ at a micromolar level, the outward current through the inwardly rectifying $K^+$ channel showed sublevels with one-third and two-thirds of the unit amplitude and fluctuated between four levels including the fully open channel current and the zero-current levels (Matsuda, 1988, 1991). Such

sublevels are also induced in the inward current by the external application of $Cs^+$ or $Rb^+$, though the effect is not as consistent as in the case of $Mg^{2+}$ block (Matsuda, Matsuura & Noma, 1989). The open-state occupancies of each current level are in reasonable agreement with the binomial theorem, suggesting that the inwardly rectifying $K^+$ channel is composed of three identical conducting subunits and each subunit is blocked independently (Matsuda, 1988; Matsuda *et al.* 1989). In the present work, neither internal nor external $Na^+$ induced the sublevel behaviour. However this does not necessarily exclude the idea that the cardiac inwardly rectifying $K^+$ channel has a triple-barrel structure. If the blocking kinetics by internal $Na^+$ are extremely rapid, not only should the discrete open and blocked states of the conducting units be obscured, but also flickerings of the open current should be averaged to reduce the current amplitude through the subunits. Thus we record the sum of the time-averaged, reduced current through the conducting units.

It has been reported that the blocking effect of internal cations is decreased by increasing external $K^+$; in $Na^+$ block of the $Ca^{2+}$-activated $K^+$ channel of bovine chromaffin cells (Marty, 1983; Yellen, 1984*b*) and $Mg^{2+}$ block of the ATP-regulated $K^+$ channel (Horie *et al.* 1987) and the inwardly rectifying $K^+$ channel (Matsuda, 1991) of guinea-pig ventricular cells. Such an effect of $K^+$ ions has been ascribed to speeding the exit of a blocker from the channel (Yellen, 1984*b*; Horie *et al.* 1987) or both competition between blocker and $K^+$ for binding to the site and unblock induced by $K^+$ (Matsuda, 1991). The very fast kinetics of $Na^+$ block of the inwardly rectifying $K^+$ channel hampered the study of the mechanism of relief of the block by external $K^+$.

The voltage dependence of the dissociation constant gave a value for the fractional electrical distance of the $Na^+$ binding site of around 0·5. The value of 0·57 was obtained for the $Mg^{2+}$ binding site (Matsuda, 1991). Thus the $Na^+$ binding site seems to be located near the $Mg^{2+}$ binding site. However the value of $K_D$ in $Na^+$ block is much larger than that in $Mg^{2+}$ block. Considering that the physiological concentration of the intracellular $Na^+$ is about 10 mM (Sheu & Fozzard, 1982; Chapman, Coroy & McGuigan, 1983; Blatter & McGuigan, 1991), the contribution of internal $Na^+$ to the inward rectification is less significant than that of $Mg^{2+}$ (Matsuda, 1988, 1991).

The effect of external $Na^+$ seems rather more complicated than that of internal $Na^+$. Macroscopic inward currents through the inwardly rectifying $K^+$ channel of skeletal muscle (Standen & Stanfield, 1979), egg cells (Ohmori, 1978, 1980) and cardiac cells (Biermans *et al.* 1987; Harvey & Ten Eick, 1988, 1989*a*) showed a more marked decay in the presence of external $Na^+$ than in the absence of $Na^+$. This effect was attributed to voltage-dependent block of the channel by external $Na^+$. Reduction of the current amplitude with substitutes for $Na^+$ such as Tris, TMA or choline was also observed, which was interpreted as a facilitating effect of external $Na^+$ (Ohmori, 1978) or as an inhibitory effect of the substitutes (Standen & Stanfield, 1979; Biermans *et al.* 1987) or as a depressive effect of intracellular protons which increased through suppression of $Na^+$–$H^+$ exchange (Harvey & Ten Eick, 1989*b*).

Recordings of unitary inward currents in the present work showed that in the presence of $Na^+$ the open time is much more dependent on voltage, the gap duration is longer and the amplitude of unitary currents is larger. These findings might be

interpreted as a voltage-dependent block by external $Na^+$ and the facilitating effect of $Na^+$ (Fukushima, 1982). However the result with NMDG suggests another possible explanation of the effects of external $Na^+$ and the substitutes.

External $Na^+$ at a concentration of 100 mM added to 150 mM $K^+$ pipette solution did not affect the single-channel conductance, excluding the facilitatory effect of $Na^+$

TABLE 2. Rate constants ($ms^{-1}$) in the inward current

| Voltage | $k_1$ | $k_{-1}$ | $k_2$ | $k_{-2}$ |
|---|---|---|---|---|
| 125 mM NMDG ($n = 4$) | | | | |
| −112 mV | 0·505±0·126 | 0·0127±0·0019 | 0·116±0·011 | 0·193±0·084 |
| −132 mV | 0·481±0·118 | 0·0224±0·0036 | 0·0754±0·0168 | 0·110±0·033 |
| −152 mV | 0·396±0·057 | 0·0409±0·0077 | 0·0581±0·0077 | 0·0469±0·0098 |
| −172 mV | 0·393±0·021 | 0·0602±0·0123 | 0·0436±0·0063 | 0·0453±0·0039 |
| 125 mM $Na^+$ ($n = 5$) | | | | |
| −109 mV | 0·103±0·025 | 0·0106±0·0024 | — | — |
| −129 mV | 0·0896±0·0130 | 0·0181±0·0061 | — | — |
| −149 mV | 0·0848±0·0134 | 0·0382±0·0185 | — | — |
| −169 mV | 0·0889±0·0219 | 0·0686±0·0130 | — | — |

Rate constants were calculated in terms of a linear sequential model ($C_2$-$C_1$-O). The closed states with external $Na^+$ fitted by a single exponential function were explained by assuming that $Na^+$ reduces the rate constant from short-lived closed states ($C_1$) to long-lived closed states ($C_2$) ($k_{-2}$) to zero.

on the cardiac inwardly rectifying $K^+$ channel (H. Matsuda, unpublished observation). Thus reduction of the current amplitude which is more prominent in the inward direction and prolongation of the open time with choline and TMA imply that these act as open-channel blockers to be poor substitutes for $Na^+$. I assume, as a last resort, that NMDG represents a control condition. On this assumption, because there is no difference between open time with $Na^+$ and with NMDG, it is unlikely that external $Na^+$ acts as an open-channel blocker.

Compared with the result with NMDG, it is the distribution of the closed states that is affected by external $Na^+$. I considered this result in terms of a linear sequential model as:

$$C_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} O,$$

where $C_2$, $C_1$ are closed states, O is the open state, and the $k$ symbols are rate constants (Kameyama, Kiyosue & Soejima, 1983; Sakmann & Trube, 1984*b*). Colquhoun & Hawkes (1981) have given the probability density function of the closed lifetime for a linear scheme with two closed states (their eqn (2.32)). Rate constants with NMDG were calculated from the observed probability density function of the closed time and the mean open time (Table 2). The closed states in the presence of $Na^+$ fitted by a single exponential function with the time constant of about 10 ms are explained in this model by assuming that external $Na^+$ reduces the rate constant, $k_{-2}$, to zero and $k_1$ to about one-fifth of that with NMDG (Table 2).

This is the simplest explanation for a majority of the present observations at the level of the single channel on the assumption that NMDG has no effects on the inwardly rectifying $K^+$ channel.

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