Kinetic properties of unitary Na^+-dependent K^+ channels in inside-out patches from isolated guinea-pig ventricular myocytes

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1. Single Na^+-activated K^+ channels (K_{Na}) were investigated by means of the inside-out patch clamp technique in ventricular myocytes isolated from the guinea-pig heart.

2. Na^+-activated K^+ channels were observed at very low density (< 9% of patches). In symmetrical (60/60 mM) K^+ solutions, K_{Na} channels had a mean slope conductance of 75 pS and in asymmetrical (150/70 mM; outside/inside) K^+ solutions, they had a mean slope conductance of 220 pS. The reversal potentials obtained under these two ionic conditions were close to the equilibrium potential for K^+, suggesting K^+ selectivity.

3. In high (98 mM) $[Na^+]_i$, the channel showed two open states and up to four closed states, and K_{Na} channels also displayed long closures (of the order of seconds). The opening probability (P_o) was not voltage dependent. Transient sublevels between 8 and 86% of the main state were identified and appeared to be a common feature of K_{Na} channels.

4. Decreasing the activating $[Na^+]_i$, reduced P_o and this was associated with both an increase in mean closed times and a decrease in mean open times. Lowering $[Na^+]_i$ also increased the longer closed-time constants and their relative proportions. The first open-time constant was more sensitive to alterations in $[Na^+]_i$.

5. Distributions of burst duration, between burst duration and openings within bursts were best described by the sum of two exponentials. Lowering $[Na^+]_i$ decreased the burst duration and the duration of openings within burst.

6. These observations show that the Na^+-activated K^+ channel from guinea-pig ventricular myocytes has complex gating and bursting behaviour.

Of the wide variety of potassium channels that are found in cell membranes, several show a high unit conductance of between 100 and 200 pS. A channel of this type activated by Na^+ (K_{Na}) at the intracellular surface has been identified in both neuronal and myocardial cells at the whole-cell and single-channel level (Kameyama, Kakei, Sato, Shibasaki, Matsuda & Irisawa, 1984; Bader, Bernheim & Bertrand, 1985; Dryer, Fujii & Martin, 1989; Haimann, Bernheim, Bertrand & Bader, 1990; Luk & Carmeliet, 1990; Rodrigo & Chapman, 1990; Sanguinetti, 1990; Wang, Kimitsuki & Noma, 1991). These channels have a relatively high incidence in neuronal inside-out patches (20–38%) with significant activation at 10 mM Na_i^+ and maximal activation at 50 mM. Consequently, they have been ascribed a role in the maintenance of the resting potential and in the repolarization of action potential (Haimann & Bader, 1989; Dryer, 1991; Haimann *et al.* 1990). The K_{Na} channels in cardiac muscle have a lower incidence (15–20%) and are less sensitive to Na^+, apparently requiring at least 30 mM Na_i^+ for activation and showing no saturation at 150 mM, features that have made their physiological role uncertain (Kameyama *et al.* 1984; Sanguinetti, 1990; Wang *et al.* 1991). Indeed the differences between neuronal and cardiac K_{Na} channels have been considered sufficient to justify their classification as separate subtypes (Haimann & Bader, 1989; Dale, 1993).

At the single-channel level, both neuronal and cardiac K_{Na} channels are typified by complex bursting behaviour with varying patterns of alternating episodes of activity and quiescence and the presence of numerous transient (probably 12 equal) substates (Kameyama *et al.* 1984; Dryer *et al.* 1989; Haimann *et al.* 1990; Luk & Carmeliet, 1990; Sanguinetti, 1990; Wang *et al.* 1991). Wang *et al.* (1991)

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suggested that a multi-barrelled channel similar to that proposed for the inward rectifier was unlikely because K^+ channel blockers revealed that the substates had similar properties, while Sanguinetti (1990) noted that their distribution failed to fit a binomial distribution as would be expected for a single pore with multiple gates.

An outward K^+ current in guinea-pig ventricular myocytes under whole-cell voltage clamp is provoked by Na^+ overload and is blocked by the agent R56865 (Rodrigo & Chapman, 1990). The same agent has been found to reduce the K^+ loss in rat heart subjected to ischaemia (Mitani & Shattock, 1992). These findings provoked a study of the behaviour of K_{Na} channels in excised patches from guinea-pig ventricular myocytes, particularly the effect of $[Na^+]_i$ on the closed times and bursting properties of the channel, which were not available. When recordings were made from patches which showed long-lasting activity with little run-down, a number of additional and novel features of the cardiac K_{Na} channel were revealed. These include periods of infrequent but protracted quiescence (of several seconds), the duration and incidence of which were affected by $[Na^+]_i$. The behaviour of the cardiac K_{Na} channel was therefore even more complex than previously thought.

METHODS

Preparation of isolated guinea-pig ventricular myocytes

Guinea-pigs were killed by cervical dislocation, the hearts were removed and mounted on Langendorff perfusion apparatus and ventricular myocytes were isolated by collagenase–protease treatment as described previously (Rodrigo & Chapman, 1990). Isolated myocytes were kept in Tyrode solution the composition of which was (mM): NaCl, 135; KCl, 5·4; $CaCl_2$, 2; $MgCl_2$, 1; NaH_2PO_4, 0·33; sodium pyruvate, 5; Hepes, 10; glucose, 10; buffered to pH 7·3 with NaOH. In some cases, 20 mM taurine was added to the solution used during the final dispersion of the myocytes and for their storage. Myocytes were kept at room temperature and were used within 2–12 h of isolation.

Recording of single K_{Na} channel activity

Ventricular myocytes were placed in a Perspex recording chamber that fitted into a disposable Petri dish. When the floor of the Petri dish had been previously etched with nitric acid, myocytes attached within a few minutes. Superfusion with Tyrode solution was then begun and the temperature of the fluid was controlled to $36 \pm 1·0$ °C by Peltier elements and a feedback circuit.

Patch pipettes were made from borosilicate glass capillaries (Clarke Electromedical, Pangbourne, Berkshire, UK) and had tip resistances of 3–6 MΩ when filled with the pipette solution. Patches were obtaining by forming a gigaohm seal to the membrane and then withdrawing the pipette (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). This was done at a holding potential of −40 mV, into a solution that contained a low $[Ca^{2+}]$, a $[Na^+]$ appropriate for activation of K_{Na} channels and ATP to inhibit ATP-sensitive K^+ channels.

Single-channel activity was recorded using a patch-clamp amplifier (Axopatch-1D, Axon Instruments, Foster City, CA, USA) coupled to an IBM-compatible PC via an analog-to-digital (AD/DA) converter (TL-1 DMA Interface; Axon Instruments). The currents were sampled at 10 kHz frequency and filtered at 2 kHz and stored on the hard disk of a 386PC using the pCLAMP 5.5.1 software package (Axon Instruments). The single-channel currents were also recorded on a VCR (NVL 20 HO; Panasonic) with the aid of a pulse code modulator (VR-10A; Instrutech, New York).

Solutions

The excised patches were exposed to solutions with either symmetrical or asymmetrical K^+ solutions. With symmetrical (60/60 mM) K^+ solutions, the solution in the pipette (extracellular solution) contained (mM): NaCl, 90; KCl, 60; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 10; adjusted to pH 7·3 with NaOH. The bath (intracellular) solution contained (mM): NaCl, 80; KCl, 60; EGTA, 1; Na_2ATP, 2; Hepes, 10; adjusted to pH 7·3 with NaOH. The asymmetrical (150/70 mM; K_o^+/K_i^+) K^+ solutions were composed of a pipette (extracellular) solution containing (mM): KCl, 150; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 10; adjusted to pH 7·3 with NaOH; and a bath (intracellular) solution containing (mM): NaCl, 96; KCl, 70; NaH_2PO_4, 0·3; Na_2ATP, 1; $MgCl_2$, 0·5; EGTA, 2·5; Hepes, 10; adjusted to pH 7·3 with NaOH.

All chemicals used were obtained from Sigma.

Data analysis and statistics

pCLAMP 5.5.1 (Fetchan) software was used to determine the open and closed times and to construct amplitude histograms. pCLAMP 6.0 (Pstat) software was used to fit open, closed and burst distributions and an F test was used to determine the number of exponential functions that best described these distributions. The appearance of a wide range of closed times with a varying incidence meant that the closed-time distributions were not adequately fitted using conventional methods. Sigworth & Sine (1987) have suggested that under these conditions a plot of the square root of the number of events against the log of time yields data that can be fitted better by least-squares methods. The data for the closed-time distributions were therefore treated in this way.

The identification of a sublevel is complicated by both the inherent noise and the filtering used. At the filter setting of 2 kHz (−3 dB) that was routinely used, a closure reached 90% of its full amplitude in 216 μs (Colquhoun & Sakmann, 1985). As a result only sublevels which lasted for longer than 0·3 ms were included in the analysis. The amplitudes of the sublevels were determined using both conventional amplitude histograms and the mean–variance technique devised by Patlak (1988, 1993). A window with a width of three points corresponding to 0·3 ms was shifted over the single-channel record. The mean and variance of the data points within each window were calculated and a 'levels' histogram constructed as described previously (Mistry, Tripathi & Chapman, 1996). Since sublevels were rare and brief (< 1% of open time), they were set to zero conductance in analyses of the open probability (P_o) and the dwell time of the channel. The time spent in sublevels was calculated from the time spent in sublevels divided by the total time spent in the open state (including all sublevels). The total time spent in the open state was determined in two stages. Firstly, total open time was calculated using the conventional 50% threshold level, and secondly, the time spent in sublevels (≤ 50% of main state) was added to this time to yield the total time spent in the open state. The time spent at the different sublevels was calculated directly using cursors.

Bursting properties were determined using the critical closed time (τ_c) (Colquhoun & Sakmann, 1985; Gibb & Colquhoun, 1992) which was obtained using the second and third closed-time constants (τ_2 and τ_3, respectively). Bursts were defined as an opening or group of openings separated by closures shorter than the specified critical

closed time, so that closures greater than τ_c were classed as interburst gaps. This method of Colquhoun & Sakmann (1985) equalizes the proportion of long closings that are wrongly classified as short with the proportion of wrongly classified short intervals. The equation solved for each patch was:

$$1 - e^{-\tau_c/\tau_3} = e^{-\tau_c/\tau_2}. \quad (1)$$

The τ_c values, determined for each patch, fell in the range of 1–5 ms. All data are expressed as means ± S.D. One-way analysis of variance (ANOVA) was used to assess statistical significance when comparing kinetics of the Na^+-activated K^+ channel under different experimental conditions. A P value of $< 0{\cdot}05$ was considered statistically significant.

RESULTS

Basic properties of K_{Na} channels

With symmetrical K^+ solutions on each side of an excised patch and with ATP absent from the intracellular solution, single-channel currents that reversed close to 0 mV and had a linear slope conductance of 43·0 ± 3·1 pS ($n = 6$) were encountered in each of four patches studied. However, when the intracellular solution contained ATP and high $[Na^+]$, a channel with this slope conductance was not seen, although another channel with a larger conductance, 75 ± 9·6 pS ($n = 15$), over the linear range of the current–voltage (I–V)

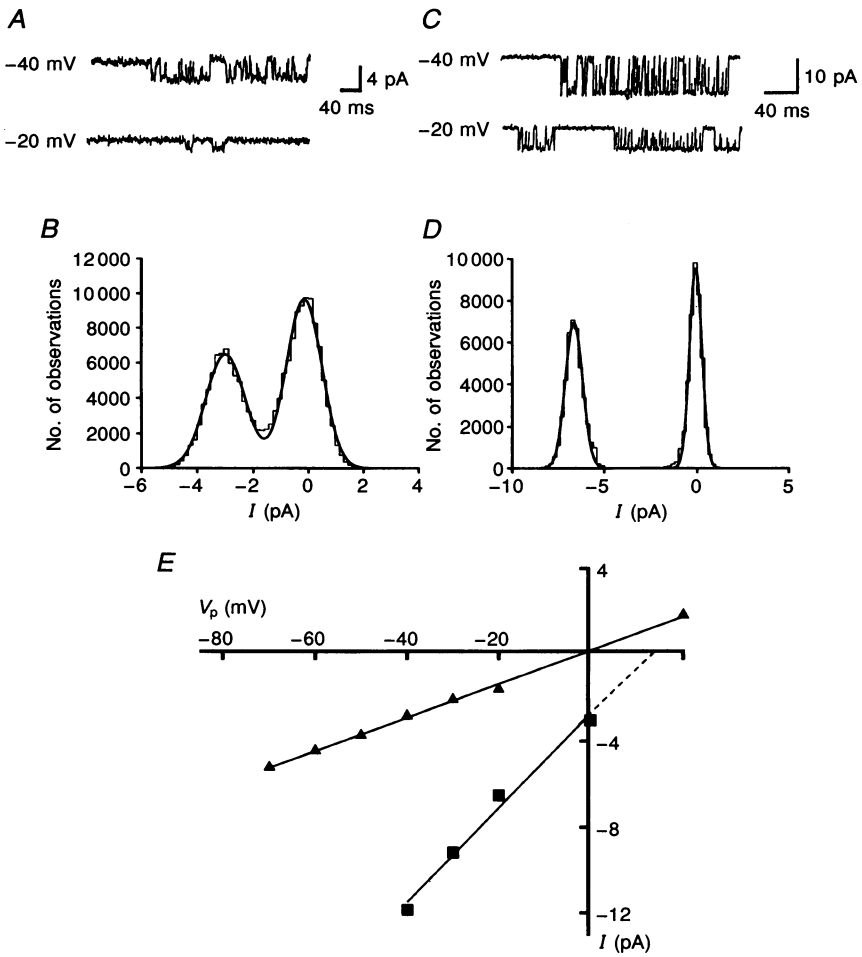


Figure 1. Unitary currents in symmetrical and asymmetrical K^+ solutions

A, examples of unitary currents obtained under symmetrical (60/60 mM) K^+ solutions at −40 and −20 mV. Openings of the channel are downwards. Unitary current amplitude increases with patch hyperpolarization. Activating $[Na^+]_i$ was 80 mM. *B*, total amplitude histogram at −40 mV (for the patch shown in *A*). The histogram has 2 peaks that were fitted by the sum of 2 Gaussians whose peaks were at 0 and 2·83 pA, which represent the closed and open level of the channel, respectively. *C*, examples of large unitary currents obtained with asymmetrical (150/70 mM K_o^+/K_i^+) K^+ solutions at −40 and −20 mV. Openings of the channel are downwards. Unitary current amplitude increases with patch hyperpolarization. Activating $[Na^+]_i$ was 98 mM. *D*, total amplitude histogram at −20 mV (for the patch shown in *C*). The histogram was fitted by the sum of 2 Gaussians whose peaks were at 0 and 6·58 pA, which represent the closed and open level, respectively. *E*, I–V relationships for the channels shown in *A* and *C*. V_p, patch potential. ▲ represents the I–V relation in symmetrical K^+ solutions; the slope conductance obtained from linear regression was 78 pS with reversal occuring at 0 mV. ■ represents the I–V relation in asymmetrical K^+ solutions; here the slope conductance was 219 pS and the extrapolated reversal potential was around 13 mV.

curve and a reversal potential of 2·2 ± 5·8 mV ($n = 15$) was encountered in the minority of patches. Typical unitary current activity from an inside-out patch is shown in Fig. 1*A*, together with an amplitude histogram (Fig. 1*B*) and the *I–V* relationship (Fig. 1*E*). The K^+ dependence of this large conductance channel was confirmed by changing to the asymmetrical K^+ solutions, which increased the mean slope conductance to 220 ± 23 pS ($n = 13$), and consistent with a high selectivity for K^+, the extrapolated reversal potential was shifted to 18 ± 7·2 mV ($n = 13$). Examples of unitary currents, the amplitude histogram and *I–V* relationship in these asymmetrical K^+ solutions are shown in Fig. 1*C*, *D* and *E*, respectively.

From a total of 858 inside-out patches, K_{Na} channel activity was detected in only seventy-six patches, with one channel present in sixty-six patches, two channels observed in seven patches and three channels observed in two patches. In the patches that showed activity of a single K_{Na} channel (no superimposition of the main state conductance was observed), twenty-four patches showed channel activity that ran down quickly or the patch was lost and fourteen showed channel activity that ran down within 2 min. In the remaining thirty-seven patches, channel activity run-down was much slower. The data from these thirty-seven patches were analysed in detail and are presented below.

Open- and closed-time distributions

The open and closed times were analysed for inside-out patches clamped at −40 mV, in asymmetrical K^+ solutions with an activating $[Na^+]_i$ of 98 mM and in symmetrical K^+ solutions with an activating $[Na^+]_i$ of 80 mM. The open-time distribution of the main state was best fitted by the sum of two exponentials under both conditions (Fig. 2*B*), with longer openings being the most common. These data agree with several previous reports (Kameyama *et al.* 1984; Sanguinetti, 1990; Veldkamp, Vereecke & Carmeliet 1994) and resemble the behaviour of the large Ca^{2+}-activated K^+ channel (Magleby & Pallota, 1983*a*). The closed times were more variable and included infrequent but prolonged closures so that if information about the number of closed-channel states is to be obtained from the distribution of all

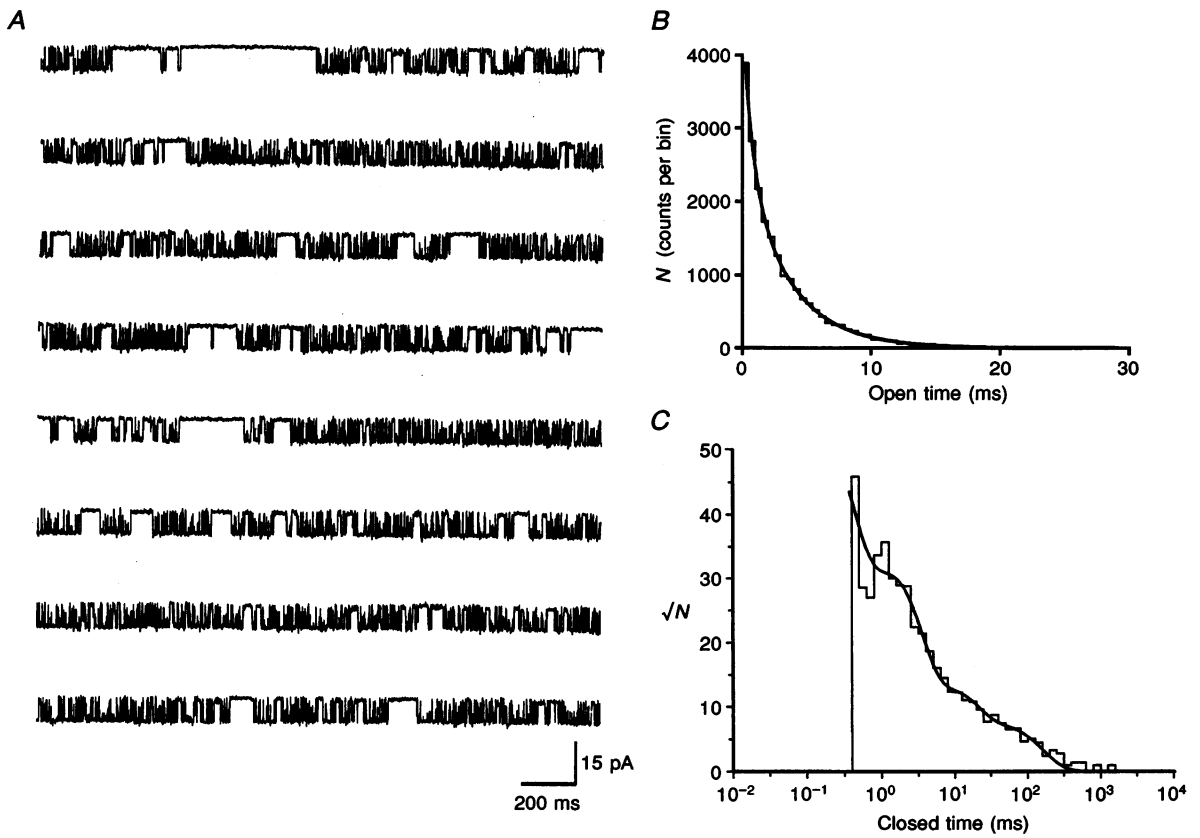


Figure 2. Open- and closed-time distributions and typical channel activity in 98 mM Na_i^+

A, examples of typical channel activity from an inside-out patch held at −40 mV. Openings of the channel occur in bursts and the openings are separated by closures of varying duration. Activating $[Na^+]_i$ was 98 mM. *B*, open-time distribution for the patch shown in *A*. Open times were best described by the sum of 2 exponentials having time constants of 0·65 and 3·45 ms. The area of the fast and slow component was 13 and 87%, respectively. *C*, closed-time distribution for the patch shown in *A*. Closed times were best described by the sum of 4 exponentials having time constants and areas, respectively, of 0·15 ms and 68%; 1·16 ms and 26%; 7·57 ms and 4%; and 42·65 ms and 2%.

Table 1. Kinetic parameters of K_{Na} channels obtained in symmetrical $[K^+]$ solutions of 60 mM

		Open				
Voltage (mV)	n	τ_1 (ms)	A_1 (%)	τ_2 (ms)	A_2 (%)	P_o
−30	7	0·40 ± 0·17	57 ± 9	3·96 ± 2·9	43 ± 9	0·32 ± 0·12
−40	7	0·33 ± 0·12	59 ± 16	4·44 ± 2·27	41 ± 16	0·34 ± 0·18
−50	7	0·31 ± 0·09	36 ± 9	4·07 ± 0·88	64 ± 9	0·37 ± 0·19
−60	5	0·35 ± 0·11	40 ± 21	3·86 ± 1·19	60 ± 21	0·51 ± 0·23
−70	3	0·33 ± 0·08	38 ± 8	4·70 ± 1·42	62 ± 8	0·45 ± 0·12

		Closed							
Voltage (mV)	n	τ_1 (ms)	A_1 (%)	τ_2 (ms)	A_2 (%)	τ_3 (ms)	A_3 (%)	τ_4 (ms)	A_4 (%)
−30	7	0·21 ± 0·08	63 ± 12	1·41 ± 0·82	29 ± 12	7·09 ± 4·77	8 ± 3	92·33 (2)	4 (2)
−40	7	0·26 ± 0·13	65 ± 8	3·08 ± 1·63	24 ± 6	13·65 ± 5·20	10 ± 5	142·31 (2)	3 (2)
−50	7	0·19 ± 0·05	59 ± 11	0·93 ± 0·18	24 ± 9	6·29 ± 2·48	15 ± 5	112·62 (2)	5 (2)
−60	5	0·20 ± 0·04	66 ± 15	1·32 ± 0·42	20 ± 8	10·68 ± 6·21	14 ± 8	—	—
−70	3	0·20 ± 0·01	54 ± 12	0·61 ± 0·15	29 ± 8	6·84 ± 2·32	16 ± 5	502·0 (2)	1 (2)

A_1, A_2, A_3 and A_4 are the corresponding areas for τ_1, τ_2, τ_3 and τ_4, respectively. Activating $[Na^+]_i$ was 80 mM. n = number of patches, except for τ_4 and A_4 where the number of patches is given in parentheses.

closed intervals (Colquhoun & Hawkes, 1981), analysis of long periods of activity is required (see Fig. 2*A*). In patches where prolonged recording with little run-down was achieved, the closed-time distributions required several exponentials to achieve a good fit. Even when three or four exponentials were derived, a few very long closures fell outside the fitted relationship (Fig. 2*C*). This suggests that the channel exhibits at least four closed states. Consistent with a catenary arrangement of states, the proportion is greatest for the closures with the shortest time constant and least for those with the longest time constant (or outside of the fitted relationship). It is reassuring that the two shorter closed-time constants are of a similar duration to those described by Sanguinetti (1990).

P_o and both the open-time constants and the first three closed-time constants and their proportions were little affected by membrane potential over the range studied (−30 to −70 mV) ($P > 0·05$). The percentage of both open-time constants showed some variability with voltage ($P < 0·05$). Table 1 shows the data obtained in symmetrical K^+ solutions. It also confirms the observation that the longer closures showed the greater variability. Similar data were also obtained in asymmetrical K^+ solutions, although the slower open-time constant (τ_2) showed a decrease with negative potentials (Fig. 3*B*). Both the first open-time constant (τ_1) and the P_o were not greatly dependent on voltage (Fig. 3*A* and *C*, respectively).

The P_o for the main state was not greatly affected by voltage in either symmetrical or asymmetrical K^+ solutions; this is consistent with previous work (Kameyama *et al.* 1984; Sanguinetti 1990; Wang *et al.* 1991) and is therefore unlike the behaviour of the Ca^{2+}-activated K^+ channel (Callewart, Vereecke & Carmeliet, 1989; McManus, 1991).

Effect of $[Na^+]_i$ on main state

The effect of $[Na^+]_i$ on channel behaviour was determined by replacing NaCl with TrisCl. When $[Na^+]_i$ was reduced from 98 mM to either 50 or 10 mM, the P_o of the channel fell: at 98 mM Na_i^+, P_o was 0·58 ± 0·09 ($n = 5$); at 50 mM, 0·23 ± 0·26 ($n = 4$, $P < 0·05$ when compared with P_o at 98 mM Na^+); and at 10 mM, 0·15 ± 0·06 ($n = 3$, $P < 0·01$ when compared with P_o at 98 mM Na_i^+). The decrease in the P_o was due to both an increase in the mean closed times and a decrease in the mean open times. An example showing the decrease of channel activity on reducing $[Na^+]_i$ from 98 to 10 mM is shown in Fig. 4*A* and *C*, and the accompanying amplitude histograms show that the single-channel current amplitude was not affected by $[Na^+]_i$ (Fig. 4*B* and *D*, respectively). Table 2 shows the collected data obtained from the fittings of both open and closed times for the three different Na_i^+ concentrations. As far as the open times were concerned, the major effect of decreasing $[Na^+]_i$ was to reduce the shorter mean open-time constant, while the relative contributions of each component were not much affected. Reduction of $[Na^+]_i$ increased the number and duration of the longer periods of quiescence, so that at least four exponentials were required to fit the closed-time distribution at 10 mM Na_i^+. The slower closed-time constants (notably τ_4) were increased and the relative contribution of

Table 2. Summary of the effect of alterations in $[Na^+]_i$ on the kinetic properties of the Na^+-activated K^+ channel

		Open			
$[Na^+]_i$ (mM)	n	τ_1 (ms)	A_1 (%)	τ_2 (ms)	A_2 (%)
98	5	0·80 ± 0·24	46 ± 13	3·51 ± 1·76	54 ± 13
50	4	0·45 ± 0·06*	35 ± 7	2·60 ± 0·65	65 ± 7
10	3	0·27 ± 0·10†	53 ± 9	2·46 ± 0·92	47 ± 9

		Closed							
$[Na^+]_i$ (mM)	n	τ_1 (ms)	A_1 (%)	τ_2 (ms)	A_2 (%)	τ_3 (ms)	A_3 (%)	τ_4 (ms)	A_4 (%)
98	5	0·14 ± 0·03	78 ± 8	0·98 ± 0·22	17 ± 5	7·43 ± 2·53	5 ± 3	—	—
50	4	0·23 ± 0·01†	48 ± 10†	1·20 ± 0·16	38 ± 7†	6·95 ± 1·13	12 ± 2†	70·86 (2)	3 (2)
10	3	0·19 ± 0·01*	58 ± 6*	1·41 ± 0·18	27 ± 3	10·37 ± 2·74	14 ± 3†	115·14 ± 0·11	2 ± 1

Significance was compared with values obtained for 98 mM Na_i^+: * $P < 0·05$; † $P < 0·01$.

the longer time constants became greater when $[Na^+]_i$ was lowered (Table 2). The fall in P_o as $[Na^+]_i$ was reduced has a complex origin which is due to the reduction in the τ_1, an increase in the slower closed-time constants and an increase in the occurrence of long periods of quiescence.

Opening to subconductance levels

General properties of subconductance states

Na^+-activated K^+ channels have been reported to show several subconductance levels (Sanguinetti, 1990; Wang *et al.* 1991). Similar behaviour is shown in Fig. 5. In the

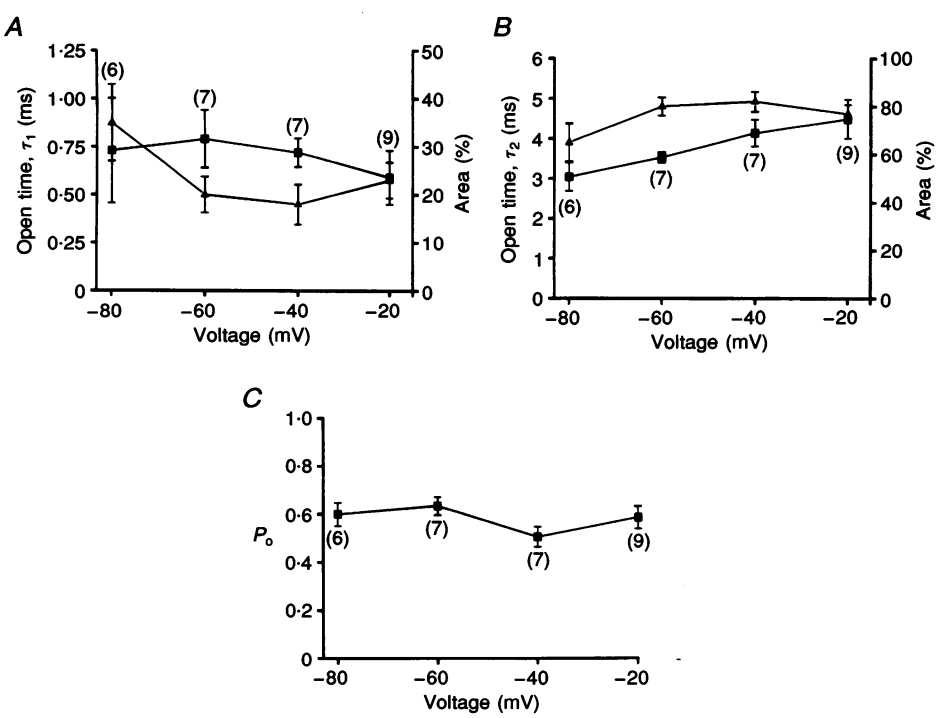


Figure 3. Effect of voltage on P_o, τ_1 and τ_2

The channel was obtained in asymmetrical K^+ solutions. Error bars shown are S.D. *A*, a graph showing τ_1 (■) and its area (▲) against voltage. No obvious relationship of τ_1 with voltage was observed. *B*, a graph showing the effect of voltage on τ_2 (■) and its area (▲). Values of τ_2 decreased with negative patch potentials. *C*, a graph showing the effect of voltage on P_o. P_o did not appear to be greatly dependent on voltage.

Table 3. Fractional amplitude of sublevels of the main state level: the occurrence of transient sublevels in 12 outside out patches

	Percentage of main state level								
Patch	A	B	C	D	E	F	G	H	I
1	—	—	27	38	44	52	68	73	87
2	—	17	25	—	48	—	—	—	84
3	—	14	28	33	46	—	65	74	—
4	—	—	24	32	—	—	—	78	85
5	—	14	29	37	—	—	—	—	84
6	—	19	25	33	—	—	66	76	—
7	—	17	26	34	43	54	67	73	88
8	9	16	26	35	46	57	66	72	89
9	—	16	24	34	43	54	66	72	89
10	—	12	25	36	44	—	—	74	—
11	—	17	26	34	42	59	64	73	—
12	7	19	24	35	49	—	68	72	86
Mean	8	16·1	25·8	34·6	45	55·2	66·3	74	85·9
S.D.	—	2·1	1·5	1·7	2·3	2·5	1·3	1·8	1·8

Only sublevels lasting $\geqslant$ 0·3 ms were accepted.

present study, sublevels were recognized by applying the following criteria: (1) sublevels should only be observed in the presence of main state activity; (2) substates should interconvert with the main state; and (3) the possibility of two independent channels comprising the main state level should be excluded (Fox, 1987). When these criteria were met, channel openings to a range of sublevels were found to occur from both the closed state and the main open state but

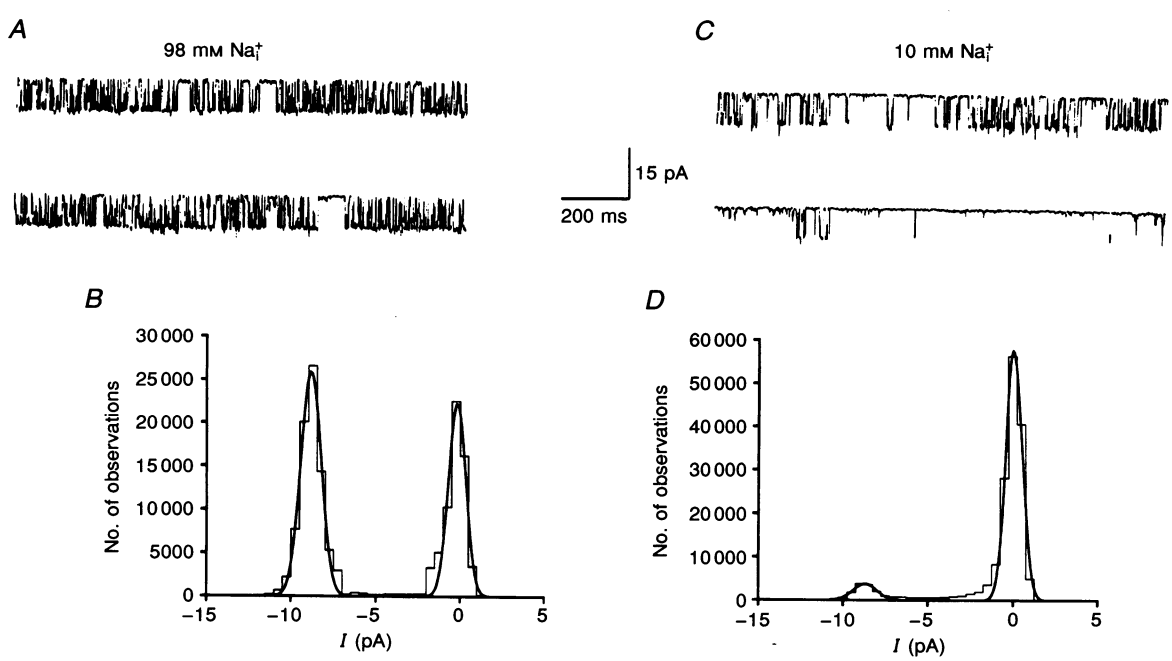


Figure 4. Effect of altering $[Na^+]_i$ on the activity of the Na^+-activated K^+ channel

A, channel activity in 98 mM Na_i^+. The channel spends most of the time in the open state. *B*, amplitude histogram for the patch shown in *A*. The histogram was fitted by the sum of 2 Gaussians whose peaks were at 0 and 8·67 pA representing the closed and open level, respectively. *C*, channel activity in 10 mM Na_i^+. The channel now spends most of its time in the closed state. *D*, amplitude histogram for the patch shown in *C*. The histogram was fitted by the sum of 2 Gaussians whose peaks were at 0 and 8·64 pA representing the closed and open level, respectively. The patch potential was −40 mV.

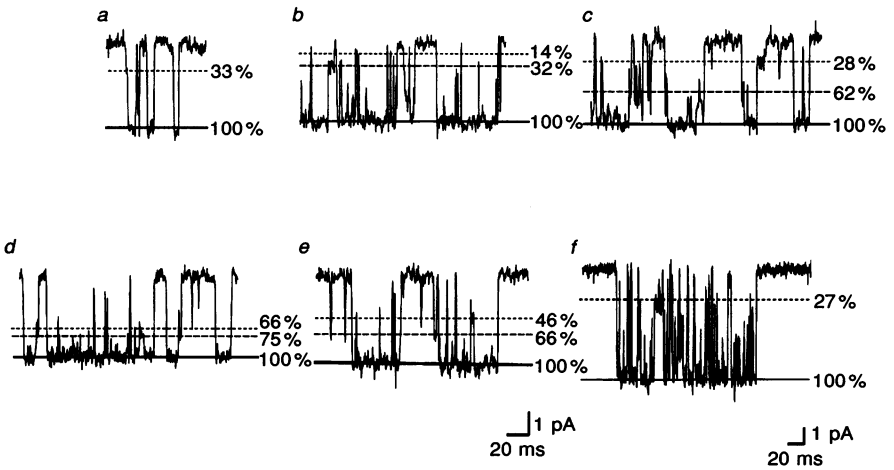


Figure 5. Examples of transient subconductance levels

Examples of openings of the channel to various different subconductance levels. Openings to the subconductance levels can occur from both the closed state (trace *a*) and from the main open state (see traces *b–f*). Openings occurred to sublevels which varied in size from 14 to 75% of the main state level. Traces *a–e* are from a patch held at −70 mV obtained in symmetrical K^+ solutions, while trace *f* was from a patch at −20 mV obtained in asymmetrical K^+ solutions.

the latter were the most common (Fig. 5, traces *b–f*). The amplitude of the subconductances, expressed as a percentage of the main state, are shown in Table 3 (12 different patches at −40 mV, activating $[Na^+]_i$ of 98 mM). Subconductance levels varying from approximately 8 to 86% of the main state were found, results which confirm previous work and suggest that there are twelve substates of equal size (Sanguinetti, 1990; Wang *et al.* 1991). However, it should be

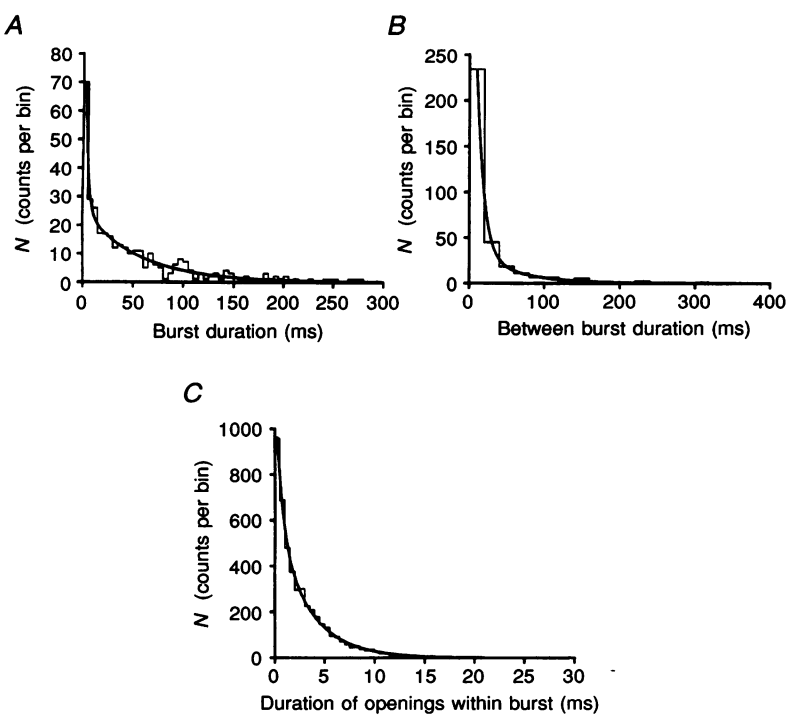


Figure 6. Distribution of burst properties of the K_{Na} channel at −40 mV

A, burst duration distribution was best described by the sum of 2 exponentials having time constants of 2·79 and 53·37 ms and areas of 18 and 82%, respectively. *B*, distribution of between burst duration was best fitted by the sum of 2 exponentials having time constants and areas of 9·18 and 52·58 ms and 74 and 26%, respectively. *C*, the distribution of duration of openings within bursts was best described by the sum of 2 exponentials having time constants and areas of 0·63 and 3·29 ms and 16 and 84%, respectively.

Table 4. Summary of the properties of channel bursting at different $[Na^+]_i$ at −40 mV

$[Na^+]_i$ (mM)	n	τ_1 (ms)	A_1 (%)	τ_2 (ms)	A_2 (%)
		Burst duration (ms)			
98	5	5·17 ± 0·63	31 ± 20	32·04 ± 10	69 ± 20
50	4	1·85 ± 0·92†	34 ± 11	15·22 ± 2·8*	66 ± 11
10	3	0·93 ± 0·24†	64 ± 13*	10·73 ± 3·56†	36 ± 13*
		Duration of openings within burst (ms)			
98	5	0·98 ± 0·19	38 ± 6	5·63 ± 1·2	62 ± 6
50	4	0·63 ± 0·11*	43 ± 17	4·34 ± 1·28	57 ± 17
10	3	0·36 ± 0·1†	64 ± 5*	3·76 ± 0·39	36 ± 5*

Significance was compared with values obtained for 98 mM Na_i^+: * $P < 0·05$; † $P < 0·01$.

remembered that the elemental current has a similar amplitude to the noise level of our recordings and this could account for the paucity of observations equivalent to a change in a single subconductance level from either the closed or fully open states. The total time spent at subconductance levels was less than 1% of the total open time (0·81 ± 0·34% for the 12 patches listed in Table 3 measured at −40 mV). The occurrence of the different subconductance levels was similar when the activating $[Na^+]_i$ was either 50 or 10 mM (data not shown).

Bursting properties of main state

General properties

The occurrence of prolonged periods of quiescence in patches containing a single K_{Na} channel suggests that openings may be collected in bursts (Fig. 2*A*). The time constants of the faster closures are around 0·2 and 1·0 ms while those for the longer closures are greater than 7 ms. This would be consistent with the brief time constants representing closed states within a burst while the longer time constants represent closed states between the bursts.

The basic bursting properties were studied at −40 mV, where the patch was stable and the unitary current amplitude was large (> 8 pA). At an activating $[Na^+]_i$ of 98 mM, the mean burst duration was 34·0 ± 7·0 ms, the mean between burst duration was 40·79 ± 43·58 ms, the mean duration of openings within burst was 3·9 ± 1·3 ms and the mean P_o within burst was 0·88 ± 0·05 ($n = 7$). The wide variation in the values for the between burst duration arose from the very long closures occasionally found in some patches.

Distributions of burst duration, between burst duration and duration of openings within burst were all best described by the sum of two exponentials. Figure 6*A*, *B* and *C* shows these distributions for a typical inside-out patch. Analysis of seven patches yielded mean values for the time constants of brief and long bursts of 5·2 ± 1·6 and 43·6 ± 11·0 ms, respectively, with the latter contributing the larger part (78 ± 3%). The data for the intervals between bursts for the same patches provided mean values to the short and long time constants of 6·3 ± 1·7 and 128 ± 47 ms, respectively, with the former contributing the larger part (77 ± 8%). In other words, the channel showed a marked tendency towards long duration bursts separated by short periods of quiescence. The existence of very occasional much longer intervals (of a second or longer, see Fig. 2*A*) suggests that the bursts may be grouped into clusters (Gibb & Colquhoun, 1992). With 98 mM Na_i^+, burst durations were best described by the sum of two exponentials with longer bursts the most prevalent, a feature common to other ion channels (Magleby & Pallota, 1983*b*; Colquhoun & Sakmann, 1985).

Effect of altering $[Na^+]_i$ on the bursting properties

Reduction of the activating $[Na^+]_i$ resulted in reduction of both P_o and the open-time constants. It also induced an increase in the slowest closed-time constants and the appearance of a significant number of much longer periods of inactivity such that the contribution of very long closed times to the closed-time distribution becomes significant (Table 2). This implies that the bursting behaviour of the channel is affected. Three different Na_i^+ concentrations of 98, 50 and 10 mM were used in a study of the bursting properties of K_{Na} channels. The change in $[Na^+]_i$ from 98 mM to either 50 or 10 mM produced the expected decrease in the mean burst duration. In 98, 50 and 10 mM Na^+, the mean burst duration was 20·7 ± 6·2 ($n = 5$), 11·8 ± 2·7 ($n = 4$) and 5·0 ± 1·2 ms ($n = 3$), respectively. Also, the mean duration of openings within the burst was also reduced with a lowering of $[Na^+]_i$, with mean values of 4·1 ± 1·4 ($n = 5$), 2·2 ± 0·7 ($n = 4$) and 1·7 ± 0·1 ms ($n = 3$) in 98, 50 and 10 mM Na_i^+, respectively. The mean P_o within bursts was 0·88 ± 0·05 ($n = 5$), 0·83 ± 0·06 ($n = 4$) and 0·81 ± 0·03 ($n = 3$) in 98, 50 and 10 mM Na_i^+, respectively, which were not significantly different ($P > 0·05$). Distributions of burst duration and duration of openings

within burst were best fitted by the sum of two exponentials at the three Na_i^+ concentrations tested. Table 4 summarizes these results and shows that on lowering $[Na^+]_i$, that both time constants associated with the burst duration are reduced. Also the fast open time constant (τ_1) in the duration of openings within bursts was decreased upon reduction of $[Na^+]$.

DISCUSSION

Many of the features of the Na^+-activated K^+ channel from guinea-pig ventricular myocytes described by previous work have been confirmed, namely the activation by Na_i^+ at the inner surface, complex bursting behaviour, a high conductance, a reversal potential consistent with a high selectivity for K^+ and the existence of many transient subconductance levels (Kameyama *et al.* 1984; Sanguinetti, 1990; Wang *et al.* 1991). The occurrence of patches with long-lived channels which showed a relatively slow run-down has allowed a more thorough analysis of the closed-time distribution and the bursting behaviour and the effects of membrane potential and $[Na^+]_i$.

Kinetic properties

In high $[Na^+]_i$, openings of the K_{Na} channel typically occur in bursts and analysis of the open times show that the distribution is best described by the sum of two exponentials (Fig. 2*B*).

The detailed closed-time distribution for the K_{Na} channel has not been previously available and either three or four exponentials are required to obtain a reasonable fit. However, a few long periods of quiescence fall outside the fitted curves (Fig. 2*C*). Typically the shortest periods of closure have the highest incidence and the longest the least. This would be expected for (but exclusive to) a catenery model of the channel which exhibited several closed states (Colquhoun & Hawkes, 1981). We found that even with high $[Na^+]_i$, the K_{Na} channel could spend a long time (of the order of seconds) in the closed state. Closed times of several seconds have been reported for the Ca^{2+}-activated K^+ channel from cultured rat muscle in the presence of high $[Ca^{2+}]_i$ (Magleby & Pallota, 1983*a*; Rothberg, Bello, Song & Magleby, 1996).

The effects of $[Na^+]_i$ on channel openings

Previous studies on the effect of $[Na^+]_i$ on the Na^+-activated K^+ channel have largely relied on measurement of the change in overall P_o of the channel (Rodrigo, 1993; Veldkamp *et al.* 1994) which falls as $[Na^+]_i$ is lowered. Kameyama *et al.* (1984) found both open and closed-time constants to be affected when $[Na^+]_i$ was reduced. We found that the shorter open-time constant was the more sensitive (Table 2). The finding that the shorter open-time constant was more sensitive to Na^+ than the longer open-time constant appears to be a property of the K_{Na} channel since both open-time constants (James & Okada, 1994) and the second (slower open-time constant) (Magleby & Pallota, 1983*a*) was increased by $[Ca^{2+}]$ in calcium-activated K^+ channels. The slower closed-time constants are increased as $[Na^+]_i$ is lowered and the increased incidence of longer periods of quiescence means that at least four exponentials were required to fit the closed-time distribution at 10 mM Na_i^+ (Table 2).

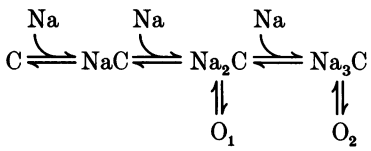
The complexity of the closed-time distribution and the changes seen when $[Na^+]_i$ is altered imply that the bursting behaviour is also affected. The majority of openings within a burst were long so that the mean was 3·87 ms, while closings tended to be brief and as a result P_o within a burst was high. The infrequent appearance of very prolonged periods without activity suggests that the bursts themselves may be collected into clusters (Gibb & Colquhoun, 1992). As would be anticipated from the changes in the open- and closed-time constants, several burst properties are affected by altered $[Na^+]_i$. Mean burst duration and the mean duration of openings within these bursts increased approximately linearly with $[Na^+]_i$. Lowering $[Na^+]_i$ reduced the time constants of both short and long bursts. The P_o during a burst showed no significant change as $[Na^+]_i$ was lowered showing that the increase in the interburst intervals and shortening of the burst length were more important in determining the fall in overall P_o.

Model

It must be stressed at the outset that the results are compatible with more than one mechanism and that we cannot present any simple and physically plausible mechanism that will account for all of our observations. Nonetheless, a preliminary kinetic model is presented.

Some requirements of the model are as follows. (1) The channel should typically bind at least three Na^+ ions for maximum activity as indicated by Hill slopes of around 3 (Rodrigo & Chapman, 1990; Kameyama *et al.* 1994; Koh *et al.* 1994). (2) The channel should have at least two open states and probably four closed states as indicated by the number of exponential components required in the dwell time distributions. (3) Both open and closed states should be capable of binding and unbinding Na^+ as revealed by the Na^+-dependent shifts in the time constants of both open and closed exponential components. (4) The gating should be consistent with a Markov process in which the transition rates from any given state depend only on the state the channel is in and not on the history of the preceding transitions (McManus & Magleby, 1989). (5) The longer duration open states should generally be connected to the brief duration closed states and the briefer duration open state should in general be connected to the longer duration closed states (McManus & Magleby, 1989). (6) Increasing $[Na^+]$ should drive the channel from the longer duration closed states to the briefer duration open states as shown by the Na^+-dependent changes in the components of the dwell time histograms.

Letting C denote the closed channel molecule and O the open channel and Na for the Na^+ ion:



There was no evidence that unoccupied channels can open in our study (cf. Luk & Carmeliet, 1990). In addition, if mono-liganded channels opened briefly, the relative frequency of fast open intervals would be expected to decrease with increasing $[Na^+]_i$ and this was not the case. The distribution of intervals with long closed time would arise mainly from closings to the compound shut state (C, NaC, Na_2C and Na_3C). The distribution of intermediate shut intervals would consist mainly of closings to the compound shut state (NaC, Na_2C and Na_3C) and the distribution of short shut intervals would consist primarily of closings to the shut states (Na_2C and Na_3C).

Similarly, a gap within a burst would consist of a sojourn in (Na_2C and Na_3C) starting with an exit from either open state O_1 or O_2, also gaps between bursts would consist of the sum of a sojourn in (C, NaC, Na_2C and Na_3C) starting in C and terminating in either open state O_1 or O_2.

At present it is not known whether the longest closed intervals seen in high $[Na^+]_i$ represent a low activity mode or an inactivated state of the channel as has been recently described in K_{Ca} channels (Rothberg *et al.* 1996). Also K_{Na} channels in different preparations appear to be quite diverse, for example, a Hill coefficient of 4·8 has been reported in spinal neurones of the frog embryo (Dale, 1993), while channel openings could occur even in the complete absence of Na^+ (Luk & Carmeliet, 1990). It is also possible that K_{Na} channels in guinea-pig ventricular myocytes are heterogeneous as evident by the highly variable rates of run-down seen upon patch excision.

Physiological and pathophysiological significance

The value of the resting intracellular sodium activity in guinea-pig myocytes has been measured at 7 mM (Rodrigo & Chapman, 1990) and is known to increase with heart rate (Cohen, Fozzard & Sheu, 1982) and on exposure to cardiac glycosides or on removal of divalent cations from the bathing fluid, conditions where a shortening of the action potential also occurs (Chapman & Tunstall, 1986; Rodrigo, 1990). With their large unitary conductance (approximately 130 pS in physiological K^+ solutions; Luk & Carmeliet, 1990), the K_{Na} channels could modulate action potential duration when $[Na^+]_i$ rises. Our data, in contrast to some previous work, show a significant activation of the K_{Na} channel in excised patches at 10 mM Na_i^+ and Rodrigo (1993) has presented evidence that P_o is more sensitive to $[Na^+]_i$ in cell-attached as opposed to excised patches. This could mean that the channel is also regulated by other agents and its activity may change when the changes in $[Na^+]_i$ are more modest. A number of observations suggest that this may be the case. Firstly, a linear relationship between measured $[Na^+]_i$, action potential duration and an outward current with characteristics of the K_{Na} channels has been reported during exposure to acetylstrophanthidin (Rodrigo, 1990). Secondly, block of the K_{Na} current with the non-specific agent R56865 (Luk & Carmeliet, 1990; Rodrigo & Chapman, 1990) reduces the loss of K^+ during an ischaemic insult (Mittani & Shattock, 1992) and the shortening of the action potential, but not the inotropic effects of strophanthidin in isolated guinea-pig ventricular myocytes (G. C. Rodrigo & R. A. Chapman, unpublished data). Lastly, large changes in total $[Na^+]$, localized to the regions just beneath the sarcolemma have been described by X-ray microanalysis of guinea-pig myocytes frozen at different times after a bout of activity (Isenberg & Wendt-Gallitelli, 1990). The organization of K_{Na} channels within the cardiac muscle cell membrane is not known but they occur in a small minority of patches. A close association with voltage-dependent Na^+ channels as would seem to occur in nodal regions of myelinated nerves, where it is involved in the repolarization of the action potential (Koh, Jonas & Vogel, 1994), would offer little functional advantage to cardiac muscle where the action potential is long. However, a close spatial association between Na^+ pump sites and K_{Na} channels has been suggested by Luk & Carmeliet (1990) and would be consistent with the effects of R56865 noted above. Moreover, the notion of a fuzzy space directly beneath the cell membrane has been proposed where the $[Na^+]_i$ is higher than that found in the rest of the cytosol (Carmeliet, 1992).

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