Journal of Physiology (1988), **402**, 219–235
With 9 text-figures
Printed in Great Britain

VOLTAGE-DEPENDENT DECREASE IN THE AVAILABILITY OF SINGLE CALCIUM CHANNELS BY NITRENDIPINE IN GUINEA-PIG VENTRICULAR CELLS

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(*Received 28 September 1987*)

SUMMARY

1. The mechanism of Ca^{2+} channel block by nitrendipine was studied by recording single-channel activity from cell-attached patches on guinea-pig ventricular cells using patch pipettes containing 50 mM-Ba^{2+}. Test depolarization pulses to around 10 mV with a duration of 100 ms were applied repetitively at 2 Hz.

2. The percentage of non-blank sweeps was maximal (about 40%) at a holding potential between −65 and −130 mV and decreased sigmoidally with its depolarization. Nitrendipine shifted the availability–voltage relationship in a hyperpolarizing direction.

3. From the number of consecutive non-blank sweeps and that of blank sweeps, the duration of the available state and that of the unavailable state were estimated.

4. The histogram of the duration of the available state showed a single-exponential distribution. Its mean duration was about 1·5 s and was shortened by nitrendipine. Correspondingly, the decay of the mean current during the depolarization step was accelerated by nitrendipine.

5. In the presence of 100 nM-nitrendipine the histogram of the duration of the unavailable state at large negative holding potentials was simulated as the sum of two exponential components, one with a time constant similar to that in the control and the other with a time constant of 6–7 s.

6. The histogram of the duration of the unavailable state at depolarized holding potentials was simulated by a double-exponential curve also in the control. The duration of the slow component was prolonged by nitrendipine.

7. The prolongation of the unavailable states initiated by drug binding during depolarization steps and maintained during depolarized holding potentials is the mechanism of the blockade. The rate constants of the state transitions between an available state and two unavailable states were estimated.

INTRODUCTION

Recordings of single-Ca^{2+}-channel activity by the patch-clamp technique from cardiac myocytes have revealed fast and slow state transitions (Reuter, Stevens, Tsien & Yellen, 1982; Cavalié, Ochi, Pelzer & Trautwein, 1983; Cavalié, Pelzer & Trautwein, 1986). One of the slow state transitions is an inactivation process which

is characterized by its strong voltage dependence. In addition, the decrease in the number of blanks by β-adrenergic stimulation suggests that there exists another slow transition process which is modulated markedly by channel phosphorylation (Ochi, Hino & Niimi, 1984; Trautwein & Pelzer, 1985; Ochi, Hino & Okuyama, 1986; Tsien, Bean, Hess, Lansman, Nilius & Nowyckey, 1986).

Organic Ca^{2+} channel blockers are widely used as therapeutic agents for diseases such as hypertension, angina and arrhythmia (Schwartz & Taira, 1983). The dihydropyridine family of blockers are highly potent and are useful for the study of Ca^{2+} channel functions. Previous patch-clamp studies of the Ca^{2+} channel in cardiac muscle have revealed an increase in the number of blank sweeps on depolarization steps by dihydropyridine Ca^{2+} channel blockers (Hess, Lansman & Tsien, 1984) and by D600, a phenylalkylamine Ca^{2+} channel blocker (Pelzer, Cavalié & Trautwein, 1985). In the present study we performed a kinetic study of the slow state transitions of Ca^{2+} channels by sustained single-channel recording to elucidate the mechanism of Ca^{2+} channel block by nitrendipine.

METHODS

Preparations. Ventricular myocytes were enzymatically isolated from the hearts of guinea-pigs, as described previously (Cavalié *et al.* 1983). Briefly, guinea-pigs weighing 250–350 g were anaesthetized with sodium pentobarbitone (50 mg/kg) and the ascending aorta was cannulated *in situ* under artificial respiration. The heart was excised and was perfused by the Langendorff method with the following solutions in the order: normal Tyrode solution, Ca^{2+}-free Tyrode solution, collagenase solution (10–15 min) and high-K^+, Ca^{2+}-free (Kraftbrühe, KB) solution. Thereafter, single cells were separated from the ventricle and stored in KB solution at 4 °C.

Chamber. For experiments, the cells were transferred to a circular chamber which had a shallow round central pool with a depth of 1 mm, a diameter of 10 mm and a bottom made of a glass microscope slide. The chamber was mounted on the mechanical stage of an inverted microscope (Diaphoto-TMD, Nikon, Japan). The superfusate flowed down to the central pool and was sucked out through a U tube, both by means of gravity. The superfusate temperature in the central pool was maintained at 30 °C with a temperature-controlling device (Shin-nihondenko, Osaka, Japan) which utilized the Peltier effect.

Patch clamp. The single-channel currents were recorded using the patch-clamp technique under the cell-attached configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). A 3 M-KCl agar electrode was dipped into the chamber as the reference electrode. Patch pipettes were made of plain haematocrit capillary tubes (Propper, New York, U.S.A.) and had a resistance of 1–3 MΩ. A gigaohm seal was established by applying gentle suction through the pipette after it had touched the cell surface. An EPC-7 patch-clamp amplifier (List-Medical, Darmstadt, F.R.G.) was used. Experiments were performed with a superfusate of high-K^+ solution. The tip potential of Ba^{2+} pipettes in the solution was −7 mV. The membrane potential was corrected for this value.

Solutions. The normal Tyrode solution used had the following composition (mM); NaCl, 135; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 1; HEPES–Tris, 5; and glucose, 10 (pH 7·4). Ca^{2+}-free solution had the same composition as the normal Tyrode solution except for the Ca^{2+} concentration, which was nominally zero. Collagenase solution was made from the Ca^{2+}-free solution by adding collagenase (type I, Sigma, U.S.A.) to a final concentration of 0·6 mg/ml and Ca^{2+} to 40 μM. KB solution contained (mM); KOH, 110; taurine, 10; oxalic acid, 10; glutamic acid, 70; KCl, 25; KH_3PO_4, 10; EGTA–Tris, 0·5; HEPES–Tris, 5; and glucose, 10 (pH 7·4). High-K^+ Tyrode solution was made by replacing the NaCl in Ca^{2+}-free Tyrode solution with equimolar KCl. The pipette solution contained (mM): $BaCl_2$, 50; choline chloride, 75; HEPES–Tris, 5; and tetrodotoxin (Sankyo, Japan), 0·03 (pH 7·4). Nitrendipine was kindly supplied by Dr Jun Inui (Yoshitomi Pharmaceutical Co. Ltd, Japan).

Data analysis. The currents obtained were filtered by a low-pass filter in the clamp amplifier with a cut-off frequency of 3 kHz and stored on video tape as digitized signals through a PCM recording system (RP880, NF Electronic Instruments, Yokohama, Japan). The analog output from the

stored data was analysed after filtering again with cut-off frequencies of 1–1·5 kHz with an 8- or 16-pole Bessel filter (FV-665, NF Electronic Instruments, Yokohama, Japan) and digitized with a 12-bit A/D converter with a sampling frequency of 5 kHz. The digitized data were analysed by personal computer (PC-9801UV2, NEC, Tokyo, Japan) after accumulation in a RAM (512 kB). The data were displayed on a cathode ray oscilloscope monitor and analysed using the graphic function of the computer. The capacitive and leak currents were digitally subtracted using the average currents of blank sweeps or of currents produced by pulses with opposite polarity. The program allowed a baseline and a single-channel amplitude to be fitted by eye. Transitions between the open state and shut state were detected as crossings at the half-amplitude level. The open-state probability of a single channel was calculated for a current-containing sweep as the ratio of the total time of opening to the total duration of test pulses. Differences in the numerical values between two groups were evaluated using Student's *t* test.

RESULTS

Effect of nitrendipine on mean Ca^{2+} channel currents

Figure 1 shows the effects of nitrendipine on the mean Ca^{2+} channel currents from a cell-attached patch containing multiple functional Ca^{2+} channels. They were obtained by averaging about 100 multi-channel currents elicited by 100 ms depolarization steps to 16 mV from various holding potentials. With the shift of the holding potential in a depolarizing direction, the current amplitude diminished due to steady-state inactivation. This 'steady-state inactivation' of the Ca^{2+} channel reflects both the inactivation developed and maintained at each holding potential and that induced by the repetitive pulses.

Upon addition of 100 nM-nitrendipine, the amplitude of the mean current was slightly depressed at large negative holding potentials between −83 and −65 mV and the depression was markedly pronounced by depolarization of the holding potential to −56 to −20 mV (Fig. 1*B*). The voltage-dependent depression was enhanced by increasing the drug concentration to 1000 nM (Fig. 1*C*). The decay of the current during the depolarization step was negligibly small in the control, as Ba^{2+} was the charge carrier. The decay was only slightly accelerated by 100 nM-nitrendipine. However, when the concentration of the drug was increased to 1000 nM, the decay was accelerated remarkably (Fig. 1*C*).

Figure 2 shows the steady-state inactivation curve which relates the peak amplitude of the mean current to the holding potential. Nitrendipine shifted the curve towards a hyperpolarized direction concomitant with some decrease in the maximum amplitude. Thus, the membrane potential at which the amplitude decreased to 50% of the maximum ($V_{\frac{1}{2}}$), was made 21 mV more negative by 100 nM-nitrendipine and a further 6 mV more negative by 1000 nM-nitrendipine.

Decrease of Ca^{2+} channel availability by nitrendipine

An available state of a single Ca^{2+} channel was defined as the state associated with a current-containing sweep, and an unavailable state as that with a blank sweep. The availability of the channel is given by the ratio of the number of current-containing sweeps to the total number of sweeps. This ratio was considerably less than 1 and was about 0·4 in the present study under control conditions at sufficiently negative holding potentials. It decreased upon depolarization of the holding potential due to an inactivation process (Reuter *et al.* 1982; Cavalié *et al.* 1983).

The effect of nitrendipine on the availability was examined by recording single-Ca^{2+}-channel currents in thirty preparations. Test depolarizations of more than 500 were applied repetitively from various holding potentials. Table 1 summarizes the results of five experiments done at varying holding potentials. The results presented in Figs 3–6 and 9 were obtained from the same preparation.

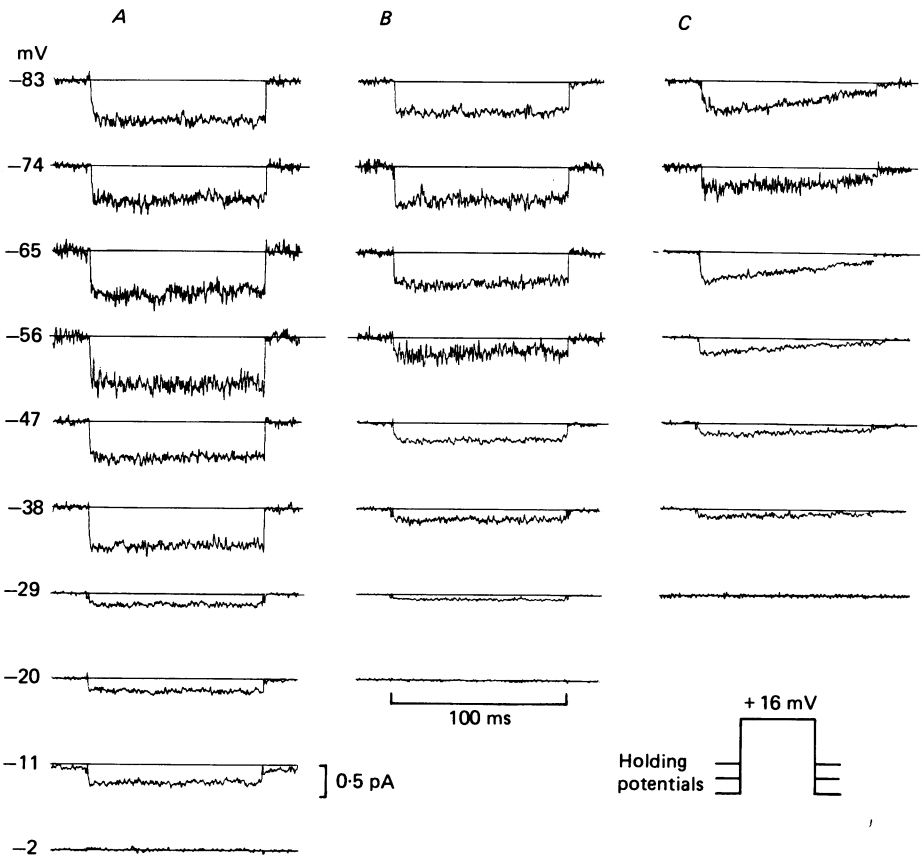

Fig. 1. Depression of mean Ca^{2+} channel currents by nitrendipine at various holding potentials. Mean currents were obtained by averaging about 100 sweeps of multi-channel currents including blanks elicited by 100 ms depolarizing steps to 16 mV applied at 2 Hz from various holding potentials as shown in the inset. 50 mM-Ba^{2+}-containing pipette. *A*, control; *B*, 100 nM-nitrendipine; *C*, 1000 nM-nitrendipine. Horizontal lines show the zero-current level.

As seen in Fig. 3*A*, a large depolarization step to 7 mV from a large negative holding potential of −65 mV did not always produce openings of the channel. The availability of the channel was 246/506 at −65 mV and decreased to 156/506 on depolarizing the holding potential to −38 mV (*B*). Nitrendipine (100 nM) decreased the number of current-containing sweeps and this action was more conspicuous at depolarized holding potentials (*C* and *D*). These decreases in the availability were responsible for the decrease in the mean current (*Cb* and *Db*).

Figure 4 shows the availability–voltage relationship of the Ca^{2+} channel. The curve follows the equation of the Boltzmann distribution with a holding potential for a half-maximal amplitude ($V_{\frac{1}{2}}$) of −43 mV. Nitrendipine affected the availability–voltage relationship in a voltage-dependent manner. At strongly hyperpolarized holding potentials the availability was decreased to about two-thirds by 100 nM-nitrendipine. Upon depolarization of the holding potential, the availability was more markedly depressed by nitrendipine. Thus, the curve was markedly shifted in a hyperpolarizing direction as seen in the steady-state inactivation curve in Fig. 2. When estimated as the hyperpolarization of $V_{\frac{1}{2}}$, the shift amounted to 16 mV at 100 nM in five preparations and 33 mV at 1000 nM in three preparations (Table 1).

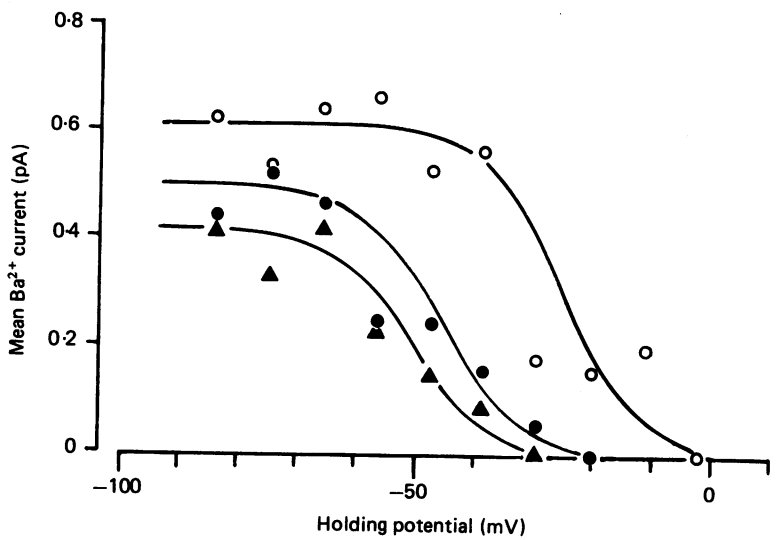

Fig. 2. effect of nitrendipine on the steady-state inactivation of Ca^{2+} channels. The amplitude of mean currents in Fig. 1 is plotted against holding potential. ○, control; ●, 100 nM-nitrendipine; ▲, 1000 nM-nitrendipine. The curves were drawn according to the equation: $I = I_{max}/(1+\exp(V-V_{\frac{1}{2}})/k)$. I_{max} was 0·62 pA in control, 0·52 pA in 100 nM-nitrendipine, 0·43 pA in 1000 nM-nitrendipine; $V_{\frac{1}{2}}$ was −25 mV in control, −45 mV at 100 nM and −51 mV at 1000 nM-nitrendipine; k was 7 in all of these curves.

Non-random appearance of current-containing sweeps and blank sweeps

As shown in consecutive records in Fig. 3, there was a tendency for blanks and non-blanks to appear in clusters both in the control and in the presence of nitrendipine. Non-randomness was examined statistically by a run analysis with the number of test pulses, number of runs and number of non-blanks (Horn, Vandenberg & Lange, 1984; Standen, Stanfield & Ward, 1985). A high degree of non-randomness was demonstrated in each experiment both at hyperpolarized and at depolarized holding potentials in the presence and absence of the drug. It suggests that the onset and the end of the runs are determined by forward and backward slow state transitions between the available state and the unavailable state. In the present study the numbers of consecutive non-blank sweeps and blank sweeps in the run were counted

and the duration of the available state and that of the unavailable state were obtained by multiplying the numbers by 0·5 s. The time resolution of this method is not sufficiently high to measure the duration of some short runs, e.g. the unavailable states shorter than 100 ms were always omitted.

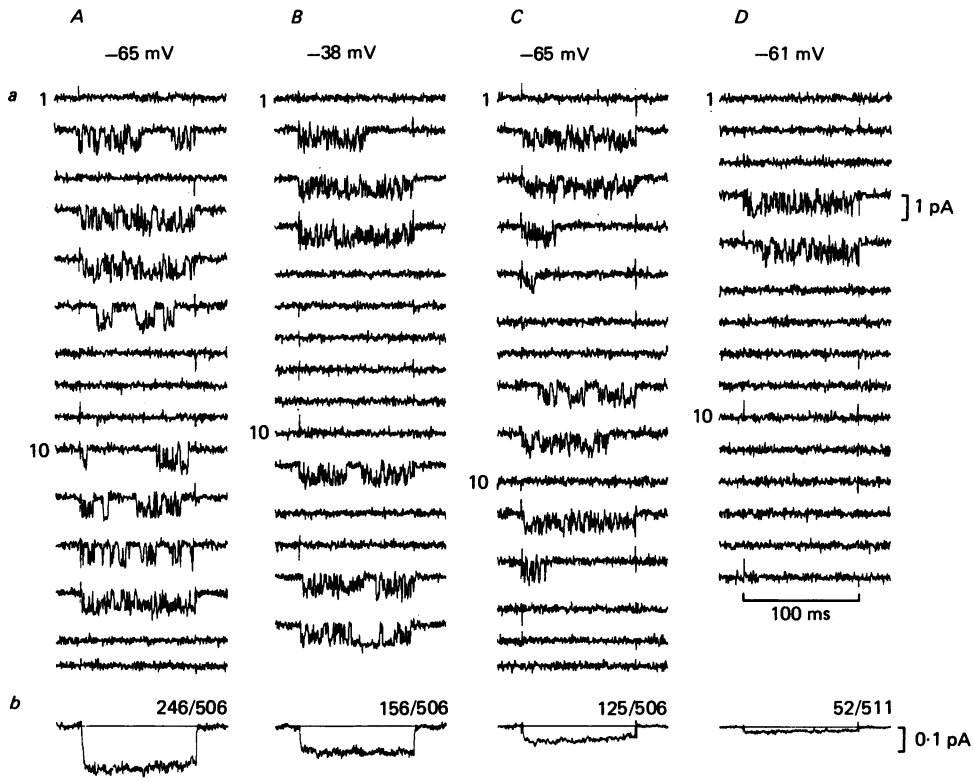

Fig. 3. Effects of nitrendipine on single-Ca^{2+}-channel currents. *A* and *B*, in control solution; holding potential = −65 mV in *A*, −38 mV in *B*; *C* and *D*, in the presence of 100 nM-nitrendipine; holding potential = −65 mV in *C*, −61 mV in *D*. About 500 depolarization steps to 7 mV with a duration of 100 ms were applied repetitively at 2 Hz from each holding potential. *a*, fifteen specimen records given in the order of depolarization sequence. Cut-off frequency was 1 kHz. *b*, mean currents from all traces including blanks, and the ratio of the number of non-blanks to total number of sweeps are given.

Effect of nitrendipine and holding potential on the duration of the available state

In Fig. 5*A* the mean number of non-blanks is plotted against the holding potential. The plot sometimes shows considerable scatter due to the limited number of clamp steps. However, the mean number of non-blanks was essentially the same at large negative holding potentials between −65 and −101 mV, the mean duration being 2·1 s. It was not significantly affected by the depolarization of the holding potential (Table 1). Nitrendipine at 100 nM slightly decreased the duration of the available state both at hyperpolarized and at depolarized holding potentials (Fig. 5*A*). The

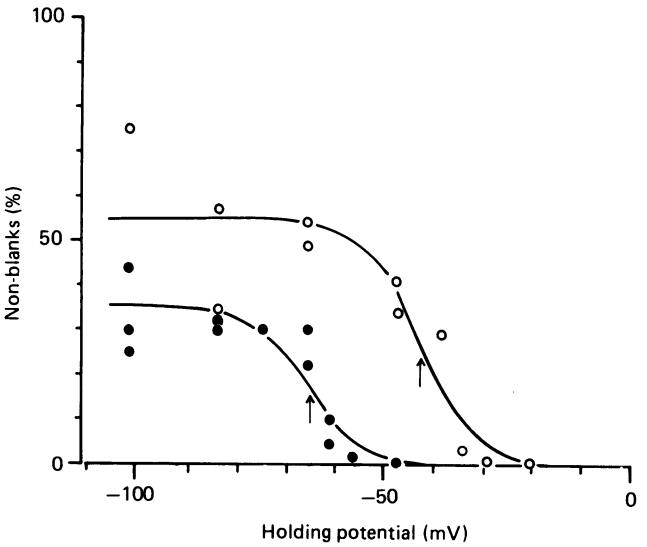

Fig. 4. The effect of nitrendipine on the availability–voltage relationship of Ca^{2+} channels. From experiments partly given in Fig. 3. Test pulses to 7 mV, with a duration of 100 ms, were applied at 2 Hz at each holding potential. The percentage of non-blanks in about 500 sweeps at each point is plotted *versus* holding potential. ○, control; ●, in the presence of 100 nM-nitrendipine. The curves were drawn according to the equation: $y = y_{max}/(1+\exp((V-V_{\frac{1}{2}})/k))$. y_{max} was 55% in the control and 35% in the presence of nitrendipine; $V_{\frac{1}{2}}$, indicated by arrows, was −43 mV in the control and −65 mV in the presence of nitrendipine; k was set at 6.

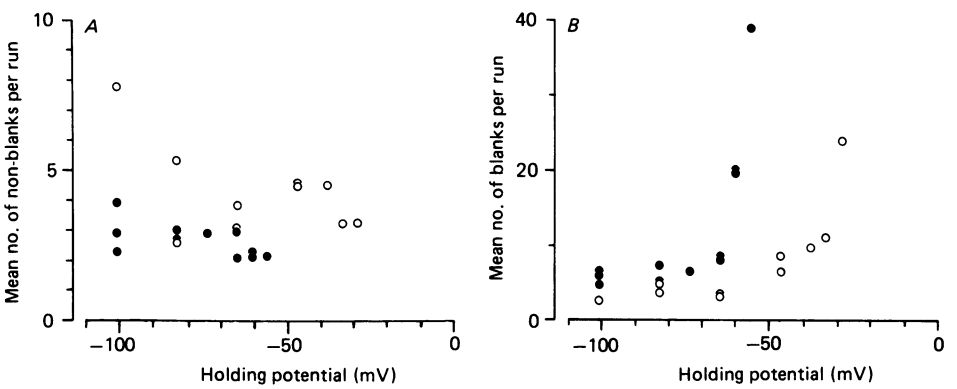

Fig. 5. The effect of nitrendipine (100 nM) on the mean number of non-blanks per run and number of blanks per run at various holding potentials. From the experiment given in Fig. 3. *A*, the mean number of non-blanks per run *versus* holding potential; *b*, the mean number of blanks per run *versus* holding potential. ○, control; ●, in the presence of 100 nM-nitrendipine. For each point about 500 depolarizing steps were applied at 2 Hz. In the control, the number of runs for each point was thirty-four to eighty-one except for the right end-point at which it was eighteen. In the presence of nitrendipine the number of runs was forty-one to sixty-four at holding potentials between −65 and −101 mV, and the right end-point represents the mean from seven runs.

TABLE 1. Effect of nitrendipine and holding potential on the slow gating process of single Ca^{2+} channels

			Holding potential −130 to −65 mV			Holding potential near $V_{\frac{1}{2}}$	
	$V_{\frac{1}{2}}$ (mV)	$\Delta V_{\frac{1}{2}}$ (mV)	P_S	m_S	m_F	m_S	m_F
Control	−39·2	—	0·43	3·20	3·96	2·87	11·60
±S.D. ($n = 5$)	4·6		0·08	0·60	0·66	0·90	2·64
Nitrendipine (100 nM)	−55·2*	−16·0	0·29*	2·51	6·02*	2·61	15·20
±S.D. ($n = 5$)	10·4	7·0	0·07	0·62	1·37	1·22	3·86
Nitrendipine (1000 nM)	−69·0**	−32·7	0·14**	1·84**	10·29**	1·56*	18·50*
±S.D. ($n = 3$)	22·6	23·7	0·04	0·12	3·12	0·40	4·68

P_S, the ratio of number of non-blanks to the number of total sweeps. $V_{\frac{1}{2}}$, potential at which P_S decreased by 50%. $\Delta V_{\frac{1}{2}}$, the shift from the control. Data at holding potentials between −65 and −130 mV were compiled and were compared with those obtained at a holding potential near $V_{\frac{1}{2}}$. More than 500 depolarizing pulses were applied at 2 Hz at each holding potential. m_S, mean number of non-blanks per run; m_F, mean number of blanks per run. The runs immediately after the onset and before the cessation of test trains were excluded in the estimation of the mean values. N, number of experiments. Five preparations were used. The statistical significance of the difference between the control was determined by Student's t test. *$P < 0.05$, **$P < 0.01$.

shortening was enhanced and became significant on increasing the concentration to 1000 nM; the mean duration in three experiments was 0·8–0·9 s (Table 1).

The kinetic parameters of the slow state transitions were determined by statistical analysis of the duration of runs obtained by hundreds or thousands of repetitive test pulses. In the histograms shown in Fig. 6, the results of all runs obtained at hyperpolarized holding potentials between −65 and −101 mV were compiled, since

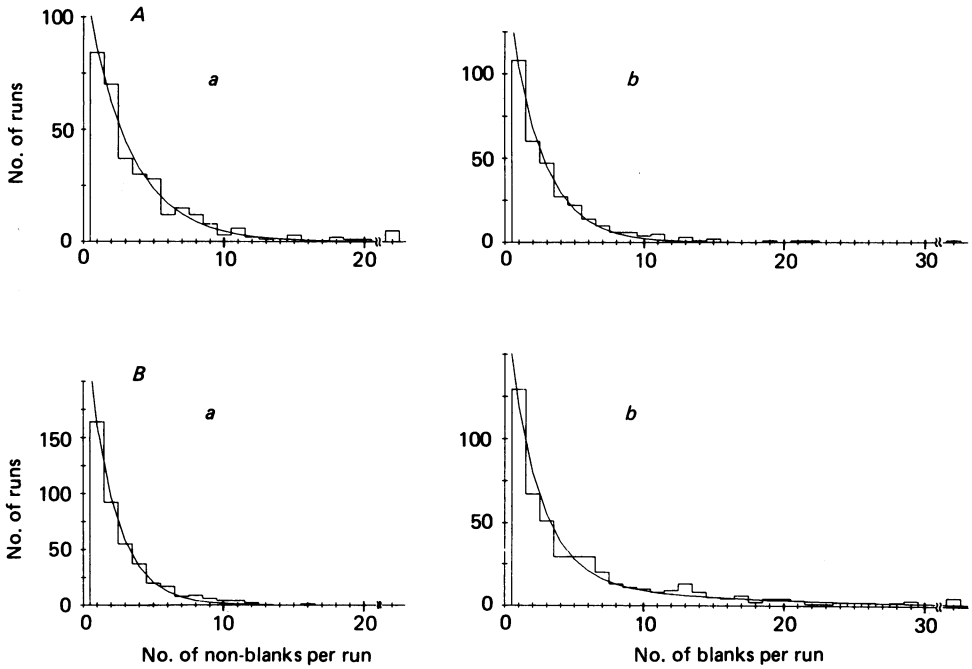

Fig. 6. Effect of nitrendipine on the distribution of the number of sweeps per run at hyperpolarized holding potentials. From the experiment given in Fig. 3. The histograms were made from all runs obtained at holding potentials between −65 and −101 mV. *A*, control; *B*, in the presence of 100 nM-nitrendipine. *a*, numbers of runs plotted against the number of non-blanks per run; *b*, numbers of runs against the number of blanks per run. Number of runs was 320 for both *a* and *b* in *A*; 420 for *a* and 417 for *b* in *B*. Histograms of *a* were approximated by a single-exponential curve with a time constant of 3·1 pulses in *Aa* and 2·0 pulses in *Ba*. The histogram of *Ab* was also approximated by a single-exponential curve with a time constant of 2·4 pulses, while that in the presence of nitrendipine (*Bb*) was approximated by a double-exponential curve: $y = 163 \exp(-x/2{\cdot}2) + 16{\cdot}8 \exp(-x/12{\cdot}0)$. The excess of the long available state (longer than 20) and that of the long unavailable state (longer than 30) are indicated in the last bins.

within these holding potentials mean values of both the duration of the available state and that of the unavailable state were not significantly affected by their changes. The total number of clamp steps was 2530 in the control and 4023 in the presence of 100 nM-nitrendipine. Figure 6*Aa* shows the histogram of the number of sweeps per run with non-blank sweeps. It was well fitted by a single-exponential curve (Ochi *et al.* 1984) with a time constant of 1·6 s, which is slightly shorter than the mean duration of the available state (2·1 s). The histogram in the presence of 100 nM-nitrendipine was also distributed single-exponentially (Fig. 6*Ba*) with a time

constant of 1·0 s which is again slightly shorter than the mean duration of the available state (1·4 s).

Effect of nitrendipine and holding potential on the duration of the unavailable state

In Fig. 5*B* the mean number of blanks per run was plotted against the holding potential. At hyperpolarized holding potentials between −65 and −101 mV, the mean value was essentially the same (2·0 s). It was prolonged with statistical significance by 100 and 1000 nM-nitrendipine to 3·0 s and to 5·1 s, respectively (Table 1).

The mean duration of the unavailable state was increased remarkably upon depolarization of the holding potential, e.g. to 13 s at a holding potential of −29 mV in the control (Fig. 5*B*). In the presence of the drug, the prolongation was more marked and pronounced prolongation occurred at a smaller depolarization than in the control, thus it approached 20 s at a holding potential of −56 mV, as shown in Fig 5*B*. Similar change was noted in all five experiments (Table 1).

Histogram at large negative holding potentials. The histogram of the duration of the unavailable state was distributed apparently single-exponentially with the time constant of 1·2 s in the control (Fig. 6 *Ab*).

Based on the single-exponential distribution of the duration of the available state and that of the unavailable state at the strongly hyperpolarized potentials, a statistical test of the existence of only one functional channel in the patch was performed (Colquhoun & Hawks, 1983). We assume that there are N channels in the patch. When the open probability for the current-containing sweeps is sufficiently large, the simultaneous occurrence of more than two available states is readily detected by the appearance of overlapped openings. In the present study the mean duration of the available state (m_S) represented that of the single channels because it occurred without overlapped openings, while the mean duration of unavailable state (m_F) could be the result of the slow transitions of N channels. The probability (P_n) of observing successively n available states with single-channel currents is given by:

$$P_n = \left(\frac{1}{1 + \left(\frac{m_S}{m_F}\right)\left(\frac{N-1}{N}\right)} \right)^n, \quad (1)$$

where $N = 2$ gives the largest P_n. When m_S is 1·6 s and m_F is 2 s (Table 1), P_n is 0·034 at $n = 10$ and 0·0012 at $n = 20$. As n was between 152 and 373 in the present study at hyperpolarized potentials, P_n becomes far smaller, i.e. the possibility of observing the consecutive available states without overlapping should be quite small, if multiple channels existed in the patch.

In the presence of nitrendipine it was possible to fit the histogram by the sum of two exponential curves. In the present study the non-linear least-squares fitting to the double-exponential curve was performed after setting the time constant of the fast component at 1·1 s, a similar value to that of the time constant from the single-exponential curve in the control. In Fig. 6*Bb* the time constant of the slow component was 6·0 s.

Figure 7 shows the histograms of the duration of runs for the total results presented in Table 1. The histogram of non-blanks per run was distributed single-exponentially and the time constant was shortened by nitrendipine from 1·3 s in the control to 1·0 s at 100 nM and further to 0·7 s at 1000 nM. The histogram of blanks per run in the control was distributed again single-exponentially, with a time

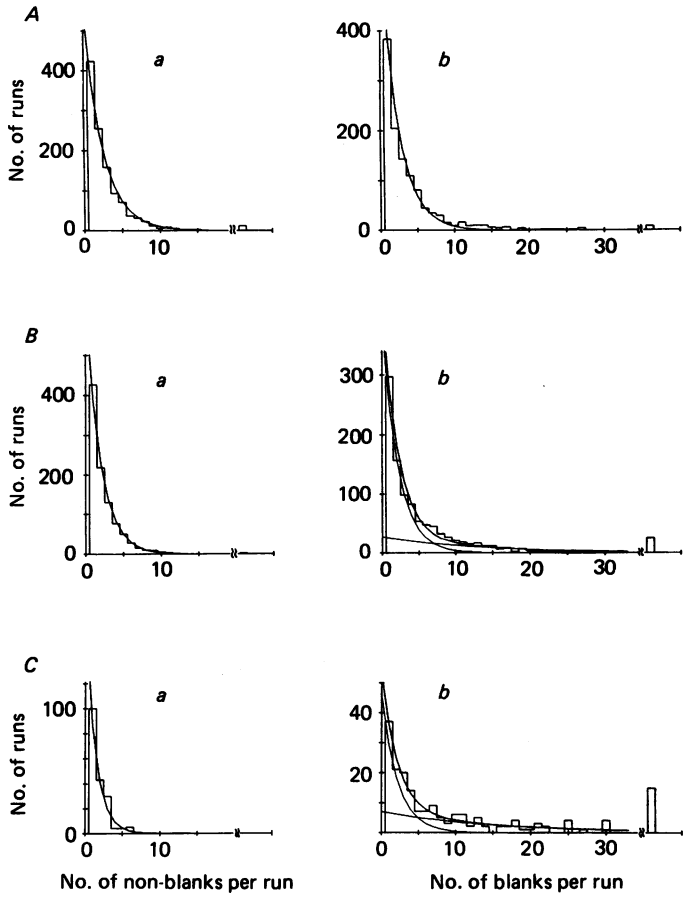

Fig. 7. Effect of nitrendipine on the distribution of number of sweeps per run for the total results given in Table 1. The number of sweeps per run at holding potentials between −65 and −130 mV compiled from five experiments in control (*A*) and in the presence of 100 nM-nitrendipine (*B*) and three experiments in the presence of 1000 nM-nitrendipine (*C*). *a*, number of runs plotted against the number of non-blanks per run; *b*, number of runs against the number of blanks per run. The histogram of number of non-blanks per run and that of blanks in control were approximated by a single-exponential curve with a time constant of 2·5 pulses in *Aa*, 2·0 pulses in *Ba*, 1·4 pulses in *Ca* and 2·33 pulses in *Ab*. Histograms of number of blanks per run in the presence of nitrendipine were approximated by a double-exponential curve; *Bb*, $y = 391{\cdot}2 \exp(-x/2{\cdot}2) + 27{\cdot}2 \exp(-x/13{\cdot}5)$; *Cb*, $y = 46{\cdot}4 \exp(-x/2{\cdot}2) + 7{\cdot}1 \exp(-x/15{\cdot}0)$; three curves for the fast and slow components and for their sum were drawn. The excess of the long available state (longer than 20) and that of the long unavailable state (longer than 30) are indicated in the last bins.

constant of 1·2 s. On the other hand the histogram in the presence of nitrendipine was approximated by a double-exponential curve. In the presence of 100 nM-nitrendipine the time constant of the slow component was 6·8 s. Increasing the drug concentration to 1000 nM did not prolong the duration of the slow component but increased the percentage of runs of this component from 30 to 51%.

Histograms at depolarized holding potentials. When the holding potential was depolarized, an exponential component with a large time constant appeared also in the control histogram together with the fast component seen at hyperpolarized holding potentials (Fig. 8*Aa*). At a holding potential of −47 mV, the time constant of the slow component was 7 s and the percentage of runs amounted to 41%. In the presence of nitrendipine the duration of the slow component was so markedly

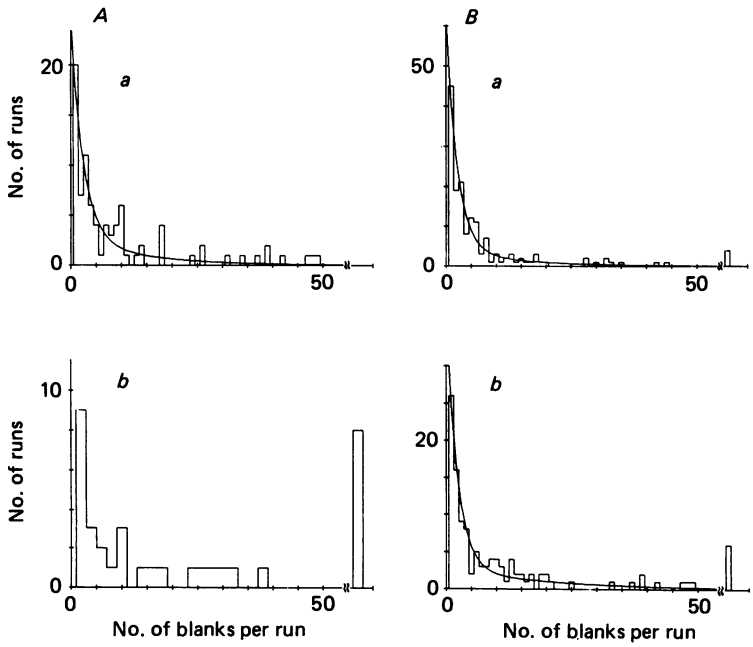

Fig. 8. Histograms of the duration of the number of blanks per run at depolarized holding potentials in the presence and absence of nitrendipine. *A*, from the experiment given in Fig. 3; *B*, combined results from three preparations. *a*, control; *b*, in the presence of 100 nM-nitrendipine. The holding potential was: *Aa* −47 mV, *Ab* −61 mV, *Ba* −38 mV, *Bb* −47 mV. Number of runs was plotted against the number of blanks per run. The number of runs having more than fifty continuous blanks is given in the last bin. A double-exponential curve was drawn for each histogram with following parameters: *Aa*, $y = 21{\cdot}8 \exp(-x/2{\cdot}5) + 2{\cdot}7 \exp(-x/14{\cdot}0)$; *Ba*, $y = 58{\cdot}1 \exp(-x/2{\cdot}2) + 4{\cdot}2 \exp(-x/13{\cdot}7)$; *Bb*, $y = 34{\cdot}6 \exp(-x/2{\cdot}2) + 2{\cdot}9 \exp(-x/20{\cdot}0)$. The time constants of corresponding histograms of the duration of available state (not shown) was 2·6 s for *Aa*, 1·8 s for *Ba* and 2·1 s for *Bb*.

prolonged that it was difficult to estimate its time constant (Fig. 8*Ab*). In order to increase the number of runs the results of three preparations with a similar voltage dependence of the mean duration of the unavailable state were compiled (Fig. 8*B*). In the control at the holding potential of −38 mV the histogram was distributed double-exponentially with a time constant of the slow component of 8·9 s (Fig. 8*Ba*), while in the presence of 100 nM at a more hyperpolarized potential (−47 mV) the time constant was 10 s (Fig. 8*Bb*). The time constant of the fast component (1·1 s)

did not appear to be affected either by the depolarization of the holding potential or by the application of nitrendipine.

Effect of nitrendipine on the fast gating process

As seen in the specimen records in Fig. 3, 100 nM-nitrendipine hardly affected the amplitude of single-Ca^{2+}-channel currents, and it little affected the fast gating process. Figure 9 shows the influence of nitrendipine on the open-time and shut-time

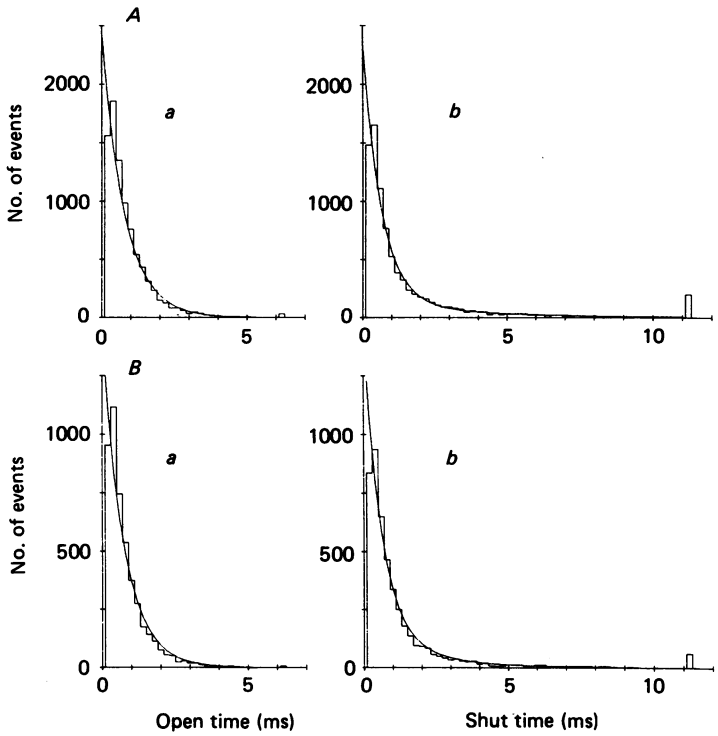

Fig. 9. Effects of nitrendipine on the open and shut time of a single Ca^{2+} channel. From the experiments shown in Fig. 3. Depolarizing steps to 7 mV with a duration of 100 ms were applied repetitively about 500 times from a holding potential of −65 mV. *A*, control; *B*, in the presence of 100 nM-nitrendipine; *a*, open-time histogram; *b*, shut-time histogram. In each histogram, the number of events are plotted *versus* the duration of the events. Cut-off frequency was 1 kHz and the bin width is 0·2 ms. Open-time histograms were fitted by exponential curves with time constants of: *Aa*, 0·78 ms; *Ba*, 0·73 ms. Shut-time histograms were fitted by the sum of two exponential curves with time constants (in ms): *Ab*, $y = 2216 \exp(x/0{\cdot}65) + 122 \exp(x/3{\cdot}9)$; *Bb*, $y = 1300 \exp(x/0{\cdot}6) + 150 \exp(x/2)$.

histograms. In the control, the open time was distributed exponentially with a time constant of 0·78 ms, which was close to the mean open time of 0·88 ms. Also, in the presence of 100 nM-nitrendipine, the open time was distributed exponentially with a time constant of 0·73 ms, which coincided with the mean open time of 0·79 ms. In all five experiments the mean open time was almost the same in the absence and presence of 100 nM-nitrendipine and was not significantly affected by depolarization

of the holding potential (Table 2). The mean open time was also little affected by 1000 nM-nitrendipine in three experiments (Table 2). Shut-time histograms were fitted by double-exponential curves, reflecting the occurrence of bursts (Fenwick, Marty & Neher, 1982), and were little affected by nitrendipine. The open-state probability calculated for current-containing sweeps obtained using 100 ms steps is determined mainly by the rapid gating process, as the decay of the mean current produced by the inactivation process is negligible in the control and in the presence of 100 nM-nitrendipine. The value was between 0·1 and 0·5 and was not significantly affected by 100 nM-nitrendipine either at strongly hyperpolarized holding potentials or at depolarized holding potentials, but was slightly diminished by 1000 nM-nitrendipine due to the acceleration of inducing unavailable states (Table 2).

TABLE 2. Effect of nitrendipine and holding potential on the open-state probability and the open time of Ca^{2+} channels

	Holding potential −130 to −65 mV		Holding potential near $V_{\frac{1}{2}}$	
	P_o	τ_o (ms)	P_o	τ_o (ms)
Control	0·29	0·96	0·33	1·03
±S.D. ($n = 5$)	0·13	0·21	0·19	0·14
Nitrendipine (100 nM)	0·29	1·06	0·25	0·99
±S.D. ($n = 5$)	0·14	0·30	0·13	0·39
Nitrendipine (1000 nM)	0·17	0·93	0·22	1·07
±S.D. ($n = 3$)	0·09	0·17	0·09	0·25

The data obtained at holding potentials between −65 and −130 mV were compiled and compared with those obtained at the depolarized holding potential near $V_{\frac{1}{2}}$. More than 500 depolarizing pulses were applied at 2 Hz at each holding potential. P_o, open-state probability calculated for the current-containing sweeps; τ_o, mean open time. From the same experiments presented in Table 1. All of the mean values obtained in the presence of nitrendipine are not significantly different from the value of the control.

DISCUSSION

The size of the macroscopic Ca^{2+} current is dependent on the open-state probability primarily determined by the activation process and the availability of the channel determined by the slow state transitions. A 1,4-dihydropyridine Ca^{2+} channel blocker, nitrendipine, decreased the channel availability without significantly affecting the activation process. We did not observe the prolongation of the open time, although it occurred at a high concentration of 5000 nM (Hess *et al.* 1984).

Drug association during depolarization

The duration of the available state was shortened by nitrendipine. Such a shortening is responsible for the accelerated decay of the mean current (Fig. 1) and of the macroscopic current (Lee & Tsien, 1983). Conversely, assuming that the channel becomes non-conductive immediately after drug binding the time course of decay can be a measure of the association. In Fig. 1, when the mean current decayed very slowly in the control, it decayed with a time constant of about 100 ms in the presence of 1000 nM-nitrendipine. As the unbinding rate for depolarization steps is

negligibly small as evidenced by the occurrence of sustained unavailable states at depolarized holding potentials, the apparent association rate for the open channel is 1×10^7 M^{-1} s^{-1}, about one-tenth of the association rate of nimodipine for the open Ca^{2+} channel in pituitary cells (Cohen & McCarthy, 1987). Since considerable transitions to the inactivated state occur during test pulses (Fig. 8*Aa*), we cannot preclude the drug association with very high affinity in the inactivated state.

Existence of multiple unavailable states

If the state transitions in the absence of nitrendipine occur by the independent movements of two slow gates, we can expect the existence of three unavailable states and one available state. In this model the distribution of the duration of the unavailable states approaches double exponential, if the rates of opening of the two gates differ by much. The present time constants of the double-exponential curves may represent the close-to-open transitions of these two gates.

The rate constants of slow state transitions were calculated from the results considering the model used for the analysis of burst kinetics (Fenwick *et al.* 1982; Sakmann & Trube, 1984). Since the duration of the available state is modulated both by application of adrenaline (Ochi *et al.* 1984) and by the increase in duration of the test pulse (R. Ochi & Y. Kawashima, unpublished results), the model which does not allow the changes of the duration of the available state by the two different interventions is not applicable. Therefore, we choose the following model which is analogous with the C–O–C model (O, open state; C, closed state) (Sakmann & Trube, 1984):

$$F_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} F_2, \qquad (2)$$

where F_1 and F_2 denote unavailable states, and S an available state. When the channel state is S, we succeed in eliciting the single-channel openings, and when it is F_1 or F_2 we fail to elicit them. The rate constants (k_1, k_2, k_3, k_4) were calculated using parameters of the double-exponential curves of the duration of unavailable state and time constant of the exponential curve of the duration of available state in Figs 6, 7 and 8 such that F_1 has a longer mean lifetime than F_2. k_1 was very small and between 0·10 and 0·17 s^{-1}. Since this transition rate is affected both by the depolarization of the holding potential and by the binding of nitrendipine, F_1 is regarded to represent the inactivated state. k_1 mainly expresses the rate at the holding potentials. k_2 was between 0·30 and 0·73 s^{-1} and when the nitrendipine concentration was increased from 100 to 1000 nM, it was increased from 0·30 to 0·73 s^{-1} by the acceleration of the drug association to the channel. The latter value could be an underestimate of the true transition rate by the limited time resolution. k_3 was between 0·45 and 0·77 s^{-1} and k_4 was set at 0·8 or 0·91 s^{-1}. Since these rate constants were little affected by depolarization of the holding potential and nitrendipine, F_2 is ascribable to the state related to the voltage-independent but phosphorylation-dependent process.

The control histogram was apparently distributed single-exponentially at strongly hyperpolarized holding potentials, so it was impossible to determine the rate constants. The single-exponential distribution is explicable if (1) k_1 coincides with

k_4 or (2) k_1 is too large to be resolved properly by the present method, as expected from the time course of restoration of the macroscopic Ca^{2+} current in cat ventricular muscle (Trautwein, McDonald & Tripathi, 1975).

Voltage-dependent decrease in the availability as the mechanism of nitrendipine-induced blockade

The well-known voltage-dependent blockade of the macroscopic current by 1,4-dihydropyridine Ca^{2+} channel blockers (Bean, 1984; Sanguinetti & Kass, 1984) is related to the increase of the time in the unavailable state at the depolarized holding potentials (Fig. 4). The voltage-dependent blockade is due to the drug-induced modulation of the transition rates between F_1 and S in the above scheme. The modulated receptor theory which describes the block of Na^{2+} channels by local anaesthetics (Hille, 1977; Hondeghem & Katzung, 1977) is useful to explain the processes involved in it. The drug-bound channel is assumed to be non-conductive and the affinity of the channel receptor to nitrendipine in the open state, one substate of S, was calculated above to be high with an apparent association rate of 1×10^7 M^{-1} s^{-1}. Analogously with the drug-unbound channel, transition from the inactivated state to the resting closed state in the drug-bound channel is assumed to be rate limited in the transition from F_1 to S. At large negative holding potentials k_1 was larger than 0·9 s^{-1}, while it was 0·13 s^{-1} in the nitrendipine-bound channel. The depolarization-dependent decrease in k_1 both in the presence and in the absence of nitrendipine suggests that the gating process of the inactivated channel is modulated by drug binding to produce this small value. After the conformational change of the channel from the inactivated state to the closed state, the drug will be rapidly dissociated from the receptor. At depolarized holding potentials the unbinding rate could be so small due to slowing of the conformational change. The binding of nitrendipine in the open state and in the inactivated state at depolarized holding potentials is supported by the radio-binding studies, which have revealed that the affinity of the receptor for 1,4-dihydropyridine is enhanced intensely by depolarization (Schilling & Drewe, 1986; Kokubun, Prod'hom, Becker, Porzig & Reuter, 1986).

In conclusion, the prolonged inactivated states induced by test potentials via drug binding, and maintained due to the decreased transition rate from the inactivated state to the resting closed state, is the mechanism of voltage-dependent blockade by nitrendipine.

The authors thank Dr Hiroshi Okuyama for making the main analysis programs. This work was supported by a Grant-in-Aid for Scientific Research No. 62570046, a Grant-in-Aid for Special Research on the Molecular Mechanism of Bioelectrical Response, a Grant-in-Aid for Special Project Research on Metabolic Researches of Blood Vessels from the Japanese Ministry of Education, Science and Culture and by a Research Grant for Cardiovascular Diseases from the Japanese Ministry of Health and Welfare.

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