Br. J. Pharmacol. (1990), **100**, 353–359

Effects of calmodulin antagonists on calcium-activated potassium channels in pregnant rat myometrium

M. Kihira, K. Matsuzawa, *H. Tokuno, & [1]*T. Tomita

Department of Obstetrics and Gynaecology, and Department of Physiology*, School of Medicine, Nagoya University, Nagoya 466, Japan

1 The effects of W-7, trifluoperazine, and W-5 on Ca^{2+}-activated K^+-channels were investigated with the inside-out patch-clamp method in smooth muscle cells freshly dispersed from pregnant rat myometrium. These drugs are known to have different potencies as calmodulin antagonists.

2 In the presence of 1 μM Ca^{2+} on the cytoplasmic side ($[Ca^{2+}]_i$), the fraction of time the channel was open (open probability, P_o) was about 0.9 and the calmodulin antagonists (1–30 μM) applied to the cytoplasmic face reduced P_o to 0.65–0.55 dose-dependently. In the presence of 0.1–0.16 μM Ca^{2+}, when P_o was very low (0.02), calmodulin antagonists increased P_o. All antagonists used produced almost identical effects at the same concentration.

3 The probability density function of the open time distribution could be described by the sum of two exponentials. W-7 decreased the time constant of the slow component of distribution and at 30 μM the slow component disappeared both at 1 and 0.25 μM $[Ca^{2+}]_i$, reflecting the appearance of flickering channel activity. The probability density function of the closed time distribution could be fitted with three exponentials. The time constants of these components were not significantly altered by W-7.

4 Internally applied calmodulin (1–5 μM) did not produce any significant effect on channel activity.

5 The effects of calmodulin antagonists are considered to be due to a direct action of these compounds on the channel, and suggest that channel activation by Ca^{2+} is not mediated by calmodulin.

Introduction

The plasma membrane of smooth muscles contains potassium (K^+) channels the activity of which is controlled by intracellular calcium (Ca^{2+}-activated K^+-channels) (Inoue *et al.*, 1985; Benham *et al.*, 1986; McCann & Welsh, 1986). Since these channels have a large conductance and are highly selective to K^+, they may play an important role in regulating electrical excitability, for example in alteration of electrical activity during pregnancy (Osa & Fujino, 1978; Kishikawa, 1981; Bengtsson *et al.*, 1984). However, the mechanism of channel activation with Ca^{2+} is still not well understood.

Since calmodulin mediates many intracellular Ca^{2+}-regulated enzymes and processes, it may also be involved in activation of the Ca^{2+}-activated K^+-channel. This has actually been suggested for some tissues (cultured fibroblast: Okada *et al.*, 1986; 1987; erythrocyte: Lackington & Orrego, 1981; Pape & Kristensen, 1984), mainly based on the action of calmodulin antagonists (e.g., phenothiazines). However, in the Ca^{2+}-activated K^+-channel in airway smooth muscle, it has been found that the order of potency as channel antagonists is haloperidol > trifluoperazine > chlorpromazine and that this is different from the order expected from the potency of these drugs as calmodulin antagonists (trifluoperazine > chlorpromazine > haloperidol), suggesting that these antagonists interact directly with the channel, not through a calmodulin-mediated process (McCann & Welsh, 1987). We have re-examined the possibility of calmodulin involvement in the Ca^{2+}-activated K^+-channel in smooth muscle cells freshly dispersed from pregnant rat myometrium, using calmodulin antagonists (a phenothiadine derivative, trifluoperazine, and a naphalenesulphonamide derivative, W-7) and a naphthalenesulphonamide of weak anticalmodulin potency (W-5) (Asano & Stull, 1985; Asano *et al.*, 1985).

Methods

Pregnant (the 14–18th day of pregnancy) Wistar rats (about 250 g) were used. After anaesthetizing the rat with pentobarbitone sodium (50 mg kg^{-1}), the uterine horn was excised and the animals were killed by bleeding. The endometrium was then carefully removed, and the circular muscle layer was separated from the longitudinal muscle layer. Single smooth muscle cells were obtained by enzymatic dissociation from the circular layer, by a technique similar to that described by Benham *et al.* (1985, 1986) and Inoue *et al.* (1985). The solution for cell dispersion contained 0.1% collagenase, 0.1% trypsin inhibitor, 0.5% bovine serum albumin and no Ca^{2+}.

The physiological solution had the following composition (mM): NaCl 127, KCl 6.0, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 12, HEPES buffer (N-2-hydroxyethylpiperazine-N'-2 ethanesulphonic acid, pH adjusted with NaOH to 7.4) 10. Pipettes used for the whole-cell clamp were filled with a solution containing (mM); K-aspartate 106.0, KCl 24.0, Na_2ATP 5.0, $MgCl_2$ 2.4, $CaCl_2$ 0.8, EGTA (ethyleneglycol-*bis*-tetraacetic acid) 5.0, HEPES 10. For the cell-detached patch clamp experiments, pipettes were filled with solution containing (mM): K-aspartate 130, NaCl 18.0, $CaCl_2$ 0.9, EGTA 1.0, HEPES 5.0. The bath solution facing the cytoplasmic surface of the plasma membrane usually contained (mM): KCl 6.0, NaCl 142.0, HEPES 5.0, EGTA 1.0 and the required concentration of Ca^{2+} ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was adjusted by adding a correct amount of Ca^{2+} in the presence of 1 mM EGTA, according to the calculation by Fabiato (1981). When the K^+ concentration was increased, NaCl was replaced with KCl isosmotically. All experiments were carried out at room temperature (22–25°C).

The methods of recording whole-cell currents and single channel currents from an inside-out isolated patch were similar to those described by Hamill *et al.* (1981). The currents were recorded with an amplifier (L/M EPC-7, List). Data were recorded on a videotape recorder (GX4, National) at a sampling rate of 28.8 kHz with an analogue-digital converter (RP-880, PCM Data Recording System, NF Electronic Instrument) and a low pass filter (3.3 kHz). Single channel activity was analysed with a computer using the pClamp programme (version 5.03, Axon Instruments, Inc.). Channel opening and closing were determined by setting the threshold at the half-value of current amplitude.

W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide) and W-5 (N-(6-aminohexyl)-1-naphthalenesulphonamide) were obtained from Seikagaku Kogyo (Japan). Other

[1] Author for correspondence.

chemicals used were all obtained from Sigma. The calmodulin used was a product from bovine brain (Sigma).

Results

Outward currents were produced with 400 ms depolarizing steps from a holding potential of −80 mV with the whole-cell clamp method in normal solution. External application of W-7 slowly inhibited the current dose-dependently. The average current was 367 ± 28 pA in normal solution and this was decreased to 232 ± 21, 187 ± 31 and 150 ± 29 pA (mean ± s.d., $n = 5$) with 1, 10 and 30 μM W-7 applied for 5 min. W-7 50 μM produced a similar degree of inhibition ($n = 2$) to 36 μM, but since the recovery became very slow, the effects of concentrations higher than 30 μM were not carefully examined. Figure 1 shows the strongest inhibitory effect on outward currents observed with 30 μM W-7. The currents were produced by constant depolarizing pulses from −80 to +20 mV applied every 12 s. The recovery was very slow, taking 40–60 min after application of 30 μM W-7.

Figure 2 shows the voltage-current relationship under whole-cell clamp conditions. Outward currents were increased with increasing membrane depolarization beyond −40 mV, showing outward-going rectification. W-7 30 μM decreased the current and reduced the rectification. Nicardipine (a Ca^{2+}-channel blocker, 1 μM) produced effects similar to W-7 and in the presence of nicardipine, the W-7 effect was greatly reduced. These results may be explained either by a direct action of W-7 on outward K^+ currents, or by an indirect action on the Ca^{2+}-dependent K^+-channel, as a result of reduced Ca^{2+}-influx produced by W-7.

In order to investigate the mechanisms underlying the inhibition of outward currents with W-7, activities of single K^+-channels were analysed. When $[Ca^{2+}]_i$ was more than 0.1 μM, three or four channels often became open sumultaneously in many membrane patches. For analysis of channel properties, however, we have chosen patches which were considered to contain only a single active channel. The amplitude of single channel currents recorded with patch pipettes containing 130 mM K^+ depended on the internal K^+ concentration ($[K^+]_i$) and the membrane potential. As shown in Figure 3, the voltage-current relationship can be described by the constant field equation (Hodgkin & Katz, 1949) assuming that only K^+ is permeable. When the permeability constant (P_K) was assumed to be 4.2×10^{-13} cm^{-3} s^{-1}, the observed points agreed reasonably well with the theoretical curves at $[K^+]_i$ higher than 65 mM. When $[K^+]_i$ was 6 mM, a P_k value of 3.8×10^{-13} cm^3 s^{-1} gave a better fit. At a symmetrical K^+ concentration (130 mM), the single channel conductance was 204 ± 4 pS ($n = 4$). These values are close to those (4.5×10^{-13} cm^3 s^{-1} for the P_k and 183–198 pS for the conductance at symmetrical 126 mM K^+) reported for the Ca^{2+}-activated K^+-channel in the smooth muscle of guinea-pig jejunum and mesenteric artery (Benham *et al.*, 1986). In the following experiments, the effects of W-7 and trifluoperazine on this type of channel were investigated at 130 mM external and 6 mM internal K^+ concentrations, because single channel currents of reasonable amplitude (about 4.5 pA) could be observed at a holding potential of 0 mV.

Figure 4a and b show the effects of W-7 (1–30 μM) applied to the cytoplasmic side on single channel currents at 1 and 0.16 μM cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_i$), respectively. The channel activity strongly depended on $[Ca^{2+}]_i$. The channel was mostly in the open state at 1 μM Ca^{2+}. The fraction of time during that the channel was in an open state (the open probability, P_o) was 0.91 ± 0.03 ($n = 4$). When 1–10 μM W-7 was applied, the channel activity was slightly decreased, as observed in airway smooth muscle cells (McCann & Welsh, 1987), and very fast flickering activity appeared at 10–30 μM (Figure 4a). The amplitude of currents was decreased at concentrations higher than 10 μM, probably due to the high frequency flickering.

The channel activity was low in the presence of a low $[Ca^{2+}]_i$ (0.16 μM, $P_o = 0.02 \pm 0.01$, $n = 3$). In this condition, the channel activity was clearly increased by W-7 dose-dependently (Figure 4b). The channel opening became not only more frequent, but also longer with flickering activity.

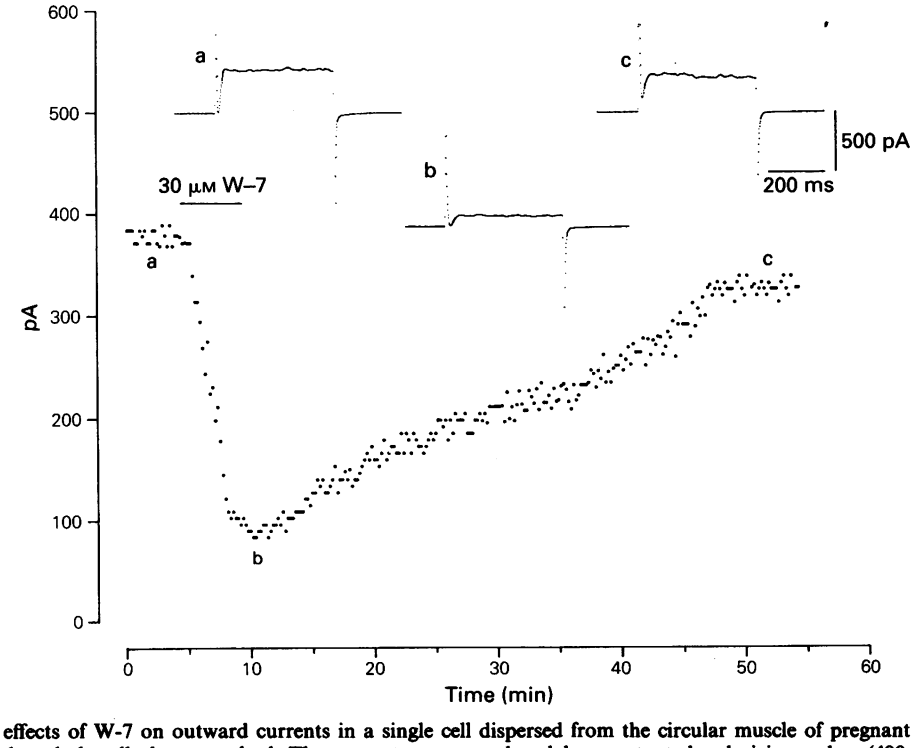

Figure 1 The effects of W-7 on outward currents in a single cell dispersed from the circular muscle of pregnant rat myometrium, recorded with the whole-cell clamp method. The currents were produced by constant depolarizing pulses (400 ms) from −80 to +20 mV every 12 s. The recording chamber (0.2 ml) was perfused with normal solution at a constant rate (2 ml min^{-1}) and 30 μM W-7 was applied to the perfusing solution for 5 min. The current traces shown above were obtained before, 2 min after, and 45 min after W-7 application corresponding to the time indicated by (a), (b) and (c).

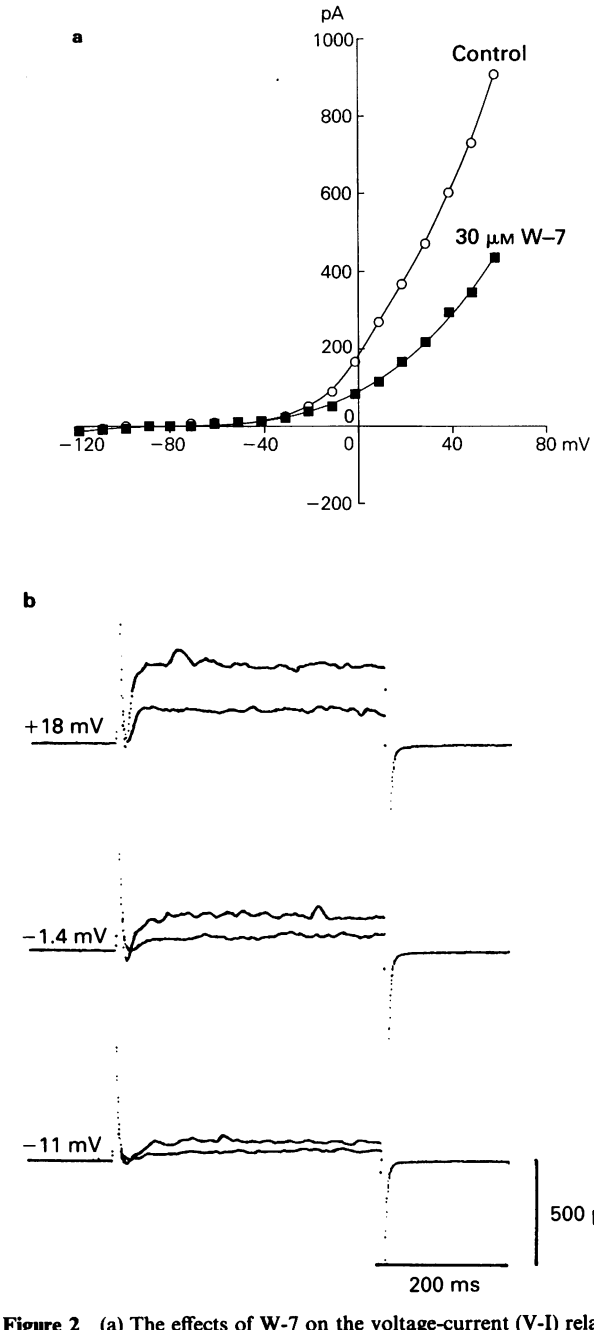

Figure 2 (a) The effects of W-7 on the voltage-current (V-I) relationship obtained with the whole-cell clamp method. After obtaining the V-I relationship in normal solution, 30 μM W-7 was applied and 5 min later the V-I relationship was again obtained in the presence of W-7. (b) Shows superimposed current tracings in the absence and the presence of W-7 at three different voltage steps. The current amplitude was measured at the end of a 400 ms pulse.

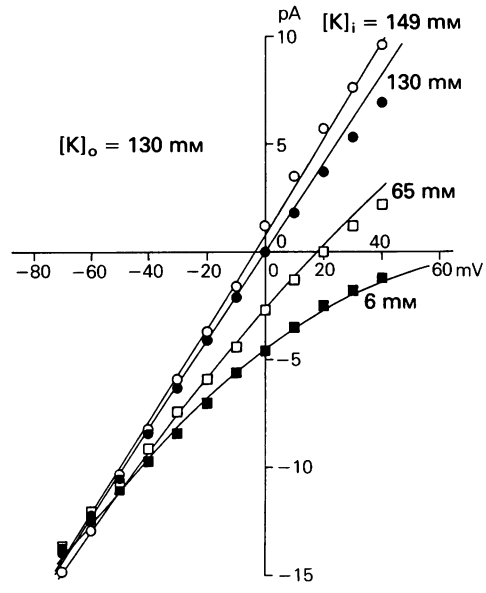

Figure 3 The voltage-current relationship obtained from a single Ca^{2+}-activated K^+ channel on an isolated inside-out patch at different cytoplasmic K^+ concentrations ($[K]_i$). The K^+ concentration in the pipette (external) solution ($[K]_o$) was kept constant at 130 mM. Lines were drawn based on the constant field equation (Hodgkin & Katz, 1949), assuming that only K^+ is permeable. The permeability constant was 4.2×10^{-13} $cm^3 s^{-1}$ for higher than 65 mM K^+ and $3.8 \times 10^{-13} cm^3 s^{-1}$ for 6 mM K^+ (23°C).

These effects were similar to those of local anaesthetics observed at the acetylcholine-receptor channel of frog muscle (Neher & Steinbach, 1978). As W-7 concentrations were increased to 10–30 μM, the current amplitude was reduced, as observed in the presence of 1 μM Ca^{2+}. Compared with the whole-cell clamp condition, recovery from W-7 was much faster in isolated membrane patches, taking about 5 min after removal of 30 μM W-7.

Nearly identical results were obtained at the same concentration (1–30 μM) with trifluoperazine, another calmodulin antagonist (not shown), and also with W-5, a derivative of W-7, which has weak potency as a calmodulin antagonist (Figure 5). In Figure 5, the effects of W-5 were compared with those of W-7 in the presence of 0.16 and 1 μM Ca^{2+} in the same channel. Calmodulin itself was applied internally to three different channels, but it produced no clear effect at a concentration of 1–5 μg ml^{-1} (not shown).

In a solution containing 5 mM EGTA and no added Ca^{2+}, channel activity was absent. Even under these conditions, however, application of 30 μM W-7 started channel activity, although at a very low rate (Figure 6). As seen in the faster current tracings, the channel activity was of a burst type in the presence of W-7. This was confirmed in three channels.

Based on experiments similar to those shown in Figure 4, the effect of W-7 on the relationship between $[Ca^{2+}]_i$ and P_o was studied (Figure 7). In the absence of W-7, the channel remained in a closed state below 0.1 μM Ca^{2+}, and its activity was sharply increased beyond 0.16 μM reaching maximum at 0.4 μM Ca^{2+}. As shown in Figure 4, at low $[Ca^{2+}]_i$ (0.1–0.16 μM), the channel activity was increased by W-7 dose-dependently, whereas at high $[Ca^{2+}]_i$ (0.25–1 μM), it was reduced by W-7. Therefore, the $[Ca^{2+}]_i - P_o$ curve was decreased in amplitude, and flattened by increasing W-7 concentrations from 1 to 30 μM, without a significant shift in the mid-point. The sensitivity to Ca^{2+} varied slightly in different channels, but the effect of W-7 was essentially the same in the three channels studied.

The effects of W-7 on the probability density function of the distributions of open time and closed time were analysed at 1 μM and 0.25 μM $[Ca^{2+}]_i$ in two channels, and the data in the presence of 1 μM are shown in Figure 8. Since the channel activity was very low at 0.16 μM $[Ca^{2+}]_i$, 0.25 μM $[Ca^{2+}]_i$ was chosen for precise analysis. In the absence of W-7, the curve could be fitted with a sum of two exponentials for the open time distribution. W-7 significantly increased the event frequency and shortened the time constant of the slow component of the distribution at both $[Ca^{2+}]_i$ (Table 1). However, the time constants in the presence of 0.25 μM $[Ca^{2+}]_i$ were very similar to those in 1 μM $[Ca^{2+}]_i$ at each W-7 concentration. At 30 μM, the slow component disappeared due to the appearance of a high frequency burst activity. Shortening of the time constant with calmodulin antagonists has previously been reported for dog airway muscle cells (McCann & Welsh, 1987).

The probability density function of closed time distribution could be fitted with three exponentials in the absence and in

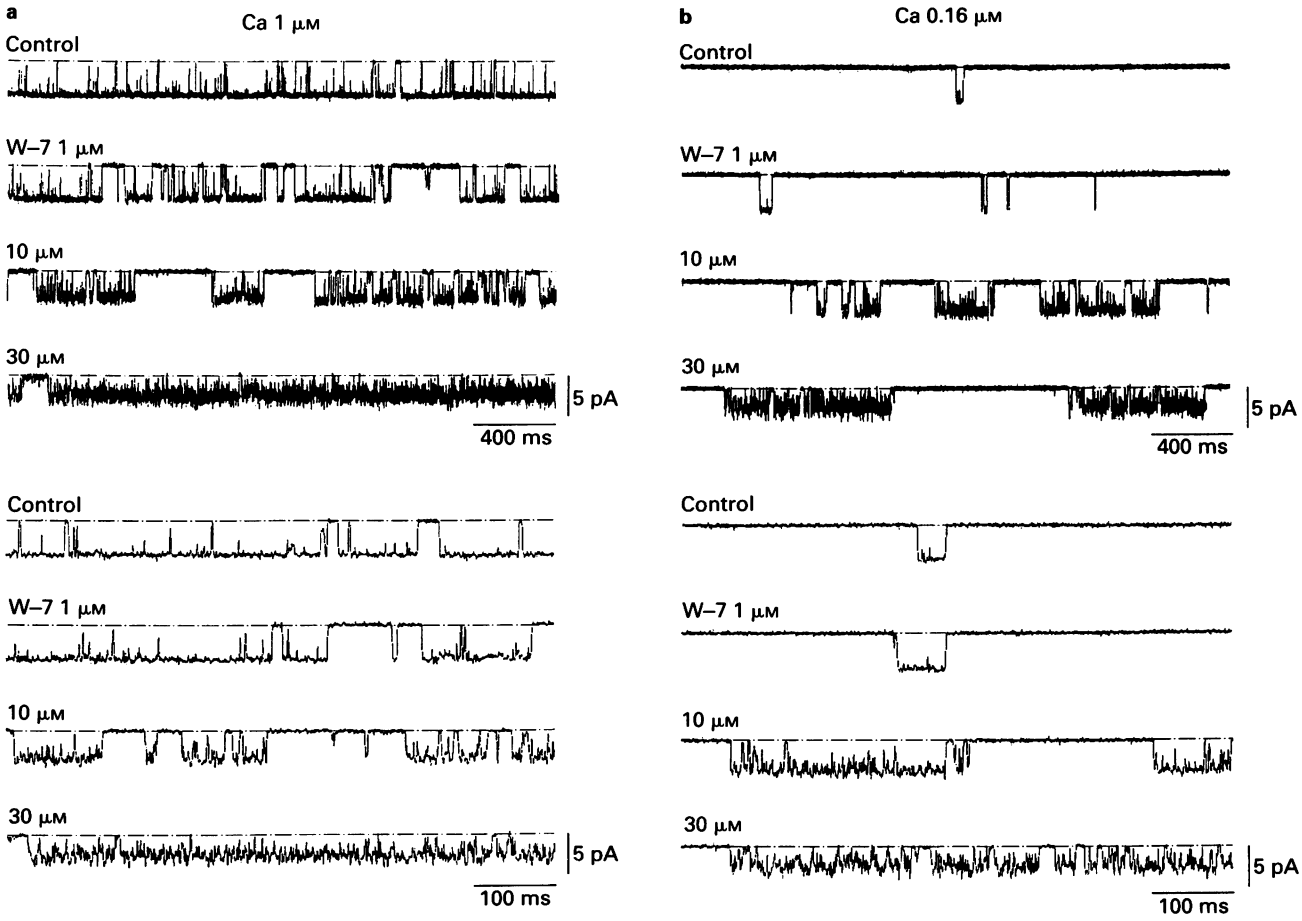

Figure 4 The effects of W-7 on a single Ca^{2+}-activated K^+ channel. $[K^+]_o = 130$ mM, $[K^+]_i = 6$ mM, $[Ca^{2+}]_i = 1\,\mu$M in (a) and $0.16\,\mu$M in (b), and the membrane potential was clamped at 0 mV. W-7 was applied to the cytoplasmic face and its concentration was increased from 1 to $30\,\mu$M stepwise, each for 5 min. The dotted lines correspond to the closed state of the channel. Lower traces are shown at a faster time scale. (a) and (b) are from the same channel. See text for further explanation.

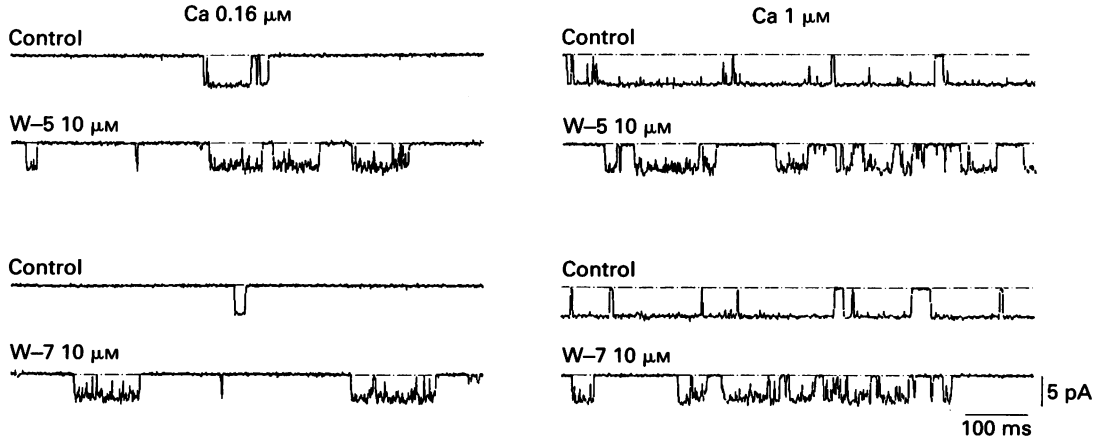

Figure 5 Comparison of the effects of W-5 and W-7 at $10\,\mu$M on channel activity, in the presence of 0.16 and $1\,\mu$M $[Ca^{2+}]_i$. The experimental conditions were the same as in Figure 4, but from a different channel. W-7 was applied 10 min after wash-out of W-5. The Ca^{2+} concentration was increased after observing the effects of $10\,\mu$M W-7 at $0.16\,\mu$M Ca^{2+}.

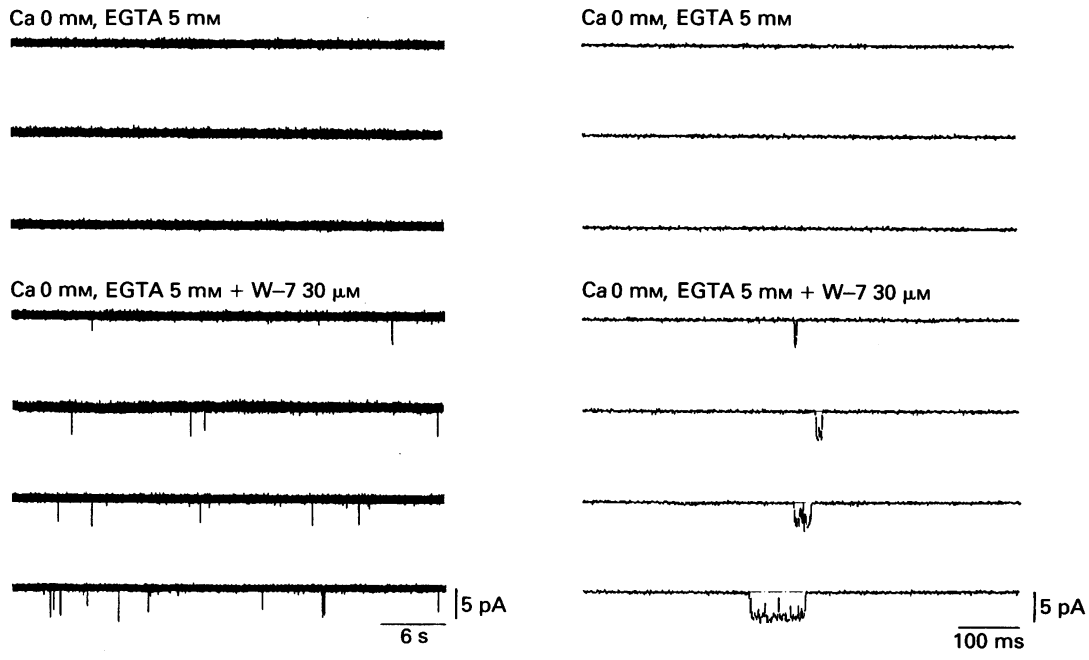

Figure 6 The effects of W-7 30 μM in the absence of Ca^{2+}. The internal solution contained no added Ca^{2+} and 5 mM EGTA. On the left, continuous recordings are shown successively at a slow speed, and W-7 was applied near the end of the third trace. The faster traces (on the right) were selected to show channel activity from the slow records shown on the left.

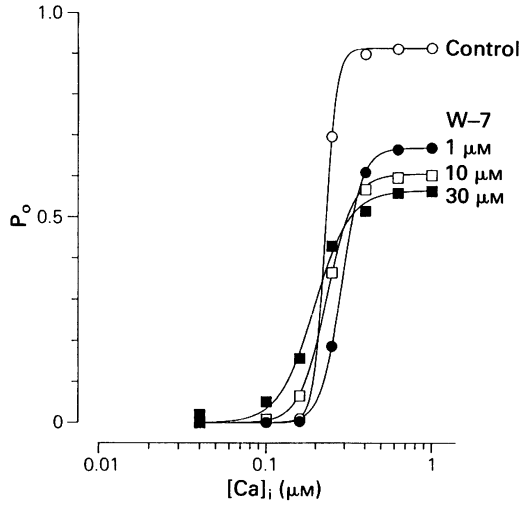

Figure 7 The relationship between open probability (P_o) and Ca^{2+} concentration at the cytoplasmic face ($[Ca]_i$), in the absence and presence of W-7 1–30 μM. All data were obtained from a single channel.

the presence of W-7 (Table 1). When P_o was reduced by decreasing $[Ca^{2+}]_i$ from 1 to 0.25 μM in the absence of W-7, the main change was a lengthening of the time constant of the slowest component. However, the effects of W-7 on the time constants of closed time distribution varied, depending on the concentration of both $[Ca^{2+}]_i$ and W-7, as shown in Table 1.

Discussion

In mouse fibroblasts, a $[Ca^{2+}]_i$-dependent hyperpolarization is reduced by calmodulin antagonists (trifluoperazine, 25 μM, or W-7, 30 μM) and intracellular application of calmodulin induces hyperpolarization of the membrane (Okada *et al.*, 1986; 1987). This result has been interpreted as suggesting that calmodulin is involved in the functioning of Ca^{2+}-activated K^+-channels. In single muscle cells freshly dispersed from pregnant rat myometrium, outward membrane currents recorded with the whole-cell clamp method are also inhibited by W-7. These currents are considered to be mainly carried by K^+ flowing through Ca^{2+}-activated K^+-channels, because the current is reduced by nicardipine. From this result alone,

Table 1 Open probability (P_o), mean open time (MOT), mean closed time (MCT) and time constants (τ) in ms of open and closed time distributions (the mean of two channels)

		Open				*Closed*		
	P_o	*MOT*	τ_1	τ_2	*MCT*	τ_1	τ_2	τ_3
$[Ca^{2+}]_i$ 1 μM								
Control	0.84	36.3	1.5	41.8	6.8	0.7	3.2	16.6
W-7 (μM)								
1	0.61	12.6	2.4	15.9	8.1	0.7	2.9	26.0
10	0.56	4.8	2.2	7.3	4.4	0.6	3.3	23.7
30	0.52	2.0	1.9	—	1.6	0.9	2.7	23.9
$[Ca^{2+}]_i$ 0.25 μM								
Control	0.70	35.8	1.7	34.8	15.2	0.3	2.6	35.3
W-7 (μM)								
1	0.20	9.9	2.4	12.9	15.3	0.6	2.5	44.8
10	0.43	7.2	1.8	8.2	8.1	0.5	2.1	15.4
30	0.47	2.1	1.9	—	2.7	0.9	3.1	19.6

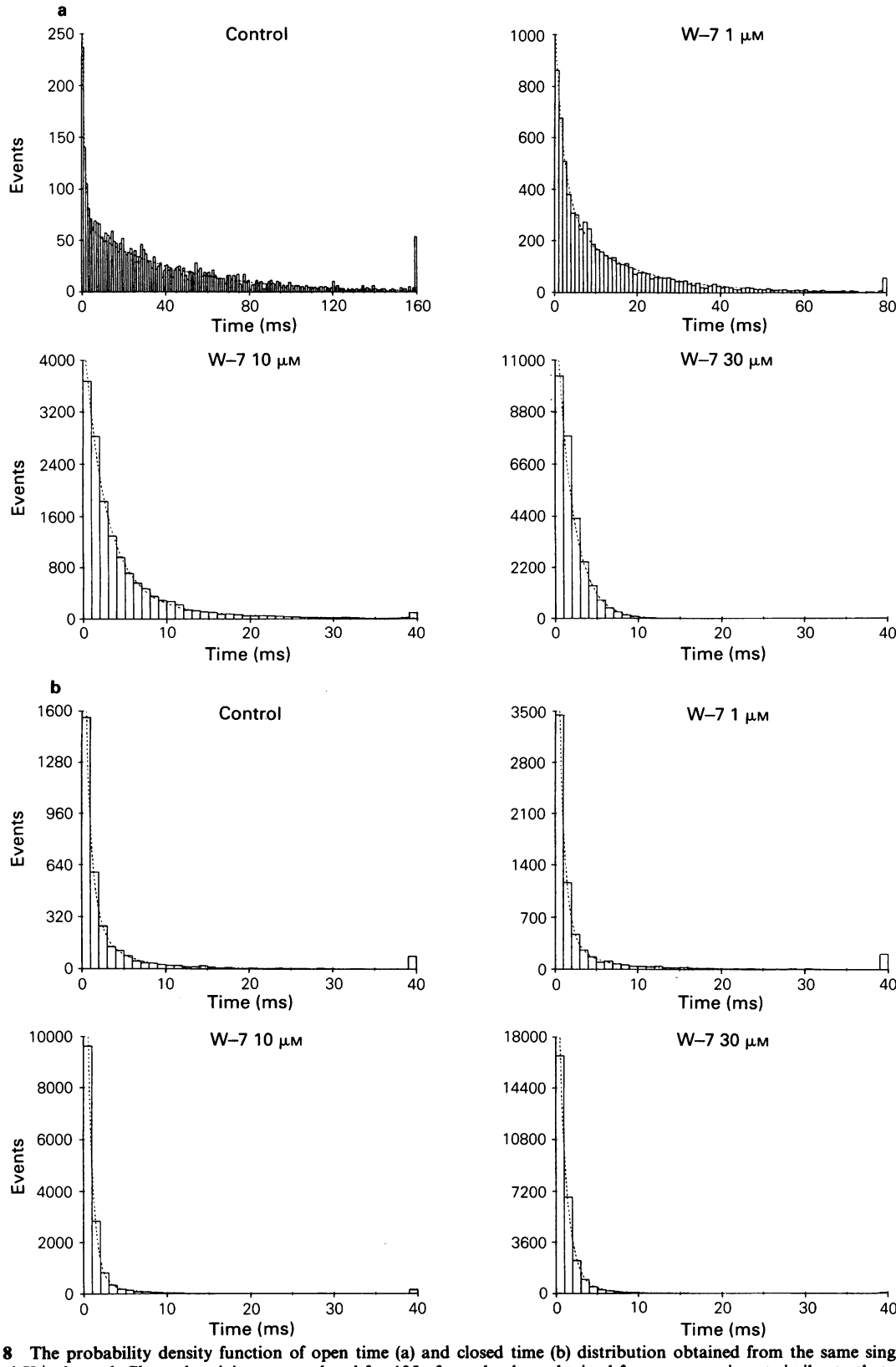

Figure 8 The probability density function of open time (a) and closed time (b) distribution obtained from the same single Ca^{2+}-activated K^+ channel. Channel activity was analysed for 135 s from the data obtained from an experiment similar to that shown in Figure 4. W-7 concentration was increased from 1 to 20 μM, each for 5 min. The effects developed fully in 1–2 min.

however, it is not certain whether W-7 is inhibiting Ca^{2+}-activated K^+-channels directly or is blocking the Ca^{2+} influx responsible for activation of the K^+-channels. It is known that calmodulin antagonists, such as trifluoperazine and W-7, reduce voltage-dependent Ca^{2+} influx (Greenberg *et al.*, 1987; Klöckner & Isenberg, 1987). In smooth muscle cells dispersed from cow portal vein, depolarization-induced outward currents are inhibited by trifluoperazine at concentrations above 1 μM, probably through blocking Ca^{2+} influx, but at a higher concentration (20 μM) the outward currents induced as a result of intracellular Ca^{2+} release by agonists (acetylcholine, prostaglandin, or angiotensin) are also reduced (Klöckner & Isenberg, 1987). Since no recovery from inhibition with trifluoperazine could be observed after dialysis with 100 μM

calmodulin, they concluded that calmodulin is not involved in the activation of K^+ channels by Ca^{2+}.

In the rat myometrium, the single channel activity of Ca^{2+}-activated K^+-channels observed in the presence of 1 μM $[Ca^{2+}]_i$ with the patch-clamp method, is also inhibited by W-7 or trifluoperazine. At a concentration of 30 μM, P_o is reduced by about 40%. However, the effect of calmodulin antagonists is different from that of lowering $[Ca^{2+}]_i$. When P_o is decreased by reducing $[Ca^{2+}]_i$ the main alteration of channel kinetics is a lengthening of the time constant of the slow component of closed time distribution, without a significant change in open kinetics. However, the decrease of P_o with W-7 or trifluoperazine is accompanied by a shortening of the time constant of the slow component of open time distributions, reflecting flickering activity of the channel. Therefore, calmodulin antagonists do not seem to be simply inhibiting the activation process of the channel with Ca^{2+}.

When P_o is low in the presence of 0.1–0.16 μM $[Ca^{2+}]_i$, channel activity is clearly increased by W-7 or trifluoperazine. Furthermore, even when no channel activity is observed in the absence of Ca^{2+}, some channel activity is started by 30 μM W-7 and the $[Ca^{2+}]_i$ for 50% activation is not shifted much by calmodulin antagonists. These results strongly suggest that W-7 and trifluoperazine exert a non-specific action on the channel, not accounted for by calmodulin antagonism.

In Ca^{2+}-activated K^+-channels in dog airway muscle cells, the potency of drugs (haloperidol, trifluoperazine, thioridazine, chlorpromazine) in reducing channel activity was found to be different from their potency as calmodulin antagonists, as mentioned in the Introduction (McCann & Welsh, 1987). The concentration of calmodulin antagonists that inhibit 50% of a calmodulin-dependent phosphodiesterase is reported to be 7–10, 26–67, and 240 μM, for trifluoperazine, W-7 and W-5, respectively (Asano & Stull, 1985; Asano *et al.*, 1985). The low potency of W-5 as a calmodulin antagonist can serve as a reasonable control compound for W-7, which has a similar structure, to specify drug effects. In the present experiments, W-5 and W-7 produced very similar effects on the single channel current of Ca^{2+}-activated K^+-channels, at the same concentration. These results suggest that the effects are not related to their calmodulin antagonistic action. They also suggest that calmodulin is not involved in the activation of the K^+-channel by Ca^{2+}, and that Ca^{2+} probably binds directly to the gating site for channel activation.

W-7, W-5, and trifluoperazine all reduced the $[Ca^{2+}]_i$-dependency of the K^+-channel. In the presence of these compounds, some channel activity continued in the absence of Ca^{2+}. A similar finding has been reported for the Ca^{2+}-activated K^+-channel in smooth muscle cells of the human aorta, following treatment with a fatty acid, 2-decanoic acid (Bregestovski *et al.*, 1989). The effect of 2-decanoic acid is interpreted as due to alteration of membrane-associated protein function. Hydrophobic compounds, such as phenothiadine and naphthalenesulphonamide, may exert a similar effect on the membrane lipid, resulting in modification of the channel activity.

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(Received January 3, 1990
Revised February 7, 1990
Accepted February 13, 1990)