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# Spironolactone inhibition of contraction and calcium channels in rat portal vein

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**1** The effects of spironolactone have been studied on the mechanical activity of rat portal vein strips and the calcium channel currents of isolated cells using the patch clamp technique (whole-cell configuration).

**2** Spironolactone (50 nM to 0.1 mM) depressed both $K^+$-induced and twitch contractions within 5–6 min. This inhibitory effect was overcome by elevating the calcium concentration in the perfusing solution.

**3** Spironolactone (60 $\mu$M) depressed the transient contractions induced in a $Ca^{2+}$-free, EGTA-containing solution by either acetylcholine (0.1 mM) or noradrenaline (10 $\mu$M). The effect of spironolactone was dependent on a reduction in the filling of the internal calcium store.

**4** Rapidly inactivating calcium channel current was maintained in the presence of spironolactone (60 $\mu$M), while slowly inactivating calcium channel current was blocked in a concentration-dependent manner. Half-inhibition of slow calcium channel current was obtained at concentrations between 5–7 $\mu$M.

**5** Administration of spironolactone (10 $\mu$M) at rest reduced calcium channel current by about 70% (tonic inhibition). Repetitive depolarizations (300 ms long pulses to zero mV, applied between 0.05 and 0.5 Hz) had no further inhibitory effect on the inward current (absence of use-dependence).

**6** When cells were held at depolarized membrane potentials at which slow calcium current was inactivated by about 80%, the inhibitory effect of spironolactone (10 $\mu$M) was similar to that obtained with cells normally polarized. Spironolactone (10 $\mu$M) had no effect on the voltage-dependence of inactivation of the calcium channel current.

**7** Our results suggest that spironolactone acts primarily on the plasma membrane by depressing inward current through slow calcium channels. This effect may be explained by a preferential binding of the drug to the resting state of the slow calcium channel. In addition, spironolactone may depress contractions dependent on the release of calcium from the sarcoplasmic reticulum.

## Introduction

Spironolactone is a mineralocorticoid that competitively inhibits the effects of aldosterone and other steroids in many tissues (Ramsay, 1982). It also possesses diuretic and antihypertensive properties which can be equivalent to those of the thiazides. The reduction in blood pressure induced by spironolactone is obtained without a significant variation in the plasma concentration of $K^+$ ions. Moreover, direct effects of spironolactone have been demonstrated in mammalian myocardium including a positive inotropic effect and an increase in both the amplitude and duration of the action potential (Coraboeuf & Deroubaix, 1974).

The purpose of the present study was to test whether spironolactone has calcium channel blocking properties in vascular smooth muscle by studying its effects on isometric contractions (in polarized and depolarized preparations), and on the calcium channel currents of isolated vascular smooth muscle cells using the patch-clamp technique. A $Ca^{2+}$-free, EGTA-containing solution was used to determine whether spironolactone may affect intracellular calcium release induced by acetylcholine or noradrenaline.

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## Methods

### *Contractile activity*

Experiments were performed on longitudinal strips (100–200 μm wide, 2–3 mm long) isolated from rat portal veins. After a stabilizing period (20 min) in the reference solution, the preparation was ready for experimental recordings. Isometric contractions were recorded in an experimental chamber which has been described previously (Mironneau *et al.*, 1984). Concentrations producing 50% inhibition of contraction ($IC_{50}$) were estimated from the concentration-response curves. Relative potency of spironolactone on polarized and depolarized preparations was calculated as the ratio of $IC_{50}$ against spontaneous contractions and against the plateau of $K^+$-induced contractions.

### *Electrical activity*

The cells were isolated from rat portal vein as described recently (Loirand *et al.*, 1986). They were maintained in primary culture and used during the first 48 h. The electrode, electronics and data recording have also been described in detail recently (Loirand *et al.*, 1986). The cells were investigated with patch electrodes in the whole-cell clamp mode (Hamill *et al.*, 1981). The patch pipettes were filled with a solution containing (mM): CsCl 130, K-pyruvate 7.5, K-succinate 7.5, HEPES 10 (pH 7.3 with KOH), EGTA 1. The CsCl equilibrated within 1 min with the internal fluid, thereby blocking the outward potassium currents. Thus, the net inward current was considered to represent the calcium channel current.

### *Solutions and drugs*

Physiological solutions had the following composition: (a) reference solution (mM): NaCl 130, KCl 5.6, $CaCl_2$ 2.1, $MgCl_2$ 0.24 and glucose 11. The solution was buffered by either Tris-HCl or HEPES-NaOH (8.3 mM) to pH 7.4. (b) Barium solutions were obtained by substituting $CaCl_2$ for $BaCl_2$ in various amounts. (c) In Ca-free solution, $CaCl_2$ was omitted and EGTA was added at 0.5 mM. (d) High-potassium solutions were obtained by substituting NaCl with KCl in equimolar amounts. (e) Cobalt chloride and nifedipine (Bayer) were used as inhibitors of the calcium inward current (Fleckenstein, 1983). All solutions were maintained at 35 ± 1°C.

Acetylcholine and noradrenaline were obtained from Sigma. Spironolactone was a gift from Searle (Paris). Spironolactone and nifedipine were prepared as a $10^{-1}$ M stock solution in dimethylsulphoxide (DMSO, Sigma) and diluted to the final concentra-

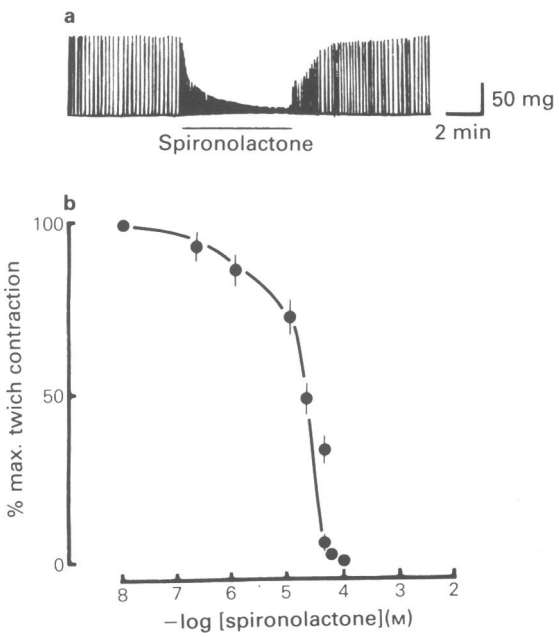


**Figure 1** Effect of spironolactone on isometric twitch contractions in a polarized rat portal vein strip bathed in the reference salt solution. (a) Spironolactone (50 μM) reversibly inhibited the contraction. (b) Concentration-response curve for the inhibitory action of spironolactone. Ordinate scale: % of maximal control contraction. Abscissa scale: −log of drug concentration (M). Each point represents the mean of 5 preparations; vertical lines show s.d.

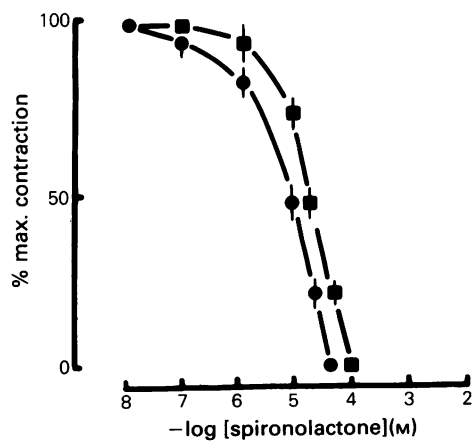


**Figure 2** Concentration-response curves for the effects of spironolactone on sustained (●) and phasic (■) contractions induced by a 60 mM $K^+$ solution. Ordinate scale: % of maximal control contraction. Abscissa scale: −log of drug concentration (M). Each point represents the mean of 4 preparations; vertical lines indicate s.d.

tion. The added amount of DMSO to the final solution had no effect on either mechanical or electrical activity.

*Statistical analysis*

The experimental results are expressed as mean ± s.d. and significance was tested by means of Student's *t* test.

## Results

*Effects of spironolactone on isometric contractions in rat portal vein*

Figure 1a shows the inhibitory effect of spironolactone (50 μM) on spontaneous contractions. In all experiments, the measurements were only made when the preparations reached a steady-state, i.e., within 5–6 min. The effect of spironolactone was completely reversed within 4–5 min in reference solution. The concentration-response curve for the inhibitory effect of spironolactone was obtained in a non-cumulative manner on 5 different preparations. The amplitude of the contraction (as %) was plotted as a function of the external drug concentration (from 50 nM to 0.1 mM) as shown in Figure 1b. Half-maximal and complete inhibition were obtained at concentrations of 20 μM and 60 μM, respectively. The inhibitory effect of spironolactone (20 μM) on spontaneous contractions was completely reversed by an increase in external calcium concentration from 2.1 to 4.2 mM.

Application of a 60 mM $K^+$ solution induced a phasic peak contraction followed by a sustained plateau the amplitude of which was dependent on the external calcium concentration (Gabella, 1968; Nanjo, 1984). Spironolactone was then added for its equilibration time either before the phasic potassium contraction or during the plateau of the potassium contraction. Figure 2 shows the depressant action of spironolactone on both potassium contractions as a function of the drug concentration. Against the plateau of the $K^+$-contraction, half-maximal and complete inhibition were obtained at 10 and 50 μM, respectively. Against the phasic peak potassium contraction, half-maximal and complete inhibition were obtained at 30 and 100 μM, respectively. From 4 different preparations, the relative potency of spironolactone calculated from the $IC_{50}$ against spontaneous contractions and against the plateau of $K^+$-induced contractions was equal to 2, indicating that spironolactone did not induce a voltage-dependent inhibitory effect ($P > 0.05$).

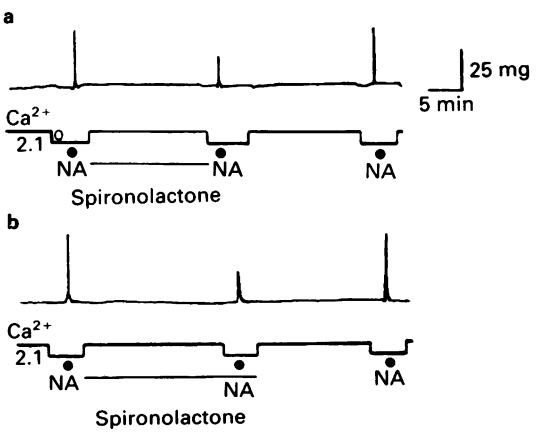


**Figure 3** Effect of spironolactone (60 μM) on transient contractions of rat portal vein induced by noradrenaline (NA, 10 μM) in a $Ca^{2+}$-free solution. (a) When spironolactone was added only during calcium loading ($Ca^{2+}$ 2.1 mM), the contraction was reduced to 51 ± 8% of control ($n = 4$). (b) When added during calcium loading and during $Ca^{2+}$-free perfusion, spironolactone reduced the transient contraction to 46 ± 7% of control ($n = 4$). NA was applied after 2 min in the $Ca^{2+}$-free solution.

*Effects of spironolactone on contractions induced in $Ca^{2+}$-free solution*

In $Ca^{2+}$-free, 0.5 mM EGTA-containing solution, the 60 mM $K^+$-induced contraction ceased within several seconds. In contrast, noradrenaline- and acetylcholine-induced contractions decreased in amplitude but persisted longer (Nanjo, 1984; Dacquet *et al.*, 1987). In the following experiments, the filling of the internal calcium store was carried out on quiescent preparations (in the presence of 50 nM nifedipine) for 10 min in 2.1 mM $Ca^{2+}$-containing solutions. When spironolactone (60 μM) was added only during calcium loading (Figure 3a), the noradrenaline-induced contraction obtained after 2 min in $Ca^{2+}$-free solution was decreased to 51 ± 8% of control ($n = 4$, $P > 0.01$). When spironolactone was applied during calcium loading and during perfusion in $Ca^{2+}$-free solution (Figure 3b), the amplitude of the transient noradrenaline-induced contraction obtained after 2 min in $Ca^{2+}$-free solution was reduced to 46 ± 7% of control ($n = 4$, $P < 0.01$). The spironolactone-induced inhibitory effects were not significantly different ($P > 0.05$). Similar experiments were made on contractions induced by acetylcholine (0.1 mM) in a $Ca^{2+}$-free solution. When spironolactone was added only during calcium loading or during calcium loading and perfusion in $Ca^{2+}$-free solution, the reductions of acetylcholine-induced contractions were not significantly different ($P > 0.05$, $n = 5$).

*Effects of spironolactone on calcium channel currents*

In rat portal vein, two distinct types of calcium channel current have been separated kinetically and pharmacologically. A rapidly inactivating current is present when cells are held at very negative potentials (−70 mV). This current is prominent for relatively small depolarizations and is insensitive to nifedipine (0.5 μM). A slowly inactivating current (slow current) is observed by stepping at a less negative holding potential (−40 mV) and it is blocked by 50 nM nifedipine (Loirand *et al.*, 1986). Both currents are unaffected by tetrodotoxin (10 μM) and both are blocked by 2.5 mM $Co^{2+}$ ions. Figure 4a shows the calcium channel current in response to a depolarization clamp pulse from a holding potential of −70 mV in a 5 mM $Ba^{2+}$-containing solution. The depolarization elicited a barium inward current which reached a peak of 950 pA within 5–7 ms. From the peak, the inward current decayed with time but was still inward at the end of a 300 ms depolarization. Application of 60 μM spironolactone reduced the peak calcium channel current to about 42 ± 6% of control ($n = 5$, $P < 0.01$) within 10 min. This reduction was associated with complete suppression of the late inward current. In the presence of spironolactone, inward current inactivated faster and the half-decay time was shortened from 100 to 45 ms. Higher concentrations of spironolactone (100 μM) had no further inhibitory action on inward calcium channel current ($n = 4$). The inhibitory effect of spironolactone (at concentrations lower than 60 μM) was completely reversible within 15 min. The influence of membrane potential pulses is illustrated by the peak current-voltage relationships obtained from a holding potential of −70 mV. By subtracting the currents obtained before and after exposure to 2.5 mM $Co^{2+}$ ions, we obtained the corrected peak inward current-voltage relationships (Figure 4b). Under control conditions, inward current started at a threshold potential of −40 mV and reached a peak near zero mV. At positive potentials, the current decreased and reversed at about +70 mV. In the presence of 60 μM spironolactone, the inward current was reduced over the whole range of clamp potentials without modification of the reversal potential. In order to verify whether the spironolactone-resistant current corresponded to the dihydropiridine-resistant current, spironolactone was added after complete inhibition of the slow inward current in the presence of 0.5 μM nifedipine (Figure 5). The fast inward current obtained by stepping from −70 mV was not significantly changed by spironolactone (3 ± 2%, $n = 4$, $P > 0.05$), suggesting that the drug exerted its main inhibitory effect on the slow inward current.

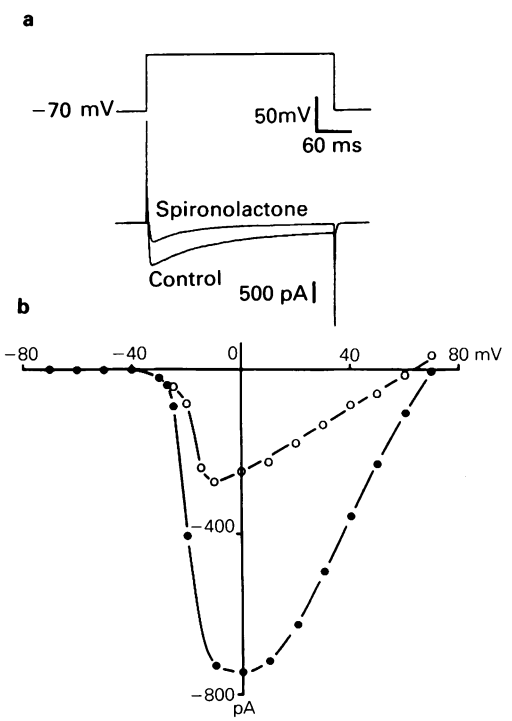


**Figure 4** Effect of spironolactone on the inward current in a single cell elicited from a holding potential of −70 mV to +10 mV in a solution containing 5 mM $Ba^{2+}$. (a) A rapidly inactivating inward current persists in the presence of a maximal concentration of spironolactone (60 μM). (b) Current-voltage relationships of the peak current obtained in another cell in the absence (●) and presence (○) of spironolactone (60 μM). Calcium channel current was defined as the inward current sensitive to 2.5 mM $Co^{2+}$.

The effects of different concentrations of spironolactone on the slow inward current obtained from a holding potential of −40 mV are illustrated in Figure 6. The maximal inward current was reduced by 60 ± 9% ($n = 6$, $P < 0.01$) at a concentration of 10 μM while 60 μM spironolactone completely abolished the inward current ($n = 4$). As shown by the current-voltage relationships (Figure 6c), the control and spironolactone peak current-voltage relationships converge to a similar potential indicating little or no change in the apparent reversal potential for the slow calcium channel current. Half-inhibition of inward current was obtained with concentrations of 5–7 μM in 6 different cells.

*Tonic and use-dependence of the effect of spironolactone on slow calcium channels*

As spironolactone exerted its main inhibitory effect on the slow calcium channel current, we used two

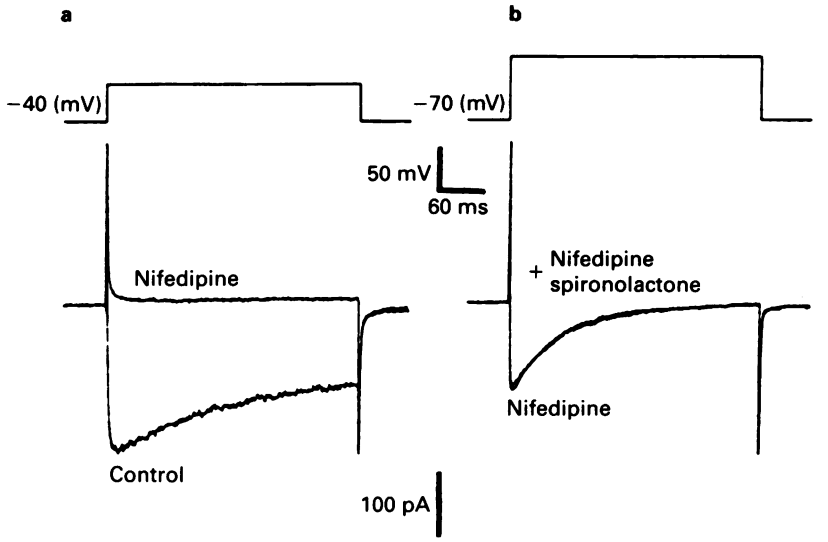


**Figure 5** Effect of spironolactone on the fast inward current of a single cell in a 5 mM $Ba^{2+}$-containing solution. (a) The slow inward current elicited from a holding potential of −40 mV to 0 mV is inhibited by the addition of 0.5 μM nifedipine. (b) After returning to a holding potential of −70 mV, the fast inward current in response to a depolarization to 0 mV is not significantly affected by 10 μM spironolactone.

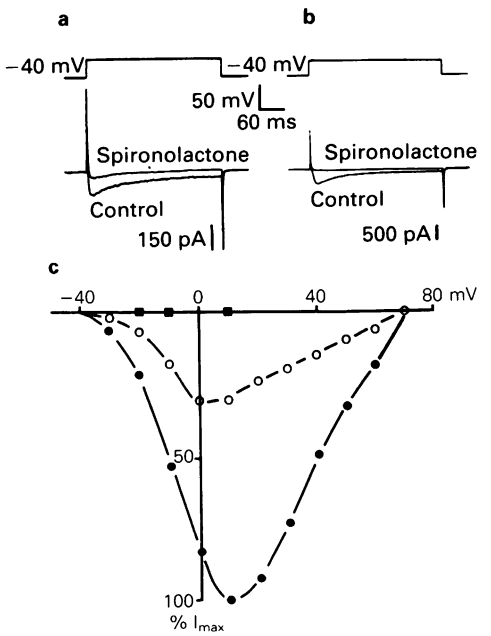


**Figure 6** Effect of spironolactone on the slow inward current of single cells elicited from a holding potential of −40 mV to 0 mV (a) and −10 mV (b) in a 5 mM $Ba^{2+}$-containing solution. Partial (a) and complete (b) inhibition of the inward current was produced by 10 and 60 μM spironolactone, respectively, in two different cells. (c) Current-voltage relationships of the peak slow inward current in control (●) and in presence of 10 μM (○) and 60 μM (■) spironolactone.

protocols to assess the relative contribution of tonic and use-dependent blockade of calcium channels. When voltage-clamp pulses were applied from a holding potential of −40 mV at a frequency of 0.05 Hz under control conditions, the decrease of maximal slow inward current within 15 min was not significant ($7 \pm 3\%$, $n = 25$, $P > 0.05$). With the first protocol, spironolactone (10 μM) was added to a cell which was stimulated at a frequency of 0.05 Hz as in control (Figure 7a). There was a progressive decline of inward current during subsequent voltage depolarizations. A steady state inhibition of $67 \pm 8\%$ ($n = 6$, $P < 0.001$) was obtained for the 18th depolarization, after 6 min of drug exposure. With the second protocol, spironolactone (10 μM) was added during a rest period of 6 min and blockade was assessed as the difference between peak inward current in the control and the first pulse after drug exposure (Figure 7b). The inward current was inhibited by $63 \pm 7\%$ ($n = 4$, $P < 0.01$) indicating that the blockade was not dependent on the number of voltage depolarizations applied at 0.05 Hz. As hyperpolarizing the membrane to −80 mV for 30 s did not restore the initial inward current, we can assume that the blockade was largely tonic.

In order to confirm that the effect of spironolactone was not use-dependent at any stimulation frequencies, the rate of pulsing was increased to 0.2 and 0.5 Hz. At these frequencies, the slow inward current became smaller even in the absence of spironolactone (Figure 8). Comparing the control slow inward current and the current obtained after 2 min during a train of repetitive pulses (−40 to 0 mV), the reduction was $40 \pm 3\%$ ($n = 3$, $P < 0.01$) at 0.2 Hz, and $58 \pm 7\%$ ($n = 4$, $P < 0.01$) at 0.5 Hz. When the stimulation was interrupted for 2–3 min, the amplitude of the inward current was completely restored. When spironolactone (10 μM) was applied under resting conditions for 3 min, the first pulse evoked a slow inward current which was significantly reduced ($45 \pm 7\%$, $n = 5$, $P < 0.01$). During stimulation at frequencies of 0.2 and 0.5 Hz, the slow inward current decreased in a beat-to-beat fashion and a steady-state was usually reached within 2 min. We compared the amplitude of the currents obtained after 2 min of stimulation in the presence and absence of spironolactone. The spironolactone-induced reductions of peak inward current at 0.2 and 0.5 Hz stimulation were $57 \pm 10\%$ ($n = 5$, $P < 0.01$) and $59 \pm 8\%$ ($n = 4$, $P < 0.01$), respectively. After spironolactone had been acting for 5 min, the differences in inhibition of the slow inward current in the absence and presence of a train of repetitive pulses for 2 min were not significant ($P > 0.05$). Thus, we can assume that the antagonistic effect of spironolactone is not changed after the depolarizing pulses have opened the slow calcium channels at frequencies between 0.05 and 0.5 Hz.

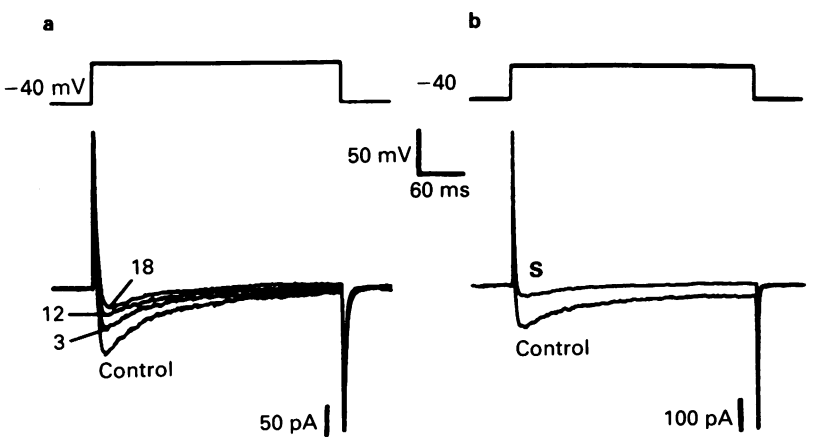


**Figure 7** Tonic and frequency-dependent inhibition of the slow inward current of single cells in 5 mM $Ba^{2+}$-containing solution by spironolactone (10 μM) at a stimulation frequency of 0.05 Hz. (a) Membrane currents elicited by repetitive voltage-clamp pulses (0.05 Hz) from a holding potential of −40 mV to 0 mV. The largest inward current was recorded under control conditions before exposure to the drug; subsequent currents were recorded during a train of voltage clamp pulses applied after the addition of spironolactone. The numbers correspond to the inward current elicited by the 3rd (1 min), 12th (4 min), 18th (6 min) pulses. (b). Membrane currents elicited under control conditions and after 6 min of perfusion in the presence of spironolactone (S) without application of any depolarizing pulse. In both cases, the steady-state inhibition of the inward current was similar.

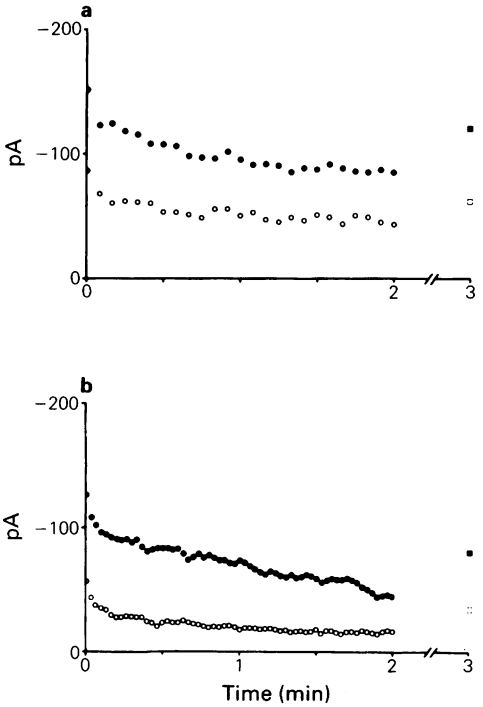


**Figure 8** Effect of frequency of the command pulse on the slow inward current of single cells recorded from a holding potential of −40 mV to 0 mV in a 5 mM $Ba^{2+}$-containing solution. Amplitudes of the inward current evoked by repetitive application of the command pulse in the absence (●) and presence (○) of spironolactone (10 μM). Spironolactone was added for 3 min before application of a train of repetitive pulses at 0.2 Hz (a) and 0.5 Hz (b). (■) and (□) correspond to a partial restoration of the inward current obtained after a resting period of 1 min in control and spironolactone-containing solutions, respectively. Steady-state blockade was evident when comparing the control current and the current obtained in the presence of spironolactone after 5 min. In (a), the slow inward current was inhibited by 43% after addition of spironolactone for 3 min without stimulation and by 50% after application of a train of repetitive pulses for 2 min at 0.2 Hz. In (b), it was inhibited by 56% after addition of spironolactone for 3 min without stimulation and 63% after application of a train of repetitive pulses for 2 min at 0.5 Hz. The command pulses were 300 ms in duration and 40 mV in amplitude.

*Voltage-dependence of the effect of spironolactone on slow calcium channels*

The next possibility we tested was that changes in membrane potential, even in the absence of repetitive stimulation, can alter the effectiveness of inward current blockade by spironolactone. In this test, calcium channel current was recorded during depolarizing voltage pulses applied from a holding potential of −40 mV or −20 mV (Figure 9). In the absence of the drug, a change in the holding potential from −40 to −20 mV resulted in a decrease in inward current (80 ± 5%, $n = 9$, $P < 0.001$) because of inactivation of the calcium channels. Then, spironolactone (10 μM) was added, and after a steady-state was reached in this solution (6 min), inward current was again recorded during a similar depolarization from the two holding potentials. Inward currents recorded during the pulses applied from both holding potentials in the presence of spironolactone were decreased by the same extent (61 ± 7%, $n = 5$, $P < 0.01$). These results clearly show that blockade of the calcium channel current by spironolactone is not affected by changes in membrane potential in the absence of repetitive stimulation.

The influence of spironolactone on the voltage-dependent inactivation of calcium channel current was also examined using a two-pulse protocol (Figure 10, inset). A test pulse to +10 mV ($V_2$) from a holding potential of −40 mV was preceded by a prepulse ($V_1$) of 300 ms duration and of variable amplitude. The two pulses were separated by a rest interval (Δ t) of 30 ms. Peak test pulse current, normalized to the peak test pulse current obtained in the absence of a prepulse ($I/I_{max}$), was plotted against the prepulse voltage and represented the calcium channel inactivation curve. No significant difference in calcium channel inactivation was consistently observed in the presence or absence of spironolactone (Figure 10). The absence of a voltage-dependent blockade of calcium channel inactivation by spironolactone was observed whatever the duration of the prepulse (from 0.3 to 5 s) in 4 other cells.

## Discussion

It is generally believed that a rise in intracellular calcium concentration is responsible for the activation of contraction in smooth muscles (Endo *et al.*, 1977; Adelstein & Hathaway, 1979). From results of voltage-clamp and contraction experiments in portal vein smooth muscle, it has been suggested that there are two sources of intracellular ionized calcium. One is the influx of $Ca^{2+}$ ions from outside through two distinct types of voltage-dependent calcium channels (Loirand *et al.*, 1986); the other is the release of calcium from intracellular storage sites (Nanjo, 1984; Dacquet *et al.*, 1987). There is little doubt that the calcium inward currents are involved in triggering a contraction (Mironneau & Gargouil, 1979; G. Loirand, P. Pacaud, C. Mironneau & J. Mironneau, unpublished observations). However, the amount of carried charges may still be at least an order of magnitude below the total amount of $Ca^{2+}$ ions required to activate a maximal

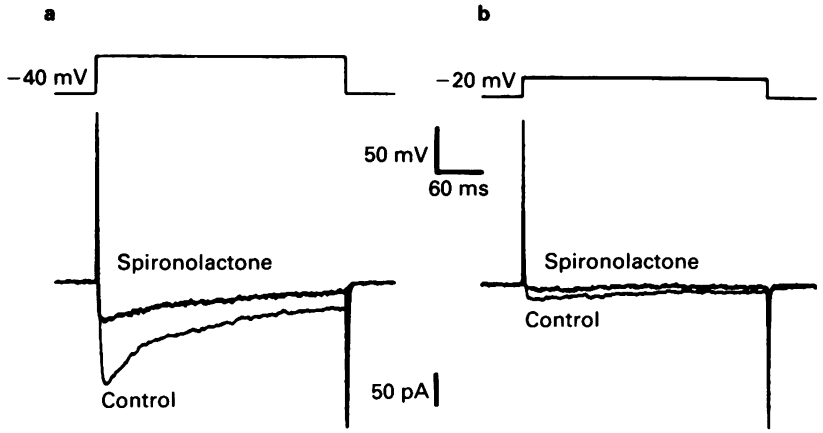


**Figure 9** Potential-dependent inhibition of the slow inward current of a single cell in a 5 mM $Ba^{2+}$-containing solution by spironolactone (10 μM). (a) Inward currents recorded from a holding potential of −40 mV to 0 mV in the absence and presence of spironolactone for 6 min. (b) Inward current recorded from a holding potential of −20 mV to 0 mV in the absence and presence of spironolactone for 6 min. In both cases, the inhibition induced by spironolactone was similar.

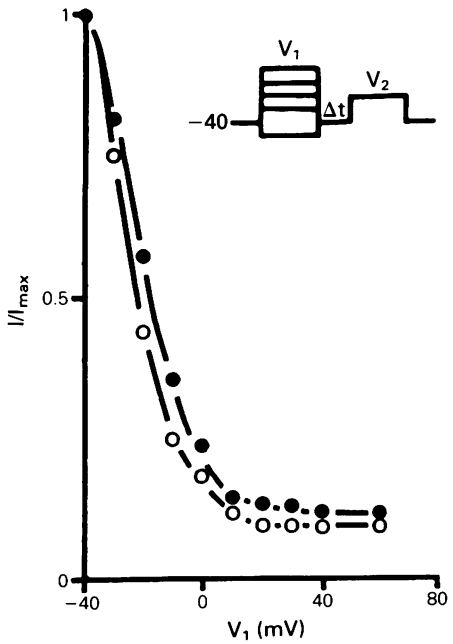


**Figure 10** Effect of spironolactone (10 μM) on the inactivation curve of the slow inward current of a single cell in a 5 mM $Ba^{2+}$-containing solution obtained with the two-pulse protocol (inset). Peak test current normalized by its value in the absence of a conditioning pulse ($I/I_{max}$) was plotted against the conditioning pulse amplitude under control conditions (●) and after addition of spironolactone (○) for 6 min. The voltage pulses were 300 ms in duration (conditioning and test pulse) and 50 mV in amplitude (test pulse) and applied from a holding potential of −40 mV.

contraction (Bolton, 1979), suggesting that the release of $Ca^{2+}$ ions from the sarcoplasmic reticulum is involved in the development of contraction. Phasic contraction induced by a 60 mM $K^+$ solution may depend on activation of both fast and slow calcium channels, while maintained contraction may depend, in part, on a calcium influx through slow calcium channels which remain largely available at membrane potentials near −20 mV (G. Loirand, P. Pacaud, C. Mironneau & J. Mironneau, unpublished observations).

The present results indicate that spironolactone, at doses between 50 nM and 0.1 mM, causes an inhibition of external calcium-dependent contractions in both polarized and depolarized portal vein preparations. The inhibitory action of spironolactone, expressed by its relative potency against spontaneous contractions and against the plateau of the $K^+$-induced contractions, was shown to be not significantly affected by the level of the membrane potential, which is in contrast to that of dihydropyridines in portal vein smooth muscle (Dacquet *et al.*, 1987). A reduced phasic $K^+$ contraction persisted in the presence of 60 μM spironolactone. Moreover, our results in single portal vein cells, also show that spironolactone, at this concentration, has little if any inhibitory effect on the rapidly inactivating calcium channel current which is insensitive to dihydropyridines (Loirand *et al.*, 1986). The parallels between contraction and electrophysiological results led us to propose that the spironolactone-resistant transient contraction may result from activation of the fast calcium channels. However, at higher concentrations of spironolactone there is a clear discrepancy

between inhibition of phasic $K^+$ contraction and persistence of fast inward current. This may be explained by taking into account the fact that spironolactone also inhibits the filling of the internal $Ca^{2+}$ store when the drug is added for 10 min before application of a high $K^+$-solution. Under these conditions, no detectable contraction is obtained, although the fast inward current is maintained. Spironolactone inhibits in a dose-dependent manner the slowly inactivating calcium channel current recorded from a holding potential of $-40$ mV. The slow inward current is reduced by spironolactone without any modification of the apparent reversal potential, suggesting that spironolactone decreases the slow calcium conductance of the vascular membrane. As the inward current through slow calcium channels is involved, in part, in spike firing and activation of the contraction, our data clearly illustate this phenomenon.

Recent studies have provided new important information regarding the mechanism of calcium channel blockade by organic calcium channel antagonists (Lee & Tsien, 1983; McDonald *et al.*, 1984; Sanguinetti & Kass, 1984; Bean, 1984; Uehara & Hume, 1985; Klöckner & Isenberg, 1986). These experiments support the 'modulated receptor hypothesis' suggesting that the inhibition of calcium channels can be described as initial, tonic and frequency-dependent mechanisms. The inhibitory effect of spironolactone was not enhanced when the cell was stimulated by repetitive depolarizing clamp pulses (0.05–0.5 Hz), indicating that the functioning of calcium channels does not play a key role in the inhibitory effect of spironolactone (absence of use-dependence). The initial block which occurs when resting tissue equilibrates with the antagonist can be reversed by hyperpolarizing the membrane to negative potentials ($-80$ mV) for 30 s, while the tonic block cannot be removed by a maximally effective unblocking potential. As the inhibitory action of spironolactone is not suppressed by long hyperpolarizations to $-80$ mV, this leaves the tonic block as the main mechanism for the spironolactone effect. Our results can be interpreted according to the 'modulated receptor hypothesis' which postulates that the binding of the antagonistic drug is dependent on the channel state (resting, open or inactivated state). When calcium channels are opened by a conditioning depolarizing pulse sequence, the binding of spironolactone is not favoured since the inhibitory effect of spironolactone was similar to that obtained in the absence of stimulation. On the other hand, increasing the number of inactivated calcium channels at depolarized holding potentials had no further effect on the spironolactone-induced blockade as assessed by the non preferential binding of the drug to the inactivated calcium channel state. The absence of effect of spironolactone on the voltage-dependent gating process is indicated by the current-voltage relationships and the inactivation curve of the slow inward current. Thus, the blocking effect of spironolactone can be explained by assuming that spironolactone binds preferentially to the resting calcium channels and, in this way, may remove calcium channels from the pool of channels available for opening.

The results of the experiments in the $Ca^{2+}$-free, EGTA-containing solution are consistent with the proposal that $Ca^{2+}$ ions may be released from an intracellular store in response to neurotransmitters or by depolarization of the membrane (Mironneau & Gargouil, 1979; Nanjo, 1984; Bond *et al.*, 1984; Dacquet *et al.*, 1987). Presumably, the addition of acetylcholine or noradrenaline releases mainly the calcium distributed both within the peripheral sarcoplasmic reticulum and on the internal surface of the plasma membrane, through activation of membrane receptors. Spironolactone depressed the transient contraction induced by both acetylcholine and noradrenaline in a $Ca^{2+}$-free solution. Spironolactone reduced the direct filling of the intracellular calcium store during calcium loading of the preparation but did not affect significantly the release of calcium from the internal store. Therefore, spironolactone resembles nicardipine and diltiazem which exert a significant effect on the filling of the intracellular internal calcium store (Dacquet *et al.*, 1987); it differs from drugs which reduce the intracellular release of calcium without affecting the direct filling of the internal store like indapamide (Mironneau *et al.*, 1986) or pinaverium bromide (Mironneau *et al.*, 1984).

In conclusion, our results demonstrate that spironolactone, at concentrations that have to be considered as pharmacological, acts on the plasma membrane of vascular smooth muscle cells by inhibiting essentially the slow calcium channels. The results also suggest that spironolactone, at maximal doses, may have an additional calcium antagonistic action, reducing contractions dependent on intracellular calcium release.

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## References


ADELSTEIN, R.S. & HATHAWAY, D.R. (1979). Role of calcium and cyclic adenosine 3': 5' monophosphate in regulating smooth muscle contraction. Mechanism of excitation-contraction coupling. *Am. J. Cardiol.*, **44,** 395–405.

BEAN, B.P. (1984). Nitrendipine block of cardiac calcium channels: high-affinity binding to the inactivated state. *Proc. natn. Acad. Sci. U.S.A*, **81,** 6388–6392.

BOLTON, T.B. (1979). Mechanism of action of transmitters and other substances on smooth muscle. *Physiol. Rev.*, **59,** 606–718.

BOND, M., KITAZAWA, T., SOMLYO, A.P. & SOMLYO, A.V. (1984). Release and recycling of calcium by the sarcoplasmic reticulum in guinea-pig portal vein. *J. Physiol.*, **355,** 677–695.

CORABOEUF, E. & DEROUBAIX, E. (1974). Effect of a spironolactone derivative, sodium canrenoate, on mechanical and electrical activities of isolated rat myocardium. *J. Pharmac. exp. Ther.*, **191,** 128–138.

DACQUET, C., MIRONNEAU, C. & MIRONNEAU, J. (1987). Effects of calcium entry blockers on calcium-dependent contractions of rat portal vein. *Br. J. Pharmac.*, **92,** 203–212.

ENDO, K., KITAZAWA, T., YAGI, S., IINO, M. & KAKUTA, Y. (1977). Some properties of chemical skinned smooth muscle fibers. In *Excitation-Contraction Coupling in Smooth Muscle*. ed. Casteels, R., Godfraind, T. & Rüegg, J.C pp. 199–209. Amsterdam: Elsevier/North-Holland.

FLECKENSTEIN, A. (1983). Calcium Antagonism in Heart and Smooth Muscle. Experimental Facts and Therapeutic Prospects. pp. 1–339. New York: John Wiley.

GABELLA, G. (1978). Effect of potassium on the mechanical activity of taenia coli, uterus and portal vein of the guinea-pig. *Q. J. exp. Physiol.*, **63,** 125–146.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, E. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membranes patches. *Pflügers Arch.*, **391,** 85–100.

KLÖCKNER, U. & ISENBERG, G. (1986). Tiapamil reduces the calcium inward current of isolated smooth muscle cells. Dependence on holding potential and pulse frequency. *Eur. J. Pharmac.*, **127,** 165–171.

LEE, K.S. & TSIEN, R.W. (1983). Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature*, **302,** 790–794.

LOIRAND, G., PACAUD, P., MIRONNEAU, C. & MIRONNEAU, J. (1986). Evidence for two distinct calcium channels in rat vascular smooth muscle cells in short-term primary culture. *Pflügers Arch.*, **407,** 566–568.

McDONALD, T.F., PELZER, D. & TRAUTWEIN, W. (1984). Cat ventricular muscle treated with D600: characteristics of calcium channel block and unblock. *J. Physiol.*, **352,** 217–241.

MIRONNEAU, J. & GARGOUIL, Y.M. (1979). Action of indapamide on excitation-contraction coupling in vascular smooth muscle. *Eur. J. Pharmac.*, **57,** 57–67.

MIRONNEAU, J., LALANNE, C., MIRONNEAU, C., SAVINEAU, J.P. & LAVIE, J.L. (1984). Comparison of pinaverium bromide, manganese chloride and D600 effects on electrical and mechanical activities in rat uterine smooth muscle. *Eur. J. Pharmac.*, **98,** 99–107.

MIRONNEAU, J., SAVINEAU, J.P. & MIRONNEAU, C. (1986). Effect of indapamide on the electromechanical properties of rat myometrium and rat portal vein. *J. Pharmac. exp. Ther.*, **236,** 519–525.

NANJO, T. (1984). Effects of noradrenaline and acetylcholine on electro-mechanical properties of the guinea-pig portal vein. *Br. J. Pharmac.*, **81,** 427–440.

RAMSAY, L.E. (1982). Clinical pharmacology and therapeutic use of aldosterone antagonists. In *Hormone Antagonism*. ed. Agarwal, M.K. pp. 335–363. Berlin: W. de Gruyter & Co.

SANGUINETTI, M.C. & KASS, R.S. (1984). Voltage-dependent block of calcium channel current in the calf cardiac Purkinje fiber by dihydropyridine calcium channel antagonists. *Circulation Res.*, **55,** 336–348.

UEHARA, A. & HUME, J.R. (1985). Interactions of organic calcium channel antagonists with calcium channels in single frog atrial cells. *J. gen. Physiol.*, **85,** 621–647.


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