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# Caffeine-induced inhibition of calcium channel current in cultured smooth muscle cells from pregnant rat myometrium

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**1** The inhibitory effect of caffeine on the calcium channel current was investigated in cultured myometrial cells isolated from pregnant rats.

**2** Caffeine inhibited the calcium channel current elicited from a holding potential of −70 mV in a concentration-dependent manner. The $IC_{50}$ was estimated to be 35 mM.

**3** The caffeine inhibition was not enhanced when calcium channels were opened by a conditioning depolarizing pulse sequence or when the number of inactivated calcium channels was increased at depolarized holding potentials.

**4** Caffeine antagonized the specific binding of (+)-[$^3$H]-isradipine to myometrial membranes. The $IC_{50}$ value found in binding experiments was similar to the $IC_{50}$ value for half-maximal inhibition of calcium channel current. Caffeine decreased the maximal binding capacity of (+)-[$^3$H]-isradipine to myometrial membranes without any significant change in the dissociation constant.

**5** The results indicate that caffeine interacts with a site closely associated with the voltage-dependent calcium channels in myometrial cells and, in turn, inhibits calcium influx.

## Introduction

In skeletal and cardiac muscle, caffeine is known to release calcium ions from the sarcoplasmic reticulum, resulting in a muscle contraction (Endo, 1977; Fabiato & Fabiato, 1977). In vascular smooth muscle, caffeine also releases cellular calcium to induce a transient contraction (Leijten & Van Breemen, 1984; Karaki *et al.*, 1987). In addition to these contractile effects, caffeine has an inhibitory action in various smooth muscles, including myometrium (Ito & Kuriyama, 1971; Ashoori *et al.*, 1985; Ahn *et al.*, 1988; Small *et al.*, 1988). Caffeine also inhibits cyclic adenosine 3′,5′-monophosphate (cyclic AMP) phosphodiesterase leading to accumulation of intracellular cyclic AMP (Ahn *et al.*, 1988; Bray *et al.*, 1989) and decrease of cytoplasmic calcium concentration (Sato *et al.*, 1988). Further, caffeine seems to inhibit stimulated calcium influx (Ahn *et al.*, 1988). However, it is not currently known whether caffeine interacts with the voltage-dependent calcium channels in the plasma membrane of smooth muscles. In the present study we have examined the inhibitory effect of caffeine on calcium channel current in the plasmalemma of isolated smooth muscle cells of rat myometrium. We have compared the ability of caffeine to inhibit calcium channel current with its ability to displace (+)-[$^3$H]-isradipine from its binding sites in the plasmalemma.

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## Methods

### *Electrophysiological experiments*

Primary cultures of single rat myometrial cells (19–20 days of pregnancy) were used for electrophysiological experiments as described previously (Amédée *et al.*, 1987). Calcium channel currents of myometrial cells were recorded by means of the whole-cell patch-clamp technique (Honoré *et al.*, 1989). The cells were bathed in an external solution containing (mM): NaCl 130, CsCl 5.6, $CaCl_2$ 2.5, $MgCl_2$ 0.24, glucose 11, Tris 8.3 (pH 7.4). In most experiments, $CaCl_2$ was substituted by $BaCl_2$ (5 mM). The bath solution was maintained at 30 ± 1°C. The patch pipettes (2–3 MΩ) contained (mM): CsCl 130, Cs-pyruvate 5, Cs-succinate 5, Cs-oxaloacetate 5,

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EGTA 10, HEPES 10 (pH 7.3 with CsOH). Calcium channel currents were digitally corrected for leakage currents by subtraction of scaled current traces obtained when hyperpolarizing or small depolarizing pulses from the holding potential were applied to the cells.

*Preparation of membranes*

Uteri were removed from pregnant rats (19–20 days of pregnancy) and the endometrium was scraped off. Membranes were prepared in the presence of 1 mM iodoacetamide and 0.1 mM phenylmethylsulphonylfluoride to minimize proteolytic degradation (Dacquet et al., 1988). Protein content was determined using gamma-globulin as standard (Bradford, 1976).

*Measurement of (+)-[$^3$H]-isradipine binding to membranes*

Membrane proteins (0.08 to 0.15 mg ml$^{-1}$) were incubated for 45 min at 25°C with various concentrations of (+)-[$^3$H]-isradipine in 1 ml of 20 mM HEPES buffer (pH 7.4) containing 0.1% bovine serum albumin (Dacquet et al., 1989). Non-specific binding was defined as the amount of radioligand bound in the presence of 2 $\mu$M unlabelled nitrendipine and subtracted from the total binding. All binding experiments were performed under a dim light to minimize photolysis of the dihydropyridines.

*Drugs*

Drugs used were: (+)-isradipine (Sandoz, Switzerland), (D)-myoinositol-1,4,5-triphosphate ($InsP_3$, Sigma, U.S.A.), (±)-propranolol (Sigma, U.S.A.), caffeine (Merck, F.R.G.), ryanodine (Calbiochem, U.S.A.) and phentolamine (Ciba-Geigy, France). (+)-[$^3$H]-isradipine (specific activity 84 Ci mmol$^{-1}$) was obtained from Amersham (France).

*Data analysis*

Scatchard plots for binding of (+)-[$^3$H]-isradipine were obtained by a non linear least square ligand programme (Munson & Rodbard, 1980). The experimental results were expressed as mean ± s.e.mean and significance was tested by Student's $t$ test.

## Results

*Effects of caffeine on the calcium channel current*

Figure 1 shows the effects of caffeine (20 mM) on the current-voltage relationships of the inward calcium channel current. The maximal inward current was generated by stepping to +20 mV (in 2.5 mM calcium-containing solution or to +5 mV (in 5 mM Ba-containing solution) from a holding potential of −70 mV. Application of caffeine (20 mM) reduced the amplitude of each current to a similar extent. Two records of the maximal inward currents, before and 5 min after caffeine application, are shown superimposed (Figure 1a,b). At 20 mM caffeine consistently reduced the amplitude of the peak inward Ba current observed at any given potential (−30 to +50 mV) without affecting the apparent reversal potential (Figure 1c). Figure 2a shows the relationship between amplitude of the inward current and concentrations of caffeine. In these experiments, the transmembrane potential stepped to zero mV from a holding potential of −70 mV. Command pulses were delivered at 20 s intervals and were of 300 ms dura-

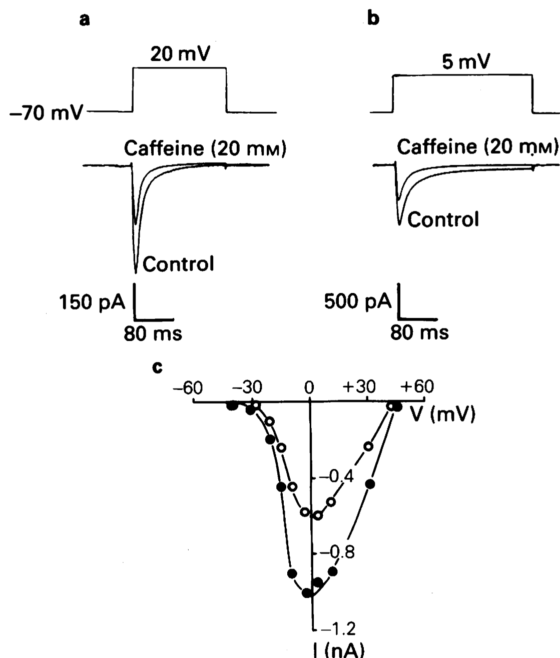


**Figure 1** Pregnant rat myometrium: effects of 20 mM caffeine on the calcium channel current recorded from single cells. Two records of the inward currents before and after application of caffeine are shown superimposed. External solutions contained 2.5 mM $Ca^{2+}$ (a) or 5 mM $Ba^{2+}$ (b). The membrane was held at −70 mV and stepped to +20 mV (a) or +5 mV (b). (c) Current-voltage relationships of the inward $Ba^{2+}$ current in the absence (●) or presence (○) of 20 mM caffeine. The amplitude of the inward current in the presence of caffeine was measured after the inhibition had reached a steady-state by repetitive applications of command pulses (20 s intervals).

tion. The peak amplitude of the inward current in the absence of caffeine was normalized as 1. The concentration-dependency of caffeine action in inhibiting the inward barium current was studied by the construction of a sequential concentration-effect curve. This curve had a sigmoidal shape (Figure 2a). The half-maximal blocking concentration for caffeine was estimated to be 35 mM. The slope of the log concentration-effect curve was not shallow, nor did the central portion of the curve exhibit points of inflection. Accordingly, it may be suggested that caffeine bound to a single binding site.

When dihydropyridine derivatives were tested for their ability to block calcium channels (Sanguinetti & Kass, 1984; Terada *et al.*, 1987; Dacquet *et al.*, 1988; Hering *et al.*, 1988), their effects were observed to depend on depolarized holding potentials rather than on the frequency or number of stimuli used to promote calcium channel opening. Accordingly, in the present study, we examined whether the action of caffeine depended on the number of command pulses applied. Command pulses (150 ms duration; 20 s intervals) were used to step membrane potential from a holding potential of −70 mV to zero mV. When such pulses were applied, amplitudes of the inward current remained constant before application of caffeine. In Figure 2b, the amplitude of inward current recorded by the last pulse before application of caffeine was normalized as 1. Caffeine (20 mM) was then added to the solution and the pattern of command pulses was either maintained (solid circles) or recommenced after a 2.8 min rest period (solid triangles). In the presence of caffeine the first post-rest peak inward current did not differ significantly from the peak inward current at the end of the maintained pulse train. A similar observation was made in 4 other experiments. After removal of caffeine, the inward current progressively recovered and 3 min after drug wash-out, its amplitude regained the control value. Addition of phentolamine (1 $\mu$M) or propranolol (100 $\mu$M) had no effect on the action of caffeine on calcium channel currents.

The effects of caffeine on the voltage-dependency of the calcium channel current were examined by studying both activation and inactivation curves. Inactivation was characterized by use of the classical two-pulse protocol. The amplitude of the current elicited by a test pulse of fixed amplitude (zero mV) and duration (150 ms) was measured when a conditioning pulse was varied. Conditioning pulses of 30 s duration were routinely used to investigate the steady-state inactivation process. The diminution of the test current was taken as an index of inactivation of the calcium channel current. Relative inactivation was expressed by plotting the test current against the level of the conditioning pulse. The amplitude of the test current was normalized by its value in the

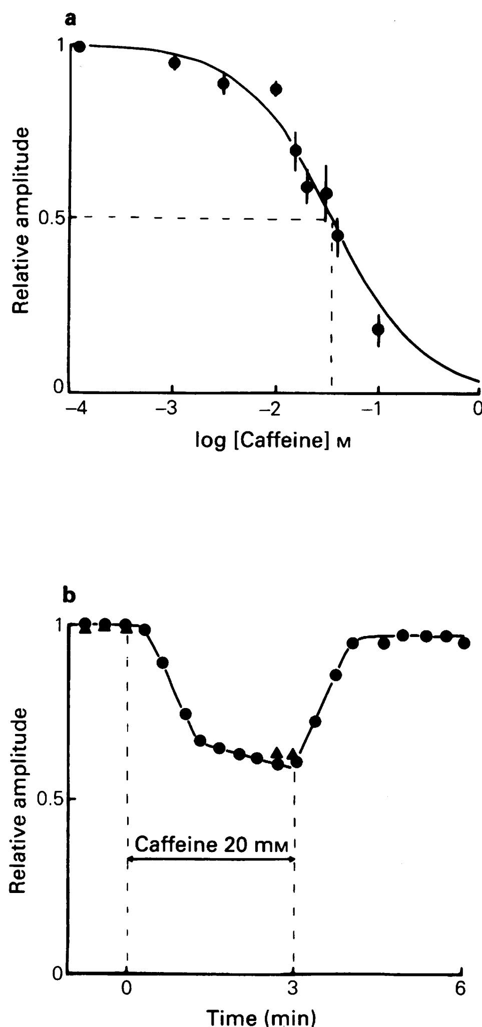


**Figure 2** Pregnant rat myometrium: effect of caffeine on the maximal amplitude of the inward current in 5 mM $Ba^{2+}$ solution. The amplitude of the inward current in the absence of caffeine was normalized as 1. The membrane was stepped to zero mV (300 ms duration) from a holding potential of −70 mV. (a) Concentration-response curve showing the inhibition of the inward current evaluated at 5 min after caffeine application with repetitive applications of command pulses (20 s intervals). Each data point is the mean of 3–12 experiments; vertical lines show s.e.mean. Glucose was added in order to have [caffeine] + [glucose] = 100 mM. Glucose by itself was without effect on the calcium channel current amplitude. (b) Effects of 20 mM caffeine with (●) and without (▲) repetitive applications of command pulses (20 s intervals). Steady-state effect of caffeine was obtained within 3 min. After 3 min of washing out, the amplitude of the current was restored to 97% of control.

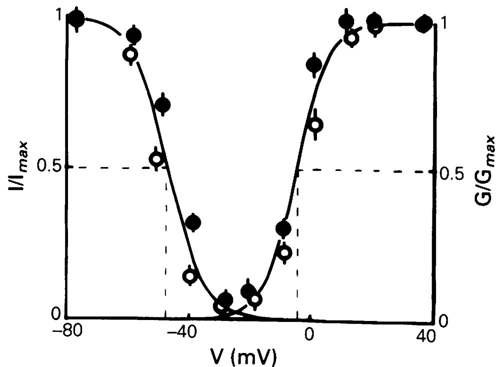


**Figure 3** Pregnant rat myometrium: steady-state inactivation and activation parameters of the inward current in 5 mM $Ba^{2+}$ solution against membrane potential in the absence (●) and presence (○) of 20 mM caffeine. The activation ($G/G_{max}$) and inactivation ($I/I_{max}$) curves are fitted by an expression of the form $1/[1 + \exp(V_m - V_h)/k]$ where $V_m$ is the membrane potential, $V_h$ is the mid potential and k is the slope factor of the curves. The parameters are: $V_h = -5.5$ mV, $k = -5.2$ mV for the activation curve; $V_h = -47$ mV and $k = 5.8$ mV for the inactivation curve. Each data point is the mean of 4 to 8 experiments; vertical lines show s.e.mean.

absence of a conditioning pulse ($I/I_{max}$). Figure 3 shows that the relative inactivation of calcium channel current was similarly enhanced in the voltage range from $-80$ to $-20$ mV in the absence and presence of 20 mM caffeine. The data were fitted by a Boltzmann distribution. Half-maximal inactivation was obtained at a membrane potential of $-45 \pm 4$ mV ($n = 8$). The activation curves were expressed by plotting the peak membrane conductance (calculated as peak current divided by the difference between membrane potential and apparent reversal potential) against voltage and fitted by a Boltzman distribution. Figure 3 shows that the activation curves are similar in the absence and presence of 20 mM caffeine. Half-maximal and complete activation were obtained at membrane potentials of $-5 \pm 3$ mV and $+35 \pm 5$ mV ($n = 7$).

*Inhibitory effect of caffeine is independent of intracellular calcium release*

In myometrium, calcium channel inactivation is largely dependent on an increase in cytoplasmic calcium concentration (Jmari *et al.*, 1986; Amédée *et al.*, 1987). It was therefore of interest to examine the inhibitory effect of caffeine on calcium channel current after depletion of intracellular calcium stores. We tested this hypothesis by applying intracellularly a mixture of 20 $\mu$M $InsP_3$ and 10 $\mu$M ryanodine. These substances are known to induce a release of calcium from the sarcoplasmic reticulum (Ohya *et al.*, 1988; Hisayama & Takayanagi, 1988; Ehrlich & Watras, 1988). In the presence of ryanodine and $InsP_3$ for 6 min, the $Ba^{2+}$ current was slightly reduced. Addition of 20 mM caffeine reduced the amplitude of the inward current by $38.6 \pm 4.5\%$ ($n = 5$), a value similar to that obtained in the absence of intracellular calcium release activators.

*Effects of caffeine on (+)-[$^3$H]-isradipine equilibrium binding*

The binding of (+)-[$^3$H]-isradipine to myometrial membranes at 25°C was saturable and specific (Honoré *et al.*, 1989). Scatchard analysis of the saturation isotherms was consistent with an interaction at a single class of high affinity sites with an equilibrium dissociation constant $K_D$ of $80 \pm 3$ pM ($n = 8$) and a binding site density of $106 \pm 4$ fmol mg$^{-1}$ of protein. Caffeine inhibited (+)-[$^3$H]-isradipine binding with an $IC_{50}$ value estimated to be 30 mM. The Hill coefficient was close to 1, suggesting that caffeine bound to a single binding site (Figure 4a). (+)-[$^3$H]-isradipine saturation isotherms were determined in the absence and in the presence of caffeine. As shown in Figure 4b, caffeine (20 and 50 mM) decreased the maximal binding capacity of the membranes to $58 \pm 3$ and $34 \pm 3$ fmol mg$^{-1}$, respectively ($n = 5$, $P < 0.001$) but did not change the apparent affinity of the radioligand ($P > 0.05$). These results indicate that caffeine behaves as a non competitive inhibitor of (+)-[$^3$H]-isradipine binding to myometrial membranes.

## Discussion

It has recently been proposed that in single myometrial cells the calcium channel inward current may be mainly an L-type current (Amédée *et al.*, 1987; Honoré *et al.*, 1989). The present results indicate that caffeine, at concentrations between 1 and 100 mM inhibits in a concentration-dependent manner the calcium channel current recorded on stepping to less negative values from a holding potential of $-70$ mV. The calcium channel current is reduced by caffeine without any modification of the apparent reversal potential suggesting that caffeine decreases the calcium conductance of the myometrial membrane. As the inward current through calcium channels is involved, in part, in spike firing and activation of the contraction (Mironneau, 1973), our data indicate that inhibition of calcium entry can contribute to the relaxant actions of caffeine observed at concentration of 1 mM and above.

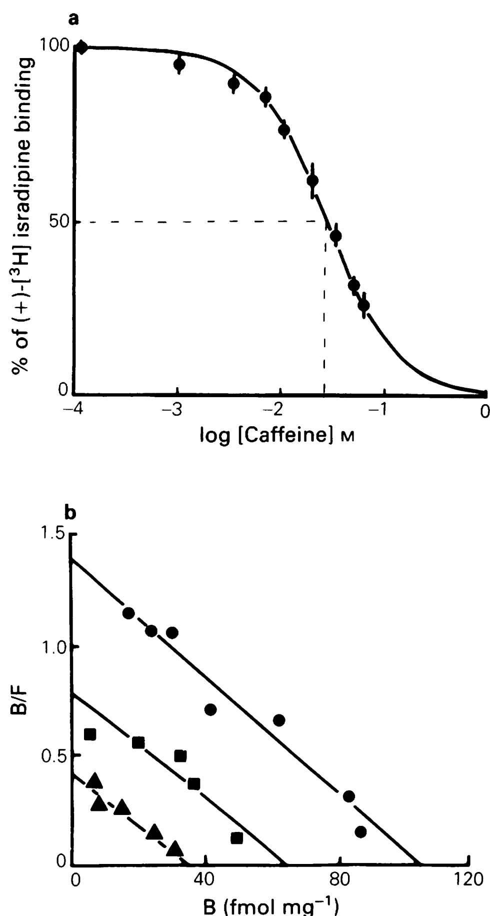


**Figure 4** Pregnant rat myometrium: effect of caffeine on the high affinity binding site for (+)-[$^3$H]-isradipine. (a) Binding of (+)-[$^3$H]-isradipine (0.1 nM; 0.08 to 0.15 mg ml$^{-1}$) in the presence of various concentrations of caffeine. Non-specific binding was defined in the presence of 2 $\mu$M nitrendipine and represented 15% of total binding. Results are expressed as a percentage of the specific binding in the absence of caffeine, and are means of 6 experiments with s.e.mean shown by vertical lines. The Hill coefficient was 1.1. Glucose was added in order to have [caffeine] + [glucose] = 100 mM. Glucose by itself was without effect on the (+)-[$^3$H]-isradipine binding assay. (b) Scatchard analysis of the specific binding in the absence (●) and presence of 20 mM (■) and 50 mM (▲) caffeine. Similar results were obtained from 4 other experiments; triplicate estimates were used for each point.

When calcium channels were opened by a conditioning depolarizing pulse sequence, the inhibitory effect of caffeine was similar to that obtained in the absence of stimulation. Increasing the number of inactivated calcium channels at depolarized holding potentials had no further effect on the caffeine-induced blockade. These results suggest that caffeine does not show preferential binding to open or inactivated calcium channels. Thus, the blocking effects of caffeine differ from these of dihydropyridines and phenylalkylamines in smooth muscles (Terada *et al.*, 1987; Dacquet *et al.*, 1988; Hering *et al.*, 1988). It can be suggested that caffeine affects all states of the calcium channels. This assumption is supported by the caffeine inhibition of specific (+)-[$^3$H]-isradipine binding for isolated membranes which are, of course, depolarized.

Several lines of evidence support the idea that caffeine has a direct action on calcium channels: (1) After depletion of internal calcium stores with $InsP_3$ and ryanodine, the inhibitory effect of caffeine was identical to that observed in the absence of intracellular calcium activators. This observation indicates that the action of caffeine is not related to the inactivation of calcium channels by calcium release from intracellular stores. In myometrium, both calcium-dependent and membrane potential-dependent mechanisms contribute to calcium channel inactivation (Jmari *et al.*, 1986). (2) The slope coefficients of the inhibition curves obtained from both electrophysiological and binding experiments were close to 1, suggesting that caffeine bound to a single binding site. (3) The apparent affinity of caffeine for the calcium channel current was nearly identical to that obtained on the high affinity site for (+)-[$^3$H]-isradipine. (4) Although caffeine completely displaced (+)-[$^3$H]-isradipine binding from its high affinity receptors, the inhibitory effect of caffeine was seen as a decrease in $B_{max}$ of isradipine binding without any significant change in affinity. These observations indicate that caffeine behaves as a non competitive inhibitor of (+)-[$^3$H]-isradipine binding to myometrial membranes.

In conclusion, our results show that caffeine, at concentrations between 1 and 100 mM, inhibits the L-type calcium current of myometrial cells by binding to a site associated with the calcium channels. This effect may contribute, in part, to the relaxant action of caffeine in myometrium.

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## References

AHN, H.Y., KARAKI, H. & URAKAWA, N. (1988). Inhibitory effects of caffeine on contractions and calcium movement in vascular and intestinal smooth muscle. *Br. J. Pharmacol.*, **93**, 267–274.

AMEDEE, T., MIRONNEAU, C. & MIRONNEAU, J. (1987). The calcium channel current of pregnant rat single myometrial cells in short-term primary culture. *J. Physiol.*, **392**, 253–272.

ASHOORI, F., TAKAI, A. & TOMITA, T. (1985). The response of non-pregnant rat myometrium to oxytocin in Ca-free solution. *Br. J. Pharmacol.*, **84**, 175–183.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–253.

BRAY, K.M., LONGMORE, J. & WESTON, A.H. (1989). Analysis of caffeine-induced responses in rabbit isolated aorta. *J. Physiol.*, **410**, 77P.

DACQUET, C., PACAUD, P., LOIRAND, G., MIRONNEAU, C. & MIRONNEAU, J. (1988). Comparison of binding affinities and calcium current inhibitory effects of a 1,4-dihydropyridine derivative (PN 200-110) in vascular smooth muscle. *Biochem. Biophys. Res. Commun.*, **152**, 1165–1172.

DACQUET, C., LOIRAND, G., RAKOTOARISOA, L., MIRONNEAU, C. & MIRONNEAU, J. (1989). (+)-[$^3$H]-PN 200-110 binding to cell membranes and intacts strips of portal vein smooth muscle: characterization and modulation by membrane potential and divalent cations. *Br. J. Pharmacol.*, **97**, 256–262.

EHRLICH, B.E. & WATRAS, J. (1988). Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. *Nature*, **336**, 583–586.

ENDO, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.*, **57**, 71–108.

FABIATO, A. & FABIATO, F. (1977). Calcium release from sarcoplasmic reticulum. *Circ. Res.*, **40**, 119–129.

HERING, D., BEECH, D.J., BOLTON, T.B. & LIM, S.P. (1988). Action of nifedipine or Bay k 8644 is dependent on calcium channel state in single smooth muscle cells from rabbit ear artery. *Pflügers Arch.*, **411**, 590–592.

HISAYAMA, T. & TAKAYANGI, I. (1988). Ryanodine: its possible mechanism of action in the caffeine-sensitive calcium store of smooth muscle. *Pflügers Arch.*, **412**, 376–381.

HONORE, E., AMEDEE, T., MARTIN, C., DACQUET, C., MIRONNEAU, C. & MIRONNEAU, J. (1989). Calcium channel current and its sensitivity to (+)isradipine in cultured pregnant rat myometrial cells: an electrophysiological and a binding study. *Pflügers Arch.*, (in press).

ITO, Y. & KURIYAMA, H. (1971). Caffeine and excitation-contraction coupling in guinea-pig taenia coli. *J. Gen. Physiol.*, **57**, 448–463.

JMARI, K., MIRONNEAU, C. & MIRONNEAU, J. (1986). Inactivation of calcium channel current in rat uterine smooth muscle: evidence for calcium and voltage mediated mechanisms. *J. Physiol.*, **380**, 111–126.

KARAKI, H., AHN, H.Y. & URAKAWA, N. (1987). Caffeine-induced contraction in vascular smooth muscle. *Arch. Int. Pharmacodyn.*, **285**, 327–339.

LEIJTEN, P.A.A. & VAN BREEMEN, C. (1984). The effects of caffeine on the noradrenaline-sensitive calcium store in rabbit aorta. *J. Physiol.*, **357**, 327–339.

MIRONNEAU, J. (1973). Excitation-contraction coupling in voltage clamp uterine smooth muscle. *J. Physiol.*, **233**, 127–141.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.*, **107**, 220–239.

OHYA, Y., TERADA, K., YAMAGUSHI, K., INOUE, R., OKABE, J., KITAMURA, K., HIRATA, M. & KURIYAMA, H. (1988). Effects of inositol phosphates on the membrane activity of smooth muscle cells of the rabbit portal vein. *Pflügers Arch.*, **412**, 382–389.

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. *Circ. Res.*, **55**, 336–348.

SATO, K., OZAKI, H. & KARAKI, H. (1988). Multiple effects of caffeine on contraction and cytosolic free $Ca^{2+}$ levels in vascular smooth muscle of rat aorta. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **338**, 443–448.

SMALL, R.C., BOYLE, J.P., CORTIJO, J., CURTIS-PRIOR, P.B., DAVIES, J.M., FOSTER, R.W. & HOFER, P. (1988). The relaxant and spasmogenic effects of some xanthine derivatives acting on guinea-pig isolated trachealis muscle. *Br. J. Pharmacol.*, **94**, 1091–1100.

TERADA, K., KITAMURA, K. & KURIYAMA, H. (1987). Blocking actions of $Ca^{2+}$ antagonists on the $Ca^{2+}$ channels in the smooth muscle cell membrane of rabbit small intestine. *Pflügers Arch.*, **408**, 552–557.

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