British Journal of Pharmacology (1996) 119, 142–148

The effect of L-type calcium channel modulators on the mobilization of intracellular calcium stores in guinea-pig intestinal smooth muscle

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1 The action of Ca^{2+} channel modulators has been examined on the intracellular Ca^{2+} signal in the longitudinal smooth muscle cells of the guinea-pig intestine after exposure to histamine and to agents known to affect intracellular Ca^{2+} stores. Isometric contraction has been measured simultaneously with front-surface fluorometry of fura 2-loaded preparations.

2 Histamine (10 μM) evoked a phasic and tonic increase in $[Ca^{2+}]_i$ and contraction which were both sensitive to the Ca^{2+} channel blockers, nimodipine and D600.

3 Caffeine (10 mM) evoked in rapid increase in $[Ca^{2+}]_i$ which was sustained as long as the preparation was exposed to the drug, whereas the contractile response was only phasic. In the presence of nimodipine 1 μM, the phasic contraction was absent although the fura 2-Ca^{2+} signal amounted to 32% of the control.

4 Ryanodine (10 μM) evoked a slow increase in $[Ca^{2+}]_i$ and a contraction, both of which were reversed after exposure to nimodipine (1 μM) or D600 (10 μM). In the presence of diazoxide (500 μM), a hyperpolarizing agent, the ryanodine-evoked increase in $[Ca^{2+}]_i$ and in muscle tone were inhibited.

5 Thapsigargin (1 μM) also produced an increase in $[Ca^{2+}]_i$ and a contraction both of which were blocked by nimodipine (1 μM).

6 In Ca^{2+}-free solution, histamine 10 μM evoked non-reproducible phasic Ca^{2+} signal and contraction. This response was recovered after refilling in Ca^{2+} containing solution. The recovery was blocked by nimodipine, D600 or diazoxide and was facilitated by the Ca^{2+} channel activator, Bay K 8644. When the refilling medium was supplemented with thapsigargin, the recovered response was significantly reduced, but Bay K 8644 still had some action.

7 The present results show that blockade of L-type Ca^{2+} channels inhibited changes in $[Ca^{2+}]_i$ evoked by histamine, caffeine and ryanodine which are generally attributed to Ca^{2+} mobilization from intracellular stores. They also show that when the tissue was exposed to nimodipine, D600 and diazoxide during the procedure of refilling after depletion of intracellular stores, the action of histamine on $[Ca^{2+}]_i$ and contraction was blocked. Bay K 8644 had an opposite effect even when the Ca^{2+} pumping activity of the sarcoplasmic reticulum was reduced by thapsigargin. This indicates that refilling of intracellular Ca^{2+} stores depleted by histamine in guinea-pig intestine mainly occurred through L-type Ca^{2+} channels.

Keywords: Internal Ca^{2+} stores; refilling of Ca^{2+} stores; Ca^{2+} antagonists; Ca^{2+} channel modulators; histamine; guinea-pig longitudinal smooth muscle

Introduction

Agonists such as histamine evoke a contraction of guinea-pig intestinal smooth muscle both by releasing Ca^{2+} from intracellular stores and by stimulating Ca^{2+} influx from the extracellular space. As shown by Morel *et al.* (1987), this Ca^{2+} influx is sensitive to nifedipine and to Bay K 8644, indicating that it occurs through activated L-type Ca^{2+} channels. Donaldson & Hill (1985) have shown that activation of H_1-receptor leads to inositol phospholipid breakdown. On the basis of studies performed mainly on saponin-permeabilized tissues, but also on isolated cells, Iino & coworkers (Iino *et al.*, 1988; Yamazawa *et al.*, 1992) proposed that intracellular Ca^{2+} could be stored in two separate compartments one, Sα equipped with ryanodine and Ins(1,4,5)P_3-receptors and another, Sβ equipped only with Ins(1,4,5)P_3-receptors.

Low *et al.* (1992) have proposed a model for the repletion of internal Ca^{2+} stores of vascular smooth muscle. They have identified two components of the contractile response of the dog mesenteric artery to phenylephrine which can be differently modulated. The initial component of the contractile response is highly sensitive to the presence of ryanodine while the second is insensitive to inhibitors of the sarcoplasmic ATPase and is stimulated in the presence of Bay K 8644. They concluded that the two components of the contractile response correspond to the release of two different internal Ca^{2+} stores with different refilling mechanisms, in agreement with the proposal of Iino *et al.* (1988) who have observed that when skinned muscle cells were exposed to ryanodine, the Ca^{2+} signal recorded in response to caffeine was blocked, whereas the signal evoked by Ins(1,4,5)P_3 was barely affected.

In a recent study, Wibo & Godfraind (1994), examined the subcellular distribution of ryanodine and Ins(1,4,5)P_3-receptors in intestinal smooth muscle. They observed that ryanodine receptors are much less numerous than Ins(1,4,5)P_3-receptors, both are associated with SR and could not be clearly separated on the basis of equilibrium density of membranes. However, the stoichiometric ratio of the two receptors is not identical when studied in various subcellular fractions separated by density equilibration in a linear sucrose gradient. Such an observation could favour an alternative hypothesis also proposed by Wong & Klassen (1993) assuming that the two Ca^{2+} stores are in communication one with the other. Thus, in Ca^{2+}-free medium, the activation of Ins(1,4,5)P_3 and of ryanodine receptors may deplete intracellular Ca^{2+} stores.

In physiological conditions, the extracellular Ca^{2+} has been clearly identified as the major source of Ca^{2+} for refilling the stores following their release by an agonist as the majority of

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Ca^{2+} released from internal pools is ejected across the plasma membrane (Putney, 1990; Kwan *et al.*, 1990). In the 'capacitative Ca^{2+} entry model', Putney proposed that the depletion of the intracellular Ca^{2+} stores by Ins(1,4,5)P_3 may induce the opening of a pathway for their replenishment from the extracellular space (Putney, 1990). The state of emptiness of the intracellular stores may be the signal that directly or indirectly initiates the Ca^{2+} entry (Zhang & Melvin, 1993) but the exact process of refilling and the mode of Ca^{2+} entry remains unclear. Some authors proposed that in canine colonic smooth muscle, Ca^{2+} entry could occur through L type Ca^{2+} channels and an additional pathway (Sato *et al.*, 1995). Recently, Ohta *et al.* (1995) have reported that in rat ileum, L-type Ca^{2+} channels are not involved in the Ca^{2+} entry activated by depletion of the intracellular stores.

The present experiments were undertaken in order to examine if the pathways used by Ca^{2+} to refill the internal Ca^{2+} stores are similar in guinea-pig and rat ileum. Therefore, we have used the fura 2 method in intact smooth muscle and have simultaneously measured the changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and in contractile tone induced by histamine and by several drugs acting either on L-type Ca^{2+} channels (diazoxide which hyperpolarizes the plasma membrane and acts indirectly, nimodipine, D600 and Bay K 8644 act directly on the channels) or on the sarcoplasmic reticulum functions (caffeine, ryanodine, thapsigargin). Our results show that blockade of L-type Ca^{2+} channels inhibits the mobilization and repletion of the Ca^{2+} stores and thus indicate that in the guinea-pig intestinal smooth muscle the refilling of the Ca^{2+} stores depleted by histamine is highly dependent on an influx through L-type Ca^{2+} channels.

Methods

Tissue preparation and fura 2 measurements

Guinea-pigs (400–500 g) were killed by stunning and decapitation. Ileum segments were isolated and immersed in physiological solution (mM: NaCl 112, KCl 5, $NaHCO_3$ 25; glucose 11.5, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, pH 7.4) at 37°C equilibrated with 95% O_2/5% CO_2. The longitudinal layer was dissected free from the circular muscle and thin sheets of about 5 mm wide and 5 mm long were prepared.

The strips were treated with a physiological solution containing fura 2-AM (3 μM) for 4 to 5 h at room temperature. The non-cytotoxic detergent, Cremophor EL (0.03%), which increases the solubility of fura 2-AM and bovine serum albumin (1%) was added. This procedure of loading was chosen to minimize the compartmentalization of the indicator (low concentration of the dye, low temperature of loading). Himpens (1988) had observed, using a very similar loading procedure, that most of the Ca^{2+} signal is cytosolic. However, we cannot exclude that a small part of the fura 2 is trapped inside organelles, therefore we use the general terminology 'intracellular' to characterize the fura 2-Ca^{2+} signal. The fluorescence of the muscle increased during the period of loading. The autofluorescence measured at the end of the experiment was equal after excitation at 340 nm and 380 nm to 21.1 ± 1.4% and to 24.7 ± 1.7% (n = 32) respectively of total fluorescence. After the loading, ileal strips were transferred to a fresh physiological solution, washed for 5 min in order to remove uncleaved fura 2-AM from the tissue and mounted between two hooks in a 7 ml-organ bath filled with physiological solution kept at 37°C and gassed with 95% O_2/5% CO_2. One end of the strip was connected to a strain gauge transducer and the muscle was maintained under a resting tension of 1 g. The organ-bath is part of a fluorometer allowing the simultaneous recording of contractile tension and fluorescence (CAF 110, JASCO, Tokyo, Japan). The muscle was alternatively illuminated (128 Hz) with two excitation wavelengths (340 ± 10 nm and 380 ± 10 nm) obtained from a xenon high pressure (75 W) lamp coupled to two monochromators. The emitted light from the muscle was collected by a photomultiplier through a 500 ± 12 nm filter. The time constant for the optical channels was 260 ms. The ratio of the fluorescence intensities due to excitation at 340 nm to that obtained after excitation at 380 nm (F_{340}/F_{380}), as it appears on the figures through the paper, was calculated from successive illumination periods. The fluorescence signals, F_{340}, F_{380} and the ratio F_{340}/F_{380} were simultaneously recorded with the contractile tension on a multichannel system (Kontron 500 SP, Switzerland).

At the end of most experiments, the fura 2-Ca^{2+} signal was calibrated. The maximal ratio was measured in a Ca^{2+}-saturating and potassium rich medium (mM: KCl 117, $NaHCO_3$ 25, glucose 11.5, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2) by addition of ionomycin, 20 μM (which was the lowest active concentration in our system). The minimum ratio was obtained by a further addition of EGTA (8 mM). In such conditions, no detectable leakage of the fura 2 from the tissue could be detected during the calibration. The autofluorescence values were obtained by quenching the fura-2 fluorescence with $MnCl_2$ (5 mM) and were subtracted from all values. In such conditions, and after autofluorescence subtraction, the mean maximum ratio was equal to 4.340 ± 0.795 (n = 32) while the mean minimum ratio amounted to 0.363 ± 0.023 (n = 32). The ratio (F_{340}/F_{380}) indicated on the figures was obtained from experimental data including autofluorescence.

The absolute $[Ca^{2+}]_i$ was calculated according to Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]cyt = \beta \times K_d \times \frac{R - R_{min}}{R_{max} - R}$$

assuming that R is the ratio of the fluorescence intensities corrected for the autofluorescence, measured at 340 nM over that measured at 380 nM. The dissociation constant for the fura 2/Ca^{2+} complex is assumed to be equal to 224 nM. The correcting factor β (3.646 ± 0.393, n = 32), which is equal to the ratio of the fluorescence intensities (after autofluorescence substraction) of the free dye (measured in Ca^{2+}-depleted medium, in the presence of EGTA) and the Ca^{2+}-saturated dye (measured in saturating Ca^{2+} medium, in the presence of ionomycin) at 380 nm, was calculated in each preparation.

Strips were stimulated twice with histamine 10 μM, applied during 5 min at intervals of 5 min at the beginning of each experiment; the response to the second stimulation was taken as a control, the effect of the different drugs was tested afterwards.

Drugs

Histamine-HCl was dissolved in distilled water as a stock solution at 10 mM. Ryanodine was prepared as a stock solution at 1 mM in ethanol 17%. Thapsigargin was dissolved in dimethyl sulphoxide (DMSO) 100% to obtain a 10 mM stock solution. Caffeine was prepared each day in physiological solution. Nimodipine and Bay K 8644 (methyl-1,4-dihydro-2-, 6-dimethy-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) gifts from Bayer AG, Germany were dissolved in ethanol as stock solution at 1 mM and 10 mM respectively. D600 (methoxy-verapamil) was dissolved each day in water to give a stock solution of 10 mM. Diazoxide was dissolved each day in NaOH 0.1 N to obtain a stock solution at 50 mM. Further dilutions were prepared in physiological solution, the pH of these dilutions being corrected when necessary.

Statistical analysis

All values are expressed as means ± s.e.mean, and n is the number of observations. Significance was tested using Student's t test and variance parameters were compared with an ANOVA one factor test.

Results

Effect of nimodipine and diazoxide on resting $[Ca^{2+}]_i$ and resting tone

As shown on Figure 1a, most preparations developed a spontaneous rhythmic activity which was superimposed on the basal Ca^{2+} signal and the basal tone. The addition of nimodipine (1 μM) rapidly inhibited this rhythmic activity. In the presence of nimodipine (1 μM), the basal $[Ca^{2+}]_i$ decreased and reached a concentration which was only $72.9 \pm 6.2\%$ ($n=4$) of the initial one. When diazoxide (500 μM), a K^+ channel opener which hyperpolarizes the smooth muscle cells and closes the L-type calcium channels (for review, Quast, 1992) was added to the bathing solution, the spontaneous rhythmic activity was also abolished and a decrease of both resting fura 2-Ca^{2+} signal and resting tone was observed (Figure 1b). This indicates that resting $[Ca^{2+}]_i$ and resting tone could be modulated through the voltage-gated Ca^{2+} channels in agreement with Himpens & Somlyo (1988).

Effect of histamine on $[Ca^{2+}]_i$ and tension

Figure 2 illustrates the increase in $[Ca^{2+}]_i$ and in muscle tone evoked by an exposure of intestinal smooth muscle to histamine (10 μM) for 5 min. Histamine produced a rapid increase in $[Ca^{2+}]_i$ from 167 ± 12 nM to 345 ± 52 nM ($n=10$) and a phasic increase in muscle tone, thereafter, $[Ca^{2+}]_i$ and muscle tone were maintained as long as the agonist was present in the bathing solution.

In preparations pretreated with nimodipine (1 μM) for 10 min, histamine (10 μM)-evoked responses were markedly reduced (Figure 2). The maximum increase in fluorescence ratio was equal to $35.1 \pm 4.7\%$ ($n=7$) of the phasic increase recorded before nimodipine and the maximum increase of muscle tone was equal to $12.8 \pm 2.0\%$ ($n=7$) of the phasic control contraction ($P<0.01$). Similar results were obtained when ileal strips were incubated for 10 min in the presence of the phenylalkylamine D600 10 μM: the maximum histamine-evoked increase in fluorescence ratio was $44.1 \pm 4.3\%$ ($n=3$) while the contractile tension reached $11.4 \pm 7.3\%$ ($n=3$) of the control response to histamine.

Effect of caffeine on $[Ca^{2+}]_i$ and tension

Caffeine (10 mM) which is known to release Ca^{2+} from the intracellular stores (Endo *et al.*, 1982) evoked a rapid increase of $[Ca^{2+}]_i$ from 125 ± 30 nM ($n=6$) to 215 ± 53 nM ($n=6$); it also produced a phasic contraction (Figure 3). When the exposure to caffeine (10 mM) was prolonged, the tone decreased rapidly to a level equal to or even lower than the initial one, while the Ca^{2+} signal diminished slightly to reach a sustained value. The maximum change in fluorescence ratio evoked by caffeine (10 mM) was $116.2 \pm 11.2\%$ ($n=13$) of the phasic response to a control response to histamine (10 μM) added before, but this was not significantly different ($P>0.05$). However, the phasic contractile response to caffeine amounted to $45.8 \pm 5.4\%$ ($n=13$) of the phasic response to histamine; this was significantly lower ($P<0.01$). For a given increase in fluorescence ratio, caffeine evoked a smaller development in

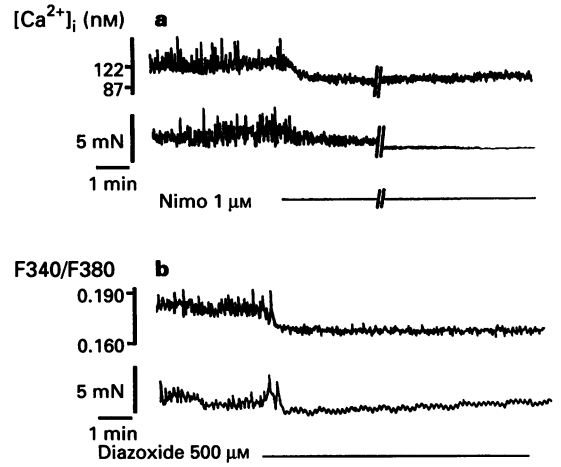

Figure 1 Recording of typical experiments showing the spontaneous rhythmic activity superimposed on the basal $[Ca^{2+}]_i$ and the resting tone of ileal strips. Addition of nimodipine (Nimo) 1 μM (a) or diazoxide 500 μM (b) completely abolished the rhythmic activity and decreased both the basal $[Ca^{2+}]_i$ and the resting tone. These recordings are representative of at least 4 different experiments.

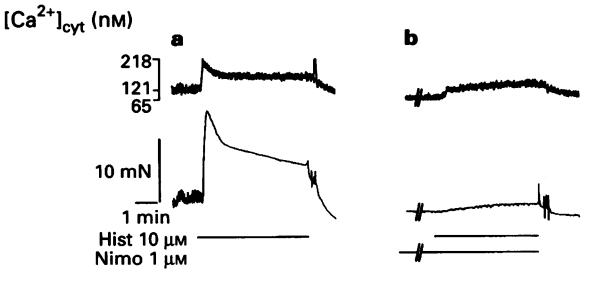

Figure 2 Recording of a typical experiment showing the effect of a stimulation with histamine 10 μM on $[Ca^{2+}]_i$ and contractile tone on guinea-pig ileal smooth muscle in the absence (a) or in the presence of nimodipine (Nimo) 1 μM preincubated for 10 min (b). This recording is representative of at least 7 different experiments.

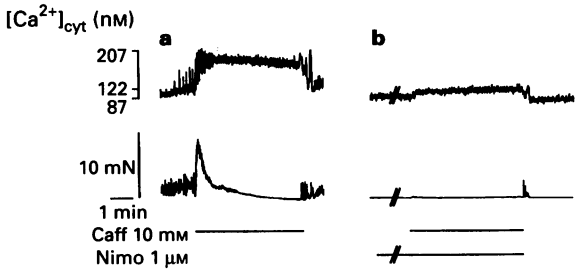

Figure 3 Typical recording showing the effect of a stimulation with caffeine (Caff) 10 mM on $[Ca^{2+}]_i$ and contractile tone on guinea-pig ileal smooth muscle in the absence (a) or in the presence of nimodipine (Nimo) 1 μM preincubated for 10 min (b). This recording is representative of at least 8 different experiments.

Table 1 The Ca^{2+} response evoked by caffeine 10 mM, ryanodine 10 μM and thapsigargin 1 μM in ileal smooth muscle

	$[Ca^{2+}]_i$ (% of the control response to histamine)		*Fura 2 ratio (% of the control response to histamine)*
Caffeine	90.3 ± 16.7 ($n=6$)	—NS—	116.2 ± 11.2 ($n=13$)
Ryanodine	78.6 ± 12.2 ($n=5$)	—NS—	79.4 ± 8.2 ($n=17$)
Thapsigargin	103.4 ± 21.3 ($n=7$)	—NS—	92.9 ± 12.6 ($n=13$)

Maximum increase in Ca^{2+} signal estimated by comparing to histamine either the $[Ca^{2+}]_i$ change or the change in fura 2 ratio. All values represent mean $\pm$ s.e.mean, n = number of experiments. NS, $P>0.1$, not significantly different.

tension than histamine did. It is noteworthy that when expressed as a percentage of the control response to histamine, experimental data were not significantly different when measuring $[Ca^{2+}]_i$ or more simply the fura 2-ratio (see Table 1).

After blockade of voltage-dependent Ca^{2+} channels by nimodipine 1 μM, the caffeine-evoked increase in fura 2-ratio was reduced to $32.3 \pm 5.4\%$ ($n=8$) of the control ($P<0.01$) while the contraction was absent (Figure 3).

Effect of ryanodine on $[Ca^{2+}]_i$ and tension

In smooth muscle cells, ryanodine has been shown to induce Ca^{2+} release from the sarcoplasmic reticulum (Hwang & Van Beemen, 1987). Figure 4 shows that in physiological solution, ryanodine 10 μM evoked a slow increase of both fluorescence ratio and muscle tone. At steady state, the increase in fluorescence signal corresponded to $79.4 \pm 8.2\%$ ($n=17$) of the phasic increase in fura 2-signal produced by the control stimulation with histamine (10 μM) ($P<0.05$), the increase in contractile tone was equal to $47.8 \pm 4.7\%$ ($n=17$) of the phasic contraction evoked by histamine (10 μM) ($P<0.01$). In 5 experiments at the end of which the fura 2-Ca^{2+} signal was calibrated, $[Ca^{2+}]_i$ increased from 180 ± 33 to 314 ± 59 nM after exposure to ryanodine 10 μM. As Figure 4a illustrates, when nimodipine 1 μM was added during the plateau of the response, both fluorescence ratio and tone returned to basal levels. A similar effect was observed after addition of D600 (10 μM). Furthermore, in preparations pretreated with nimodipine 1 μM, ryanodine 10 μM was without detectable effect ($n=4$) on $[Ca^{2+}]_i$ and muscle tone (data not shown). When histamine was added to the bath solution, as soon as the fura 2-Ca^{2+} signal and the muscle tone elevated by ryanodine (10 μM) had returned to basal values in the presence of nimodipine 1 μM, there was no detectable change in Ca^{2+} signal. However, a small increase in muscle tone corresponding to $2.8 \pm 0.9\%$ ($n=9$) of the control value was observed.

At variance with reports in vascular tissues (Newgreen *et al.*, 1990; Antoine *et al.*, 1992), diazoxide (500 μM) does not inhibit high KCl-evoked contraction in the guinea-pig ileum smooth muscle (data not shown). When ileal preparations were preincubated for 15 min with diazoxide 500 μM, the ryanodine-evoked increase in fluorescence ratio and contractile tension amounted respectively to $7.4 \pm 4.3\%$ and 0% of the responses measured in the absence of diazoxide, indicating that hyperpolarization attenuated the action of ryanodine ($n=4$).

Effect of thapsigargin on $[Ca^{2+}]_i$ and tension

Below 1 μM, thapsigargin is a specific inhibitor of the sarcoplasmic reticulum Ca^{2+} pump; at higher concentrations it also blocks L-type Ca^{2+} channels (Buryi *et al.*, 1995). Representative time course changes in fura 2-ratio and tone of ileal strips treated with thapsigargin 1 μM are shown in Figure 4b. The mean increase in fluorescence ratio and contractile tension were equal to $92.9 \pm 12.6\%$ ($P>0.1$) and to $67.3 \pm 6.96\%$ ($P<0.01$) ($n=13$) respectively of the phasic control response to histamine, 10 μM. In 7 experiments at the end of which the fura 2-Ca^{2+} signal was calibrated, thapsigargin evoked an increase in $[Ca^{2+}]_{cyt}$ from 191 ± 20 to 377 ± 34 nM. The addition of nimodipine 1 μM when the responses had reached a steady state induced a rapid decline of both Ca^{2+} signal and tension to a level not different from the basal line.

Depletion and refilling of the stores

After a control stimulation with histamine (10 μM), ileal preparations were washed for 2 min with a Ca^{2+}-free solution ($CaCl_2$, 0 mM; EGTA, 0.3 mM, other components as before) and re-exposed to histamine (10 μM) for 3 min in the Ca^{2+}-free medium. This procedure allowed the complete discharge of the internal stores as indicated by the absence of responses to further addition of histamine in Ca^{2+}-free solution (Figure 5).

Figure 6 illustrates another set of experiments: ileal strips were first exposed to a control stimulation with histamine 10 μM (Hist 1). The intracellular Ca^{2+} stores were then emptied by a 2 min incubation in a Ca^{2+}-free medium followed by a 3 min stimulation with histamine 10 μM (Hist 2). After a further period of washing in the absence of Ca^{2+} (2 min), the Ca^{2+}-depleted preparations were re-exposed to a Ca^{2+}-containing medium in the absence of histamine for 5 min in order to allow the stores to be refilled. This Ca^{2+} readmission evoked an increase of the fura 2 fluorescence, but almost no change in muscle tone. To determine the extent of repletion of the stores, the preparations were exposed during 1 min to a Ca^{2+}-free solution and then to histamine 10 μM (Hist 3). This last histamine-exposure elicited a phasic increase of the fluorescence ratio corresponding to $38.5 \pm 8.0\%$ ($n=8$) of the response

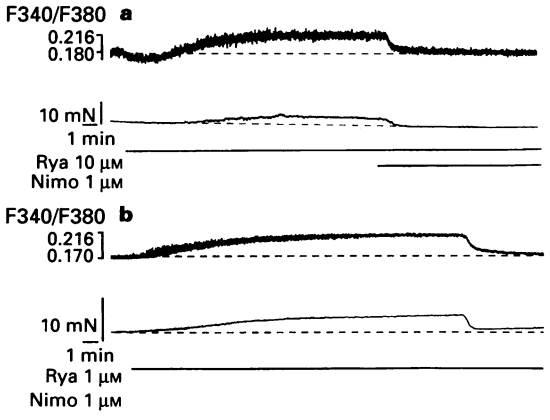

Figure 4 (a) Changes in the fura 2 fluorescence ratio (F_{340}/F_{380}) and in contractile tone evoked by ryanodine (Rya) 10 μM. Nimodipine (Nimo) 1 μM added when the responses had reached a steady state completely reversed both increase in fura 2-Ca^{2+} signal and contraction. (b) Changes in the fura 2 fluorescence ratio (F_{340}/F_{380}) and in the contractile tone evoked by thapsigargin (Thaps) 1 μM. Nimodipine 1 μM added when the responses had reached a steady state reversed both increase in fura 2-Ca^{2+} signal and contraction nearly to the basal levels. These recordings are representative of respectively 6 and 5 different experiments.

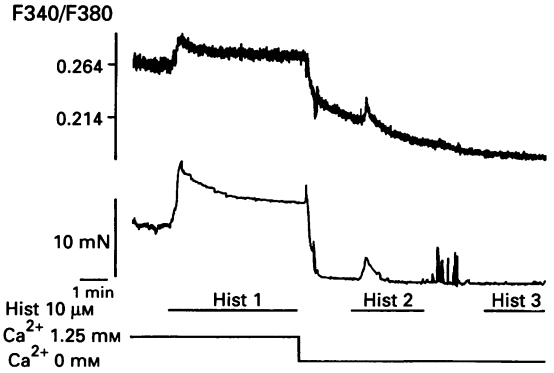

Figure 5 Typical recording showing the experimental procedure followed to deplete the histamine-sensitive Ca^{2+} stores. After a control stimulation with histamine 10 μM, ileal preparations were washed with a Ca^{2+}-free solution (Ca^{2+}, 0 mM, EGTA 300 μM), 2 min later, a second stimulation with histamine 10 μM was performed. A further addition of histamine 10 μM in Ca^{2+}-free solution was without detectable effect of both fura 2 Ca^{2+} signal contraction, suggesting the complete depletion of the intracellular Ca^{2+} stores.

measured in the presence of external Ca^{2+} and a contraction amounting to 14.6±2.7% ($n=8$), indicating that a 5 min period in the presence of Ca^{2+} was long enough to allow a significant refilling of the stores.

Effect of L-type Ca^{2+} channels modulators on the refilling process

To test whether voltage-dependent Ca^{2+} channels modulators could interact with the refilling process of the Ca^{2+} stores, we have exposed the ileal strips to Bay K 8644 1 μM, nimodipine 1 μM or D600 10 μM during the period of Ca^{2+} loading.

When Bay K 8644 (1 μM) was added to the bath solution during the re-exposure to external Ca^{2+}, we observed an increase in the basal fluorescence ratio and the development of a contractile tone amounting to 96.7±16.0% and 41.0±13.8% ($n=8$) respectively of the control response to histamine 10 μM (in the presence of Ca^{2+} 1.25 mM) which were abolished after a washout of the ileal preparation with a Ca^{2+}-free medium.

The treatment with Bay K 8644 (1 μM) enhanced the recovery of the response to histamine after the procedure of depletion/refilling of the stores since in such conditions, Hist 3 (histamine 10 μM added after a 1 min wash in Ca^{2+}-free medium), evoked a significantly higher increase of the fura 2 fluorescence signal ($P<0.05$) and of the contractile tension ($P<0.01$) than in controls (see Table 2).

When the refilling procedure occurred in the presence of nimodipine 1 μM or D600 10 μM, the increase of the fluorescence ratio observed after readmission of Ca^{2+} was absent and the response evoked by hist 3 (in Ca^{2+}-free medium) was nearly abolished (Table 2).

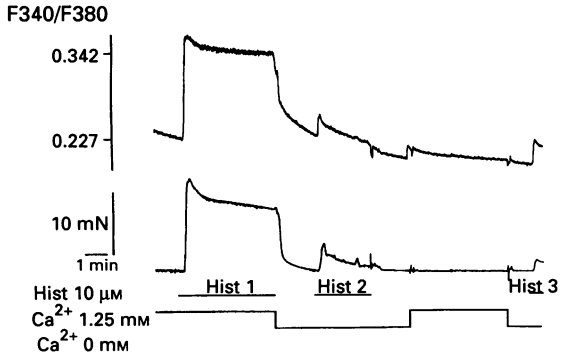

Figure 6 Recording of a typical experiment showing the experimental procedure used to analyse the refilling process of the intracellular Ca^{2+} stores sensitive to histamine in guinea-pig ileum smooth muscle in the absence of any testing agent. After a control response to histamine 10 μM (Hist 1), preparations were washed with a Ca^{2+}-free solution (Ca^{2+}, 0 mM, EGTA 300 μM), 2 min later, a stimulation with histamine 10 μM was performed (Hist 2). After a further washout with a Ca^{2+}-free medium (2 min), the stores were allowed to refill in the presence of a Ca^{2+} containing medium for 5 min. Thereafter, the extent of refilling was evaluated by the amplitude of the transient fura 2-Ca^{2+} signal and contractile tension elicited by histamine 10 μM (Hist 3) in a Ca^{2+}-free medium. This recording is representative of at least 8 different experiments.

Effect of thapsigargin on the refilling process

Because of the slow onset of its action, thapsigargin was added for a 30 min preincubation period before the depletion/refilling procedure. This time seemed sufficient to reach a steady state both for the fura 2-signal and for the contraction (see Figure 4b in physiological solution). The increase of the fluorescence ratio evoked by Hist 3 after the depletion/refilling process was significantly diminished ($P<0.01$) indicating that thapsigargin (1 μM) inhibited this process (see Table 2).

When the refilling was performed in the presence of Bay K 8644, 1 μM, after pretreatment with thapsigargin (1 μM), the response to Hist 3 was reduced as compared to that elicited by Bay K 8644 alone ($P<0.01$).

Effect of diazoxide on the refilling process

When the refilling procedure was performed in the presence of diazoxide, the readmission of Ca^{2+} to the bathing solution did not evoke an increase of the fura 2-Ca^{2+} signal. Furthermore, the fura 2-Ca^{2+} signal and the contractile responses elicited by Hist 3 were totally blocked (Table 2).

Discussion

We have investigated the effect of modulators of L-type Ca^{2+} channels on the mobilization of intracellular Ca^{2+} stores in the longitudinal smooth muscle of guinea-pig isolated ileum. Using the fura 2 technique allowing the simultaneous measurement of $[Ca^{2+}]_i$ and contractile tone in isolated ileal strips, we have observed that the responses elicited by histamine or by drugs directly acting on the intracellular Ca^{2+} stores, were highly dependent on an influx through the L-type Ca^{2+} channels.

Casteels & Raeymaekers (1979) have reported that the intracellular Ca^{2+} stores in the guinea-pig longitudinal smooth muscle cells are particularly labile since in Ca^{2+}-free solution the response of this muscle to agonists or to caffeine declines rapidly. In Ca^{2+}-containing solution, the responses to histamine, to ryanodine, to thapsigargin and to caffeine were highly sensitive to blockade of voltage-dependent Ca^{2+} channels. In 1981, Bolton & Clark showed that histamine (100 μM) depo-

Table 2 Increase in fura 2 ratio and contractile response to histamine (10 μM) after depletion of Ca^{2+} stores and their refilling in various conditions

Agent in refilling medium	(M)	n	*Hist 3* *Fura 2 ratio* (% Hist 1)	*Contraction* (% Hist 1)
Control	–	8	38.5±8.0	14.6±2.7
Nimodipine	1 μM	5	1.5±1.5**	0.6±0.3
D600	10 μM	7	2.1±1.5**	0±0*
Bay K 8644	1 μM	8	59.9±5.0*	51.2±6.5**
Thapsigargin	1 μM	6	14.7±4.2**	15.1±2.5
Thapsigargin	1 μM	5	30.3±6.9††	44.2±9.4**
+Bay K 8644	1 μM			
Diazoxide	500 μM	6	1.0±1.0**	0±0*

Increases in fura 2 ratio and in contractile tone are expressed as percentage of the histamine (10 μM) responses evoked in physiological solution (Hist 1). All values represent mean±s.e.mean, $n=$ number of experiments. *$P<0.05$, **$P<0.01$, significantly different from control, ††$P<0.01$ significantly different from response elicited after treatment with Bay K 8644 alone. Hist 3: addition of histamine 10 μM in Ca^{2+}-free solution, after depletion of the stores in Ca^{2+}-free solution by Hist 2 and re-exposure to a Ca^{2+}-containing solution for 5 min in the presence or not of testing agents.

larizes the membrane of the guinea-pig ileum smooth muscle cells to a value of about -44.3 ± 1.6 mV which is close to the threshold potential for activation of the L-type Ca^{2+} channels. The inhibition of the sustained response to histamine by the Ca^{2+} entry blockers, nimodipine and D600, is consistent with this observation. However, the initial phasic response to histamine elicited in Ca^{2+} free solution, which is believed to be related to the activation of Ins(1,4,5)P_3 receptors (Donaldson & Hill, 1985), was also abolished. This indicates that Ca^{2+} entry blockade could affect the contractile response evoked by the release of intracellular Ca^{2+} stores.

Caffeine is known to evoke a direct and specific action on sarcoplasmic reticulum (Endo *et al.*, 1982). In the longitudinal smooth muscle of the guinea-pig ileum, caffeine evoked a smaller contractile response than histamine did while the Ca^{2+} signals were similar. This observation may be related to some other actions of caffeine. Indeed, Watanabe *et al.* (1992) have shown that in vascular smooth muscle, caffeine may directly influence the Ca^{2+}-tension relationship through an increase of cyclic AMP level caused by phosphodiesterase inhibition. However, Prestwich & Bolton (1995) have reported that up to 30 mM, caffeine had no effect on either basal or stimulated levels of cyclic AMP in the guinea-pig ileum. Rembold *et al.* (1995) have shown in swine carotid artery that caffeine evokes a relatively inhomogeneous cellular increase in $[Ca^{2+}]_i$, suggesting that caffeine may inhibit the contraction by localizing increases in $[Ca^{2+}]_i$ to small cytoplasmic regions distant from the contractile apparatus, a view in agreement with the observation of Chen & Van Breemen (1993). The dissociation that we observed between the Ca^{2+} signal and the contraction in the guinea-pig longitudinal smooth muscle exposed to caffeine could be related to such an effect, but this hypothesis needs further studies.

The high sensitivity of the caffeine response to the calcium entry blocker nimodipine is in agreement with the previous observations of Watson *et al.* (1988) who reported that the contractile response of the guinea-pig ileum smooth muscle to caffeine is completely abolished by nifedipine. The action of caffeine (at concentrations higher than 5 mM) may involve a depolarizing effect as reported by Itoh *et al.* (1983) in the rabbit mesenteric artery. This effect could lead to the activation of L-type Ca^{2+} channels producing a caffeine-evoked contraction sensitive to the action of nimodipine. However, caffeine still evoked a transient contraction in Ca^{2+} free solution (data not shown) indicating that part of the response was mediated through the release of intracellular Ca^{2+} stores. As for histamine, the Ca^{2+}-releasing action of caffeine was sensitive to nimodipine pretreatment confirming the functional similarity between the two intracellular Ca^{2+} releasing processes resulting respectively from Ins(1,4,5)P_3 and ryanodine receptors, a conclusion in agreement with the observation that those receptors are associated with a common subcellular structure (Wibo & Godfraind, 1994).

Ryanodine and thapsigargin have been used by several authors to characterize the function of intracellular Ca^{2+} stores. Thapsigargin is a specific inhibitor of the SR Ca^{2+}-ATPase. It prevents the filling of the Ca^{2+} stores, but when used at concentrations higher than 1 μM, it interacts with L-type Ca^{2+} channels (Buryi *et al.*, 1995). Ryanodine has been shown to lock the sarcoplasmic reticulum (SR) Ca^{2+} release channels in an open state (Hwang & Van Breemen, 1987), and thereby inhibits or attenuates agonist-induced contraction related to Ca^{2+} release (Kanmura *et al.*, 1988; Ashida *et al.*, 1988). The action of ryanodine appears to be complex (Wagner-Mann *et al.*, 1992; Lynn *et al.*, 1993) although the drug acts on a well identified receptor associated with SR (Wibo & Godfraind, 1994). In the presence of extracellular Ca^{2+}, ryanodine always evoked a rise in $[Ca^{2+}]_i$ in vascular smooth muscle preparations (Hisayama *et al.*, 1990; Wagner-Mann, 1992) but its action on the contractile tone is more controversial: some authors reported a large ryanodine-evoked contraction (Hisayama *et al.*, 1990) while others observed no contraction (Julou-Schaeffer & Freslon, 1988) or a small development of tension (Shima & Blaustein, 1992). At variance with observations done in rat aortic smooth muscle cells where the ryanodine- and the thapsigargin-evoked increases in tone were resistant to the L-type Ca^{2+} channel blocker, nicardipine (Shima et Blaustein, 1992; Xuan *et al.*, 1992), in guinea-pig intestinal smooth muscle, both ryanodine and thapsigargin evoked increases in $[Ca^{2+}]_i$ and contraction were blocked by nimodipine, by D600 and furthermore attenuated by diazoxide, an agent known to hyperpolarize smooth muscle cells. Since nimodipine and D600 are Ca^{2+} channel blockers and since a hyperpolarizing stimulus may close Ca^{2+} channels, this indicates that those responses were dependent on the entry of Ca^{2+} through L-type Ca^{2+} channels.

The transient fura 2-Ca^{2+} signal evoked by histamine in Ca^{2+}-free medium was used as an index of the extent of refilling of the Ca^{2+} stores previously emptied by stimulation with histamine in the absence of external Ca^{2+}. When the refilling medium contained a Ca^{2+} channel modulator, this response was modified: blocked by nimodipine or D600 and augmented by the Ca^{2+} channel activator Bay K 8644, suggesting the implication of a Ca^{2+} influx from the extracellular space through the L-type Ca^{2+} channels in the refilling process. The refilling process was prevented by a hyperpolarizing agent such as diazoxide, which closes Ca^{2+} channels. Furthermore, thapsigargin inhibition of stores refilling was overcome by Bay K 8644, a dihydropyridine with Ca^{2+} channel activating properties. This indicates that Ca^{2+} intracellular stores may be refilled through a thapsigargin-sensitive Ca^{2+} pump activated by Ca^{2+} entering the cytoplasm through L-type Ca^{2+} channels.

Ohta *et al.* (1995) have reported that in the longitudinal ileum of the rat, the emptying of intracellular Ca^{2+} stores evokes a Ca^{2+} influx and a contraction which are insensitive to D600 suggesting that the entire refilling of the intracellular Ca^{2+} stores in the rat ileum occurs through a pathway different from L-type Ca^{2+} channels. As shown in this paper, both histamine-evoked contraction and Ca^{2+} signal measured in guinea-pig ileum after refilling of depleted stores were absent when the refilling medium was supplemented with Ca^{2+} entry blocker. This indicates that the mode of refilling of intracellular Ca^{2+} stores in ileum may be different among animal species.

We are indebted to Greet Leonardy for her valuable technical assistance. This work was supported by the Fonds de la Recherche Scientifique Médicale (grant FRSM n° 3.4597.93), and the Ministère de l'Education et de la Recherche Scientifique (grant n° 89/95-135).

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(Received January 10, 1996
Revised May 28, 1996
Accepted May 29, 1996)