L-Type Calcium Channels May Fill Directly the IP_3-sensitive Calcium Store

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Abstract. The relationship between mositol-1 4 5-trisphosphate-sensitive Ca^{2+} stores and Ca^{2+} entry through potential-dependent L-type Ca^{2+} channels was examined using whole-cell voltage-clamp technique in cells of longitudinal muscle layer of guinea-pig ileum It was found that heparin (10^{-10} mol/l) in the pipette rapidly inhibited the current through L-type Ca^{2+} channels Neither an inhibitor of the sarcoplasmic reticulum Ca^{2+} pump (cyclopiazonic acid) nor blockers of Ca^{2+}-induced Ca^{2+} release (ryanodine or ruthenium red) affected the Ca^{2+} current The failure of heparin to affect Ca^{2+}-currents through L-type Ca^{2+} channels in cells from circular muscle of the same organ suggested that heparin had no direct effect on L-type Ca^{2+} channels Thus the inhibition of the latter in heparin-loaded cells from the longitudinal layer is supposed to be Ca^{2+}-dependent due to the overfilling of the mositol-1,4 5-trisphosphate-sensitive Ca^{2+} store

Key words: Heparin Cyclopiazonic acid Ruthenium red — Ca^{2+} stores L-type Ca^{2+} channels — Smooth muscle

Introduction

It is widely accepted that Ca^{2+} which triggers contraction in smooth muscle enters the cell from the outside or is derived from the mositol-1 4 5-trisphosphate-(IP_3)-sensitive Ca^{2+}-pool (Berridge and Irvine 1989, Walsh 1991 for review see Missiaen et al 1992) But the feedback mechanisms by which cells regulate the quantity of depolarization-evoked calcium entry depending on the concentrations of Ca^{2+} ions stored still remain unclear (Casteels et al 1992) The existence of a close contact between plasmalemma and the membranes of IP_3-sensitive store (Somlyo 1985 van Breeman and Saida 1989 Stehno-Bittel and Sturek 1992) indicate the possibility of direct refilling of this pool by voltage-dependent Ca^{2+} entry (Bourreau

et al. 1991; Daniel et al. 1992). The latter could be provided by the L-type Ca^{2+} channels, known to undergo, in a number of tissues, a very weak Ca^{2+}-dependent inactivation, even at relatively high densities of Ca^{2+} currents (Ganitkevich et al. 1987; Yamamoto et al. 1989). We evaluated this possibility on guinea-pig ileal smooth muscle cells freshly isolated from the longitudinal layer. The ionic currents in membranes of these cells have been characterized previously (Droogmans and Callewaert 1986; Duridanova et al. 1992), and the L-type Ca^{2+} channels were found to be the only voltage-dependent Ca^{2+} channels there. Recently Zholos et al. (1991) reported that procaine, which is known to block the Ca^{2+}-induced Ca^{2+} release (Endo 1977), caused progressive decrease of inward Ca^{2+} current (I_{Ca}) in the same cells, but did not discuss this finding. Uyama et al. (1993) showed that the cyclopiazonic acid, an inhibitor of sarcoplasmic Ca^{2+} pump, increases the influx of calcium through voltage-gated calcium channels, and supposed "a direct regulation of Ca^{2+} channels activity by the intracellular calcium storage sites". In our experiments we tried to manipulate the functional availability of the stored calcium by "locking" it separately: into IP_3-sensitive stores located near the sarcolemma (see Missiaen et al. 1992), and the Ca^{2+}-induced release sites which may be located deeper in the cells (Zholos et al. 1992; Low et al. 1992). The data presented suggest that L-type Ca^{2+} channels conduct Ca^{2+} directly into the IP_3-sensitive pool, whose overloading leads to the Ca^{2+}-dependent inhibition of I_{Ca}.

Materials and Methods

Experiments were performed on single smooth muscle cells freshly isolated from the longitudinal or circular layer of the guinea-pig ileum. The animals weighed 300-400 g. Isolated muscle strips were placed in physiological salt solution (PSS) and cut into small pieces. The pieces were then transferred into the PSS without the addition of Ca^{2+} at 37 °C and containing 0.5 g/l collagenase (type 1A, Sigma), 1.5 g/l soybean trypsin inhibitor and 2 g/l bovine serum albumin. After 30–40 min of incubation at 37 °C the enzyme was carefully washed from the pieces with 20 ml prewarmed Ca^{2+}-free PSS. Single smooth muscle cells were then obtained by gentle agitation of the pieces with two Pasteur pipettes with different tip openings in 1 ml fresh modified "KB" solution until the solution became cloudy. In this solution cells were stored up to 12 h at 6 °C for this study. For the purpose of the study only fully relaxed cells were used. Cells which reduced their length by more than 35% or did not contract at all after application of 10^{-6} mol/l acetylcholine were rejected, as well as those that did not relax after acetylcholine-induced contraction. PSS and drugs were perfused continuously to the bath chamber where cells adhered to the glass bottom.

The whole-cell mode of the patch-clamp technique was used. The patch electrodes were from borosilicate glass (Jencons) which, when filled with the internal solution, had resistances of approximately 1.5 MOhms. Membrane currents were recorded using an EPC-7 (List Electronics) amplifier. The capacitive and series resistance compensations were made by the use of potentiometer furnished at the amplifier. Current signals were

recorded and further analysed on an AT 286 PC through a TL-1 DMA (AXOPATCH) interface, and pCLAMP software, respectively, and simultaneously displayed on an oscilloscope monitor.

The physiological salt solution (PSS) in the experiments was of the following composition (in mmol/l): 110 NaCl, 12 KCl, 10 HEPES, 20 taurine, 20 glucose, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 5 Na-pyruvate, pH 7.4. I_{Ca} were recorded in the presence of 5 mmol/l tetraethylammonium. The modified "KB" medium, used in cell isolation consisted of 85 KCl, 30 KH_2PO_4, 5 $MgCl_2$, 20 taurine, 5 Na_2-ATP, 5 Na-pyruvate, 5 creatine, 5 oxalacetate, 1 g/l bovine serum albumin (pH 7.2). The internal solution into the recording pipette contained: 105 KCl (or CsCl instead of KCl while recording inward currents), 10 HEPES, 11 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 4 Na-pyruvate, 4 succinic acid, 4 oxalacetic acid, 1.5 Na_2-ATP, 0.001 cyclic AMP, pH 7.2. Ruthenium red or light heparin (Sigma) were diluted into the internal solution. The cyclopiazonic acid (Sigma) and ryanodine (Calbiochem) were added to the bath solution. All experiments were carried out at room temperature (25 ± 2 °C).

Results

Effects of heparin on I_{Ca}

Fig. 1*A* (inset) shows typical I_{Ca} waveforms, elicited by depolarization in cell from the longitudinal layer, recorded by a caesium-loaded pipette to block potassium outward currents and the corresponding current-voltage relation curve (I/V-curve), obtained under the holding potential (V_h) of -50 mV (Fig. 1*A*), which is close to the resting potential of these cells (Droogmans and Callewaert 1986). In agreement with earlier studies on the same cells (Duridanova et al. 1993), this inward current is carried exclusively through the L-type (dihydropyridine-sensitive, long-lasting, high-threshold) Ca^{2+} channels (Tsien 1983), because it can be strongly and reversibly inhibited by nifedipine (Fig. 1*A*), or activated by micromolar amounts of the dihydropyridine BAY K 8644 (Droogmans and Callewaert 1986). On dialysing cells with heparin-containing internal solution (10^{-10} mol/l and above) a fast decrease of inward current amplitude developed (Fig. 1*B*), and complete and irreversible block of I_{Ca} was reached in about 6 min after the beginning of the dialysis. A subsequent change of bathing solution to Ca^{2+}-free or Ba^{2+}-containing (instead of Ca^{2+}) solutions (Fig. 1*C*), or changes in V_h to various more negative values up to -120 mV did not recover from inactivation the inward current in cells from longitudinal layer (not shown). This led us to the suggestion that the block of I_{Ca} was caused by elevated intracellular calcium concentration – $[Ca^{2+}]_i$, which stopped the ion entry into the channel, according to the model proposed for neurons and heart (Hess et al. 1989; Rosenberg and Chen 1991).

Effect of blocking intracellular Ca^{2+} stores

In control experiments (cells from longitudinal layer dialysed with heparin-free solution), I_{Ca} was not affected by ryanodine (10^{-6} mol/l) or when Ca^{2+} uptake into

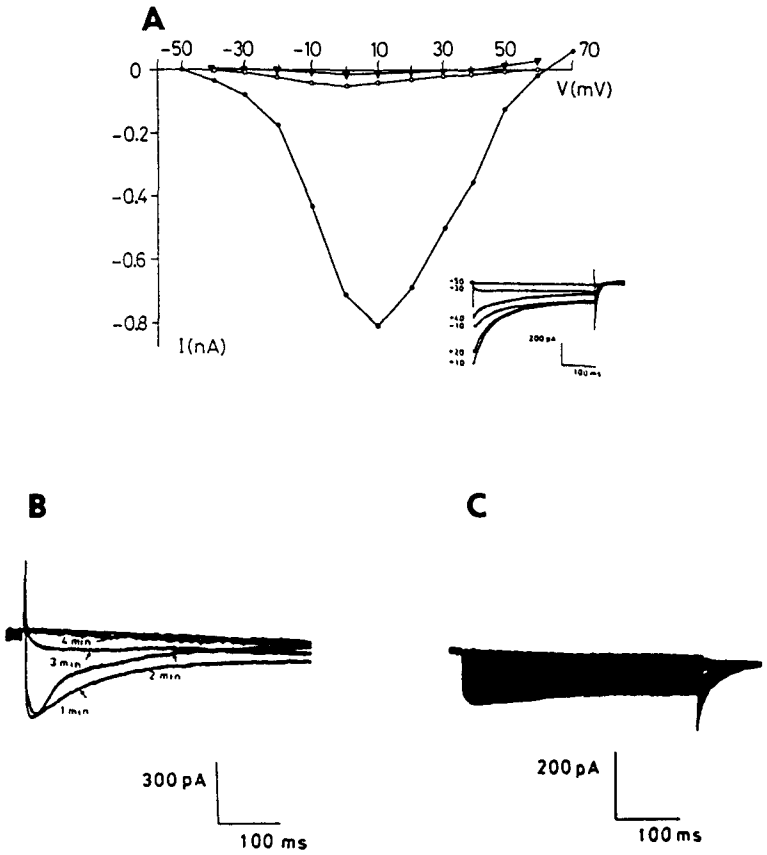

Figure 1. Comparison of the ability of nifedipine and heparin to block Ca^{2+} current in cells isolated from longitudinal layer (*A*) Current-voltage (*I/V*) relation curve of I_{Ca}, obtained under $V_H = -50$ mV control (closed circles) in the presence of 0 03 mmol/l nifedipine (open circles) or with 0 1 mmol/l heparin into the pipette solution (triangles) measured 10 ms after the voltage step Cell with input impedance 1 2 GOhms and capacitance 13 pF Inset original records of depolarization-elicited I_{Ca} waveforms of the same cell (photographed from the oscilloscope monitor) Depolarization steps applied to the potentials signed from $V_h = -50$ mV (potential applied here and at the next figures are marked with digits) (*B*) Blocking effect of heparin (0 1 mmol/l) on I_{Ca} in the same cell as in (*A*) $V_h = -50$ mV, test potential to +10 mV min after the beginning of the dialysis are signed at the figure (*C*) The same voltage protocol as in (*A*) was applied at the 12th min after the beginning of the dialysis in the same cell and at the 6th min after replacing the Ca^{2+}- with Ba^{2+} containing (2 5 mmol/l) external solution

internal stores was inhibited by 10^{-6} mol/l cyclopiazonic acid (Fig 2), a selective inhibitor of the Ca^{2+} pump of the sarcoplasmic reticulum in striated (Goeger et

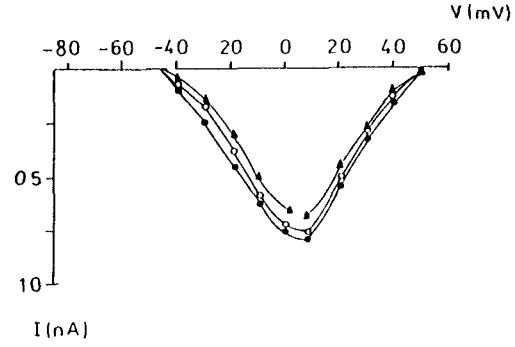

Figure 2. I/V-relation curves of I_{Ca} in cells from longitudinal layer voltage clamped at $V_h = -50$ mV in control solution (filled circles) in the presence of cyclopiazonic acid 10^{-6} mol/l (triangles) or ryanodine (10^{-6} mol/l) (open circles). Rectangular depolarizing pulses of 400 ms duration were applied at 10 mV increments. Data are means for 10 cells with similar passive electrical properties. There were no significant differences. Deviation bars are not presented for clarity.

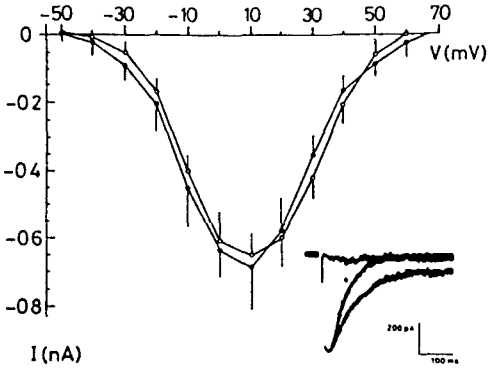

Figure 3. Effect of ruthenium red (0.1 mmol/l) on I_{Ca} in cells from longitudinal layer. I/V curve obtained as described in legends of the previous figures. Before (control, closed circles) and after (open circles) dialysis with ruthenium red containing internal solution. Data are means ± S.E.M. for 12 cells. $V_h = -50$ mV. Inset: acceleration of whole cell inward current inactivation by ruthenium red. Original records from cell with input impedance of 1.1 GOhms and capacitance 39 pF. $V_h = -50$ mV, test potential to +10 mV before and after (marked with circle) 10 min of dialysis with ruthenium red containing internal solution. Constants of inactivation estimated: 102.6 ms without and 73.9 ms in the presence of ruthenium red (104.3 ± 6.7 ms and 72.7 ± 7.2 ms, respectively, are means ± S.E.M. for 7 cells).

al. 1988; Seidler et al. 1989) and smooth muscle (Bourreau et al. 1991; Imaizumi et al. 1992; Uyama et al. 1992; Low et al. 1992). Similar lack of effects on the

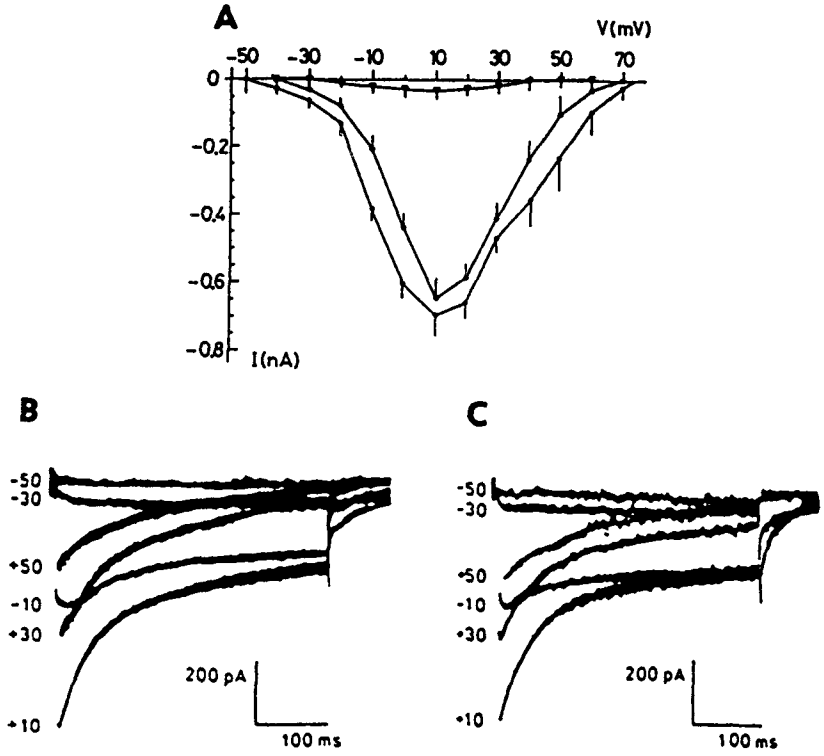

Figure 4. Effect of heparin on I_{Ca} in cells from the circular layer of the ileum. (*A*) I/V-curve of I_{Ca}, elicited by depolarization from $V_h = -50$ mV (closed circles), in the presence of 0.03 mmol/l nifedipine (triangles) or after dialysis with heparin-containing (1 mmol/l) internal solution (open circles). Data are means ± S.E.M. for 6 cells. (Half-bars are drawn in opposite directions for clarity). (*B*) Original records of depolarization-evoked I_{Ca} in cell from circular layer with input impedance of 1.9 GOhms and capacitance 62 pF. Depolarization steps were applied to potentials signed. (*C*) Current records in the same cell after 10 min of dialysis with heparin-containing (1 mmol/l) internal solution.

amplitudes, activation threshold and reversal potential of depolarization-elicited I_{Ca} (Fig. 3) was obtained when 10^{-7} to 10^{-4} mol/l ruthenium red, a widely used blocker of Ca^{2+} release channels of Ca^{2+}-sensitive Ca^{2+} pool (Moutin et al. 1992) was present in the pipette. Thus the inhibition of Ca^{2+}-induced Ca^{2+} release or emptying of Ca^{2+} stores did not inhibit I_{Ca}. This implies that only stores with IP_3-sensitive release sites are near plasmalemma (the inner mouth of the L-type Ca^{2+} channels) and that there is an overfilling of the stores rather than general elevation of $[Ca^{2+}]_i$ which influences I_{Ca}. However, an acceleration of whole-cell

I_{Ca} inactivation was observed to increase with time of dialysis (Fig. 3, inset). The latter was taken as an evidence for Ca^{2+}-dependent inactivation of L-type channels (Eckert and Chad 1984; Campbell et al. 1988), caused by the relative increase in the concentration of Ca^{2+} in the IP_3-sensitive Ca^{2+} pool, due to the block of the Ca^{2+}-induced Ca^{2+} release mechanism. It can be suggested that both pools may have area of close contact where they exchange Ca^{2+}. Ruthenium red holds the deeply situated Ca^{2+}-sensitive Ca^{2+} pool (Zholos et al. 1991) full and "locked" which hinders the entry of Ca^{2+}, released from the IP_3-sensitive Ca^{2+} pool in that regions, when depolarization activates I_{Ca}.

Does heparin bind the L-type Ca^{2+} channels or does it block the L-type channels indirectly?

We examined further the possibility for heparin to interact directly with the channel pore from the inside. For this purpose we isolated cells from the circular layer of the same organ (guinea-pig ileum), using the procedure described above. Under the same V_h depolarization-evoked I_{Ca} in these cells had very similar I/V-relation properties and dihydropyridine sensitivity as the one observed in cells from longitudinal layer (Fig. 4*A*, *B*; the low-threshold dihydropyridine-resistant component of I_{Ca} in cells, isolated from circular layer was negligible if $V_h = -50$ mV was held – for more details see Duridanova et al. 1993). But this current could not be affected by heparin even at 1 mmol/l concentration (Fig. 4*C*). In some heparin-loaded cells I_{Ca} tended to accelerate its inactivation course, but this effect was not significant. It was concluded that heparin is not able to interact with L-type Ca^{2+} channels. The lack of action of heparin in cells from circular layer may reflect the absence of a close connection between IP_3-sensitive stores and L-type Ca^{2+} channels.

Discussion

The above data were taken as a proof that effects of heparin in cells from longitudinal layer of guinea-pig ileum are specific for some cell types and do not include a general ability of heparin to interact with the channel molecule. The latter conclusion is consistent with the experimental results on cardiac myocytes (Lacinova et al. 1993) and is also in agreement with the high efficiency of the heparin-induced I_{Ca} blockade observed in cells from longitudinal layer at concentrations of 100 times lower than expected (Kobayashi et al. 1988). As heparin is known to be the most powerful and selective inhibitor of IP_3-induced Ca^{2+} release in smooth muscle cells (Ghosh et al. 1988; Kobayashi et al. 1989, Chadwick et al. 1990), where it prevents the interaction of IP_3 with Ca^{2+} release channels (Kobayashi et al. 1989), the most obvious explanation of the above data is that the inner mouth of L-type Ca^{2+} channels opens directly into this pool. In this way, the Ca^{2+} ions stored and "locked" into the pool by the heparin blockade, cannot be released by conventional

stimuli, so the pool remains heavily loaded with Ca^{2+} which inhibits subsequent movements of ions through L-type channels from the outside This explanation is also consistent with cytomorphological data, which have shown IP_3-binding sites on sarcoplasmic reticulum to be placed close to the inner face of plasmalemma (van Breeman and Saida 1989 Missiaen et al 1992)

Effectiveness of cyclopiazonic acid to raise $[Ca^{2+}]_i$ in cells from longitudinal layer initially was shown on K^+ current measurements (Gagov et al 1993b) It was also found that the long-lasting (10 min and more) exposure of ileal cells to cyclopiazonic acid (10^{-6} mol/l) leads to a selective blockade of the Ca^{2+}-sensitive K^+ conductance (Suzuki et al 1992, Gagov et al 1993a) Our experiments have also shown that in heparin loaded cells from longitudinal layer of the ileum cyclopiazonic acid causes almost a total inhibition of K^+ currents in 5 min (Gagov et al 1993b) The Ca^{2+} supporting K^+ outward currents in heparin-loaded cells enter through L-type Ca^{2+} channels even though I_{Ca} could not be recorded in these conditions (Gagov et al 1993b) These data showed that when Ca^{2+} was locked into IP_3-sensitive stores by heparin during the depolarization there was an elevation of $[Ca^{2+}]_i$ near the membrane which presumably originated from leakage out of overfilled stores through the cyclopiazonic acid-sensitive Ca^{2+} pump Such mechanism has been proposed in regulation of Ca^{2+} store refilling in tracheal smooth muscle (Janssen and Sims 1993) Thus we supposed that this enormous (due to the overfilling of the stores) Ca^{2+} leak from the IP_3 sensitive Ca^{2+}-store was directed to the inner mouths of the Ca^{2+}-sensitive K^+ channel pores It is also consistent with evidence that emptying the Ca^{2+} pool promotes Ca^{2+} entry into the pool (Bourreau et al 1991)

In conclusion the data obtained provide evidence that in some smooth muscles the IP_3-sensitive Ca^{2+}-pool undergoes a voltage-dependent Ca^{2+} refilling through L-type Ca^{2+}-channels This mechanism can be effectively antagonized by intracellularly applied heparin

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