Participation of Calcium, Released from the IP_3-Sensitive Ca-Store in Activation of Ca-Dependent Potassium Conductance of Ileal Smooth Muscle Cells

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Abstract. The apamin-, charibdotoxin- (CTX) and glibenclamide- (GLB) sensitive components, which form the active part of the net potassium outward current (I_K) in single smooth muscle cells from the longitudinal layer of guinea-pig ileum (LC), were investigated for their sensitivity to calcium. The experiments were carried out by the whole-cell voltage-clamp method. A successful block of all Ca-sources (with heparin and nifedipine; heparin and cyclopiazonic acid while the intracellular Ca-concentration – $[Ca^{2+}]_i$-was kept at $3 \cdot 10^{-8}$ mol/l by 11 mmol/l EGTA into the pipette solution) led to the complete inhibition of I_K. The deeply located Ca-sensitive Ca-pool was effectively isolated by the high concentration of the chelator, which was proved by the fact that ruthenium red and ryanodine failed to affect I_K. The GLB-sensitive component of I_K demonstrated Ca-gated properties, while both the other components were activated most probably by calcium, released form the IP_3-sensitive Ca-pool. It was concluded that the IP_3-induced Ca-release mechanism plays an important role in the regulation of K^+-conductivity in LC.

Key words: Calcium stores — Potassium outward currents — IP_3-induced calcium release — Heparin — Ileum

Introduction

Recently it has been widely accepted that intracellular calcium regulates the membrane potassium conductance in a variety of tissues (McCann and Welsh 1986; Ohya et al. 1987; Latorre et al. 1989; Kato et al. 1992; for review see McManus 1991). The general properties of Ca-dependent K-channels in smooth muscle cell membranes have been extensively investigated and thoroughly described (McManus 1991; see also Singer and Walsh 1986). It seems that these K-channels may be

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"suitable intracellular calcium indicators near the membrane intracellular surface" (Zholos et al. 1991). However, it is still unclear where the calcium, needed for the activation of this type of K-conductance ($I_{K(Ca)}$) comes from. For example, in some cell types there is a component of the Ca-dependent K-current ($I_{K(Ca)}$), activated directly by Ca-influx (Zholos and Baidan 1989; Hisada et al. 1990). But there are also a number of studies on cardiac (Maylie and Morad 1984; Hiraoka and Kawano 1989) and intestinal (Zholos et al. 1991) muscles, which describe a transient $I_{K(Ca)}$, whose activation has been related to a Ca-induced Ca-release (CICR) from the sarcoplasmic reticulum (SR). The participation of the calcium released by the IP_3-induced Ca-release mechanism (IICR) seems to be poorly investigated at that moment. Some cytomorphological data showed that the IP_3-sensitive Ca-pool in a number of smooth muscle cells is most probably placed close to the inner surface of the plasmalemma (Somlyo 1985; van Breemen and Saida 1989). Recent data showed that the basal IP_3-turnover may provide a negative feed-back control of contraction (Ozaki et al. 1992). It is established that the IP_3-sensitive Ca-pool is a distinct membrane compartment, separated from the Ca-sensitive one (see Berridge 1984 for review; Berridge 1992) and that both pools participate in contraction development by different pathways (Kobayashi et al. 1988). The fact that the IICR mechanism is involved in receptor activation by a number of agonists seems to be undoubted. But Somlyo and Somlyo (1992) showed also that in permeabilized ileal smooth muscle cells the heparin-induced inhibition of IICR led to 2-fold decrease in the force of the depolarization-induced contraction, while the intracellular calcium concentration rose 1.5-fold at the same time. Thus, it seemed interesting for us to study the role of IICR mechanism in regulation of K-conductance in smooth muscle cell membranes. For this purpose we used a high EGTA-containing internal solution to isolate the more deeply situated cisternae of the SR by the superficial ones. The data obtained suggests that Ca-dependent K-conductance in ileal smooth muscle cells could be regulated by Ca^{2+} derived from the IP_3-sensitive Ca-store.

Materials and Methods

Experiments were performed on single smooth muscle cells, freshly isolated from the longitudinal layer of the guinea-pig ileum. The animals weighted 300–400 g.

Cell isolation. Isolated muscle strips were placed in physiological salt solution (PSS), without Ca^{2+} in it, and cut into small pieces. The pieces were then transferred into the fresh Ca^{2+}-free PPS, containing 1 g/l collagenase (type A1, Sigma), 1.5 g/l soybean trypsin inhibitor and 2 g/l bovine serum albumin, and temperated at 37 °C. After 30–40 min of incubation at this temperature the enzyme was carefully washed from the pieces with 20 ml prewarmed PPS. Single smooth muscle cells were then obtained by gentle agitation of the pieces with two Pasteur pipettes with different tip opening in 1 ml fresh modified "KB" solution (Isenberg and Klockner 1982), containing (in mmol/l): 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) until the solution became cloudy. In this solution cells were stored up to 12 hours at 6 °C for the study. In control experiments LC exhibited outward current amplitudes varying from cell to cell, but generally at +40 mV test potential (from $V_h = -50$ mV) they were between 0.9 and 2.3 nA. In the majority of cells the outward current amplitudes at this test potential were about 1.0–1.2 nA, and membrane capacitances between 38 and 45 pF. Only these cells were used in the present study. All experiments were performed under $V_h = -50$ mV, which was assumed as a value close to the resting potential of these cells (Droogmans and Callewaert 1986).

Electrical recordings. Whole-cell mode of the patch-clamp technique was used (Hamill et al. 1981). The patch electrodes from borosilicate glass (Jencons), filled with the internal solution had resistance approx. 1.5 MΩ. Membrane currents were recorded using a EPC-7 (List Electronic) amplifier. The capacitive and series resistance compensations, were made by the use of potentiometers furnished at the amplifier. The leakage current component was subtracted electronically before amplification. Current signals were recorded and further analyzed on an AT 286 PC through a TL-1 DMA (AXOPATCH) interface and pCLAMP software resp., and simultaneously displayed on an oscilloscope monitor.

Solution and drugs. The PSS usually used as a bathing solution, 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, 5 tetraethylammonium, 0.01 tetrodotoxin, pH 7.4. Into the Ca-free PSS $CaCl_2$ was replaced by NaCl. The internal solution for recording of the outward currents contained: 105.5 KCl, 10 HEPES, 11 K_2-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-sodium salt, pH 7.22. While the inward currents were recorded, K^+ into the pipette solution was repaced by Cs^+. Ruthenium red (Sigma) or light heparin (Sigma) were diluted into the internal solution. The latter was exchanged as it has been described by Zholos et al. (1991). The concentrations of every drug, needed to cause the total block of the corresponding I_K -component was accepted as maximally effective if further elevation of its concentration into the bath solution failed to cause further decrease in I_K amplitudes. All experiments were carried out at 32 °C temperature.

Results

1. Pharmacological dissociation of I_K into three different components.

It has been previously reported, that components of the outward current in LC could not be separated by their different activation threshold or voltage-dependence (Droogmans and Callewaert 1986; Duridanova et al. 1992). Under our experimental conditions ($V_h = -50$ mV, 11 mmol/l EGTA into the K-pipette solution) the whole-cell current of isolated smooth muscle cells from the longitudinal layer of guinea-pig ileum exhibited generally the same pattern, as it was described previously by Droogmans and Callewaert (1986). It consisted of one transient inward current, whose development was interrupted early by an outward current. At membrane potentials over +30 mV, where the inward current contaminations were negligible, it peaked at 15–20 ms after the beginning of the pulse (Fig. 1*A*(*a*)) and then decayed slowly (Fig. 1*A*(*b*)). This net outward current (I_K) was dissected

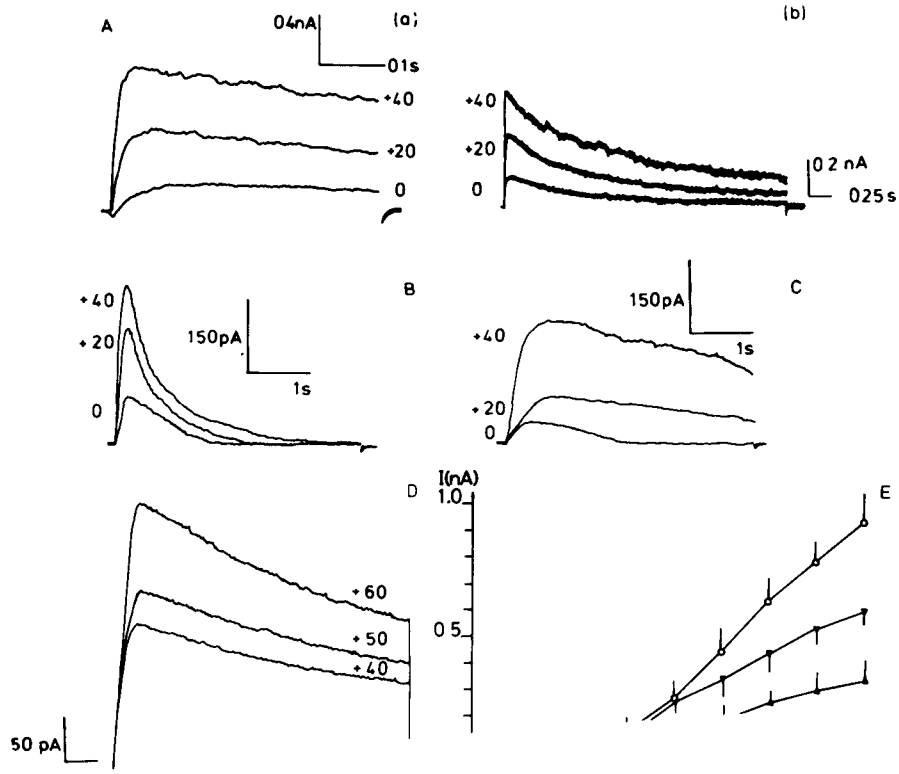

Figure 1. Whole cell outward current (I_K) in LC. (A) Original records of depolarization-elicited I_K waveforms in LC with 39 pF membrane capacitance, bathed in PSS. V_h = −50 mV. Depolarization steps applied here and in the next figures are signified with digits. Duration of the pulses 400 ms in (a) and 4 s in (b). (B − D) Time course of activation and inactivation of the three pharmacologically separated components of I_K in LC (current waveforms were obtained as described in the text): (B) The component, which was completely blocked by charibdotoxin (10^{-6} mol/l), had inactivation constants τ_{in} = 580 ± 69 ms at +40 mV, 652 ± 34 ms at +20 mV, and 950 ± 76 ms at 0 mV depolarizations; data are means ± S.E.M. for 12 LC; (C) The component, which was completely blocked by apamin ($5 \cdot 10^{-5}$ mol/l) was slowly activating one, with τ_{in} = 1960± 59 ms at +40 mV, 2153 ± 87 ms at +20 mV and 2980 ± 108 ms at 0 mV test potentials; data are means ± S.E.M. for 8 LC; (D) The component, which was completely inhibited by glibenclamide (GLB) (10^{-5} mol/l) had a fast activation course, and τ_{in} = 122.1 ± 3 ms at +60 mV, 191.8 ± 6 ms at +50 mV, and 157 ± 4 ms at +40 mV test potentials; data are means ± S.E.M. for 6 LC. (E) Current-voltage (I/V) relations of the net I_K (open circles) in LC and of the current, which remained after addition of CTX (open triangles), apamin (solid triangles) and GLB (solid circles) in 6 LC of good quality, kept at V_h = −50 mV. Half-bars representing the S.E.M. from the mean value, are drawn this way for clarity.

at separate components on the basis of its sensitivity to three selective K-channel blockers (Fig.1*B–E*): charibdotoxin (CTX), apamin and glibenclamide (GLB). Every one of these pharmacologically separated components was obtained by subtraction of the depolarization-elicited waveforms, recorded in the presence of the corresponding drug by the total I_K, elicited by the corresponding depolarization steps (Fig. 1*B–D*). The GLB-sensitive component of I_K was measured at +60, +50 and +40 mV test potentials, because it developed at the same time as the inward current did, but the contamination on the latter in I_K over +40 mV depolarizations is negligible (see Droogmans and Callewaert 1986). The analysis of tail currents, performed before and after the application of the drugs showed that all components of I_K, separated by such a procedure, possessed high selectivity to potassium (not shown). Fig. 1*E* shows that up to 0 mV the net I_K consisted of GLB- and apamin-sensitive components, which inhibited about 48% and 50% resp. of the net I_K, while CTX was ineffective at negative membrane voltages. Into the potential range from +10 to +40 mV GLB blocked 33–23%, apamin - 32–21%, and CTX - 19 to 40 % of I_K resp. More than that, each one of the drugs blocked the same percent of I_K if added into the solution containing one or both the other drugs, and the order of addition did not affect its blocking ability. It was suggested that every one of the K-channel blocking drugs tested inhibited a separate population of K-channels, as it has been expected. The part of I_K, which could not be blocked by the antagonists (see Fig. 1*E*) reflected the linear leakage of the cell membrane.

2. The usage of a high concentration of EGTA for the removal of calcium, released by the Ca-dependent Ca-store.

The reliability of the high EGTA-containing pipette solution (11 mmol/l EGTA, together with 1 mmol/l Ca^{2+} gave a final intracellular calcium concentration $[Ca^{2+}]_i$ about $3 \cdot 10^{-8}$ mol/l, which is close to the resting levels of $[Ca^{2+}]$ in smooth muscle cells, (see Bond et al. 1984) for isolation of Ca-sensitive Ca-store from the superficially situated IP_3-sensitive one (to eliminate the effects of CIRC) was tested by the use of ruthenium red and ryanodine (see Iino et al. 1988; also van Breemen and Saida 1989). Fig. 2*A* shows that neither ruthenium red ($10^{-6} - 10^{-4}$ mol/l), nor ryanodine ($10^{-6} - 10^{-5}$ mol/l) influenced the amplitudes and current-voltage (I/V) relations of the net I_K in LC under these conditions. The dialysis of cells with heparin-containing internal solution ($10^{-10} - 10^{-6}$ mol/l) was without significant effects on I_K amplitudes and I/V relations either. However, it turned out that 10^{-10} mol/l heparin was sufficient to block almost entirely the inward Ca-current, recorded by the use of Cs^+-containing internal solution (Fig. 2*B*). The situation became more complicated with the finding that I_K in heparin-loaded cells could be almost totally abolished by externally applied nifedipine ($5 \cdot 10^{-5}$ mol/l), which left only the linear leakage current (Fig. 2*C*).

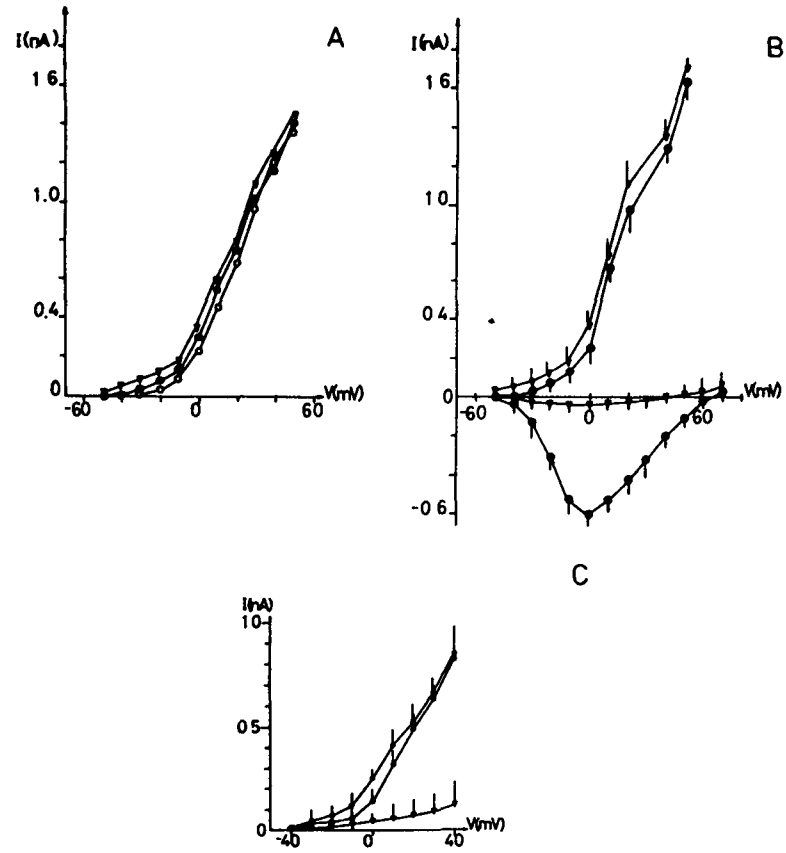

Figure 2. Effect of ruthenium red (10^{-5} mol/l), or heparin (10^{-10} mol/l), applied intracellularly, or ryanodine (10^{-6} mol/l) into the bathing solution on I_K in LC (*A*) I/V-relation curves of the net I_K, obtained by conventional voltage protocol in PSS (open circles), in the presence of ryanodine (solid circles), and in cells, dialyzed with ruthenium-red containing internal solution (solid triangles) Data are means ± S E M of 12 LC, bars are not represented for the clarity of the drawings (*B*) I/V-relations of I_K and I_{Ca} in 5 LC, kept at $V_h = -50$ mV in control conditions (solid circles) and after dialysis with heparin-containing internal solution (solid triangles) (*C*) I/V-relation curves of I_K in 5 cells, obtained under $V_h = -50$ mV in PSS (open circles), at 5^{th} min of dialysis with heparin-containing internal solution (solid circles), and at the 8^{th} min after the addition of 5 10^{-6} mol/l nifedipine into the bath (open triangles)

3. In order to explain the interrelations between calcium, which enters the cell from the outside and calcium, stored into the IP_3-sensitive pool, and the participation of both these Ca-sources in activation of the Ca-dependent potassium

conductance, we studied the effects of nifedipine and heparin on the three pharmacologically separated components of I_K.

3.1. Dependence of different I_K components on Ca entry.

The I/V relations of the CTX-sensitive component of I_K obtained by conventional voltage protocol in PSS, and in the presence of $5 \cdot 10^{-5}$ mol/l nifedipine into the bath solution (Fig. 3*A*) revealed the independence of this component from the calcium entry under our experimental conditions. Current amplitudes were measured at 200th ms of the pulses with increasing voltage when this component reached its maximum (see also Fig. 1*B*).

The apamin-sensitive component of I_K, which showed slow course of activation (see Fig. 1*C*) appeared too dependent on the Ca-entry (Fig. 3*B*). Under our experimental conditions nifedipine ($5 \cdot 10^{-5}$ mol/l) inhibited almost totally this component of I_K.

The effect of nifedipine on the I/V relation of the GLB-sensitive part of the I_K was observed measuring the maximum amplitudes at 20th ms of the pulses. That is why a slight increase in amplitudes of this component was observed at membrane potentials between – 40 and +20 mV due to the block of I_{Ca} which was reflected at the I/V curve as a relative increase of the current amplitudes in the presence

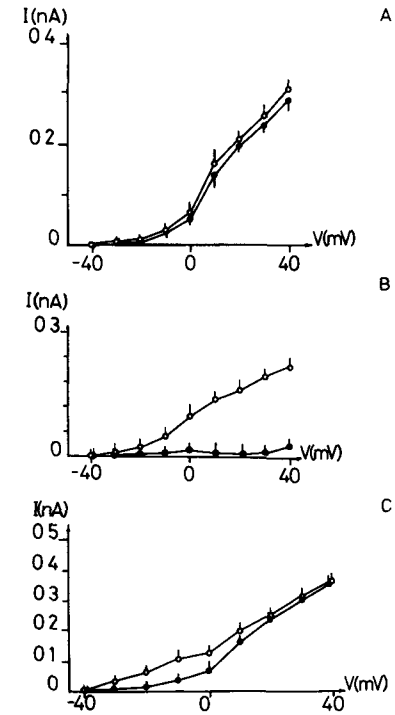

Figure 3. Dependence of the three pharmacologically separated components of I_K on Ca-entry, inhibited by nifedipine, as compared to their controls, obtained in the absence of the drug. The amplitudes of every one of the components were measured as follows: In (*A*) and (*B*) – at 200th ms, and in (*C*) – 20th ms after the beginning of the pulse. (*A*) Effect of nifedipine on the I/V relations of the CTX-sensitive component of I_K (solid circles), as compared to its control amplitudes (open circles). Data are means ± S.E.M. from 4 LC of good quality, kept at $V_h = -50$ mV. (*B*) Effect of nifedipine on the I/V-relations of the apamin-sensitive component of I_K (solid circles), as compared to its control amplitudes (open circles). Data are means ± S.E.M. for 5 LC. (*C*) Effect of nifedipine on I/V-relations of the GLB-sensitive component of I_K (open circles), as compared to its control amplitudes (solid circles). Data are means ± S.E.M. for 6 LC.

of nifedipine (Fig. 3*C*). However, at more positive membrane voltages the block of the calcium entry was without effect on the amplitudes of the GLB-sensitive current.

3.2. Dependence of different I_K components on the availability of calcium, stored into the IP_3-sensitive Ca pool – effects of heparin.

The effect of intracellularly applied heparin on the apamin-sensitive component of I_K was more considerable. No apamin-sensitive current remained at the 5^{th} min of dialysis, and apamin was unable to reduce the I_K amplitudes after dialysis with heparin-containing solution (Fig. 4*B*).

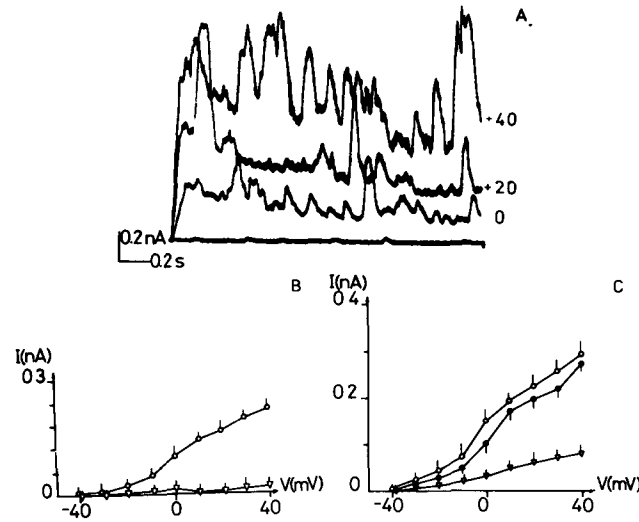

Figure 4. Dependence of different components of I_K on the availability of calcium stored into the heparin-blockable (IP_3-sensitive) Ca-pool. V_h was −50 mV. (*A*) The picture of STOCs with large amplitudes, usually observed at the 2^{nd} min after the beginning of dialysis with heparin-containing internal solution. LC with 43 pF membrane capacitance. (*B*) Heparin-induced block of the apamin-sensitive component of I_K as could be seen from the latter's amplitudes, measured at the 3^{rd} min of dialysis (open triangles) and represented at its I/V-relation curve, compared to that, obtained before dialysis (open circles). Data are means ± S.E.M. for 4 LC. (C) Effect of the heparin induced block of IICR on I/V-relations of the GLB-sensitive component of I_K: current amplitudes were measured at 3^{rd} min (solid circles), or at 12^{th} min of dialysis (open triangles) and compared to those, obtained before heparin (open circles). Data are means ± S.E.M. for 4 LC.

In cells, dialysed with heparin-containing internal (10^{-9} mol/l) solution, the most obvious change was the appearance of large spontaneous transient outward

currents (STPOs), having amplitudes comparable to those of the net current waveforms (Fig. 4*A*). Besides, the I/V relations of the net I_K did not show significant changes if compared to the controls (see Fig. 2*B*). The influence of heparin (10^{-10} mol/l heparin into the pipette solution was found to cause the same effect as higher doses up to 10^{-4} mol/l) on the amplitudes of the GLB-sensitive part of I_K was negligible at the 3rd min after the beginning of dialysis. However, the amplitudes of this component decreased slowly with time of dialysis, reaching 20% of initial values at 12th minute (Fig. 4*C*).

Surprisingly, the dialysis with heparin-containing solution led to an increase of amplitudes of the CTX-sensitive part of the current. We are unable to measure these amplitudes directly because in the presence of CTX cells, loaded with heparin, contracted in 3 min after the addition of CTX (10^{-8} - 10^{-6} mol/l), and changed their capacitive parameters and impedance. Thus, the amplitudes of the CTX-sensitive current were estimated as a difference between current amplitudes measured at 5th min after the beginning of the dialysis, and the current, which remained after the block of the GLB-sensitive component (Fig. 5*A*). The large STOCs, which superimposed at I_K in heparin-loaded cells, could lead to overestimation of its amplitudes, made us to investigate their nature. It turned out that cyclopiazonic acid (CPA), a potent and selective inhibitor of Ca^{2+}-ATPase of SR, could diminish the STOCs'amplitudes to their control levels (Fig. 5*B*). However, in the presence of CPA the amplitudes of the net I_K in heparin-loaded cells decreased quickly and in 12 minutes only the linear leakage current remained (Fig. 5*C*).

4. In some of the cells a fast transient component of I_K was observed, which

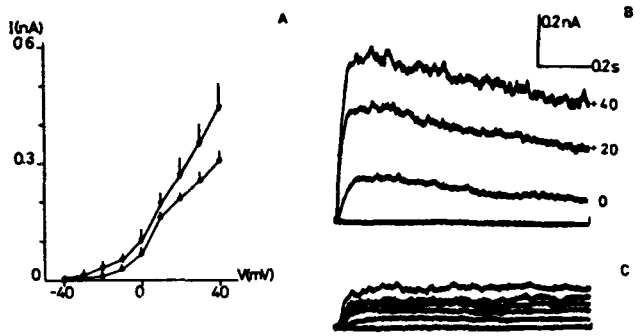

Figure 5. (*A*) I/V-relations of the CTX-sensitive component of I_K in heparin-loaded LC (open triangles), as compared to the same, obtained in the absence of the drug (open circles). (*B*) Blocking effect of CPA (10^{-6} mol/l) on STOCs' amplitudes in heparin-loaded cell with 43 pF membrane capacitance (the same cell as in Fig. 4*A*). $V_h = -50$ mV. (*C*) Depolarization-elicited I_K waveforms in the same heparin-loaded cell at 12th min after bath application of CPA. Potentials applied from 0 to +40 mV in 10 mV increments, beginning from $V_h = -50$ mV. Calibration bars are the same as in (*B*).

was insensitive to all known selective blockers of K-channels, could not be affected by dihydropyridines (not shown) and persisted even in the absence of Ca^{2+} outside (Fig. 6*A*). The changes of inactivation constants (τ_{in}) at different membrane potentials suggested a voltage-dependent mechanism of inactivation of this component (Fig. 6*B*). Because of its calcium insensitivity more detailed data on this component are not presented.

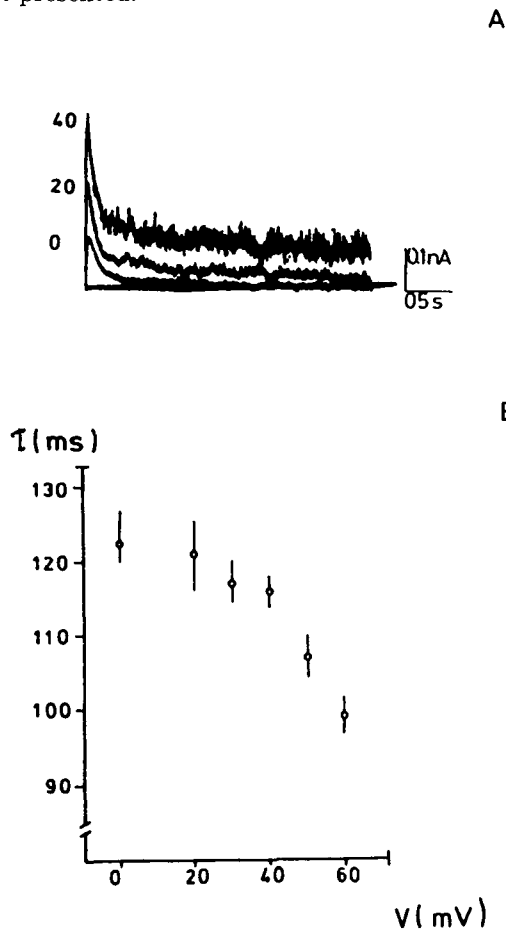

Figure 6. (*A*) The depolarization-elicited waveforms of a fast transient component of $I_K - K_{ft}$ which remained in the presence of CTX (10^{-5} mol/l), apamin ($5 \cdot 10^{-5}$ mol/l), GLB (10^{-4} mol/l), quinine (10^{-5} mol/l), 4-amino-pyridine ($5 \cdot 10^{-3}$ mol/l), added into the nominally Ca^{2+}-free PSS. $V_h = -50$ mV. LC with capacitance 37 pF. (*B*) Voltage dependence of the inactivation constants τ_{in} of this component. $V_h = -50$ mV. Data are obtained in the presence of the above mentioned drugs, applied in the same doses from 5 LC, expressed K_{ft} (means ± S.E.M.).

Discussion

The data presented show that the main part of the I_K in LC was conducted through Ca-sensitive potassium channels. The high sensitivity of the apamin-blockable component to the calcium entry observed is in agreement with single channel experiments made on other cell types (for review see Latorre et al. 1989), assuming that the apamin blocked preferably the so called "small conductive calcium dependent potassium channels" (SK), known as highly sensitive to Ca^{2+} (Ewald et al. 1985; Blatz and Magleby 1986). The slowly developed block of the GLB-sensitive component in heparin-loaded cells, and its insensitivity toward the inhibition of Ca-entry by nifedipine suggests a calcium-dependent mechanism of opening of ATP-sensitive K-channels (K_{ATP}-channels). The latter is in accordance with the hypothesis of Krippeit-Drews and Lonnendonker (1992), in which the elevation of the $[Ca^{2+}]_i$ close to the inner mouth of these channels help their removement from ATP-induced inactivation. According to it the GLB-blockable K_{ATP}-channels can be described as Ca-gated potassium channel type, but this definition needs to be specified by further investigations.

The finding that nifedipine blocked all components of I_K in heparin-loaded cells is not unexpected, bearing in mind that under our experimental conditions heparin totally inhibited the inward I_{Ca}. It was considered that the dihydropyridine-sensitive type Ca-channels (which are the only type in LC – see Droogmans and Callewaert 1986) fill directly the IP_3-sensitive Ca-store, placed close to the inner surface of the plasmalemma. The same organization of the "superficial calcium signalling" is proposed to exist in tracheal smooth muscle (Daniel et al. 1992), and in other smooth muscle cells (Missiaen et al. 1990; for review see Casteels et al. 1992). Assuming this, it becomes easy to explain the total block of all Ca-sensitive components of I_K by nifedipine in LC in situation when the IP_3-sensitive Ca-release channels are "locked", and the store could not be refilled by Ca^{2+} from the outside because of the Ca-dependent block of Ca-channels, directly connected with it. During depolarization pulses this Ca-pool looses calcium through Ca^{2+}-ATPase (Imaizumi et al. 1992). The cyclic activity of this enzyme could be visualized through the periodical opening of BK^+-channels by the locally released Ca^{2+}, forming the general picture of the STOCs. The latter was confirmed by the fact that STOCs disappeared immediately after the addition of CPA, and later the entire K-conductivity diminished – a phenomenon, which has been recently reported for other smooth muscle cells as well (see Low et al. 1992). This observation was considered as another confirmation of the suggestion that BK^+ could be activated only by Ca^{2+}, released from the intracellular stores in a way, which has been proposed by Komori and Bolton (1991). As ruthenium red and ryanodine failed to affect I_K at all, neither in the control experiments, nor in heparin-loaded cells (not shown) it was suggested that under our experimental conditions (high concentra-

tion of EGTA into the pipette solution) CICR mechanism could not influence the Ca-activated K-conductance.

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