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Characteristics of Potassium and Calcium Currents of Hepatic Stellate Cells (Ito) in Rat

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Hepatic stellate cells (HSCs) are known to play a role in the pathogenesis of the increased intrahepatic vascular resistance found in chronic liver diseases. The aim of this study was to evaluate the K^+ and Ca^{2+} currents in cultured HSCs from rat liver, through the patch-clamp technique. Most cells were positive for desmin immunostain after isolation and in α-smooth muscle actin immunostain after 10 - 14 days of culturing. Outward and inward rectifying K^+ currents were confirmed. Two different types of K^+ currents were distinguished: one with the inward rectifying current and the other without. The outward K^+ currents consisted of at least four components: tetraethylammonium (TEA)-sensitive current, 4-aminopyridine (4-AP)-sensitive current, pimozide-sensitive current and three blocker-resistant current. The peaks of the outward K^+ currents evoked by a depolarizing pulse were decreased to 32.0 ± 3.0, 62.8 ± 3.7 and 32.8 ± 3.5% by 5 mM TEA, 2 mM 4-AP and 15 μM pimozide, respectively. Moreover, the combined application of three blockers caused 86.6 ± 4.8% suppression. The inward currents evoked hyperpolarizing pulses were inwardly rectifying and almost blocked by Ba^{2+}. Elevation of external K^+ increased the inward current amplitude and positively shifted its reversal potential. Voltage-dependent Ca^{2+} currents which were completely abolished by Cd^{2+} and nimodipine were detected in 14 day cultured HSCs. In this study, the cultured HSCs were found to express outward K^+ currents composed of multiple pharmacological components, Ba^{2+}-sensitive inward rectifying K^+ current and L-type Ca^{2+} current.

Key Words: Hepatic stellate cells, outward K^+ currents, inward K^+ currents, L-type Ca^{2+} current

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INTRODUCTION

Hepatic stellate cells (HSCs), also called Ito cells, fat storing cells or lipocytes, play a major role in storage and metabolism of vitamin A. HSCs are microvascular pericytes situated in the space of Disse and possess many long cytoplasmic processes that embrace the sinusoid over endothelial cells and have contact with hepatocytes. HSCs have been regarded as an important cell type that are responsible for extracellular matrix accumulation during liver repair reactions, including fibrosis.[1,2] Some studies indicate that HSCs play a role in the pathogenesis of the increased intrahepatic vascular resistance found in advanced chronic liver diseases.[3,4] In these conditions, the basic features of HSCs phenotypically and functionally change, a process also called "activation".[1,2,4] Activation includes HSC proliferation and the transformation of star-shaped cells rich in vitamin A to vitamin A-deficient cells of myofibroblast-like appearance (activated HSC) that display contractile properties. Studies using *in vitro* and *in vivo* models of HSC activation have constantly shown that activated HSCs express high amounts of α-smooth muscle actin (α-SMA), myosin and cytosolic proteins essential for cell contractility, which are absent in quiescent HSCs.[5,6] Due to their anatomical location, the contraction and relaxation of activated HSCs are believed to regulate sinusoidal resistance to blood flow and thus, play an important role in the pathogenesis of portal hypertension in advanced chronic liver diseases.[3,7]

Membrane potential primarily regulates muscle

contractility through alterations in the Ca^{2+} influx across voltage-dependent Ca^{2+} channels in the vascular smooth muscles and pericytes.[8,9] In addition, several studies have discovered that the membrane potential in vascular smooth muscle cells is regulated through the modulation of a variety of K^+ channels that serve as important determinative factors of vascular tone,[10] suggesting the physiological significance of these channels. In HSCs, recent electrophysiological studies for the investigation of ionic channels have suggested electrophysiological profiles, such as inward and outward voltage dependent K^+ currents, hemi gap-junctional channel allowing cation to pass nonspecifically,[11] Ca^{2+}-activated K^+ currents modulated by endothelin-1 and nitric oxide[12] and *de novo* expressed L-type Ca^{2+} channels.[13] However, the properties and components of K^+ and Ca^{2+} currents remain to be fully studied in order to explain all the electrophysiological characteristics of HSCs. Therefore, in the present study, our aims were to examine the electrophysiological properties of cultured HSCs, to reveal the exact components of K^+ and Ca^{2+} currents.

MATERIALS AND METHODS

Preparation of primary cultured HSCs

HSCs were isolated from male Sprague Dawley rats (150 - 250g) by means of in situ perfusion using the collagenase digestion methods and nycodenz gradient centrifugation, as described elsewhere,[14] after intraperitoneal anesthetization with ketamine at 5 mg/100g. Briefly, the rat liver was perfused with Hank's buffer containing 0.02% pronase (Boehringer Mannheim, Mannheim, Germany) and 0.015% collagenase (Boehringer Mannheim) at 37℃. After digestion, the liver was rapidly excised, transferred onto a dish, cut into small pieces, ground with a grinder set and redigested with Hank's buffer containing 0.02% pronase in a 37℃ shaking water bath for 20 minutes. The suspension was filtered through nylon gauze and the filtrate centrifuged at 450 × g for 10 min at 4℃. After several washes with Hank's buffer, the cells were mixed with Hank's buffer and 28.7% nycodenz (Sigma Chemical Co, St Louis, MO, U.S.A.). Hank's buffer was layered on top of the cell mixture. After centrifugation (1000× g, 4℃, 20 min), HSCs were isolated from the floating band, then washed with DMEM (GIBCO BRL Life Technologies, Grand Island, NY, U.S.A.) and cultured in 5% CO_2, 95% air, using DMEM containing antibiotics-antimycotics and 10% fetal bovine serum, at 37℃. After plating the cells, the culture medium was changed every third day.

Electrophysiology

The macroscopic currents were recorded using the whole-cell variant of the patch clamp technique, with a patch clamp amplifier (EPC9, Instrutech Corp., NY, U.S.A.). For each experiment, glass coverslips were transferred to a 0.2 ml recording chamber mounted on the stage of an inverted microscope (Eclipse TS100, Nikon, Tokyo, Japan) and superfused by gravity at 2 ml/min with bath solution. Patch electrodes were fabricated from a borosilicate glass capillary (Garner Glass Co., Claremont, CA, U.S.A.) using a P-97 Flaming Brown micropipette puller (Sutter Instrument Co., San Rafael, CA, U.S.A.). The patch electrodes were fire polished on a microforge (Narishige, Tokyo, Japan) to give resistances of 1 to 3 MΩ when filled with the internal solutions described below. An Ag/AgCl wire was used to earth the bath. For voltage clamp measurement, the cell membrane capacitance and series resistance were compensated (>80%) electronically using the amplifier. Voltage protocol generation and data acquisition were performed using the Pulse/Pulsefit v8.50 software (Heka Elektronik, Lambrecht, Germany). The sampling rate was 1 - 3 kHz and the current traces low-pass filtered at 2 to 5 kHz using the four-pole Bessel filter within the amplifier and stored on a computer for later analysis. All experiments were performed at room temperature (20 - 24℃). Drugs were applied to single cells via a gravity-fed fused silica capillary tube connected to six polyethylene tubes located within 100 μm of the cells.

Immunocytochemistry

The HSCs cultured on coverslips were rehy-

drated in PBS, fixed in 4% paraformaldehyde, rinsed in PBS for 10 min and then stained. Immunocytochemistry was performed using the avidin-biotin complex-alkaline phosphatase system. After blocking with normal goat serum, samples were treated with primary antibodies of desmin or α-SMA (Sigma Chemical Co). After treating with biotinylated secondary antibodies and avidin-biotin alkaline phosphatase complex, NBT-BCIP was used as a cytochrome.

Solution and drug

The K^+ currents were recorded in a pipette solution of (in mM) 100 K^+-gluconate, 30 KCl, 10 EGTA, 1.2 $MgCl_2$, 10 HEPES, 5 MgATP, 0.3 Na_2GTP, 10 Tris-phosphocreatine and 10 glucose (pH 7.2 with KOH). The external solution for measuring K^+ currents (normal PSS; mM) was comprised of 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 10 glucose (pH 7.4 with Tris). The Ca^{2+} currents were isolated using patch electrodes filled with an internal solution containing 120 mM N-methyl-D-glucamine-methanesulfonate, 20 mM tetraethylammonium-methanesulfonate, 20 mM HCl, 11 mM EGTA, 1 mM $CaCl_2$, 10 mM HEPES, 4 mM Mg-ATP, 0.3 mM Na_2-GTP and 14 mM creatine phosphate (pH 7.4). The external recording solution for Ca^{2+} currents contained 145 mM tetraethylammonium-methanesulfonate, 10 mM HEPES, 10 mM $CaCl_2$, 15 mM glucose and 0.001 tetrodotoxin (TTX) (pH 7.4 with TEA-OH). The TTX, tetraethylammonium chloride (TEA), pimozide, 4-aminopyridine (4-AP), $CdCl_2$, nimodipine and Bay K8644 were obtained from Sigma Chemical Co. The fetal bovine serum, DMEM and antimycotic-antibiotics were purchased from GIBCO. Stock solutions (10 - 100 mM) of all drugs were prepared in distilled water.

Data analysis

For the Ca^{2+} and K^+ currents, the amplitudes of step currents were usually determined isochronally at 10 ms or at peak current after the onset of a test pulse. In some cases the amplitudes of currents were normalized to the membrane capacitance and expressed as pA/pF. Data are presented as the mean values ± S.E.M. The inhibition percentage of the currents was quantified with using the equation $[1\text{-}I_{test}/I_{control}]\cdot 100$. Statistical significance was determined using Student's t-test and one-way ANOVA, with a value of $p<0.05$ considered significant.

RESULTS

Immunocytochemistry

Identification of the cells as HSCs was routinely carried out by the detection of desmin filaments as a marker for HSCs.[15] The vast majority of purified HSCs (95%) in our culture displayed strong intracytoplasmic staining with a desmin antibody (Fig. 1A). As culturing progresses, isolated HSCs are known to change their contractile phenotypes, as indicated by the increasing expression of α-SMA.[5] The α-SMA expression was prominent in those cells cultured for over 10 days after cell dissociation (Fig. 1B).

Different two type of K^+ currents in HSCs

To investigate the K^+ currents, patch-clamp experiments using the whole-cell configuration were performed. The patch pipette contained 10 mM EGTA to block Ca^{2+}-activated ion currents, such as K^+ and Cl^-. In the K^+ currents experiments, cells were used within 6 days of culturing. Fig. 2 illustrates the K^+ currents in HSCs, elicited by a voltage ramp from -140 to 60 mV, from a holding potential of -80 mV. In a subpopulation of the tested HSCs, there were inward rectifying currents between -140 and -80 mV on the current-voltage (I-V) curves (B). Based on the presence of inward rectifying K^+ currents, two different subpopulations of HSCs, the 'outward only type' and 'outward and inward type', could be distinguished. The outward and inward type occupied 21 of 73 HSCs (28.8%) and had a mean capacitance of 24.8 ± 6.6 pF (n=19), while the outward only type accounted for 71.2% of the tested HSCs, and had a mean capacitance of 9.7 ± 1.9 pF (n= 36). The former was larger than the latter in size ($p<0.01$), although the distribution of the two types of HSCs overlapped considerably.

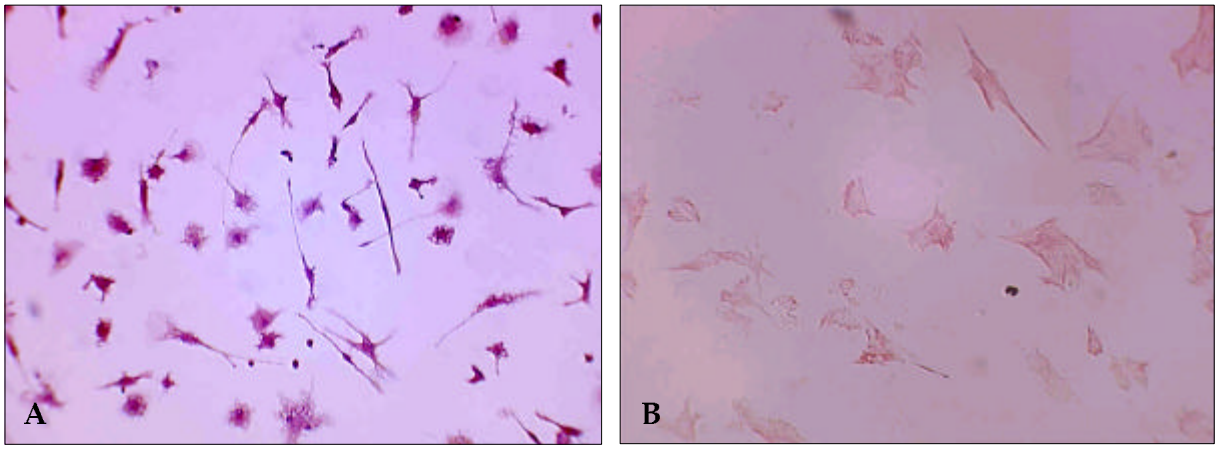

Fig. 1. Immunocytochemistry for desmin and α-SMA in cultured hepatic stellate cells (HSCs). Cells were prepared and processed as described in MATERIALS AND METHODS. Nearly all cells exhibit cytoplasmic immunocytochemical staining for desmin by the 5th day of culturing (A, magnification × 100). The cytoplasmic expression of α-smooth muscle actin (α- SMA) was detected by immunocytochemistry in cultured HSCs at the 14th day of culturing (B, magnification ×150).

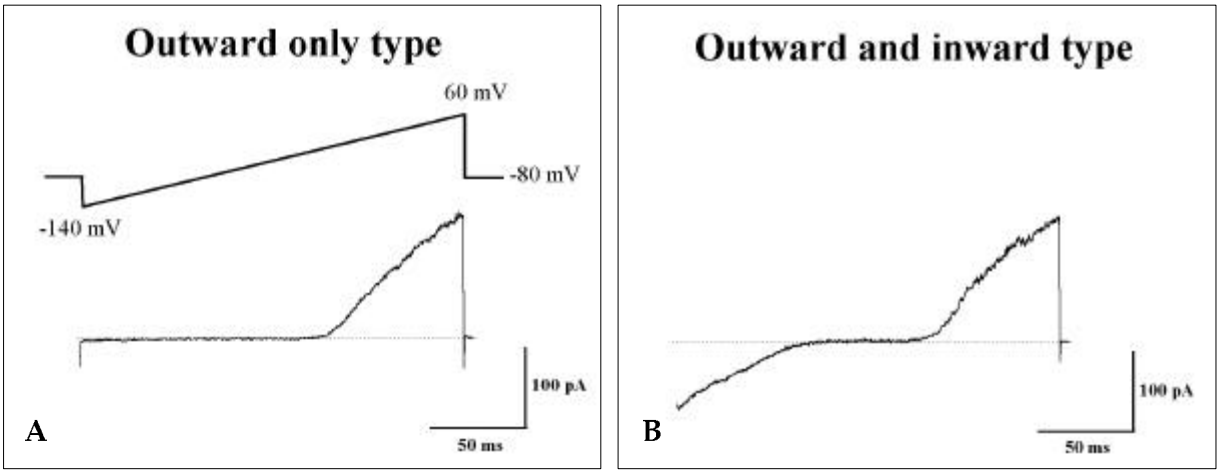

Fig. 2. Different whole-cell K^+ currents in cultured HSCs. Representative traces of K^+ currents are illustrated in A (outward only type) and B (outward and inward type). Voltage-dependent alterations in the membrane currents in a single HSC cultured for 2 days, measured under voltage-clamp conditions, using the whole cell configuration of the patch-clamp technique. The K^+ currents were elicited by voltage ramps, from -140 to +60 mV, in cells held at -80 mV and cultured for 3 days. Note that the inward current between -140 and -80 mV (B), indicating an inward rectifier K^+ current.

Outward voltage-dependent K^+ currents in HSCs

At first, the outward K^+ currents were studied to identify their components and were evoked in the HSCs by depolarizing pulses ranging from -80 to 70 mV in 10 mV increments from an initial holding potential of -80 mV. Depolarizing to voltages more positive than -40 mV produced outward K^+ currents, which declined only slightly over the period of a 200-ms step (Fig. 3, Left), with an average current amplitude of 17.9 ± 1.9 pA/pF at 20 mV (n=8). The I-V relationships measured soon after the command onset (outward peak in A) showed outward rectification (Fig. 3, Right).

Tetraethylammonium (TEA) and 4-aminopyridine (4-AP) are well known as traditional blockers of K^+ currents. TEA has been regarded as a blocker for delayed rectifier K^+ channels rather

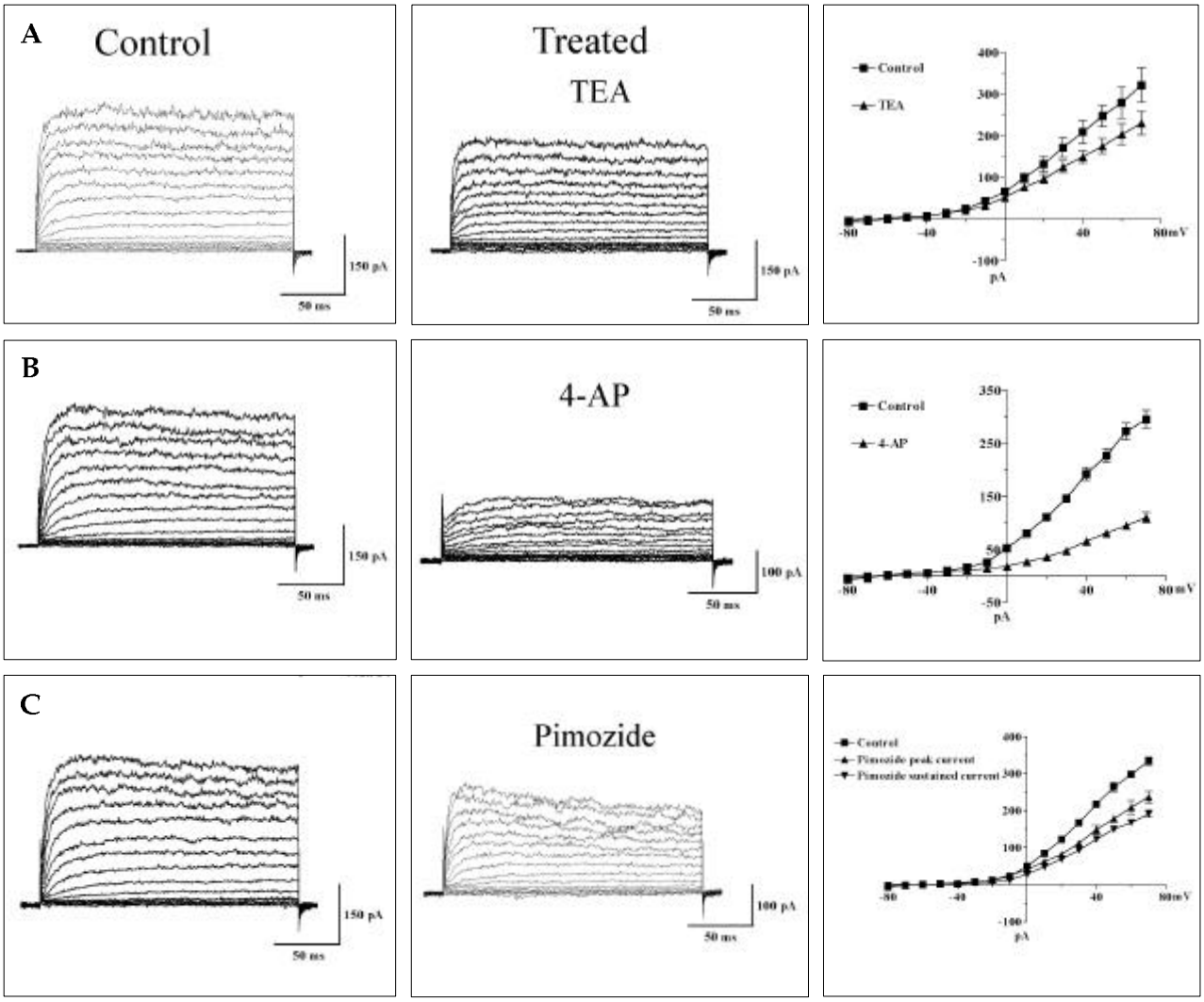

Fig. 3. Effect of tetraethylammonium (TEA), 4-aminopyridine (4-AP) and pimozide on the outward K^+ currents. Representative traces were measured before (Left) and after (Middle) application of 5 mM TEA (A), 2 mM 4-AP (B) and 15 μM pimozide (C) in a HSC cultured for 2 days. The peak amplitudes of the currents (A, B), or both the peak amplitudes of the currents, and the currents measured at the end of pulses (C), were plotted as a function of the voltage (Right). The I-V relationships show inhibition of the outward K^+ currents due to the blockers (A, B; Control (■), Blocker (▲), C; Peak current (▲), Sustained current (▼) after pimozide applied). Data are shown as the mean ± S.E.M. (n=5).

than for transient outward (A-type) K^+ channels. 4-AP is a more potent blocker of voltage-dependent K^+ channels than TEA.[16] Pimozide is an antipsychotic agent useful in the management of the motor and phonic tics associated with Tourette syndrome. It is known that pimozide has an antagonistic effect on HERG (ether-a-go-go-related gene) and delayed rectifier, such as Kv1.5, Kv2.1 and KvLQT1/mink.[17,18] Three agents were utilized to try and analyze the constituents of the outward K^+ currents pharmacologically. TEA, 4-AP and pimozide blocked the outward K^+ currents at several concentrations (100 μM - 10 mM, 100 μ -5 mM and 0.1 - 30 μM, respectively) and had maximal effects at concentrations of 5 and 2 mM and 15 μM, respectively. Fig. 3 shows the effects of three blockers on the outward K^+ currents. Voltage-dependent outward K^+ currents were found at the beginning of the voltage pulses, which did not decline until the end of the pulses (Left). The currents after the application of 5 mM TEA (A), 2 mM 4-AP (B) and 15 μM pimozide (C)

decreased at most of the test voltage (Middle), indicating that the K^+ currents were mediated by the opening of TEA-, 4-AP- and pimozide-sensitive K^+ channels. The I-V relationships demonstrate the effect of each blocker and the similar shape of the remaining current (Right). The degree of inhibition at 20 mV with TEA (n=17, $p < 0.001$) and 4-AP (n=15, $p < 0.001$) were 32.0 ± 3.0 and 62.8 ± 3.7%, respectively. Somewhat different from TEA and 4-AP, pimozide induced a reduction in the peak current as well as an additional time-dependent block of the current, which developed over the course of the depolarizing step (Fig. 3C, Middle). The I-V relationship shows the effect of pimozide at the peak and with sustained currents (Right). The currents elicited by a depolarizing pulse to +20 mV, measured at the peak and at the end of these pulses, were reduced by 32.8 ± 3.5 and 44.1 ± 4.2%, respectively (n=8, $p < 0.001$).

The combined application of the three drugs almost completely blocked the outward K^+ currents (Fig. 4). The remaining current resistant to the three agents was 13.4 ± 4.8% of total currents (n=8, $p < 0.001$), which declined only slightly. Combined application suppressed the outward K^+ current more than a single application. The applications of TEA + 4-AP, TEA + pimozide and 4-AP + pimozide blocked the outward K^+ current by 83.5 ± 5.0 (n=5), 55.0 ± 2.1 (n=4) and 73.4 ± 8.6% (n=4), respectively.

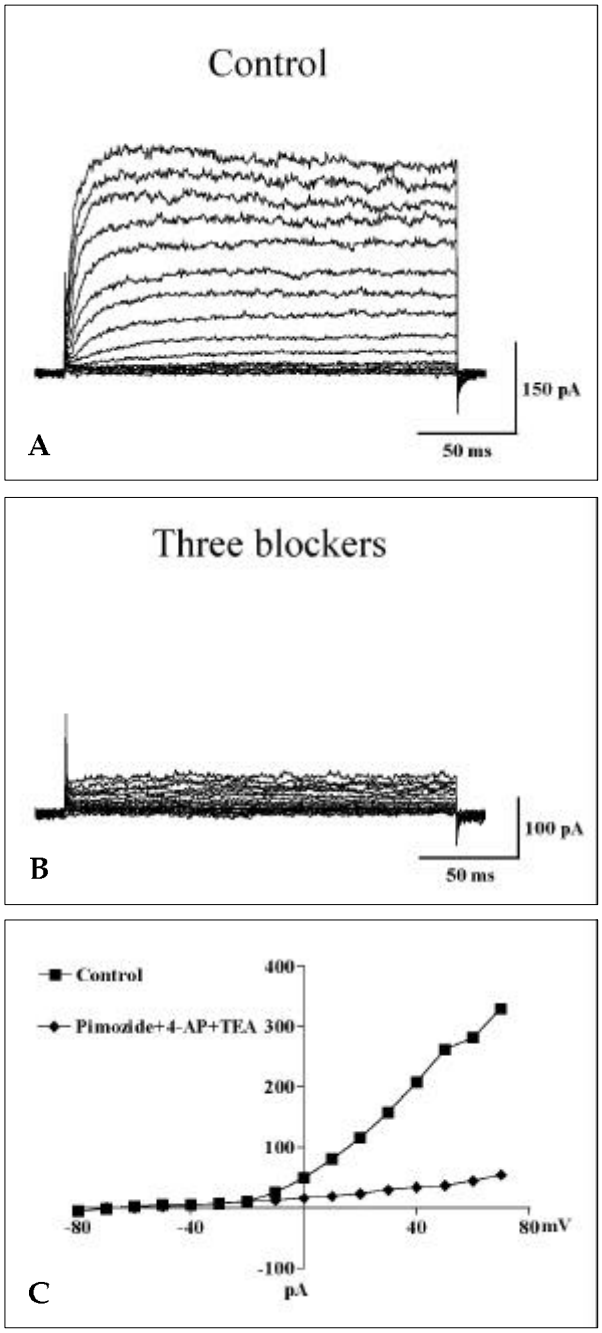

Fig. 4. The effect of co-application of TEA, 4-AP, and pimozide on the outward K^+ currents. Representative traces were measured before (A) and after (B) application of 5 mM TEA, 2 mM 4-AP and 15 μM pimozide in a HSC cultured for 2 days. C; the I-V relationship shows inhibition of the outward K^+ currents due to the three drugs (Control (■), three drugs (◆)).

Inward voltage-dependent K^+ currents in HSCs

Hyperpolarizing pulses elicited inward currents, which were either sustained or decayed, and the amplitude increased with further hyperpolarization. At hyperpolarized pulses of more than -100 mV, the inactivation kinetics of the inward current became faster, producing a "criss-crossing pattern" by the voltage pulse protocol (Fig. 5A). The I-V relationships, measured immediately after the beginning of the voltage command, exhibited inward rectification within command voltages of less than -70 mV (Fig. 5B). However, the steady-state current reached a maximum at -120 mV and the I-V relation had a region of a negative slope at more negative potentials. When the external K^+ concentration ($[K^+]_o$) was increased from 5 to 45, 90, and 130 mM (Fig. 5C), the I-V relations crossed the zero current level at -70 mV in the 5 mM $[K^+]_o$, -24 mV in 45

mM $[K^+]_o$, -12 mV in 90 mM $[K^+]_o$ and -1 mV in 130 mM $[K^+]_o$. Furthermore, the current was blocked by the addition of 200 μM Ba^{2+} to the bathing solution (Fig. 5D, Left). Application of Ba^{2+} suppressed 92.2 ± 7.9% of the inward rectifying current evoked by voltage pulses to -120 mV

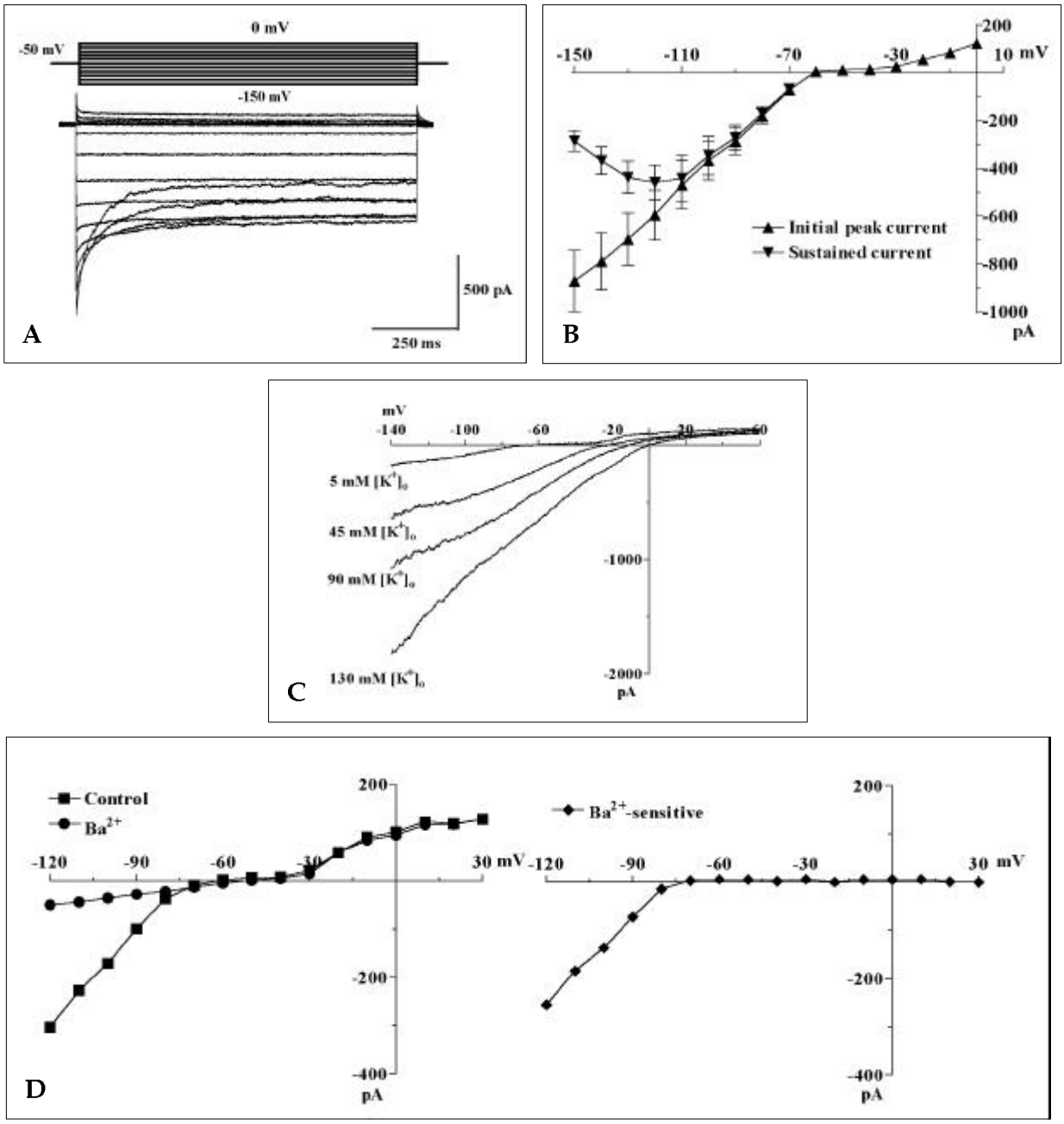

Fig. 5. Hyperpolarization-evoked inward K^+ current in cultured HSCs. A; Representative traces show inward K^+ currents evoked by a series of pulses, ranging from -150 to 0 mV, with 10 mV increments for 1 s, from a holding potential of -50 mV. B; Relationships between the command voltages and the inward currents at the peak (triangle) and those sustained (inverted triangle) show inward rectifying K^+ currents. C; Effects of changing the external concentration of $[K^+]_o$ from 5 to 45, 90 and 130 mM on the I-V relationships measured during ramp pulses, from -140 mV to +60 mV, at the holding potential of -60 mV. D; the I-V relationships show the inhibitory effect of 200 μM Ba^{2+} (Left, square; control, circle; Ba^{2+}) and the Ba^{2+}-sensitive current (Right, diamond). Data are shown as the mean ± S.E.M. (n=4). HSCs cultured for 4 days were used.

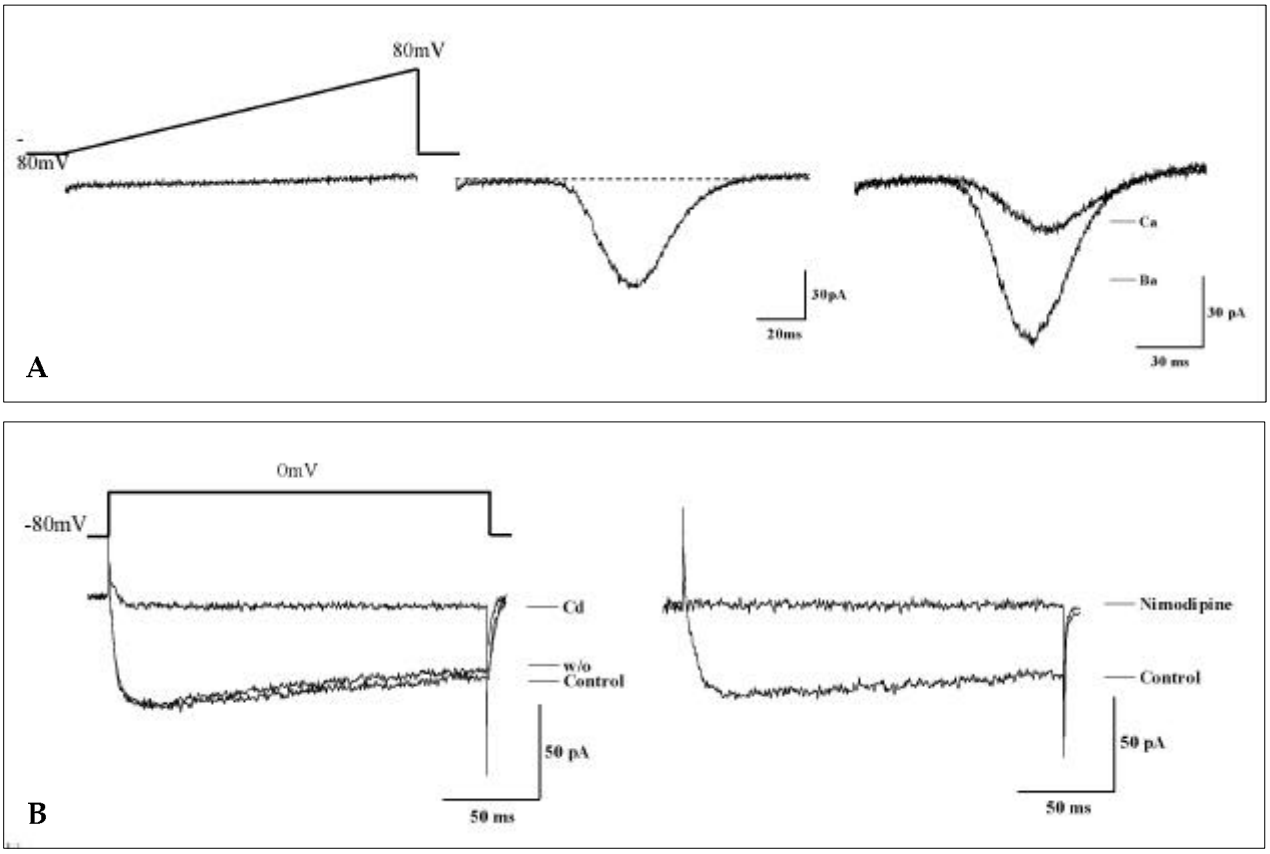

Fig. 6. The voltage-dependent inward Ca^{2+} currents in cultured HSCs. A; No Ca^{2+} currents were detected in a quiescent HSC (cultured for 3 days) after the cell was depolarized using the ramp protocol (Left), whereas a marked Ca^{2+} current was found in cells activated *in vitro* (cultured for 14 days) when depolarized (Middle). Representative Ca^{2+} currents were elicited by voltage ramps from -80 to +80 mV in cells held at -80 mV. I-V curves of Ba^{2+} and Ca^{2+} currents, elicited using the ramp protocol, are illustrated (Right). B; Typical Ca^{2+} currents were recorded in activated HSCs after cell depolarized by a pulse to 0 mV, from a holding potential of -80 mV. Representative Ca^{2+} current traces were superimposed before and after the administration of 100 μM Cd^{2+}, a non-selective antagonist of Ca^{2+} channels (Left). Representative Ca^{2+} current traces were superimposed before and after the addition of 10 μM nimodipine, a selective antagonist of L-type Ca^{2+} channels (Right) (Control - trace represent the current before drug application, w/o - trace represent the current when drug was removed by washing).

(n=5). The Ba^{2+}-sensitive inward current, calculated from the difference in the currents before and after the application of Ba^{2+}, reversed near -80 ~ -70 mV - close to the K^+ equilibrium potential (Fig. 5D, Right). Ba^{2+} did not significantly affect the outward current component.

Voltage-dependent Ca^{2+} current in HSCs

Ramp depolarization to 80 mV from a holding potential set at -80 mV, revealed inward Ca^{2+} currents. Fig. 6A illustrates that the Ca^{2+} currents of 3-day cultured HSCs (Left) were not measured, while those of 14-day cultured HSCs (Middle) had developed enough to be recorded. Ca^{2+} currents were observed frequently in the cells cultured for more than 10 days. 13 of 23 (56.5%) HSCs activated *in vitro* had Ca^{2+} currents. By contrast, no Ca^{2+} currents were detected in any of the 22 quiescent HSCs studied. These results indicated that voltage-dependent Ca^{2+} current appeared in activated HSCs. Charge carrier substituted due to 10 mM Ba^{2+} enhanced the currents by 166.9 ± 8.4% in the ramp protocol (Right; n=5). The maximum current density varied from cell to cell (range between 0.3 and 10.8 pA/pF), with a mean value of 2.8 ± 0.9 pA/pF at the 14th day of culturing (n=13). The membrane capacitance in the cells

associated with inward Ca^{2+} currents ranged from 13.8 to 142 pF, with a mean of 45.0 ± 11.4 pF. To analyze the effect of Ca^{2+} channel blockers, Ca^{2+} currents were elicited by step pulses from, a holding potential of -80 mV to 0 mV. The currents were typically fast activating and slowly inactivating over the course of a 200 ms step pulse (Fig. 6B). The representative Ca^{2+} currents in the presence of Cd^{2+}, a non-selective Ca^{2+} channel blocker, are superimposed in the left of Fig. 6B. 100 μM Cd^{2+} completely decreased the currents (92.3 ± 1.2%, n=7, $p<0.001$). When 10 μM nimodipine, a specific L-type Ca^{2+} channel blocker, was applied to the bath solution, the Ca^{2+} currents were also completely decreased (91.7 ± 1.9%, n=5, $p<0.001$, right). However, applying Bay K8644 (10 μM), an agonist of L-type Ca^{2+} channels, enhanced the Ca^{2+} currents two-fold (94.3 ± 7.9%, n=5, $p<0.01$, data not shown). Also, the N-type Ca^{2+} channel blocker, ω-conotoxin GVIA (1 μM), P/Q-type blocker, ω-agatoxin IVA (0.5 μM), and the N-, P- and Q-type blocker, ω-conotoxin MVIIC (1 μM), had no effect on the Ca^{2+} current (data not shown). These results indicate that the Ca^{2+} current detected in activated HSCs was caused by the opening of L-type Ca^{2+} channels.

DISCUSSION

This study has characterized the K^+ currents that would be responsible for determining the membrane potential of rat HSCs in culture. At least two types of K^+ channels were identified, that is, the voltage-dependent outward K^+ channel, activated by depolarization, and the voltage-dependent inward rectifier K^+ channel, activated by hyperpolarization. K^+ channels were found commonly in smooth muscles and pericytes, but their components were somewhat different to those in tissues.[9,10,19] For example, the inward rectifying K+ current was found in smooth muscle cells of the cerebral and coronary arteries, but not in other smooth muscle cells.[20-22] In this study, the described characteristics of the K^+ currents are generally similar to those found in other contractile cells, and corresponded to previous results obtained in rat HSCs.[11,23] However, a detailed view of K^+ currents raised several differences from the previous studies. Therefore, 4 topics relating to the different properties were described.

First, individual HSCs express different K^+ currents. Inward rectifier K^+ currents were measured in some portions of the HSCs. In the previous electrophysiological study,[11] K^+ currents were described as outward and inward rectifying currents, which would be expressed together in most HSCs. However, our results suggest two different groups of HSCs, one with inward and outward rectifying K^+ currents and the other with only outward rectifying K^+ currents. This means that one group has an inward rectifier and the other does not. The inward rectifier K^+ channels are known to be present in a variety of excitatory and non-excitatory cells. The physiological roles of the inward rectifier include regulating the resting membrane potential, preventing membrane hyperpolarization to values more negative than the E_K, minimizing cellular K^+ loss and mediating K^+-induced dilations.[10,20] The inward K^+ channel in HSCs, therefore, serves as a device responsible for maintaining the resting membrane potential. Apart from the inward rectifier, outward K^+ currents exist in both types of HSC. The outward K^+ channels observed in HSCs have also been identified widely in other types of cells. In action potential-generating cells, this channel is considered to play an important role in repolarization of the action potential and the regulation of the membrane potential.[10,20] In vascular smooth muscle cells the outward K^+ channel act mainly to limit membrane depolarization.[24] At present, the physiological functions of the outward K^+ channel are unknown in HSC-like inward rectifiers. Although the actual physiological role of voltage-dependent K^+ channels should further be examined, previous studies collectively suggest that the expressed outward and inward K^+ channels are two major factors regulating the membrane potential of HSCs.

Second, outward K^+ currents in HSCs were composed of TEA- and 4-AP-sensitive currents, in addition to currents resistant to both. In previous studies on HSCs, Gasull et al.[12] demonstrated the TEA-sensitive outward currents, including Ca^{2+}-activated K^+ channel, in contrast to Kashiwagi et al.,[11] who showed no TEA-sensitive outward current. Voltage-dependent outward K^+ currents

have been classified as fast inactivating A-type and slowly inactivating delayed-rectifier currents. TEA has been traditionally regarded as a blocking agent for native delayed rectifier currents. The sensitivity to block is not a very useful criterion for classifying voltage-dependent K^+ channels.[25] 4-AP is a more potent blocker of voltage-dependent K^+ channels than TEA,[16] which is known as a selective blocker to A-type currents. However, the selectivity for A-type currents over a delayed rectifier does not always manifest.[25] Pimozide, an antipsychotic agent, has an antagonistic effect on HERG and delayed rectifier.[17,18] At nanomolar concentrations, sufficient to inhibit HERG, the effect of pimozide on K^+ currents in HSCs was to small to be detected, but at micromolar concentrations, appropriate for inhibiting delayed rectifier, an inhibitory effect was shown. Most of the outward peak currents were sensitive to three blockers. Each blocker suppressed a separated current, and overlapped with the other agents. Based on the gradual slant in I-V relation, the slight inactivation in the shape of currents, and the above pharmacological results, it was thought that most of the outward currents might be a delayed rectifier and that they were sensitive to all three agents to different degrees.

Third, typical A-type K^+ currents were rarely recorded within 6-day cultures, in contrast to the study of Kashiwagi et al.[11] Most of the K^+ currents recorded in this study were slowly inactivated, with linear rectification in their I-V relationships, which was similar to the characteristics of the delayed rectifier and in the study of Gasull et al.[12] on human HSCs. The A-type has been shown to be activated by depolarizing pulses to -60 mV or over, which decay quickly with time, with a -75 mV half maximal inactivation value.[11,20] Thus, a depolarized membrane potential over -75 mV causes steady-state inactivation of most A-type currents. Kashiwagi et al.[11] showed that changing the holding potential to -30 mV inactivated the typical A-type K^+ currents, satisfying the above property. However, in this study, changing the holding potential to -40 mV elicited no effect on the kinetics of inactivation or the I-V relationship (data not shown).

Fourth, the inward rectifier K^+ currents evoked by hyperpolarizing pulses from -120 to -150 mV inactivated for about 300 ms, in contrast to those of Kashiwagi et al.[11] which were not inactivating in the same voltage ranges. From this point of view, only Kir 2.2 inactivates among all the cloned inward rectifiers (Kir),[26] and some studies dealing with native cells expressing endogenous inward rectifier demonstrated that inactivating inward rectifying currents made a "criss-crossing pattern".[27,28] Although the relation of the inactivation property and subtype is unclear, it would be a clue to characterize the inward rectifier.

Voltage-dependent Ca^{2+} currents were found, which commonly play a role in cell contraction in myocytes and smooth muscle cells,[10] in accordance with previous studies. The voltage-dependent Ca^{2+} current, which was not detected in quiescent HSCs isolated from rats, consistently appeared in those undergoing activation *in vitro* after 1 - 2 weeks of culturing.[13,29] After inoculation of cells onto glass coverslips, the HSCs showed active proliferation. During this phase, no HSCs showed Ca^{2+} current or α-SMA. After the 7th - 10th day of culturing, the Ca^{2+} current was accompanied by an apparent production of α-SMA. The observation that Ca^{2+} currents in activated HSCs were abolished by specific L-type blocker indicates that most voltage-dependent Ca^{2+} channels belong to the L-type. HSCs are known to proliferate around injured sites and to activate from the quiescent to the myofibroblast-like phenotypes. The process of activation is characterized by morphological and functional changes, including a decrease in the number of vitamin A droplets, increased size of the endoplasmic reticulum, increased expression of cytosolic proteins, such as α-SMA and myosin, production of cytokines, high proliferative activity and increased production of extracellular matrix components.[30,31] The HSCs observed in this study showed spontaneous conversion to the myofibroblast-like phenotype during culturing following active proliferation.

In conclusion, the results of the current study indicate that cultured HSCs express outward K^+ currents, composed of multiple pharmacological components and Ba^{2+}-sensitive inward rectifying K^+ currents and activated HSCs express L-type voltage-dependent Ca^{2+} currents. These electrophysiological results can provide basic properties

of HSCs for new pharmacotherapeutic trials in the management of portal hypertension.

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