Characteristics of hyperpolarization-activated cation currents in portal vein smooth muscle cells

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Received 10 August 2001; accepted in final form 21 November 2001

Greenwood, I. A., and S. A. Prestwich. Characteristics of hyperpolarization-activated cation currents in portal vein smooth muscle cells. Am J Physiol Cell Physiol 282: C744–C753, 2002. First published November 27, 2001; 10.1152/ajpcell.00393.2001.—Voltage-clamp studies of freshly isolated smooth muscle cells from rabbit portal vein revealed the existence of a time-dependent cation current evoked by membrane hyperpolarization (termed Ih). Both the rate of activation and the amplitude of Ih were enhanced by membrane hyperpolarization. Half-maximal activation of Ih was about −105 mV with conventional whole cell and −80 mV when the perforated patch technique was used. In current clamp, injection of hyperpolarizing current produced a marked depolarizing “sag” followed by rebound depolarization. Activation of Ih was augmented by an increase in the extracellular K+ concentration and was blocked rapidly by externally applied Cs+ (1–5 mM). The bradycardic agent ZD-7288 (10 μM), a selective inhibitor of Ih, produced a characteristically slow inhibition of the portal vein Ih. The depolarizing sag recorded in current clamp was also abolished by application of 5 mM Cs+. Cs+ significantly decreased the frequency of spontaneous contractions in both whole rat portal vein and rabbit portal vein segments. Multiplex RT-PCR of rabbit portal vein myocytes using primers derived from existing genes for hyperpolarization-activated cation channels (HCN1–4) revealed the existence of cDNA clones corresponding to HCN2, 3, and 4. The present study shows that portal vein myocytes contain genes shown to encode for hyperpolarization-activated channels and exhibit an endogenous current with characteristics similar to Ih in other cell types. This conductance appears to determine, in part, the rhythmicity of this vessel.

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mg/ml collagenase type 1A for 10 min. Cells were released by gentle agitation with a wide-bore Pasteur pipette. Cells were stored at 4°C and used within 6 h.

Functional studies. Portal veins have been shown to exhibit regular, spontaneous contractions under isometric conditions (31), and therefore functional experiments were undertaken using conventional organ baths (20-ml volume). However, due to the large size of the whole rabbit portal vein, transverse segments ~5 mm in length were taken from either the hepatic end or the middle of the vessel. Each segment was attached to an isometric force transducer by surgical thread at a resting tension of 1.5 g and was bathed in normal aerated Krebs solution at 37°C. Regular, spontaneous activity developed within 90 min and was stable for over 2 h. Control mechanical activity was recorded for 30 min, and then either Cs+ (1–5 mM) or ZD-7288 (10 μM) was added to the organ bath. Mechanical activity was recorded for 15 min in the presence of these agents. The mean amplitude and intercontraction interval were determined by measuring the amplitude of each individual contraction and also the interval between each contraction over a 5-min period before CsCl or ZD-7288 was added to the bath (control value) and for a 5-min period immediately before the blocker was washed from the bath (drug value). The intercontraction interval was determined as the time between the termination of one contraction and the onset of the next contraction (taken as a 0.02-g increase from baseline). In addition, experiments were performed on complete portal veins excised from Wistar-Kyoto rats (250 g) to determine how addition of Cs+ to the bathing solution affected the spontaneous activity of a complete portal vein. Animals were killed by cervical dislocation and exsanguination, and portal veins were ligated at the veins were bathed in aerated Krebs in the organ bath. All data were collected and analyzed by MacLab software on an Apple Macintosh computer.

Electrophysiology. Whole cell currents were recorded at room temperature (21–24°C) using CED software (Cambridge Electronic Design, Cambridge, UK) and a List amplifier (Heka Electronics). Analysis was performed using CED software as well as Origin (Microcal, Northampton, MA). The characteristics of \( I_h \) were investigated using protocols adapted from Refs. 25 and 35. Cells were held at −50 mV and initially stepped to −30 mV for 200 ms to fully inactivate any contaminating voltage-dependent currents and to ensure full activation of \( I_h \). Cells were then pulsed to various potentials between −30 mV and −150 mV for either 4 s or 10 s to activate \( I_h \). The amplitude of \( I_h \) was taken as the current amplitude immediately after stepping to the test potential subtracted from the amplitude of the inward current at the end of the test pulse. Current activation was fitted by a single exponential in most cases using a nonlinear least-squares fitting routine where the time course of activation was determined by the equation

\[
I = I_e \exp(-t/\tau)
\]

where \( I_e \) is current amplitude at time \( t \) and \( \tau \) is the time constant.

In the present study, reversal potential \( E_{rev} \) was determined by the method of Mayer and Westbrook (22) at negative test potentials at which the influence of voltage-dependent currents would be minimal (see also Ref. 13). In this method, it is assumed that the instantaneous current amplitude recorded on stepping from −30 mV to various hyperpolarized test potentials was composed of a background conductance plus an unknown but negligible contribution from \( I_h \). In comparison, the instantaneous current at various negative test potentials following maximal activation of \( I_h \) caused by hyperpolarization to −150 mV for 4 s would be dominated by \( I_h \). \( E_{rev} \) was calculated by plotting the amplitude of the current at test potentials between −150 mV and −70 mV following an initial 4-s step to either −30 mV or −150 mV. The voltage at which the two lines intersect was taken as \( E_{rev} \). In two cells, \( E_{rev} \) was also calculated by using a conventional tail current protocol, which involved stepping the cell from the holding potential of −50 mV to −120 mV for 4 s to maximally activate \( I_h \). The cell was then stepped to various test potentials (−100 to −20 mV), and the amplitude of the instantaneous current was plotted against the test potential. Identical values for \( E_{rev} \) were obtained with both methods.

\( I_h \) in other cell types has been shown to be a mixed cation current with an \( E_{rev} \) around −35 mV (e.g., Refs. 13, 25, and 35), a calculated permeability of Na+ relative to K+ of −0.3 (see Ref. 26), and a minimal contribution from divalent cations. The relative permeability of K+ to Na+ (\( P_{K}/P_{Na} \)) was calculated using the Goldman-Hodgkin-Katz (GHK) equation as in a previous study (25)

\[
E_{rev} = RT/F \ln \left[ \frac{[(K^+)]_o + P_{Na}/P_{K}[Na^+]]/[K^+]_i + P_{Na}/P_{K}[Na^+] \right]
\]

where \( R, T, F \) and \( H \) have their usual meanings.

Solutions. The external solution for electrophysiological experiments contained (in mM) 126 NaCl, 6 KCl, 10 HEPES, 20 glucose, 1.8 CaCl₂, and 1.2 MgCl₂, and pH was set to 7.2 with NaOH. Tetraethylammonium-Cl (10 mM) and 4-aminopyridine (5 mM) were also included in the external solution to block any ion flux through either Ca²⁺-activated K⁺ channels or voltage-dependent K⁺ channels that are present in these cells (3, 17). The pipette solution contained (in mM) 126 KCl, 10 HEPES, 20 glucose, 0.1 EGTA, and 1.2 MgCl₂, and pH was set to 7.2 with KOH. In some experiments, the perforated patch configuration was used, which was achieved by including 600 μg/ml amphotericin A in the same pipette solution used in conventional whole cell experiments (shown above). The Krebs solution for the functional studies contained (in mM) 117 NaCl, 4.7 KCl, 25 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, 11 glucose, and 2.6 CaCl₂. All reagents used in this study were purchased from Sigma (Poole, UK) except for ZD-7288 (Tocris, Avonmouth, UK).

Molecular biology. Single cells were prepared from rabbit portal vein as described in Isolation of vascular myocytes. Cells were plated onto glass coverslips and allowed to attach for 10 min. They were then rinsed with sterile PBS solution, and ~50 single smooth muscle cells were sucked into a large-diameter micropipette under a microscope. The pipette was then transferred to a Microfuge tube and frozen at −70°C until use. Total RNA was isolated using the RNAqueous-4PCR Kit from Ambion (Austin, Texas) as per the manufacturer’s instructions with the DNase treatment step. First-strand cDNA was prepared using Superscript II (Life Technologies, Paisley, UK) and 500 ng of oligo(dT) primers in a total volume of 20 μl.

Multiplex and nested PCR. The HCN1–4 were amplified simultaneously in the mPCR using HCN1–4 primers as previously described (11, 30). The primer sequences were taken from the following sequences in GenBank: HCN1 primers from HAC2, accession no. AJ2255123; HCN2 primers from HAC1, accession no. AJ2255122; HCN3 primers from HAC3, accession no. AJ2255124; and HCN4 primers from mBCNG-3, accession no. AF064874. The primer pairs (5′ to 3′) in the first PCR were: HCN1 sense, TCTTGGGCTATTACGCCCT (position 985), antisense, TTTTCTTGGCTATCCGATCG (position 1983); HCN2 sense, TACTTGCCGTCAGTGGTCTG.
(position 810), antisense, GAAATAGGACCATCCGACA (position 1775); HCN3 sense, CGCATCAAGATCTACGA (position 1242), antisense, CACTCCAAGGCTTTTACGC (position 2322); and HCN4 sense, TCTGTATCATACCCGTG (position 295), antisense, GAAGACCTGAAACGGC-CACT (position 1315). For mPCR, the final volume of each sample was 100 µl containing 10 µl of the RT reaction, 100 pmol of each primer, 0.2 mM each dNTP, 1.5 mM MgCl₂, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), and 3.5 units of Platinum Taq polymerase (Life Technologies). Amplification was performed according to the following schedule using a Hybaid Touchdown PCR machine (Hybaid, Ashford, UK): 94°C, 3 min; 35 cycles of 94°C, 30s; 50°C, 60s; and 72°C, 3 min, followed by a final elongation period of 7 min at 72°C. The nested PCR amplifications were carried out in four individual reactions for each tissue type, in each case with 2.5 µl of the first PCR product, 50 pmol of each primer, and 2.5 units of Platinum Taq polymerase; the extension time was shortened to 60 s. For the nested PCR amplifications, the primer pairs used were HCN1 sense, CCCTTTTTTGCTAACGCCGAT (position 1612), antisense, CATTGAATTTGTCACCCGAA (position 1902); HCN2 sense, GTGGAGGCGACCTCTACTCGT (position 1181), antisense, GTCACAAATCTTCCTACCGA (position 1550); HCN3 sense, GCAGGCTATTGGTACAACCG (position 1808), antisense, AGCGTCTAAGGATCGAGCT (position 2040); and HCN4 sense, GACAGCGCATCCATGACTAC (position 1110), antisense, ACAAGGTGGGATCTGAGCC (position 1278). The PCR products were separated and visualized in an ethidium bromide-stained 2% agarose gel by electrophoresis. The PCR products were then excised from a holding potential of −70 mV for 4 s evoked a slowly developing inward current (termed Iₐ) after an initial delay (see Fig. 1). This current did not inactivate during prolonged test pulses (see Fig. 5, for example) and did not exhibit “rundown” over the course of an experiment. Progressive membrane hyperpolarization shortened the duration of the delay and increased the amplitude of the current at the end of a 4-s step (Fig. 1B). The enhancement of current amplitude was associated with an enhanced rate of activation. Figure 1 shows that, at −110 mV, the development of Iₐ could be fitted by a single exponential with a time constant (τ) of 6,000 ms. At −150 mV, the current was also well fitted by a single exponential with a τ of 371 ms. Figure 1C shows the mean data from 13 cells showing that τ decreased exponentially for a 18-mV hyperpolarization. These values for the activation of Iₐ at room temperature are comparable with time constants recorded in other smooth muscle cell types (e.g., Refs. 23, 25, and 35). Figure 1 also shows that, following the test step to negative potentials, stepping back to the holding potential revealed a slowly declining inward “tail” current that represented the closure of Iₐ channels opened by the previous membrane hyperpolarization. The amplitude of the tail current following each hyperpolar-

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RESULTS

Iₐ has distinctive characteristics that allow this conductance to be identified from other inward rectifiers. These characteristics include slow, sigmoidal activation on hyperpolarization, permeability to Na⁺ and K⁺, and blockade by Cs⁺ and ZD-7228. We have used these distinctive features to compare Iₐ in the present study with similar currents recorded in previous studies.

Hyperpolarization of portal vein smooth muscle cells from a holding potential of −50 mV to potentials more negative to −70 mV for 4 s evoked a slowly developing inward current (termed Iₐ) after an initial delay (see Fig. 1). This current did not inactivate during prolonged test pulses (see Fig. 5, for example) and did not exhibit “rundown” over the course of an experiment. Progressive membrane hyperpolarization shortened the duration of the delay and increased the amplitude of the current at the end of a 4-s step (Fig. 1B). The enhancement of current amplitude was associated with an enhanced rate of activation. Figure 1 shows that, at −110 mV, the development of Iₐ could be fitted by a single exponential with a time constant (τ) of 6,000 ms. At −150 mV, the current was also well fitted by a single exponential with a τ of 371 ms. Figure 1C shows the mean data from 13 cells showing that τ decreased exponentially for a 18-mV hyperpolarization. These values for the activation of Iₐ at room temperature are comparable with time constants recorded in other smooth muscle cell types (e.g., Refs. 23, 25, and 35). Figure 1 also shows that, following the test step to negative potentials, stepping back to the holding potential revealed a slowly declining inward “tail” current that represented the closure of Iₐ channels opened by the previous membrane hyperpolarization. The amplitude of the tail current following each hyperpolar-
ization relative to the amplitude of the maximum tail current gives an index of the current availability. Fitting of the Boltzmann function to these data (see Fig. 2C) gives a potential of half-maximal activation \( V_{0.5} \) of \(-112 \pm 11 \text{ mV} \) and a slope factor of \( 12 \pm 2 \text{ mV} \) \((n = 7)\). When the duration of the test step was prolonged to 10 s to ensure greater activation of \( I_h \) at each test potential, \( V_{0.5} \) was shifted to \(-108 \pm 5 \text{ mV} \) and the slope factor was unchanged \((n = 4)\). However, when similar experiments were performed using the perforated patch configuration of the whole cell voltage-clamp technique, \( V_{0.5} \) was calculated to be \(-83 \pm 3 \text{ mV} \) and the slope \( 13 \pm 3 \text{ mV} \) \((n = 6)\). These values are similar to \( I_h \) recorded in other cell types. Moreover, because the perforated patch configuration minimizes perturbation of the intracellular compartment, these data suggest that intracellular regulators govern the availability of \( I_h \). Overall, the data show that a current activated by membrane hyperpolarization with characteristics similar to \( I_h \) recorded in other cell types is present in vascular smooth muscle cells isolated from rabbit portal vein.

**Effect of extracellular K\(^+\) and Na\(^+\) on \( I_h \).** A feature of \( I_h \) that distinguishes it from conventional K\(^+\) inward rectifiers is that it is carried by Na\(^+\) and K\(^+\) and has a concomitant \( E_{\text{rev}} \) that is between \(-25 \text{ mV} \) and \(-40 \text{ mV} \) \((\text{e.g., Refs. } 4 \text{ and } 23)\). Application of the modified GHK to these reversal potential measurements shows that the relative permeability of Na\(^+\) to K\(^+\) \((P_{\text{Na}}/P_{\text{K}})\) is between 0.2 and 0.5 \((26)\). In portal vein cells, \( E_{\text{rev}} \) was \(-37 \pm 2 \text{ mV} \) \((n = 7)\), which was markedly different from the theoretical potentials of K\(^+\) and Cl\(^-\) \((E_K \text{ and } E_{\text{Cl}})\) with the solutions used \((-78 \text{ mV} \) and \(-3 \text{ mV})\) and shows that \( I_h \) in portal vein myocytes is also carried by K\(^+\) and Na\(^+\). A similar value for \( E_{\text{rev}} \) was obtained in two cells where \( E_{\text{rev}} \) was also calculated by determining the reversal potential of the tail current following maximal activation at \(-120 \text{ mV} \). Similar to previous reports \((4, 23, 25)\), the amplitude of \( I_h \) in portal vein myocytes was not affected by complete removal of external Ca\(^{2+}\) \((n = 3)\). Calculation of \( P_{\text{Na}}/P_{\text{K}} \) in the portal vein myocytes using the reversal potential values and the modified GHK equation gave a value of 0.4 under physiological ion gradients that is similar to values reported for \( I_h \) in jejunal, lymphatic, and uterine smooth muscle.

The generation of inward current was highly dependent on the extracellular K\(^+\) concentration. Thus \( I_h \) could not be evoked when the external solution contained no K\(^+\) \((n = 3)\). Moreover, when the external K\(^+\) concentration was increased from 6 to 40 mM with a concomitant reduction in extracellular Na\(^+\) concentration, there was a marked enhancement of \( I_h \) \((2)\). In this series of experiments, the amplitude of \( I_h \) at \(-110 \text{ mV} \) was \( 35 \pm 18 \text{ pA} \) \((n = 7)\) in 6 mM external K\(^+\) and \( 114 \pm 58 \text{ pA} \) in 40 mM external K\(^+\) \((n = 5)\). The mean data from seven such experiments is shown in Fig. 2C, and it can be seen that the amplitude of \( I_h \) was augmented at every potential at which \( I_h \) was activated. This was associated with an acceleration of the rate of activation at each test potential. For example, the mean \( \tau \) for current activation at \(-110 \text{ mV} \) was \( 5.8 \pm 1.2 \text{ s} \) in 6 mM external K\(^+\) and \( 1.9 \pm 0.19 \text{ s} \) in 40 mM K\(^+\) \((n = 5)\). Figure 2C shows that the augmentation of current amplitude by an increase in the external K\(^+\) concentration was associated with a shift in the voltage dependence of activation to less negative potentials. Reduction of external Na\(^+\) by 36 mM without a concomitant increase in K\(^+\) \((\text{equimolar replacement with the impermeant cation Tris})\) did not augment \( I_h \) \((\text{see Fig. 3 for example})\). In four cells, the mean amplitude of \( I_h \) at \(-110 \text{ mV} \) was \(-27 \pm 7 \text{ and } -20 \pm 2 \text{ pA} \) with 136 mM and 102 mM external Na\(^+\), respectively. These data show that, similar to \( I_h \) in other studies \((26)\), the activation of this conductance in portal vein smooth muscle is dependent on the extracellular K\(^+\) concentration.

**Fig. 2.** Effect of raising external K\(^+\) concentration on \( I_h \). A: increasing external K\(^+\) from 6 (a) to 40 mM (b) markedly increased the amplitude of \( I_h \). The mean data from 7 such experiments are shown in B. Each point is the mean amplitude of \( I_h \) calculated as the amplitude of the instantaneous current subtracted from the current at the end of the test step. \( I_h \) was recorded in an external solution containing either 6 (○) or 40 (○) mM K\(^+\). C: Boltzmann analysis of tail currents recorded at \(-50 \text{ mV} \) following test steps between \(-30 \text{ and } -150 \text{ mV} \) in the presence of either 6 (○) or 40 (○) mM K\(^+\).
muscle cells is markedly accentuated by an increase in external K⁺ concentration.

**Inhibition of Iₜ by extracellular Cs⁺ and ZD-7288.** Previous studies have shown that Iₜ is rapidly inhibited by extracellular Cs⁺ (e.g., Refs. 4 and 25) and slowly blocked by the bradycardic agent ZD-7288 (5, 13). Figure 4A shows an example of the effect of bath-applied Cs⁺ on Iₜ activated in portal vein myocytes. Addition of 2 mM Cs⁺ to the bathing solution produced a rapid and complete inhibition of the time-dependent current (see Fig. 4B) that was fully reversible after 5-min wash with normal extracellular solution (mean data are shown in Fig. 4B). In comparison, application of Ba²⁺ up to 1 mM had no effect on the amplitude of Iₜ. In three cells, the mean amplitude of Iₜ at −110 mV in the absence and after 2-min incubation in 1 mM Ba²⁺ was −120 ± 47 pA and −119 ± 43 pA, respectively. Application of 10 μM ZD-7288 to the bathing solution produced a characteristically slow inhibition of Iₜ (see Fig. 4C), in contrast to the rapid effect of Cs⁺. Thus after 20-min application of 10 μM ZD-7288, the amplitude of Iₜ at −130 mV was inhibited by 30 ± 5% (n = 3). The pharmacological profile of Iₜ in portal vein smooth muscle cells is therefore similar to Iₜ recorded in other cell types.

**Effect of dibutyryl cAMP on Iₜ.** It has been shown in numerous studies that activation of Iₜ is rapidly (within 1 min) augmented by maneuvers that increase cAMP concentration, such as stimulation of adenyl cyclase by forskolin or inhibition of phosphodiesterases (10, 19, 26). In the present study, the cell-permeable analog of cAMP, dibutyryl cAMP (10–100 μM), was applied to cells exhibiting Iₜ. It can be seen from Fig. 5 that application of 100 μM dibutyryl cAMP for 8 min did not enhance the activation of Iₜ but actually diminished the current amplitude. In Fig. 5, the amplitude of Iₜ at −150 mV was decreased from −93 to −76 pA after...
8-min application of 100 μM dibutyryl cAMP, and the mean inhibition in five such experiments was 20 ± 7%. Application of dibutyryl cAMP at 40 μM also inhibited the amplitude of \( I_h \) (n = 3). However, Fig. 5 shows that application of dibutyryl cAMP did increase the amplitude of the outward K⁺ current evoked by depolarization to −30 mV. In these experiments, tetraethylammonium and 4-aminopyridine were excluded from the external solution to allow outward K⁺ currents to be recorded. Overall, the data suggest that, compared with \( I_h \) recorded in cardiac and neuronal cells, \( I_h \) in portal vein smooth muscle cells is not enhanced by an increase in cAMP concentration.

**Functional role of \( I_h \).** Previous studies have shown that \( I_h \) contributes to the depolarizing “sag” in membrane voltage produced in response to injection of hyperpolarizing current (e.g., Refs. 4, 23, and 25). This depolarizing potential can elicit overshoot action potentials and underpins cellular rhythmicity. Similar anomalous rectification of membrane potential was observed in the present study. Figure 6 shows an example of a current-clamp recording from a portal vein smooth muscle cell made using the perforated patch technique. In the cell shown, the resting membrane potential was −36 mV and injection of depolarizing current evoked a rapid depolarizing action potential followed by a sustained depolarization. Injection of 20-pA hyperpolarizing current produced an initial deflection to −87 mV that gradually relaxed to a level of −67 mV. Figure 6 also shows that the amplitude and rate of sag was enhanced by injection of more hyperpolarizing current. Termination of the current pulse was accompanied by an immediate rebound depolarization, and similar effects were observed in four other

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**Fig. 5.** Effect of dibutyryl cAMP on \( I_h \). Representative currents evoked by stepping from −50 mV to test potentials between −30 and −150 mV for 10 s in control conditions (A) or after 8-min application of 100 μM dibutyryl cAMP (db-cAMP; B) are shown. The amplitude of the outward current (\( I_{out} \)) evoked by depolarization from −50 to −30 mV is also shown. Neither tetraethylammonium nor 4-aminopyridine was included in the bathing solution to allow outward currents to be recorded in these experiments.

**Fig. 6.** Membrane potentials recorded in current clamp mode exhibit inward rectification. Recordings of action potentials and electrotonic potentials evoked by various intensities of depolarizing and hyperpolarizing pulses for 4 s are shown. Under control conditions (A), the electrotonic potentials elicited by the injection of hyperpolarizing current exhibit pronounced “sag” that was not apparent in the presence of 5 mM CsCl (B). Lines highlighted by an asterisk show the lag between the termination of the injection of hyperpolarizing current and the initiation of rebound depolarization.
cells. In the presence of 5 mM Cs\(^+\), which abolishes \(I_h\), the voltage response to injection of depolarizing current was unaffected, but the voltage sag seen following injection of hyperpolarizing current was completely absent and the extent of membrane hyperpolarization was more marked (Fig. 6B). Moreover, the generation of rebound depolarization after termination of the current pulse was markedly slowed. These data show that a hyperpolarization-activated and Cs\(^+\)-sensitive conductance limits the duration and extent of membrane hyperpolarizing responses and provides a depolarizing influence.

A role for \(I_h\) in the generation of spontaneous contractile activity has been implicated in other smooth muscle preparations (23, 25). Because portal veins exhibit spontaneous mechanical activity (31), we used conventional isometric tension recordings to study the possible contribution of \(I_h\) to the contractile activity of rabbit portal vein. Because the whole rabbit portal vein is too large to be bathed in a conventional organ bath, we used transverse segments taken from different areas of the vein. Under control conditions, there was a marked difference in the frequency of spontaneous contraction depending on the anatomic position of the segment (see Fig. 7A for example). Segments close to the hepatic end of the vessel exhibited contractions every 33 ± 5 s (n = 3 animals), whereas more central segments had a shorter intercontraction interval of 10 ± 2 s (n = 3 animals). Application of 5 mM Cs\(^+\) prolonged the interval between respective contractions in all segments of rabbit portal vein (mean increase in duration was 36 ± 4%; n = 6 segments from 3 rabbits). Addition of 20 μM ZD-7288 to the organ bath produced complicated effects on the spontaneous activity recorded in rabbit segments (n = 3 animals) that were difficult to quantify. This was characterized by a loss of synchrony, resulting in frequent small contractions at irregular intervals similar to previous observations on rat detrusor muscle (13). We also performed similar experiments on whole portal veins excised from rats to investigate whether Cs\(^+\) could modulate the spontaneous activity of a complete vessel. Under control conditions, the spontaneous activity of rat whole portal veins had a frequency similar to that of the faster contracting rabbit segments (compare Fig. 7, A and B). In Fig. 7B, the vessel exhibited regular, spontaneous contractions every 11 s under control conditions and the mean interval in tissues from seven animals was 10 ± 1 s. Application of 2 mM CsCl to the bathing solution slowed the frequency of contraction, and the mean interval in the presence of Cs\(^+\) (19 ± 3 s; n = 7) was significantly (\(P < 0.01\)) longer than the control interval. The effects of Cs\(^+\) were fully reversible after 5-min wash with normal bathing solution (Fig. 7C). Application of Cs\(^+\) also increased the mean amplitude of contraction from 0.5 ± 0.12 to 0.69 ± 0.17 g, which was reversed by washout of Cs\(^+\). Overall, the data show that agents that block \(I_h\) modulate the spontaneous contractile activity observed in segments of rabbit portal vein as well as complete portal veins isolated from rats.

**Molecular biology of the underlying channel.** The characteristics of \(I_h\) in rabbit portal vein resemble those of ion channels expressed by HCN genes. Consequently, we used mRT-PCR to determine the molecular identity of the channel underlying \(I_h\). Three rabbit genes encoding regions homologous to HCN2, HCN3, and HCN4 were found in rabbit portal vein smooth muscle cells (Fig. 8).

![Image](http://ajpcell.physiology.org/)

**Fig. 7.** Effect of extracellular Cs\(^+\) on the spontaneous contractile activity portal vein tissues. Examples of isometric tension recordings from segments of rabbit portal vein (A) or complete rat portal veins (B) are shown. Aa: example of a rabbit segment that contracted at a faster frequency than segments typified in Ab. B: spontaneous contractions in rat portal vein under control conditions (a) and after 5-min application of 2 mM CsCl to the bathing solution (b). In all panels, upward deflections represent an increase in tension produced by contraction of the tissue. C: mean data from 7 different rats showing the interval between each contraction under control conditions, after 5-min application of 2 mM CsCl, and after 10-min washout of CsCl.

![Image](http://ajpcell.physiology.org/)

**Fig. 8.** Hyperpolarization-activated cation (HCN) channels in rabbit portal vein smooth muscle cells. 2% Agarose gel shows HCN2–4 PCR amplicons from a multiplex PCR with secondary nested PCR using cDNA made after 50 single rabbit portal vein smooth muscle cells were collected.
The clones isolated from the smooth muscle cells (rpvsHHCN2, 3, and 4) showed significant homology with those already published from rat and rabbit. rpvsHHCN2 was 95% homologous to accession no. AF067815, rpvsHHCN3 was 97% homologous to accession no. AF247452, and rpvsHHCN4 was 95% homologous to accession no. AB022927. These data show that cDNA corresponding to HCN is amplified by mRT-PCR in single cells collected from rabbit portal vein smooth muscle.

**DISCUSSION**

We show in the present study that in rabbit portal vein smooth muscle cells an ion current carried by monovalent cations is elicited by progressive membrane hyperpolarization. This current, termed $I_h$, was not affected by 100 μM Ba$^{2+}$ but was rapidly inhibited by external Cs$^+$ and slowly blocked by a specific inhibitor, ZD-7288. The degree of inhibition and slowness of effect of ZD-7288 was similar to that reported in rat detrusor myocytes (13). These kinetics and pharmacological properties of $I_h$ are similar to those of $I_h$ reported in other spontaneously active, visceral smooth muscles (see Introduction) and are clearly different from the features of the time-independent, Ba$^{2+}$-sensitive inward rectifier K$^+$ channels (Kir) that have been reported in some arterial smooth muscle cells (6). Although this current was described briefly by Kamouchi et al. (18), the present study represents the first extensive characterization of this conductance in vascular smooth muscle cells. Moreover, mRT-PCR reactions using primers specific to HCN1–4 revealed the existence of three HCN clones in portal vein myocytes that have been shown to encode for hyperpolarization-activated cation currents (9, 19, 29). A functional role for this conductance was implicated from current clamp experiments that showed that injection of hyperpolarizing current produced a marked Cs$^+$-sensitive depolarizing sag. In addition, blockers of $I_h$ modified the spontaneous contractile activity of rabbit and rat portal veins.

**Voltage-dependence of $I_h$ in portal vein myocytes.** In the present study, the activation of $I_h$, at room temperature was relatively slow, differentiating this conductance form a conventional, pure K$^+$ inward rectifier (e.g., Kir). The activation of $I_h$ was described by a single exponential, and the time constant for activation was similar to values reported for $I_h$ in other smooth muscle cells (see Refs. 23, 25, and 35). However, the $V_{0.5}$ of $I_h$ using the whole cell configuration (approximately −108 mV) was more negative than values in other studies. Thus in other smooth muscle cell types, the $V_{0.5}$ values for activation of $I_h$ range between −74 mV (rat bladder; Ref. 13) and −91 mV (guinea pig ileum; Ref. 35). The negative $V_{0.5}$ of $I_h$ in portal vein cells suggests that the physiological contribution of $I_h$ would be negligible. However, Yanagida et al. (35) have shown that the $V_{0.5}$ for activation becomes 12 mV less hyperpolarized per 10°C increase in temperature, and therefore the involvement of $I_h$ at physiological temperatures would be greater. Moreover, in the present study the $V_{0.5}$ of $I_h$ was shifted to less negative potentials (−83 mV) when the perforated patch configuration was used. Because the intracellular ionic conditions are the same with this technique but loss of intracellular regulators is minimized, these data suggest that a cytosolic mediator that is washed out by the conventional whole cell configuration regulates the activation of $I_h$ in portal vein smooth muscle cells. Hisada et al. (15) also proposed that the activation of hyperpolarization-gated, but not stretch-activated, cation currents in toad stomach myocytes was dependent on a soluble mediator. In portal vein cells, the regulator seems not to be cAMP (discussed in Modulation of $I_h$ in portal vein cells by cAMP), because, in general, binding of cAMP only shifts $V_{0.5}$ by about +11 mV, which is insufficient to explain the +30-mV shift in $V_{0.5}$ observed in the present study. Interestingly, cation currents generated by the heterologous expression of HCN clones have $V_{0.5}$ values of about −100 mV (19, 24, 28), and the activation of $I_h$ is exquisitely dependent on the local environment (see Ref. 29). It remains the focus of future studies to determine what is the exact nature of the regulator concerned in portal vein myocytes.

**Modulation of $I_h$ in portal vein cells by cAMP.** One of the distinctive properties of endogenous hyperpolarization-activated cation currents in cardiac and neuronal preparations is that the voltage dependence is shifted to less negative potentials by the direct binding of cAMP to the channel (10, 26). In the present study, application of a cell-permeable analog of cAMP (dibutyryl cAMP) did not enhance $I_h$ and actually reduced the amplitude of $I_h$ slightly. However, dibutyryl cAMP did augment the outward current in portal vein cells, consistent with the effects of cAMP on delayed rectifier currents reported previously in this cell type (1). A similar lack of effect of cAMP has been observed on native $I_h$ in octopus cells of the mammalian cochlear (2) and anterior pituitary cells (32), as well as some heterologously expressed HCN isoforms (8, 19). Moreover, it is significant that only one study of $I_h$ in smooth muscle cells has reported a small stimulatory effect of cAMP on $I_h$ (35). These data suggest that the channel underlying $I_h$ may have a different molecular composition from $I_h$ channels in cardiomyocytes and various neurons, where the current is highly sensitive to cAMP. Alternatively, the basal level of cAMP in portal vein smooth muscle cells may be sufficient to saturate the cyclic nucleotide binding site similar to the situation in mouse anterior pituitary cells (32). Future studies will attempt to elucidate the precise regulatory pathways that govern the activation of $I_h$ in portal vein smooth muscle cells.

**Identification of HCN in rabbit portal vein.** The facts that the native $I_h$ in portal vein myocytes is activated by hyperpolarization, that its selectivity for K$^+$ over Na$^+$ is low, and that it can be blocked by Cs$^+$ all suggested that its molecular structure would be similar to that encoded by the HCN gene family. Consequently, we isolated partial cDNA clones for three
rabbit portal vein smooth muscle cells. The rate of activation of $I_h$ in portal vein cells is comparable to the slow kinetics of heterologously expressed HCN4 that has an activation time constant at $-150$ mV of a few hundred milliseconds (19, 29), which is considerably slower than that of other HCN isoforms. However, activation of HCN4 is augmented markedly by cAMP (19, 29), in contrast to the relative insensitivity of the native $I_h$ to cAMP in portal vein cells. HCN1 channels are almost unaffected by cAMP (8, 19, 29), but the rate of activation of HCN1 channels is very fast, and no expression of HCN1 gene products was detected in the single-cell PCR of portal vein myocytes.

HCN channel subunits assemble to form either homotetramers or heterotetramers, and recent studies suggest that when different members of the HCN family are coexpressed the heterologous channels have distinct channel characteristics in terms of kinetics and sensitivity to cAMP compared with homologous combinations (8, 33). Moreover, the characteristics of HCN channel subunits can be modulated by auxiliary, non-pore-forming β-subunits, such as minK-related peptide 1 (MiRP1; Ref. 36), similar to the situation with K$^+$ channels (e.g., Ref. 34). Consequently, which isoform (or isoforms) contribute to the channel protein underlying $I_h$ can only be guessed until the portal vein transcripts are cloned and expressed in heterologous expression systems. These experiments should also highlight whether the features of the native current are the product of heterogeneous HCN combinations alone or due to modulation by subunits such as MiRP1. Additional PCR experiments to identify the presence of regulatory subunits in the portal vein should also be undertaken. In addition, antibodies to various HCN isoforms have been raised, and these could be used to elucidate functional HCN isoforms.

**Putative role of $I_h$ in the generation of spontaneous contractile activity of the portal vein.** In current-clamp experiments, injection of hyperpolarizing current evoked an electrotonic potential that had a pronounced depolarizing sag that was followed immediately on termination of current injection by a marked depolarization. Application of Cs$^+$ abolished the anomalous rectification and increased the latency of the rebound depolarization. Moreover, Cs$^+$ markedly slowed the spontaneous contractile activity of segments of rabbit portal vein as well as the rhythmic activity of whole rat portal veins. Similar effects of external Cs$^+$ were observed on contractile activity in sheep lymphatic vessels (23) and rat uterine tissues (25). This effect is consistent with the blockade of an ion conductance that provides a depolarizing influence sufficient to activate voltage-dependent Ca$^{2+}$ influx. The voltage dependence, activation kinetics, and lack of inactivation of $I_h$ give this conductance the unique property of activating on membrane hyperpolarization that follows action potential discharge (19, 26). Hence, $I_h$ has been implicated as a pacemaker current. This proposal is supported by the observation that $I_h$ has so far been reported in smooth muscle cells that exhibit regular spontaneous contractions analogous to the rhythmic firing and contraction of thalamic neurons and cardiomyocytes (26). However, the fact that Cs$^+$ did not abolish spontaneous contractile activity in either rat or rabbit portal vein suggests that $I_h$ is not the sole mechanism responsible for generating spontaneous activity in these preparations. The precise mechanism that governs rhythmicity in the portal vein is unknown and would require the use of various agents such as tetraethylammonium and 4-aminopyridine in functional studies to tease apart the contributions of different ion channels to normal electrical activity. Moreover, it is a worthwhile caveat that application of Cs$^+$ externally might also affect other ion channels involved with the normal electrical activity of smooth muscle cells. Ideally, a more selective and specific agent would be used to probe the functional role of $I_h$ in the generation of spontaneous activity. However, application of the specific inhibitor of $I_h$, ZD-7288 (see Refs. 5 and 13), produced more complicated effects on the spontaneous activity of rabbit portal vein segments, including an increase in basal tension, that were comparable to the actions of this agent on spontaneous contractile activity in rat detrusor muscle (13). The mechanism by which ZD-7288 increases contractile activity was not determined in the previous study (13) but precluded the use of this agent to probe for a role of $I_h$ in the generation of spontaneous activity.

In conclusion, the present study shows that a hyperpolarization-activated cation current with characteristics similar to $I_h$ reported in cardiomyocytes, neurons, and visceral smooth muscle cells (26) is present in a vascular smooth muscle cell type. However, it is worth noting that the portal vein is a relatively specialized blood vessel in that it has a predominant longitudinal layer of smooth muscle (16, 31) that contributes to its function as a pumping unit exhibiting regular, coordinated contractions. It is therefore possible that the hyperpolarization-activated current studied in the present investigation may not exist in quiescent “conventional” blood vessels. It remains the aim of future experiments to determine the molecular and regulatory determinants of this conductance in smooth muscle and whether such a conductance exists in other blood vessels.

We acknowledge the helpful comments of Dr. Mark Hollywood, Queen’s University, Belfast and thank Professor Tom Bolton at St. George’s Hospital Medical School for his advice and support.

I. A. Greenwood is a Wellcome Trust Research Career Development Fellow (grant no. 053794/Z/98S). S. A. Prestwich is a Research Fellow at St. George’s Hospital Medical School.

**REFERENCES**

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