Ca^{2+}-Sensitive K^+ Channel in Aortic Smooth Muscle of Rats (43196)

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Abstract. **We measured K^+ channel activity in inside-out patches of cell membrane from aortic vascular smooth muscle cultured (Passages 1–3) from Wistar, Wistar-Kyoto, and spontaneously hypertensive rats (SHR). With $[Ca^{2+}]_i$ between 25 and 100 n*m* and 150 m*m* K^+ on both sides of the membrane, the conductance of this channel was 55 ± 7 pS (slope of current-voltage curve through 0 mV) and the current was outwardly rectified. There was no difference in single-channel conductance among the three rat strains. Increasing negative holding voltages or increasing $[Ca^{2+}]_i$, increased the probability of this type channel being open (Np_o; $P < 0.01$); SHR had a larger NP_o ($P < 0.01$). Compared with cells from Wistar and Wistar-Kyoto, cells from SHR also had the longest mean open time. The increased NP_o and mean open time we observed in this K^+ channel of cells from SHR could contribute, at least in part, to the increased membrane K^+ permeability, reported previously.**
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Specific potassium channels are found in vascular smooth muscle (VSM), and other cell membranes (1), and they are involved in the modulation of cell membrane potential and contractile state. A large proportion of the resting membrane potential (V_m) is attributable to the K^+ electrochemical gradient (diffusional forces), and the V_m is near E_k (2–4). Whole cell current recordings have provided evidence for Ca^{2+}-activated K^+ channels in several cell types, including arterial smooth muscle (5). Single-channel studies of Ca^{2+}-activated K^+ channels in VSM have been reported by Sadoshima *et al.* (6, 7), Benham *et al.* (8), Williams *et al.* (9), and Inoue *et al.* (10, 11); using 100 n*M* (or greater) $[Ca^{2+}]_i$, these investigators observed channels with conductances ranging from 92 to 200 pS. Under the above conditions, the K^+ channels probably are involved in repolarization of the membrane. Ca^{2+}-activated single K^+ channels have also been found in intestinal smooth muscle (12, 13), gastric smooth muscle (14–16), as well as other types of cells (17).

An increased cell membrane permeability to K^+ in aortic strips from spontaneously hypertensive rats (SHR) was initially reported by Jones (18). Aviv and his co-investigators (19, 20) have observed similar ion transport abnormalities in VSM cells cultured from SHR aorta. However, we find no previous patch clamp studies comparing the characteristics of single K^+ channels in SHR, Wistar-Kyoto (WKY), and unrelated Wistar VSM. Thus, the objectives of the present study were (i) to use patch-clamp techniques to characterize the K^+ channel found in rat aortic vascular smooth muscle cells cultured with resting levels of $[Ca^{2+}]_i$; (ii) to determine the sensitivity of activation of this channel to change in holding voltage and $[Ca^{2+}]_i$; and (iii) to determine if there are differences in the characteristics of this channel in cells cultured from Wistar, WKY, or SHR aortic VSM cells.

Materials and Methods

Vascular Smooth Muscle Cell Cultures. Thoracic aortic smooth muscle cells were obtained from each of five batches of SHR, with each batch composed of three rats; all rats had systolic blood pressures > 150 mm Hg. VSM cells were also obtained from each of four batches of WKY and six batches of unrelated Wistar rats, each including three rats; the WKY and Wistar rats had systolic blood pressures below 125 mm Hg. The rats (SHR and WKY from Taconic, Germantown, NY, and Wistar from Harlan Laboratories, Indianapolis, IN) weighed 175–250 g (8–12 weeks old). We obtained cells by enzyme (collagenase-elastase) digestion using methods described previously (21). Routinely, $>10^6$ cells/aorta were harvested, and more than 90% of the cells

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were viable (trypan blue exclusion). Dissociated cells were seeded at $0.5 \times 10^4/cm^2$ on plastic coverslips in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (Hyclone, Logan, UT) with penicillin (100 units/ml) and streptomycin (100 μg/ml). Approximately 60–90% of the cells attached to the coverslips. Thereafter, cells were fed every second day. Cells from Passages 1–3 were used in experiments 2–5 days after plating; cells from nonconfluent areas of the coverslip were patched.

Patch Clamp Techniques. We pulled pipettes from glass capillary tubing (8161 type; Garner Glass Co., South Indian Hills, CA), using a two-stage (Narishige PP 83; Medical Systems Corp., Greenvale, NY) puller. The taper of the electrode was insulated (Q-Dope; GC Electronics, Rockford, IL) and the tips were fire polished and produced a tip resistance of 2–5 MΩ when filled with 150 m*M* KCl. The pipette was connected to the head stage of an amplifier (List EPC-7; Medical Systems Corp.) by an Ag:AgCl electrode-agar-0.1 *M* KCl bridge (22–24); the reference electrode (Ag:AgCl pellet) was similarly connected to the bath. A Nikon Diaphot microscope with Hoffman modulation optics (×400) final magnification; Nikon, New York, NY) on a "vibration-free" table (Micro-G, Woburn, MA) enclosed in a Faraday cage, was used to visualize the cells; a micromanipulator (Newport Corp., Fountain Valley, CA) with motorized drive was attached to the microscope stage and used for controlling the pipette.

We prepared all solution with Milli Que water (Millipore, Bedford, MA). We used a Ringer-type solution (containing in m*M*: 150 NaCl, 5 KCl, 1 $MgCl_2$, 1 Hepes, 1 EGTA, and 0.1 $CaCl_2$ with a pH of 7.25) in the chamber (bath) until we obtained an inside-out patch, at which time we filled the chamber with the following solution (in m*M*): 145 K^+ glutamate (stock solution prepared by titrating KOH with glutamic acid), 5 Hepes, 5 $MgCl_2$, and 1 EGTA, pH 7.25 (with NaOH). Ca^{2+} was added to the solutions to produce a concentration of 25, 50, or 100 n*M* by the procedures described by Fabiato and Fabiato (25) and Burgess *et al.* (26). Chamber fluid (0.5 ml) was changed by a peristaltic pump at a rate of 2.5 ml/min. The pipette contained the same potassium glutamate solution with 0.4 m*M* Ca^{2+}. All solutions were prepared weekly, stored in a refrigerator until the day of the experiment, and then brought to room temperature and filtered with a 0.2-μm filter.

We checked $[Ca^{2+}]$ of the solutions by the Fura-2 method (27). The range of bath $[Ca^{2+}]$ that was used equaled that present during resting conditions of the VSM cells (28, 29). With these $[Ca^{2+}]$ single channels were usually observed, and the data were reproducible; in contrast, $[Ca^{2+}]_i$ greater than 100 n*M* produced multiple-type flickering channels, as observed by Sadoshima *et al.* (6, 7). For preparing current voltage plots, holding voltages (V_h) of ±50, ±40, and ±20 were used. During experiments to test the effect of $\Delta[Ca^{2+}]_i$ or holding voltage on NP_o, etc., only −20, −40, and −50 mV were used (inside of the membrane in reference to the outside), with N being the number of channels active per patch, and P_o being the probability of a channel being open. We conducted all experiments at room temperature (≈20 °C). Cells were patched within 1 hr after removal from the incubator; after exposure to a high K^+ experimental solution, the cells were discarded.

As a final procedure, to estimate channel selectivity (K^+ selective versus nonselective cation channel) and relative permeabilities (P_K/P_{Na}), we used solutions having a 10-fold gradient across the membrane for K^+ inward and Na^+ outward (changed chamber solution and kept pipette solution constant). The predicted reversal potential versus the actual E_{Rev} gave an estimated P_K/P_{NA} ratio. The $E_{Rev} \approx E_K+$ in these inside-out patches.

We used an inside-out membrane patch configuration (30) in all experiments reported. We used K^+ glutamate solutions in the pipette and chamber to eliminate anion currents. At each holding voltage, we recorded K^+ channel activity for 20–25 sec; from these data we plotted current-voltage relations (for estimating conductance and rectification), open time histograms (t_o), closed time histograms (t_c), and calculated the NP_o. For each holding voltage at each $[Ca^{2+}]_i$, we obtained NP_o by dividing the time that channels were open by the total time of the record analyzed, and we analyzed records with one active channel per patch ($n = 1$).

Patch clamp currents (filtered at 3 kHz) and the holding voltage were recorded on video tape (Panasonic PV-1364 video recorders and Sony PCM-502ES adapter; A. R. Vetter, Rebersburg, PA). We analyzed recorded data by the "P-Clamp-5.0" program (Axon Inc., Burlingame, CA), and the data obtained from this analysis were tested by the use of analysis of variance for unequal groups, using a 3 × 3 factorial, making preplanned comparisons among strains, i.e., testing the effects of change in $[Ca^{2+}]_i$, or change in V_h (between and within strains of rats) on NP_o, T_o, and so forth. The hypothesis rejection was set at $P < 0.05$. Because of the unequal variances among groups, we also analyzed log-transformed data. However, conclusions were similar, so data are reported with the simpler analysis.

Results

Under the conditions used in growing the VSM, this type of K^+ channel was present in cell membrane patches of most cells studied. Within each strain, results from first to third passaged cells were similar. We observed no significant differences in channel charac-

teristics related to time after plating (2–5 days), but the cells became more difficult to patch (form a GΩ seal) 4 days after plating. Overall, passage number, days after plating, and patches per coverslip were similar for cells from WKY, SHR, and Wistar strains.

Shown in Figures 1 and 2 are sample recordings of inside-out patches with 145 m*M* K^+ glutamate, 2.5 μM Ca^{2+} solution in the pipette, and 145 m*M* K^+ glutamate in the chamber; the bath $[Ca^{2+}]$ is given for each condition in the various figures: patch V_h are given for the cell interior in reference to the outside of the cell. In all figures, a downward deflection of the current trace indicates an inward current flow. All sample current traces were filtered at 250 Hz. Sample records showing channel activity using 100 n*M* of $[Ca^{2+}]_i$ are shown in Figure 1. Currents from an inside-out patch of a VSM

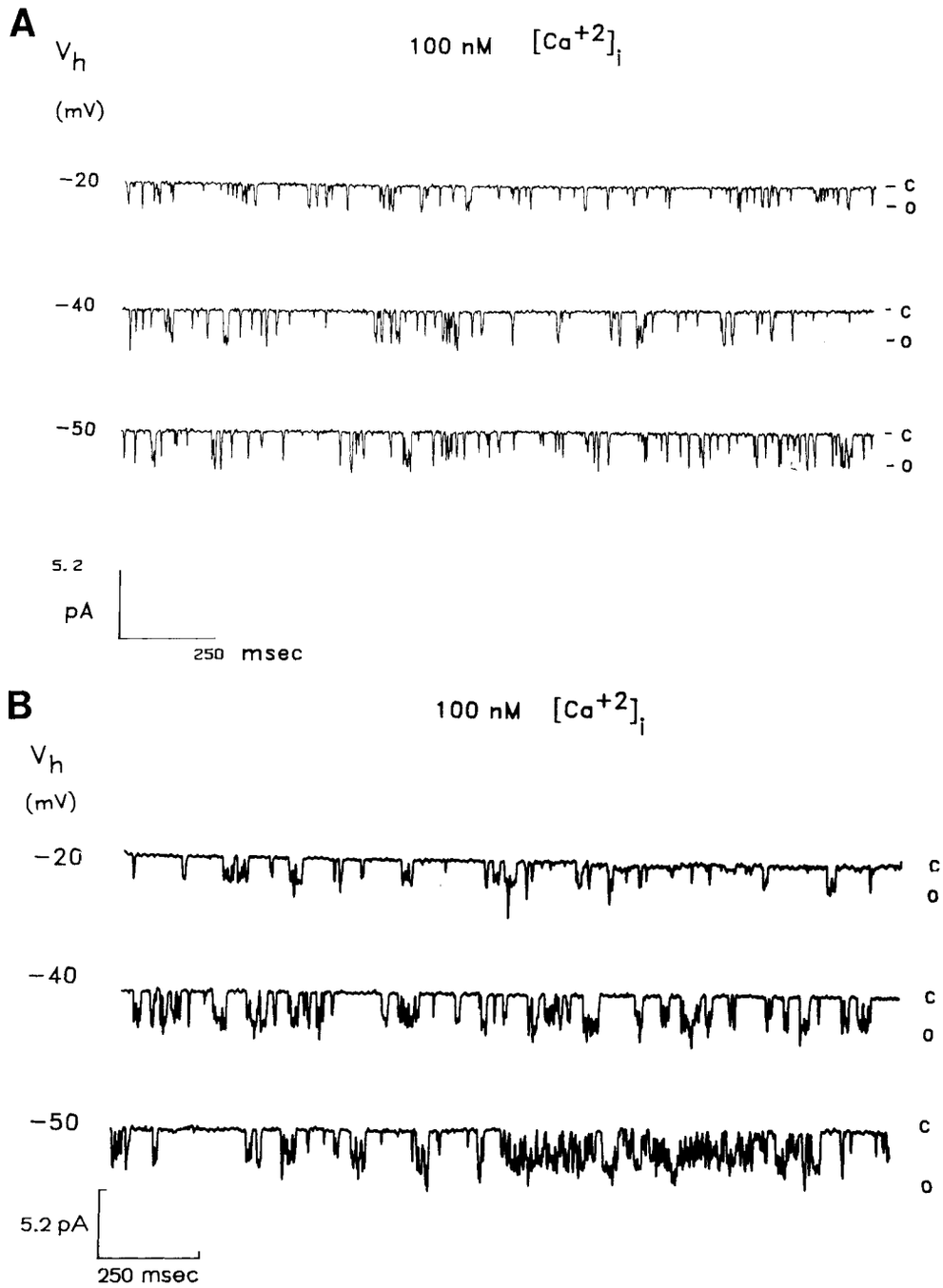

Figure 1. Sample records of K^+ channels in VSM using inside-out patches. K^+-glutamate (145 m*M*) was in all solutions ($E_K = 0$); these records (and all records shown) were filtered at 250 Hz. (A) K^+ channel in a WKY cell when 100 n*M* $[Ca^{2+}]_i$ was in the bath. C, the channels are closed; o, current flow when the channels are open. The holding voltages are given as the inside of the cell in reference to the outside; a downward deflection indicates inward current flow. (B) A sample record of K^+ channels from a SHR when 100 n*M*$[Ca^{2+}]_i$ was in the bath.

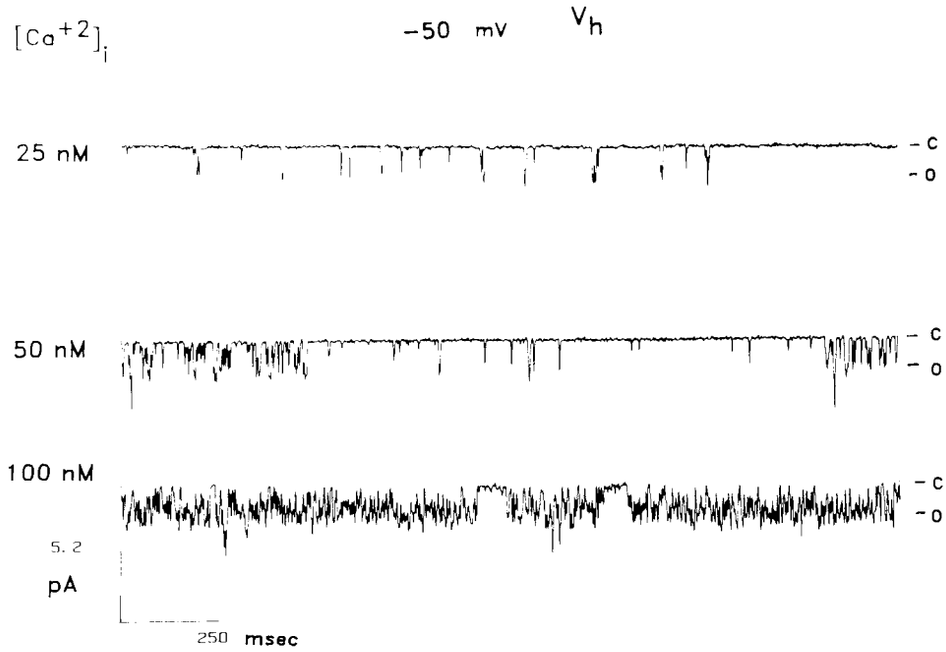

Figure 2. Sample records of K^+ channel current with the V_h held at −50 mV in Wistar cells and the $[Ca^{2+}]_i$ varied. The other conditions were the same as in Figure 1.

cell from a WKY rat are shown; the holding voltages were changed from −20 to −40 and −50 mV. In Figure 1B, channels from SHR VSM are shown; the $[Ca^{2+}]_i$ was 100 n*M*. Probability of a channel being open (NP_o) varied between patches, and Figure 1 illustrates the response of the test conditions to change in holding voltage and a given $[Ca^{2+}]_i$, and the tracings are not intended to illustrate the difference between strains, i.e., there were variations among NP_o between patches, and the differences between strains of cells could only be determined by using patch clamp analysis and statistics. The 100 n*M* Ca^{2+} response was chosen because it was near physiologic resting levels.

Figure 2 is a current record taken from a Wistar VSM inside-out patch. The holding voltage was −50 mV, and the $[Ca^{2+}]_i$ was varied. Figure 2 shows a sample current trace with some channel openings occurring in bursts. This type record was also obtained in some patches using WKY or SHR cells.

Shown in Figure 3 is a current-voltage plot obtained from the three rat strains (chamber contained 50 n*M* Ca^{2+}). For each voltage point, the mean channel current amplitude from 10 or more patches, each with greater than 500 channel openings, were averaged. With an equal concentration of K^+ on both sides of the membrane, there was a slight outward rectification. The single channel conductance with 150 m*M* K^+ glutamate on both sides of the membrane was 55 ± 7 pS when measured from −20 to +20 mV, i.e., slope of the current-voltage curve through zero but note the outward rectification. Although the single-channel conductance did not increase as the $[Ca^{2+}]_i$ increased, the

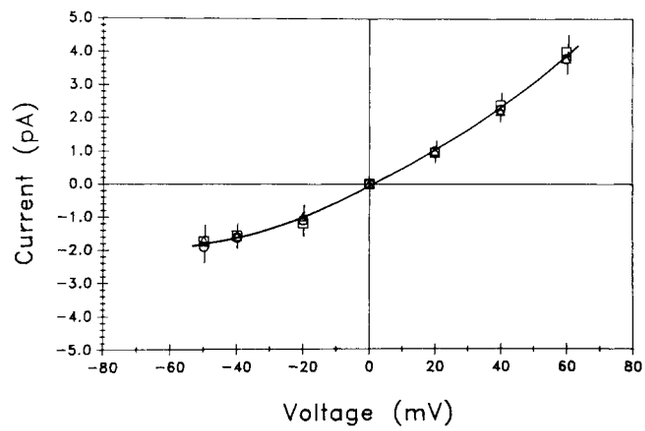

Figure 3. Current-voltage plot obtained from inside-out patches of Wistar (○), SHR (△), and WKY (□). Each point represents a mean of 10 patches ± SE; 500 or more channel openings were analyzed in each patch.

probability of a channel being open (NP_o) increased. During this project, 25 patches of Wistar and 40 of WKY and SHR were made, but due to noisy recordings or multiple channel activity, only the number of patches shown in Table I were analyzed. At each $[Ca^{2+}]_i$, we detected no significant difference in the single channel conductance among Wistar, SHR, and WKY VSM cells.

Mean and SE of the NP_o values are summarized in Table I. Analysis of variance of the data in Table I provided the following statistically significant comparisons: NP_o increased with increasing $[Ca^{2+}]_i$ ($P < 0.01$); NP_o increased with an increase in hyperpolarization

Table I. Open Channel Probability (NP_o)[a]

$[Ca^{2+}]_i$ (n*M*)	V_h (mV)	*n*[b]	Wistar	*n*	WKY	*n*	SHR
25	−20	11	7.5 ± 3.1	13	25.5 ± 6.5	18	32.7 ± 5.1
	−40	11	7.9 ± 3.1	15	31.7 ± 6.4	16	36.2 ± 5.4
	−50	11	7.7 ± 2.7	14	40.7 ± 6.6	14	46.8 ± 5.7
50	−20	13	22.8 ± 4.8	9	25.7 ± 5.9	12	39.6 ± 8.9
	−40	12	34.9 ± 6.9	10	34.4 ± 8.0	9	42.2 ± 8.0
	−50	12	36.7 ± 6.8	8	36.6 ± 8.6	11	53.9 ± 7.1
100	−20	10	27.9 ± 5.8	10	37.1 ± 6.2	10	52.4 ± 10.1
	−40	10	34.2 ± 6.8	8	34.4 ± 8.8	6	44.8 ± 11.7
	−50	10	34.6 ± 7.5	7	48.4 ± 9.7	7	62.1 ± 8.9

[a] NP_o in inside-out patches. Data were analyzed for a 20-sec segment at each voltage to estimate the NP_o.
[b] Indicates number of patches.

change in V_h ($P < 0.002$); NP_o differed due to strain of rats ($P < 0.01$); and preplanned comparisons among strains indicated that the NP_o of the cells of the three groups significantly differed as follows: SHR > WKY > Wistar. In all tables, *n* represents number of patches from which data were analyzed, and data from each patch contained >500 channel openings. At any given condition, the NP_o of channels in cells from SHR was significantly greater ($P < 0.001$) than that for cells from WKY and Wistar; the NP_o of cells from WKY was significantly greater ($P < 0.01$) than that for cells from unrelated Wistar rats.

Open time (t_o) and closed time(t_c) histograms were plotted, and time constants for each histogram were obtained by fitting exponentials to the plot (as shown in Fig. 4). The channel open time histograms were best fit by a single exponential (see Fig. 4A), and the time constants for mean t_o are shown in Table IIA. Analysis of variance of the data in Table IIA provided the following comparisons that were significant: t_o differed among strain ($P < 0.001$) and t_o differed when the effects of strain X $[Ca^{2+}]_i$ were totaled ($P < 0.02$). Preplanned comparisons among strains indicated that t_o of the cells of the three groups significantly differed as follows: SHR > WKY = Wistar. Significant inter-strain differences were observed for the t_o. The mean open time of the channel was longest in the SHR cells, compared with cells from unrelated Wistar and WKY ($P < 0.001$). Four of 11 patches in cells from Wistar rats had no channel activation at 25 n*M* $[Ca^{2+}]_i$, but channels in all patches activated at 50 n*M* $[Ca^{2+}]_i$. The best fit for closed time histograms required two exponentials, t_{c1} and t_{c2} (see Fig. 4B for typical histogram); t_{c1} represents the time a channel is closed within a burst of channel activity, and t_{c2} represents the time between bursts (see Table IIB for the t_{c1} values). Analysis of variance of the data shown in Table IIB provided a significant change in t_{c1} due to different strains ($P < 0.02$); preplanned comparisons among strains indicated that t_{c1} of the cells of the three groups significantly differed as follows: Wistar > WKY = SHR. Regarding t_{c1}, channels in the unrelated Wistar cells had the greatest mean closed time compared with cells from either WKY or SHR. There were no significant differences in t_{c2} among strains, and these values will not be discussed further. To ensure that we were measuring currents from a K^+ selective channel, the relative permeabilities

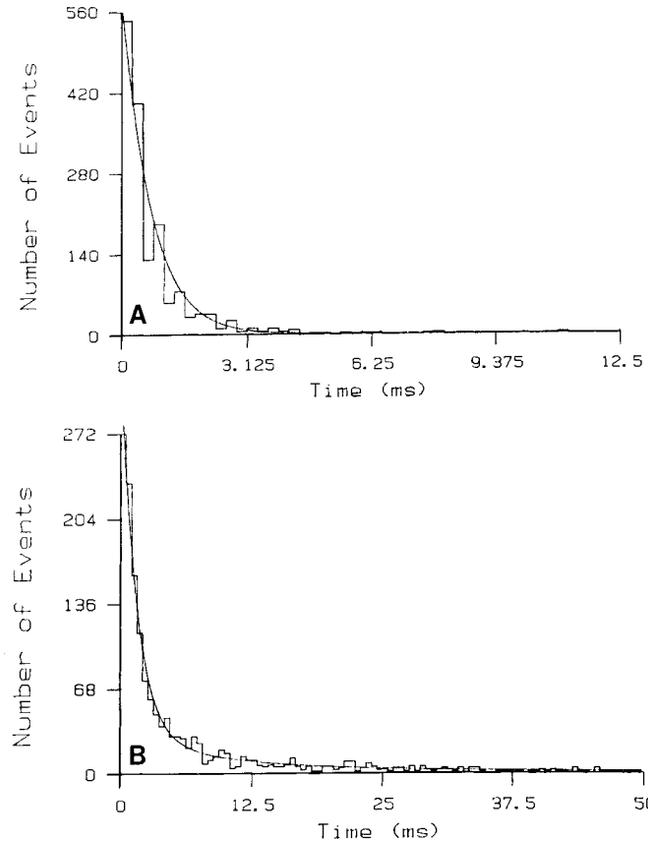

Figure 4. Sample histogram of channel open and closed times, WKY cells, −20 mV, 50 n*M* $[Ca^{2+}]$. (A) Open time histogram that was best fit by a single exponential that gave a mean t_o of 0.886 msec. (B) Closed times histogram that was best fit with two exponentials that gave a mean t_{c1} of 1.523 msec and t_{c2} of 9.236 msec.

Table II. Mean t_o and t_{c1} (msec)

$[Ca^{2+}]_i$ (n*M*)	V_h (mV)	*n*	Wistar	*n*	WKY	*n*	SHR
A. Mean t_o[a]							
25	−20	11	0.59 ± 0.31	11	1.09 ± 0.31	12	1.80 ± 0.30
	−40	11	0.59 ± 0.31	9	1.15 ± 0.34	12	2.10 ± 0.30
	−50	11	0.57 ± 0.31	8	1.28 ± 0.37	11	2.30 ± 0.31
50	−20	13	1.27 ± 0.29	7	1.09 ± 0.39	7	1.94 ± 0.39
	−40	12	1.55 ± 0.30	8	1.08 ± 0.37	9	2.00 ± 0.34
	−50	12	1.57 ± 0.30	9	1.08 ± 0.34	8	2.17 ± 0.37
100	−20	10	1.46 ± 0.33	7	1.08 ± 0.39	8	2.08 ± 0.37
	−40	10	1.34 ± 0.33	8	0.97 ± 0.37	6	1.51 ± 0.42
	−50	10	1.25 ± 0.33	8	1.04 ± 0.37	6	1.72 ± 0.42
B. Mean t_{c1}[b]							
25	−20	5	1.08 ± 0.41	9	0.69 ± 0.31	12	1.90 ± 0.26
	−40	4	1.12 ± 0.46	9	0.72 ± 0.30	10	1.14 ± 0.29
	−50	5	1.24 ± 0.41	9	0.55 ± 0.30	12	1.00 ± 0.26
50	−20	11	0.96 ± 0.28	6	1.69 ± 0.37	8	1.24 ± 0.32
	−40	12	0.99 ± 0.26	7	1.09 ± 0.35	8	1.08 ± 0.32
	−50	11	2.10 ± 0.28	6	1.13 ± 0.37	8	1.04 ± 0.32
100	−20	10	1.65 ± 0.29	7	1.01 ± 0.35	8	0.94 ± 0.33
	−40	10	1.44 ± 0.29	7	0.51 ± 0.35	6	1.38 ± 0.37
	−50	9	1.10 ± 0.30	6	0.65 ± 0.37	4	1.01 ± 0.46

[a] Mean t_o of inside-out patches expressed in msec. The mean t_o was calculated by exponential fit.
[b] Mean t_{c1} of inside-out patches expressed in msec. Closed time histograms were constructed for each condition (V_h and $[Ca^{2+}]_i$) for each strain and the data fit with a double exponential. The t_{c1} (for the first exponential) represents the time the channel is closed within a burst of channel activity.

were determined; this type of K^+ channel had a $(P_K/P_{Na}) > 5$, and a $(P_K/P_{CL}) > 5$, $n = 4$ for each ratio.

Discussion

Ca^{2+}-activated K^+ channels serve to restore cell membrane potential (repolarize) toward normal values after excitation involving increases in $[Ca^{2+}]_i$ (31). Thus, abnormalities in the activity of these channels could contribute to the disease mechanism of hypertension. We compared Ca^{2+}-sensitive K^+ channels in isolated aortic smooth muscle cells from SHR, WKY, and unrelated Wistar rats. Using $[Ca^{2+}]_i$ levels within resting concentration ranges, we assessed the physiologic activity of a single type of Ca^{2+}-activated K^+ channel, i.e., role of this channel under resting conditions (muscle tone and Ca^{2+} levels), and postulate that this type channel can help modulate the resting membrane potential under resting conditions.

The characteristics of Ca^{2+}-activated K^+ channels in several smooth muscle cell types were reviewed by Tomita (32) and Latorre *et al.* (33). The magnitude of the conductance of the channel we observed, 55 pS, is different from that measured in bovine aortic smooth muscle (266 pS) (9), guinea pig mesenteric artery (200 pS) (8), rat aortic cells (135 or 140 pS) (6, 7), rabbit portal vein (273, 180, and 92 pS) (10, 11), or striated muscle (300 pS) (34). Thus, it appears that several varieties of Ca^{2+}-activated K^+ channels exist within vascular smooth muscles. Although less likely, it is also possible that under different conditions($[Ca^{2+}]_i$, temperature, voltage, and chamber solutions), a single K^+ channel may conduct in several substrates. The NP_o of K^+ channels we observed in cultured rat aortic cells is slightly sensitive to $[Ca^{2+}]_i$ [over the range studied (25–100 n*M*)] and holding voltages; there was no steep change in slope of the NP_o over the Ca^{2+} or V_h used. We assumed $N = 1$ (number of channels open per patch), but this gave a maximum estimate of NP_o. When we increased Ca^+ to >100 n*M*, we observed a larger conductance K^+ channel (≈150 pS) in a few patches. This channel is presently being further characterized.

As has been shown in other cells, we observed that the conductance of the individual channels did not change as a function of the $[Ca^{2+}]_i$, but instead raising $[Ca^{2+}]_i$ increases the probability of a channel being open. Wistar VSM cells were more sensitive to the $[Ca^{2+}]_i$ in the 25–50 n*M* range, but as shown in Tables I and IIA, both the probability of channel being open and mean open time in aortic cells from SHR are increased, compared with cells from WKY and Wistar rats. At similar levels of $[Ca^{2+}]_i$, these two altered channel characteristics would lead to an increased K^+ permeability of the membranes of SHR cells. Thus, the increased K^+ channel activity we observed could contribute to the increased K^+ permeability of SHR aortic

strips and cultured VSM cells reported by Jones (18) and by Aviv and his co-investigators (20), respectively. Although increased levels of $[Ca^{2+}]_i$ in vascular smooth muscle of SHR could also be involved, there is presently no convincing evidence for such elevated levels, although several laboratories have investigated this possibility (35, 36).

Isolated cells were used from the first through the third passage. The cells showed no difference in the characteristics of the parameters tested due to passage number used.

The mechanisms underlying the increase in the sensitivity of this K^+ channel to $[Ca^{2+}]_i$ in SHR VSM are not known. Although we observed a slight change in sensitivity to activations by Ca^{2+} or voltage in membrane patches isolated from different sources, the mechanism may involve abnormalities, alteration, or change in sensitivity in the channel protein itself.

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