Up-regulation of pressure-activated Ca^{2+} -permeable cation channel in intact vascular endothelium of hypertensive rats

(mechanosensitive channel/vascular endothelium/hypertension)

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ABSTRACT In endothelial cells, stretch-activated cation channels have been proposed to act as mechanosensors for changes in hemodynamic forces. We have identified a novel mechanosensitive pressure-activated channel in intact endothelium from rat aorta and mesenteric artery. The 18-pS cation channel responded with a multifold increase in channel activity when positive pressure was applied to the luminal cell surface with the patch pipette and inactivated at negative pipette pressure. Channel permeability ratio for K^+ , Na^+ , and Ca^{2+} ions was 1:0.98:0.23. Ca^{2+} influx through the channel was sufficient to activate a neighboring Ca^{2+} -dependent K^+ channel. Hemodynamic forces are chronically disturbed in arterial hypertension. Endothelial cell dysfunction has been implicated in the pathogenesis of arterial hypertension. In two comparative studies, density of the pressure-activated channel was found to be significantly higher in spontaneously hypertensive rats and renovascular hypertensive rats compared with their respective normotensive controls. Channel activity presumably leads to mechanosensitive Ca^{2+} influx and induces cell hyperpolarization by K^+ channel activity. Both Ca^{2+} influx and hyperpolarization are known to induce a vasodilatory endothelial response by stimulating endothelial nitric oxide (NO) production. Up-regulation of channel density in hypertension could, therefore, represent a counterregulatory mechanism of vascular endothelium.

The endothelium plays a pivotal role in regulation of vascular tone (1, 2). In response to stimulation by hemodynamic forces such as shear stress or stretch forces the endothelium liberates nitric oxide or prostacyclin inducing a relaxation of underlying smooth muscle cells. The mechanism of mechanotransduction of hemodynamic forces into vasoregulatory responses by the endothelium is incompletely understood. Cytoskeletal elements, integrins, focal adhesions, or plasma membrane proteins like G protein-linked receptors are possibly involved in this process (3). Also, stretch-activated cation channels have been identified in vascular endothelial cells and presumably act as sensors for hemodynamic forces that induce Ca^{2+} influx or cell hyperpolarization (3–5). The studies on endothelial mechanosensitive ion channels were performed in cultured or isolated endothelial cells. However, cell isolation and culture of endothelial cells may lead to alterations in endothelial cell characteristics. To provide observations under more physiological conditions, we performed patch-clamp experiments in tissue preparations with an intact endothelium.

Arterial hypertension is characterized by an increase in peripheral vascular resistance associated with altered shear forces and elevated intravascular pressure (6). An endothelial dysfunction has been implicated in human and experimental hypertension and might contribute to the increased peripheral resistance and elevated blood pressure (7–9). Therefore, we

examined whether properties of mechanosensitive endothelial ion channels are altered under the chronic changes of hemodynamic forces present in hypertension.

MATERIALS AND METHODS

For transmission electron microscopy and patch-clamp experiments, rats were killed by cervical dislocation and exsanguinated. The thoracic aorta and mesenteric artery were excised and cleaned of connective tissue. The vessels were cut open longitudinally and small tissue slices about 4-mm long were dissected without destroying the luminal surface. Transmission electron microscopy was performed as described (10). Briefly, tissue slices were fixed by immersion in 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 7.2) for about 7 h. After a short wash with Ringer's solution, the tissue was refixed in buffered osmium tetroxide for 1 h, incubated in 2% uranyl acetate for 2 h, then dehydrated by ethanol, and embedded in Spurr's epoxy resin. After polymerization, ultrathin sections were cut and examined in a Philips electron microscope CM 12 operated at 60 kV.

For patch-clamp experiments, tissue slices were placed in a chamber on a stage of an inverted Zeiss Axiovert microscope with the luminal surface facing the bath solution. This enabled a direct approach to the endothelial cells with the patch pipette. Patch-clamp experiments and data analysis were carried out as described (10, 11). Membrane currents were recorded with a HEKA Electronics (Lambrecht/Pfalz, Germany) EPC-9 patch-clamp amplifier using patch pipettes prepared from borosilicate glass with a pipette resistance of 6–8 M Ω . Seal resistance ranged from 8 to 20 G Ω . Data were low-pass-filtered (–3 dB, 800 Hz) at a sample frequency of 2 kHz. The given potential values resemble the pipette clamp potential and the sign of the potential refers to the cytosolic side. If not otherwise stated, the pipette solution contained 140 mM KCl, 1.3 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM Hepes, adjusted to pH 7.4 with KOH. Bath solution contained 140 mM NaCl, 4.3 mM KCl, 1.3 mM CaCl_2 , 1 mM MgCl_2 , adjusted to pH 7.4 with NaOH. Experiments were performed at 35°C. In multichannel patches, the number of channels was determined by the maximum number of superimposed channel openings during maximal mechanical activation. Single-channel open probability (P_o) in multichannel patches was calculated as described (12). The relation between P_o and applied pipette pressure was fitted to the following Boltzmann equation: $P_o = P_{o\text{max}}/[1 + \exp(P - P_{0.5})/k]$, where P_o was the channel open probability, $P_{o\text{max}}$ was the maximum P_o , P is the applied pipette pressure, $P_{0.5}$ is the pipette pressure inducing half-maximal P_o , and k is the slope of the curve.

Abbreviations: WKY, Wistar-Kyoto rat, SHR, spontaneously hypertensive rat; PAC, pressure-activated channel; P_o , open probability.

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FIG. 1. Transmission electron micrograph of intact endothelium at the luminal surface of tissue slices from rat thoracic aorta. From the perinuclear region of the monolayered endothelial cells, the cell height decreases to the periphery where only a flat cytoplasmic sheet covers the underlying connective tissue. At the intercellular clefts, neighboring cells are connected by junctional structures. Arrows point to tight junctions; arrow heads point to Weibel-Palade bodies. L, Vascular lumen; N, nucleus; SE, subendothelial space; IEL, internal elastic lamina.

For a comparative study, male Wistar-Kyoto rats (WKYs) and spontaneously hypertensive rats (SHRs) were obtained from Møllegaard Breeding and Research Center (Skensved, Denmark). Systolic blood pressure, monitored by tail cuff sphygmomanography (Harvard Apparatus) was 118 ± 8 mmHg (mean $\pm$ SEM; 1 mmHg = 133 Pa) in young SHRs (5–6 weeks old; $n = 6$) and 191 ± 10 mmHg in adult SHRs (14–18 weeks old; $n = 7$) compared with 122 ± 8 mmHg in young WKYs (5–6 weeks old; $n = 6$) and 134 ± 7 mmHg in adult WKYs (14–18 weeks old; $n = 7$). For induction of renovascular hypertension, i.e., two-kidney one-clip model, 8-week-old WKYs ($n = 8$) were anesthetized with pentobarbital (30 mg/kg i.v.) and the left renal artery was exposed and dissected free of the renal vein. A silver clip with an internal diameter of 0.23 mm was placed around the artery, causing partial occlusion and the wound was closed. For a control group, sham operation was carried out in WKYs ($n = 8$) of same age and body weight. Rats were allowed to recover, and after 8 weeks, experiments were conducted as detailed below. Systolic blood pressure was 207 ± 10 mmHg (mean $\pm$ SEM) in 2K1C ($n = 7$) and 126 ± 8 mmHg in sham-operated controls ($n = 7$). Differences between groups have been calculated by use of Friedman's two-way ANOVA.

RESULTS AND DISCUSSION

Transmission electron microscopy was performed to ascertain that the luminal surface of freshly dissected tissue slices from rat arteries were covered by a morphologically intact layer of endothelial cells. As shown in Fig. 1, tight junctions in the intercellular cleft between two endothelial cells and Weibel-Palade bodies indicated that the endothelial monolayer remains morphologically intact during preparation procedure. The micrograph closely resembles the results of electron microscopy studies performed in tissue slices from rat thoracic aorta (13, 14).

In cell-attached patches, a mechanosensitive nonselective cation channel with a conductance of 17.8 ± 4.5 pS (mean $\pm$ SD; $n = 34$) for K^+ was identified. The channel had low basal channel activity with a channel open probability (P_o) of less than 0.1 in the absence of mechanical stimulation. Channel activity showed a multifold increase when positive pressure was applied to the cell membrane via the patch pipette (Fig. 2). Channel activity was immediately suppressed in the presence of negative pipette pressure (Fig. 2). Channel activity was characterized by the pressure responsiveness as P_o correlated with an increase in positive pipette pressure. As shown in Fig. 3A, the application of positive pressure of 5 mmHg resulted in

FIG. 2. Increase in low spontaneous channel activity of pressure-activated channel in intact aortic endothelium from WKYs in the presence of positive pipette pressure (+20 mmHg) and inactivation of channel activity in the presence of negative pipette pressure (−30 mmHg). Activation or inactivation responses were immediately reversed when pipette pressure was stopped. A total of five channels was present in this cell-attached patch. Pipette holding potential was −80 mV. c, Closed state of channels.

FIG. 3. (A) Gradual increase of open probability (P_o) from 0.21 to 0.36 and 0.62 in response to a pressure of 5 mmHg, 10 mmHg, and 30 mmHg applied to the rear of the patch pipette, respectively. A total of three channels were simultaneously active in this cell-attached patch. c, Closed state of channels. (B) Relationship between applied pipette pressure and channel activity. Data were fitted to the Boltzmann equation.

a mean P_o of 0.21 and gradually increased to 0.36, 0.47, and 0.62 in the presence of 10, 20, and 30 mmHg, respectively. The resting activity of the channel in the absence of pipette pressure was $P_o = 0.021$. The channel was completely inactivated by pipette suction or negative pipette pressures between -5 and -20 mmHg. Fig. 3B illustrates the relationship between the applied pipette pressure and P_o . The increase in P_o showed a steep pressure dependence of P_o at pipette pressures of 20 mmHg or less. At high pipette pressures, 30 mmHg and more, the pressure response saturated. This pressure-activated channel (PAC) was often arranged in clusters of three to nine channels per patch. Ca^{2+} permeability of PAC was demon-

strated by an inward current in cell-attached patches performed with pipette solutions containing only Ca^{2+} as the permeant cation (Fig. 4A). In general, PAC activity showed a rapid run-down within 1–2 sec after excising the membrane patch from the cell. However, in few experiments ($n = 12$), channel activity was preserved in inside-out patches and ionic substitution protocols revealed a channel permeability ratio of 1:0.98:0.23 for $\text{K}^+/\text{Na}^+/\text{Ca}^{2+}$ (Fig. 4B) as determined by the reversal potentials and use of the Goldman-Hodgkin-Katz equation (15), respectively. Channel conductance was 3.9 ± 0.6 pS (mean $\pm$ SD; $n = 4$) in experiments performed with symmetrical CaCl_2 solutions in bath and pipette. When glu-

FIG. 4. (A) Ca^{2+} influx through PAC in cell-attached patch performed with a Ca^{2+} pipette solution containing 90 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM HEPES- NaOH (pH 7.4). Pipette holding potential was -80 mV. (B) Determination of cation selectivity with cation substitution protocol. Current-voltage (I - V) relation of PAC in inside-out patches in the presence of following pipette solutions: 90 mM CaCl_2 /1 mM MgCl_2 /10 mM HEPES, pH 7.2 (open circles [$n = 4$] and squares); 140 mM KCl /1 mM MgCl_2 /1.3 mM CaCl_2 /10 mM HEPES, pH 7.2 (solid circles, $n = 12$). Bath solution contained 145 mM NaCl , 4.3 mM KCl , 1.3 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM HEPES (pH 7.4, adjusted with NaOH). Data are given as mean $\pm$ SEM and were fitted to the Goldman-Hodgkin-Katz equation (15). Reversal potentials were -22.1 mV (open circles) and $+1.1$ mV (solid circles). (C) Simultaneous opening of PAC and Ca^{2+} -dependent K^+ channel and PAC in cell-attached patch of intact endothelium from WKYs. Channel activity of PAC was followed by opening of a Ca^{2+} -dependent 170-pS K^+ channel that was not mechanosensitive itself. This coactivation suggests that Ca^{2+} influx through PAC is sufficient to increase Ca^{2+} concentration at the cytosolic face of the K^+ channel inducing its activation. Pipette holding potential was -80 mV. c, Closed state of PAC; o, open state of PAC. Inactivation of the PAC led to simultaneous inactivation of the K^+ channel.

tamate ($n = 2$) was substituted for chloride, channel current and reversal potential were not altered, thus excluding appreciable chloride permeability. Channel activity was independent of membrane potential in the range from -70 to $+20$ mV. Also, changes in cytosolic Ca^{2+} concentration on the cytosolic side of the channel from $0.1 \mu\text{M}$ to 1.3 mM had no effect on pressure activation of the channel.

The activation mechanism of mechanosensitive ion channels has been proposed to involve cytoskeletal structures (16–18). Studies using videomicroscopy have shown that in the presence of positive pipette pressure, patch-clamped membranes become spherical and compress the submembranous cytoskeleton (19). Negative pipette pressure leads to stretch of the membrane and membrane-coupled cytoskeleton (19). Therefore, the opposing response of PAC to positive and negative pressure might be explained by the different effect of pressure on submembranous cytoskeleton associated with the channel. Mechanosensitive ion channels have been reported in a variety of nonvertebrate and vertebrate tissues (17). Stretch-inactivated channels have been described as a K^+ -selective neuronal channel in snails (20), as

a 8-pS nonselective cation channel in smooth muscle cells from toad stomach (21), or as a nonselective cation channel preferentially permeable to divalent cations (17 pS in 110 mM BaCl_2 solution) in *mdx* myotubes (22). These channels did not display any distinct responsiveness to positive pipette pressure. In vascular endothelial cells, only mechanosensitive ion channels that were activated by membrane stretch or negative pipette pressure have been reported (4, 10, 12). A mechanosensitive channel with an opposite response to positive and negative pressure has not been described in endothelial cells or cells of other origin, so far. The fact that we performed the patch-clamp experiments in tissue slices with an intact endothelium may have contributed to this channel's identification since isolation of endothelial cells presumably leads to disruption of the cytoskeleton and subsequently altered activation characteristics of mechanosensitive channels. The PAC was almost exclusively observed in intact endothelium as the channel was recorded in only 6 out of 320 patches (1.9%) from freshly isolated aortic endothelial cells. It should be pointed out, that the pressure applied to the rear of the patch pipette

FIG. 5. Density of PAC in intact aortic endothelium from adult SHR or renovascular hypertensive rats (2K1C) compared with their normotensive controls, WKYs, or sham-operated rats, respectively. SHR ($n = 7$) and control WKYs ($n = 7$) were 14–18 weeks old. Note that channel density of all individual hypertensive rats was higher compared with the normotensive controls. Channel density was determined as percentage of patches with PAC activity in each rat. Data are given as mean $\pm$ SD.

to stimulate is not equivalent to the pressure occurring in blood vessels *in vivo*.

In excised inside-out patches from intact endothelium of rat aorta, we also identified a Ca^{2+} -dependent K^+ channel with a channel conductance of 170.7 ± 23.7 pS (mean $\pm$ SD; $n = 5$) and a nonselective cation channel with a channel conductance of 31.3 ± 3.4 pS ($n = 12$). The Ca^{2+} -dependent K^+ channel proved to be selective for K^+ over Na^+ ions. In experiments with a KCl pipette solution and a NaCl bath solution, reversal potential was 48 mV in inside-out patches corresponding to a permeability ratio $P_{\text{K}}/P_{\text{Na}}$ of 10.1:1 as calculated by use of the Goldman-Hodgkin-Katz equation (15). Both channels were not mechanosensitive as channel activity was not affected by positive or negative pipette pressure. However, activity of these channels was strongly dependent of the Ca^{2+} concentration at the cytosolic face of the channel. Half-maximal activation of the K^+ channel and nonselective cation channel in inside-out patches occurred at a Ca^{2+} concentration of 6.5 ± 1.5 μM (mean $\pm$ SD; $n = 3$) and 7.8 ± 1.2 μM ($n = 6$), respectively. Electrophysiological properties were similar to those reported earlier for large conductance K^+ channels (10, 23) and nonselective cation channels in endothelial cells (24). In a series of cell-attached patches ($n = 5$) performed with KCl pipette solution, activation of PAC was followed by opening of a 170-pS K^+ channel present in the same patch. This coactivation indicates that the Ca^{2+} influx through the PAC was sufficient to activate the neighboring Ca^{2+} -dependent K^+ channel (Fig. 4C). After cessation of PAC activity the 170-pS K^+ channel inactivated as well. Therefore, mechanosensitive activation of PAC might lead to an increase in intracellular Ca^{2+} concentration as well as a cell hyperpolarization by concomitant K^+ channel activation. A similar coactivation in response to opening of PAC was observed for the 31-pS cation

channel in cell-attached patches ($n = 4$), additionally suggesting a sufficient Ca^{2+} influx through the PAC to elevate intracellular Ca^{2+} concentration. However, it cannot be excluded that a depolarizing effect of the cation influx through PAC itself impairs endothelial function. The role of PAC activation remains to be determined by use of a specific channel blocker.

Endothelial NO production has been reported to be regulated by intracellular Ca^{2+} concentration (25) and membrane potential (26). Therefore, activation of PAC could stimulate Ca^{2+} -dependent NO production by Ca^{2+} influx and concomitant cell hyperpolarization and subsequently induce an endothelium-dependent vasodilation.

Impaired endothelial function has been demonstrated in human and experimental hypertension (7, 8, 9). In SHRs and rats with renovascular hypertension, a decreased endothelium-dependent vasodilation has been observed (27–30). However, in some studies a normal endothelium-dependent vasodilation has been observed in experimental hypertension (31, 32) and in human hypertension (33). Data on endothelial NO release in experimental hypertension is also controversial with studies reporting either decreased or unchanged NO synthase activity (34).

We were interested to determine whether properties of endothelial PAC are altered in experimental hypertension. We studied channel properties in young (5–6 weeks old) and adult (14–18 weeks old) SHR compared with normotensive age-matched WKYs. Furthermore, we compared channel properties between renovascular (two kidney, one clip) hypertensive rats (2K1C) and sham-operated WKY controls (Sham). In each rat, 20 tight-seal cell-attached patch-clamp experiments were performed in intact aortic endothelium. Channel density was determined as the percentage of patches with PAC activity in each rat (35). PAC density was significantly higher in adult SHR with established hypertension (PAC activity in $48.1 \pm 9.6\%$ of patches; mean $\pm$ SD) compared with WKYs ($22.4 \pm 8.9\%$ of patches; $P < 0.008$; Fig. 5). However, when young prehypertensive SHR and young WKYs were compared, no difference in channel density was observed ($22.0 \pm 6.7\%$ versus $23.0 \pm 7.6\%$). PAC density was also increased in intact aortic endothelium of 2K1C ($55.0 \pm 9.3\%$ of patches) compared with sham-operated controls ($24.4 \pm 3.2\%$ of patches; $P < 0.005$; Fig. 5). As illustrated in Fig. 5, all individual adult SHR and 2K1C had a higher PAC density than the respective controls. Therefore, in these experiments PAC density discriminated hypertensive rats from normotensive rats. In mesenteric arteries overall PAC density was lower in 2K1C and sham-operated rats compared with the density observed in aortic endothelium, respectively. However, as for aortic endothelium channel density was higher in 2K1C ($27.5 \pm 11.3\%$) than in sham-operated controls ($7.2 \pm 9.5\%$; $P < 0.008$).

The observation of an increased channel density in adult SHR but not in prehypertensive young SHR implies that the up-regulation of PAC density in hypertensive rats may be a consequence, rather than a cause, of hypertension. This is supported by the results of the experiments involving renovascular hypertensive rats. The up-regulation of PAC density in renovascular hypertensive rats after induction of hypertension strongly suggests the changes in channel density to be secondary to hypertension.

In conclusion, the study demonstrates the importance of experiments performed in intact tissue preparations since a new type of mechanosensitive ion channel was identified. The Ca^{2+} -permeable pressure-activated channel described herein may participate in the endothelium-dependent vasodilation in response to hemodynamic stimulation.

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