Chloride Regulates Afferent Arteriolar Contraction in Response to Depolarization

Pernille Bjørg Hansen, Boye L. Jensen, Ole Skøtt

Abstract—Renal vascular reactivity is influenced by the level of dietary salt intake. Recent in vitro data suggest that afferent arteriolar contractility is modulated by extracellular chloride. In the present study, we assessed the influence of chloride on K⁺-induced contraction in isolated perfused rabbit afferent arterioles. In 70% of vessels examined, K⁺-induced contraction was abolished by acute substitution of bath chloride. Consecutive addition of Cl⁻ (30, 60, 80, 100, 110, and 117 mmol/L) restored the sensitivity to K⁺, and half-maximal response was observed at 82 mmol/L chloride. The calcium channel antagonist diltiazem (10⁻⁶ mol/L) abolished K⁺-induced contractions. Bicarbonate did not modify the sensitivity to chloride. Norepinephrine (10⁻⁶ mol/L) induced full contraction in depolarized vessels even in the absence of chloride. Iodide and nitrate were substituted for chloride with no inhibitory effect on K⁺-induced contraction. Approximately 30% of the vessels constricted in response to K⁺ in the absence of chloride. This response was reversibly blocked by the α₁-blocker phentolamine (PA) (10⁻⁵ mol/L) and, with PA present, the dependence on chloride was similar to the above series. The results show that K⁺-induced contraction of smooth muscle cells in the afferent arteriole highly sensitive to chloride, whereas neurotransmitter release and ensuing contraction is not dependent on chloride. Thus, there are different activation pathways for depolarizing vasoconstrictors and for the sympathetic nervous system in renal afferent arterioles. This could be of physiological relevance for the resetting of afferent arteriolar sensitivity during changes in salt intake. (Hypertension. 1998;32:1066-1070.)

Key Words: kidney ■ calcium ■ hypertension, arterial ■ diltiazem ■ resistance

The afferent glomerular arterioles are major regulatory sites of renal vascular resistance, and a functional decrease in vessel diameter may be involved in the development and maintenance of arterial hypertension. The tone of these resistance vessels is controlled by several mechanisms that include renal perfusion pressure, renal sympathetic nerve activity, circulating and local vasoactive substances, and the tubuloglomerular-feedback mechanism. A common feature in the excitation-contraction coupling that is initiated by these signals is the dependence on Ca²⁺ influx through voltage-gated Ca²⁺ channels in the afferent arteriolar smooth muscle cells. Data show that vasoconstrictor peptides depolarize the smooth muscle cells mainly by Ca²⁺-activated chloride conductance. Because chloride is maintained above equilibrium in smooth muscle cells, the activation of chloride channels leads to depolarization. In addition to this well-characterized role of chloride ions, a second step in the excitation-contraction coupling is modulated by chloride. Acute substitution of extracellular chloride ions with impermeable anions for 1 minute totally blocks afferent contraction in response to depolarizing concentrations of K⁺. This implies that chloride ions directly control voltage-dependent afferent arteriolar contraction. Recent electrophysiological data support this hypothesis, and therefore our goal in the present study was to characterize the physiological significance of these observations at the functional level of contraction. We found that K⁺-induced afferent arteriolar contraction is sensitive to extracellular chloride ions in a concentration range very close to the physiological level, which implies that this mechanism could play a role in the control of afferent arteriolar tone in vivo.

Methods

Solutions

Physiological salt solution (PSS) had the following composition (in mmol/L): NaCl 115, NaHCO₃ 25, K₂HPO₄ 2.5, CaCl₂ 1.3, MgSO₄ 1.2, and glucose 5.5. K⁺ solution (100 mmol/L) was generated by substitution of 95 mmol/L NaCl with KCl. Anion substitution was performed by using gluconate salts of all chloride salts. One liter of Dulbecco’s modified Eagle’s medium (DMEM) was supplied with 1.2 g NaHCO₃, 100 000 U penicillin, and 100 mg streptomycin. All bicarbonate-buffered solutions were equilibrated with 5% CO₂ in air. Then BSA was added to a final concentration of 0.1%, and pH was adjusted to 7.4. Perfusate was PSS with 1% BSA, pH 7.4. BSA, DMEM, gramicidin D, diltiazem, phentolamine (PA), norepinephrine, and HEPES were obtained from Sigma Chemical Co.

Isolation and Microperfusion Procedure of Rabbit Afferent Arterioles

All animal procedures conformed with the Danish law on experiments on animals and with the guidelines for the care and handling
of animals established by the US Department of Health and Public Services and published by the National Institutes of Health. The experiments were performed on afferent arterioles dissected from 1.5- to 3-kg rabbits (Kolding Technical School, Kolding, Denmark) that were fed standard rabbit chow and allowed free access to tap water. A total of 72 animals were used in the present study. The preparation took place as described before in detail.26 Afferent arterioles were dissected, transferred to a thermoregulated chamber on an inverted microscope, and perfused with concentric glass pipettes at a pressure of 60 to 80 mm Hg and a temperature of 37°C. If perfusion was not achieved within 120 minutes after the rabbit was killed, the experiment was stopped. A test stimulus of 100 mmol/L K+ assured viability of the vessels. The bath contained 1 mL, and all exchanges were made with 10 mL of bathing fluid.

**Experimental Protocols**

**Series 1**
The dependence of K+ responses on Cl− concentration and the equilibration time were determined. First, the bath was exchanged with Cl−-free solution. After 10 minutes, K+ (100 mmol/L) was added for 1 minute. Three series were performed, during which the vessels equilibrated for 1, 5, and 10 minutes, respectively, at each of the following Cl− concentrations (in mmol/L): 0, 30, 60, 80, 100, 110, and 117. At the end of each period, K+ was added for 1 minute.

**Series 2**
To test whether Cl−-dependent contraction was caused by Ca2+ influx through voltage-gated Ca2+ channels, we determined chloride dependence as in series 1, and then the experiment was repeated in the presence of diltiazem (10−6 mol/L) and PA (10−5 mol/L, to exclude nerve-mediated effects).

**Series 3**
A subgroup of afferent vessels constricted in response to K+ in the absence of bath chloride. To test whether Cl− in the luminal perfusate contributed to the atypical response, we determined chloride dependence in vessels in which only bath chloride was substituted and compared the result with the response obtained by combined bath and perfusate chloride substitution. In a second series, we ascertained whether release of endogenous norepinephrine from preserved nerve terminals was involved in the Cl−-insensitive contraction. K+ was added at a range of chloride concentrations before and after addition of the α1-receptor antagonist PA (10−5 mol/L). Because PA blocked the response, a third series was used to test whether addition of exogenous norepinephrine could contract vessels that were insensitive to K+ during the same experimental conditions.

**Series 4**
To assess the anion specificity of the response, we substituted chloride with iodide or nitrate in 2 separate series and tested the reactivity to K+.

**Series 5**
To exclude tachyphylaxis to the action of K+, we stimulated the vessels repeatedly with 100 mmol/L K+ for 45 seconds, and the vessels were allowed to recover each time for 15 minutes. We then determined whether smooth muscle cell alkalinization caused the reduced K+ response in zero ambient Cl−. The sensitivity to K+ in the absence of chloride was compared in bicarbonate-buffered and in HEPES-buffered solutions. To rule out that the reduced response to K+ in zero chloride was because of chelation of calcium by gluconate, we added 10 mmol/L calcium in one series, and we substituted chloride with methanesulfonate, which does not chelate calcium, in a second series.

**Series 6**
In the last series, we tested whether Cl− ions may function as compensatory charge carriers to allow a continuous Ca2+ influx during depolarization.22 Vessels that were insensitive to K+ in the absence of Cl− were exposed to the cation-ionophore gramicidin for

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**Figure 1.** Influence of chloride concentration in the bath on K+-action in afferent arterioles. Bath chloride was totally substituted with gluconate for 10 minutes. Chloride was added to the bath in graded steps; sensitivity to K+ was tested at each level of chloride. The response at 60 mmol/L was significantly different from the response at 110 mmol/L when tested with ANOVA and Newman-Keuls test.

2 minutes in a buffer in which Na+ and Cl− were substituted with N-methyl-D-glucamine (NMDG−) and gluconate−, respectively. This way, gramicidin may allow an efflux of cations in exchange for Ca2+ influx during depolarization.

**Analytic Methods**
Experiments were recorded on videotape at magnification ×400. The video sequences of interest were digitized with a Matrox frame grabber, and vessel diameters were assessed by imaging software (Metamorph, Universal Imaging).

**Statistics**
The arteriolar diameters were compared by Student’s t test on paired data in the individual arterioles before and after addition of an agonist, with appropriate reduction for multiple comparisons. Only the maximal responses were tested. When comparing several treatments at the same time, ANOVA was used and then a Newman-Keuls test. P<0.05 was considered significant.

**Results**

**Chloride Dependence of K+-Induced Contraction**
Extracellular substitution of chloride by gluconate for 10 minutes did not change basal vessel diameters but did abolish reactivity in response to K+ (100 mmol/L). K+ did not evoke contraction at any concentration of Cl− when vessels equilibrated for 1 or 5 minutes (data not shown; n=5 and 6, respectively). When vessels equilibrated for 10 minutes between depolarizations, a striking dependence on extracellular Cl− became evident. Half-maximal contraction in response to K+ was observed at a Cl− concentration of 82 mmol/L, and total occlusion was seen at 110 mmol/L chloride (Figure 1, n=6).

**Role of Voltage-Gated Ca2+ Channels**
In this series, K+-induced half-maximal contraction at a Cl− concentration of 94 mmol/L (Figure 2). Diltiazem had no effect on basal diameter but reversibly abolished all K+-induced responses (n=6). Thus, the Cl−-sensitive component of depolarization-induced afferent arteriolar contraction is dependent on intact function of voltage-gated Ca2+ channels.
Chloride-Independent, K⁺-Evoked Contraction of Afferent Arterioles

A subgroup (~30%) of vessels reacted in a qualitatively different way to K⁺ by a maximal contraction in the absence of chloride. A simultaneous substitution of Cl⁻ in bath and perfusate fluid did not change K⁺-induced contraction compared with bath substitution alone (data not shown, n=5). The potential release of endogenous norepinephrine from nerve terminals by K⁺ was ascertained. PA (10⁻⁵ mol/L) did not affect basal diameter but reversibly blocked the chloride-independent response to K⁺ (Figure 3). Thus, the marked response to K⁺ was reversed by PA to the typical Cl⁻-sensitive response found earlier (Figure 4, n=5) with a half-maximal contraction in response to K⁺ at 73 mmol/L chloride. In other vessels, with a chloride-dependent response to K⁺, exogenous norepinephrine (1 μmol/L) caused maximal constriction in the absence of chloride during exposure to K⁺ (Figure 3, n=4). Diltiazem (10⁻⁶ mol/L) had no effect on norepinephrine-induced contraction (n=3). Altogether, these data suggest that the chloride-independent response to K⁺ is induced by release of endogenous norepinephrine from sympathetic nerve terminals. Moreover, norepinephrine responses are not dependent on intact function of voltage-gated Ca²⁺ channels.

Anion Specificity of the Response

Acute substitution of extracellular chloride with iodide or nitrate (n=3) had no effect on basal diameters, and contrary to the responses with impermeable anions, these anions fully supported contraction in response to K⁺ (Table 1).

Pathways for Regulation of K⁺ Sensitivity by Chloride

Two consecutive exposures to K⁺ contracted the vessels to 0 μm and to 1.2±1.2 μm. There was no significant difference between the results at first and second addition of K⁺ and hence no tachyphylaxis to K⁺ (data not shown, n=4). Gluconate, which was substituted for chloride in most series, can potentially chelate extracellular Ca²⁺ and thereby inhibit vasoreactivity. However, addition of extra Ca²⁺ (10 mmol/L) during gluconate substitution did not restore sensitivity to K⁺ (data not shown, n=3). In a second series in which chloride was substituted with methanesulfonate, which does not chelate calcium, half-maximal contraction was observed at 102 mmol/L chloride, which is not different from the value obtained in the gluconate series (n=3, data not shown). Thus, chelation of calcium is not likely to have contributed significantly to the present results.

Substitution of extracellular chloride could indirectly affect vasoreactivity by cellular alkalinization. However, K⁺-induced contraction was inhibited in a similar way by

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<th>Sensitivity to K⁺ of Afferent Arterioles After Total Substitution of Chloride With NO₃⁻ and I⁻, Respectively</th>
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Luminal diameter in μm (n=3).
substitution of chloride in HCO₃-buffered PSS (diameter, 14.8±1.3 μm) compared with HEPES-buffered PSS (diameter, 15.4±1.4 μm; n=6). Smooth muscle cell alkalinization therefore is not likely to cause the inhibition of K⁺-induced contraction in Cl⁻-depleted media.

A steady Ca²⁺ influx during depolarization might depend on regulated charge compensation by concomitant flux of a counter ion, eg, Cl⁻, to maintain electroneutrality. This hypothesis was tested in 7 experiments. Exchange of extracellular permeable ions (Na⁺ with NMDG⁺ and Cl⁻ with gluconate) did not change the internal diameter of the vessels. Subsequent addition of the ionophore gramicidin (2 and 20 μg/mL) had no effect on basal diameter, but in the presence of gramicidin, K⁺ induced a small but significant contraction (Figure 5). After recovery in PSS, K⁺ occluded the lumen totally. Thus, an artificial pathway for charge compensation during Ca²⁺ influx does not fully restore the K⁺ sensitivity in a Cl⁻-free medium.

Discussion

In a previous study, we demonstrated that contraction of afferent arterioles evoked by depolarization was directly modulated by extracellular chloride. This could be of functional significance for afferent arterioles in vivo in which variations in the interstitial Cl⁻ concentration may occur and in which the NaCl concentration in the tubular fluid at the macula densa affects arteriolar sensitivity to agonists. Therefore, this dependence was investigated in more detail.

A striking dependence of the K⁺ response on the concentration of chloride in the bathing fluid was apparent with an estimated threshold for contraction at 70 mmol/L chloride, a half-maximal response at ~80 mmol/L, and a maximal response at 110 mmol/L (Figure 1). Moreover, an equilibration time of 10 minutes at each level of chloride was necessary to allow the arteriole to regain sensitivity to K⁺. The chloride-sensitive contraction in response to K⁺ could be attributed to voltage-gated Ca²⁺ channels because it was blocked by diltiazem (Figure 2). Therefore, we suggest that a physiologically relevant concentration of chloride ions is required to sustain K⁺-induced, diltiazem-sensitive contraction of rabbit renal afferent arterioles. This conclusion is consistent with data in which comparable substitutions of chloride led to inhibition of the sequence of events distal to activation of voltage gated Ca²⁺ channels (Ca²⁺ currents, cytosolic Ca²⁺ increases and contraction) in response to agonists or K⁺ depolarization. The requirement for chloride is not absolute since other permeable anions supported K⁺-induced vasoconstriction in a way similar to chloride (Table 1). However, under physiological conditions, the requirement for permeable anions is satisfied by the prevalence of chloride in the extracellular fluid.

The experiments defined a population of vessels (~30%) in which K⁺ constricted the arterioles independently of extracellular Cl⁻ ions (Figure 3). The α₂-receptor antagonist PA converted this Cl⁻-independent response to K⁺ to a typical Cl⁻-dependent pattern (Figure 4). Furthermore, exogenous norepinephrine constricted vessels in which K⁺ had no effect after substitution of chloride (Figure 3). Together, these data suggest that the occasional chloride-insensitive contraction represents a K⁺-mediated release of endogenous norepinephrine from intact sympathetic nerve endings.

As to the mechanism of the vascular dependence on extracellular chloride, we could exclude several factors. The data exclude tachyphylaxis to the action of K⁺, an intracellular pH change, or chelation of extracellular calcium as causes of the reduction in vasoreactivity in low external Cl⁻. Moreover, smooth muscle cell membrane potential remains constant at negative values after Cl⁻ substitution, and K⁺ elicits depolarization as in Cl⁻ media. Previous observations suggest that the voltage dependence of channel activation may control the voltage dependence of channel activation. These anion shifts the voltage dependence of gating in a more positive direction, possibly through local effects of anion adsorption to the cell membrane that decrease the local steepness of the voltage gradient. A modification of the Ca²⁺ channel gating characteristics by a change in surface charges at, or close to, the channel protein is therefore a relevant possibility. According to a second hypothesis, Cl⁻ acts as an obligate counter ion for Ca²⁺ influx to proceed. By use of a protocol that allowed cellular cation efflux as charge compensation for calcium, we did not find experimental support for this idea in afferent arterioles. In smooth muscle, voltage-gated Ca²⁺ channels are modulated by G proteins that may be chloride sensitive. The present experimental design does not exclude that the cytosolic concentration of chloride changes. This effect could explain why a 10-minute equilibration period was required for alterations in extracellular chloride to influence the response to K⁺. This suggestion would be consistent with data obtained in the perfused hydrophobic kidney in which an acute increase in the chloride gradient out of the cell enhances vasoreactivity in response to vasoconstrictors.

The sensitivity to chloride may have physiological relevance in several ways. The interstitial concentration of chloride as determined by macula densa epithelial transport could directly determine the reactivity of the vessel. Consistent with this proposal, afferent arteriolar sensitivity to angiotensin II is markedly enhanced when the thick ascending limb is perfused with a high NaCl solution. On the other hand, variations in plasma chloride concentrations could potentially influence vessel reactivity in vivo because the
sensitivity to depolarization is maximal in a range of chloride concentrations very close to physiological levels. In this setting, it is interesting to note that ingestion of a low salt diet usually modulates (reduces) the sensitivity of isolated smooth muscle strips in vitro and of the renal vasculature in vivo to angiotensin II, whereas the sensitivity to norepinephrine is largely unchanged.23 Our observations of the afferent arteriole are consistent with this response.

In summary, the data show that K\textsuperscript+ -induced contraction of smooth muscle cells in the afferent arteriole is highly sensitive to chloride, whereas K\textsuperscript+ -induced sympathetic transmitter release and ensuing norepinephrine-mediated contraction is not chloride dependent. Thus, there are different pathways for the action of depolarizing vasoconstrictors and for the sympathetic nervous system in renal afferent arteriolar excitation-contraction coupling.

Acknowledgments

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References

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