Chloride-dependent calcium transients induced by angiotensin II in vascular smooth muscle cells

Yun-Hwa Ma, Hsiao-Wen Wei, Kwan-Hwa Su, Harlan E. Ives, and R. Curtis Morris, Jr.
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Ma, Yun-Hwa, Hsiao-Wen Wei, Kwan-Hwa Su, Harlan E. Ives, and R. Curtis Morris, Jr. Chloride-dependent calcium transients induced by angiotensin II in vascular smooth muscle cells. Am J Physiol Cell Physiol 286: C112–C118, 2004; 10.1152/ajpcell.00605.2002.—Cl− is essential for the vasoconstrictive response to angiotensin II (ANG II). In vascular smooth muscle cells (VSMC), we determined whether ANG II-induced transient increase in intracellular Ca2+ concentration ([Ca2+]i) is Cl− dependent. After incubating the cells at different extracellular Cl− concentration ([Cl−]e) for 40 min, the ANG II-induced Ca2+ transients at 120 meq/l Cl− were more than twice those at either 80 or 20 meq/l Cl−. Replacing Cl− with bicarbonate or gluconate yielded similar results. In addition, after removal of extracellular Ca2+, ANG II-induced as well as platelet-derived growth factor-induced Ca2+ release exhibited Cl− dependency. The difference of Ca2+ release with high vs. low [Cl−]e, was not affected by acutely altering [Cl−]e. 1 min before administration of ANG II when [Cl−]e was yet to be equilibrated with [Cl−]i. Pretreatment of a Cl− channel inhibitor, 5-nitro-2-(3-phenylpropylamino)benzoic acid, increased ANG II-induced Ca2+ release and entry at 20 meq/l Cl− but did not alter those at 120 meq/l Cl−. However, after equilibration, a reduced [Cl−]e did not affect thapsigargin-induced Ca2+ release, suggesting that Cl− may not affect the size of intracellular Ca2+ stores. Nevertheless, at high [Cl−]e, the peak increase of inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] induced by ANG II was approximately sixfold that at low [Cl−]e. Thus the Cl−-dependent effects of ANG II on Ca2+ transients may be mediated, at least in part, by a Cl−-dependent Ins(1,4,5)P3 accumulation in VSMC.

THE IMPORTANCE OF Cl− in the regulation of vascular function has traditionally received much less consideration than has Na+. However, some studies have revealed that Cl− can participate in the regulation of vasoconstrictor responses in vascular smooth muscles. Selectively increasing [Cl−]e in the renal artery of the intact dog (27) and the isolated perfused rat kidney (28) induced acute renal vasoconstriction and augmented that induced by angiotensin II (ANG II), phenylephrine, and vasopressin (19). In the isolated perfused afferent arteriole of the rabbit, abluminal Cl− was found to be essential for the vasoconstrictive response to ANG II (9).

ANG II is a potent vasoconstrictor and growth factor in vascular smooth muscle (26). The renin-angiotensin system plays an important role in the regulation of blood pressure. Activity of the renin-angiotensin system is modulated by various factors, including Cl−. Elevated extracellular Cl− concentration ([Cl−]e) may stimulate the renin-angiotensin system by acting directly on juxtaglomerular cells to stimulate renin release (10, 23). Cl− also increases the activity of angiotensin-converting enzyme (21). In a Cl−-sensitive hypertensive model described by Tanaka et al. (25), it has been found that supplemental dietary KCl exacerbated hypertension, and supplemental potassium citrate or bicarbonate attenuated hypertension in the stroke-prone spontaneously hypertensive rat (SHR). Interestingly, elevated plasma renin activity was associated with exacerbated elevation of blood pressure in this rat model with a high-Cl− diet (25). Thus enhanced ANG II conversion may further increase peripheral resistance. In addition, ANG II-induced vasoconstriction of the resistant arterioles may be further enhanced by Cl−(9) and contribute to the Cl−-sensitive hypertension. Although ANG II has been considered as a paradigm of receptor signaling in the vasculature, it is not known how Cl− enhances ANG II-induced vasoconstriction.

The mechanism by which receptor activation entrains contraction of vascular smooth muscle cells (VSMC) involves a transient increase in the intracellular concentration of Ca2+ ([Ca2+]i) (12). ANG II stimulates phospholipase C (PLC) which acts on phosphatidyl inositol to release inositol 1,4,5-trisphosphate [Ins(1,4,5)P3]. Binding of Ins(1,4,5)P3 to a specific receptor on the sarcoplasmic reticulum releases stored Ca2+ into cytoplasm (12). The released Ca2+ may activate a Ca2+-sensitive Cl− channel on the plasma membrane that sequentially entrains Cl− efflux and membrane depolarization, which may be strong enough to cause Ca2+ influx through voltage-sensitive Ca2+ channels (11). Thus ANG II-stimulated Ca2+ transient results from both increase of intracellular Ca2+ stores and influx of extracellular Ca2+ (12). However, it is not known whether [Cl−]e affects ANG II-induced Ca2+ signaling in vascular smooth muscle. We report a positive test of the hypothesis that Cl− enhances ANG II-induced Ca2+ transients in VSMC. The magnitude of ANG II-induced Ca2+ transients varies directly with [Cl−]e over a physiological and lower range in both the absence and presence of extracellular Ca2+. To our knowledge, these results are the first to demonstrate in vascular smooth muscle that extracellular Cl− can modulate intracellular Ca2+ release induced by receptor activation. This modulating effect of Cl− on ANG II-induced Ca2+ transients may be mediated by a mechanism involving Ins(1,4,5)P3 metabolism via altering intracellular Cl− concentration ([Cl−]i). Some of the results have been presented previously as an abstract (16).

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MATERIALS AND METHODS

**Materials.** ANG II (acetate salt, human, synthetic), d-glucuronic acid (sodium salt), saponin, thapsigargin, ethylene glycol-bis(B-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and elastase (EC 3.4.21.36, type III, porcine pancreas) were obtained from Sigma (St. Louis, MO). Recombinant platelet-derived growth factor (PDGF) B/B was from Boehringer Mannheim (Indianapolis, IN). Fura 2-acetoxybenzyl ester (fura 2-AM), N-ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE), 4-bromo-A23187 (free acid), and Pluronic F127 were from Molecular Probes (Eugene, OR) and were dissolved in concentrated form and stored in dimethyl sulfoxide before experimentation. Collagenase (CLSIII) and soybean trypsin inhibitor were obtained from Worthington Biochemical (Freehold, NJ). Culture medium components were obtained from Gibco-BRL (Rockville, MD).

**Cell culture.** From abdominal aortas of male SHR (364 ± 14 g, at least 16 wk old, n = 14; Taconic Farms) anesthetized with halothane, primary cultures of VSMC were obtained by enzyme digestion. Cells from SHR were used because of our previous observation of C1 sensitivity in stroke-prone SHR (25). Slices of arteries were incubated with collagenase (402 U/ml), elastase (1.3 U/ml), and soybean trypsin inhibitor (1 mg/ml) for 30-30 min intervals. Cells suspensions were pooled and cultured in minimum essential medium with fetal bovine serum (10%), tryptose phosphate broth (2%), glutamine (20 mM), penicillin (50 U/ml), and streptomycin (50 U/ml) in a humidified atmosphere of 5% CO2-95% air at 37°C for at least 4 days before experimentation to ensure establishment of appropriate cell-matrix interaction. Cells not used in primary culture were used for experiments within five passages if not otherwise mentioned. In addition, immortalized newborn human VSMC (HNB18E6E7, male) obtained from Dr. Karen Yee (University of Washington, Seattle, WA) were used at passage 7. These cells were maintained in Waymouth's medium containing 10% fetal bovine serum and were handled similarly to VSMC from rats. Identification of cells as smooth muscle was determined by immuno fluorescence with a fluorescein isothiocyanate-conjugated anti-α-actin antibody.

**Composition of solutions.** All salt solutions (pH 7.2) used in the experiments contained NaCl (111 mM), NaHCO3 or Na-glucuronic (29 mM), KCl (5 mM), MgSO4 (1 mM), NaHPO4 (1 mM), CaCl2 (0.2 mM), glucose (25 mM), N-2-hydroxyethylpiperazine-N′,N′-2-ethanesulfonic acid (25 mM), and bovine serum albumin (0.05%). Where [Cl−] was varied, NaCl was replaced isosmotically with NaHCO3, if not otherwise mentioned. The amount of Cl− in other ingredients was taken into consideration for calculation of the final [Cl−] reported in the study. The pH of solutions was titrated to 7.2 with NaOH or acetic acid. The concentration of ionized, free Ca2+ in various salt solutions was measured using a Ca2+/pH analyzer (Ciba Corning).

**[Ca2+]i measurement.** [Ca2+]i was determined by using a Nikon inverted epifluorescence microscope with the UMANS analytic software (Bio-Rad) or an SLM 8000 spectrofluorometer (Urbana, IL) at 37°C. Cells plated on a coverslip were incubated with a mixture containing fura 2-AM (4.2 μM), Pluronic F127 (0.03%), and bovine serum albumin (0.4%) in salt solutions with various [Cl−] (as described above) at room temperature for 30 min. The ratio of fluorescence intensity was recorded at excitation wavelengths of 340 and 380 nm was used to determine [Ca2+]i, as previously described (4). Each cell preparation was exposed to ANG II or PDGF once only. In some experiments, VSMC was incubated with 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPBA), a Cl− channel inhibitor, for 40 min before the addition of ANG II and throughout the experiment.

**[Cl−]i measurement.** Relative [Cl−] changes were assessed by fluorescence dye MQAE, as described previously (13). Briefly, VSMC were incubated with 5 mM MQAE at room temperature with 120 or 20 meq/l Cl− for 30 min. After removal of extracellular MQAE and equilibration at 37°C for 10 min, solutions were changed to alter [Cl−], for 1-2 min before the fluorescence intensity was recorded with a spectrofluorimeter (Hitachi F4500) with an excitation wave-length of 350 nm. The intensity of fluorescence emitted at a wavelength of 460 nm during a period of 30 s was averaged for each preparation.

**Ins(1,4,5)P3 measurement.** VSMC were equilibrated with salt solutions containing 120 vs. 20 meq/l Cl− for 30 min before the addition of ANG II. LiCl (10 mM) was added 10 min before the addition of ANG II. The response was terminated by 0.2 volume of 50% ice-cold perchloric acid at the indicated time. After 20-min incubation on ice, the cells and medium were collected and the mixture was centrifuged at 2,000 g for 15 min at 4°C. Supernatants were titrated to pH 7-8.5 with 10 N KOH and kept on ice. After centrifugation, the total mass of Ins(1,4,5)P3 was assayed by using an assay kit from Amersham (TRK 1000). The levels of Ins(1,4,5)P3 with both samples and standards were determined in duplicates.

**Statistical analysis.** Data are presented as means ± SE. Significant differences were established using Student's t-test or two-way analysis of variance followed by Duncan's post hoc test.

**RESULTS**

[C1−]i modulates intracellular Ca2+ transients. The ANG II-induced Ca2+ transients varied directly with [C1−], over a range extending from 20 to 120 meq/l in primary culture of VSMC from SHR (Fig. 1A) and in human VSMC (Fig. 1B). The Ca2+ transient induced by ANG II (2 μM) at the [Cl−]i of 120 meq/l was more than twice that at the lower [Cl−]i, of 80 or 20 meq/l. In VSMC from SHR, the effect of [Cl−]i on ANG II-induced Ca2+ transients was similar in primary culture (Fig. 1A) and in cells up to passage 5 (Fig. 1C). No obvious morphological changes were observed after cells were incubated for 1 h in physiological salt solutions with [Cl−]i ranging from 20 to 120 meq/l.

When bicarbonate replaced Cl−, the response to ANG II at a [Cl−]i of 80 and 20 meq/l was 42 ± 12% (n = 11, from 4 rats) and 25 ± 16% (n = 9, from 3 rats), respectively, of that at a [Cl−]i of 120 meq/l (n = 15, from 4 rats). When gluconate replaced Cl−, the response to ANG II (2 μM) at a [Cl−]i of 80 and 20 meq/l was 36 ± 14% (n = 7, from 5 rats) and 15 ± 5% (n = 9, from 6 rats), respectively, of that at a [Cl−]i of 120 meq/l (n = 11, from 6 rats) (Fig. 1C). These results suggest that the effect of Cl− replacement was independent on the anion used for replacement. Given that the resolution for measuring [Ca2+]i, above 1 μM is reduced (4), most of the following experiments measuring ANG II-induced Ca2+ transients were conducted with a smaller concentration of ANG II, i.e., 100 nM, to induce a smaller amplitude of responses.

The effects of [Cl−]i described above were not limited to cells obtained from SHR; VSMC from Sprague-Dawley (SD) rats of a different genetic origin exhibited similar results. With VSMC from SD rats, the ANG II (100 nM)-induced Ca2+ transient with a [Cl−]i of 120 meq/l (283 ± 29 nM; n = 10) was significantly higher than that with a [Cl−]i of 20 meq/l (176 ± 29 nM; n = 10; P < 0.05). Corresponding values were not different from those induced by 100 nM ANG II in VSMC from SHR (data not shown, n = 18).

**Assessment of effect of Cl− on Ca2+ measurement.** When Cl− was replaced with bicarbonate, extracellular free Ca2+ measured 1.3, 1.1, and 0.9 mM in solutions containing 120, 80, and 20 meq/l Cl−, respectively. For gluconate replacement, these values were 1.0, 0.6, and 0.3 mM. Thus free [Ca2+]i did vary significantly with anion composition. However, when additional CaCl2 (3.5 mM) was added to increase free [Ca2+]i to that measured with a [Cl−]i of 120 meq/l, the amplitude of...
the ANG II (2 μM)-induced Ca\(^{2+}\) transient remained greatly reduced in solutions containing 20 meq/l Cl\(^{-}\), i.e., 16 ± 6% (n = 3) and 23 ± 11% (n = 3) of that in a [Cl\(^{-}\)]\(_e\) of 120 meq/l. Addition of CaCl\(_2\) to control for ionized [Ca\(^{2+}\)]\(_i\) in solution did not reverse the effect of low [Cl\(^{-}\)]\(_e\) on ANG II-induced Ca\(^{2+}\) transients. Thus the results with different [Cl\(^{-}\)]\(_e\) in Fig. 1 cannot be explained by differences in extracellular ionized [Ca\(^{2+}\)]\(_i\) in the solutions.

To assess whether cellular handling of fura 2-AM may be modulated by Cl\(^{-}\), the maximal-to-minimal fluorescence ratio (R\(_{max}\)/R\(_{min}\)) was determined with different [Cl\(^{-}\)]\(_e\). R\(_{max}\)/R\(_{min}\) in 120 meq/l Cl\(^{-}\) was 7.2 ± 0.7 (n = 22) for bicarbonate replacement and 7.2 ± 1.1 (n = 12) for gluconate replacement. This ratio was unaffected by changes in [Cl\(^{-}\)]\(_e\). (see MATERIALS AND METHODS; data not shown). Finally, Cl\(^{-}\) did not affect the Ca\(^{2+}\) transient-induced by the Ca\(^{2+}\) ionophore A-23187 (1 μM) with either bicarbonate or gluconate replacing Cl\(^{-}\) (n = 5–11, data not shown). Therefore, it is unlikely that the effects of Cl\(^{-}\) on ANG II-induced Ca\(^{2+}\) transient were due to an effect on fluorescent characteristics of fura 2.

Effects of [Cl\(^{-}\)]\(_e\) on Ca\(^{2+}\) release and entry. To determine how [Cl\(^{-}\)]\(_e\) modulates ANG II-induced Ca\(^{2+}\) transients, ANG II (2 μM) was first added in the absence of extracellular Ca\(^{2+}\) to induce intracellular Ca\(^{2+}\) release; after [Ca\(^{2+}\)]\(_i\) recovered to near basal level (2–8 min), CaCl\(_2\) (2 mM) was added to the medium to examine Ca\(^{2+}\) entry in a primary culture of VSMC from SHR. ANG II-induced intracellular Ca\(^{2+}\) release and the subsequent Ca\(^{2+}\) entry were both attenuated when [Cl\(^{-}\)]\(_e\) was reduced to 20 meq/l, as shown in Fig. 2A. This Cl\(^{-}\)-dependent Ca\(^{2+}\) release was observed with a wide range of ANG II concentrations from 10\(^{-8}\) to 3 × 10\(^{-6}\) M in subsequently cultured VSMC (passages 8–10; P < 0.05; n = 13–30, 2 rats), as shown in Fig. 3. Our results demonstrate that Cl\(^{-}\) replacement dramatically reduced the efficacy of ANG II to induce Ca\(^{2+}\) release from intracellular stores in VSMC from SHR. Although the amplitude of ANG II-induced Ca\(^{2+}\) release might be blunted with time in culture, the effect of Cl\(^{-}\) persisted to at least passage 10 of cultured VSMC. Similar results were observed with PDGF (25 ng/ml) (Fig. 2C). Ca\(^{2+}\) release and entry induced by PDGF (25 ng/ml) in a [Cl\(^{-}\)]\(_e\) of 80 meq/l were 15 ± 7 and 50 ± 19% (n = 4, 2 rats) of those in a [Cl\(^{-}\)]\(_e\) of 120 meq/l (n = 5, 3 rats).

With a [Cl\(^{-}\)]\(_e\) of 120 meq/l, thapsigargin (2 μM) almost completely prevented Ca\(^{2+}\) release induced by ANG II (2 μM) (n = 3, data not shown). Although intracellular Ca\(^{2+}\) stores were believed to be heterogeneous in nature (3, 12), the results suggest that thapsigargin-sensitive stores were responsible for most, if not all, of the Ca\(^{2+}\) released by these agents in our preparation. Figure 2B shows that, in a primary culture of VSMC, the magnitude of the Ca\(^{2+}\) release induced by thapsigargin (2 μM) was similar with a [Cl\(^{-}\)]\(_e\) of both 120 and 20 meq/l. After passages in culture, VSMC also exhibited similar Ca\(^{2+}\) release to thapsigargin in high and low [Cl\(^{-}\)]\(_e\) (total n = 13, from 6 rats). In addition, we found no difference in the subsequent Ca\(^{2+}\) entry with a [Cl\(^{-}\)]\(_e\) of 120 vs. 20 meq/l when extracellular Ca\(^{2+}\) was restored (Fig. 2B). Cl\(^{-}\) did not affect Ca\(^{2+}\) release induced by a submaximal concentration (1 μM) of ionophore A-23187 (n = 6, data not shown).

To determine how Cl\(^{-}\) channel may participate in the Cl\(^{-}\)-sensitive Ca\(^{2+}\) signaling, a Cl\(^{-}\) channel inhibitor, NPPB (10 μM), was added 40 min before the addition of ANG II, and the treatment was sustained throughout the experiment. As shown in Fig. 4, NPPB increased ANG II-induced Ca\(^{2+}\) release and the subsequent Ca\(^{2+}\) entry at low [Cl\(^{-}\)]\(_e\) (P < 0.05). The results indicate that only with low [Cl\(^{-}\)]\(_e\) did the NPPB-sensitive Cl\(^{-}\) channel have a role in modulating ANG II-induced Ca\(^{2+}\) signaling.

Mechanism of [Cl\(^{-}\)]\(_e\) effect on intracellular Ca\(^{2+}\) release. First, to examine how [Cl\(^{-}\)]\(_e\) modulates the amplitude of ANG II-induced Ca\(^{2+}\) release, [Cl\(^{-}\)]\(_e\) was acutely altered before the addition of ANG II. VSMC were preincubated with solutions containing 120 or 20 meq/l Cl\(^{-}\) for 40 min before [Cl\(^{-}\)]\(_e\) was changed. ANG II (100 nM) was added to cells 1 min after extracellular Ca\(^{2+}\) was removed and [Cl\(^{-}\)]\(_e\) was changed. As shown in Table 1, ANG II-induced Ca\(^{2+}\) release in VSMC...
preincubated with 120 meq/l Cl\(^{-}\) (group A) was >1.6-fold that in VSMC preincubated with 20 meq/l Cl\(^{-}\) (group B). Acutely increasing [Cl\(^{-}\)]\(_e\) from 20 to 120 meq/l 1 min before the addition of ANG II did not restore the peak of released Ca\(^{2+}\) (group D); acutely decreasing [Cl\(^{-}\)]\(_e\) from 120 to 20 meq/l 1 min before the addition of ANG II did not attenuate the peak of released Ca\(^{2+}\) (group C). However, addition of ANG II 30 min after [Cl\(^{-}\)]\(_e\) was increased from 20 to 120 meq/l restored ANG II-induced Ca\(^{2+}\) release (116 ± 19 nM; n = 6). ANG II-induced Ca\(^{2+}\) release correlated well with MQAE fluorescence intensity under these situations. When the MQAE fluorescence intensity was low, such as in groups A and C, indicating relative high intracellular [Cl\(^{-}\)]\(_i\)], the amplitude of Ca\(^{2+}\) release was significantly larger; and vice versa. These results demonstrated that the capacity of Cl\(^{-}\) to modulate the ANG II-induced increase in [Ca\(^{2+}\)]\(_i\)] was associated with the intracellular level of [Cl\(^{-}\)].

To determine whether ANG II-induced Ca\(^{2+}\) entry as well as release was affected by acute changes of [Cl\(^{-}\)]\(_e\). Ca\(^{2+}\) was maintained at 2 mM throughout the experiment and [Cl\(^{-}\)]\(_e\) was acutely altered 1 min before ANG II was added. After incubation with a [Cl\(^{-}\)]\(_e\) of 20 meq/l for 40 min, the amplitude of ANG II (100 nM)-induced Ca\(^{2+}\) transients, 198 ± 23 nM (n = 18), was 67% of that with a [Cl\(^{-}\)]\(_e\) of 120 meq/l (n = 18, P < 0.05). After 40-min preincubation with a [Cl\(^{-}\)]\(_e\) of 20 meq/l, an acute increase of [Cl\(^{-}\)]\(_e\) to 120 meq/l 1 min before the addition of ANG II did not amplify the ANG II-induced Ca\(^{2+}\) transients, which remained unchanged (198 ± 27 nM, n = 18). These results suggest that ANG II-induced release and entry of Ca\(^{2+}\) might be modulated by intracellular [Cl\(^{-}\)].

In our preparation of VSMC, ANG II-induced Ca\(^{2+}\) release appears to be stimulated primarily by an increase in Ins(1,4,5)P\(_3\) that is produced by PLC activation, given that exogenous ryanodine (10 μM), an Ins(1,4,5)P\(_3\)-independent Ca\(^{2+}\)-releasing agent, induced no Ca\(^{2+}\) release (data not shown). Table 2 shows the effects of a PLC inhibitor, U-73122, on ANG II-induced Ca\(^{2+}\) release at 120 vs. 20 meq/l Cl\(^{-}\). U-73122 nearly completely blocked ANG II-induced Ca\(^{2+}\) release with either [Cl\(^{-}\)]\(_e\), whereas U-73343, an inactive analog of U-73122, did not.

In our preparation, ANG II induced a peak increase in Ins(1,4,5)P\(_3\) level 15 s after its initiation (Fig. 5A). Total
Ins(1,4,5)P₃ level in cells was significantly higher with high [Cl⁻] (15 and 30 min after the addition of ANG II (P < 0.05).

The peak increase that occurred with 120 meq/l Cl⁻ was approximately sixfold that with 20 meq/l Cl⁻. Similar results were observed with gluconate replacement of Cl⁻ (n = 5, P < 0.05, data not shown). Furthermore, in the absence of extracellular Ca²⁺, the ANG II-induced peak increase in Ins(1,4,5)P₃ level with 120 meq/l Cl⁻ was approximately sixfold that with 20 meq/l Cl⁻ (Fig. 5B). In addition, PDGF (25 ng/ml for 30 s)-induced peak increase in Ins(1,4,5)P₃ (2.7 ± 0.2 pmol/well, n = 3) with 120 meq/l Cl⁻ was completely blocked with 80 meq/l Cl⁻ (P < 0.05, data not shown), suggesting that both the ANG II- and PDGF-induced increase in Ins(1,4,5)P₃ was Cl⁻ dependent.

DISCUSSION

We have demonstrated, for the first time, a time-dependent effect of Cl⁻ on ANG II-induced Ca²⁺ release, as assessed by the Ca²⁺ transient induced in the absence of extracellular Ca²⁺. Because acutely changing the [Cl⁻]ₐ 1 min before adding ANG II did not affect the Ca²⁺ transient it induced, the modulating effect of Cl⁻ on the Ca²⁺ transient requires that [Cl⁻]ₐ be maintained at the level examined for longer than 1 min (Table 1). Therefore, the enhanced Ca²⁺ release with higher [Cl⁻]ₐ is unlikely to be a result of a direct membrane effect, e.g., an alteration of membrane potential or ligand-receptor interaction. Rather, [Cl⁻]ₐ appeared to be associated with the amplitude of Ca²⁺ transients. This idea is further supported by the observation that inhibition of Cl⁻ efflux by NPPB significantly increased ANG II-induced Ca²⁺ release and entry with reduced [Cl⁻]ₐ (Fig. 4). Furthermore, in the presence of extracellular Ca²⁺, an acute increase of [Cl⁻]ₐ from a reduced level did not restore the ANG II-induced Ca²⁺ transient responses occurring with the prolonged higher level of [Cl⁻]ₐ. This observation suggests that the entry component of the Ca²⁺ transient may also depend on more prolonged increase of [Cl⁻]ₐ before the addition of ANG II. Although cultured cells are not necessarily representative of those found in intact artery, these findings are consistent with observations in the isolated renal afferent arteriole indicating that a preincubation of at least 10 min is required for an increased [Cl⁻]ₐ to enhance vasoconstriction induced by depolarization with K⁺ (5).

In our preparation, Ins(1,4,5)P₃ production appears to be a major immediate upstream event of intracellular Ca²⁺ release, because inhibition of PLC by U-73122 completely blocked Ca²⁺ release in both 120 and 20 meq/l Cl⁻ (Table 2). An effect of Cl⁻ on the levels of Ins(1,4,5)P₃ after ANG II stimulation may be contributed to the effect of Cl⁻ on ANG II-induced Ca²⁺ release. Total Ins(1,4,5)P₃ at [Cl⁻]ₐ of 20 meq/l was less than that at 120 meq/l both 15 and 30 min after addition of ANG II. This result is consistent with the previous finding that Cl⁻ may modulate Ins(1,4,5)P₃ level in rat mesangial cells (18). Cl⁻ may increase production of Ins(1,4,5)P₃ by modulating G protein-mediated PLC activation. It has been shown that Cl⁻ increased affinity of Gα₁ with GTP to decrease subsequent hydrolysis of the nucleotide, which increases the population of activated G proteins (6). However, the amplitude of Ins(1,4,5)P₃ increase and Ca²⁺ release induced by PDGF also depends on [Cl⁻]. It is unlikely that the site of action is

Table 1. Amplitude of ANG II-induced Ca²⁺ release is determined by [Cl⁻]ₐ that was sustained during preincubation and is independent of [Cl⁻]ₐ that was attained only immediately before addition of ANG II

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Addition of ANG II</th>
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<tbody>
<tr>
<td>Group</td>
<td>[Cl⁻]ₐ, meq/l</td>
</tr>
<tr>
<td>A</td>
<td>120</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
</tr>
<tr>
<td>C</td>
<td>120</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
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</table>

For each group (A–D), solutions with different extracellular Cl⁻ concentrations ([Cl⁻]ₐ) during preincubation were replaced with solutions of the same or differing [Cl⁻]ₐ 1 min before addition of ANG II (100 nM; n=19–21). Extracellular Ca²⁺ was also removed 1 min before addition of ANG II, to determine the effect of Cl⁻ on ANG II-induced intracellular Ca²⁺ release. In separate vascular smooth muscle cell (VSMC) preparations (n=9) with the same strategy of changing [Cl⁻]ₐ, MQAE was employed to compare relative intracellular [Cl⁻] changes that were inversely associated with the fluorescence intensity of the dye. *P < 0.05 compared with group A; †P < 0.05 compared with group B; ‡P < 0.05 compared with group C.
Cl⁻-DEPENDENT CA²⁺ TRANSIENTS

Table 2. ANG II-induced Ca²⁺ release is blocked by phospholipase C inhibitor U-73122 but not by inactive analog U-73343

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>[Cl⁻]o, meq/l</th>
<th>Basal [Ca²⁺]i, nM</th>
<th>Δ[Ca²⁺]i, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-7343</td>
<td>120</td>
<td>99±7</td>
<td>99±11</td>
</tr>
<tr>
<td>U-7343</td>
<td>20</td>
<td>102±10</td>
<td>55±11*</td>
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<td>U-73122</td>
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<td>169±14†</td>
<td>1±2†</td>
</tr>
<tr>
<td>U-73122</td>
<td>20</td>
<td>159±19†</td>
<td>4±3†</td>
</tr>
</tbody>
</table>

Both U-73122 and U-73343 (10 µM) were incubated with VSMC for 12 min before addition of ANG II (100 nM). Extracellular Ca²⁺ was removed 1 min before addition of ANG II (n=10). *P < 0.05 compared with that of corresponding group treated with U-73343.

primarily or solely on G proteins. Alternatively, Cl⁻ may increase the level of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] to facilitate conversion of Ins(1,4,5)P₃; however, a previous study (2) has demonstrated that 1.1 M NaCl does not alter the level of PtdIns(4,5)P₂, which makes it unlikely that a concentration of 120 meq/l Cl⁻ may alter PtdIns(4,5)P₂ in the present study. Nevertheless, the current results do not rule out the possibility that Cl⁻ might either increase the total Ins(1,4,5)P₃ level by reducing Ins(1,4,5)P₃ catabolism or modulate the capacity of Ins(1,4,5)P₃ generated in response to ANG II to induce the release of Ca²⁺ from intracellular stores.

Alternatively, increased [Cl⁻]o might increase the size of intracellular Ca²⁺ stores, which could contribute to enhancement of ANG II-induced Ca²⁺ release (12). Despite the heterogeneous nature of intracellular Ca²⁺ stores (3), Ca²⁺ released by ANG II was primarily from thapsigargin-sensitive stores in our preparation; however, [Cl⁻]o did not affect thapsigargin-induced Ca²⁺ release in the absence of extracellular Ca²⁺ (Fig. 2B), suggesting that the size of this ANG II- and thapsigargin-sensitive Ca²⁺ stores may not be altered by Cl⁻. Furthermore, Ca²⁺ entry following thapsigargin-induced Ca²⁺ release was also unaffected by [Cl⁻]o (Fig. 2B), suggesting that Cl⁻ does not alter the capacitative Ca²⁺ entry induced by thapsigargin.

In addition to Ca²⁺ release, Ca²⁺ entry may contribute to the effects of Cl⁻ on ANG II- and PDGF-induced Ca²⁺ transients (Fig. 2, A and C). It has been proposed that receptor activation-induced Ca²⁺ release may trigger Cl⁻ efflux via a Cl⁻ channel, resulting in depolarization and subsequent Ca²⁺ entry through a voltage-sensitive Ca²⁺ channel in smooth muscles (15). ANG II-induced Ca²⁺ entry may thus be affected by [Cl⁻]o. However, an inhibitor of Cl⁻-channel, NPPB, exhibited no effect on ANG II-induced Ca²⁺ release or entry with a [Cl⁻]o of 120 meq/l (Fig. 4), suggesting that Cl⁻ efflux may not play a significant role in Ca²⁺ movement in our preparation with a [Cl⁻]o of 120 meq/l. In contrast, NPPB increased Ca²⁺ transients at a [Cl⁻]o of 20 meq/l, suggesting Cl⁻ efflux may play a role in the reduced Ca²⁺ transients with low [Cl⁻]o, probably due to a Cl⁻ gradient between intracellular and extracellular space that may serve as a driving force to induce Cl⁻ efflux with low [Cl⁻]o. Furthermore, the magnitude of Ca²⁺ entry may depend on the amount of Ca²⁺ released from the intracellular store, and hence the extent to which it is depleted of Ca²⁺ (24). It is possible that the reduced Ca²⁺ entry with lower [Cl⁻]o is secondary to the effect of lower [Cl⁻]o on Ca²⁺ release. On the other hand, Ca²⁺ entry may occur independently of Ca²⁺ store depletion (8, 17), and [Cl⁻]o could have an effect on Ca²⁺ entry independent of intracellular Ca²⁺ release. Previous studies have demonstrated that in isolated perfused afferent arterioles of rabbit, Cl⁻ was essential for vasoconstrictor responses to K⁺ (5), which increases intracellular Ca²⁺ solely by an entry mechanism. Nevertheless, our results are consistent with previous findings that [Cl⁻]o is essential for the Ca²⁺ influx induced in mesangial cells by vasopressin and endothelin (14), and for that induced in endothelial cells by histamine and ATP (7).

Finally, both intracellular alkalization and acidification have been found to acutely increase basal [Ca²⁺]i (1, 22). Possibly, replacing Cl⁻ with other anions might alter ANG II-mediated Ca²⁺ transients by altering intracellular pH (pHi). However, previous studies with VSMC have shown that complete replacement of Cl⁻ in the medium with either gluconate or aspartate induces only a minimal reduction in pHi of 0.05 pH units (20). In addition, replacement of extracellular Cl⁻ with gluconate, acetate, or methanesulfonate has no significant effect on the basal pH of mesangial cells (14). Thus, in the present study, altering pHi is unlikely to mediate the effects of partial replacement of Cl⁻ on ANG II- or PDGF-induced Ca²⁺ transients.

In conclusion, our results provide evidence for a modulable mechanism for ANG II-induced Ca²⁺ signaling by Cl⁻ in VSMC. Cl⁻ may play a role in modulating both intracellular
Ca\(^{2+}\) release and Ca\(^{2+}\) entry induced by ANG II and, perhaps, other vasoconstrictors. The possible mechanism(s) for the effect of Cl\(^{-}\) on Ca\(^{2+}\) release includes modulation of ANG II-induced Ins(1,4,5)P\(_3\) increase. Our results provide a potential mechanism for Cl\(^{-}\)-dependent responses to vasoconstrictors observed in isolated arteries (9) and for Cl\(^{-}\)-sensitive hypertension described in stroke-prone SHR with high plasma renin activity (25).

REFERENCES