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Intracellular alkalinization augments α_1-adrenoceptor-mediated vasoconstriction by promotion of Ca^{2+} entry through the non-L-type Ca^{2+} channels

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Abstract

Modulation by intracellular pH of the vasoconstriction induced by α-adrenoceptor agonists was investigated in isolated guinea pig aorta. NH_4Cl (15 mM) increased intracellular pH of aortic smooth muscle cells by about 0.2 pH unit and significantly augmented KCl-induced contraction of aortic strips, whereas simultaneous administration of NH_4Cl (15 mM) plus Na^+ propionate (30 mM) failed to affect intracellular pH or contractility. NH_4Cl (15 mM) potentiated contractions induced by α-adrenoceptor agonists, norepinephrine, phenylephrine and clonidine. Contraction induced by α_1-selective adrenoceptor agonist, phenylephrine, but not that induced by norepinephrine or clonidine, was insensitive to inhibition by verapamil (1 μM). Phenylephrine-induced contraction was not affected by NH_4Cl in Ca^{2+}-free medium whereas extracellular Ca^{2+}-induced contraction of phenylephrine-stimulated aorta was significantly augmented by NH_4Cl. Consistently, $^{45}Ca^{2+}$ uptake into phenylephrine (1 μM)-stimulated aortic strips was increased by incubation with NH_4Cl. The potentiating effects of NH_4Cl on both phenylephrine-induced Ca^{2+} entry and contraction were antagonized by Na^+ propionate. These results suggest that intracellular alkalinization facilitates α_1-adrenoceptor-mediated vasoconstriction by facilitation of an agonist-induced Ca^{2+} entry pathway that is independent of L-type Ca^{2+} channels.

Keywords: Cytosolic alkalosis; Na^+/H^+ exchange; Ammonium chloride; Ca^{2+} channel, receptor-operated; Smooth muscle, vascular; Adrenoceptor agonist

1. Introduction

Various vasocontractile agonists such as angiotensin II, vasopressin, endothelin and thrombin stimulate sarcoplasmic Na^+/H^+ exchange and elevate intracellular pH (Berk et al., 1987, 1991; Kikeri et al., 1990; Koh et al., 1990). Intracellular alkalinization has been recognized as a factor which affects the contractility of vascular smooth muscle (Smith et al., 1998), and it has consequently been hypothesized that agonist-induced contraction of vascular smooth muscle may be mediated in part by intracellular alkalinization. However, the regulatory mechanisms which link changes in intracellular pH and vascular tone have not yet been unequivocally identified. Moreover, the results of the previous studies on the significance of intracellular alkalinization in agonist-induced vasoconstrictions are controversial: Intracellular alkalosis potentiates subsequent agonist-induced vasocontraction (Wakabayashi et al., 1991, 1996), whereas acute intracellular alkalinization during tonic vasocontraction has been reported to result in relaxation (Furtado, 1987; Feletou et al., 1989). One of the reasons for these discrepancies may be the different experimental approaches, such as the use of different animal species and blood vessels as well as different tools and interventions for inducing intracellular alkalinization.

In rat aortas and porcine coronary arteries, low concentrations of NH_4Cl, a weak base, which is frequently used as a pharmacological tool to induce intracellular alkalosis (Thomas, 1974), potentiated contractile responses by increasing nifedipine-sensitive transplasmalemmal Ca^{2+} entry (Wakabayashi et al., 1991, 1996). In ferret pulmonary arterial smooth muscles, intracellular alkalinization by NH_4Cl resulted in an initial increase of both intracellular

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free-Ca^{2+} and pulmonary arterial pressure, which was in a large part due to elevated Ca^{2+} influx, and also resulted in a potentiation of KCl-induced contraction (Farrukh et al., 1996). An enhancement of L-type Ca^{2+} channel currents by elevation of intracellular pH has been observed in porcine coronary arterial cells and rabbit portal venous cells (Iino et al., 1994; Klöckner and Isenberg, 1994). Consistently, we have shown that KCl-induced depolarization of A7r5 smooth muscle cells is promoted by NH_4Cl (Tanaka et al., 1996). Taken together, these findings suggest that intracellular alkalinization results in activation of voltage-dependent Ca^{2+} channels and facilitation of transplasmalemmal Ca^{2+} entry. However, vasoconstrictions induced by stimulation of phospholipase-C-coupled receptors such as adrenoceptors are at least in part mediated by virtually voltage-independent, so-called "receptor-operated Ca^{2+} channels" (van Breemen and Saida, 1989). The molecular nature of these channels is so far elusive. Few studies have examined whether intracellular pH affects the vasoconstriction induced by activation of receptor/phospholipase C-activated Ca^{2+} entry. In vascular endothelial cells, NH_4Cl-induced intracellular alkalinization rather inhibited a store-operated Ca^{2+} entry pathway that is usually activated in response to stimulation of phospholipase C-coupled receptors (Wakabayashi and Groschner, 1997). In guinea-pig aortas, norepinephrine-induced contraction is known to be due to Ca^{2+} entry through non-L-type Ca^{2+} channels (Gouw et al., 1990). Thus, in the present study using guinea pig aortas, we investigated the effects of intracellular alkalosis on the vasocontractile responses to α-adrenoceptor agonists, with particular focus on the non-L-type Ca^{2+} channel-mediated Ca^{2+} entry pathway that is initiated due to stimulation of phospholipase C.

2. Materials and methods

2.1. Tissue preparation

The study protocols regarding treatment of animals were in accordance with the "Guide for the experiments using laboratory animals in Yamagata University School of Medicine". Male Hartley guinea-pigs (400–450 g) were anesthetized with intraperitoneal injection of sodium pentobarbital (25 mg/kg) and killed by exsanguination. The aorta was rapidly excised and placed in physiological salt solution [PSS: 10 mM HEPES/Tris, pH 7.4, containing (in mM) 135 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 1 KH_2PO_4 and 10 glucose at 37 °C]. After removal of excess fat and connective tissue, helical strips of aorta (2×12 mm) were prepared for tension studies. The endothelium was removed by gentle abrasion of the intimal surface with filter paper and the removal was confirmed functionally by the disappearance of the acetylcholine (1 μM)-induced relaxing response of the norepinephrine (0.5 μM)-precontracted strip.

2.2. Contraction study

The strips were mounted in 10-ml organ baths containing PSS maintained at 37 °C and gassed constantly with 100% O_2. Tension was recorded isometrically by means of force transducers (Nippon Kohden Kogyo, Tokyo, Japan) connected to a multichannel recorder. Each strip was stretched to an initial tension of 9.8 mN and allowed to equilibrate for approximately 1 h.

First, the muscle strips were contracted with 60 mM KCl and then washed with PSS. This procedure was repeated twice before each experimental protocol until a reproducible constant contractile force was obtained. Addition of a single dose of 60 mM KCl produced maximum contraction, which we used as a reference contraction of each aortic strip, and the contractile force in each experimental condition was expressed in terms of the percentage of the KCl (60 mM)-induced contractile force in each strip. The mean contractile force induced by 60 mM KCl was 9.40 ± 0.17 mN. The concentration–response relationship

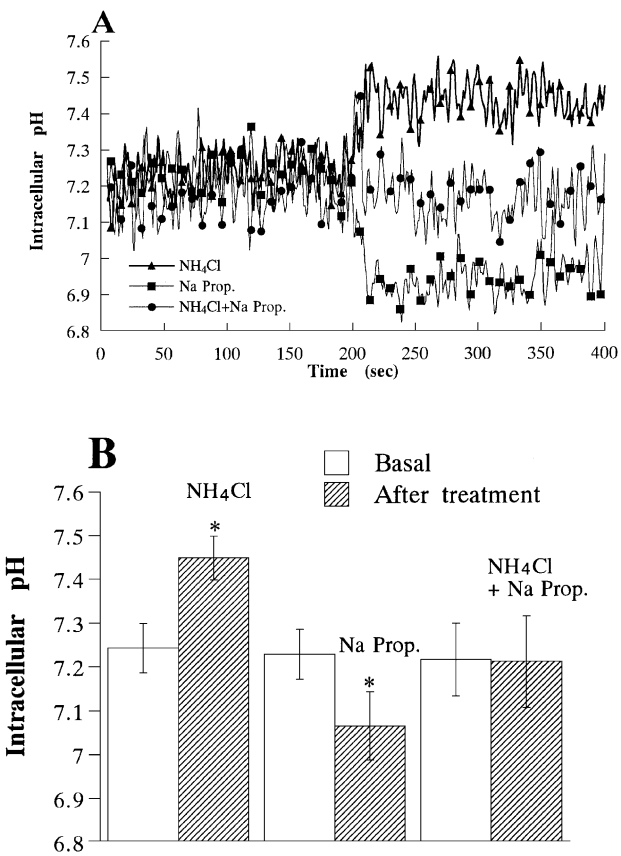

Fig. 1. Changes in intracellular pH of guinea-pig aortic smooth muscle cells. (A) Representative chart of intracellular pH changes after NH_4Cl and propionate addition. When NH_4Cl (15 mM) or Na^+ propionate (30 mM, Na Prop.) was added to the cell suspension, the intracellular pH was immediately elevated or dropped, respectively. (B) Mean values of peak intracellular pH after the addition of NH_4Cl (15 mM), Na^+ propionate (30 mM, Na Prop.), and their combination. Asterisks denote significant differences compared to the basal intracellular pH levels ($n = 5$).

of each contraction was obtained by adding the drug in a cumulative manner.

2.3. Measurement of $^{45}Ca^{2+}$ uptake

The strips were equilibrated for 1 h in 5-ml physiological solution of the following composition (mM): 10 HEPES/Tris (pH 7.4), 135 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$ and 10 glucose at 37 °C. The solution was bubbled with 100% O_2 and maintained at 37 °C. Then, the strips were treated with 0.4 μCi/ml of $^{45}CaCl_2$ together with a vehicle or phenylephrine. After 5-min incubation, the strips were taken out and washed for 45 min (15 min each × 3 times) with ice-cold Ca^{2+}-free EGTA solution [composition (mM): 10 HEPES/Tris (pH 7.4), 135 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, 2 EGTA]. The tissues were then blotted, weighed and digested with addition of 400-μl tissue solubilizer (Soluene-350 from Packard) at 70 °C for 2 h. Following acidification and addition of scintillation fluid, the radioactivity remaining in the tissue was detected with a liquid scintillation counter. The rate of Ca^{2+} uptake was calculated as c.p.m./nmol of the EGTA-resistant $^{45}Ca^{2+}$ fraction divided by c.p.m./nmol Ca^{2+} of the specific activity of $^{45}Ca^{2+}$-containing medium and expressed as percentage (%) of the basal Ca^{2+} uptake without stimulation in each strip.

2.4. Culture of guinea pig aortic smooth muscle cells

Smooth-muscle cells were isolated from the thoracic aorta of guinea pig by outgrowth of explants according to the method of Ross (1971). Briefly, a segment of thoracic aorta was dissected and placed on 60-mm plastic dishes containing Ca^{2+}, Mg^{2+}-free phosphate buffer saline (PBS). Subsequently, fat and connective tissues were removed carefully. After longitudinal dissection, the intima was removed by a scraper. Then 1–2 mm^2 sections of the media were prepared and transferred into 35-mm wells in a

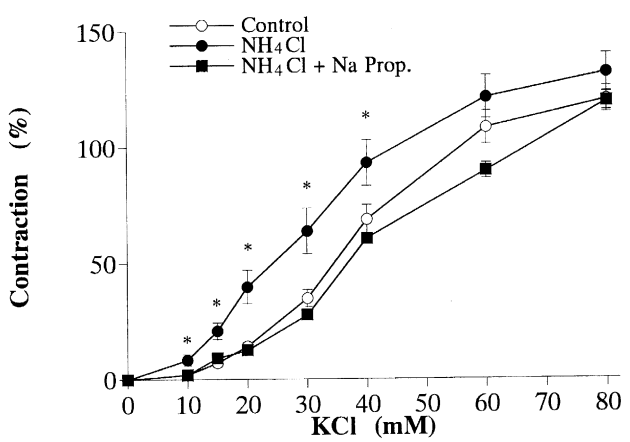

Fig. 2. Effects of NH_4Cl pretreatment on KCl-induced contraction. NH_4Cl (15 mM), NH_4Cl (15 mM) plus Na^+ propionate (30 mM, Na Prop.), or a vehicle was added to each organ bath 10 s before the aortic strips were stimulated with KCl. Asterisks denote significant differences compared with the controls treated with a vehicle ($n = 5$). The controls were the vessels pretreated with a vehicle of NH_4Cl.

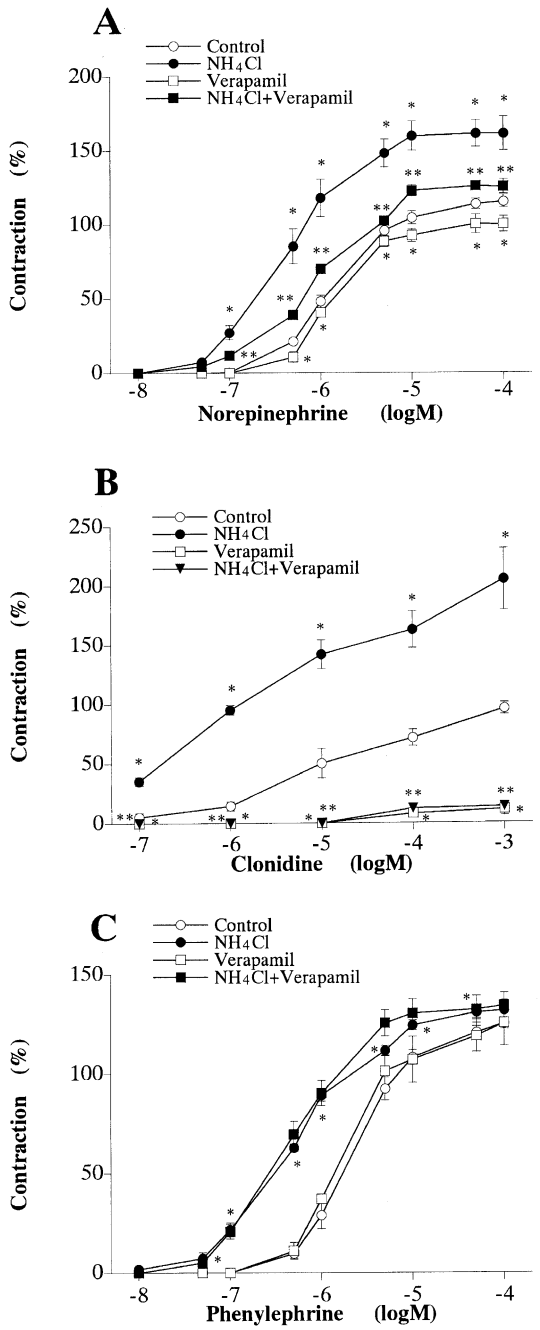

Fig. 3. Effects of pretreatment with NH_4Cl and verapamil on contractile responses to α-adrenoceptor agonists. The aortic strips were pretreated with verapamil (1 μM) or a vehicle for 60 min, and then contracted by norepinephrine (A), clonidine (B) and phenylephrine (C) in a cumulative manner. NH_4Cl (15 mM) or a vehicle was added 10 s before the vessels were stimulated with the contractile agonists. Asterisks denote significant differences compared with the controls pretreated with a vehicle (*) and the strips pretreated with NH_4Cl in the absence of verapamil (**) ($n = 5$).

6-well plate containing Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal calf serum, 4 mM glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin. The dishes were incubated in a humidified atmosphere at 37 °C under 5% CO_2–95% air. After 10–14 days of incubation, cell layers showing the hills and valleys pattern characteristic of smooth muscle cells grew to confluency. Cells from passages 5–10 were used for experiments. The cells were cultured in DMEM containing 5% fetal calf serum, 4 mM glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere at 37 °C under 5% CO_2–95% air, and then spread in a 100-mm culture dish and cultured until reaching confluency.

2.5. Measurement of intracellular pH

Intracellular pH was ascertained using a pH indicator, 2′7′-bis-(2-carboxyethyl)-5(6)-carboxy-fluorescein (BCECF). BCECF/AM (acetoxymethyl ester) was initially dissolved in dimethyl sulfoxide at 2.5 mM, and was used at a final concentration of 5 μM. Confluent cells from one dish were collected and suspended in 6.5 ml of physiological salt solution [PSS: 10 mM HEPES/Tris, pH 7.4, containing (in mM) 135 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 1 KH_2PO_4 and 10 glucose at 37 °C]. The suspended cells were loaded with BCECF for 60 min at 37 °C. After loading, the cells were washed with PSS and then resuspended in PSS to perform the intracellular pH measurement.

Fluorescence measurement was carried out with a dual-wavelength spectrophotometer (Hitachi F2000) using a 2-ml cuvette maintained at 37 °C. Excitation wavelengths used were 506 and 455 nm, and emission was collected at 530 nm. Using ratio (R) of fluorescence intensity (F) of F_{506}/F_{455}, the fractional change in intracellular pH was determined. Calibration of intracellular pH measurements was performed in a nigericin (7 μM)-containing high-K^+ solution at various extracellular pH values.

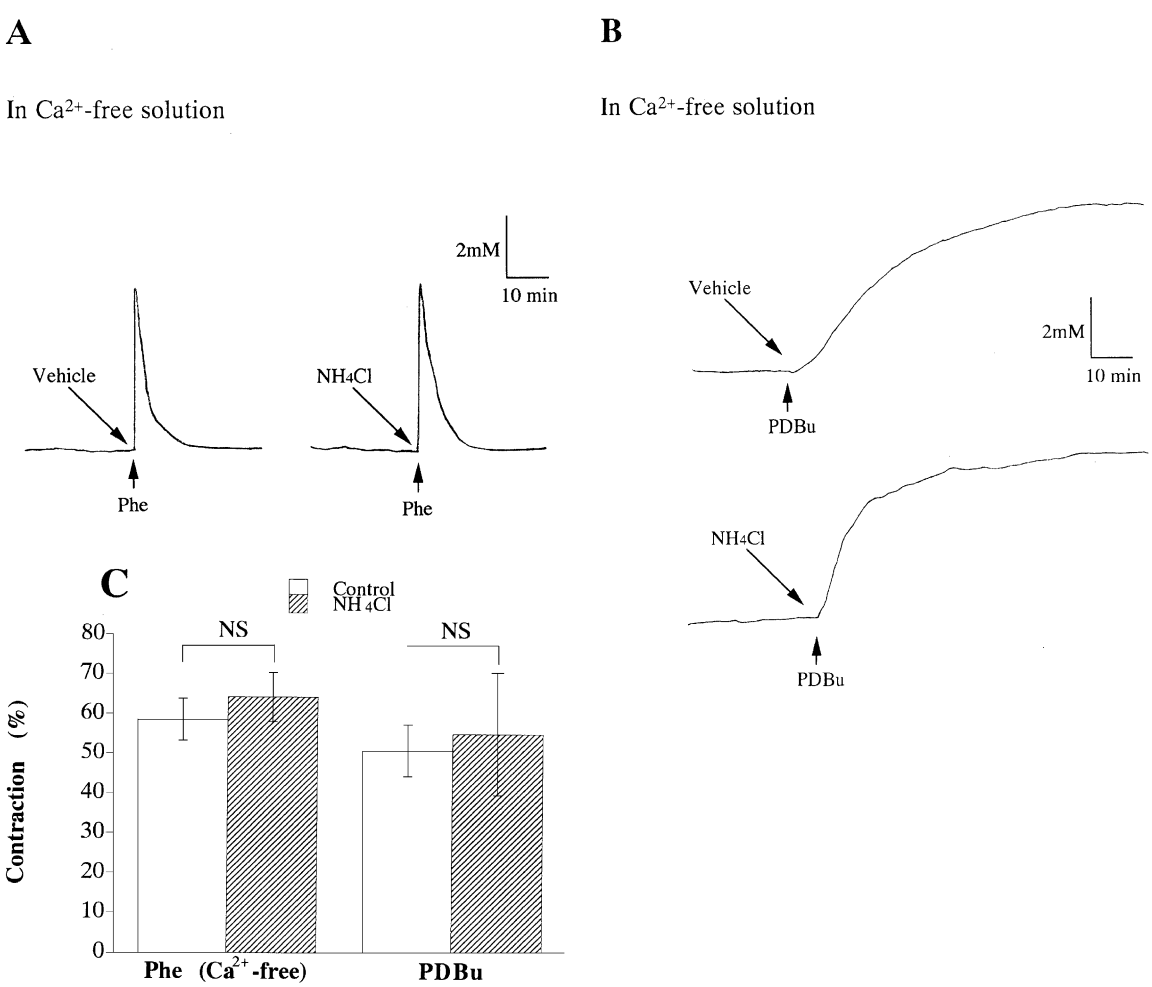

Fig. 4. Effects of NH_4Cl pretreatment on contractile responses to phenylephrine and phorbol 12,13-dibutyrate in Ca^{2+}-free medium. NH_4Cl (15 mM) or a vehicle was added to the organ bath 10 s before stimulation with phenylephrine (Phe, 10 μM) or phorbol 12,13-dibutyrate (PDBu, 1 μM). (A, B) Representative tension recordings. (C) Comparison of mean levels of contractile forces induced by phenylephrine (10 μM) ($n = 7$) and phorbol 12,13-dibutyrate (1 μM) ($n = 5$) in Ca^{2+}-free medium. NS, not significant.

2.6. Chemicals

NH_4Cl, Na^+ propionate, acetylcholine chloride (Wako), phenylephrine hydrochloride, clonidine hydrochloride and norepinephrine bitartrate (Sigma) were dissolved in distilled water, and these stock solutions were stored at 4 °C. Phorbol 12,13-dibutyrate (PDBu) was dissolved in dimethylsulfoxide to make a stock solution of 1 mM and kept at −20 °C. An addition of stock solutions of norepinephrine (100 μM), clonidine (1 mM) or phenylephrine (100 μM) did not affect the pH of PSS buffered with HEPES. The concentration of each drug was expressed as the final concentration in the organ chamber.

2.7. Statistical analysis

The data were expressed as means with standard error. Statistical analysis was performed with two-way ANOVA (analysis of variance) and subsequent Scheffé *F*-test for the data from concentration–response relationships and with Student's *t*-test for the data from the experiment using single doses of the drugs. *P* values less than 0.05 were considered significant.

3. Results

3.1. NH_4Cl and propionate-induced changes in intracellular pH

The effects of the maneuvers used to alter intracellular pH of guinea-pig aortic smooth-muscle cells were tested using the pH-sensitive dye, BCECF. As shown in Fig. 1, application of NH_4Cl or propionate resulted in fairly instantaneous changes and stable changes in intracellular pH (Fig. 1A). NH_4Cl (15 mM) increased intracellular pH by about 0.20 pH unit, while propionate (30 mM) decreased it by about 0.17 pH unit (Fig. 1B). When NH_4Cl (15 mM) and propionate (30 mM) were simultaneously added to the cell suspension, intracellular pH was not affected.

3.2. Modulation by intracellular pH of depolarization (KCl)-induced contraction

NH_4Cl at 15 mM did not affect basal tension of the aortic strips (data not shown). Nonetheless, KCl (10–40 mM)-induced contraction of guinea pig aorta was significantly increased by NH_4Cl (15 mM), and the concentration–response curve was shifted to the left by NH_4Cl (Fig. 2). This potentiating effect of NH_4Cl was eliminated when propionate (30 mM) was added together with NH_4Cl (Fig. 2).

3.3. NH_4Cl potentiates verapamil-sensitive as well as verapamil-insensitive contractions induced by α-adrenoceptor receptor stimulation

Treatment of the aortic strips with NH_4Cl (15 mM) increased contractile responses to norepinephrine, clonidine and phenylephrine (Fig. 3A–C). Verapamil (1 μM) pretreatment partially antagonized the contraction induced by norepinephrine (Fig. 3A), abolished the response to α_2-adrenoceptor-selective agonist, clonidine (Fig. 3B), but failed to affect the contraction induced by α_1-adrenoceptor-selective agonist, phenylephrine (Fig. 3C). These results demonstrate the existence of a verapamil-insensitive vasoconstriction which is induced by stimulation of α_1-adrenoceptors. This verapamil-insensitive contraction as well as verapamil-sensitive contraction was clearly promoted by NH_4Cl-induced intracellular alkalinization (Fig. 3A–C). The potentiating effect of NH_4Cl on phenylephrine contraction was not changed in the presence of verapamil compared with those in the absence of vera-

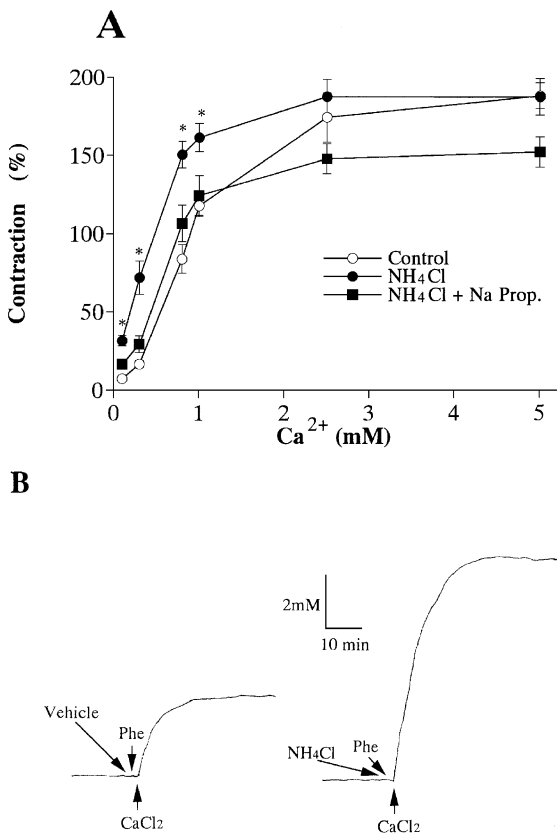

Fig. 5. Effects of NH_4Cl pretreatment on Ca^{2+}-induced contraction in phenylephrine-stimulated vessels. The aortic strips were washed three times with the Ca^{2+}-free PSS containing 1 mM EGTA and then contracted by phenylephrine (10 μM) in the Ca^{2+}-free PSS containing 0.1 mM EGTA. Then, the strips were washed with the Ca^{2+}-free PSS containing 1 mM EGTA again. After repeating this procedure once, the vessels were preincubated with verapamil (1 μM) or a vehicle in the Ca^{2+}-free PSS for 30 min. Next, NH_4Cl (15 mM) alone, NH_4Cl (15 mM) plus Na^+ propionate (30 mM, Na Prop.), or a vehicle was added and 10 s later the vessels were immediately stimulated with phenylephrine (10 μM), but no contraction was observed. Next, the vessels were contracted by cumulative addition of Ca^{2+} to the medium (A). Representative tension recordings of contraction induced by a single addition of Ca^{2+} at 0.3 mM after the above treatment (B). Asterisks denote significant differences compared with the controls pretreated with a vehicle ($n = 5$).

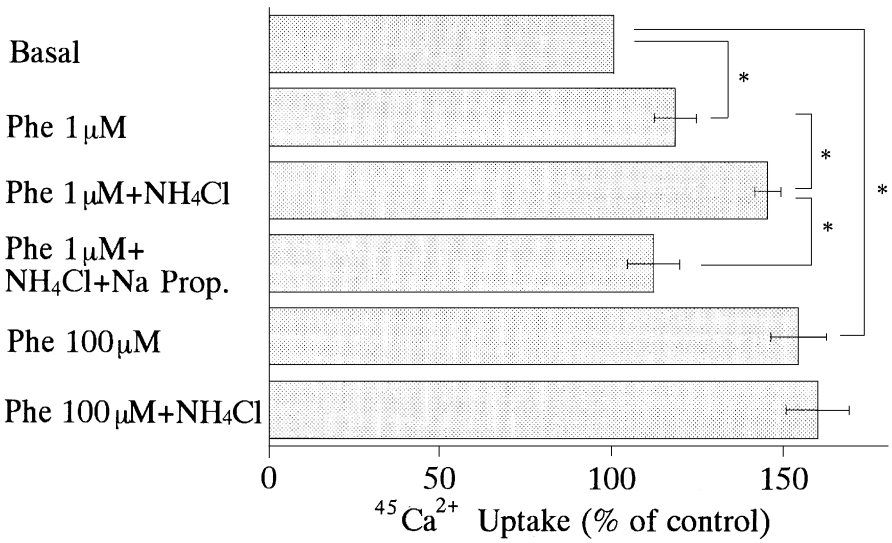

Fig. 6. Effects of pretreatment with NH_4Cl on $^{45}Ca^{2+}$ uptake in guinea-pig aortic strips. NH_4Cl (15 mM), NH_4Cl (15 mM) plus Na^+ propionate (30 mM, Na Prop.) or a vehicle was added 10 s before the aortic strips were stimulated with phenylephrine (1 μM or 100 μM). $^{45}Ca^{2+}$ uptake for 5 min was measured as described in the Materials and methods. Basal $^{45}Ca^{2+}$ uptake was obtained by an addition of a vehicle instead of phenylephrine. The data were expressed as percentage (%) of the basal Ca^{2+} uptake (control). Asterisks denote significant differences among the indicated data sets ($n = 5$).

pamil (Fig. 3C), while NH_4Cl did not significantly affect the contractile response to clonidine in the presence of verapamil (Fig. 3B). Increased contractile response to norepinephrine in the vessels pretreated with NH_4Cl was in part attenuated in the presence of verapamil (Fig. 3A).

3.4. NH_4Cl does not affect the phenylephrine- and PDBu-induced contractions in Ca^{2+}-free medium

In Ca^{2+}-free medium, phenylephrine (10 μM) induced a transient phasic contraction in both NH_4Cl (15 mM)-pretreated and control aortic strips (Fig. 4A). This extracellular Ca^{2+}-independent contraction was not affected by NH_4Cl (15 mM) (Fig. 4A,C). Moreover, PDBu-induced contraction in Ca^{2+}-free medium was not affected by NH_4Cl (Fig. 4B,C). Thus, potentiation of phenylephrine contraction by NH_4Cl was clearly restricted to extracellular Ca^{2+}-dependent contractions, indicating a modulation of voltage-independent, receptor-operated Ca^{2+} entry. To test this hypothesis further, we studied the effects of NH_4Cl and Na^+ propionate under conditions which are expected to perfectly dissect the component of receptor-operated Ca^{2+} entry-mediated contraction.

3.5. NH_4Cl potentiates extracellular Ca^{2+}-induced contraction of phenylephrine-stimulated aortic smooth muscle in the presence of verapamil

Intracellular Ca^{2+} stores were initially depleted by stimulation with phenylephrine in the absence of extracellular Ca^{2+}, and the smooth muscle strips were then washed with Ca^{2+}-free medium. This procedure was repeated once. Subsequent stimulation of the vessels with phenylephrine in the presence of verapamil failed to produce any increase in isometric tension (data not shown), however, further addition of Ca^{2+} to the medium resulted in a profound tonic contraction. These Ca^{2+} (0.1–1 mM)-induced contractions were significantly augmented in the presence of NH_4Cl (15 mM), but not affected when both NH_4Cl (15 mM) and propionate (30 mM) were administrated (Fig. 5A). A single-step elevation of Ca^{2+} (0.3 mM) induced a sustained contractile response in the above-conditioned strips, which was markedly increased by pretreatment with NH_4Cl (15 mM) (Fig. 5B).

3.6. Intracellular alkalinization augments phenylephrine-induced $^{45}Ca^{2+}$ uptake

Fig. 6 illustrates the effects of NH_4Cl on the ^{45}Ca uptake induced by phenylephrine. Phenylephrine (1 and 100 μM) significantly increased $^{45}Ca^{2+}$ uptake compared with the basal level. The basal $^{45}Ca^{2+}$ uptake was not affected by NH_4Cl (15 mM) [100 % (control) vs. 102.1 ± 1.7% (NH_4Cl-treated)]. NH_4Cl (15 mM) augmented the $^{45}Ca^{2+}$ uptake induced by the low concentration (1 μM) of phenylephrine, but not that induced by the higher concentration (100 μM). This result agrees with the NH_4Cl effects on phenylephrine-induced contraction (Fig. 3C). The potentiation by NH_4Cl of $^{45}Ca^{2+}$ uptake stimulated with 1 μM of phenylephrine was cancelled by propionate (30 mM) (Fig. 6).

4. Discussion

With the present study, we demonstrate that the vasoconstriction induced by norepinephrine, a nonselective α-adrenoceptor agonist, is substantially promoted by intra-

cellular alkalinization. We provide evidence that this alkalosis-induced facilitation of vasoconstriction is in part based on the modulation of α_1-adrenoceptor-regulated, non-L-type Ca^{2+} channels. Adrenoceptors of vascular smooth muscle cells are of both the α_1 and α_2 subtypes (Ruffolo et al., 1991). In the present study, we observed that the contractile response of the guinea pig aorta to clonidine, a selective α_2-adrenoceptor agonist, was abolished by pretreatment with verapamil, whereas the response to phenylephrine, a selective α_1-adrenoceptor agonist, was not affected. In our preliminary experiments, prazosin (10 μM), a selective antagonist of the α_1-adrenoceptor, completely inhibited phenylephrine-induced contraction of guinea pig aorta, and yohimbine (10 μM), a selective antagonist of the α_2-adrenoceptor, completely inhibited contraction induced by clonidine at 0.1–10 μM and strongly inhibited (by 80–90%) the contraction induced by clonidine at 0.1 and 1 mM. Thus, it appears reasonable to conclude that contractile responses to phenylephrine and clonidine are mainly due to their action on α_1- and α_2-adrenoceptors, respectively. These results suggest that in guinea-pig aorta, Ca^{2+} entry through voltage-dependent Ca^{2+} channels is centrally involved in α_2-adrenoceptor-mediated vasoconstriction, but not in the vasoconstriction induced by stimulation of α_1-adrenoceptors. NH_4Cl pretreatment potentiated the contractile response to phenylephrine and augmented that to clonidine, indicating that intracellular alkalinization affects both types of vasocontractile responses. Modulation by intracellular pH of voltage-gated Ca^{2+} channels has previously been demonstrated in various smooth muscle tissues (Wakabayashi et al., 1991, 1996; Iino et al., 1994; Klöckner and Isenberg, 1994; Farrukh et al., 1996). This concept is in line with the clear potentiation of depolarization (KCl)-induced contraction of the guinea-pig aorta observed in the present study. Moreover, KCl-induced contraction was not affected by NH_4Cl, when propionate, which antagonizes the effects of NH_4Cl on intracellular pH, was added simultaneously with NH_4Cl. This result further confirmed that the NH_4Cl effects on voltage-dependent Ca^{2+} entry and contraction are indeed due to elevation of intracellular pH. The potentiation of voltage-dependent L-type Ca^{2+} currents by elevated intracellular pH has been attributed to a direct modulation of class C L-type Ca^{2+} channels, which appears to be strictly dependent on the interaction of the α_1 and β subunits of this Ca^{2+} channel (Schuhmann et al., 1997). Hence, the present findings of a clear potentiation by intracellular alkalinization of the α_2-adrenoceptor-mediated, verapamil-sensitive contraction of the guinea-pig aorta strongly suggest that both α_2-adrenoceptors and intracellular alkalinization affect Ca^{2+} entry into this tissue through voltage-dependent L-type Ca^{2+} channels. In our study, verapamil (1 μM) slightly but significantly attenuated the maximum contraction induced by norepinephrine and caused a slight rightward shift of its concentration–response curve. This may be explained by the stimulation of both α_1- and α_2-adrenoceptors by norepinephrine and by only a minor contribution of L-type Ca^{2+} channels to the norepinephrine-induced contraction. Our results are different from those of Gouw et al. (1990), who reported that norepinephrine-induced contraction was insensitive to organic Ca^{2+} entry blockers. Possible explanations for this discrepancy are the differences in the methods of norepinephrine application (single addition vs. cumulative addition) and the kind of L-type Ca^{2+} channel antagonists.

By contrast, the contractile response to the α_1-selectve adrenoceptor agonist, phenylephrine, was significantly attenuated by removal of extracellular Ca^{2+} but completely insensitive to the L-type Ca^{2+} channel blocker. This result was interpreted in terms of a significant role of Ca^{2+} entry pathway through non-L-type Ca^{2+} channels in α_1-adrenoceptor agonist-induced contractions of guinea-pig aorta. The mechanisms by which stimulation of the phospholipase C-coupled receptors is linked to Ca^{2+} entry are still elusive. Nonetheless, these mechanisms may involve G-protein coupling (Macrez-Leprêtre et al., 1997), protein phosphorylation (Tsunoda, 1998; Lagaud et al., 1999), or inositol 1,4,5-trisphosphate (IP_3)-mediated depletion of intracellular Ca^{2+} stores (store-operated Ca^{2+} entry) (Noguera et al., 1997; Yoshimura et al., 1997; Gibson et al., 1998). Moreover, G protein-mediated activation of phospholipase C is in addition associated with an enhancement of smooth muscle contraction which is independent of Ca^{2+} entry (Rokolya et al., 1994). However, NH_4Cl pretreatment failed to affect the phenylephrine contraction in Ca^{2+}-free medium, indicating that the α_1-adrenoceptor-mediated Ca^{2+} influx is promoted by elevation of intracellular pH. Moreover, a sustained contraction induced by PDBu in the Ca^{2+}-free medium was not affected by pretreatment with NH_4Cl. Since phorbol ester-induced contraction in Ca^{2+}-free medium was reported to be due to an increase in Ca^{2+} sensitivity of the contractile apparatus of vascular smooth muscle (Jiang and Morgan, 1987), the augmenting effects of NH_4Cl-induced intracellular alkalinization on α_1- and α_2-adrenoceptor-mediated contraction were not due to its nonspecific action on vascular smooth muscle contractility (e.g., direct action on cytoskeletal components). NH_4Cl increased extracellular Ca^{2+}-induced contraction as well as Ca^{2+} uptake into guinea-pig aortic smooth muscle strips challenged with phenylephrine. The potentiating effect of NH_4Cl on phenylephrine contraction was not changed in the presence of verapamil compared with those in the absence of verapamil, implying that the NH_4Cl effect on α_1-adrenoceptor-mediated contraction is not due to its action on voltage-dependent L-type Ca^{2+} channels. In contrast, NH_4Cl did not significantly affect the contractile response to clonidine in the presence of verapamil, suggesting that the augmenting effect of NH_4Cl on α_2-adrenoceptor-mediated contraction results mainly from an increase in Ca^{2+} entry through voltage-dependent L-type Ca^{2+} channels. Increased contractile response to norepinephrine in the vessels pretreated with NH_4Cl was

in part attenuated in the presence of verapamil, which may well be explained by the fact that norepinephrine acts on both α_1- and α_2-adrenoceptors. NH_4Cl enhanced contractile responses to low concentrations of phenylephrine or low concentrations of Ca^{2+} in the presence of phenylephrine but not those to higher concentrations of these stimulants. Similarly, NH_4Cl enhanced $^{45}Ca^{2+}$ uptake initiated with a low concentration of phenylephrine but not that induced by a higher concentration of phenylephrine, which elicited maximum contraction. These results suggest that NH_4Cl increases the potency of α_1-adrenoceptor-mediated vasoconstriction by Ca^{2+} entry through the receptor-operated Ca^{2+} channels, while it enhances both the potency and maximum response of α_2-adrenoceptor-mediated vasoconstriction by Ca^{2+} entry through the voltage-dependent Ca^{2+} channels.

Stimulation of rat and rabbit aortic smooth-muscle cells with angiotensin II and phorbol esters, both of which activate protein kinase C, results in facilitation of Na^+/H^+ exchange and was reported to induce an increase of intracellular pH by 0.1–0.2 unit (Danthuluri et al., 1987). This degree of increased intracellular pH is similar to that induced by an application of NH_4Cl to guinea-pig aortic smooth muscle cells in the present study. Hence, the observed NH_4Cl-induced facilitation of vasoconstrictions may well be of (patho)physiological significance. Weak acids such as Na^+ propionate are known to induce intracellular acidification (Thomas, 1984), and thus, to counteract the effects of NH_4Cl on intracellular pH. This phenomenon was confirmed in guinea-pig aortic smooth muscle cells by measurements of intracellular pH, which demonstrated that elevation of intracellular pH by NH_4Cl (15 mM) was completely cancelled in the presence of propionate (30 mM). We used this ability of propionate to cancel the effect of NH_4Cl on intracellular pH in order to further test the significance of intracellular pH changes in the NH_4Cl effects on vasoconstriction and to rule out any nonspecific, intracellular pH-independent effects of NH_4Cl. In the presence of propionate, NH_4Cl affected neither extracellular Ca^{2+}-induced contraction nor Ca^{2+} uptake into phenylephrine-stimulated vessels. Thus, our results suggest that, indeed, elevation of intracellular pH potentiates the vasoconstriction mediated by the α_1-adrenoceptor agonist-induced Ca^{2+} entry. To our knowledge, this is the first study reporting facilitation by intracellular alkalosis of vasospasms elicited by activation of a phospholipase-C-coupled receptor such as the α_1-adrenoceptor.

A recent study has reported that trimethylamine, a weak base, did not affect norepinephrine-induced contraction of rat tail artery, but by contrast even reduced the sensitivity of KCl-induced depolarization (Achike et al., 1996). The discrepancy between these findings and our study may be due to the different vessels used. Although the mechanism for facilitation of receptor-operated Ca^{2+} entry by intracellular alkalinization remains elusive, it appears tempting to speculate that the gating of receptor/phospholipase C-regulated Ca^{2+} channels in vascular smooth muscle cells is dependent on intracellular pH. Nonetheless, another possible mechanism is that intracellular alkalinization facilitates the coupling between receptor stimulation and activation of receptor-operated Ca^{2+} channels. However, neither the cascade of events leading to channel opening nor the molecular nature of these receptor-operated ion channels has been identified. Enhanced phosphoinositide hydrolysis might not be involved in the effects of intracellular alkalinization since extracellular Ca^{2+}-independent vasocontraction, which is in large part due to Ca^{2+} mobilization from intracellular Ca^{2+} stores triggered by IP_3 (Abdel-Latif, 1986), was not affected by NH_4Cl.

In conclusion, intracellular alkalinization facilitates vasoconstriction due to enhanced Ca^{2+} entry through non-L-type, receptor/phospholipase C-dependent Ca^{2+} channels, and may therefore play a role in (patho)physiology of vascular smooth muscle. Our present findings strongly support the hypothesis that intracellular pH controls receptor-operated Ca^{2+} entry, which is a critical determinant of vascular tone. Further studies are needed to clarify the molecular basis of the intracellular pH sensitivity of receptor-operated Ca^{2+} channels in vascular smooth muscle.

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