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NH_4Cl-induced contraction of porcine coronary artery involves activation of dihydropyridine-sensitive Ca^{2+} entry

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Abstract

The role of voltage-dependent, dihydropyridine-sensitive Ca^{2+} channels in NH_4Cl-induced vasoconstriction was investigated in isolated porcine coronary arteries by measuring in parallel isometric tone and $^{45}Ca^{2+}$ uptake. NH_4Cl (10–80 mM) concentration dependently induced tonic contractions which were preceded by a time lag of several minutes. Contractile responses to high (60 mM) as well as low (25 mM) concentrations of NH_4Cl were markedly inhibited by 1 μM nifedipine or removal of extracellular Ca^{2+}. The contractile effect of 25 mM NH_4Cl was substantially enhanced by increasing extracellular K^+ to 14.7 mM or by pretreatment of coronary arteries with either 5 mM tetraethylammonium chloride or 0.1 μM 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester (BAY K8644). NH_4Cl (60 mM) significantly increased $^{45}Ca^{2+}$ uptake with a lag time of more than 5 min. The increase in $^{45}Ca^{2+}$ uptake induced by 60 mM NH_4Cl was abolished in the presence of 1 μM nifedipine. Although NH_4Cl (25 mM) did not detectably stimulate $^{45}Ca^{2+}$ uptake in normal K^+ solution, it significantly augmented $^{45}Ca^{2+}$ uptake when extracellular K^+ was increased to 14.7 mM. Furthermore, NH_4Cl (20 mM) potentiated histamine-induced contraction of coronary arteries. This potentiating effect of NH_4Cl was completely antagonized by nifedipine. Our results suggest an involvement of nifedipine-sensitive Ca^{2+} channels in NH_4Cl-induced vasoconstriction of porcine coronary artery.

Keywords: NH_4Cl; Vasoconstriction; Coronary artery; Intracellular pH

1. Introduction

Intracellular pH has recently been recognized as an important determinant of cellular functions. In vascular smooth muscle cells, intracellular alkalinization is thought to control growth and contractile function (Berk et al., 1988; Aalkjaer, 1990). Agonists such as angiotensin II, thrombin, vasopressin, endothelin as well as protein kinase C-activating phorbol ester were reported to induce vasoconstriction and concomitant intracellular alkalinization in vascular smooth muscle cells (Berk et al., 1987, 1991; Danthuluri et al., 1987; Kikeri et al., 1990; Koh et al., 1990).

NH_4Cl, a weak base, is often used as an experimental tool to directly elevate intracellular pH. NH_4Cl has been reported to increase the basal tone of various kinds of isolated vessels (Danthuluri and Deth, 1989; Krampetz and Rhoades, 1991; Wakabayashi et al., 1992; Nguyen-Duong, 1993). This finding has led to the hypothesis that intracellular pH is intimately linked to contractile tone. However, it still remains unclear how intracellular alkalinization controls smooth muscle contraction. Moreover, the mechanisms of NH_4Cl-induced vasoconstriction have not been clarified in detail. To date, there are only a few studies showing effects of NH_4Cl on coronary artery tone. In isolated porcine coronary arteries, NH_4Cl-induced contraction was found to be clearly dependent on extracellular Ca^{2+} (Nguyen-Duong, 1993). However, in a recent investigation NH_4Cl failed to increase the intracellular free Ca^{2+} level, measured by a fluorescent method, at concentrations which induced contraction in isolated porcine coronary arteries (Nagesetty and Paul, 1994). Thus, the role of Ca^{2+} entry in NH_4Cl-induced vasoconstriction as well as the nature of the possibly involved Ca^{2+} entry mechanism is still a matter of controversy. The present study was designed to rigorously test for a possible contribution of dihydropyridine-sensitive Ca^{2+} influx in NH_4Cl-induced contraction of porcine coronary artery.

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2. Materials and methods

2.1. Tissue preparation

Porcine hearts were obtained from the local slaughterhouse and immediately transported to the laboratory in closed plastic sacs. Right coronary arteries were removed and placed in fresh Krebs-Henseleit physiological salt solution of the following composition (mM); NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $CaCl_2$ (2.5), $MgCl_2$ (1.2), $NaHCO_3$ (25) and glucose (10), previously gassed with 5% CO_2-95% O_2. The vessels were dissected free from surrounding tissues and cut into ring-shaped vascular strips 3–4 mm long. The endothelium of the strips was removed by gentle abrasion of the intimal surface with a wooden stick.

2.2. Contraction study

The vascular strips were mounted in 5 ml organ baths containing the above solution maintained at 37°C and gassed constantly with 5% CO_2-95% O_2 (pH 7.3–7.4). Tension was recorded isometrically by means of F30 force transducers connected to a multichannel recorder. Each ring strip was stretched to an initial tension of 19.6 mN, and allowed to equilibrate for approximately 1 h.

First, the vessels were contracted by 40 mM KCl, and then washed with normal solution. This procedure was repeated several times before each experimental protocol until a reproducible constant contractile force was obtained. Only one concentration of NH_4Cl was applied per ring strip. The contractile force was expressed as a percentage of the 40 mM KCl-induced contractile force in each vessel. Some experiments were performed under conditions corresponding to those of the $^{45}Ca^{2+}$ uptake studies, which were done in a solution not containing KH_2PO_4 (see below).

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

The ring strips were equilibrated for 1 h in 5 ml of the above solution, which did not contain KH_2PO_4, and gassed with 5% CO_2-95% O_2 at 37°C. Then, the strips were stimulated with 40 mM KCl for 20 min, followed by washout with normal solution for 20 min. This KCl stimulation and the following washout procedure were repeated once again. Subsequently, the strips were transferred to a tube containing 5 ml of conditioning solution according to the experimental protocol. After various times of incubation, 0.4 μCi/ml of $^{45}CaCl_2$ was added to the tubes. After 5 or 10 min of incubation with $^{45}CaCl_2$, the strips were washed for 40 min with ice-cold Ca^{2+}-free EGTA solution of the following composition (mM); NaCl 118, KCl 4.7, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 10, EGTA 2 and *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (Hepes) 5, pH 7.4 at 4°C. The tissues were then blotted, weighed and digested with 0.8 ml tissue solubilizer (Soluene-350 from Packard Instrument) at 60°C for 1 h. Following acidification and addition of scintillation fluid, the radioactivity remaining in the tissue was detected with a liquid scintillation counter (Packard Tri-carb Model 4530 liquid scintillation spectrometer). The rate of Ca^{2+} uptake was calculated as cpm/g of the EGTA-resistant $^{45}Ca^{2+}$ fraction divided by cpm/nmol Ca of the specific activity of $^{45}Ca^{2+}$-containing medium. The level of Ca^{2+} uptake in each experimental condition was expressed as a percentage of basal Ca^{2+} uptake.

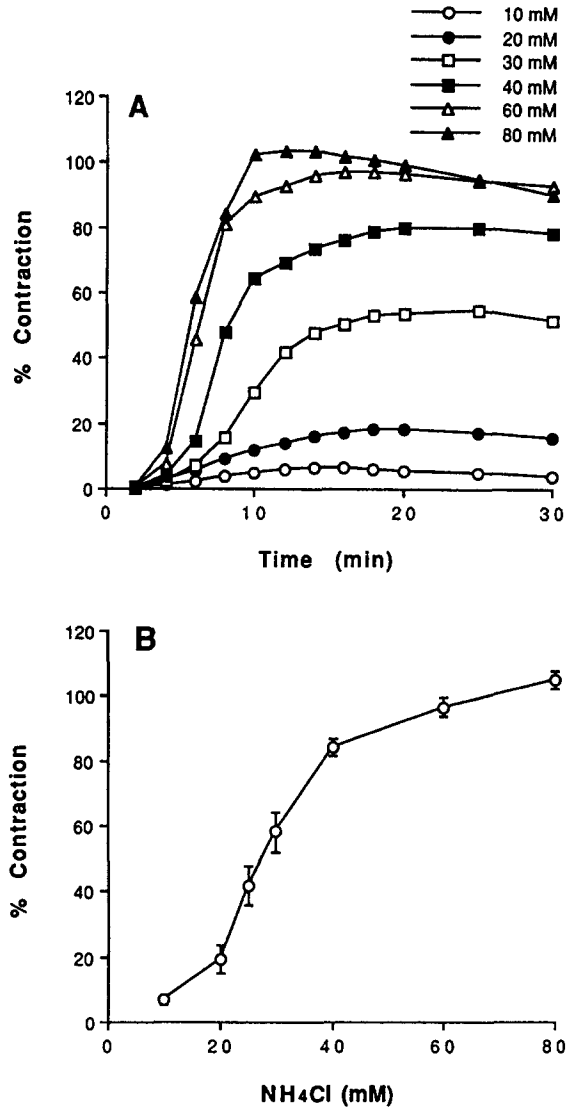

Fig. 1. NH_4Cl-induced contraction of porcine coronary artery. (A) Time courses of contractions induced by various concentrations of NH_4Cl (open circles, 10 mM; closed circles, 20 mM; open squares, 30 mM; closed squares, 40 mM; open triangles, 60 mM; closed triangles, 80 mM). Only mean values ($n = 7$) are given: error bars are omitted to avoid overcrowding of the graph. (B) Concentration-response relationship obtained for maximum contractile force induced by NH_4Cl.

2.4. Drugs

Drugs used in this study were: nifedipine, tetraethylammonium chloride and histamine (Sigma), 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine carboxylic acid methyl ester (BAY K8644) (Bayer), and $^{45}CaCl_2$ (New England Nuclear). Nifedipine and BAY K8644 were dissolved in dimethylsulfoxide to make up stock solutions of 10 mM and 4 mM, respectively, and kept at 4°C in the dark. Tetraethylammonium chloride and histamine were dissolved in distilled water to make up stock solutions of 0.5 M and 0.1 M, respectively, and kept at 4°C. The solution containing high K^+ (14.7 or 40 mM) or NH_4Cl was made by replacing the additive amount of KCl or NH_4Cl with an equal amount of NaCl.

2.5. Statistical analysis

Data are expressed as means with standard errors. Statistical analysis of contraction data was performed with analysis of variance and subsequent Scheffé F-test. Data

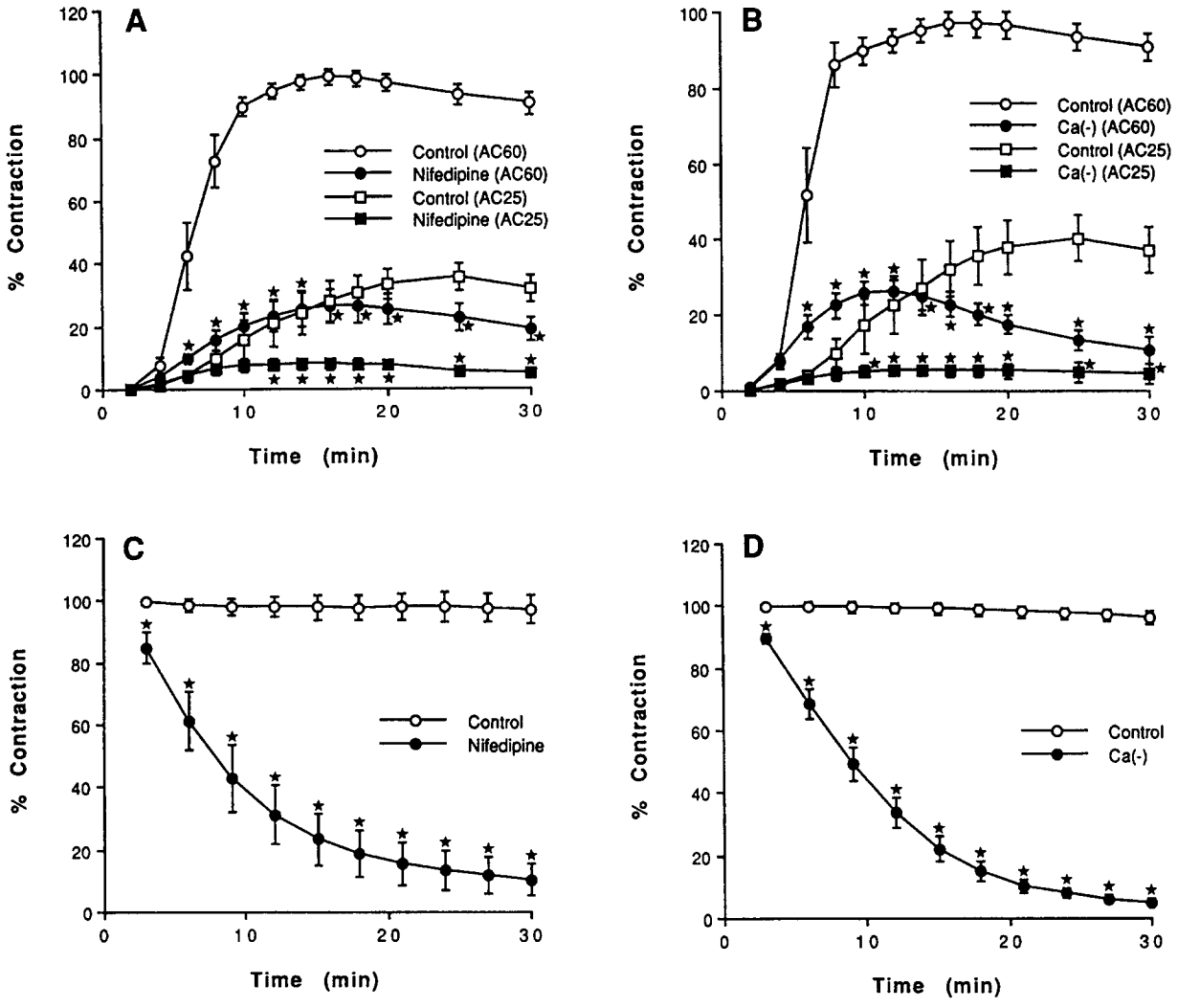

Fig. 2. Suppression of NH_4Cl-induced vasoconstriction by nifedipine (A) or removal of extracellular Ca^{2+} (B), and relaxation of NH_4Cl-contracted vessels by nifedipine (C) and extracellular Ca^{2+} removal (D). (A) Time courses of contractions induced by 25 mM (squares, AC25) and 60 mM (circles, AC60) NH_4Cl in the absence (open symbols) and presence (closed symbols) of nifedipine (1 μM). Vessels were incubated with nifedipine (1 μM) or vehicle (0.01% dimethylsulfoxide) for 30 min before NH_4Cl (25 mM or 60 mM) was administered. Asterisks denote statistically significant difference ($P < 0.05$) between the nifedipine-treated and control tissues ($n = 7$). (B) Time courses of contractions induced by 25 mM (squares, AC25) and 60 mM (circles, AC60) NH_4Cl in the presence (open symbols) and absence (closed symbols) of extracellular Ca^{2+}. Vessels were rinsed rapidly 3 times with the Ca^{2+}-free Krebs-Henseleit solution containing 1 mM EGTA and allowed to equilibrate in the Ca^{2+}-free solution for 10 min before administration of NH_4Cl. Asterisks denote statistically significant differences ($P < 0.05$) between the responses in the Ca^{2+}-free solution and the control ($n = 7$). (C,D) Vessels were contracted by NH_4Cl (60 mM) for 20 min. Then, nifedipine (1 μM) or vehicle (0.01% dimethylsulfoxide) was added to the organ bath (C). Extracellular Ca^{2+} removal (D) was performed by exchanging the bath solution rapidly 3 times with Ca^{2+}-free Krebs-Henseleit solution containing 1 mM EGTA and 60 mM NH_4Cl, 20 min after administration of 60 mM NH_4Cl. Open circles, controls (vehicle addition or no treatment); closed circles, experiments in which nifedipine was added or extracellular Ca^{2+} was removed. Asterisks denote statistically significant differences ($P < 0.05$) between the responses obtained in the presence of nifedipine or in the Ca^{2+}-free condition, and controls ($n = 7$).

of $^{45}Ca^{2+}$ uptake were analyzed using Student's *t*-test. *P* values less than 0.05 were considered significant.

3. Results

3.1. NH_4Cl-induced contraction in porcine coronary artery is inhibited by nifedipine and is dependent on the presence of extracellular Ca^{2+}

NH_4Cl (10–80 mM) induced tonic contraction of coronary artery rings in a concentration-dependent manner (Fig. 1A,B). Contraction was preceded by a time lag of several minutes (Fig. 1A).

Pretreatment of the vessels with nifedipine (1 μM) markedly inhibited the contractile responses to 25 mM and 60 mM NH_4Cl (Fig. 2A). Mean values of maximum contractile tone induced by 25 mM and 60 mM NH_4Cl in the presence of nifedipine (7.9 ± 2.0% and 26.1 ± 5.3%, respectively) were significantly lower than in controls (vehicle-treated; 35.3 ± 4.4% and 98.8 ± 2.5%, respectively, $P < 0.01$). Removal of extracellular Ca^{2+} attenuated the contractile responses to 25 mM and 60 mM NH_4Cl (Fig. 2B). The maximum contractile responses to 25 mM and 60 mM NH_4Cl obtained in nominally Ca^{2+}-free solution (5.5 ± 1.4% and 26.2 ± 3.2%, respectively) were significantly lower than in controls (40.4 ± 6.2% and 96.9 ± 3.2%, respectively, $P < 0.01$).

When either nifedipine (1 μM) was added, or the bath solution was replaced by a nominally Ca^{2+}-free buffer after the contractile response to 60 mM NH_4Cl had reached a plateau level, the tension of the vessels decreased gradually (Fig. 2C,D). The residual levels of contractile tension at 30 min after nifedipine addition and replacement with Ca^{2+}-free solution were 10.3 ± 5.2% and 5.0 ± 1.2%, respectively [$P < 0.01$ from the control without the addition and replacement (97.1 ± 4.6% and 95.8 ± 2.0%, respectively)].

3.2. NH_4Cl-induced contraction in porcine coronary artery is augmented by increases in extracellular K^+, tetraethylammonium chloride and BAY K8644

Increasing the extracellular K^+ concentration to 14.7 mM induced a small contraction (9.2 ± 1.6%). In the

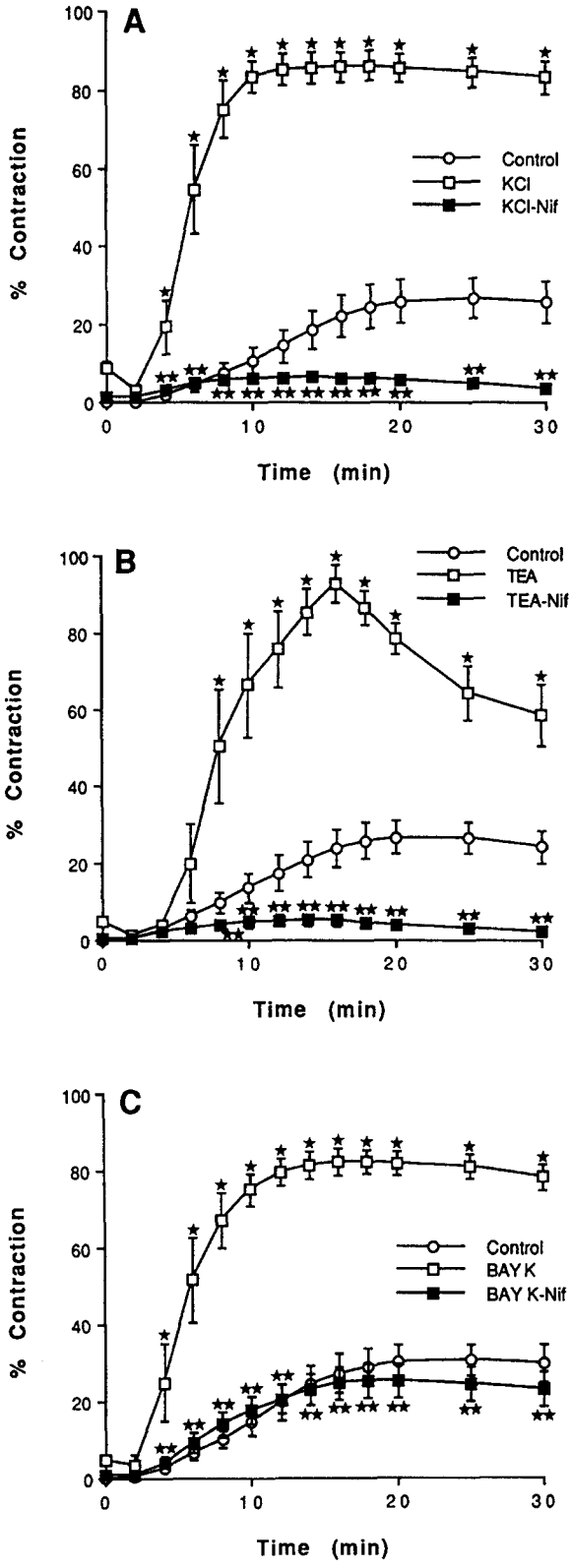

Fig. 3. Potentiation of NH_4Cl-induced contraction by a moderate increase of extracellular K^+ concentration to 14.7 mM (A), pretreatment with 5 mM tetraethylammonium chloride (B) or pretreatment with 0.1 μM BAY K8644 (C). Vessels were pretreated with 1 μM nifedipine (Nif) or vehicle for 30 min, and subsequently preincubated in either control, 14.7 mM K^+ (KCl)-, 5 mM tetraethylammonium chloride (TEA)- or 0.1 μM BAY K8644 (BAY K)-containing solutions for 15 min. Then, NH_4Cl (25 mM) was administered (time 0). Open circles, control contractile response to 25 mM NH_4Cl; open squares, 25 mM NH_4Cl-induced contraction in the presence of high K^+, tetraethylammonium chloride or BAY K8644; closed squares, 25 mM NH_4Cl-induced contraction in the presence of high K^+, tetraethylammonium chloride or BAY K8644 after nifedipine treatment. Asterisks denote statistically significant differences ($P < 0.05$) between responses in the presence of 14.7 mM KCl, tetraethylammonium chloride or BAY K8644, and the controls (*), and between the groups with and without nifedipine pretreatment (**). $n = 7$ (A), $n = 7$ (B) and $n = 9$ (C).

presence of 14.7 mM KCl, the effect of NH_4Cl (25 mM) on contractile force was markedly potentiated, and this potentiation was abolished in the presence of 1 μM nifedipine (Fig. 3A). Also, tetraethylammonium chloride (5 mM) and BAY K8644 (0.1 μM) alone caused small contractile responses (6.5 ± 2.8% and 5.0 ± 1.3%, respectively), and pretreatment of the vessels with either tetraethylammonium chloride or BAY K8644 markedly potentiated the contractile response to NH_4Cl (25 mM). These potentiating effects were again abolished in the presence of nifedipine (1 μM) (Fig. 3B,C).

3.3. NH_4Cl (60 mM) increases $^{45}Ca^{2+}$ uptake in porcine coronary artery

No significant increase in $^{45}Ca^{2+}$ uptake above basal value was detected within an initial phase of 5 min after addition of 60 mM NH_4Cl (initial phase). In contrast, high K^+ (40 mM) significantly increased $^{45}Ca^{2+}$ uptake in this initial phase (Fig. 4A). The mean maximum contractile force induced by 60 mM NH_4Cl and 40 mM KCl, under experimental conditions corresponding to those used in

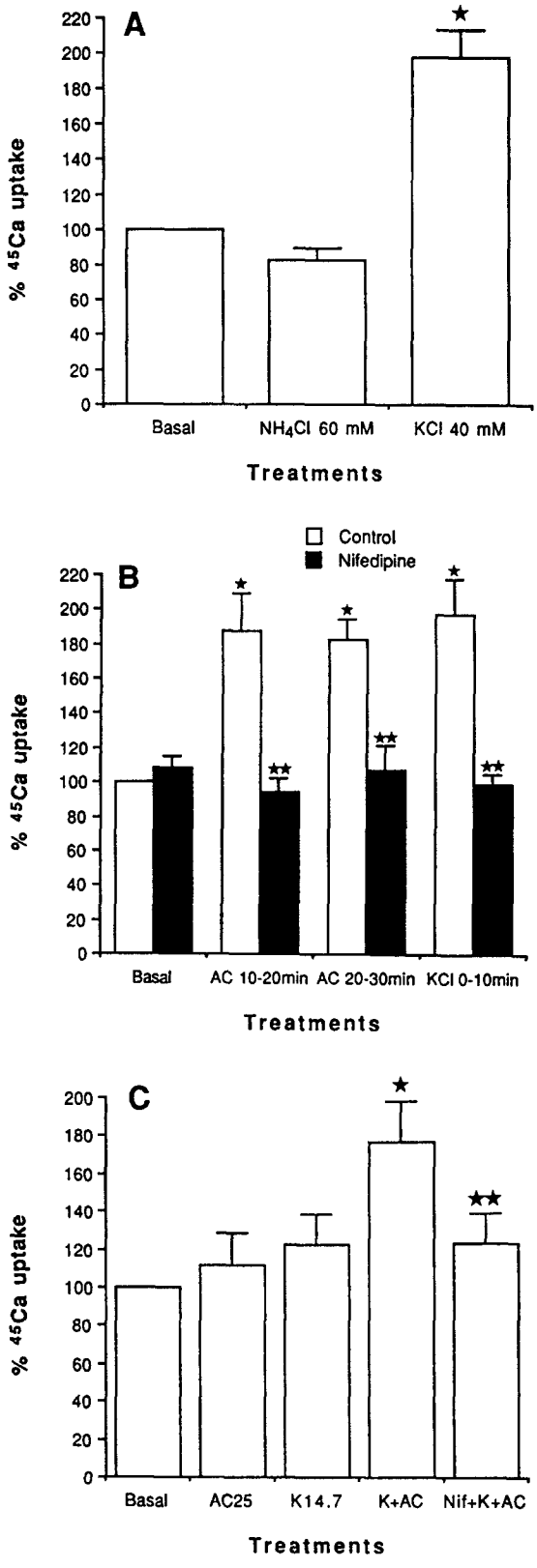

Fig. 4. $^{45}Ca^{2+}$ uptake in the initial (A) and late (B) phases after stimulation with NH_4Cl, and effects of increasing extracellular K^+ concentration to 14.7 mM on NH_4Cl (25 mM)-induced $^{45}Ca^{2+}$ uptake (C). (A,B) $^{45}Ca^{2+}$ uptake during initial 5 min (A) or $^{45}Ca^{2+}$ uptake during a later period of 10 min (B) was measured and is expressed as the percentage of basal uptake in the normal solution. For measurement of initial $^{45}Ca^{2+}$ uptake after NH_4Cl stimulation (A), 60 mM NH_4Cl and $^{45}Ca^{2+}$ were simultaneously administered. For measurement of $^{45}Ca^{2+}$ uptake in the late phase after NH_4Cl (AC) stimulation (B), vessels were incubated in the solution containing 60 mM NH_4Cl for 10 or 20 min, and then $^{45}Ca^{2+}$ was added. In the case of KCl stimulation (A,B), 40 mM KCl and $^{45}Ca^{2+}$ were simultaneously administered. In the case of nifedipine (1 μM) pretreatment (stippled columns), vessels were incubated with the dihydropyridine for 30 min and then stimulated with NH_4Cl or KCl. Mean basal $^{45}Ca^{2+}$ uptake was 224.1 ± 18.0 nmol/g/10 min. Basal, no stimulation; AC 10–20 min, from 10 to 20 min after stimulation with 60 mM NH_4Cl; AC 20–30 min, from 20 to 30 min after stimulation with 60 mM NH_4Cl; KCl 0–10 min, from 0 to 10 min after stimulation with 40 mM KCl. Asterisks denote statistically significant differences ($P < 0.01$) compared to the values of the basal level (*) and the nifedipine-untreated control (**) ($n = 7$). (C) Vessels were pretreated with nifedipine (1 μM) or vehicle (0.01% dimethylsulfoxide) for 30 min, and then preincubated in the normal or 14.7 mM KCl-containing solution further for 15 min. Subsequently, NH_4Cl (25 mM) was administered. At 10 min after the NH_4Cl administration, $^{45}Ca^{2+}$ was added and $^{45}Ca^{2+}$ influx into the vessels was measured during the following 10 min. Basal $^{45}Ca^{2+}$ uptake was measured after pretreatment with vehicle (0.01% dimethylsulfoxide) for 55 min. In case of only KCl (14.7 mM) stimulation (K14.7), $^{45}Ca^{2+}$ was added 10 min after the end of the preincubation period in high K^+ (14.7 mM) solution. Mean basal $^{45}Ca^{2+}$ uptake was 212.5 ± 23.9 nmol/g/10 min. AC25, uptake of the vehicle-pretreated vessels in the 25 mM NH_4Cl containing solution; K + AC, uptake of the vehicle-pretreated vessels in the 25 mM NH_4Cl- and 14.7 mM KCl-containing solution; Nif + K + AC, uptake of the nifedipine-pretreated vessels in the 25 mM NH_4Cl- and 14.7 mM KCl-containing solution. Asterisks denote statistically significant differences ($P < 0.05$) compared to the value of the basal $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ uptake in the solution containing NH_4Cl (25 mM) or KCl (14.7 mM) (*), and that in the solution containing 25 mM NH_4Cl and 14.7 mM KCl without nifedipine treatment (**) ($n = 7$).

$^{45}Ca^{2+}$ uptake experiments, was 1.8 ± 0.8% and 95.2 ± 2.0%, respectively, suggesting that this period after NH_4Cl stimulation corresponded to the lag time of NH_4Cl contraction.

Fig. 4B shows $^{45}Ca^{2+}$ uptake in later phases after NH_4Cl stimulation, i.e. from 10 min to 20 min and from 20 min to 30 min after the addition of 60 mM NH_4Cl. In these later phases, $^{45}Ca^{2+}$ uptake significantly increased compared to basal levels. The increase in $^{45}Ca^{2+}$ uptake induced by NH_4Cl was abolished by pretreatment with nifedipine (1 μM). $^{45}Ca^{2+}$ uptake measured within 10 min after stimulation with KCl (40 mM) increased about twice with respect to the basal level. This increase was abolished in the presence of 1 μM nifedipine (Fig. 4B). Mean maximum contractile tensions induced by NH_4Cl (within the period of 10–20 min and 20–30 min after the stimulation) and by KCl (within 10 min after the stimulation) observed under conditions corresponding to those of $^{45}Ca^{2+}$ uptake were 104.8 ± 2.0%, 105.8 ± 4.0% and 100%, respectively, and were also inhibited by pretreatment with 1 μM nifedipine (13.9 ± 2.1%, 14.6 ± 2.5% and 4.6 ± 1.7%, respectively).

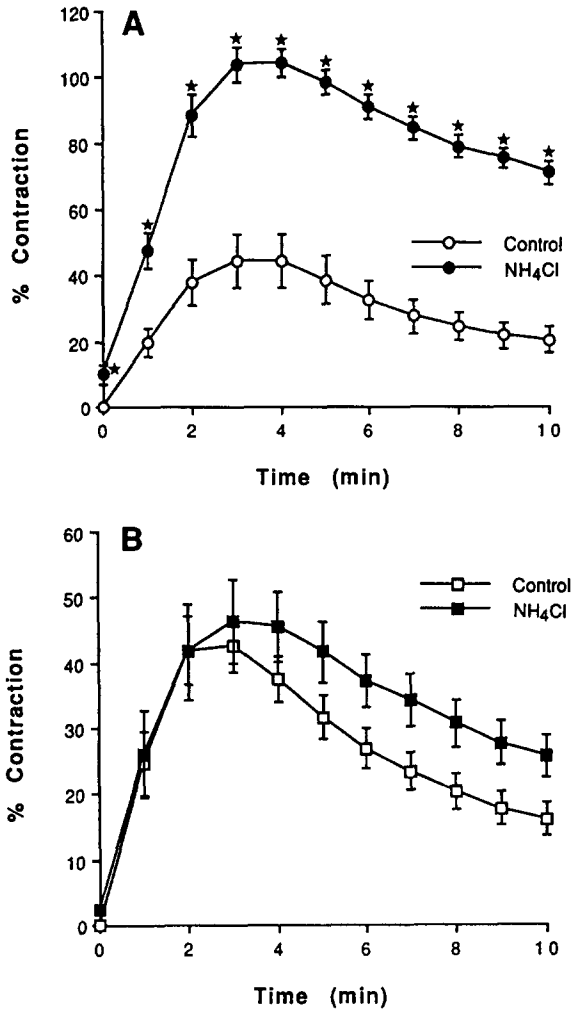

Fig. 5. Potentiation of histamine-induced contractile effects by NH_4Cl. (A) Time courses are shown for histamine (5 μM) effects in the absence (open circles) and presence (closed circles) of NH_4Cl (20 mM). (B) Time courses are shown for histamine (10 μM) effects in the absence (open squares) and presence (closed squares) of NH_4Cl after nifedipine pretreatment. Vessels were pretreated with vehicle (A) or 1 μM nifedipine (B) for 30 min, and subsequently incubated in normal or NH_4Cl (20 mM)-containing solution further for 15 min. Then, histamine at 5 μM (A) or 10 μM (B) was added to the organ bath. Statistical analyses were done after subtracting the contractile force induced by 20 mM NH_4Cl alone from the contractile force induced by histamine after NH_4Cl pretreatment. Asterisks denote statistically significant differences ($P < 0.05$) between the control and NH_4Cl-preincubated groups ($n = 7$).

3.4. Effects of 25 mM NH_4Cl on $^{45}Ca^{2+}$ uptake in porcine coronary artery

NH_4Cl (25 mM) or KCl (14.7 mM) by itself slightly increased the mean level of $^{45}Ca^{2+}$ uptake compared to basal uptake, however, these increases were not statistically significant. When 25 mM NH_4Cl and 14.7 mM KCl were combined, $^{45}Ca^{2+}$ uptake increased significantly compared to the basal level, and this increase was inhibited by pretreatment with 1 μM nifedipine (Fig. 4C).

3.5. Pretreatment of coronary artery with a low concentration (20 mM) of NH_4Cl augments histamine-induced contraction

Histamine (5 μM) induced a phasic contraction of pig coronary artery exhibiting a maximum of 46.6 ± 8.1% of the KCl (40 mM)-induced reference contraction (Fig. 5A). NH_4Cl (20 mM) by itself induced only a small tonic contractile response (Fig. 1A). The contractile effect of 5 μM histamine was dramatically potentiated in the presence of 20 mM NH_4Cl. Histamine (5 μM) induced a maximum contraction of 107.0 ± 4.8% in NH_4Cl-pretreated vessels. In another set of experiments we tested the sensitivity of this potentiating effect of NH_4Cl to nifedipine. In the presence of nifedipine, the histamine-induced contraction was suppressed, and thus we increased the histamine concentration to 10 μM in order to adjust contractile tension to the level observed in the absence of nifedipine. In the presence of nifedipine (1 μM), NH_4Cl (20 mM) failed to potentiate the contractile effect of 10 μM histamine (Fig. 5B). In nifedipine-pretreated vessels, there was no significant difference in the maximum contractile force elicited by 10 μM histamine in the absence and presence of NH_4Cl (20 mM) [44.9 ± 4.6% (control) vs. 48.1 ± 6.4% (NH_4Cl-pretreated)].

4. Discussion

Extracellular application of NH_4Cl is known to induce vasoconstriction, however, the mechanism(s) of the contraction has not been clarified. One candidate mechanism

is stimulation of voltage-dependent Ca^{2+} entry via L-type Ca^{2+} channels. A number of previous reports have demonstrated a dependence of the contractile effects of NH_4Cl on the presence of extracellular Ca^{2+}: in rat aorta, rat portal vein, canine pulmonary artery and also porcine coronary artery, the contractile responses to NH_4Cl were inhibited by removal of extracellular Ca^{2+} (Danthuluri and Deth, 1989; Krampetz and Rhoades, 1991; Wakabayashi et al., 1992; Nguyen-Duong, 1993). However, divergent results have been obtained regarding the sensitivity of NH_4Cl-induced vasoconstriction to classical blockers of voltage-sensitive Ca^{2+} channels. In a previous study, nifedipine failed to inhibit 20 mM NH_4Cl-induced contraction in rat aorta (Danthuluri and Deth, 1989), whereas in another study verapamil partially inhibited 40 mM NH_4Cl-induced contraction (Horie et al., 1995). In canine pulmonary artery, contractile force induced by high concentrations (60 mM, 120 mM) of NH_4Cl, but not that induced by low concentrations (30 mM or less), was inhibited by nifedipine (Krampetz and Rhoades, 1991). In rat portal vein, contractile responses to both low (20 mM) and high (60 mM) concentrations of NH_4Cl were abolished by nifedipine (Wakabayashi et al., 1992; Taggart et al., 1995). These discrepancies might be explained by the existence of multiple mechanisms of NH_4Cl-induced vasoconstriction, with Ca^{2+} channels being activated only at high concentrations of NH_4Cl in some tissues. Recently, Nagesetty and Paul (1994) reported that NH_4Cl (30 mM) induced a tonic contraction of porcine coronary artery without any increase in intracellular Ca^{2+} concentration as evidenced by fura-2 fluorescence. These authors speculated that the mechanisms underlying NH_4Cl-induced vasoconstriction are mainly related to changes in the Ca^{2+} sensitivity of the contractile apparatus in vascular smooth muscle. Thus, low concentrations of NH_4Cl might induce contraction without stimulation of transmembrane Ca^{2+} influx. However, this speculation is clearly contradicted by the findings of the present study demonstrating that nifedipine pretreatment or removal of extracellular Ca^{2+} clearly inhibit the contraction induced by a low concentration (25 mM) of NH_4Cl. Moreover, it has recently been reported that the Ca^{2+} sensitivity of the contractile apparatus in vascular smooth muscle is not potentiated by elevation of intracellular pH (Crichton et al., 1994). In the present study, contractile responses to both high and low concentrations of NH_4Cl were found to depend largely on the presence of extracellular Ca^{2+} and were markedly inhibited by pretreatment of the vessels with nifedipine. Consistently, removal of extracellular Ca^{2+} or addition of nifedipine, subsequent to the induction of contractile tone by NH_4Cl, resulted in a clear relaxation of coronary vessels. Thus, the results of the present study unequivocally demonstrate that NH_4Cl induces contraction in porcine coronary arteries via a nifedipine-sensitive Ca^{2+} entry pathway.

Vasoconstrictor effects involving nifedipine-sensitive Ca^{2+} channels are expected to be potentiated by any intervention which increases the activity of these ion channels. Indeed, we have observed potentiation of NH_4Cl-induced vasoconstriction by increasing extracellular K^+ or by adding the K^+ channel blocker tetraethylammonium chloride, both of which are known to induce membrane depolarization (Bolton, 1979; Haeusler and Thorens, 1980), or by adding the Ca^{2+} channel agonist BAY K8644 (Schramm et al., 1983a, b). Consistently, $^{45}Ca^{2+}$ uptake induced by 25 mM NH_4Cl was markedly potentiated by increasing extracellular K^+. Thus, NH_4Cl-induced contraction is clearly augmented by agents which directly or indirectly activate the voltage-dependent Ca^{2+} channel, suggesting that the mechanism of NH_4Cl-induced contraction is closely related to the function of the voltage-dependent Ca^{2+} channel. In the present study, we tested whether NH_4Cl could potentiate receptor-mediated contraction of coronary artery, which involves L-type Ca^{2+} channels. In porcine coronary artery, histamine causes contraction through both extracellular and intracellular Ca^{2+}-dependent pathways, and the former is sensitive to verapamil (Mori et al., 1990). Pretreatment of the vessels with a lower concentration of NH_4Cl (20 mM) significantly potentiated the contractile response to histamine in the absence of nifedipine, but not in its presence. This implies that NH_4Cl potentiates the component of histamine contraction related to Ca^{2+} influx through the nifedipine-sensitive Ca^{2+}-channel.

Our results obtained in functional experiments strongly support the view that NH_4Cl-induced vasoconstrictor effects are in large part due to Ca^{2+} entry. As a next step, we tested whether NH_4Cl-induced, nifedipine-sensitive Ca^{2+} entry can be demonstrated in $^{45}Ca^{2+}$ flux experiments. There are only a few previous reports describing the effects of NH_4Cl on transplasmalemmal Ca^{2+} influx in vascular smooth muscle. In rat aorta, 30 mM NH_4Cl did not affect $^{45}Ca^{2+}$ uptake whereas it elicited a clear extracellular Ca^{2+}-dependent contractile response (Danthuluri and Deth, 1989). Consistently, we found in the present study that 25 mM NH_4Cl did not significantly increase $^{45}Ca^{2+}$ uptake despite induction of a discrete increase in tension. Similarly, moderate depolarization of coronary vessels by 14.7 mM K^+, which is well known to increase vascular tone via activation of voltage-dependent Ca^{2+} channels (Bolton, 1979), also failed to produce detectable increases in $^{45}Ca^{2+}$ uptake. Thus, the negative result obtained with the low concentration of NH_4Cl does not exclude the involvement of Ca^{2+} entry but may rather be explained by the low sensitivity of the $^{45}Ca^{2+}$ uptake assay. However, a higher concentration of NH_4Cl (60 mM) produced a significant increase in $^{45}Ca^{2+}$ influx which was comparable to that obtained in high K^+ (40 mM) solution. To our knowledge, this is the first demonstration of NH_4Cl-induced stimulation of transplasmalemmal Ca^{2+} influx by NH_4Cl. Interestingly, there was a clear difference in the time required for detection of NH_4Cl and KCl-induced stimulation of $^{45}Ca^{2+}$ uptake. NH_4Cl-in-

duced stimulation of $^{45}Ca^{2+}$ uptake was statistically significant only after a time lag which was consistent with that observed in tension experiments. This initial phase, in which no $^{45}Ca^{2+}$ uptake was detected, corresponded to the time lag of the NH_4Cl-induced contraction. Both the increase in $^{45}Ca^{2+}$ uptake and contractile force induced by NH_4Cl were abolished by nifedipine. Thus, the results obtained with $^{45}Ca^{2+}$ flux experiments further confirm the idea that NH_4Cl contracts porcine coronary artery mainly by activation of dihydropyridine-sensitive Ca^{2+} channels.

NH_4Cl incubation is a popular maneuver to directly elevate intracellular pH (Thomas, 1974). Although intracellular pH was not measured in the present study, it is known that intracellular pH immediately rises to a peak value after NH_4Cl incubation and then gradually declines, as measured in vascular smooth muscle cells from various vessels including porcine coronary artery (Danthuluri and Deth, 1989; Siskind et al., 1989; Krampetz and Rhoades, 1991; Nagesetty and Paul, 1994). The relationship between changes in intracellular pH and contractile force is complicated. From previous reports, it is known that NH_4Cl immediately induces a transient relaxation when it is administered to precontracted vessels (Andersson et al., 1981; Furtado, 1987; Feletou et al., 1989; Nguyen-Duong, 1993). In line with these reports we observed that NH_4Cl induced an initial transient relaxation of vessels which were slightly precontracted by elevation of extracellular K^+ to 14.7 mM, or by addition of tetraethylammonium chloride or BAY K8644 (Fig. 3). Furthermore, there was a clear time lag of several minutes between addition of NH_4Cl and onset of contraction. Thus, the change in intracellular pH to alkaline itself may not be directly related to the contractile response. Dissociation of changes in intracellular pH and tension has been demonstrated recently in porcine carotid artery (Chen and Rembold, 1995). Recently, NH_4Cl has been reported to increase the availability of L-type Ca^{2+} channel activity in porcine coronary smooth muscle and this increase occurs almost immediately upon addition of NH_4Cl (Klöckner and Isenberg, 1994). However, in the present study, NH_4Cl-induced vasoconstriction and $^{45}Ca^{2+}$ uptake were detected only after a lag phase of several minutes. The reason for this time lag preceding the NH_4Cl effects is as yet unclear. The mechanism of such a slowly developing activation of smooth muscle L-type Ca^{2+} channels by NH_4Cl, as well as its possible relation to changes in intracellular pH, remains to be clarified.

In summary, NH_4Cl induces a tonic vasoconstriction mainly by facilitating transplasmalemmal Ca^{2+} influx through a nifedipine-sensitive Ca^{2+} entry pathway in porcine coronary artery. Further investigation is required to elucidate the relationship between NH_4Cl-induced changes in intracellular pH and the contractile response.

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References

Aalkjaer, C., 1990, Regulation of intracellular pH and its role in vascular smooth muscle function, J. Hypertens. 8, 197.

Andersson, K.E., L. Brandt, B. Hindfelt and B. Ljunggren, 1981, Cerebrovascular effects of ammonia in vitro, Acta Physiol. Scand. 113, 349.

Berk, B.C., M.S. Aronow, T.A. Brock, E. Cragoe, Jr., M.A. Gimbrone, Jr. and R.W. Alexander, 1987, Angiotensin II-stimulated Na^+/H^+ exchange in cultured vascular smooth muscle cells, J. Biol. Chem. 262, 5057.

Berk, B.C., M. Canessa, G. Vallega and R.W. Alexander, 1988, Agonist-mediated changes in intracellular pH: role in vascular smooth muscle cell function, J. Cardiovasc. Pharmacol. 12 (Suppl. 5), S104.

Berk, B.C., M.B. Taubman, K.K. Griendling, E.J. Cragoe Jr., J.W. Fenton II and T.A. Brock, 1991, Thrombin-stimulated events in cultured vascular smooth-muscle cells, Biochem. J. 274, 799.

Bolton, T.B., 1979, Mechanisms of action of transmitters and other substances on smooth muscle, Physiol. Rev. 59, 606.

Chen, X.L, and C.M. Rembold, 1995, pHi, $[Ca^{2+}]i$, and myosin phosphorylation in histamine- and NH_4^+-induced swine carotid artery contraction, Hypertension 25, 482.

Crichton, C.A., A.G.B. Templeton and G.L. Smith, 1994, Effect of altered bathing pH on calcium activated force in α toxin permeabilised rat portal vein and human umbilical artery, Cardiovasc. Res. 28, 1378.

Danthuluri, N.R. and R.C. Deth, 1989, Effects of intracellular alkalinization on resting and agonist-induced vascular tone, Am. J. Physiol. 256, H867.

Danthuluri, N.R., B.C. Berk, T.A. Brock, E.J. Cragoe, Jr. and R.C. Deth, 1987, Protein kinase C-mediated intracellular alkalinization in rat and rabbit aortic smooth muscle cells, Eur. J. Pharmacol. 141, 503.

Feletou, M., C.T. Harker, K. Komori, J.T. Shepherd and P.M. Vanhoutte, 1989, Ammonium ions cause relaxation of isolated canine arteries, J. Pharmacol. Exp. Ther. 251, 82.

Furtado, M.R., 1987, Effect of NH_4Cl on the contractility of isolated vascular smooth muscle, Life Sci. 41, 95.

Haeusler, G. and S. Thorens, 1980, Effects of tetraethylammonium chloride on contractile, membrane and cable properties of rabbit artery muscle, J. Physiol. (London) 303, 203.

Horie, S., S. Yano and K. Watanabe, 1995, Intracellular alkalinization by NH_4Cl increases cytosolic Ca^{2+} level and tension in the rat aortic smooth muscle, Life Sci. 21, 1835.

Kikeri, D., M.L. Zeidel, B.J. Ballermann, B.M. Brenner and S.C. Hebert, 1990, pH regulation and response to AVP in A10 cells differ markedly in the presence vs. absence of CO_2-HCO_3^-, Am. J. Physiol. 259, C471.

Klöckner, U. and G. Isenberg, 1994, Intracellular pH modulates the availability of vascular L-type Ca^{2+} channels, J. Gen. Physiol. 103, 647.

Koh, E., S. Morimoto, S. Kim, T. Nabata, Y. Miyashita and T. Ogihara, 1990, Endothelin stimulates Na^+/H^+ exchange in vascular smooth muscle cells, Biochem. Int. 20, 375.

Krampetz, I.K. and R.A. Rhoades, 1991, Intracellular pH: effect on pulmonary arterial smooth muscle, Am. J. Physiol. 260, L516.

Mori, T., T. Yanagisawa and N. Taira, 1990, Histamine increases vascular tone and intracellular calcium level using both intracellular and extracellular calcium in porcine coronary arteries, Jpn. J. Pharmacol. 52, 263.

Nagesetty, R. and R.J. Paul, 1994, Effects of pHi on isometric force and $[Ca^{2+}]i$ in porcine coronary artery smooth muscle, Circ. Res. 75, 990.

Nguyen-Duong, H., 1993, Effects of manipulations of cytoplasmic pH on the mechanical responses of isolated porcine coronary arteries, Arch. Int. Physiol. Biochem. Biophys. 101, 207.

Schramm, M., G. Thomas, R. Towart and G. Franckowiak, 1983a, Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels, Nature 303, 535.

Schramm, M., G. Thomas, R. Towart and G. Franckowiak, 1983b, Activation of calcium channels by novel 1,4-dihydropyridines, Arzneim-Forsch./Drug Res. 33, 1268.

Siskind, M.S., C.E. McCoy, A. Chobanian and J.H. Schwartz, 1989, Regulation of intracellular calcium by cell pH in vascular smooth muscle cells, Am. J. Physiol. 256, C234.

Taggart, M.J., C. Austin and S. Wray, 1995, Contribution of extracellular calcium to intracellular pH-induced changes in spontaneous force of rat portal vein, Exp. Physiol. 80, 69.

Thomas, R.C., 1974, Intracellular pH of snail neurones measured with a new pH-sensitive glass micro-electrode, J. Physiol. (London) 238, 159.

Wakabayashi, I., K. Hatake and K. Sakamoto, 1992, Ammonium ion increases the tone of rat portal vein, Gen. Pharmacol. 23, 1189.