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Acidosis-induced protein tyrosine phosphorylation depends on Ca^{2+} influx via voltage-dependent Ca^{2+} channels in SHR aorta

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Abstract

The contractile response to acidosis in isolated aorta from spontaneously hypertensive rat (SHR) depends upon tyrosine phosphorylation of phosphatidylinositol 3 kinase (PI3-kinase) and Ca^{2+} influx via voltage-dependent Ca^{2+} channels (VDCC). In this study, verapamil, a VDCC inhibitor, was shown to markedly inhibit acidic pH-induced contraction, whereas the residual contraction in the presence of verapamil was unaffected by the PI3-kinase inhibitor, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one hydrochloride (LY-294002). Interestingly, the LY-294002-insensitive component of contraction was further inhibited by verapamil in the presence of LY-294002. Western blotting revealed that acidosis stimulated tyrosine phosphorylation of p85, which was abolished when tissues were pretreated with tyrphostin 23, a tyrosine kinase inhibitor, verapamil or EGTA. In fura-2-loaded aortic strips, acidosis induced a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) that was partially inhibited by LY-294002. The residual increase in $[Ca^{2+}]_i$ caused by acidosis in the presence of LY-294002 was abolished by verapamil. These findings suggest that acidosis-induced Ca^{2+} influx through VDCC is the upstream event leading to the tyrosine phosphorylation of PI3-kinase, which in turn contributes to the enhancement of Ca^{2+} entry to some extent in SHR aorta.

Keywords: Acidosis; Tyrosine phosphorylation; Voltage-dependent calcium channel; Phosphatidylinositol 3 kinase; Spontaneously hypertensive, rat; Aorta

1. Introduction

Tyrosine phosphorylation of proteins is widely recognized for its role in numerous cellular processes, such as cell growth, differentiation or proliferation (Ullrich and Schelessinger, 1990). It is now appreciated that this pathway also contributes to the contractility of a variety of smooth muscle preparations from vascular tissue (Hollenberg, 1994; Zheng et al., 1998; Rohra et al., 2002b; Suenaga and Kamata, 2002). Tyrosine kinase inhibitors cause an inhibition of contraction (Rohra et al., 2002b; Sun et al., 1999). However, the mechanism by which tyrosine phosphorylation is translated into contraction in vascular smooth muscle is not completely resolved. Since tyrosine kinase inhibitors have been reported to decrease Ca^{2+} channel currents, a tyrosine kinase-dependent pathway is thought to enhance Ca^{2+} influx through L-type Ca^{2+} channels leading to the contraction (Wijetunge and Hughes, 1996; Carter and Kanagy, 2002; Che and Carmines, 2002). In a few studies, the contractile response resulting from agonist-induced tyrosine phosphorylation was reported to involve Ca^{2+}-sensitization of the contractile proteins in vascular smooth muscle (Martínez et al., 2000; Masui and Wakabayashi, 2000).

The tone of vascular smooth muscle can be modified by several factors, including pH (Chen and Rembold, 1995; Rohra et al., 2003a, 2004), and various clinical situations

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like ischemia and metabolic disorders may cause extracellular as well as intracellular acidosis (Komukai et al., 1998). Acidosis exerts unique contractile effects on the isolated aortas from SHR and WKY rats (Komukai et al., 1998; Rohra et al., 2002a). The present study is the continuation of our previous reports studying the mechanisms underlying the unique constrictor effects of acidosis on the isolated aortas from SHR and WKY strains only (Rohra et al., 2002a,b, 2003a,c). It is worth noting that although exaggerated in SHR aorta, in principle, the mechanisms underlying the acidic pH-induced contraction are essentially similar in both the strains. For example, acidosis causes activation of Cl^- channels (Rohra et al., 2002a), and stimulation of protein tyrosine phosphorylation (Rohra et al., 2002b), leading to contraction in both SHR and WKY aortas. The difference in the magnitude of contraction between SHR and WKY aortas is due to the difference in the steady state of intracellular acidosis attained following extracellular acidosis (Rohra et al., 2003c). Considering that the difference between the two strains regarding the difference in sensitivity towards acidosis is already known, the present study was conducted on the SHR only. We have recently reported that a major mechanism underlying the acidic pH-induced contraction in isolated aorta from spontaneously hypertensive rats (SHR) is the tyrosine phosphorylation of phosphatidylinositol 3 kinase (PI3-kinase; Rohra et al., 2002b). In another study, we have also shown that the acidosis-induced Ca^{2+} mobilization in SHR and Wistar-Kyoto (WKY) rat aortas is due to Ca^{2+} influx via voltage-dependent L-type Ca^{2+} channels (Rohra et al., 2002a,b). The relationship between the protein tyrosine phosphorylation and the Ca^{2+} influx via voltage-dependent Ca^{2+} channels (VDCC), both taking place at acidic pH conditions, is unclear. The goal of the present study was to investigate the relationship between two major mechanisms underlying the acidic pH-induced contraction in SHR aorta i.e. the protein tyrosine phosphorylation and Ca^{2+} influx via VDCC.

2. Materials and methods

2.1. Animals

The procedures used in this study were approved by the Committee on the Care and Use of Experimental Animals, Tohoku University and were in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health. Experiments were performed on thoracic aortas from 12- to 13-week-old male SHR. Before the sacrifice, the systolic blood pressure of SHR was determined by tail cuff method in order to make sure that at the age of 12–13 weeks, SHR have actually become hypertensive. The mean systolic blood pressure calculated from three consecutive steady readings was found to be 180±7 mm Hg.

2.2. Tissue preparation

The animals were decapitated and exsanguinated. Open rings (cut longitudinally to convert them into strips) of approximately 0.3 cm width were made from the aorta. The endothelium was removed by gently rubbing the endothelial surface with cotton pellets. The lack of endothelium was confirmed by observing the failure of carbachol (1 μM) to cause relaxation of the phenylephrine (1 μM)-induced contraction.

2.3. Measurement of isometric contraction

The aortic strips were suspended vertically in organ baths, containing aerated (95% O_2/5% CO_2) HEPES-buffered physiological salt solution of the following composition (in mM): NaCl 120, KCl 4.8, $MgSO_4$ 1.3, $CaCl_2$ 1.2, $NaHCO_3$ 25.2, glucose 5.8, KH_2PO_4 1.2 and HEPES 20. The tissues were adjusted to a preloaded resting tension of 1 g and equilibrated for at least 1 h. Isometric contraction was measured by a force-displacement transducer (Nihon Kohden, Tokyo, Japan). The aorta was contracted three times by 64.8 mM KCl before starting the actual experiments. All experiments were carried out at 37 °C. The pH of the solution was changed from the control value of 7.4 by addition of HCl and the tissue was exposed to the acidic pH by exchanging the bathing solution.

2.4. Simultaneous measurement of intracellular Ca^{2+} and contraction

The level of intracellular Ca^{2+} $[Ca^{2+}]_i$ was monitored by using fura-2 as a fluorescent Ca^{2+} indicator. Aortic strips were loaded with 5 μM fura-2 acetoxymethyl ester overnight (12–15 h) at 4 °C in physiological salt solution containing a non-cytotoxic detergent cremphor EL (0.05%) and mounted horizontally in a bath attached to a fluorimeter (CAF 100, Japan Spectroscopic, Tokyo, Japan), and a force displacement transducer. Viability of aorta was tested by inducing contraction with 64.8 mM KCl twice. Contraction and fura-2 fluorescence were measured simultaneously. The intensity of fluorescence at 500 nm induced by excitation at 340 and 380 nm was monitored. The ratio of these two fluorescence values (R340/380) was calculated as an indicator of the relative $[Ca^{2+}]_i$ level. The absolute $[Ca^{2+}]_i$ was not calculated because the dissociation constant of fura-2 and Ca^{2+} in cytosol may be different from that obtained in vitro (Mitsui and Karaki, 1990). Since fura-2 leaks out from the rat aortic strips during the experiment, strict care was taken to finish the experiment before the mirror image response of 340 and 380 fluorescence signals was lost. It is likely that acidic pH might interfere with the fura-2 signal by affecting the affinity of fura-2 with Ca^{2+}. However, in this study, we were only concerned with the relative $[Ca^{2+}]_i$ level at acidic pH in the presence or absence of inhibitors. Since acidosis was present throughout the experimental

period and the data were generated by using different inhibitors on the same tissue in the continuous presence of acidosis, we assume that the confounding effect of acidic pH on the fura-2 signal (if any) was not contributing to the misinterpretation of the results.

2.5. Tyrosine phosphorylation assay

Analyses of the expression of tyrosine phosphorylated proteins in SHR aorta were conducted by Western blotting. Aortic homogenates were prepared as described in detail in our previous paper (Rohra et al., 2002b). Fifty micrograms of proteins from each tissue homogenate were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% gel) and transferred to polyvinylidene difluoride membrane. Identical loading of protein content in each lane was confirmed by the appearance of similar density band of myosin heavy chain. Blotted membrane was blocked in 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 for 2 h at room temperature. The membrane was incubated with the anti-phosphotyrosine antibody (Zymed Laboratories, CA, USA), in a dilution of 1:1000 overnight at 4 °C. After washing, the membrane was reacted with anti-rabbit IgG antibody linked to horseradish peroxidase (HRP; Cell Signaling, USA), in a dilution of 1:2000 for 1 h at room temperature. Following incubation with enhanced chemiluminescence reagent (Pierce, IL, USA), immunoreactive proteins were visualized by exposing the blots to Hyper-film ECL.

2.6. Materials

SHR were purchased from Charles River (Kanagawa, Japan). Tyrphostin 23, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one hydrochloride (LY-294002), and verapamil were from Sigma (St. Louis, MO, USA). Fura-2 acetoxymethyl ester and cremphor EL were obtained from Dojindo (Kumamoto, Japan) and Nakarai Tesque (Kyoto, Japan), respectively. All other reagents used in the study were of analytical grade.

2.7. Analysis of results

All values are expressed as means±S.E.M. *n* represents the number of experiments as indicated in figure legends. Statistical significance was evaluated using Dunnett's multiple comparison tests. The results were considered significant at $P<0.05$. The density of the signals corresponding to tyrosine-phosphorylated proteins was quantitatively analysed by densitometry (Advanced American Biotechnology, Fullerton, CA, USA). Tyrosine phosphorylation of proteins was expressed as the fold of control.

3. Results

3.1. Characterization of the acidic pH-induced contraction

Decreasing extracellular pH from 7.4 to 6.5 by simply exchanging the bathing solution produced a slowly developing, marked and sustained contraction of SHR aorta, as shown in Fig. 1A. The magnitude of contraction was 121.2±5.1% (*n*=8 from 4 different animals) of the 64.8 mM KCl-induced contraction. The effect of acidosis was reversible since the tone returned to resting conditions, when pH of the bathing solution was retrieved to 7.4. The acidic pH-induced contraction was reproducible after washout (data not shown).

Verapamil, a VDCC inhibitor at a concentration of 1 μM markedly inhibited the acidic pH-induced contraction (Fig. 1B). This concentration of verapamil was able to abolish the 64.8 mM KCl-induced contraction (data not shown). SHR aorta was treated with verapamil 15 min before the change of pH from 7.4 to 6.5. When the contraction in the presence of verapamil attained a steady level, 10 μM LY-294002, a PI3-kinase inhibitor was added to the bathing solution. This agent did not produce any significant inhibition of the residual contraction in the presence of verapamil, as shown in Fig. 1B. In reciprocal experiments, SHR aorta was pretreated with 10 μM LY-294002 and the pH was changed to 6.5 in the presence of this inhibitor. As shown in Fig. 1B,

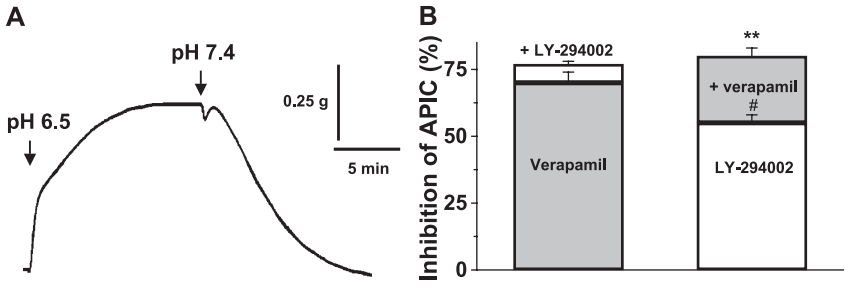

Fig. 1. Effects of inhibitors of voltage-dependent Ca^{2+} channels (VDCC), and phosphatidylinositol 3 kinase (PI3-kinase) on the acidic pH-induced contraction in SHR aorta. (A) Representative recording showing the effect of acidic pH on SHR aorta. The pH of the bathing solution was changed from 7.4 to 6.5. (B) Aorta was pretreated with either 1 μM verapamil (a VDCC inhibitor) or 10 μM LY-294002 (a PI3-kinase inhibitor). When the contraction reached a steady level in the presence of verapamil, LY-294002 was added to the medium. Reciprocally, when the contraction reached a steady level in the presence of LY-294002, verapamil was added to the bathing solution. $^{**}P<0.01$ vs. contraction in the presence of LY-294002 alone; $^{\#}P<0.05$ vs. contraction in the presence of verapamil alone. *n*=6−7 in each group from three to four different animals.

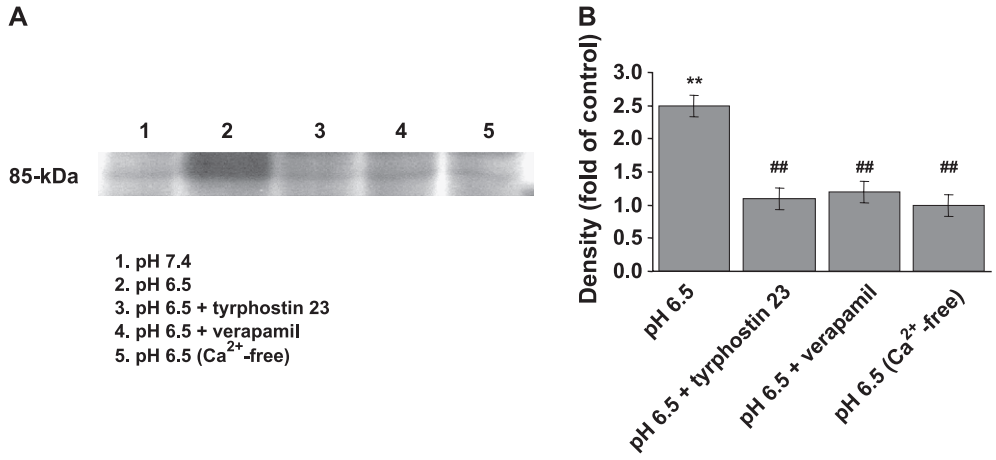

Fig. 2. Effects of acidic pH on protein tyrosine phosphorylation in aortic tissue from SHR in the absence or presence of inhibitors. Samples were prepared as described under Materials and methods and resolved by SDS-PAGE. Immunoblotting was performed with anti-phosphotyrosine antibody and immunoreactive proteins were visualized by enhanced chemiluminescence. (A) Representative immunoblot showing the acidic pH-induced tyrosine phosphorylation in the absence or presence of 20 μM tyrphostin 23 (a tyrosine kinase inhibitor), 1 μM verapamil (a voltage-dependent Ca^{2+} channel inhibitor) and under the Ca^{2+}-free conditions. (B) Quantitative analysis of the density of the bands corresponding to the tyrosine phosphorylated 85-kDa protein in aortic tissue from SHR. Data is expressed as-fold of control at pH 7.4. $^{**}P<0.01$ versus pH 7.4 (at resting conditions); $^{\#\#}P<0.01$ versus pH 6.5 in the absence of any drug. n=3 independent experiments on tissues from different animals.

LY-294002 also markedly inhibited the acidic pH-induced contraction; nevertheless, the extent of inhibition of acidic pH-induced contraction by LY-294002 was significantly less than that caused by verapamil ($P<0.05$). At the steady level of acidic pH-induced contraction in the presence of LY-294002, 1 μM verapamil was added to the bathing medium. Interestingly, verapamil was able to inhibit further the residual contraction in the presence of LY-294002 (Fig. 1B). LY-294002 was found to be highly selective at the concentration used in the present study since it did not affect the contraction induced by high KCl solution (data not shown).

3.2. Acidic pH-induced protein tyrosine phosphorylation

Consistent with our previous report (Rohra et al., 2002b), Western blotting of tissue extracts prepared from SHR aorta showed that acidic pH (pH 6.5) stimulated the tyrosine phosphorylation of 85-kDa protein (Fig. 2A), which has been earlier identified as the regulatory subunit of PI3-kinase (Rohra et al., 2002b). As shown in Fig. 2A and B, the increase in tyrosine phosphorylation of p85 induced by acidosis was completely prevented by pretreatment with 20 μM tyrphostin 23 and 1 μM verapamil or when the pH was decreased in Ca^{2+}-free solution. Ca^{2+}-free solution was made by omitting $CaCl_2$ from the physiological salt solution and the residual Ca^{2+} was chelated with the addition of 0.5 mM EGTA.

3.3. Acidic pH-induced Ca^{2+} mobilization

In the fura-2-loaded SHR aorta, pH 6.5 induced a sustained increase in $[Ca^{2+}]_i$ followed by a slowly developing contraction (Fig. 3A). When the rise in $[Ca^{2+}]_i$ reached a

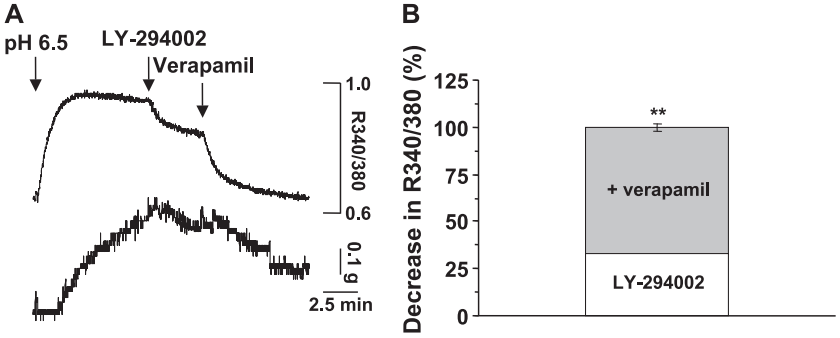

Fig. 3. Effects of LY-294002 and verapamil on acidic pH-induced Ca^{2+} mobilization and contraction in aortic strips from SHR. Aortic strips from SHR were loaded with fura-2 acetoxymethyl ester, and then cytosolic $[Ca^{2+}]_i$ (upper trace in A) and contraction (lower trace in A) were measured simultaneously. Strips were contracted by changing the pH from 7.4 to 6.5. When the $[Ca^{2+}]_i$ attained a steady level, 10 μM LY-294002 was added to the bathing solution, and when the inhibitory effect of LY-294002 reached a maximum level, 1 μM verapamil was added. (B) Quantification of the inhibitory effects of LY-294002 and verapamil on acidosis-induced increase in $[Ca^{2+}]_i$. '0%' and '100%' correspond to resting level (at pH 7.4) and the peak level of $[Ca^{2+}]_i$ at pH 6.5, respectively. $^{**}P<0.01$ between LY-294002 alone and combination of LY-294002 and verapamil. n=4 independent experiments from three different animals.

plateau, 10 μM LY-294002 was added to the bathing solution. Treatment with LY-294002 resulted in a partial reversal of $[Ca^{2+}]_i$ level (Fig. 3A). As far as the relaxant effect of LY-294002 on the acidic pH-induced contraction is concerned, it was very slow. Moreover, at the time of administration of LY-294002, the acidic pH-induced contraction being a slowly developing contraction was still in the process of rising. Together, it was difficult to examine the inhibitory effect of LY-294002 on the acidic pH-induced contraction, quantitatively, by this protocol. The reason why we could not wait for the acidic pH-induced contraction to attain maximum level or LY-294002 to produce maximum inhibition is due to the fact that using fura-2 in intact aortic strips has certain limitations. Among them the most important one is the time available for the experiments because fura-2 rapidly leaks from the rat aorta (Mitsui et al., 1993). However, the main purpose of these experiments was to observe the effect of PI3-kinase inhibitor on $[Ca^{2+}]_i$. The residual increase in $[Ca^{2+}]_i$ induced by acidosis in the presence of LY-294002 was abolished when the aorta was treated with 1 μM verapamil (Fig. 3A). Fig. 3B represents the effects of LY-294002 on acidosis-induced increase in $[Ca^{2+}]_i$, alone as well as in combination with verapamil.

4. Discussion

Phosphorylation of a tyrosine residue of many vascular smooth muscle cellular proteins may participate in contractile responses (Hollenberg, 1994; Ohanian et al., 1997). Like agonists, acidosis also stimulates tyrosine phosphorylation of proteins. The major substrate protein to be phosphorylated was identified as the PI3-kinase (Rohra et al., 2002b), therefore the present study deals primarily with the role of PI3-kinase under the acidic pH conditions. Both a PI3-kinase inhibitor, LY-294002 and a VDCC inhibitor, verapamil, markedly inhibited the acidic pH-induced contraction albeit verapamil was more efficacious. A very important observation obtained from the present pharmacological experiments was that whereas LY-294002 could not affect the verapamil-insensitive component of the acidic pH-induced contraction, verapamil did inhibit further the residual contraction in the presence of PI3-kinase inhibitor. One may argue that the two inhibitors used in the concentrations shown in the present study may have a submaximal inhibitory effect. Furthermore, verapamil may not show an additive effect to LY-294002 if the later would have been used in a higher concentration. However, we have shown conclusively in our previous study that the maximum inhibitory effect of LY-294002 on the acidic pH-induced contraction in SHR aorta is observed at the concentration of 5 μM and, and increasing the concentration further does not increase its inhibitory efficacy (Rohra et al., 2002b). Nevertheless, a concentration of 10 μM was used in the present study, which rules out the possibility of LY-294002 exerting a submaximal inhibitory effect in the present protocol. From these results, two conclusions can be drawn. First, the PI3-kinase-dependent component of the acidic pH-induced contraction shares a similar pathway with the VDCC-dependent component. Second, PI3-kinase-dependent contraction is a part of the Ca^{2+} influx-dependent contraction. These conclusions were further supported by the results of the Western blotting experiments. The main finding from these experiments was that acidosis-induced tyrosine phosphorylation of p85 was equally inhibited by a tyrosine kinase inhibitor, tyrphostin 23 and verapamil. In this study, we also evaluated the effect of extracellular Ca^{2+}-free conditions on the tyrosine phosphorylation profile under acidosis. Acidic pH failed to induce tyrosine phosphorylation of p85 in Ca^{2+}-free solution. Together, from the results of tension recording and Western blotting experiments, it is plausible to interpret that acidosis-induced tyrosine phosphorylation of p85 is dependent upon the Ca^{2+} entry which occurs through VDCC.

Having observed the Ca^{2+}-dependence of tyrosine phosphorylation of PI3-kinase stimulated by acidosis, the next question to be addressed was to evaluate the effect of the tyrosine phosphorylated PI3-kinase on the Ca^{2+} mobilization induced by acidosis. The acidosis-induced rise in $[Ca^{2+}]_i$ and contraction was partially inhibited by PI3-kinase inhibitor, LY-294002. Interestingly, further addition of verapamil brought the $[Ca^{2+}]_i$ level to basal level. One may argue that the inhibitory effects on $[Ca^{2+}]_i$ mobilization exhibited by LY-294002 and verapamil might be on the different Ca^{2+} entry pathways. However, we have noted that almost all the detectable rise in $[Ca^{2+}]_i$ caused by acidosis is susceptible to the inhibitory effect of verapamil (Furukawa et al., 1996). From these observations, it is reasonable to conclude that although Ca^{2+} entry via VDCC is an upstream event of the stimulation of tyrosine kinases and hence the activation of PI3-kinase, tyrosine phosphorylation of PI3-kinase acts in a positive feed back fashion to stimulate the Ca^{2+} entry. Stimulation of Ca^{2+} entry via VDCC caused by agonists also has been shown to be mediated by tyrosine kinase-regulated PI3-kinase in vascular smooth muscle cells by other researchers (Touyz and Schiffrin, 2000; Macrez et al., 2001; Quignard et al., 2001). From all these observations, it can be concluded that the stimulation for Ca^{2+} influx under the acidic pH conditions is dual; mainly as a result of depolarization elicited by the activation of Cl^- channels (Rohra et al., 2002a), and partially due to activation of PI3-kinase. Furthermore, a rise in $[Ca^{2+}]_i$ through VDCC caused by acidosis, in addition to the PI3-kinase-dependent component also mediates the PI3-kinase-independent but verapamil-sensitive component of the acidic pH-induced contraction.

The finding of Ca^{2+}-dependent tyrosine phosphorylation of PI3-kinase enhancing Ca^{2+} influx in a positive feed back manner, further presents a question that whether this is the sole mechanism of contraction underlying the PI3-kinase-dependent component of the acidic pH-induced contraction? As shown in Figs. 1B and 3B, there seems to be a little

discrepancy between the extent of inhibition of Ca^{2+} entry (approximately 30%) and the contraction (approximately 55%) caused by LY-294002. This discrepancy suggests the possibility that PI3-kinase pathway utilized by acidosis may involve some other mechanism(s) of contraction in addition to enhancement of Ca^{2+} influx. It is acknowledged however that this suggestion is based on the assumption that there is a linear relationship between the fura-2 signal and $[Ca^{2+}]_i$, and between force and $[Ca^{2+}]_i$. Neither of which will necessarily be true. As mentioned in Materials and methods, we cannot rule out the possibility that the relationship between both the fura-2 and $[Ca^{2+}]_i$, and between force and $[Ca^{2+}]_i$ was confounded by the acidosis. Nevertheless, the contractile response resulting from the agonists-induced tyrosine phosphorylation has been reported to involve Ca^{2+}-sensitization of contractile proteins in vascular smooth muscle (Martínez et al., 2000; Masui and Wakabayashi, 2000; Ohanian et al., 1997; Sasaki et al., 1998). Furthermore, PI3-kinase specifically has been shown to mediate contraction through a variety of signaling pathways. For example, PI3-kinase acting via its substrate Akt causes cyclic nucleotide-dependent contraction (Komalavilas et al., 2001). Recently, PI3-kinase has been also shown to be involved in endothelin-1-induced contraction by activation of the Rho kinase pathway (Miao et al., 2002). One more recent study has described an enhanced role of PI3-kinase in contraction in hypertensive rats (Northcott et al., 2002). From all these observations, it is speculated that PI3-kinase, in addition to the stimulation of Ca^{2+} influx, causes the contraction induced by acidosis by some other as yet unidentified mechanism(s).

In conclusion, the present study provides evidence for the first time that acidosis-induced tyrosine phosphorylation of PI3-kinase is dependent upon an increase in $[Ca^{2+}]_i$ due to Ca^{2+} influx through VDCC. The opening of VDCC is brought about mainly by depolarization caused by Cl^- channels (Rohra et al., 2002a) and partially by PI3-kinase in a positive feed back manner. Furthermore, the PI3-kinase-dependent component of the acidic pH-induced contraction seems to involve some other mechanism(s) in addition to Ca^{2+} influx via VDCC.

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