Stimulated Tyrosine Phosphorylation of Phosphatidylinositol 3-Kinase Causes Acidic pH-Induced Contraction in Spontaneously Hypertensive Rat Aorta

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ABSTRACT

Acidic pH induced a contraction (APIC) in isolated aortas from spontaneously hypertensive (SHR) and Wistar Kyoto rats, but failed to produce any response in age-matched Wistar rat aorta. This study was conducted to test the hypothesis that tyrosine phosphorylation of proteins is a molecular mechanism underlying the APIC. Tyrosine kinase inhibitors, genistein and tyrphostin 23 inhibited the APIC in a concentration-dependent manner. APIC was inhibited by phosphatidylinositol 3-kinase (PI3-kinase) inhibitors, LY-294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride] and wortmannin. Consistent with the results from tension measurement experiments, Western blot analysis showed that acidic pH induced an appreciable increment of tyrosine phosphorylation of 85-kDa protein (p85) in SHR aorta, which was completely inhibited by tyrphostin 23, whereas in Wistar rat aorta, the protein tyrosine phosphorylation was not observed. Further investigations using immunoprecipitation followed by Western blotting confirmed an increase in the tyrosine phosphorylation of p85. Analysis by SDS-polyacrylamide gel electrophoresis followed by silver staining of the gel revealed that amounts of multiple proteins with molecular sizes of 120, 130, 210, and 225 kDa were increased at acidic pH, which were immunoprecipitated with anti-phosphotyrosine antibody. Western blotting using a specific anti-PI3-kinase antibody identified the p85 as the regulatory subunit of PI3-kinase, whereas 120-, 130-, and 225-kDa proteins were identified by mass spectrometry as pro-α2 (I) collagen, collagen α1 (I) chain, and fibronectin I, respectively. As assayed by Western blotting using anti-myosin light chain (MLC) antibody, acidic pH induced a stimulation of MLC phosphorylation, and the stimulated MLC phosphorylation was abolished by tyrphostin 23 and LY-294002. These results suggest that acidic pH induces an increase in tyrosine phosphorylation of PI3-kinase, resulting in the MLC phosphorylation-dependent contraction of SHR aorta.

Multiple signaling pathways have been shown to be involved in the mechanism of vasoconstriction (Somlyo and Somlyo, 1998). Ca2+ is a major determinant of contractile force in all types of vascular smooth muscle (VSM). Apart from that, there is accumulating evidence for additional regulatory mechanism(s) in smooth muscle contraction (for review, see Horowitz et al., 1996). Tyrosine kinase-mediated pathways have also been shown to cause agonist-stimulated VSM contractile processes (Khalil et al., 1995; Banes et al., 1999). Consistently, tyrosine kinase inhibitors have been shown to attenuate vasoconstriction caused by a number of agents, including noradrenaline and angiotensin II in intact arteries (Di Salvo et al., 1993, 1994; Laniyonu et al., 1994; Carmines et al., 2001). Recent studies have shown that the voltage-dependent Ca2+ channels (VDCCs), activated by many vasoactive agents, are inhibited by tyrosine kinase inhibitors via tyrosine kinases (Wijetunge et al., 1992; Wijetunge et al., 1998; Lagaud et al., 1999). In addition to the effects on Ca2+ channels, tyrosine kinases may regulate...
other mechanisms controlling the contractile state of smooth muscle cells. For example, mitogen-activated protein kinase, which itself is partly activated by tyrosine phosphorylation, has been suggested to modulate the activity of the actin- and myosin-binding protein caldesmon (Adam et al., 1995).

The contractile state of the VSM can be modified by several factors, including pH (Chen and Rembold, 1995). Ischemia and metabolic disorders, such as diabetes mellitus, cause various disturbances, including hypoxia and acidosis in the circulatory system (Levine, 1993; Leach et al., 1998; Komukai et al., 1998). During ischemia or hypoxia, alterations in pH may play a significant role in the changes in vessel wall function (Nagesetty and Paul, 1994; Nishiyama et al., 1999). Although physiological pH of the body fluids is maintained at around 7.4, ischemia easily decreases the pH value significantly (Butwell et al., 1993). We have described previously that acidic pH induces a contraction (APIC) in spontaneously hypertensive (SHR) and Wistar Kyoto (WKY) rat aortas (Fu-rukawa et al., 1996); however, the molecular mechanism underlying the APIC is not clear. We have also observed that acidic pH fails to produce any contractile response in Wistar rat aorta. This study was conducted to test the hypothesis that tyrosine phosphorylation of proteins may be a difference between SHR and Wistar aortas in the sensitivity toward acidic pH. In this article, we present the first direct evidence that acidic pH stimulates the tyrosine phosphorylation of phosphatidylinositol 3-kinase (PI3-kinase) in isolated aorta from SHR but not in that from Wistar rat, and this enhanced tyrosine phosphorylation is the major mechanism underlying the contractile response to acidic pH.

### Materials and Methods

**Measurement of Isometric Tension.** Male SHR and WKY, both NCrj strain, and Wistar rats (all strains 13–14 weeks old) were stunned and killed by cervical dislocation. Thoracic aorta was re-moved and placed in ice-cold physiological salt solution (PSS) of the following composition: 120 mM NaCl, 4.8 mM KCl, 1.5 mM MgSO4, 1.2 mM CaCl2, 25.5 mM NaHCO3, 5.8 mM glucose, 1.2 mM KH2PO4, and 20 mM HEPES. Aorta was dissected into helical strips (2.0 × 1.7–1.9 cm). The endothelial cell layer was removed by rubbing the luminal side of the vessel with a moistened cotton swab. Tissues were placed in organ baths filled with warmed (37 °C) and aerated (95% O2, 5% CO2) PSS. pH of the bathing solution was strictly maintained at 7.4. One end of the preparation was attached to a force transducer (Nihon Kohden, Tokyo, Japan). The tissues were then placed under resting tension of 10 mN. Changes in isometric force were recorded on a polygraph. After equilibration for 1 h, the lack of the endothelium was checked by observing the failure of 1 μM carbachol to induce relaxation in tissues contracted with 1 μM phenylephrine. After washing multiple times, the tissues were equilibrated by contracting with 64.8 mM KCl three times and the last contractile response was taken as a standard and later all the contractions were normalized with it. Acidic solutions were prepared by the addition of HCl to PSS and pH of the bathing solution was changed from 7.4 to desired pH by simply replacing the bathing PSS. For experiments in which inhibitors of tyrosine kinase and PI3-kinase were used, tissues were incubated with these compounds for 20 min before the induction of contraction with acidic pH or other agonists. In control, 0.1% dimethyl sulfoxide was used instead. When using different concentrations of the drugs, separate tissue was used for each concentra-

**Tyrosine Phosphorylation Assay.** Analyses of the expression of tyrosine-phosphorylated proteins in SHR and Wistar rat aorta were conducted by Western blotting as follows. Helical strips (0.3 × 2.5 cm) of aorta with wet weight of approximately 12 mg were treated according to the same procedure as for tension recording experiments. Thereafter, the aortic strips were immediately frozen in liquid nitrogen and kept at −80 °C until subsequently used.

Segments of aorta were homogenized in 10 mM HEPES buffer, pH 7.5, containing 1 mM EDTA, protease inhibitors (leupeptin, antipain, pepstatin A, chymostatin, and phosphoramidon (all 10 μg/ml−1), and 250 μM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (0.32 μM okadaic acid, 10 nM calyculin A, 10 mM sodium fluoride, 50 μM bisperoxy(1,10-phenanthroline)oxovanadate, and 1 mM sodium orthovanadate) at 4 °C using a glass–glass tissue homogenizer. Then 10% SDS was added in the homogenate (final concentration of SDS was 1%), mixed, and boiled for 5 min. After centrifugation (18,000g) for 30 min, the supernatant was used as a tissue extract. Protein content was determined by BCA protein analysis kit (Pierce Chemical, Rockford, IL) using bovine serum albumin (BSA) as a standard. Proteins (50 μg) in each sample were separated by SDS-PAGE (7.5% gel) and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Blotted membrane was blocked in 5% BSA 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, South San Francisco, CA), 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 Technology Inc., Beverly, MA) in a dilution of 1:2000 for 1 h at room temperature. After incubation with enhanced chemiluminescence reagent (Pierce Chemical, Rockford, IL) immunoreactive proteins were visualized by exposing the blots to Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK).

**Myosin Light Chain (MLC) Phosphorylation Assay.** MLC phosphorylation assay using glycerol-PAGE followed by Western blotting was performed as follows. The frozen tissues were treated with dry ice-cold acetone containing 10% trichloroacetic acid and 10 mM dithiothreitol (DTT). Trichloroacetic acid was washed out with acetone-10 mM DTT. The tissues were mixed with urea-sample buffer (20 mM Tris, 22 mM glycine, 10 mM DTT, 8.3 M urea, and 0.1% bromphenol blue) and allowed to stand at room temperature for 1 h, while vigorously shaking the mixture intermittently. The samples were then filtered through a 0.45 μM membrane filter (Millipore Corporation, Bedford, MA), and the filtrates were subjected to glycerol-PAGE. Proteins were transferred to polyvinylidene difluoride membrane. The blotted membrane was blocked in 1% BSA in phosphate-buffered saline containing 0.1% Tween 20 for 2 h at room temperature. Later, the blot was incubated with monoclonal anti-MLC antibody (Sigma-Aldrich, St. Louis, MO) in a dilution of 1:200 overnight at 4 °C. After washing with phosphate-buffered saline containing 0.1% Tween 20, the blot was incubated with anti-mouse IgG linked to HRP (Sigma-Aldrich) in a dilution of 1:2000 for 90 min at room temperature. Immunoreactive proteins were visualized as described above.

**Immunoprecipitation Followed by Western Blotting.** Anti-phosphotyrosine antibody (5 μg) was added to tissue extract containing 150 μg of protein and mixed by rotation at room temperature for 2 h. Then, 40 μL of protein A-Sepharose (50% slurry) was added to each sample and mixed by rotation at room temperature for 1 h. The samples were centrifuged (5000g) for 1 min and the supernatant was discarded. The pellet was washed with 10 mM HEPES containing 1% Nonidet P-40 five times, mixed with sample buffer, and heated at 100°C for 5 min. The mixture was centrifuged (12,000g) for 5 min and the supernatant was subjected to SDS-PAGE, as described above. The blot was blocked in blocking solution (5% skimmed milk in Tris-buffered saline containing 0.05% Tween 20 for PI3-kinase and 3% skimmed milk in phosphate-buffered saline for cortactin) for 20 min at room temperature and incubated with either 0.8 μg/ml anti-PI3-kinase p85α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or 0.5 μg/ml anti-cortactin (p80/85), clone 4F11 antibody (Upstate Biotechnology, Lake Placid, NY), for 2 h at room temperature.
temperature. After washing, the blot was incubated with either anti-rabbit IgG for PI3-kinase or anti-mouse IgG for cortactin, both linked to HRP, in a dilution of 1:2000 for 45 min at room temperature. The immunoreactive proteins were visualized as described above.

Identification of Proteins by Mass Spectrometry. To identify the proteins undergoing tyrosine phosphorylation at acidic pH by mass spectrometry, tissue extract immunoprecipitated with anti-phosphotyrosine antibody was subjected to SDS-PAGE and the gel was silver-stained. Silver-stained protein bands were excised from the gel, in-gel digested with trypsin, and subjected to nano-flow liquid chromatography-tandem mass spectrometry (MS/MS) analysis for protein identification. The chromatography was performed on a nano ESI column (inside diameter, 120 μm × 30 mm) packed with a C_{18} reversed phase medium (Mightysil-C18; Kanto Chemicals, Tokyo, Japan) using a linear gradient from 0 to 70% acetonitrile in 0.1% formic acid at 100 nL/min, and the separated peptides were directly sprayed into a hybrid mass spectrometer equipped with an electrospray source (Q-Tof 2; Micromass, Manchester, UK). Electrospray ionization was carried out at a voltage of 1.5 kV, and MS/MS spectra were automatically acquired in data-dependent mode during the entire run. All MS/MS spectra were correlated by search engine, Mascot program (Matrixscience, London, UK), against the non-redundant protein sequence database at the National Center for Biotechnology Information (National Institutes of Health). Each high-scoring peptide sequence was confirmed by manual inspection of the corresponding MS/MS spectrum to ensure that the match was correct.

Materials. SHR and WKY were purchased from Charles River (Kanagawa, Japan) and Wistar rats were from Kumagai (Sendai, Japan). Tyrphostin 23 and phenylephrine were from Sigma-Aldrich. Genistein, LY-294002, and wortmannin were obtained from Wako Pure Chemicals (Osaka, Japan), BIOMOL Research Laboratories (Plymouth Meeting, PA), and Calbiochem (San Diego, CA), respectively. Genistein, tyrphostin 23, LY-294002, and wortmannin were dissolved in dimethyl sulfoxide, whereas phenylephrine was dissolved in water. Other reagents used for this study were all of biochemical grade.

Statistics. All values are expressed as mean ± S.E.M. n represents the number of experiments as indicated in figure legends. Statistical significance was evaluated using Students’ t test, when two groups were compared and in case of more than two groups Dunnett’s multiple comparison test was used. The results were considered significant at p < 0.05. The density of the signals corresponding to tyrosine-phosphorylated proteins and MLC was quantitatively analyzed by densitometry (Advanced American Biotechnology, Ful-
lerton, CA). Tyrosine phosphorylation of proteins was expressed as the -fold of control, whereas MLC phosphorylation was quantified with the formula [monophosphorylated MLC/(nonphosphorylated MLC + monophosphorylated MLC)].

**Results**

**Effect of Acidic pH on Isolated Aortas from SHR, WKY, and Wistar Rats.** Decreasing pH of the bathing medium from 7.4 to 6.5 by exchanging the PSS produced persistent contractions in isolated aortas from SHR (Fig. 1A) and WKY (Fig. 1B) that were 122.0 ± 4.9% (n = 12) and 82.4 ± 3.8% of the 64.8 mM KCl-induced contraction, respectively. Interestingly, unlike SHR and WKY, acidic pH failed to produce any contractile response in aorta from Wistar rat (Fig. 1C). In separate experiments, pH-contractile response showed a direct relationship with the level of decrease in pH in both SHR and WKY; however, at each pH level, the contractile response in SHR was significantly higher than that in WKY (Fig. 1D).

**Effects of Tyrosine Kinase Inhibitors on APIC.** To evaluate the involvement of tyrosine kinases in the APIC, two structurally unrelated tyrosine kinase inhibitors, genistein and tyrphostin 23, were used. These agents were added to the bathing medium 20 min before the change of pH from 7.4 to 6.5. Figure 2, A and B, show the representative recordings of the effects of 30 μM genistein and 30 μM tyrphostin 23, respectively, on the APIC in SHR aorta. As shown in Fig. 2, C and D, both genistein (10, 30, and 100 μM) and tyrphostin 23 (10, 30, and 50 μM) inhibited the APIC in a concentration-dependent manner. Tyrphostin 23 at a concentration of 50 μM nearly abolished the APIC.

To test the selectivity of genistein and tyrphostin 23, we...
analyzed the inhibitory effects of these agents on 10 μM phenylephrine (PE)- and 64.8 mM KCl-induced contractions in aorta from SHR. The same concentrations of genistein and tyrphostin 23 were used in these experiments as in the APIC. As shown in Fig. 3, genistein and tyrphostin 23 both inhibited the PE-induced contractile response. The IC50 values of genistein and tyrphostin 23 on the PE-induced contraction were 29.62 and 29.96 μM, respectively, whereas those on the APIC were 27.93 and 19.71 μM, respectively. Tyrphostin 23 had no effect on the KCl-induced contraction, whereas genistein inhibited this contractile response at higher concentration (100 μM). Nevertheless, the IC50 values of both the inhibitors in KCl-induced contraction could not be determined because 50% inhibition did not occur, even at higher concentrations.

Effects of PI3-Kinase Inhibitors on APIC. To evaluate the involvement of PI3-kinase in the APIC in SHR, two different PI3-kinase inhibitors, LY-294002 (5 and 10 μM) and wortmannin (50 and 100 nM), were used. Pretreatment with these inhibitors significantly blunted the magnitude of contractile response induced by pH 6.5 (Fig. 4A). However, the magnitude of inhibition by PI3-kinase inhibitors was significantly less compared with that produced by the tyrosine kinase inhibitors: the degree of inhibition of APIC by 30 μM tyrphostin 23 and 10 μM LY-294002 was 66.5 ± 7.0 and 46.8 ± 4.5%, respectively (p < 0.05).

To investigate further the selectivity of the inhibitory activity of LY-294002, we examined the effect of this compound on 10 μM PE- and 64.8 mM KCl-induced contractile responses. LY-294002 at the concentrations of 5 and 10 μM had no effect on these contractions as shown in Fig. 4, B and C.

Acidic pH Induced Tyrosine-Phosphorylation of Proteins in SHR and Wistar Aortas. Consistent with the results of tension recording experiments, 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. 5A), and this increase in tyrosine phosphorylation of p85 was completely prevented by pretreatment with 30 and 50 μM tyrphostin 23 (Fig. 5, B and C). Parallel with the failure of acidic pH to induce contraction, tyrosine phosphorylation was not observed to be stimulated by pH 6.5 in tissue extracts prepared from Wistar rat aorta (Fig. 5D).

Furthermore, to check the specificity of the observed immunoreaction for p85, immunoprecipitates of the tissue extract from the acidic pH treated sample with either normal rabbit serum or anti-phosphotyrosine antibody were analyzed by Western blotting using the anti-phosphotyrosine antibody. Normal rabbit serum did not immunoprecipitate the p85 (data not shown), whereas the anti-phosphotyrosine antibody immunoprecipitated the immunoreactive protein with a molecular size corresponding to that of p85 detected in whole tissue extract by Western blotting (Fig. 6, A and B). These results demonstrated that p85 was specifically immunoreactive for the anti-phosphotyrosine antibody. To analyze further the proteins contained in the immunoprecipitate with the anti-phosphotyrosine antibody, the immunoprecipitate was subjected to SDS-PAGE followed by silver staining of the gel. These experiments revealed increases in the amounts of p120, p130, p210, and p225 in acidic pH treated sample compared with that at pH 7.4 (Fig. 6, C and D). Conversely, the p85 showing immunoreactivity for phosphotyrosine in Western blotting could not be detected by silver staining.

**Identification of Proteins Undergoing Stimulated Tyrosine-Phosphorylation at Acidic pH.** We further investigated the proteins undergoing stimulated tyrosine phosphorylation at acidic pH by two methods: Western blotting using specific antibodies of the candidate proteins or by mass spectrometry. For the p85 band, we considered PI3-kinase and cortactin as two candidate proteins, because both have the molecular size of approximately 85 kDa, and the two have been shown to be tyrosine-phosphorylated (Okada et al., 1994; Huang et al., 1998). Western blotting of the immunoprecipitate

![Figure 5](https://i.imgur.com/3jQ5.png)

Fig. 5. Effect of acidic pH on protein tyrosine phosphorylation in aortic tissue from SHR and Wistar rats. 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, lanes correspond to tissue extract from SHR aorta at pH 7.4 and 6.5, respectively. B, lanes from left to right are pH 7.4, pH 6.5, pH 6.5 in the presence of 30 μM tyrphostin 23, and pH 6.5 in the presence of 50 μM tyrphostin 23, respectively, in tissue extract from SHR aorta. C, quantitative analysis of the density of the bands corresponding to the tyrosine phosphorylated 85-kDa protein in aortic tissue from SHR at pH 7.4 and at pH 6.5 in the absence and presence of 30 or 50 μM of tyrphostin 23. Data are expressed as-fold of control at pH 7.4. #, p < 0.001 versus control; **, p < 0.01 versus pH 6.5 in the absence of tyrphostin 23. n = 5. D, lanes correspond to pH 7.4 and 6.5, respectively, in tissue extract from Wistar rat aorta (n = 3). The equal intensity of myosin heavy chain (MHC) bands in all samples, stained by Amido Black 10B indicates that each lane contains an equal amount of proteins.
with the anti-tyrosine antibody using anti-PI3-kinase p85α antibody showed a much stronger immunoreactive signal at pH 6.5 compared with that at pH 7.4 (Fig. 7, A and B), whereas the immunoreactive signal for cortactin was not much different at the two pH points (Fig. 7, C and D). These results demonstrate that the p85 of which tyrosine phosphorylation was stimulated at acidic pH was the 85-kDa regulatory subunit of PI3-kinase. Furthermore, the other proteins detected by silver staining were identified by mass spectrometry, as described under Materials and Methods. MS/MS spectra generated from the excised protein bands were compared against the National Center for Biotechnology Information sequence database by the Mascot algorism. This procedure assigned the proteins of 120, 130, and 225 kDa as Pro-collagen I (I) chain, collagen α1 (I) chain, and fibronectin I, respectively.

**Effects of Acidic pH on MLC Phosphorylation.** The level of MLC phosphorylation regulates the contractile state of VSM cells (Begum et al., 2000). MLC phosphorylation by acidic pH in SHR aorta was thus analyzed by glycerol-PAGE followed by Western blotting using anti-MLC antibody, which can separate and detect the nonphosphorylated and phosphorylated forms of MLC. pH 6.5 significantly stimulated the monophosphorylation of MLC, which was prevented when the tissue was pretreated with 30 or 50 μM tyrphostin 23 (Fig. 8, A and B). Tyrphostin 23 (50 μM) decreased the MLC phosphorylation even below the resting level. Similarly, acidic pH induced MLC phosphorylation was also inhibited by pretreatment with 10 μM LY-294002 (Fig. 8, C and D).

**Discussion**

pH regulates various important cellular functions in VSM cells (for review, see Smith et al., 1998). Ischemia and metabolic disorders, such as diabetes mellitus and renal dysfunction, cause extracellular and intracellular acidification. In this study, acidic pH produced an exaggerated contractile response in SHR compared with that in WKY at all pH levels, even at pH that is close to resting level (7.2), suggesting an important influence of acidosis in hypertension. Interestingly, acidification failed to cause contraction in aorta from...
Wistar rat. These results point to the importance of acidic pH-contraction coupling mechanism in SHR and WKY, which is lacking in Wistar rat. From all these findings, two conclusions can be drawn. First, the magnitude of the APIC is dependent on blood pressure in the case of SHR and WKY and is consistent with our previous report (Furukawa et al., 1996). Second, APIC is strain-specific because acidic pH did not induce any contraction in Wistar rat aorta.

Other important findings from the tension recording experiments are that tyrosine kinase inhibitors genistein and tyrphostin 23 both caused a marked inhibition of the APIC, suggesting the involvement of tyrosine kinases in the APIC. Genistein and tyrphostin 23 are two structurally unrelated tyrosine kinase inhibitors that act through different mechanisms: genistein exerts its inhibitory action by binding to the ATP-binding site (Akiyama et al., 1987), whereas tyrphostin 23 interacts with the substrate-binding site of the enzymes (Levitzki and Gazit, 1995). In the present study, genistein and tyrphostin 23 showed a differential inhibitory effect on the acidic pH, PE-, and KCl-induced contractions, that is, the drugs inhibited the acidic pH and PE-induced contractions markedly compared with that induced by KCl. Similar relaxant effects of tyrosine kinase inhibitors, including tyrphostin 23 and genistein, on a variety of agonists-induced contractions in vascular tissues have been reported through inhibition of tyrosine kinases (Di Salvo et al., 1993; Jin et al., 1996; Watts et al., 1996; Yang et al., 2000b; Carmines et al., 2001).

Consistent with the results of the tension recording experiments, Western blotting of the tissue extract from acidic pH treated aorta from SHR demonstrated the stimulation of tyrosine phosphorylation at acidic pH, which was prevented by pretreatment with tyrphostin 23. Because tyrosine phosphorylation has been implicated in several signaling pathways, one may argue that the contraction and stimulation of tyrosine phosphorylation observed at acidic pH in SHR might be unrelated to each other. Therefore, to confirm the parallelism between the two events, tissue extract from Wistar rat aorta was used in which APIC failed to occur. Acidic pH failed to induce tyrosine phosphorylation in aorta from Wistar rat. These observations demonstrate that the tyrosine kinase-mediated protein phosphorylation is mainly responsible for the contractile response induced by acidic pH in SHR aorta and that failure of acidic pH to produce contraction in Wistar rat is in parallel with the lack of protein tyrosine phosphorylation.

For the identification of p85, we targeted two candidate proteins with molecular size of approximately 85 kDa. First, the p85 regulatory subunit of PI3-kinase, which has been shown to be tyrosine phosphorylated upon activation of several receptor tyrosine kinases in various types of cells (Okada et al., 1994). Second, cortactin, a cytoskeleton-associated protein with two isoforms of 80 and 85 kDa (Wu and Parsons, 1993), which has been recently identified as a substrate of p60 V (Huang et al., 1998). In Western blotting experiments,
PI3-kinase, but not cortactin, exhibited much stronger signal at pH 6.5 than that at pH 7.4, indicating that p85 showing stimulated tyrosine-phosphorylation at acidic pH was the p85 regulatory subunit of PI3-kinase. To evaluate the functional role of PI3-kinase in the APIC, we carried out the tension recording experiments using two specific inhibitors of PI3-kinase, LY-294002 (Vlahos et al., 1994) and wortmannin (Okada et al., 1994). Both the agents markedly attenuated the contractile response, providing evidence for the involvement of PI3-kinase in the APIC. Interestingly, LY-294002 did not affect the contractile response to PE and KCl, suggesting that the APIC, but not PE- and KCl-induced contractions are PI3-kinase-dependent. Other investigators have also reported the implication of PI3-kinase in the contractile response induced by various pathological stimuli, such as hydrogen peroxide (Yang et al., 1999) and low extracellular magnesium associated with ischemic heart disease and hypertension (Yang et al., 2000a). The extracellular acidic pH is also an abnormal situation associated with many clinical conditions (Levine, 1993; Komukai et al., 1998). An attractive interpretation is that the APIC and the contractile response of VSM cells to pathological stimuli described above share a common PI3-kinase-dependent mechanism.

Regarding the involvement of PI3-kinase in the APIC, two observations merit discussion. First, the APIC was not completely inhibited by PI3-kinase inhibitors. Second, the stimulation of tyrosine phosphorylation of p85 identified as PI3-kinase was completely inhibited by 30 μM tyrphostin 23 (Fig. 5), whereas some part of the APIC still persisted at this concentration of the tyrosine kinase inhibitor (Fig. 2). These results suggest the involvement of PI3-kinase-dependent as well as PI3-kinase-independent mechanisms in tyrosine kinase-mediated contraction by acidic pH. In fact, we found multiple proteins shown to be increased in amount under the acidic pH conditions by silver staining and could identify two more proteins other than PI3-kinase, i.e., collagen I and fibronectin I. These proteins did not show immunoreactivity for anti-phosphotyrosine antibody. Therefore, we have no direct evidence that collagen I and fibronectin I were actually tyrosine-phosphorylated. It is likely that these proteins were coimmunoprecipitated in association with tyrosine-phosphorylated PI3-kinase. It has been shown that collagen I is tyrosine phosphorylated to play an important role in the contractility of VSM cells (Lee et al., 1998). This protein has been reported to increase Ca^{2+} influx in VSM cells of hypertensive SHR but not in those of normotensive WKY rats (Bouillier et al., 2001). Therefore, it is probable that collagen I may contribute to the contractile response produced by acidic pH.

How the stimulation of tyrosine phosphorylation is translated into contraction is not well understood. Tyrosine phosphorylation has been shown to be associated with Ca^{2+} entry through VDCC (Wijetunge et al., 1992, 1998; Lagaud et al., 1999). A recent study has specifically shown the involvement

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**Fig. 8.** Effect of acidic pH on MLC phosphorylation at acidic pH in aortic tissue from spontaneously hypertensive rat. Samples were prepared as described under Materials and Methods and resolved by glycerol-PAGE. Immunoblotting was performed with anti-MLC antibody, and immunoreactive proteins were visualized by enhanced chemiluminescence. A and C, representative immunoblots showing the effect of acidic pH on MLC phosphorylation in the absence and presence of 30 and 50 μM tyrphostin 23 and 10 μM LY-294002, respectively. NonP, nonphosphorylated MLC; mono-P, monophosphorylated MLC. B and D, analysis of MLC phosphorylation profiles with densitometry, as outlined under Materials and Methods. #, p < 0.05 versus control at pH 7.4; *, p < 0.05 versus pH 6.5 in the absence of tyrphostin 23 (B) or LY-294002 (D). n = 2 to 3 independent experiments.
of PI3-kinase in the mediation of Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels in vascular myocytes (Quignard et al., 2001). Indeed, we have also reported that a major component of the APIC is associated with depolarization and Ca\(^{2+}\) entry through VDCC (Furukawa et al., 1996). Therefore, it is reasonable to assume that the acidic pH induced tyrosine phosphorylation results in the Ca\(^{2+}\) influx via VDCC and hence the contraction.

It is well known that the contractile state of the VSM cells is usually dependent upon the phosphorylated level of MLC (Karaki, 1987). In the present study, MLC phosphorylation was shown to be stimulated at the acidic pH, and both tyrosinase 23 and LY-294002 inhibited the phosphorylazation of MLC. Tyrophostin 23 at a concentration of 50 \(\mu\)M completely prevented the stimulation of MLC phosphorylation, complementing the results of tension recording experiments where tyrophostin 23 nearly abolished the APIC. From these observations, it is suggested that the acidic pH activates these tyrosine kinases and causes enhancement of MLC phosphorylation, and thus the contraction of SHR aorta.

In summary, the present study is the first to demonstrate that acidic pH stimulates tyrosine kinases to enhance tyrosine phosphorylation of PI3-kinase, resulting in the MLC phosphorylation-dependent contraction of isolated aorta from SHR but not from Wistar.

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