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Differential regulation of Na^+/H^+ exchange and DNA synthesis in vascular smooth muscle cells

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Abstract

Na^+/H^+ exchange has been proposed to be involved in the regulation of cell growth. However, little is known about the regulatory pathway and relationship between Na^+/H^+ exchange and DNA synthesis. In vascular smooth muscle cells, platelet-derived growth factor (a tyrosine kinase-coupled receptor agonist) and thrombin (a G protein-coupled receptor agonist) stimulate both activation of Na^+/H^+ exchange and DNA synthesis. In this study, we compared the effect of platelet-derived growth factor (PDGF) and thrombin on the signal transduction pathway leading to the activation of these responses in A10 cells, clonal rat thoracic aortic smooth muscle cells. To investigate the role of mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase as potential mediators, we examined the effect of phar-macological kinase inhibitors on these responses. The Na^+/H^+ exchange activity induced by thrombin was inhibited by a specific inhibitor of MAPK kinase, 2′-amino-3′-methoxyflavone (PD98059), but was not affected by a specific phosphatidylinositol-3-kinase inhibitor, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002). Thrombin-induced DNA synthesis was inhibited by LY294002, but not by PD98059. In contrast, the Na^+/H^+ exchange activity induced by PDGF was inhibited by neither LY294002 nor PD98059, but PDGF-induced DNA synthesis was inhibited by both LY294002 and PD98059. These data suggest that, in A10 cells, Na^+/H^+ exchange activation and DNA synthesis are differently regulated by the two extracellular stimuli. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: PDGF (platelet-derived growth factor); Thrombin; Na^+/H^+ exchange; Smooth muscle cell, vascular

1. Introduction

Na^+/H^+ exchangers are expressed at the surface of all mammalian cells, where they have been shown to regulate cell volume, intracellular pH, and transepithelial ion transport. Na^+/H^+ exchanger isoform-1, the first Na^+/H^+ exchanger isoform to be cloned (Sardet et al., 1989), is the predominant type in vascular smooth muscle cells and is thought to be an important regulator of intracellular pH. It can be activated by a number of stimuli that include growth factors, hormones, and hyperosmolality (Lucchesi and Berk, 1995).

Many studies have shown that Na^+/H^+ exchange can be modulated by various growth factors through tyrosine kinase-coupled receptors and G protein-coupled receptors (Wakabayashi et al., 1997). However, the mechanisms that regulate Na^+/H^+ exchange activity are not fully understood. The kinases that regulate Na^+/H^+ exchange activity have not yet been identified. It has been postulated that there is an important relationship between intracellular pH and cell growth, since an inhibitor of Na^+/H^+ exchange, amiloride, inhibits growth factor-induced activation of Na^+/H^+ exchange and cell growth in several cell types, including vascular smooth muscle cells. Activation of Na^+/H^+ exchange and DNA synthesis requires activation of the phosphorylation cascade. In this context, it has been proposed that there is a mutual regulatory pathway for Na^+/H^+ exchange and DNA synthesis. The mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase pathway may be involved in this response. Recently, MAPK has been suggested to play a major role in regulating Na^+/H^+ exchange activity. In fibroblasts, MAPK mutants cause significant inhibition of growth factor-induced Na^+/H^+ exchanger isoform-1 activity (Pages et al., 1993). Phosphatidylinositol-3-kinase activity has been implicated in the activation of Na^+/H^+ exchanger isoform-1 in Chinese hamster ovary cells (Ma et al., 1994). It has yet to be determined whether these results can be generalized to other receptors or other cell types.

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In this study, we examined the possible role of MAPK and phosphatidylinositol-3-kinase on Na^+/H^+ exchange activation and DNA synthesis induced by platelet-derived growth factor (PDGF, a tyrosine kinase-coupled receptor agonist) and thrombin (a G protein-coupled receptor agonist), which are known to activate distinct signaling pathways, in cells of the clonal vascular smooth muscle cell line, A10. These agonists cause a rapid increase in intracellular pH via Na^+/H^+ exchange and DNA synthesis in A10 cells. 2′-Amino-3′-methoxyflavone (PD98059) is a specific inhibitor of MAPK kinase and is used to evaluate the role of the MAPK pathway (Pang et al., 1995). PD98059 blocked the activation of Na^+/H^+ exchange induced by thrombin and DNA synthesis induced by PDGF, but did not block the activation of Na^+/H^+ exchange induced by PDGF or the DNA synthesis induced by thrombin. In contrast, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) (Vlahos et al., 1994), an inhibitor of phosphatidylinositol-3-kinase, inhibited DNA synthesis induced by thrombin and PDGF, but did not inhibit the activation of Na^+/H^+ exchange by thrombin and PDGF. Our data suggest that Na^+/H^+ exchange activity and DNA synthesis are regulated differently in vascular smooth muscle cells.

2. Materials and methods

2.1. Materials

2′,7′-Bis(carboxyetyl)-5(6)-carboxyfluorescein–acetoxymethyl ester (BCECF-AM) was from Molecular Probe. Amiloride, nigericin, bovine serum albumin and thrombin were from Sigma. LY294002 was from Biomol. [^{3}H]Thymidine was from Amersham. PD98059 was from Calbiochem. PDGF-BB was from Cosmo Bio. Cell culture materials were from Gibco BRL. All other reagents were of analytical grades and obtained from commercial sources.

2.2. Cell culture

A10 cells (rat thoracic aortic smooth muscle) were provided by American Type Cell Collection. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air in 100 mm dishes. The growth medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Medium was changed twice a week and cell passages 18–22 were used for all experiments.

2.3. Measurement of intracellular pH

Na^+/H^+ exchange activity was measured by determination of amiloride-sensitive alkalization as described previously (Mitsuhashi and Ives, 1988). Briefly, cells were loaded with 5 μM BCECF-AM and bovine serum albumin (0.38%) at 30°C for 30 min. Extracellular BCECF-AM was removed by washing with a solution (pH7.0) containing NaCl (140 mM), KCl (5 mM), $MgSO_4$ (1 mM), Na_2HPO_4 (1 mM), $CaCl_2$ (2 mM), glucose (25 mM), HEPES (25 mM), and bovine serum albumin (0.05%). Following a 15-min equilibration at 30°C, fluorescence was measured with a Shimadzu spectrophotometer with wavelength settings of 495 and 525 nm for excitation and emission, respectively. At the end of each experiment, calibration was performed using nigericin (10 μM) in calibration buffer containing 140 mM KCl and 5 mM NaCl. The fluorescence of BCECF-AM was approximately linear between pH 6.6 and 7.6.

2.4. Determination of [^{3}H]thymidine incorporation

The assay was performed by measuring the incorporation of [^{3}H]thymidine into acid-insoluble materials as previously described (Nishio and Watanabe, 1997). Cells

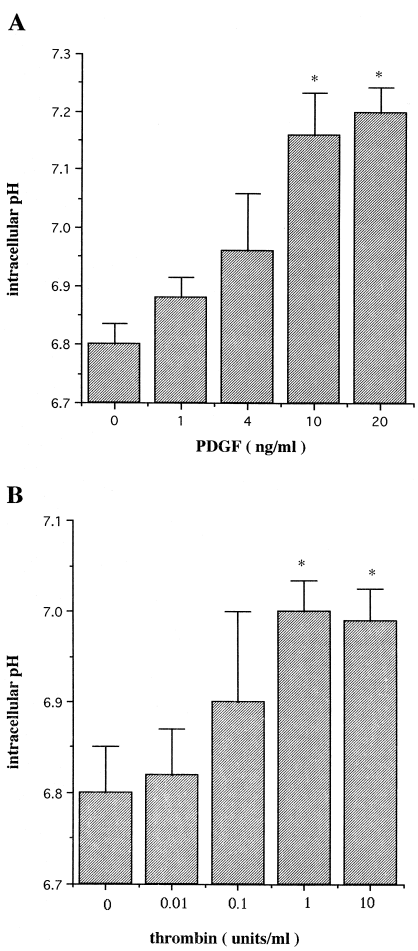

Fig. 1. Effect of various concentrations of PDGF and thrombin on intracellular pH responses in A10 cells. Intracellular pH was measured using BCECF-AM as described in Section 2. Each value represents the mean ± S.D. for three independent experiments performed in duplicate. *$P < 0.05$ as compared with the basal pH.

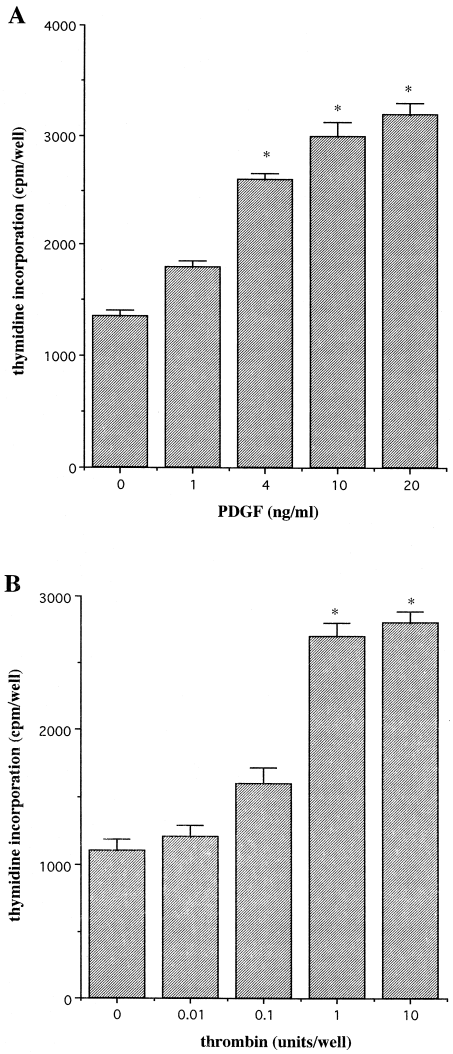

Fig. 2. Effect of various concentrations of PDGF and thrombin on DNA synthesis in A10 cells. [^{3}H]Thymidine incorporation was measured as described in Section 2. Each value represents the mean ± S.D. for three independent experiments performed in duplicate. $^*P < 0.05$ as compared with the basal DNA synthesis.

were seeded into 24-well culture plates, allowed to grown to 70–80% confluence and then growth-arrested by incubation in serum-free DMEM. Growth-arrested cells were exposed to growth factors at the indicated concentrations for 24 h. Cells were pulse-labeled with 1 μCi/ml [^{3}H]thymidine for 2 h just before the end of the incubation period and were incubated at 4°C for 2 h in 10% trichloroacetic acid. Acid-insoluble material was extracted with 0.1 N NaOH and incorporated radioactivity was measured by liquid scintillation spectroscopy. All experiments were performed in triplicate.

2.5. Statistics

Values are expressed as the arithmetic means ± S.D. Statistical analysis of the data was performed by the use of one-way analysis of variance (ANOVA), followed by Scheffe test when F ratios were significant ($P < 0.05$).

3. Results

3.1. PDGF- and thrombin-induced increase in intracellular pH and DNA synthesis in A10 cells

We first measured intracellular pH, using the fluorescent pH-sensitive dye BCECF-AM. As shown in Fig. 1A, A10 cells exposed to various concentrations of PDGF showed a concentration-dependent increase in intracellular pH. Maximum increases of intracellular pH occurred with 10 ng/ml final concentration of PDGF. The basal pH was

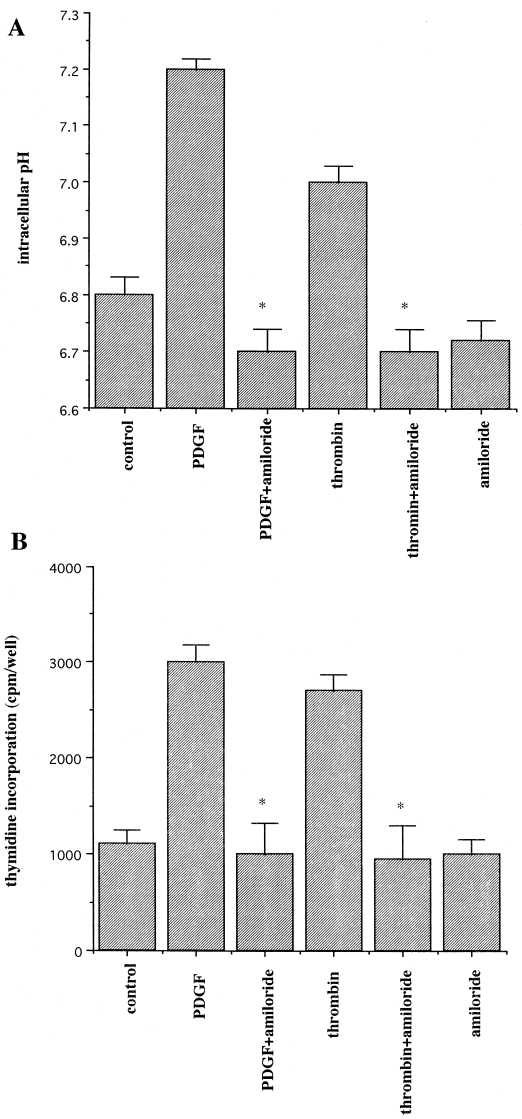

Fig. 3. Effect of amiloride on PDGF- and thrombin-induced intracellular pH and DNA synthesis in A10 cells. (A) Intracellular pH was measured using BCECF-AM as described in Section 2. Cells were pretreated with 10 μM of amiloride for 30 min before addition of either 10 ng/ml PDGF or 1 unit/ml thrombin. (B) Growth-arrested cells were pretreated with 10 μM amiloride for 30 min before addition of either PDGF or thrombin. [^{3}H]Thymidine incorporation was measured as described in Section 2. Each value represents the mean ± S.D. for three independent experiments performed in duplicate. $^*P < 0.01$ as compared with the respective control.

6.80, and in response to 10 ng/ml PDGF, an increase of 0.41 pH units was observed. The dose-dependent increase in intracellular pH was similar for cells treated with thrombin (Fig. 1B). The basal pH was 6.80, and in response to 1 unit/ml thrombin, a maximum increase of 0.20 pH units was observed. Next we measured PDGF- and thrombin-induced DNA synthesis. As shown in Fig. 2, both agonists induced activation of DNA synthesis in a dose-dependent manner. The maximal response was observed with 1 unit/ml thrombin and 10 ng/ml PDGF, respectively, and the time course of stimulation of DNA synthesis was similar to that for stimulation of Na^+/H^+ exchange activity.

3.2. Effect of amiloride on the PDGF- and thrombin-induced Na^+/H^+ exchange activity and DNA synthesis

To determine whether the increase in intracellular pH mediates activation of the Na^+/H^+ exchanger, we examined the effects of the Na^+/H^+ exchange inhibitor, amiloride, on the PDGF- and thrombin-induced Na^+/H^+ exchange activity and DNA synthesis. Amiloride did not alter the basal pH, but completely blocked the PDGF- and thrombin-induced increase in intracellular pH at a concentration of 10 μM (Fig. 3A). This shows these agonists increased the intracellular pH via the amiloride-sensitive Na^+/H^+ exchanger. Amiloride also blocked both PDGF-

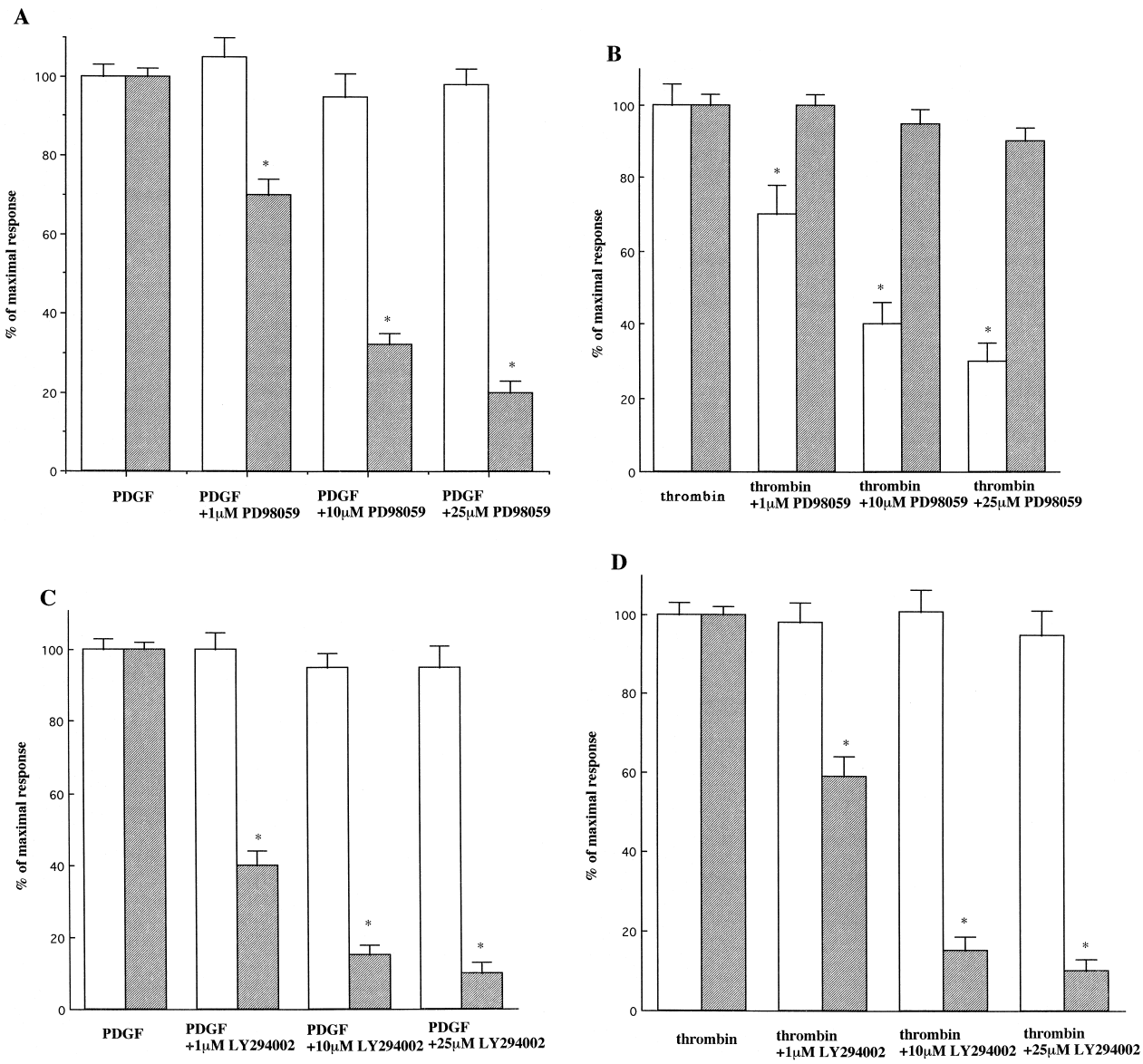

Fig. 4. Effect of PD98059 or LY294002 on PDGF- and thrombin-induced intracellular pH and DNA synthesis in A10 cells. Cells were stimulated with PDGF (10 ng/ml) or thrombin (1 unit/ml) in the absence or presence of increasing concentrations of PD98059 or LY294002 (1 to 25 μM). Both inhibitors were added for 30 min before addition of the agonists. The changes in intracellular pH (open column) and [^{3}H]thymidine incorporation (shared column) were measured as described in Section 2. Values given on the *y*-axis correspond to percentage of maximal responses obtained in the absence of inhibitors. Each value represents the mean ± S.D. for three independent experiments performed in duplicate. $^*P < 0.05$ as compared with the control (agonist alone).

and thrombin-induced DNA synthesis (Fig. 3B). Amiloride did not reduce the basal DNA synthesis. These data suggest a correlation between Na^+/H^+ exchange activity and DNA synthesis.

3.3. Effects of PD98059 on the PDGF- and thrombin-induced stimulation of Na^+/H^+ exchange activity and DNA synthesis

To investigate the involvement of the MAPK pathway in the regulation of Na^+/H^+ exchange and DNA synthesis, we examined the effects of PD98059, a specific MAPK kinase inhibitor. As shown in Fig. 4A, PD98059 was found to significantly inhibit the response of the Na^+/H^+ exchanger to thrombin in a dose-dependent manner. Inhibition was almost maximal with 10 mM of this inhibitor. In contrast, the stimulation of DNA synthesis by thrombin remained unaffected by the same pretreatment with PD98059 (1 to 25 μM). These results indicate that the MAPK cascade plays a role only in the activation of Na^+/H^+ exchange induced by thrombin in A10 cells. Moreover, PD98059 inhibited PDGF-induced DNA synthesis while having little effect on Na^+/H^+ exchange. These data indicate that the MAPK cascade is not a mutual pathway involved in Na^+/H^+ exchange and DNA synthesis.

3.4. Effects of LY294002 on the PDGF- and thrombin-induced activation of Na^+/H^+ exchange

To investigate the involvement of the phosphatidylinositol-3-kinase pathway in the regulation of Na^+/H^+ exchange and DNA synthesis, we examined the effect of LY294002, an inhibitor of phosphatidylinositol-3-kinase. As shown in Fig. 4C and D, LY294002 (1 to 25 μM) had little effect on Na^+/H^+ exchange activity. In contrast, agonist-induced DNA synthesis was substantially inhibited by pretreatment of the cells with LY294002. Similar results were obtained using wortmannin, a structurally distinct inhibitor of phosphatidylinositol-3-kinase (data not shown). These results indicate that phosphatidylinositol-3-kinase plays a role in DNA synthesis but not in Na^+/H^+ exchange, further distinguishing between Na^+/H^+ exchange and DNA synthesis.

4. Discussion

In the present study, we showed that the signaling pathways mediating stimulation of Na^+/H^+ exchanger activity and DNA synthesis are different in A10 cells when the two responses are stimulated by either thrombin or PDGF.

Many studies have reported a relationship between the regulation of Na^+/H^+ exchange activity and DNA synthesis, because amiloride analogues inhibit both the growth factor-induced stimulation of Na^+/H^+ exchange and the proliferation of several cell types. We also observed that amiloride blocked agonist-induced stimulation of Na^+/H^+ exchange activity and DNA synthesis (Fig. 3). However, since it has been reported that amiloride affects the activity of several protein kinases (Presek and Reuter, 1987; Grinstein et al., 1989), amiloride is not a suitable tool to investigate their role in Na^+/H^+ exchange. Therefore, we tested the effects of several kinase inhibitors to examine the role of Na^+/H^+ exchange in DNA synthesis in vascular smooth muscle cells. A10 cells have been shown to be similar to primary vascular smooth muscle cells and are proposed to be a good model because of their biochemical and morphological stability (Cascieri et al., 1986). Like A10 cells, stimulation of Na^+/H^+ exchange activity and DNA synthesis was also observed in primary cultures isolated from rat thoracic aorta (data not shown).

The MAPK pathway is associated with cell growth in many cell types. MAPK is a good candidate regulator of Na^+/H^+ exchange activity and DNA synthesis. Although both PDGF and thrombin can activate MAPK, a specific inhibitor of MAPK kinase (PD98059) was effective only against thrombin-induced Na^+/H^+ exchange activation and PDGF-induced [^{3}H]thymidine incorporation (Fig. 4). The results suggest that MAPK has a role in Na^+/H^+ exchange activated by thrombin and do not support the possibility that MAPK is involved in a regulatory pathway between Na^+/H^+ exchange and DNA synthesis. Consistent with our data, recent studies suggest that MAPK might regulate Na^+/H^+ exchange. It has been shown that arginine vasopressin and phorbol-12-myristate-13-acetate rapidly activate Na^+/H^+ exchange by a PD98059-sensitive pathway (Aharonovitz and Granot, 1996). Furthermore, Bianchini showed that a dominant negative of p44 MAPK reduced Na^+/H^+ exchange activated by growth factors (Bianchini et al., 1997). The difference between thrombin and PDGF might reflect the receptor type. Since the effects of both thrombin and arginine vasopressin are mediated through their G-coupled receptors, MAPK might be a regulator of Na^+/H^+ exchange activated by G-coupled receptors.

The regulatory mechanism of Na^+/H^+ exchange by MAPK is not still understood. Since it has been suggested that the Na^+/H^+ exchanger is phosphorylated in response to extracellular stimuli, thrombin might activate Na^+/H^+ exchange through a mechanism that leads to phosphorylation of the exchanger. Moreover, PD98059 inhibited thrombin-induced activation of Na^+/H^+ exchange by only 50%, indicating the existence of an additional pathway that does not require MAPK.

Phosphatidylinositol-3-kinase activity has also been implicated in the activation of Na^+/H^+ exchanger isoform-1 of fibroblasts. We examined the effect of LY294002, a phosphatidylinositol-3-kinase inhibitor, on the activation of Na^+/H^+ exchange and DNA synthesis induced by PDGF and thrombin. LY294002 inhibited PDGF- and thrombin-induced [^{3}H]thymidine incorporation, whereas activation

of Na^+/H^+ exchange was not inhibited. The results provide further evidence for the differential regulation of Na^+/H^+ exchange activation and DNA synthesis by PDGF and thrombin. In Chinese hamster ovary cells expressing the PDGF receptor mutant, phosphatidylinositol-3-kinase is involved in the activation of Na^+/H^+ exchange (Ma et al., 1994). The difference between our results and those of Ma et al. may be explained by the different cell types used. The mechanism of Na^+/H^+ exchange activated by PDGF is not still clear. It might be mediated by a mechanism other than phosphatidylinositol-3-kinase. Previous studies have shown that PDGF might activate Na^+/H^+ exchange by both protein kinase C-dependent and protein kinase C-independent pathways (Rozengurt, 1986; Lowe et al., 1990). The protein kinase C-independent pathway has not yet been determined. Our data indicate that, in A10 cells, phosphatidylinositol-3-kinase is not involved in the activation of Na^+/H^+ exchange. It remains to be determined which kinase is responsible for this activation.

Thrombin is thought to transmit mitogenic effects through its receptors, which are coupled to G_i, G_q, and G_{12} classes of heterodimeric G proteins (LaMorte et al., 1993; Offermanns et al., 1994). Pretreatment of cells with pertussis toxin (100 ng/ml) did not inhibit thrombin-induced activation of Na^+/H^+ exchange and DNA synthesis (data not shown). These results indicate that pertussis toxin-sensitive G proteins, G_i, do not mediate these responses. G_{12} have been recently shown to increase Na^+/H^+ exchange activity through a protein kinase C-dependent pathway (Dhanasekaran et al., 1994). Further studies will be needed to investigate the possible involvement of G_q or G_{12}.

These results suggest that MAPK and phosphatidylinositol-3-kinase are not directly linked to activation of Na^+/H^+ exchange and DNA synthesis in A10 cells stimulated by PDGF and thrombin. Therefore, activation of Na^+/H^+ exchange and DNA synthesis are regulated differently in A10 cells.

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