Activation of Na⁺/H⁺ Exchange by Platelet-derived Growth Factor Involves Phosphatidylinositol 3' '-Kinase and Phospholipase Cy⁺

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The effect of site-specific mutations in the mouse platelet-derived growth factor (PDGF) β-receptor on activation of the Na⁺/H⁺ exchanger was examined in normal murine mammary gland epithelial (NMuMG) and Chinese hamster ovary (CHO) cells. These cells, which do not normally express PDGF receptors, were stably transfected with PDGF β-receptor cDNA. Intracellular pH and Ca²⁺ were monitored using fluorescent probes. In both NMuMG and CHO cells expressing wild-type PDGF β-receptors, PDGF B/B activated the amiloride-sensitive Na⁺/H⁺ exchanger. In both cell types, cell alkalinization was reduced by approximately 50% with a receptor mutant Y708F,Y719F which cannot bind phosphatidylinositol (PI) 3'-kinase. An inhibitor of PI 3'-kinase, LY294002, also inhibited alkalinization by 48% in cells with wild-type, but not Y708F,Y719F receptors. PDGF-induced intracellular Ca²⁺ release was not affected by this mutation. Both alkalinization and Ca²⁺ release were reduced by nearly 100% with the mutant Y977F,YSSSF, which cannot bind phospholipase Cy. In both cell types, cell alkalinization was reduced by approximately 50% with a receptor mutant Y708F,Y719F which cannot bind phosphatidylinositol (PI) 3'-kinase. An inhibitor of PI 3'-kinase, LY294002, also inhibited alkalinization by 48% in cells with wild-type, but not Y708F,Y719F receptors. PDGF-induced intracellular Ca²⁺ release was not affected by this mutation. Both alkalinization and Ca²⁺ release were reduced by nearly 100% with the mutant Y977F,YSSSF, which cannot bind phospholipase Cy (PLCγ). Y739F, a mutant that fails to bind the GTPase-activating protein did not affect PDGF-induced alkalinization. In protein kinase C (PKC) down-regulated NMuMG cells (wild-type receptor), PDGF no longer activated the Na⁺/H⁺ exchanger. In contrast, in PKC down-regulated CHO cells (wild-type receptor), PDGF-induced alkalinization was attenuated by only 37%. This residual activity was unaffected by the Y708F,Y719F mutation, but was completely eliminated by removal of medium Ca²⁺. These findings indicate that phospholipase Cy is essential for activation of Na⁺/H⁺ exchange. PI 3'-kinase participates in PKC-dependent activation of Na⁺/H⁺ exchange by PDGF. In CHO cells, there is a second, Ca²⁺-dependent mechanism for activation of the exchanger.

Among its many activities, platelet-derived growth factor (PDGF) activates the Na⁺/H⁺ exchanger, NHE-1 (1–3). Biochemical studies suggest that PDGF may be capable of activating Na⁺/H⁺ exchange by both protein kinase C (PKC)-dependent (4) and independent pathways (5, 6). The PKC-dependent pathway and PLC-γ follow the activation of phospholipase Cy (PLCγ) which results in production of diacylglycerol.

The mechanism(s) responsible for activation of Na⁺/H⁺ exchange by the PKC-independent pathway(s) remain unclear. In particular, it has not yet been determined whether the PKC-independent pathway requires PLC activity or whether it results from alternative signaling molecules. One candidate for an alternative signaling pathway is the phosphatidylinositol (PI) 3'-kinase which is activated by the PDGF receptor (1, 7). By producing novel lipid intermediates, PI 3'-kinase could participate in activation of membrane transporters. One approach to distinguishing these possibilities is to analyze PDGF receptor mutants that are deficient in one or more of the signaling pathways of interest.

Binding of PDGF to its receptor initiates autophosphorylation on multiple tyrosine residues, including tyrosines in the kinase insert (Ki) region, and the C-terminal domain. After phosphorylation, the receptor physically associates with signaling molecules, including PLCγ-1 (8–13), PI 3'-kinase (1, 7, 14–16). Binding sites for PI 3'-kinase and GAP are located in the Ki region, while PLCγ binds to the C-terminal domain of the receptor (12). Specific Tyr/Phe mutations prevent binding of these signaling molecules to the PDGF receptor (17).

We examined the effect of Tyr/Phe signaling mutations on activation of the Na⁺/H⁺ exchanger by the PDGF receptor. The data indicate that both PLCγ and PI 3'-kinase are involved in PDGF-induced activation of Na⁺/H⁺ exchange.

EXPERIMENTAL PROCEDURES

Materials—2',7'-Bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein acetoxymethyl ester (BCECF-AM) and Fura-O/AM were obtained from Molecular Probes, Inc. (Eugene, OR). Dulbecco’s modified Eagle medium (H-21, Ham’s F-12 medium, bovine calf serum, penicillin, and streptomycin were obtained from the University of California, San Francisco tissue culture facility. G418 sulfate (Genetica) is from Life Technologies, Inc. Nigericin, phorbol 12-myristate 13-acetate (PMA), A23187, and transferrin (human) were purchased from Sigma. PDGF B/B (human, recombinant) was obtained from Boehringer Mannheim. Bovine serum albumin (fraction V, fatty acid-poor) was obtained from Miles (Naperville, IL). LY294002 was the kind gift of Chris Vlahos, from Lilly Research Laboratories (Indianapolis, IN).

PDGF receptor mutants—The PDGF receptor mutants were constructed by site-directed mutagenesis (18, 19). Plasmids containing the mutated receptor cDNA were used to stably transfect the normal murine mammary gland epithelial cells (NMuMG) or Chinese hamster ovary cells (CHO) which normally lack PDGF receptors (17). Mutation of Tyr-739 → Phe (Y739F) prevents binding of GAP; Y708F,Y719F prevents the association of PI 3'-kinase (17); Y977F

ethyl)-5-(and-6)carboxyfluorescein acetoxymethyl ester; LY294002, 2-(4-morpholino)-8-phenyl-4H-1-benzopyran-4-one.

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FIG. 1. Immunoblot analysis of PDGF receptor mutants expressed in NMuMG cells. Expression of mouse wild-type PDGF receptor and mutants in NMuMG cells was assessed by immunoblot with anti-PDGFR receptor antibodies as described under “Experimental Procedures.” Arrow indicates PDGF receptor band.

and/or Y989F interfere with binding of PLC\(\gamma\) (based on homology with Tyr-1009 and Tyr-1021 of the human receptor (12)). AKi, a deletion of 82 of the 104 amino acids in the kinase insert region, was produced as described previously (2).

Cell Culture—NMuMG or CHO cells transfected with PDGF receptors were grown in Dulbecco’s modified Eagle’s medium or Ham’s F-12 medium, respectively, in a humidified atmosphere of 5% CO\(_2\), 95% air at 37 °C. Both media contain 10% (v/v) calf serum, penicillin (50 units/ml), and streptomycin (50 units/ml). Medium for stable transfectants was supplemented with 800 μg/ml G418. Culture medium was changed every 2–3 days until cells were confluent. For measurement of intracellular pH (pHi), cells were transferred and grown to confluence in Dulbecco’s modified Eagle’s medium or Ham’s F-12 medium, respectively, in a humidified atmosphere of 5% CO\(_2\), 95% air at 37 °C. Both media contain 10% (v/v) calf serum, penicillin (50 units/ml), and streptomycin (50 units/ml). Medium for stable transfectants was supplemented with 800 μg/ml G418. Culture medium was changed every 2–3 days until cells were confluent. For measurement of intracellular pH (pHi), cells were transferred and grown to confluence in Leighton tubes (Costar, Cambridge, MA). Cells were made quiescent by replacement of serum with bovine serum albumin (0.38%). Medium for stable transfectants was prepared previously (17). The expression of the PDGF receptor mutants was quantitated using laser densitometry (Zeineh Soft Laser Scanning Densitometer, model SLR-504-XL, Biomed Instruments, Inc., Fullerton, CA).

Measurements of Intracellular pH—Na\(^+\)/H\(^+\) exchange activity was measured by determination of amiloride-sensitive alkalization as described previously (20). Briefly, cells were loaded with 5 μM BCECF-AM and bovine serum albumin (0.38%) at room temperature for 15 min. Extracellular BCECF-AM was removed by washing with a solution (pH 7.0) containing: NaCl (140 mM), KCl(5 mM), MgSO\(_4\) (1 mM), Na\(_2\)HPO\(_4\) (1 mM), CaCl\(_2\) (2 mM), glucose (25 mM), HEPES (25 mM), and bovine serum albumin (0.05%). Following a 15-min equilibration at 32 °C, fluorescence (excitation wavelength = 440 or 505 nm, emission wavelength = 525 nm) was measured with an SLM 8000 spectrofluorometer (Urbana, IL). At the end of each experiment, the fluorescence signal was calibrated using nigericin (10 mM) in calibration medium containing 140 mM KCl, 5 mM NaCl (pH 6.5–7.4). Cell preparations with basal pH\(_i\) > 7.3 were discarded (approximately 20%).

Measurements of Intracellular Ca\(^{2+}\)—Cells grown on coverslips were incubated with 4.2 μM fura-2/AM and 0.02% pluronic detergent at room temperature for 1 h. Fura-2 fluorescence was measured using a Nikon epifluorescence inverted microscope as described previously (3, 21). After equilibration, cells on coverslips were washed with solution containing 0 Ca\(^{2+}\) and EGTA (2 mM) to remove extracellular Ca\(^{2+}\). PDGF (5 ng/ml) was then added immediately in the absence of Ca\(^{2+}\).

Statistics—Values presented are means ± S.E. or (where indicated) S.D. Differences in mean values between groups were examined using analysis of variance followed by individual contrast (22). Student’s t test was used when appropriate. p values < 0.05 were considered statistically significant.

RESULTS

Expression of the PDGF β-receptor mutants in NMuMG cells was analyzed by Western blot (Fig. 1). PDGF receptor protein was undetectable in nontransfected cells (lane 1). Cells transfected with PDGF receptor mutants Y739F, Y989F, and Y977F,Y989F expressed similar amount of receptor protein as cells transfected with the wild-type receptor. Mutants AKi, Y977F,Y708F,Y719F expressed 2–3-fold greater PDGF receptor protein than cells transfected with wild-type receptor.

Coupling of Transfected Receptors and Endogenous Na\(^+\)/H\(^+\) Exchanger—Activation of Na\(^+\)/H\(^+\) exchange by PDGF BB was examined in NMuMG cells by measurement of pH\(_i\). PDGF (20 ng/ml) did not alter pH\(_i\) in untransfected cells (Fig. 2A), while PMA (250 ng/ml) caused pH\(_i\) to increase by 0.21 pH units. In NMuMG cells expressing wild-type PDGF β-receptor, PDGF elicited an increase in pH\(_i\) of 0.15 ± 0.09 (n = 22) pH units (Fig. 2B). Amiloride (250 μM) completely blocked this pH change (Fig. 2C). In CHO cells expressing wild-type PDGF β-receptor, PDGF also induced amiloride-sensitive alkalization (see below).

Mutation at the GAP Binding Site—We next studied the pH\(_i\) effects of PDGF in cells expressing PDGF receptors with mutations in the kinase insert region. A mutation at the GAP-binding site (Y739F) did not alter the PDGF response. PDGF (20 ng/ml) elicited a rise in pH\(_i\) of 0.13 ± 0.02 (n = 13, Fig. 3A), not different from that in cells expressing wild-type receptor. In CHO cells expressing the Y739F mutation, PDGF caused pH\(_i\) to rise 0.16 ± 0.02 (n = 9) from a basal of 7.04 ± 0.02 (not shown).

Mutations at PI 3'-Kinase Binding Sites—Mutation of the PDGF receptor at amino acids Y708F,Y719F prevents binding of PI 3'-kinase (17). In NMuMG cells expressing this mutation, PDGF (20 ng/ml) caused pH\(_i\) to rise much less than in cells with
wild-type receptor (Figs. 2B and 3B). PDGF raised pHi by 0.07 ± 0.01 (n = 18), 49% of that in cells with wild-type receptors (p < 0.05, Fig. 4A). Similar results were obtained in CHO cells, where alkalization with Y708F,Y719F was 56% of the response elicited by wild-type receptor (n = 20, p < 0.05, Fig. 4A).

The Y708F,Y719F mutation could potentially cause PDGF to elicit additional acid generation, thus giving the appearance of diminished alkalization. This was not the case, as application of PDGF following amiloride (100 μM) inhibition of Na+/H+ exchange resulted in indistinguishable rates of pHi change in wild-type and Y708F,Y719F receptors (wild type, 0.02 ± 0.01 pH unit/15 min, n = 4; Y708F,Y719F, 0.0 ± 0.1 pH unit/15 min, n = 5).

Since even point mutations in the PDGF receptor could theoretically confer multiple defects in signaling, the role of PI 3'-kinase was further explored using a recently discovered inhibitor of the enzyme LY294002 (23). This compound, when added to NMuMG cells expressing PI 3'-kinase (LY294002 (20 ng/ml)) was added to NMuMG wild-type (n = 4) or Y708F,Y719F (n = 5) cells 5 min before addition of PDGF. The basal pHi for NMuMG cells (which was unaffected by LY294002) with wild-type and Y708F,Y719F receptors was 7.07 ± 0.15 (S.D.; n = 30) and 7.10 ± 0.15 (S.D., n = 29), and for CHO cells, 7.02 ± 0.10 (S.D., n = 29) and 6.96 ± 0.08 (S.D., n = 20), respectively. *p < 0.05 compared with wild-type receptors. **p < 0.05 compared to wild-type receptor. 

Effect of Mutations at PLCγ Binding Site—As for the Ca²⁺ response the pHi response to PDGF (20 ng/ml) was unaltered in six of nine NMuMG cell preparations expressing Y977F (Fig. 6A); conversely, in mutant Y989F the PDGF response was absent in 7 out of 10 NMuMG preparations (Fig. 6B). In CHO cells, PDGF failed to raise pHi, significantly with either point mutation (not shown). The double mutation, Y977F,Y989F, reduced activation of Na+/H+ exchange by PDGF from 0.15 pH unit in wild-type to nearly zero in both NMuMG cells (Fig. 6C) and CHO cells (not shown). As for the Y708F,Y719F mutation, the Y977F,Y989F mutation did not alter the Na+/H+ exchanger per se, as osmotic agents activated the exchanger in cells expressing this mutation (not shown). These results indicate that PDGF activation of Na+/H+ exchange is entirely dependent upon activation of PLCγ in both cell types.

Involvement of PKC in PDGF-induced Alkalization—The results of Fig. 6 clearly implicate PLCγ as playing a critical role in activation of the Na+/H+ exchanger. To assess the role of PKC, pHi was measured after acute administration of PMA or after PKC down-regulation by pre-treatment of the cells with 20 ng/ml PMA for 24 h (6, 24). In control preparations, PMA (250 ng/ml) increased pHi by 0.13 ± 0.03 pH units (n = 9) from 7.14 ± 0.04 in NMuMG cells expressing the wild-type receptor (Fig. 7A). A similar response was observed in CHO cells expressing wild-type receptors and Y708F,Y719F receptors. After PKC down-regulation (which does not alter basal pH), PMA failed to elicit alkalization in all three cell types (Fig. 7A).

In contrast, the effect of PDGF in PKC down-regulated cells varied with cell type (Fig. 7B). PMA pre-treatment almost completely blocked PDGF-induced alkalization of NMuMG cells.
expressing wild-type PDGF receptors \((p < 0.05, \ n = 10, \ \text{left hand bars, Fig. 7B})\), suggesting a major role for PKC in PDGF-induced alkalization in NMuMG cells. On the other hand, in CHO cells with wild-type receptors, PKC down-regulation reduced the alkalization after PDGF by only 37\% \((n = 8, \ p < 0.05, \ \text{center bars, Fig. 7B})\), suggesting a second mechanism other than PKC in mediating activation of Na+/H+ exchange. In CHO cells expressing the Y708F,Y719F mutant, PKC down-regulation did not affect the PDGF-induced alkalization response at all \(\text{(right-hand bars, Fig. 7B)}\). Finally, in cells lacking both PI 3'-kinase and PKC activity, the residual activation of Na+/H+ exchange was completely eliminated when cells were depleted of Ca\(^{2+}\) by removal of Ca\(^{2+}\) from the extracellular medium for 15 min. Removal of Ca\(^{2+}\) does not affect Na+/H+ exchange activity per se, since osmotic agents can activate the exchanger normally following Ca\(^{2+}\) removal both in wild type cells \(\text{(20, 25)}\) and in Y708F/Y719F cells lacking PKC \(\text{(not shown)}\). These results suggest the existence of two pathways for activation of Na+/H+ exchange by PDGF in CHO cells. The first requires both PI 3'-kinase and PKC for maximal activity; the second is dependent upon changes in Ca\(^{2+}\).

**Fig. 5. Effect of mutations in the binding sites of PI 3'-kinase \((Y708F,Y719F)\) and PLC\(\gamma \) \((Y977F,Y989F)\) on PDGF-induced increase in Ca\(^{2+}\). To measure the release of intracellular Ca\(^{2+}\), NMuMG cells carrying PDGF receptors with the indicated mutations were acutely placed in nominally Ca\(^{2+}\)-free medium containing PDGF \((5 \text{ ng/ml})\) as indicated by the arrow. Intracellular Ca\(^{2+}\) was monitored for 10 min. For numbers of experiments, see Table I.

**Fig. 6. Mutations in the PLC\(\gamma\) binding domain of the PDGF receptor block PDGF activation of Na+/H+ exchange. PDGF \((20 \text{ ng/ml})\) was administered to NMuMG cells expressing single- or double-point mutations in the PLC\(\gamma\) binding site \((Y977F,Y989F)\) of PDGF receptor. A, representative tracing of 6 in 9 of Y977F preparations that responded to PDGF, pH increased by an average of 0.15 ± 0.04 pH unit \((n = 9)\) from a basal pH of 7.06 ± 0.04 \((n = 9)\). B, 7 in 10 of Y989F did not respond to PDGF. C, 10 of 11 preparations of Y977F,Y989F did not respond to PDGF, increasing pH, only 0.04 ± 0.02 pH unit \((n = 11, \ p < 0.05\) compared with wild type).**

**DISCUSSION**

When CHO or NMuMG cells, which both lack native PDGF receptors, were stably transfected with a cDNA for the wild-type PDGF \(\beta\)-receptor, PDGF was able to activate the Na+/H+ exchanger in these cells. This observation was exploited to study the effect of signaling mutations on the coupling of the PDGF receptor to Na+/H+ exchange. In both cell lines, PDGF B/B consistently failed to activate Na+/H+ exchange with mutants that cannot bind PLC\(\gamma\) \((\text{Fig. 6})\). Tyr 989 appears to be more important in the interaction with PLC\(\gamma\) than Tyr-977, since residual Ca\(^{2+}\) \((\text{Table I})\) and pH \((\text{Fig. 6})\) signals were present with Y977F, but not with Y989F. This finding is consistent with previous work with the human receptor, in which Ty-1021, the homolog of mouse Tyr-989, is more important for binding of PLC\(\gamma\) than is Tyr-1009 \((\text{12})\).

Activation of PLC\(\gamma\) results in production of diacylglycerol which stimulates PKC, and production of inositol triphosphate which induces Ca\(^{2+}\) release. Direct activation of PKC by PMA also induces Na+/H+ exchange in both cell lines \((\text{Fig. 7A})\), consistent with a vital role for this enzyme in activation of the exchanger. Moreover, in PKC down-regulated NMuMG cells expressing wild-type receptors, activation of Na+/H+ exchange by PDGF was absent \((\text{Fig. 7B})\), suggesting that the PKC pathway is both sufficient and necessary for activation of the exchanger by PDGF in NMuMG epithelial cells. However, in CHO cells this pathway is not the sole mechanism for PDGF induced Na+/H+ exchange, because PKC-down-regulation attenuated the response to PDGF by only 37\% in this cell line \((\text{Fig. 7})\). This result is consistent with findings made with other ligands and cell types \((5, 6, 26)\). In these cell types, there is a pathway for...
activation of Na\(^+\)/H\(^+\) exchange that is not mediated by any isoform of PKC that can be down-regulated by PMA.

Since activation of Na\(^+\)/H\(^+\) exchange appears to require PLC\(_{\gamma}\), the "PKC-independent" pathway for activation of Na\(^+\)/H\(^+\) exchange most likely involves an alternative action of diacylglycerol or an inositol phosphate. Synthetic diacylglycerols do not activate the exchanger in PKC down-regulated cells (2). In subsequent work with the wild-type PDGF receptor or a mutant unable to bind to PI 3'-kinase (Y708F,Y719F), PLC\(_{\gamma}\) was absolutely required for activation of Na\(^+\)/H\(^+\) exchange, leading to activation of PKC and Ca\(^{2+}\) mobilization which independently activate the exchanger in CHO cells. PI 3'-kinase appears to act through a pathway similar to the PKC pathway, perhaps by activating an alternative isoform of the enzyme.

Previous studies showed that a large deletion of the Ki region (ΔKi) did not interfere with activation of Na\(^+\)/H\(^+\) exchange when the mutant receptor was expressed at levels 5-fold higher than wild type (2). In subsequent work with the ΔKi mutant expressed at levels closer to wild type, activation of Na\(^+\)/H\(^+\) exchange was reduced (data not shown). Examination of point mutations in the Ki region was used to determine whether Na\(^+\)/H\(^+\) exchange is actually signaled by a molecule that binds in that region.

Tyr→Phε mutations at residues 708 and 719 prevent binding of PI 3'-kinase to the receptor (17). Receptors carrying this double mutation were clearly defective in activation of Na\(^+\)/H\(^+\) exchange (Figs. 3 and 4). This was not due to abnormally low expression of these receptors (Fig. 1) and was observed in two different cell lines. Moreover, the PI 3'-kinase mutants exhibited a normal response to phorbol esters (Fig. 7) and a normal Ca\(^{2+}\) response to PDGF (Fig. 5). Thus, the partial blockade in activation of Na\(^+\)/H\(^+\) exchange in the Y708F,Y719F mutant was not part of a global defect in early signaling events.

It has recently been found that certain PDGF receptor residues, including 708 and 719, may bind multiple signaling molecules. For example, Nck (29) and Shc (30) share binding sites with the PI 3'-kinase on the human receptor. Therefore, the unambiguous assignment of functions to specific sites on the PDGF receptor is not always possible. It seems unlikely that our results with Na\(^+\)/H\(^+\) exchange are due to one of these alternative interactions since a new inhibitor of PI 3'-kinase (LY294002) closely mimicked the effect of the Y708F,Y719F mutation. This similarity in the effect of LY294002 and the Y708F,Y719F mutation leads us to conclude that PI 3'-kinase (in addition to PLC\(_{\gamma}\)) plays an important role in activation of Na\(^+\)/H\(^+\) exchange.

PI 3'-kinase specifically phosphorylates the 3' position of the inositol ring of various phosphatidylinositols to form 3-phosphorylated compounds, including PI 3,4,5-triphosphate (31, 32). PI 3,4,5-triphosphate is a poor substrate for PLC\(_{\gamma}\) (33–35), but has recently been found to activate PKC\(\zeta\) (36), a ubiquitous isoform of PKC that does not respond to DAG or phorbol esters (37, 38). It is conceivable that the "PKC-independent" activation of Na\(^+\)/H\(^+\) exchange is through activation of PKC\(\zeta\) by PI 3,4,5-triphosphate. However, activation of the Na\(^+\)/H\(^+\) ex-
changer by PDGF was unaffected by prolonged PMA treatment of cells with the Y708F,P719Y mutation (Fig. 7H). This suggests that PI 3'-kinase actually participates in the "PKC-dependent" pathway (Fig. 8), perhaps through synergistic activation of the same isoform by PI 3,4,5-triphosphate and diacylglycerol.

In summary, we demonstrate that PLCγ is essential for activation of Na+/H+ exchange by PDGF. Activation of PKC and Ca2+ mobilization following PLCγ activation both appear to activate the exchanger, and in CHO cells, do so independently (Fig. 8). PI 3'-kinase appears to play an important modulatory role in the PKC-dependent pathway. To our knowledge, this is the first demonstration of an involvement of PI 3'-kinase in regulation of an ion transport event.

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