Up-regulation of the Pit-2 Phosphate Transporter/Retrovirus Receptor by Protein Kinase C ε*

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The membrane receptors for the gibbon ape leukemia retrovirus and the amphotropic murine retrovirus serve normal cellular functions as sodium-dependent phosphate transporters (Pit-1 and Pit-2, respectively). Our earlier studies established that activation of protein kinase C (PKC) by treatment of cells with phorbol 12-myristate 13-acetate (PMA) enhanced sodium-dependent phosphate (Na/Pi) uptake. Studies now have been carried out to determine which type of Na/Pi transporter (Pit-1 or Pit-2) is regulated by PKC and which PKC isotypes are involved in the up-regulation of Na/Pi uptake by the Na/Pi transporter/viral receptor. It was found that the activation of short term (2-min) Na/Pi uptake by PMA is abolished when cells are infected with amphotropic murine retrovirus (binds Pit-2 receptor) but not with gibbon ape leukemia retrovirus (binds Pit-1 receptor), indicating that Pit-2 is the form of Na/Pi transporter/viral receptor regulated by PKC. The PKC-mediated activation of Pit-2 was blocked by pretreating cells with the pan-PKC inhibitor bisindolylmaleimide but not with the conventional PKC isotype inhibitor Gö 6976, suggesting that a novel PKC isotype is required to regulate Pit-2. Overexpression of PKCε, but not of PKCα, -δ, or -ζ, was found to mimic the activation of Na/Pi uptake. To further establish that PKCε is involved in the regulation of Pit-2, cells were treated with PKCε-selective antisense oligonucleotides. Treatment with PKCε antisense oligonucleotides decreased the PMA-induced activation of Na/Pi uptake. These results indicate that PKCε-induced stimulation of Na/Pi uptake by Pit-2 is specifically mediated through activation of PKCε.

Protein kinases, including members of the protein kinase C family, regulate numerous biological functions, including intracellular protein trafficking and the activities of different ion transporters (1). Previously, we showed that sodium-dependent phosphate (Na/Pi)1 transport was stimulated by protein kinase C (PKC) and inhibited by protein kinase A in NIH 3T3 cells.

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‡‡ The abbreviations used are: Na/Pi, sodium-dependent phosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; A-MuLV, amphotropic murine Phorbol 12-myristate 13-acetate (PMA), an activator of PKC, was found to cause a rapid (within 10 min) stimulation of short term Na/Pi uptake. However, at that time the identity of the Na/Pi transporter stimulated in response to PMA was not known, and this prevented further characterization of the PKC-mediated activation of the transporter. More recently, cell surface receptors for the gibbon ape leukemia virus (Glvr-1 and Pit-1) and the amphotropic murine leukemia virus (Ram-1, Ear, and Pit-1) were demonstrated to serve as Na/Pi transporters in the normal cellular physiology of diverse cell types (3–5).

Amino acid sequence data obtained for the Pit-1 and Pit-2 receptor-transporters has revealed multiple sites potentially susceptible to phosphorylation by protein kinases, including PKC, which are found within the hydrophilic cytoplasmic domain of both transporters between residues 250 and 450. Indeed, parathyroid hormone-induced regulation of transporter function mediated through activation of protein kinase A and PKC has been described for type I and II Na/Pi transporters present in kidney brush-border membranes (6). In addition to parathyroid hormone, prostaglandin E2, insulin-like growth factor 1, and vitamin D3 all have been reported to regulate Na/Pi uptake through activation of PKC in osteoblasts, another cell type that uses high levels of inorganic phosphate (7–10). It also has been suggested that a phospholipase Cγ-PKC signaling pathway is responsible for the up-regulated P, transport observed with platelet-derived growth factor treatment of osteoblast-like cells. (11–13). In osteoblasts it is possible that the more ubiquitously expressed type III Na/Pi transporters, such as Pit-1 and Pit-2, might be involved in the PKC-regulated uptake of P, Converversely, activation of PKC has been reported to inhibit P, uptake in opossum kidney cells, which may indicate different regulation of the renal type I and II transporters (6, 14, 15). These differences in PKC-mediated regulation of P, uptake may be a consequence of different expression patterns of either PKC and/or Na/Pi transporter isoforms in different cell lines.

One of the major difficulties in determining which PKC isoforms are involved in regulating Na/Pi transport is that different cell types express various combinations of PKC isoforms. Protein kinase C is a family of at least 11 serine- and threonine-specific phosphotransferase isoenzymes that are characterized by a high degree of homology in their catalytic and cysteine-rich domains (1). Although the possible role(s) of different PKC isoforms in cell growth and differentiation has been well studied (for review, see Ref. 1), much less is known of their potential involvement in modulating intracellular trafficking of transport membrane receptors and up-regulation of ion transporters. In retroviruses; E-MuLV, ecotropic MuLV; GALV, gibbon ape leukemia retrovirus; AON, antisense oligonucleotide; pe-MTH, pe-metallothionein.
this study we have sought to determine which type of Na/Pi transporter/viral receptor is regulated by PMA activation of PKC and which PKC isotype(s) may be involved in the up-regulation of Na/Pi uptake in NIH 3T3 cells. The results presented in this communication indicate that the Pit-2 Na/Pi transporter/viral receptor is specifically activated by PMA stimulation of PKC.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were purchased from Biofluids Inc. (Rockville, MD). PMA, bisindolylmaleimide, and Gö 6976 were products of Calbiochem (San Diego, CA). [32P]-Labeled mononucleotide phosphate was from ICN (Costa Mesa, CA). The PKC isotype-specific antisense and scrambled oligonucleotides (ISIS 17260, PKCe antisense; ISIS 17261, PKCe scrambled control) were from ISIS Pharmaceuticals. PKCe isotype-specific polyclonal antibodies were purchased from Life Technologies, Inc.; PKCα-specific monoclonal antibodies were from Upstate Biotechnology (Lake Placid, NY); and PKCe- and PKCε-specific monoclonal antibodies were from Transduction Laboratories (Lexington, KY).

Cell Culture—Retrovirus-infected and vector-transduced NIH 3T3 cells were cultured in DMEM supplemented with 10% fetal calf serum. After the cells reached confluency, the medium was changed to serum-free DMEM for 24 h. To induce overexpression of any ectopic gene products, the cells were incubated in the presence or absence of 20 μM zinc acetate, as indicated, to induce the up-regulation of the metallothionein promoter of the pMTH vector (16).

Generation of Overexpressor Cell Lines—The construction of expression vectors and establishment of PKC overproducer cell lines were carried out as described previously (17). The PKCa, -δ, -ε, and -ζ plasmid constructs were prepared in the pMTH vector, and overexpressor cell lines were established following protocols described elsewhere (18). Individually picked colonies (10 from each transfection) were selected and combined for further studies to eliminate potential cloning artifacts. These mixed populations of overexpressor cells were used only through 12-14 passages in culture to negate possible outgrowth of one particular clone.

Phosphate Uptake Measurement—Sodium-dependent phosphate uptake was determined as described previously (2). Retrovirus Infections—NIH 3T3 murine fibroblasts and mink lung fibroblasts (ATCC CCL 64) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum. NIH 3T3 cells were infected with wild type amphotropic murine retrovirus (A-MuLV) strain 4070A or the 57A Friend strain of ecotropic MuLV (E-MuLV). Mink fibroblasts expressing gibbon ape leukemia virus (GALV)-competent PIT-1 were infected with wild type A-MuLV strain 4070A or infected with a GALV strain (SEATO), as described previously (4). Productive infection was monitored by measuring the reverse transcriptase activity found in the cell media of the infected cells (19).

Antisense Oligonucleotide Treatment—NIH 3T3 fibroblasts were cultured in 150-mm tissue culture dishes until they reached ~80% confluency. The cells were then harvested by trypsinization, washed with DMEM, and resuspended in 400 μl of cytosalt electroporation buffer (75% cytosol (120 mM KCl, 0.15 mM CaCl2, 10 mM K2HPO4, pH 7.6, 6.5 mM MgCl2) and 25% Opti-MEM I). Twenty-μl aliquots of PKC isotype-specific or scrambled control oligonucleotides were added to the cells resuspended in prechilled BTX disposable electroporation cuvettes (BTX 640; 4-mm gap) to reach the indicated concentrations and then incubated on ice for 5 min. The oligonucleotides indicated were introduced into the cells by electroporation with a BTX Electro Square Porator (settings: low voltage mode, 99 mesc; charge voltage, 475 V; pulse length, 1 msec; number of pulses, 4). The electroporated cells were kept at room temperature for 10 min and then seeded onto 100-mm tissue culture plates for immunoblot studies and onto 24-well plates for Pi uptake measurements. Western blot analysis of PKC isotypes and Pi transporters, including Pit-1, Pit-2, and the cationic amino acid transporter CAT/y+, was carried out 24 h after introduction of the antisense oligonucleotides.

Western Blot Analysis—Proteins were separated by precast 4–20% SDS-polyacrylamide gel (Owl Separation Systems, Portsmouth, NH) electrophoresis and electrophoretically transferred from the gel onto Protran membranes (Schleicher & Schuell, Keene, NH), and immunoreactive proteins were detected as described elsewhere (16, 18).

RESULTS AND DISCUSSION

Most retroviruses have been found to use distinct cell surface receptors for specific cellular recognition and infection (for review, see Ref. 20). Furthermore, studies have revealed that the normal cellular function of a number of viral receptors is to serve as membrane transport proteins (20). NIH 3T3 cells have been found to express a number of these viral receptor/transporters, including Pit-1, Pit-2, and the cationic amino acid transporter CAT/y+, as determined by viral infection studies and reverse transcription-polymerase chain reaction analysis. NIH 3T3 cells are susceptible to infection by A-MuLV via Pit-2 and to infection with E-MuLV via the CAT/y+ amino acid transporter. However, because of the presence of specific point mutations in the endogenous murine Pit-1, Pit-1 is not functional as a GALV receptor in NIH 3T3 cells.

Effect of Viral Infection on PMA-induced Activation of Phosphate Transport—Previous studies have established that infection of cells with retroviruses that selectively recognize either Pit-1 or Pit-2 resulted in the specific down-modulation of phosphate uptake mediated by that receptor/transporter (3, 5). A similar phenomenon has been observed to occur with the E-MuLV CAT/y+ receptor (21, 22). To examine which of the viral receptor/Pi transporters are subject to regulation by PMA activation of PKC, studies were carried out with NIH 3T3 cells infected with different C-type retroviruses. The results presented in Fig. 1A demonstrate that infection with A-MuLV decreased short term (2-min) basal Na/Pi transport (from 900 ±
Thus, to control for possible pleiotropic effects of retrovirus distinct cell surface cationic amino acid transporter (21, 22). PKC isozymes (PKC\(\alpha\), PKC\(\beta\), PKC\(\epsilon\), PKC\(\zeta\), PKC\(\delta\), and PKC\(\gamma\)), isotype(s) are involved in mediating the PMA-induced up-regulation of the Pit-2 transporter/receptor in NIH 3T3 cells. NIH 3T3 cells express a limited, but representative, set of different PKC isoforms (PKC\(\alpha\), PKC\(\beta\), PKC\(\epsilon\), and PKC\(\zeta\)) (18, 23). Because PKC\(\alpha\) does not directly activate atypical PKC\(\zeta\), this isoform would not appear to be involved in the short term PMA-induced activation of Pit-2 P\(i\) transport. Thus, experiments were initiated to address which class of PKC isoform stimulates P\(i\) transport in response to activation by PMA. Two different PKC inhibitors were used to determine whether the PMA-induced activation of P\(i\)/P\(m\) transport in NIH 3T3 cells was mediated via a conventional, Ca\(^{2+}\)-dependent (PKC\(\alpha\)), or a novel, Ca\(^{2+}\)-independent (PKC\(\delta\) and PKC\(\epsilon\)) isotype. Addition of the pan-specific bisindolylmaleimide inhibitor, which inhibits both classical and novel isoforms, resulted in pronounced inhibition of the PMA-induced activation of P\(i\)/P\(m\) uptake (Fig. 2). Conversely, treatment of the cells with the Gö 6976 PKC inhibitor, which selectively inhibits only the classical PKC isoforms, did not cause significant inhibition of the PMA-induced up-regulation of P\(i\)/P\(m\) transport. These results suggested that the classical PKC isoforms were not involved in mediating the activation of P\(i\)/P\(m\) uptake by Pit-2 with exposure of NIH 3T3 cells to PMA.

Effect of Overexpression of PKC Isoforms on PMA-induced Stimulation of Pit-2 P\(i\)/P\(m\) Uptake—To further resolve which of the PKC isoform(s) may be involved in mediating the PMA activation of the Na/P\(i\)/P\(m\) uptake, we used NIH 3T3 cells overexpressing PKC\(\alpha\), -\(\epsilon\), -\(\delta\), or -\(\zeta\) isozymes to determine the ability of each isotype to enhance Pit-2 P\(i\)/P\(m\) transport activity in the absence of PMA treatment. The cell cultures were shifted to serum-free media and incubated in the presence of 20 \(\mu\)M zinc acetate for 24 h to enhance expression of the indicated PKC isotype directed by the metallothionein promoter of the phagemid MTH vector. Overexpression of PKC\(\epsilon\) was found to increase Na/P\(i\) uptake, whereas overexpression of PKC\(\alpha\), PKC\(\delta\), and PKC\(\zeta\) did not appreciably alter the level of P\(i\)/P\(m\) transport relative to the level determined in vector control cells (Fig. 3A). Exposure of the PKC\(\epsilon\) overexpressor cells to 1 \(\mu\)M PMA resulted in only an additional 15–20% increase in P\(i\)/P\(m\) transport. Western blot analysis showed that the level of expression was similar for each of the \(\epsilon\) epitope-tagged PKC isoforms (Fig. 3B). These results indicate that the selective overexpression of PKC\(\epsilon\) alone can mimic the stimulation of Na/P\(i\)/P\(m\) uptake observed with PMA treatment of wild type cells.

Effect of PKC- and PKC\(\delta\)-Specific Antisense Oligonucleotides on PMA-induced Stimulation of Pit-2 P\(i\)/P\(m\) Transport—To further support the findings that PKC\(\epsilon\) is the isoform involved in mediating the PMA-induced activation of the Pit-2 P\(i\)/P\(m\) transporter, studies were carried out with PKC\(\epsilon\)-selective antisense oligonucleotide (AON) to specifically inhibit PKC\(\epsilon\) in the cell. As shown in Fig. 4A, pretreatment of NIH 3T3 cells with increasing concentrations of PKC\(\epsilon\)-AON significantly decreased the expressed levels of PCK\(\epsilon\) and had no effect on the levels of PKC\(\alpha\), PKC\(\delta\), or PKC\(\zeta\). Densitometric scanning to quantitate the intensity of the PKC bands of cells treated with PKC\(\epsilon\)-AON relative to AON scrambled controls indicated relative band densities of 1.0, 0.59, and 0.28 with 0.24, 1.2, and 2.4 \(\mu\)M oligonucleotide treatment, respectively. Importantly, treatment of the cells with PKC\(\epsilon\)-AON did result in inhibition of PMA-induced up-regulation of P\(i\)/P\(m\) transport (Fig. 4B). Treatment of cells with scrambled oligonucleotides did not have any effect on either the intracellular level of PKC\(\epsilon\) or PMA activation of Na/P\(i\)/P\(m\) uptake. Similar experiments with PKC\(\delta\)-AON (ISIS 17254) caused a significant decrease in the levels of PKC\(\delta\) but had no effect on PMA-induced stimulation of P\(i\)/P\(m\) uptake (data not shown). These data provide additional evidence to support the exclusive involvement of PKC\(\epsilon\) in mediating PMA activation of Pit-2.

The suggestion that different PKC isoforms play distinct functional roles in the cell by phosphorylating either isoform- or subcellular compartment-specific substrates is widely accepted. However, few studies have been reported that establish...
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that a specific PKCe isotype may selectively regulate a given biological function. Although a role for PKCe has been implicated in the regulation of numerous membrane transport mechanisms (10, 24–27), little information is available on the specific PKCe isotype(s) involved in the regulation of these transport activities. Karim et al. (28) attributed the modulation of the Na/H antiport to both PKCe and PKCd. PKCe also has been implicated in the stimulation of anionic amino acid transport (29), and treatment with antisense oligonucleotides to PKCe has been shown to block the α1-adrenergic activation of Na-K-2Cl cotransport (30). The evidence reported here indicates that PKCe is involved in mediating the PMA-induced up-regulation of Na/Pi uptake by the Pit-2 transporter/viral receptor.

The Pit-1 and Pit-2 viral receptor/Pit transporters share 56% amino acid identity (31). Hydropathy analysis of Pit-1 and Pit-2 suggested the presence of at least two clusters of putative transmembrane helices, along with a large intracellular hydrophilic domain located between the sixth and seventh transmembrane helices (4, 19). There are a number of consensus phosphorylation sites in both Pit-1 and Pit-2, particularly within the hydrophilic loop domain. Thus, it is likely that PKCe may directly phosphorylate Pit-2 to stimulate Na/Pi uptake.

However, another mechanism of regulation found with other transporters is induced redistribution of the transporter from intracellular stores to the plasma membrane. For example, insulin has been reported to regulate the intracellular trafficking of glucose transporter 4 (32). Previously, we have shown that PKCe can regulate Golgi-related functions, including protein trafficking and secretion (17). To address this possibility, studies were carried out to determine whether PMA still was able to enhance Pi uptake under conditions in which vesicle trafficking from the Golgi to the plasma membrane was blocked by incubating cells at room temperature and by treatment of cells with nocodazole (to disrupt microtubules) and cytochalasin D (to disrupt actin filaments). It was found that these treatments did not block PMA-induced activation of Pi uptake. Although these results indicate that PKCe does not act by modulating the trafficking of Pit-2 from the Golgi to the plasma membrane, they do not fully rule out the possibility that PKCe might act to mediate the rapid recruitment (fusion) of an existing pool of Pit-2-containing vesicles to the plasma membrane.

In addition to their role as representative members of an important family of phosphate transporters, Pit-1 and Pit-2 are...
of particular interest as the cell surface receptors for the GALV and A-MuLV retroviruses, respectively. Many current gene therapy protocols use GALV- or A-MuLV-enveloped vectors (33, 34). A basic knowledge of murine leukemia virus receptor regulation and trafficking is likely to be useful in the development and improvement of gene therapy protocols based on the use of these retroviral vectors. Although our results clearly indicate that PKCe is involved in regulating Na/Pi uptake by Pit-2, it remains to be determined whether PKCe-mediated regulation of the Pit-2 transporter/viral receptor might influence recognition of the viral envelope protein and viral entry into the cell.

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