Platelet-derived growth factor regulates K-Cl cotransport in vascular smooth muscle cells

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Zhang, Jing, Peter K. Lauf, and Norma C. Adragna. Platelet-derived growth factor regulates K-Cl cotransport in vascular smooth muscle cells. Am J Physiol Cell Physiol 284: C674–C680, 2003; 10.1152/ajpcell.00312.2002.—Platelet-derived growth factor (PDGF), a potent serum mitogen for vascular smooth muscle cells (VSMCs), plays an important role in membrane transport regulation and in atherosclerosis. K-Cl cotransport (K-Cl COT/KCC), the coupled-movement of K and Cl, is involved in ion homeostasis. VSMCs possess K-Cl COT activity and the KCC1 and KCC3 isoforms. Here, we report on the effect of PDGF on K-Cl COT activity and mRNA expression in primary cultures of rat VSMCs. K-Cl COT was determined as the Cl-dependent Rb influx and mRNA expression by semiquantitative RT-PCR. Twenty-four-hour serum deprivation inhibited basal K-Cl COT activity. Addition of PDGF increased total protein content and K-Cl COT activity in a time-dependent manner. PDGF activated K-Cl COT in a dose-dependent manner, both acutely (10 min) and chronically (12 h). AG-1296, a selective inhibitor of the PDGF receptor tyrosine kinase, abolished these effects. Actinomycin D and cycloheximide had no effect on the acute increase in K-Cl COT activity but had inhibitory effects on the chronic increase in K-Cl COT activity. PDGF-induced increased KCC1 mRNA expression was concentration dependent, whereas PDGF-induced decreased KCC3 mRNA expression occurred in a time-dependent manner. These results indicate that chronic activation of PDGF-COT by PDGF may involve regulation of the two KCC mRNA isoforms, with KCC1 playing a dominant role in the mechanism of PDGF-mediated activation.

K-CL COT, the electroneutral coupled movement of K and Cl driven by their chemical gradients (21–23), plays an important role in maintaining cell volume and ion homeostasis by mediating the efflux of K and Cl and obligatory water outflow under most physiological conditions (22, 23). Regulation of K-Cl COT has been extensively studied in erythrocytes and other cell types (22, 23). We recently found that K-Cl COT is expressed in vascular smooth muscle cells (VSMCs) and is activated by the NO/cGMP/PKG pathway in both low-K sheep red blood cells and VSMCs (2–4). Furthermore, we found that two of the five mRNA isoforms, KCC1 and KCC3, are present in cultured VSMCs and that their expression is also regulated by the NO/cGMP/PKG pathway (6, 7).

Serum plays multiple roles in cultured cells, including regulation of membrane transporters (26, 29). Serum was reported to stimulate the Na/H exchanger through phosphorylation in resting hamster fibroblasts and A431 human epidermoid cells (29). In quiescent human fibroblasts, serum and growth factors stimulated the bumetanide-sensitive Na-K-2Cl cotransporter in a dose-dependent manner (26). However, there is little knowledge on the regulation of K-Cl COT by these effectors.

Platelet-derived growth factor (PDGF) is a major serum growth factor for mesenchymal cells (13, 27). It is known as a potent chemoattractant and mitogen for VSMCs (13) and is involved in the migration and proliferation of VSMCs during atherosclerosis (28). The PDGF protein molecule is composed of two polypeptide chains linked by an intermolecular disulfide bond (14). To date, five isoforms, PDGF-AA, -AB, -BB, -CC, and DD, have been identified (10, 14, 20). They bind differentially to two monomeric receptor subunits, PDGF-α and PDGF-β. The PDGF-A and -C selectively bind to the α-receptor (10), whereas the PDGF-B chain can bind to both α- and β-receptors (14). PDGF-D binds to PDGF-β but not to α in cells expressing individual PDGF receptors (20). Upon PDGF binding, the receptor monomers dimerize and autophosphorylate on tyrosine residues, which in turn relay the signals to intracellular mediators (12). AG-1296, a protein tyrosine kinase inhibitor of the tyrophostin family, selectively inhibits signaling of PDGF-α and β receptors (18).

PDGF induces the expression of unique genes in different cell types. For instance, PDGF induces an increase in c-myc mRNA expression in fibroblasts (17) and c-fos gene expression in BALB/c-3T3 cells (5). Induction of voltage-dependent sodium channel mRNA by PDGF has been studied in PC12 cells stably expressing mutant PDGF-β receptors (9). PDGF also upregulates the transport of L-ornithine, the cationic amino acid precursor of polyamines, by inducing gene expression of the cationic amino acid transporter (CAT)-1 and -2B in VSMCs (8). Here, we study the regulation of K-Cl COT activity and KCC gene expres-

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sion by PDGF in primary cultures of VSMCs. We found that PDGF increased K-Cl COT activity both acutely and chronically through its membrane receptor. In addition, PDGF increased KCC1 but decreased KCC3 mRNA expression. These results indicate that the acute stimulation of K-Cl COT activity by PDGF occurs at the posttranslational level, whereas the chronic stimulation may involve regulation of the two KCC mRNA isoforms, with KCC1 playing a dominant role in the mechanism of PDGF-mediated activation.

MATERIALS AND METHODS

Materials. PDGF-BB was from Sigma (St. Louis, MO). Dulbecco's modified Eagle's culture medium (DMEM), TRIZol reagent for total RNA extraction, and all tissue culture grade or molecular biology reagents were purchased from Life Technologies (Grand Island, NY). The access RT-PCR kit and the specific rat actin primer set were from Promega (Madison, WI). AG-1296, actinomycin D, and cycloheximide were from Calbiochem (La Jolla, CA). The bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL).

Primary culture of rat VSMCs. Published procedures were used to obtain primary cultures of rat aortic SMCs (3). Briefly, aortas from Sprague Dawley rats were provided by the Wright State University Animal Facilities. Aortas were digested in collagenase for 30 min at 37°C and the tunica adventitia was peeled away. The remaining intima and medial layers were cut into small segments and placed in a collagenase/trypsin solution. These segments were incubated in a 37°C shaking water bath until a single cell suspension was obtained. Cells were spun down and the pellet was resuspended in DMEM with 10% FBS. The cells were plated into T-75 tissue culture flasks and the medium was changed gently after 3–4 h. VSMCs were grown in DMEM + 10% fetal bovine serum (FBS), penicillin (50 units/ml), and streptomycin (50 μg/ml) and maintained in a fully humidified incubator supplied with 5% CO2 at 37°C. VSMCs at passages 5–6 were used in this study.

K-Cl COT measurement. A previously published flux protocol has been modified for this study (3). Cells were grown on 12-well plates until 90% confluence and then serum deprived for 24 h before the experiments were done. Cells were washed and preincubated for 15 min in balanced salt solution (BSS) containing (in mM) 130 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, and 20 HEPES/Tris (pH 7.4 at 37°C). Sulfamate (Cl-free) solutions contained Na- and K-sulfamate and Ca- and Mg-gluconate salts. Rb influx was performed at 37°C in BSS solution containing 10 mM Rb in the presence of 1 mM ouabain, 30 μM bumetanide, 2 mM BaCl2, and 10 μM GdCl3 as inhibitors of the Na/K pump, Na-K-2Cl cotransport, and channel fluxes, respectively. The difference between the Rb fluxes in Cl and sulfamate media was calculated as the Cl-dependent Rb influx or K-Cl COT. Rb uptake was measured by flame emission spectrophotometry in a Perkin-Elmer 5000 atomic absorption spectrophotometer. Rb influx was finally normalized by the amount of protein measured by the BCA method (33, 34).

Total RNA extraction, RT-PCR analysis, and KCC mRNA expression in VSMCs. Cells were grown on six-well plates until confluence and then serum deprived for 24 h before starting the experimental protocol. Total RNA extraction and RT-PCR conditions were as described elsewhere (7). Total RNA from primary cultures of rat VSMCs were obtained by using TRIzol reagent following the instructions of the manufacturer. RNA (0.5 μg) was used for each reaction. Specific sets of primers for KCC1 and KCC3 mRNA were synthesized according to the sequences previously published (7). Negative RT-PCR controls were performed in the absence of reverse transcriptase (data not shown). The RT-PCR products were analyzed by 2% agarose gel electrophoresis.

Statistical analysis. A minimum of two independent experiments with triplicate samples/condition was assayed for all the results, which were expressed as mean ± SD or SE. Statistical significance between two groups was conducted by the Student's t-test. A P value of <0.05 was used as the criterion of statistical significance.

RESULTS

Basal K-Cl COT activity. Basal K-Cl COT activity is defined as the Cl-dependent Rb influx and is calculated as the difference between Rb influx in Cl and sulfamate. However, in some of the experiments reported in this study, the basal Rb influx in sulfamate was higher than in Cl under certain experimental conditions. The absence of a basal Cl-dependent flux has been reported before in different cell types and species and with different techniques used to determine K-Cl COT activity (1, 16, 24, 30). The reason for the lack of basal Cl-dependent flux under certain experimental conditions is not clear at this point and is under investigation. In the present study, we tested different Cl substitutes, and sulfamate was chosen as the replacement anion that gave the highest Cl-dependent Rb influx with PDGF under our experimental conditions (results not shown). As will be shown below, it appears that the basal component behaves differently from the PDGF-activated cotransporter after short- and long-term treatment with the drug.

Serum factors are important to maintain basal K-Cl COT activity. Serum plays multiple roles in the regulation of the activity of ion channels and transporters in cultured cells (26, 29). To examine whether serum has an effect on basal K-Cl COT activity in primary cultures of rat VSMCs, cells were serum deprived for 24 h and K-Cl COT activity was subsequently measured. Figure 1 shows the Rb influx in VSMCs in the presence of 10% FBS and after serum deprivation for 24 h. Serum deprivation abolished basal K-Cl COT activity. This result indicates that serum factors are important to maintain basal K-Cl COT activity in VSMCs.

PDGF increased the total protein content of cultured VSMCs. PDGF is a major growth factor in serum. It is also a potent chemoattractant and mitogen for VSMCs. We examined the effect of PDGF on the total protein content, which is used to normalize K-Cl COT activity in flux experiments. Figure 2 shows the effect of PDGF (100 ng/ml) on the total protein content of cultured VSMCs as a function of time. PDGF significantly increased the total protein content after 12 h of treatment with the drug. Controls were incubated for the same period of time with vehicle alone. No statistically significant effect with respect to zero time was found (data not shown).
Increase in K-Cl COT activity by PDGF as a function of time. Flux experiments were then conducted to examine the effect of PDGF (100 ng/ml) on RbCl influx as a function of time (Fig. 3). PDGF increased K-Cl COT activity after 10 min of treatment, and the activation remained up to 12 h. To understand the mechanism of activation by PDGF, K-Cl COT activity was studied after short-term (in minutes, acute) and long-term (in hours, chronic) treatment with the growth factor.

Acute increase in K-Cl COT activity by PDGF in VSMCs. The acute effect of PDGF on K-Cl COT activity was studied by incubating VSMCs in the presence of 0–100 ng/ml PDGF for 10 min. Figure 4 shows the Rb influx measured in Cl and sulfamate as a function of the PDGF concentration. As shown in the figure, PDGF increased K-Cl COT activity in a dose-dependent manner, with an EC50 around 5 ng/ml. Coincubation of PDGF with AG-1296, a selective inhibitor of the PDGF receptor tyrosine kinase, abolished the PDGF-induced increase in K-Cl COT activity (Fig. 5). AG-1296 alone did not have any effect on K-Cl COT activity as a function of time and concentration (data not shown).

Fig. 1. Effect of serum on Rb influx in primary cultures of rat vascular smooth muscle cells (VSMCs). Culture of cells and determination of Rb influx was as described in MATERIALS AND METHODS. Cells 90% confluent were serum deprived for 24 h before the flux experiment. After the initial wash, cells were preincubated in isotonic buffer saline salt (BSS) (See MATERIALS AND METHODS for composition) for 15 min. Rb uptake was measured for 5 min at 37°C. Data represent means ± SE from 5 independent experiments. K-Cl COT, K-Cl cotransport.

Fig. 2. Effect of platelet-derived growth factor (PDGF)-BB on the total protein content in rat VSMCs. Cells at 90% confluence were serum deprived for 24 h. The cells were then exposed to PDGF (100 ng/ml) during the indicated time points, and the total protein content was determined by the bicinchoninic acid (BCA) method following the manufacturer's instruction. Data represent means ± SE, n = 6. *P < 0.05 with respect to time 0.

Fig. 3. Effect of PDGF-BB on K-Cl COT activity as a function of time in rat VSMCs. Culture of cells and determination of Rb influx was as described in MATERIALS AND METHODS. Cells were serum deprived for 24 h and then incubated with PDGF (100 ng/ml) for the indicated periods of time. Rb uptake was measured for 5 min at 37°C. Data represent means ± SE from 3 independent experiments.

Fig. 4. Acute effect of PDGF-BB on K-Cl COT activity in rat VSMCs. Culture of cells and determination of Rb influx was as described in MATERIALS AND METHODS. Cells were serum deprived for 24 h before the experiment. After the initial wash and preincubation in isotonic BSS for 10 min, cells were preincubated with PDGF for 5 min at the indicated concentrations. Rb uptake was measured for 5 min at 37°C in the presence of PDGF at the same concentrations as in the preincubation. Data represent means ± SE from 3 independent experiments. ●, Cl; ▼, sulfamate; ●, Cl-dependent Rb influx or K-Cl COT.
These results indicate that PDGF acutely activated K-Cl COT through its membrane receptor.

To examine whether the acute regulation involves nongenomic action of PDGF, we preincubated the cells with actinomycin D or cycloheximide. As shown in Figs. 6 and 7, neither actinomycin D (10 μg/ml) nor cycloheximide (10 μg/ml) affected PDGF-stimulated K-Cl COT activity. Preincubation with inhibitors alone did not have any effect on the activity of the cotransporter (data not shown).

Chronic increase in K-Cl COT activity by PDGF in VSMCs. The chronic effect of PDGF on K-Cl COT activity was studied by incubating VSMCs with PDGF for 12 h. Figure 8 shows the Rb influx measured in Cl and sulfamate as a function of the PDGF concentration. PDGF increased K-Cl COT activity in a dose-dependent manner, with an EC50 of ~10 ng/ml. In contrast to its acute effect, chronic treatment of VSMCs with PDGF activated K-Cl COT by decreasing the Rb uptake in sulfamate, i.e., by maintaining Rb flux constant in Cl medium (see DISCUSSION for interpretation of these results). Coincubation of PDGF with AG-1296 abolished the PDGF-induced K-Cl COT activation (Fig. 9). These results indicate that chronic...
treatment of VSMCs with PDGF activated K-Cl COT also through its membrane receptor but appeared to behave differently from its acute effect on noncotransporter-mediated Rb in flux.

Effect of PDGF on KCC1 and KCC3 mRNA expression in VSMCs. To examine whether the chronic effect of PDGF involves regulation of mRNA expression, we performed semiquantitative RT-PCR. Figure 8 shows the effect of PDGF (20 ng/ml) on the KCC1 and KCC3 mRNA expression levels in VSMCs. PDGF increased KCC1 mRNA expression in a time-dependent and saturating fashion, by 50% above the level of the control after 12-h treatment with the drug. In contrast, PDGF decreased KCC3 mRNA expression with increasing incubation times, by ~35%. These results suggest that chronic activation of K-Cl COT activity by PDGF may involve both upregulation of KCC1 mRNA expression and downregulation of KCC3 mRNA expression, but the KCC1 isoform may play a dominant role in the chronic effect of PDGF on K-Cl COT activation (Fig. 10).

This is the first report on regulation of K-Cl COT by growth factors and, specifically, by PDGF in primary cultures of VSMCs. In conclusion, PDGF activated K-Cl COT both acutely and chronically through its membrane receptor and oppositely modulated the two KCC isoforms by increasing KCC1 and decreasing KCC3 mRNA expression in VSMCs.

DISCUSSION

Serum regulates membrane channels (25) and transporters (26). In the present study, we found that serum factors were important to maintain the basal activity of K-Cl COT in VSMCs because serum deprivation for 24 h abolished this activity (Fig. 1). These results indicated that serum factors might play a role in the regulation of the cotransporter. A similar phenomenon was observed in the regulation of the Na-K-2Cl cotransporter in human skin fibroblasts (26). In that study, both the ouabain-sensitive and the bumetanide-sensitive Rb fluxes were decreased by almost 50% after 1 day of serum deprivation compared with zero time.

PDGF, a major growth factor in serum for mesenchymal cells (13), increased the total protein content of VSMCs (Fig. 2) and activated K-Cl COT both acutely and chronically (Figs. 4 and 6). Activation of K-Cl COT by PDGF is significantly faster than the increase of protein content, suggesting a possible role of K-Cl COT in cell proliferation. This result is in agreement with a recent report proposing that in transfected NIH/3T3 cells, the K-Cl COT isoform KCC3 plays an important role in cell growth regulation (30).
Signal transduction of PDGF is through its membrane receptor. Binding of PDGF to its receptor rapidly activates the receptor tyrosine kinase through dimerization and autophosphorylation (14). AG-1296 is a member of the quinoxalin-type tyrphostin family. It selectively inhibits the PDGF receptor tyrosine kinase and PDGF-dependent DNA synthesis in Swiss 3T3 cells and porcine aorta endothelial cells (18). AG-1296 abolishes the autophosphorylation of the PDGF receptor without interfering with PDGF binding or receptor dimerization, indicating that its specific inhibitory effect is on the catalytic activity of the receptor tyrosine kinase (19). In the present work (Figs. 5 and 9), the IC\textsubscript{50} of AG-1296 on both acute and chronic PDGF-induced K-Cl COT activity in rat aortic SMCs was about 5 \( \mu \)M, which is higher than that reported for Swiss 3T3 cells (0.3–0.5 \( \mu \)M) (18). This difference may be due to the different target, cell system, and/or methodology.

In VSMCs, PDGF activated K-Cl COT both acutely and chronically (Figs. 4 and 8), but the activation pattern was different. PDGF increased Rb influx in Cl during the acute effect (Fig. 4), whereas it decreased Rb influx in sulfamate during the chronic effect (Fig. 8). Others have also reported different cellular effects induced by short- and long-term exposure to PDGF. Short-term exposure (3–60 min) of VSMCs to PDGF induces only \(^{[3]}\)H\textsubscript{thymidine} incorporation without an increase in protein and cell number, whereas long-term exposure results in proliferation (11). Short-term regulation of membrane transporters by PDGF was reported to occur through phosphorylation and translocation of the protein (15, 31), whereas long-term regulation is through gene expression (8, 32). Our results indicate that acute stimulation of K-Cl COT activity by PDGF occurs at the posttranslational level (Figs. 6 and 7), whereas the chronic stimulation may involve regulation of the two KCC mRNA isoforms (Fig. 10), with KCC1 playing a dominant role in the mechanism of PDGF-mediated activation. We found that actinomycin D and cycloheximide had no effect on the acute activation of K-Cl COT by PDGF (Figs. 6 and 7), strongly suggesting a posttranslational regulation mechanism of the growth factor.

In the present study, existence of an unidentified VSM cationic channel/s through which Rb permeates may explain the different phenomena described in Figs. 4 and 8. This putative Rb channel/s, which mediates Rb influx in both Cl and sulfamate, appears to be inhibited by long-term incubation with PDGF but not affected by short-term exposure to the drug. After acute treatment, PDGF activated K-Cl COT in Cl, but not in sulfamate, in which the cotransporter is not functional. However, chronic treatment with PDGF not only activated K-Cl COT but also inhibited the putative Rb channel. This inhibition appeared to mask the activation of K-Cl COT in Cl and cause the decrease of Rb influx in sulfamate. The nature of this putative Rb channel is still unknown and is under further investigation. The above results are summarized in Fig. 11.

Furthermore, it is important to note that the absence of a Cl-dependent Rb influx, described in RESULTS, was only observed under baseline conditions [no serum (Fig. 1) or 0 ng/ml PDGF (Figs. 4 and 8)]. However, PDGF induced Cl-dependent Rb influxes that were always positive (Figs. 4 and 8).

Regulation of Na/Ca exchanger mRNA expression was reported in VSM (32). In primary cultures of rat VSMCs, two KCC isoforms KCC1 and KCC3 are detected by RT-PCR (7). In the present study, PDGF regulated both KCC1 and KCC3 mRNA expression in VSMCs (Fig. 10). It is interesting to note that PDGF regulated these two isoforms in opposite directions. KCC1 is claimed to be the housekeeping isoform that exists in all the cell types reported so far. In VSMCs, basal mRNA expression of KCC1 is twofold higher than KCC3, as determined by RT-PCR in our laboratory (7). In the present study, PDGF activated VSMC K-Cl COT to saturation after 10 min of treatment (Fig. 3), commensurate with its regulation of KCC1 mRNA expression (Fig. 10). These results indicate that up-regulation of KCC1 mRNA expression may be more important for the increase in K-Cl COT activity by PDGF. It is worth noting that the magnitude of increase in KCC1 mRNA expression is less than the increase in K-Cl COT activity, so the investigation of additional mechanisms at the posttranslational level may be reasonable in a future study.

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REFERENCES


