Identification of the Mechanism for the Inhibition of Na^+,K^+-Adenosine Triphosphatase by Hyperglycemia Involving Activation of Protein Kinase C and Cytosolic Phospholipase A_2

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Abstract

Inhibition of Na^+,K^+-ATPase activity by hyperglycemia could be an important etiological factor of chronic complications in diabetic patients. The biochemical mechanism underlying hyperglycemia's inhibitory effects has been thought to involve the alteration of the protein kinase C (PKC) pathway since agonists of PKC can normalize hyperglycemia-induced inhibition of Na^+,K^+-ATPase activity. Paradoxically, elevated glucose levels and diabetes have been shown to increase PKC activities in vascular cells. The present study tested the hypothesis that the inhibition of Na^+,K^+-ATPase activity is mediated by the sequential activation of PKC and cystolic phospholipase A_2 ($cPLA_2$). In cultured rat vascular smooth muscle cells (VSMC), increasing glucose levels in the medium from 5.5 to 22 mM elevated $cPLA_2$ activity and increased [^{3}H]arachidonic acid release and PGE_2 production by 2.3-, 1.7- and 2-fold, respectively. Similar increases in $cPLA_2$ activity were also induced by elevated glucose levels in human VSMC and rat capillary endothelial cells. The activation of $cPLA_2$ was mediated by PKC since the increases in $cPLA_2$ phosphorylation and enzymatic activity were inhibited by the PKC inhibitor GFX. In contrast, elevation of glucose levels decreased Na^+,K^+-ATPase activity as measured by ouabain-sensitive ^{86}Rb uptake by twofold in rat VSMC. Surprisingly, both PMA, a PKC agonist, and GFX, a PKC inhibitor, were able to prevent glucose-induced decreases in ^{86}Rb uptake. Further, the PLA_2 inhibitor $AACOCF_3$ abolished both glucose-induced activation of $cPLA_2$ and the decrease in ^{86}Rb uptake. These data indicated that hyperglycemia is inhibiting Na^+,K^+-ATPase activity by the sequential activation of PKC and $cPLA_2$, resulting in the liberation of arachidonic acid and increased the production of PGE_2, which are known inhibitors of Na^+,K^+-ATPase.(*J. Clin. Invest.* 1995. 96:733–740.)

Key words: diabetes • vascular cells • protein kinase C • phospholipase A_2 • Na^+,K^+-ATPase

Introduction

Na^+,K^+-ATPase is an integral component of the sodium pump and is critically involved in the maintenance of cellular integrity as well as the regulation of cellular activities and functions, such as contractility, growth, and differentiation (1). Numerous reports have documented that Na^+,K^+-ATPase activity is decreased significantly in the vasculature of diabetic patients and experimental diabetic animals (2–5), suggesting abnormalities in metabolic parameters as causal factors in regulating this essential enzyme activity. Amongst the numerous metabolic factors which are altered in the diabetic state, the most important causal factor for the development of vascular complications is probably hyperglycemia as indicated by multiple clinical and epidemiological studies (2, 6). Normalization of hyperglycemia by insulin therapy has also been reported to prevent the reduction in Na^+,K^+-ATPase activity in vivo (7, 8). Using cultures of both vascular and nonvascular cells, multiple reports have found that elevated glucose concentration can reduce Na^+,K^+-ATPase activity, further supporting the involvement of hyperglycemia (3–5).

The biochemical mechanism by which elevated glucose level reduces Na^+,K^+-ATPase activity has been the topic of multiple studies, but the results have been confusing with regard to the role of protein kinase C (PKC)[1]. Earlier studies have reported that the supplementation of myoinositol or PKC agonist, such as phorbol ester, was able to prevent hyperglycemia's inhibitory effect on Na^+,K^+-ATPase, suggesting that PKC activity in the vascular tissue may be reduced by elevated glucose level (3, 9). However, recent reports from multiple groups have clearly documented that diabetes or elevated glucose level will increase PKC as defined by both activity and phosphorylation of PKC's cellular targets in vascular tissues or cells in culture (2, 10–13). These apparent paradoxical findings suggest that hyperglycemia may affect Na^+,K^+-ATPase in the vasculature both directly and indirectly via other yet unknown metabolic pathways which will ultimately result in the inhibition of Na^+,K^+-ATPase activity.

Arachidonic acid and its products, eicosanoids, have been recently shown to regulate Na^+,K^+-ATPase activity in many cell types (14–16). Furthermore, there is evidence that elevated glucose level in the culture media enhances the cellular production of arachidonic acid which could be the result of activation of PKC (17). Recently, other studies have indicated that the cytosolic phospholipase A_2 ($cPLA_2$) is activated by phosphorylation on serine residues through PKC-and/or mitogen-activated protein kinase–dependent pathways (18–21). These observations have suggested to us that hyperglycemia-induced inhibition of Na^+,K^+-ATPase activity could be the result of increases in the release of arachidonic acid derived from PKC-mediated activation of $cPLA_2$. To test this hypothesis, we have examined

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1. *Abbreviations used in this paper:* $cPLA_2$ cytosolic phospholipase A_2; GFX, GF109203X; PKC, protein kinase C; REC, retinal endothelial cells; VSMC, vascular smooth muscle cells.

in the present study whether elevation of glucose level could induce activation and phosphorylation of $cPLA_2$ via PKC pathway to inhibit Na^+,K^+-ATPase activity in the vascular cells. In addition, we have determined whether the glucose-induced decrease in Na^+,K^+-ATPase activity could be prevented by the inhibition of PKC or $cPLA_2$.

Methods

Cell culture. Rat aortic vascular smooth muscle cells (VSMC) were harvested from aortae of Sprague-Dawley rats and cultured in DME (GIBCO, Grand Island, NY) containing 10% calf serum. Human VSMC were derived from minced pieces of a human aorta and cultured in DME containing 20% FBS (HyClone Laboratories, Logan, UT). Bovine retinal capillary endothelial cells (REC) were isolated by homogenization and a series of filtration steps and subsequently cultured in DME containing 10% plasma-derived horse serum (Lampire Biological Laboratories, Pipersville, PA) and endothelial cell growth factor as described previously (11).

For the experimental studies, cultured cells were allowed to reach confluence in above mentioned regular growth medium. Then the medium was changed to either control medium (DME containing 1% serum and 5.5 mM glucose), or high glucose medium (DME containing 1% serum and 22 mM glucose). Incubation of VSMC or REC with increasing glucose levels from 5.5 to 22 mM for up to 4 d did not alter the cell number and protein concentration significantly. Protein concentration was measured using a protein assay kit (Bio-Rad Laboratories, Richmond, CA) with BSA as standard.

Assay of PLA_2 activity. PLA_2 activity was determined in cell lysates using 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (PAPC) (57 mCi/mmol; Dupont/NEN, Boston, MA) as substrate as described previously (22). Since PAPC vesicles are readily hydrolyzed by $cPLA_2$ but a poor substrate for secretory PLA_2 (23), this assay is best suited to determine enzymatic activity of $cPLA_2$ in cell lysates. A 50-μl aliquot of cell lysate was incubated with 0.5 nmol of [^{14}C]PAPC in a total volume of 0.2 ml of 0.1 M Tris-HCl, pH 8.5, containing 5 mM $CaCl_2$, 0.5 mg/ml BSA, and 1 mM 2-mercaptoethanol. Incubations were carried out at 37°C for 30 min and terminated by adding 2 ml of Dole's reagent (2-propanol/heptane/0.5 N H_2SO_4, 40:10:1, by vol). After addition of 10 μg of arachidonic acid as carrier, 1 ml of heptane and 1 ml of water were added. The mixtures were briefly vortex-mixed and the upper phase transferred to tubes containing 2 ml of heptane and 150 mg of 200 mesh-Silica gel (Sigma Chemical Co., St. Louis, MO). After vortex-mixing and centrifugation, the supernatants were transferred to scintillation vials containing 10 ml of Hydrofluor (National Diagnostics, Inc., Atlanta, GA), and the radioactivity quantified by scintillation counting. Released [^{14}C] arachidonic acid was quantitatively recovered by this procedure, and in control assays (containing no Ca^{2+} or heat-denatured samples), $< 0.2\%$ of the total [^{14}C]-radioactivity was detected in the heptane phase after adsorption with silica gel. $cPLA_2$ activity was expressed as picomoles of arachidonic acid produced per milligram of protein per minute. To establish that the PLA_2 activity detected with this assay system was due to $cPLA_2$ we studied the effects of monoclonal antibodies M12 and M3-1 prepared as described (24). As shown previously (19, 25) M12 inhibits the activity of $cPLA_2$ whereas M3-1 does not.

Release of [^{3}H]arachidonic acid by VSMC. Cultured cells were allowed to reach confluence in 12-well plates when the medium was maintained at 5.5 mM (low) or changed to 22 mM (high) glucose. After 2 d of incubation exposed to low or high glucose, the cells were labeled with 0.5 μCi/ml [^{3}H]arachidonic acid (221 Ci/mmol; DuPont/NEN) for 20 h at 37°C. The radioactive medium was then removed and the cells were washed rapidly four times with DME containing 0.1% BSA. The radiolabeled cells were incubated with control or high glucose medium for another 3 h at 37°C, and the medium was replaced with 0.5 ml of fresh conditional medium with or without desired agents for an additional 30 min of incubation. The medium was then collected and the amount of [^{3}H] arachidonic acid released by VSMC into the medium over 30 min was determined by scintillation counting as described previously (17). The cells were washed again and solubilized with 0.5 ml of 1 N NaOH. A 15-μl aliquot was retained for protein determination and the remaining solubilized cells were subjected to scintillation counting to determine the [^{3}H]arachidonic acid content of the cells. The data of [^{3}H]arachidonic acid release were presented as the percentage of total counts (radioactivity in the released fraction plus those in the solubilized cells) released per 30 min. No significant differences were found in the amount of incorporation of [^{3}H] arachidonic acid by the cells exposed to low or high glucose for 3 d.

Immunoblotting analysis of $cPLA_2$. After 3 d of incubation in low or high glucose medium, the cultured cells were lysed with ice-cold lysis buffer (20 mM Hepes, pH 7.5, 1% Triton X-100, 150 mM NaCl, 25 mM KCl, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.1 mM leupeptin, 100 μg/ml aprotinin, 10 μM pepstatin A, 50 mM NaF, and 0.2 mM Na_3VO_4), and homogenized by being passed five times through a 28.5-gauge needle. After centrifugation at 10,000 *g* for 10 min at 4°C, equivalent protein aliquots were dissolved in SDS-sample buffer and electrophoresed in 10% Tris/glycine gels as previously described (19). Then the resolved proteins were electroblotted to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH). The blots were blocked in Tris-buffered saline with 3% BSA for 2 h at room temperature, and then hybridized for 2 h with anti-$cPLA_2$ antiserum (diluted 1:10,000). A detailed description of the antibody preparation has been reported previously (24). The immunoreactive bands were detected with ^{125}I-labeled protein A (Amersham Corp., Arlington Heights, IL) and quantitated by phosphoimager.

^{32}Pi labeling and immunoprecipitation. Rat VSMC were grown to confluence in P-100 dishes and incubated with low or high glucose medium for 3 d. After serum-starved overnight, cells were labeled with 1 mCi/ml of ^{32}Pi in Hepes-buffered phosphate-free DME for 3 h at 37°C and treated with or without the PKC activator PMA (100 nM) or the PKC-specific inhibitor GF109203X (GFX) (Calbiochem-Novabiochem Corp. La Jolla, CA) (5 μM) for 30 min. The reaction was terminated by removing medium and adding 0.6 ml of a mixture containing 20 mM Hepes, pH 7.4, 1% Triton X-100, 0.5% SDS, 0.75% deoxycholate, 10 mM EDTA, 1 mM PMSF, 0.1 mM leupeptin, 100 μg/ml aprotinin, 10 μM staurosporin, 10 mM $Na_4P_2O_7$, 50 mM NaF, 0.2 mM Na_3VO_4, and 1 μM microcystin-LR. Solubilized cells were centrifuged at 15,000 *g* for 10 min at 4°C, and the supernatants were immunoprecipitated by incubation with anti-$cPLA_2$ antibody bound to protein A-Sepharose beads (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) overnight at 4°C. The immunoprecipitates were washed three times with 50 mM Hepes, pH 7.4, 500 mM NaCl, 0.1% SDS, 0.2% Triton X-100, 5 mM EGTA, and three additional times with the same buffer containing 150 mM NaCl. The amount of protein was assessed by determination of trichloroacetic acid–precipitated radioactivity. The samples were analyzed by SDS-PAGE using 10% gels. The ^{32}Pi-labeled $cPLA_2$ was visualized and quantitated by phosphoimager.

Assay of PGE_2 production by VSMC. The amount of PGE_2 produced by VSMC was determined in nonextracted cell supernatants using radioimmunoassay kit (Amersham Corp.) following the instructions of the manufacturer.

Assay of Na^+,K^+-ATPase activity. Na^+,K^+-ATPase activity was determined by measuring ouabain-sensitive ^{86}Rb uptake based on the assumption that ^{86}Rb transport by Na^+,K^+-ATPase showed nearly identical kinetics to the normal substrate K^+ (26). Rat VSMC were grown in 12-well dishes and exposed to indicated conditions. The cells were washed twice and preincubated for 10 min in serum-free DME supplemented with or without 2 mM ouabain ^{86}Rb (67.8 μCi/mmol; DuPont/NEN) was added to a final concentration of 1 μCi/ml for 10 min. ^{86}Rb uptake was terminated by aspirating the medium and rapidly washing four times with ice-cold 100 mM $MgCl_2$. The cells were then extracted with 0.5 ml of 10% trichloroacetic acid to measure the radioactivity incorporated into cells by scintillation counting. The ouabain sensitive portion of the ^{86}Rb uptake, which presumably represents Na^+,K^+ATPase–mediated transport activity (26), was calculated by subtracting the portion of the uptake observed in cells preincubated with ouabain from the total uptake. The time course of ^{86}Rb uptake has been

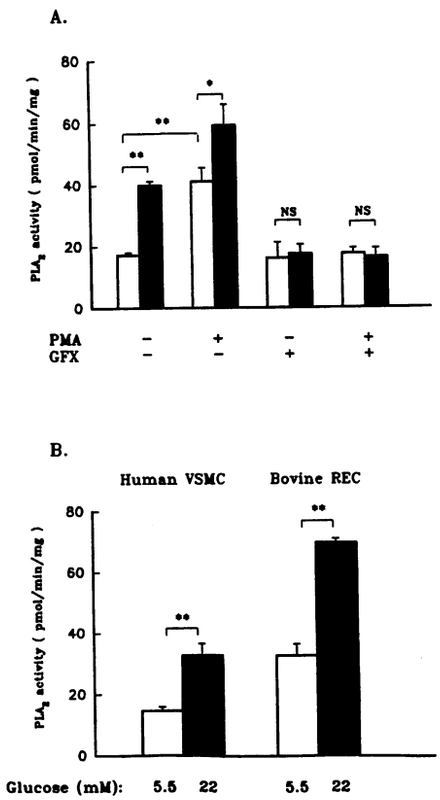

Figure 1. Effect of elevated glucose levels and regulators of PKC on $cPLA_2$ activity. (*A*) after a 3-d incubation with 5.5 mM (*open bars*) or 22 mM glucose (*hatched bars*), rat VSMC were treated with or without PMA (100 nM) or GFX (5 μM) for 30 min and then lysed as described in Methods. (*B*) $cPLA_2$ activity was assayed in human VSMC and bovine REC incubated in 5.5 or 22 mM glucose for 3 d. $cPLA_2$ activity was measured in the cellular lysates (50 μl) and expressed as picomoles arachidonic acid released per minute per milligram protein. * $P < 0.05$, ** $P < 0.01$. The results are expressed as the mean±SEM derived from three separate experiments.

characterized and found to be linear for up to 45 min in the VSMC. The data were normalized to cellular protein concentration.

Statistical analysis. All data are expressed as mean±SEM. Comparison of the two groups was calculated by the unpaired Student's *t* test; ANOVA and Newman-Keuls tests were used to identify statistical significance of multiple comparisons.

Results

Effect of elevated glucose levels on enzymatic activity of cPLA. After 3 d of incubation in medium containing glucose levels of 5.5 and 22 mM, $cPLA_2$ activity in rat VSMC was 17.2±1.4 and 39.9±2.4 pmol/min per mg protein, respectively, and was significantly increased by 2.3-fold ($P < 0.01$) with the elevation of glucose levels. Addition of the PKC activator PMA (100 nM) caused an increase in $cPLA_2$ activity by 2.4-fold ($P < 0.01$) and 1.6-fold ($P < 0.05$) in VSMC incubated at 5.5 or 22 mM glucose, respectively (Fig. 1 *A*). When the PKC-specific inhibitor GFX was added at 5 μM, which was shown to completely prevent PKC activation in response to high glucose or PMA-stimulation in intact cells (data not shown), the high glucose-induced increase in $cPLA_2$ activity was inhibited. However, the addition of GFX did not affect basal $cPLA_2$ activity

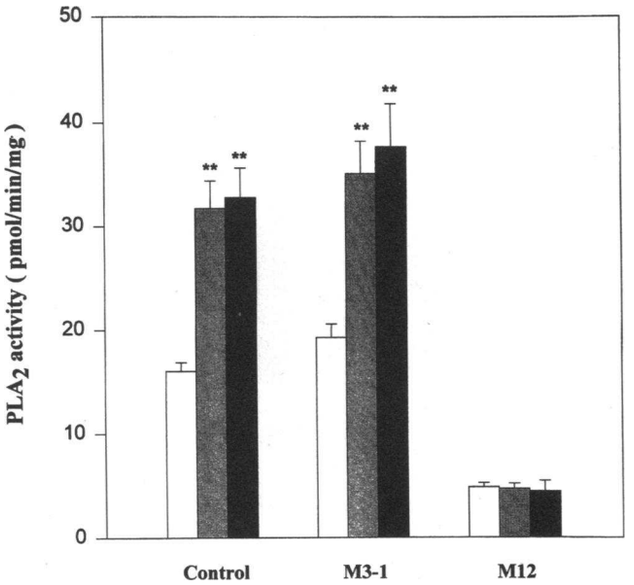

Figure 2. Effect of anti-$cPLA_2$ monoclonal antibodies on PLA_2 activity. Incubation with 5.5 mM (*open bars*) or 22 mM glucose (*gray bars*) for 3 d and treatment with PMA (100 nM) for 30 min (*dark bars*) as described in legend of Fig. 1. The lysates of VSMC were preincubated for 60 min on ice with buffer (*Control*) or 1 mg/ml monoclonal antibody M3-1 (*control antibody*), or M12 (*neutralizing antibody*) and then assayed for PLA_2 activity. The results are expressed as the mean±SD derived from four separate sets of dishes in two different experiments. ** $P < 0.01$.

observed at 5.5 mM glucose, but inhibited the PMA-induced activation of $cPLA_2$ (Fig. 1 *A*).

To determine whether an increase in $cPLA_2$ activity can be induced by an elevation of glucose levels in vascular cells from other species as well as in capillary endothelial cells, we compared the effect of 5.5 and 22 mM glucose on the $cPLA_2$ activity in cultures of human aortic VSMC and bovine REC. As shown in Fig. 1 *B*, exposure of human VSMC and bovine REC to 22 mM glucose for 3 d increased $cPLA_2$ activity in both cell types by 1.9- and 2.5-fold, respectively.

Since several forms of PLA_2, both calcium-dependent and -independent, have been described in vascular cells, we verified that the increase in PLA_2 activity induced by high glucose or PMA was due to $cPLA_2$. As shown in Fig. 2, basal levels as well as increased PLA_2 activity induced by high glucose or PMA were abolished after preincubation with the neutralizing anti-$cPLA_2$ monoclonal antibody M12. In contrast, no inhibitory effect was observed with the control antibody M3-1 which recognizes $cPLA_2$, but does not affect its enzymatic activity as described previously (19, 24, 25).

Effect of elevated glucose levels on [^{3}H]arachidonic acid release by VSMC. To examine whether high glucose-induced activation of $cPLA_2$ affects the cellular metabolism of arachidonic acid, we measured the release of [^{3}H]arachidonic acid from prelabeled rat VSMC. As shown in Fig. 3, after 3 d of incubation with elevated glucose levels (22 mM), the release of [^{3}H]arachidonic acid increased 1.8-fold ($P < 0.05$) by VSMC compared to control medium. Likewise, in the presence of the PKC agonist PMA, the release of [^{3}H]arachidonic acid was further increased in both 5.5 and 22 mM glucose–containing medium. On the other hand, the PKC inhibitor GFX prevented the high glucose-induced increase in [^{3}H]arachidonic acid re-

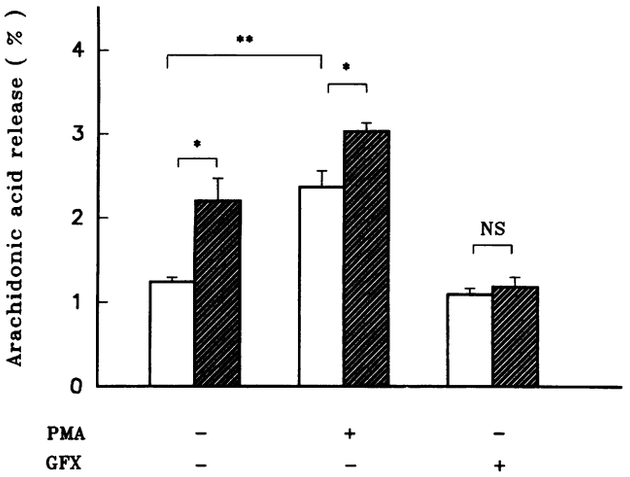

Figure 3. Effect of elevated glucose level on arachidonic acid release by rat VSMC. VSMC were prelabeled with [^{3}H]arachidonic acid after 3 d of exposure to 5.5 mM (*open bars*) or 22 mM (*hatched bars*) glucose. The cells were treated with or without PMA (100 nM) or GFX (5 μM) for 30 min before measuring the [^{3}H]arachidonic acid release. * $P < 0.05$, ** $P < 0.01$. The results are expressed as the mean±SEM derived from four separate experiments.

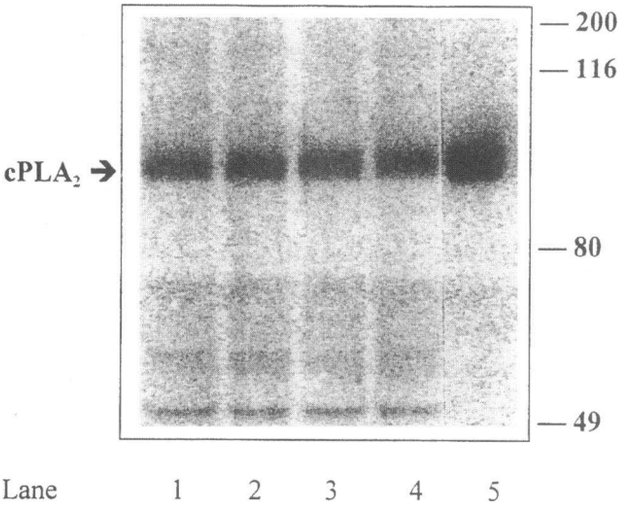

Figure 4. Immunochemical detection of $cPLA_2$ in VSMC. Equivalent protein aliquots of lysates (40 μg) from cultured rat VSMC exposed to 5.5 mM (lane *1*) or 22 mM glucose (lane *2*) for 3 d were analyzed by SDS-PAGE (10% gel) and immunoblotted with anti-$cPLA_2$ antibody. (Lanes *3* and *4*). Cells were treated with 100 nM PMA or 5 μM GFX for 30 min, respectively. (Lane *5*). Purified $cPLA_2$ (1 ng) produced in a baculovirus/insect cell expression system.

lease. To verify that high glucose or PMA increased the release of arachidonic acid by VSMC and did not act by inhibiting the reacylation of arachidonic acid, the reincorporation of [^{3}H]-arachidonic acid released by VSMC was determined. No significant differences were found in the reincorporation of released [^{3}H]arachidonic acid by rat VSMC exposed to 22 mM glucose or treated with PMA or GFX when compared with control medium (data not shown).

Immunochemical detection of $cPLA_2$ in VSMC. To establish the presence of $cPLA_2$ in VSMC, immunoblotting analysis was carried out in rat VSMC using rabbit polyclonal antibodies raised against purified human $cPLA_2$ (24). Fig. 4 shows that VSMC contain a single major band of protein recognized by the anti-$cPLA_2$ antibody that comigrates with human $cPLA_2$ purified from a baculovirus/insect cell expression system. When VSMC were exposed to 5.5 or 22 mM glucose for 3 d, or to PMA (100 nM) or GFX (5 μM) for 30 min, the amount of $cPLA_2$ present in the cells remained unchanged.

Effect of elevated glucose levels on $cPLA_2$ phosphorylation. Based on the results in Fig. 1 showing that $cPLA_2$ activity was increased by elevation of glucose, and the data of Fig. 4 demonstrating that the amount of $cPLA_2$ did not change, we characterized the phosphorylation state of $cPLA_2$ which may affect $cPLA_2$ activity in VSMC. The rat VSMC were metabolically labeled with ^{32}Pi for 3 h after 3 d of incubation with 5.5 or 22 mM glucose. The labeled cells were then incubated for 30 min in the absence and presence of 100 nM PMA or 5 μM GFX. As demonstrated in Fig. 5, the extent of $cPLA_2$ phosphorylation was markedly increased by about two-fold in rat VSMC exposed to 22 mM glucose or treated with PMA. In contrast, the PKC inhibitor GFX prevented the effect of elevated glucose levels on increasing the phosphorylation of $cPLA_2$.

Effect of elevated glucose levels on PGE_2 production by VSMC. The findings so far have demonstrated that elevated glucose levels can induce the activation of $cPLA_2$ via PKC-dependent pathway. Since arachidonic acid is the precursor for the biosynthesis of eicosanoids, with PGE_2 being the predominant prostaglandin in VSMC (27), we measured the production of PGE_2 by VSMC. As shown in Fig. 6, increasing glucose from 5.5 to 22 mM for 3 d induced a marked and significant increase (1.98-fold) in PGE_2 production by VSMC. Exposure to PMA further increased the production of PGE_2 by VSMC, and the increases induced by glucose or PMA-stimulation were inhibited by GFX.

Effect of elevated glucose levels and PKC regulators on Na^+,K^+-ATPase activity. Since arachidonic acid has been reported to inhibit Na^+,K^+-ATPase (14), we evaluated the effect of glucose and various enzymatic inhibitors on this enzyme activity. After 3 d of increasing glucose levels from 5.5 to 22 mM, ouabain-sensitive ^{86}Rb uptake by VSMC was decreased two-fold, from 3.89±0.21 to 1.94±0.18 nmol/min per mg protein (Fig. 7 *A*). The decrease in Na^+,K^+-ATPase activity induced by elevated glucose levels was normalized by the addition of the PKC inhibitor, GFX. The PKC agonist PMA (100 nM) increased the Na^+,K^+-ATPase activity by 1.3-fold ($P < 0.05$) in medium containing 5.5 mM glucose and normalized the decrease in Na^+,K^+-ATPase activity observed in 22 mM glucose. However, even at low concentration of PMA (6.25 nM), no decreases in the Na^+,K^+-ATPase activity were observed. In contrast, a diacylglycerol analog (1, 2-dioctanoylglycerol [DiC8]) was able to decrease ouabain-sensitive ^{86}Rb uptake in VSMC by 50–80% in a dose-dependent manner (from 0.1 to 20 μM).

As previously reports in fibroblasts (28), Na^+,K^+-ATPase can be regulated by PKC-dependent activation of the Na^+,H^+-antiporter. Table I showed that amiloride, a known inhibitor of the Na^+,H^+-antiporter, did not alter the effects of both high glucose level and PMA on the Na^+,K^+-ATPase activity in rat VSMC. This finding indicated that high glucose level or PMA induced alteration in Na^+,K^+-ATPase did not involved alterations of Na^+,H^+-antiporter activity.

Characterization of $cPLA_2$-mediated inhibition of Na^+,K^+-ATPase by high glucose. Both high glucose and PMA activate

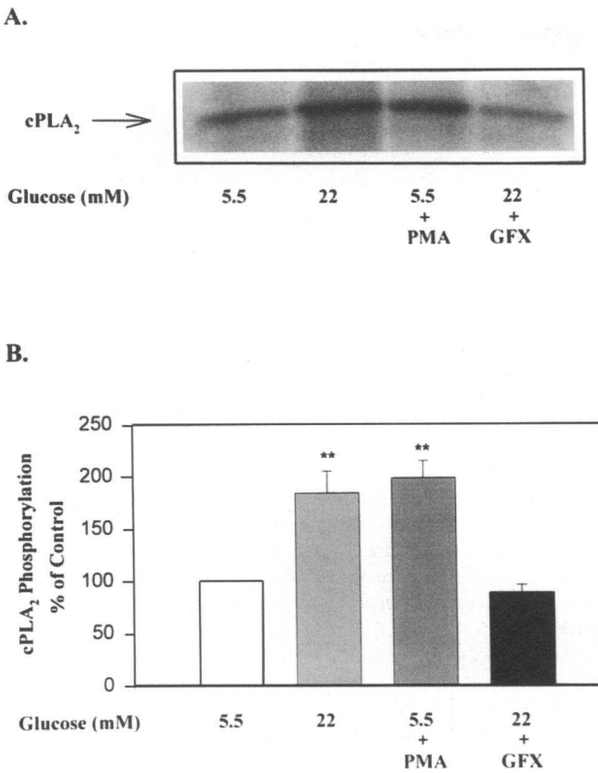

Figure 5. Regulation of $cPLA_2$ phosphorylation by glucose levels and PKC regulators. (*A*) autoradiogram depicting ^{32}Pi-labeled $cPLA_2$ in rat VSMC. Comparable numbers of cells were incubated with 5.5 or 22 mM glucose for 3 d, treated with or without PMA (100 nM) or GFX (5 μM) for 30 min, and labeled with 1 mCi/ml of ^{32}Pi for 3 h. $cPLA_2$ was then immunoprecipitated and subjected to SDS-PAGE as described in Methods. (*B*) Graph depicting the relative phosphorylation of $cPLA_2$. The results are quantitated by phosphoimager analysis and expressed as percentage of the control. Vertical bars represent the SD derived from three independent experiments. ** $P < 0.01$.

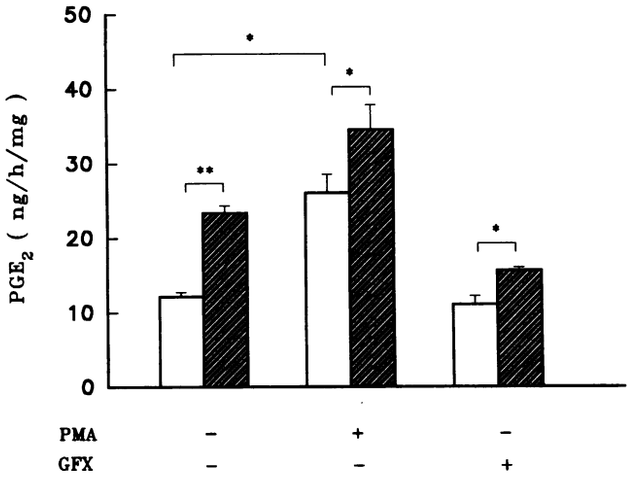

Figure 6. Effect of elevated glucose levels on PGE_2 production by VSMC. Rat VSMC were incubated with 5.5 mM (*open bars*) or 22 mM (*hatched bars*) glucose for 3 d and treated with or without PMA (100 nM) or GFX (5 μM) for 30 min before the measurement of PGE_2 production. * $P < 0.05$, ** $P < 0.01$. The results are expressed as the mean±SEM derived from three separate experiments.

PKC yet appear to have opposite effects on Na^+,K^+-ATPase activity. These data suggest the involvement of other cellular pathways besides direct activation of Na^+,K^+-ATPase by PKC. To examine the possible role of $cPLA_2$ in mediating the glucose-induced inhibition of Na^+,K^+-ATPase, we used the PLA_2 inhibitor arachidonyl trifluoromethyl ketone ($AACOCF_3$) (29, 30). $AACOCF_3$ was found to prevent the increase in arachidonic acid release induced by high glucose or PMA, but did not alter PKC activity in rat VSMC exposed to 5.5 or 22 mM glucose (Table II). As shown in Fig. 7 *B*, elevation of glucose levels from 5.5 to 22 mM decreased ouabain-sensitive ^{86}Rb uptake by two-fold. In the presence of the PLA_2 inhibitor $AACOCF_3$, the high glucose-induced inhibition of Na^+,K^+-ATPase activity was completely reversed. Moreover, $AACOCF_3$ further elevated the PMA-induced increases in Na^+,K^+-ATPase activity both in the presence of 5.5 and 22 mM of glucose.

Discussion

Hyperglycemia causes vascular dysfunctions and pathologies in diabetic patients and animals, probably by multiple biochemical mechanisms. One of these mechanisms is likely to involve the generation of diacylglycerol and activation of PKC, which are known to affect vascular contractility, permeability, coagulation, and cell growth (31, 32), all of which have been found to be abnormal in the diabetic state (2). Increases in diacylglycerol levels and PKC activity have been documented in the aorta, heart, renal glomeruli, and retina from diabetic animals as well as in cultured vascular cells exposed to elevated levels of glucose (10–13, 33). However, the cellular targets of PKC activation induced by high glucose level have not been defined clearly. In this study we have characterized two important regulatory enzymes, $cPLA_2$ and Na^+,K^+-ATPase, which are altered in

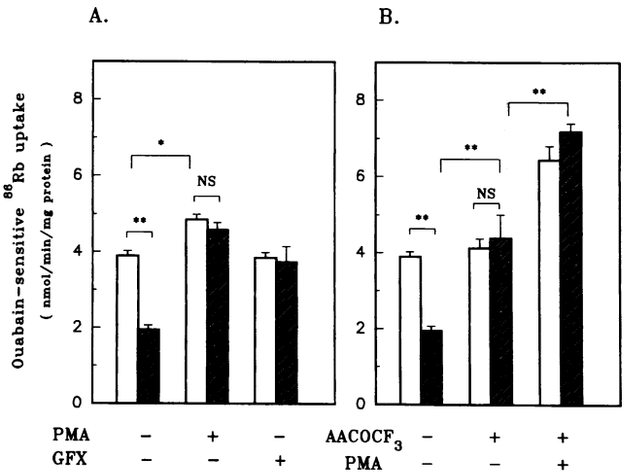

Figure 7. Effect of elevated glucose levels on Na^+,K^+-ATPase activity as measured by ouabain-sensitive ^{86}Rb uptake. Rat VSMC were incubated with 5.5 mM (*open bars*) or 22 mM (*hatched bars*) for 3 d and treated with indicated agents for 30 min. ^{86}Rb uptake was corrected for ouabain sensitivity and determined after 10-min incubation with ^{86}Rb as described in Methods. The concentrations of agents used were 100 nM for PMA, 5 μM for GFX, and 10 μM for AACOCF. * $P < 0.05$, ** $P < 0.01$. The results are expressed as the mean±SEM derived from four separate experiments.

Table I. Effects of Amiloride on the Elevated Glucose Levels and PMA-induced Alterations in Na^+, K^+-ATPase Activity by Rat VSMC

Condition	Ouabain-sensitive ^{86}Rb uptake	
	Absence of amiloride	Presence of amiloride
	nmol/min per mg protein	
Control	4.03±0.17	3.27±0.21
22 mM glucose	1.99±0.21*	1.67±0.19*
PMA, 100 nM	5.12±0.33‡	4.38±0.24‡

Cultured cells were incubated in the presence or absence of 400 μM amiloride for 10 min before the addition of ^{86}Rb. Ouabain-sensitive ^{86}Rb uptake was measured as described in Fig. 7 legend. Data are expressed as the mean±SD derived from three separate experiments with duplicates performed in each experiment. $*P < 0.01$, $‡P < 0.05$, vs control medium (5.5 mM glucose).

Table II. The Effects of $AACOCF_3$ on Arachidonic Acid Release and PKC Activity in Cultures of Rat VSMC

Condition	Arachidonic acid release		PKC activity	
	$-AACOCF_3$	$+AACOCF_3$	$-AACOCF_3$	$+AACOCF_3$
	%		*pmol/min per mg*	
Control	1.24±0.09	1.08±0.26	18.64±1.64	17.56±2.04
22 mM glucose	2.21±0.47*	1.13±0.32	28.07±2.08‡	27.67±2.51‡
PMA, 100 nmol	2.37±0.34‡	1.09±0.17	85.03±6.53‡	86.75±6.04‡

Rat VSMC were cultured in 5.5 mM (*control*) or 22 mM glucose for 3 d and treated with or without PMA (100 nM), $AACOCF_3$ (10 μM), or DMSO (0.01%) alone before the determination of arachidonic acid release and PKC activity. PKC activity was measured in digitonin-permeabilized VSMC using the specific peptide substrate RKRTLRRL as described previously (31). PKC activity was expressed as picomoles of peptide phosphorylated per minute per milligram cellular protein. $*P < 0.05$, $‡P < 0.01$ vs control medium (mean±SEM, $n = 3$). There was no significant difference in arachidonic acid release among these experimental groups in the presence of $AACOCF_3$, and there was no significant difference in PKC activity between presence and absence of $AACOCF_3$ for each group.

their activities by glucose-induced PKC activation in a variety of vascular cells from human, bovine, and rat.

The finding that $cPLA_2$ can be activated via glucose-induced PKC activation could be potentially important because the product of $cPLA_2$ is arachidonic acid, the precursor for the synthesis of prostaglandins, thromboxanes, and leukotrienes, all of which are mediators with potent vasoactive effects (34, 35). In addition, the other product of $cPLA_2$ reaction, lysophospholipid, can be converted to platelet-activating factor which is a potent mediator of inflammation (36). In the present study, multiple lines of evidence support the notion that glucose is inducing the activation of $cPLA_2$ via activation of PKC. First, our results show that the enzymatic activity of $cPLA_2$ is increased in lysates derived from VSMC after exposure to elevated levels of glucose. This effect can be mimicked by the PKC agonist PMA. Conversely, the PKC inhibitor GFX prevents the stimulatory effect of glucose. Second, an increase in arachidonic acid release by VSMC was observed after elevation of glucose, which could be mimicked by a PKC agonist and prevented by a PKC inhibitor. Third, the effect of glucose in VSMC is most likely directed at $cPLA_2$ since we could readily detect $cPLA_2$ in VSMC immunologically and found that $cPLA_2$ is phosphorylated by elevation of glucose levels. Further, the inhibition of PLA_2 activity by the neutralizing anti-$cPLA_2$ antibody M12 strongly supported the fact that the increase of PLA_2 activity was specifically due to $cPLA_2$. This effect of glucose can also be mimicked by PMA and are prevented by the PKC inhibitor GFX. It is well-known that phosphorylation of $cPLA_2$ by PKC and/or (MAP) kinase can increase its catalytic activity (18–21). Thus, the finding that elevated glucose levels increases the phosphorylation of $cPLA_2$ provides evidence that increasing glucose may activate $cPLA_2$ via PKC activation. Further studies on the effect of glucose levels on MAP kinase are needed to evaluate whether in VSMC PKC or MAP kinase can directly phosphorylate $cPLA_2$. Our data are in agreement with a recent study reporting that elevation of glucose level increase arachidonic acid release in cultured glomerular mesangial cells (17).

The finding that high glucose level induces an increase in PGE_2 production provides another example of cellular consequences of glucose-induced PKC activation in vascular cells. This conclusion is supported by the observation that the PKC activator PMA mimics the effect of glucose and that, conversely, a PKC inhibitor diminishes the glucose-induced production of PGE_2. Many investigators have reported that prostaglandin synthesis in vascular tissues is altered in experimental diabetic animals or vascular cells in response to elevated glucose levels (17, 36–39). Our results are consistent with previous reports showing that PGE_2 synthesis was increased in perfused hearts (36), or glomeruli (37) prepared from streptozotocin-induced diabetic rats, as well as platelets from diabetic patients (38). On the other hand, there are reports showing that in the vasculature-elevated glucose levels may decrease PGE_2 production (39), and arachidonic acid release was decreased by stimulated porcine aortic endothelial cells (40). One possible explanation for these discrepancies could be that different types of vascular cells and conditions were used amongst the various studies which may result in different patterns of arachidonic acid metabolism.

Another important consequence of the activation of $cPLA_2$ via glucose-induced PKC activation in vascular cells is the change in Na^+,K^+-ATPase activity. This transporter is known to be important for maintenance of cell viability and multiple metabolic and growth functions (1). Numerous studies have shown that in both neurological and vascular tissues from the diabetic animals Na^+,K^+-ATPase activity is decreased as assessed by measuring enzymatic activity directly or determining ouabain-sensitive ^{86}Rb uptake, a physiological measurement for Na^+,K^+-ATPase activity (3–5). Incubation of cells from vascular or neurological origin with the PKC agonist PMA normalized the inhibition of Na^+,K^+-ATPase activity induced by high glucose levels (3, 9). Our results confirm that elevation of glucose levels can decrease the ouabain-sensitive ^{86}Rb uptake in VSMC, and that PMA normalizes such glucose-induced changes in Na^+,K^+-ATPase activity. However, the finding that the PKC inhibitor GFX also normalizes glucose-induced decrease in ouabain-sensitive ^{86}Rb uptake is unexpected. We believe that this apparent paradoxical finding is due to the fact that the activation of PKC induced by hyperglycemia can regulate Na^+,K^+-ATPase through multiple pathways. The main effect is inhibitory and may be due to PKC-dependent activation of $cPLA_2$. This is substantiated by the use of the $cPLA_2$ inhibitor

$AACOCF_3$, which normalizes the inhibitory effect of glucose on Na^+,K^+-ATPase. The involvement of $cPLA_2$ in the regulation of Na^+,K^+-ATPase is via the release of arachidonic acid from phospholipids for prostaglandin production. Satoh et al. (16) have reported that Na^+,K^+-ATPase activity can be inhibited by arachidonic acid metabolites generated via the cytochrome P450-dependent monooxygenase pathway. In addition, PGE_2 was found to inhibit Na^+,K^+-ATPase activity in cortical collecting duct cells (15) as well as in Madin–Darby canine kidney cells (41). Others have reported that nonesterified fatty acid and lysophosphatidylcholine also inhibit Na^+,K^+-ATPase (42). Collectively, these studies indicate that metabolic products derived from $cPLA_2$ catalytic activity can cause inhibition of Na^+,K^+-ATPase. Therefore, we propose that elevation of glucose levels in vascular cells is inhibiting Na^+,K^+-ATPase via activation of PKC and $cPLA_2$. Hence, high glucose levels activate PKC which will then mediate the activation of $cPLA_2$ to increase the production of eicosanoids, such as PGE_2 causing an inhibition of Na^+,K^+-ATPase activity.

Our data showed that in rat VSMC, PMA through PKC activation increased Na^+,K^+-ATPase activity. These findings agreed with those of previous observations in several types of cells (43–45). However others have reported that the activation of PKC can phosphorylate the catalytic subunit of Na^+,K^+-ATPase to either stimulate or inhibit its activity depending on the system studied (45, 46). In view of these conflicting observations, it is likely that PKC can regulate Na^+,K^+-ATPase activity by multiple pathways depending on the physiological state and the type of cells studied. A possible mechanism by which phorbol ester's effect could inhibit Na^+,K^+-ATPase activity was suggested by Gupta et al., who postulated that the activation of PKC by PMA or hyperglycemia could be inhibiting Na^+,K^+-ATPase activity by decreasing the synthesis and release of endothelium-derived nitric oxide (47). Since nitric oxide may regulate phospholipase A_2 activity, further studies will be needed to delineate whether the decrease in nitric oxide level induced by hyperglycemia will act in series or in parallel with $cPLA_2$ activation to inhibit Na^+,K^+-ATPase.

The variety of PMA's effects on the Na^+,K^+-ATPase activity is probably due to its wide range of actions on many biochemical pathways. PMA, a nonphysiological activator of PKC, is capable of stimulating biological actions either through the PKC or non-PKC–mediated pathways (32). Our findings that PKC inhibitor (GFX) was able to prevent the activation of $cPLA_2$ and inhibition of Na^+,K^+-ATPase activity induced by PMA suggests that these effects of PMA are mediated by PKC activation. However, PMA apparently can have both stimulatory and inhibitory effects on Na^+,K^+-ATPase as shown by the results of adding PMA singularly and by jointly adding PMA and PLA_2 inhibitor ($AACOCF_3$). Furthermore, phorbol esters (PMA, phorbol 12,13-dibutyrate) and diacylglycerol and its analogs (*sn*-1,2-dioctanoylglycerol) may activate PKC's various isoforms differently to mediate either stimulatory or inhibitory effects. In preliminary studies we have found that in VSMC overexpressing PKC β isoforms, phorbol ester will inhibit Na^+,K^+-ATPase activity suggesting that the regulation of Na^+,K^+-ATPase by PKC could be isoform specific.

Our results also suggest that the regulation of Na^+,K^+-ATPase by PKC activation induced by elevated glucose levels and phorbol ester may not be the same. Previously we have reported that hyperglycemia will activate PKC βII isoform predominantly whereas PMA can activate almost all PKC isoforms (2, 10). In this study, the difference between the effects of

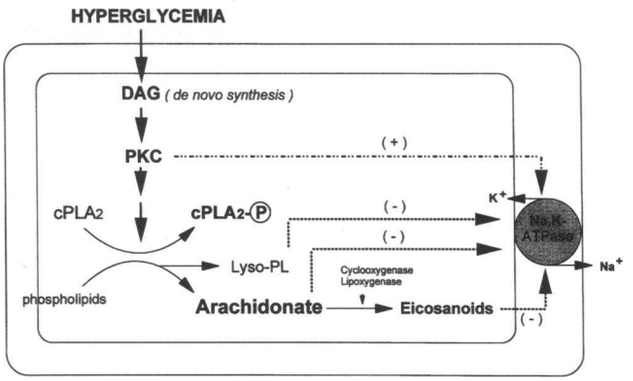

Figure 8. Schematic diagram of the proposed intracellular mechanisms for the inhibition of Na^+,K^+-ATPase activity in vascular cells by the glucose-induced activation of PKC and $cPLA_2$.

hyperglycemia and PMA on Na^+,K^+-ATPase can be observed in the action of PLA_2 inhibitor ($AACOCF_3$) on the activity of Na^+,K^+-ATPase. The addition of PMA showed an enhancement with $AACOCF_3$ whereas it did not enhance Na^+,K^+-ATPase activity above the low glucose level (5.5 mM) when added to cells incubated in high glucose medium. One possible difference between PMA and high glucose level is that the PKC can increase PKC activities 5–10 times greater than those induced by elevated glucose levels (2, 10). This could explain the apparent isoform-specific activation induced by hyperglycemia. In addition, glucose's effect on PKC activation is mediated by increasing the levels of palmitate-labeled diacylglycerol (10, 11), which may provide a greater specificity in biological actions than the addition of PMA. Therefore, it is not surprising that similar effects on Na^+,K^+-ATPase activity were induced by high glucose level and diacylglycerol analog, *sn*-1, 2-dioctanoylglycerol, as compared to PMA. Further studies are in progress to determine whether the activation of $cPLA_2$ and Na^+,K^+-ATPase can be regulated differently by the various PKC isoforms.

In summary, the present study demonstrates that in a variety of vascular cells the glucose-induced activation of PKC leads to activation of $cPLA_2$, which then mobilizes arachidonic acid, resulting in the inhibition of Na^+,K^+-ATPase (Fig. 8). These results provide an explanation for the current paradoxical findings of the effect of glucose on the activation of PKC and the inhibition of Na^+,K^+-ATPase in vascular cells. Further studies will have to examine whether these findings are applicable to all the vascular tissues in diabetes. Our preliminary data showed that $cPLA_2$ activity in aorta from diabetic rats is increased providing in vivo correlative data to our studies with cultured vascular cells. On the other hand, insulin has been recently reported to increase Na^+,K^+-ATPase activity via PKC pathway in rat skeletal muscle cells (44). The involvement of insulin's effect in the inhibition of Na^+,K^+-ATPase by high glucose or diabetes will also need to be resolved. Our findings could have clinical implications for determining the site for therapeutic intervention to prevent diabetic vascular complications.

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