High Glucose Induces Alteration of Gap Junction Permeability and Phosphorylation of Connexin-43 in Cultured Aortic Smooth Muscle Cells

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Gap junction is thought to have a crucial role in maintaining tissue homeostasis. We examined the effect of a high glucose level on gap junctional intercellular communication (GJIC) activity in cultured vascular smooth muscle cells (VSMCs) using the fluorescent dye transfer method. After a 48-h incubation with 22 mmol/l glucose (high glucose level), GJIC activity of VSMCs was significantly reduced compared with incubation with 5.5 mmol/l glucose (normal glucose level) (P < 0.05). Treatment of the cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 5×10^{-8} mol/l), a protein kinase C (PKC) activator, for 1 h also reduced GJIC activity (P < 0.01). In addition, treatment of the cells with calphostin C, a specific PKC inhibitor, for 3 h completely restored the GJIC activity inhibited by the high glucose level. Western blot analysis showed that connexin 43 (Cx43), which is the major functional protein of gap junction, is present in multiphosphorylated forms: a nonphosphorylated form (P0) and phosphorylated forms (P1, P2, and P3). Incubation of VSMCs with a high glucose level significantly increased the density ratio of P3/P0 compared with a normal glucose level (P < 0.05). Similarly, treatment of the cells with TPA significantly increased the P3/P0 ratio compared with controls (P < 0.01). In addition, the increase in the P3/P0 density ratio induced by a high glucose level was restored to the control level by both staurosporine and calphostin C. These results suggest that the high glucose level induced the inhibition of GJIC activity in cultured VSMCs through excessive phosphorylation of Cx43, mediated by PKC activation. This may contribute to the development of the macroangiopathy associated with diabetes. *Diabetes* 47:931–936, 1998

Hyperglycemia appears to be a significant etiologic factor in the development of micro- and macrovascular complications in diabetic patients (1). Various pathophysiological and biochemical mechanisms have been proposed to explain the adverse effect of hyperglycemia on vascular resident cells (2,3). We and other investigators previously demonstrated that hyperglycemia induces a persistent activation of protein kinase C (PKC) in vascular tissues and cells in culture (4–8). Because PKC is a key enzyme regulating various cellular functions, one might expect that PKC activated by hyperglycemia in diabetic patients would thus alter vascular functions (9–11).

Gap junctions are clusters of transmembrane channels that permit the intercellular exchange of ions and second messengers between adjacent cells (12). Intercellular communication via gap junctions (GJIC) has been considered to play a role in the maintenance of tissue homeostasis. GJIC is implicated in the regulation of cell growth, migration, differentiation, and electronic coupling (13). In particular, vascular cells, such as smooth muscle cells (VSMCs), and endothelial cells, formed both in vivo and in vitro, are linked by these structures. GJIC as well as diffusible growth factors and extracellular matrix components take part in the stringent growth control of VSMCs (18–21) in vasculature. Alteration of GJIC activity in VSMCs might be involved in the vascular remodeling of macroangiopathy.

To our knowledge, no study has yet been done regarding the effect of the diabetic state on GJIC activity in VSMCs. We expected that the GJIC activity in VSMCs might be altered by a high glucose level through the activation of PKC, since it has been shown that PKC functionally regulates the GJIC activity in many tissues (14–17).

In the present study, we examined the effect of a high glucose level on GJIC activity in cultured VSMCs using the dye transfer method. Recent biochemical and molecular studies have demonstrated that the gap junction is composed of highly evolutionary conserved proteins called connexins (22–24). GJIC can be regulated at the transcriptional, translational, and posttranslational levels of connexins (25–27). To characterize the biochemical mechanism responsible for the alteration of the GJIC activity by a high glucose level, we studied the phosphorylation state of connexin 43 (Cx43), which is a dominant connexin in VSMCs related to the activation of PKC.

RESEARCH DESIGN AND METHODS

Cell culture. VSMCs were obtained from a bovine thoracic aorta according to the method previously described by Ross (28). Intima-media segments cut into small pieces were explanted into dishes with Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS) (Gibco), 100 mU/ml penicillin, and 100 mg/ml streptomycin (Gibco). The VSMCs were then cultured at 37°C under an atmosphere of 95% O_2/ 5% CO_2, as described previously (29). Every 5–10 days, the cells were subcultured after 0.05% (wt/vol) trypsin (Difco, Detroit, MI) harvesting. For all experiments, the third through seventh subcultured VSMCs were used.

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Cx43, connexin 43; FCS, fetal calf serum; GJIC, gap junctional intercellular communication; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VSMC, vascular smooth muscle cell.

Measurement of GJIC activity. VSMCs were grown to ~90% confluence in the above-described culture medium in 35-mm plastic dishes (FALCON 3001; Falcon, Oxnard, CA). The medium was then removed and changed to a test medium containing 5.5 or 22 mmol/l glucose with concomitant lowering of the FCS concentration in the medium to 1% to keep the cells in the quiescent state. Following a 48-h exposure to each test medium, the activity of GJIC was measured as follows: Using a phase-contrast microscope, 0.5 pl of 10% Lucifer Yellow CH (Sigma, St. Louis, MO) dissolved in 0.33 mol/l lithium chloride solution was microinjected into a single cell with a pneumatic picopump (PV-800; World Precision Instruments, New Haven, CT) assembled in an injectscope system (Olympus IMT-2; Olympus, Tokyo, Japan), as previously described (30). After microinjection, the extent of dye transfer was recorded with a videosystem under fluorescence microscopy. There was no leakage of injected dye into the medium. The dye was then transferred into neighboring cells. The number of dye-transferred cells increased progressively and reached the steady state 20 s after microinjection. The GJIC activity was assessed by counting dye-transferred cells at 20 s after microinjection. For the treatment of cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma), after a 48-h exposure to a test medium containing 1% FCS and 5.5 mmol/l glucose, TPA was applied to the cells at a final concentration of 5×10^{-8} mol/l, as previously described. Following a 1-h incubation with TPA, the dye was then microinjected into a single cell and the GJIC activity was measured as described above. For the treatment of the cells with PKC inhibitor, after a 48-h exposure to a test medium containing 1% FCS and 22 mmol/l glucose, calphostin C (Wako, Osaka, Japan) at a final concentration of 5×10^{-8} mol/l was applied to the cells for 3 h.

Assay of PKC activity. VSMCs were washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+}. The sample was homogenized at 4°C in buffer (20 mmol/l Tris-HCl [pH 7.5], 2 mmol/l EDTA, 0.5 mmol/l EGTA, 330 mmol/l sucrose, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], and 0.025% leupeptin). PKC proteins in the membranous and cytosolic fractions were partially purified, and PKC activity was determined as previously described (5,6,31).

Analysis of protein level and phosphorylation of connexin. For the Western blot analysis, the VSMCs grown in each test medium described above in 100-mm plastic dishes (FALCON 3003; Falcon) were rinsed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and then lysed in a sample buffer comprised of 0.125 mol/l Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2 mmol/l EDTA, and 4 mmol/l PMSF, and then sonicated (three times, 10 s, midpower) on ice. The protein concentration was determined with a DC protein assay kit (Bio-Rad, Richmond, CA). The samples were boiled for 4 min and applied (10 µg of protein/lane) on 7.5% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Hybond-C super; Amersham, Buckinghamshire, U.K.). The detection of the Cx43 protein was performed by an immunoblot analysis using specific anti-Cx43 monoclonal antibodies (Chemicon, Themecula, CA), as described previously by Matesic et al. (32). The detection of the Cx43 protein-antibody complexes was performed using an enhanced chemiluminescence Western blotting detection reagents kit (Amersham), according to the manufacturer's protocol. For the treatment of the samples with alkaline phosphatase, the samples were, in part, pre-incubated with alkaline phosphatase (10 U/ml calf intestine phosphatase; Boehringer Mannheim, Mannheim, Germany) for 2 h at 37°C, and then subjected to Western blot analysis, as described above. The results were then analyzed by autoradiography and video densitometry.

Statistical analysis. Comparison of the two groups was calculated by Student's *t* test. Analysis of experiments containing more than two groups was calculated by Fisher's protected least significant difference test.

RESULTS

Effects of a high glucose level and TPA on GJIC activity. After microinjection, Lucifer Yellow rapidly transferred to neighboring cells (Fig. 1), and the number of Lucifer Yellow–transferred cells reached a steady state at 20 s after the microinjection in both normal glucose (5.5 mmol/l) and high glucose (22 mmol/l) level culture conditions. The GJIC activity was then assessed by counting the number of dye-transferred cells at 20 s after microinjection. The number of dye-transferred cells was significantly inhibited in the high glucose level culture condition as compared with that in the normal glucose level culture condition (11.75 ± 0.64 vs. 15.83 ± 1.36 cells; $P < 0.05$; $n = 12$) (Fig. 2). The addition of mannitol (16.5 mmol/l) with 5.5 mmol/l glucose did not affect the GJIC activity (12.01 ± 0.62) (Fig. 2). The total protein content and cell number were not significantly different between cells exposed to a normal glucose level and those exposed to a high glucose level for 48 h in 1% FCS. In addition, the treatment of the VSMCs with 5×10^{-8} mol/l TPA for 1 h significantly inhibited the number of dye-transferred cells, decreasing it from 15.83 ± 1.36 to 6.25 ± 0.76 cells ($P < 0.01$; $n = 12$) (Fig. 2).

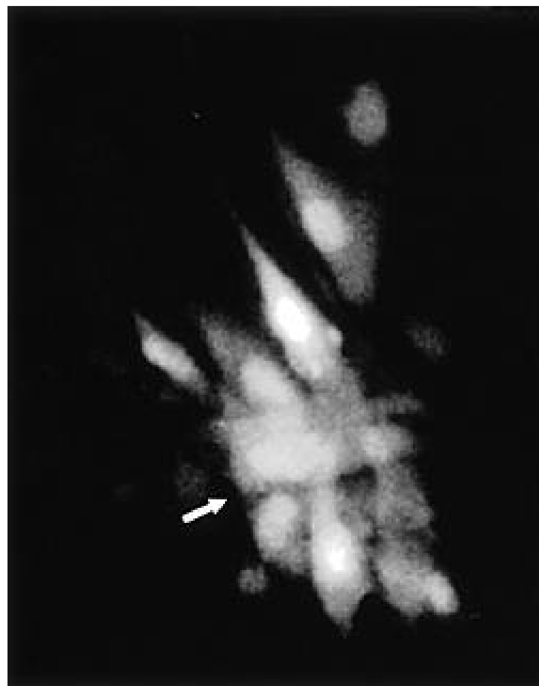

FIG. 1. A fluorescent micrograph showing the dye transfer via gap junction between VSMCs in ~90% confluence in culture. Using a phase-contrast microscope, 0.5 pl of 10% Lucifer Yellow CH was microinjected into a single cell (arrow showing a microinjected cell) with a pneumatic picopump assembled in an injectscope system. After microinjection, the extent of the dye transfer was recorded with a videosystem under fluorescence microscopy. Lucifer Yellow microinjected into a cell was then transferred to neighboring cells.

Effect of calphostin C on GJIC activity in VSMCs. To confirm the role of PKC in the high glucose–induced inhibition of GJIC activity, after a 48-h exposure of the VSMCs to a high glucose level, calphostin C (5×10^{-8} mol/l) was added to the culture medium for 3 h. As shown in Fig. 3, calphostin C completely restored the GJIC activity inhibited by a high glucose level to the control level (12.3 ± 1.1 [normal glucose level] vs. 5.5 ± 0.4 [high glucose level] vs. 10.5 ± 1.0 [high glucose level + calphostin C]; $P < 0.01$; $n = 6$). Calphostin C did not affect the GJIC activity in 5.5 mmol/l glucose condition (12.3 ± 1.1 vs. 11.6 ± 0.8; $n = 6$).

Effect of high glucose level on activation of PKC in VSMCs. The specific activities of PKC in the membranous and cytosolic fractions of VSMCs were measured after a 48-h incubation in normal glucose (5.5 mmol/l) and high glucose (22 mmol/l) level conditions. When the VSMCs were cultured with a high glucose level for 48 h, the PKC-specific activities in the membrane fraction were significantly increased versus when cultured with a normal glucose level (100 vs. 134.2 ± 8.6%; $P < 0.05$; $n = 4$) (Fig. 4). There was no significant difference in PKC activities in the cytosolic fractions between the normal glucose and high glucose levels (100 vs. 98.1 ± 12.1%; $n = 4$) (Fig. 4).

Detection of Cx43. A Western blot analysis revealed that Cx43 proteins are present in cultured bovine VSMCs in four

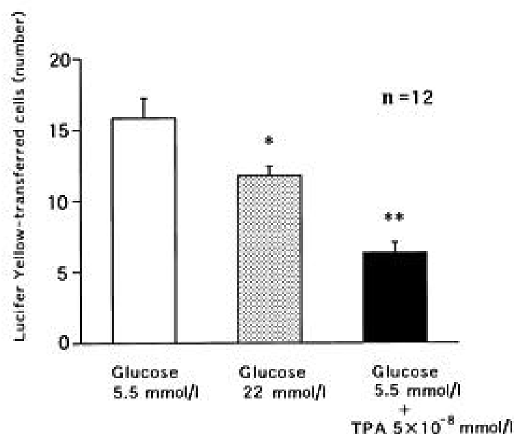

FIG. 2. Effect of a high glucose level and TPA on GJIC activity in VSMCs. VSMCs were incubated with a test medium containing 5.5 or 22 mmol/l glucose without TPA for 48 h or 5.5 mmol/l glucose with TPA at a final concentration of 5×10^{-8} mol/l for 1 h. After the treatments, the GJIC activity was measured (see METHODS). Data are means ± SE. *n*, number of experiments. *$P < 0.05$; **$P < 0.01$.

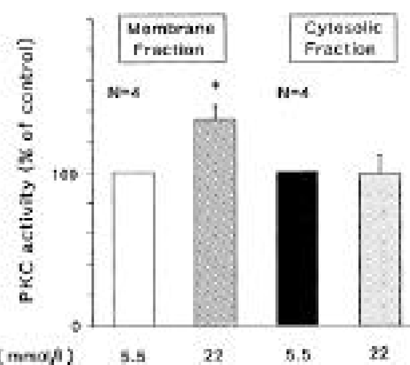

FIG. 4. Effect of a high glucose level on PKC activities. PKC-specific activities were measured in membranous and cytosolic fractions from cultured VSMCs exposed to 5.5 or 22 mmol/l glucose for 48 h. Data are means ± SE. *n*, number of experiments. *$P < 0.05$.

forms in the 40–45 kDa range (Fig. 5). Alkaline phosphatase treatment increased the density of the lowest molecular weight form of the protein at the expense of the other three forms with the higher molecular weights that were totally eliminated (Fig. 5). These results suggest that Cx43 proteins exist in multiphosphorylated forms, consisting of a nonphosphorylated form (P0) and phosphorylated forms (P1, P2, and P3) (Fig. 5).

Effects of high glucose level and TPA on the phosphorylation of Cx43. The exposure of cultured VSMCs to a high glucose level (22 mmol/l) for 48 h increased the density of P3, but decreased the density of P0 (Fig. 6). However, there was no significant difference in the density of P1 or P2 between the cells exposed to high glucose and normal glucose levels. Similarly, an increased density ratio of P3/P0 was observed when the cells were treated with 5×10^{-8} mol/l TPA for 1 h (Fig. 6). The density of P1 and P2 did not change after treatment with TPA. Therefore, we applied the density ratio of P3/P0 as a parameter for phosphorylation in Cx43 proteins. When VSMCs were cultured with a high glucose (22 mmol/l) level for 48 h, the P3/P0 ratio significantly increased as compared with the normal glucose (5.5 mmol/l) level (1.89 ± 0.16 vs. 0.81 ± 0.06; $P < 0.01$; $n = 5$) (Fig. 7). Similar to the high glucose results, a culture of the VSMCs with 5×10^{-8} mol/l TPA significantly increased the P3/P0 ratio as compared with the control level (2.38 ± 0.26 vs. 0.81 ± 0.06; $P < 0.01$; $n = 5$) (Fig. 7). The total amount of Cx43 (P0 + P1 + P2 + P3) was not significantly affected by the high glucose or TPA culture conditions.

Effect of staurosporine and calphostin C on phosphorylation of Cx43. To confirm the role of PKC in the high glucose–induced phosphorylation of Cx43, after a 48-h exposure of the VSMCs to a high glucose level, staurosporine (10^{-7} mol/l) and calphostin C (5×10^{-8} mol/l) were added to the culture medium for 3 h. As shown in Fig. 8, staurosporine and calphostin C completely restored the increased P3/P0 ratio induced by a high glucose level to the control level (2.08 ± 0.16

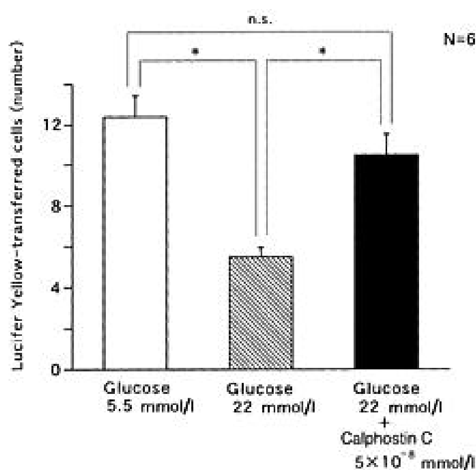

FIG. 3. Effect of calphostin C on GJIC activity in VSMCs induced by a high glucose level.VSMCs were incubated with a test medium containing 5.5 or 22 mmol/l glucose without calphostin C for 48 h or 22 mmol/l glucose with calphostin C at a final concentration of 5×10^{-8} mol/l for 3 h. After the treatments, the GJIC activity was measured (see METHODS). Data are means ± SE. *n*, number of experiments. *$P < 0.01$.

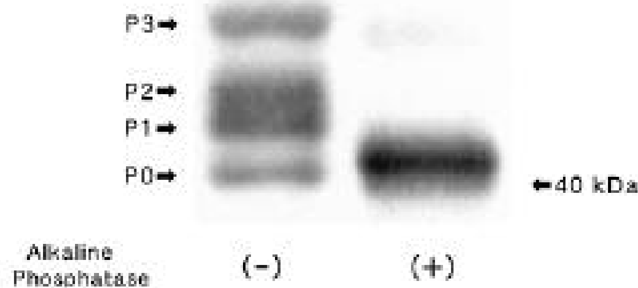

FIG. 5. Identification of Cx43 in the cultured VSMCs by Western blot analysis. Protein (10 μg) extracted from cultured VSMCs was treated with or without alkaline phosphatase (10 U/ml). Following the treatments, the samples were subjected to SDS-PAGE, followed by Western blot analysis using mouse anti-Cx43 monoclonal antibodies (see METHODS). This is a representative autoradiogram of three experiments.

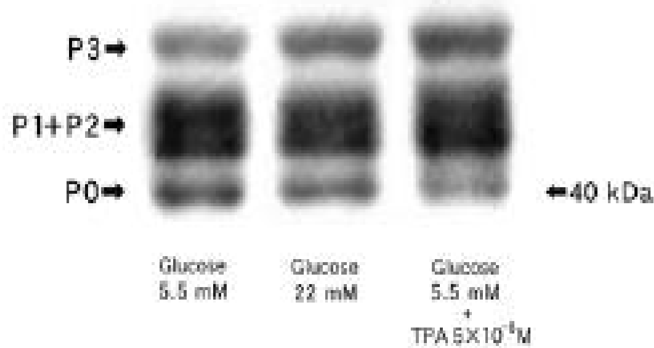

FIG. 6. Effect of a high glucose level and TPA on Cx43 phosphorylation state in cultured VSMCs. VSMCs were incubated with a medium containing 5.5 or 22 mmol/l glucose without TPA or 5.5 mmol/l glucose with TPA at a final concentration of 5×10^{-8} mol/l for 1 h. After the treatments, the samples were subjected to SDS-PAGE, followed by Western blot analysis using mouse anti-Cx43 monoclonal antibodies (see METHODS). This is a representative autoradiogram showing changes in the amount of Cx43 bands (P0, P1, P2, and P3).

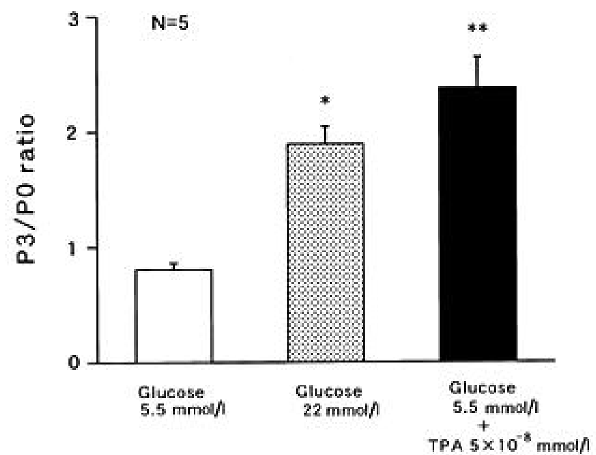

FIG. 7. Analysis of the changes in the ratio of P3/P0 by a high glucose level or TPA. Data are means ± SE. n, number of experiments. $^*P < 0.05$; $^{**}P < 0.01$.

vs. 1.23 ± 0.18; $n = 3$, 1.53 ± 0.16 vs. 0.74 ± 0.09; $n = 3$, respectively). Addition of calphostin C to 5.5 mmol/l glucose for 3 h did not change the P3/P0 ratio as compared with the control level (1.26 ± 0.08 vs. 1.17 ± 0.08; $n = 4$). Addition of 16.5 mmol/l mannitol to 5.5 mmol/l glucose did not affect the P3/P0 ratio as compared with the control level (1.16 ± 0.15 vs. 1.32 ± 0.19; $n = 3$) (Fig. 8*A*).

DISCUSSION

In the present study, we demonstrated that GJIC activity in cultured bovine aortic smooth muscle cells was inhibited by a high glucose level. This finding was consistent with our previous results demonstrating that GJIC activity in bovine aortic endothelial cells is inhibited by a high glucose level (33). The present results showed that the inhibition of the GJIC activity induced by a high glucose level is mimicked by TPA and restored to normal by calphostin C, a PKC inhibitor, thus suggesting PKC-mediated inhibition of GJIC activity. GJIC activity is generally regulated in many ways at the transcriptional, translational, and posttranslational levels of connexins, which make up the gap junctions (25–27). One mechanism responsible for regulating GJIC activity is the PKC-dependent gating of the channels of gap junctions by phosphorylating connexins posttranslationally (32,34,35). Matesic et al. (32) and others (36–38) have reported that PKC regulates GJIC activity by directly phosphorylating connexins in rat liver epithelial cells or other cells in culture. Gap junction protein connexins are encoded by a gene family; members of this family are differentially expressed in various types of tissue (23,24). In VSMCs, Cx43 is a major protein of gap junctions. It has been clearly established that Cx43 is a phosphoprotein; in many fibroblasts and epithelial cell lines, Cx43 exists in nonphosphorylated and numerous phosphorylated forms (32,36–38). Northern blot analysis has revealed that vascular cells expressed RNA homologous to Cx43, which contains many serines with a number of potential PKC phosphorylation sites (16,39). In the present study, we observed four forms of the Cx43 protein (P0, P1, P2, and P3) in cultured VSMCs. After treatment of alkaline phosphatase, the P1, P2, and P3 bands disappeared but the P0 band increased concomitantly, suggesting that the P1, P2, and P3 bands may represent phosphorylated connexin, and the P0 band may represent nonphosphorylated connexin. When the cells were cultured with TPA for 1 h, the relative densities of P3 intensified, whereas those of P0 diminished; as a result, the P3/P0 ratio significantly increased. There were no changes in the densities of the P1 and P2 bands. In line with our results, Berthoud et al. (36) also observed four forms of phosphorylated Cx43, and found that the P3 band in C9 rat liver epithelial cells increased after treatment with TPA (36). Matesic et al. (32) reported that TPA induces the appearance of a P3 band resulting from the phosphorylation of P0, since a concomitant approximate stoichiomeric loss of P0 staining intensity occurred (32). All these data suggest that the P3 band may represent PKC-dependent phosphorylated Cx43 converted from the nonphosphorylated connexin P0. However, it is still possible that after TPA treatment, P0 might be first converted to P1 or P2 and then be further phosphorylated to P3. The present study demonstrated that a high glucose level may also induce a significant increase in the P3/P0 ratio in cultured VSMCs. This finding suggests that a high glucose level may induce the PKC-mediated phosphorylation of Cx43 protein in VSMCs. This suggestion is supported by the fact that the increased P3/P0 ratio induced by a high glucose level was restored to a normal level by staurosporine and calphostin, both PKC inhibitors. In addition, in this study, we demonstrated that a high glucose level induces PKC activation in cultured VSMCs as previously reported (5,6,8). The mechanism responsible for PKC activation by a high glucose level has been considered to be due to an increase in the diacylglycerol level, a physiological activator of PKC (4–8). High glucose increases the de novo synthesis of diacylglycerol (4–8). Taken together, all these data suggest that a high glucose level may inhibit the GJIC activity by phosphorylating Cx43 directly via the activation of PKC in cultured VSMCs.

VSMCs in large vessels of the mature vasculature are under stringent growth control. The migration and overproliferation of VSMCs are key events in developing atherosclerotic lesions. With regard to diabetes, various abnormalities—such as increased levels of platelet-derived growth factor (40), hyperinsulinemia (41), or the overproduction of extracellular matrix components (18)—have been found to

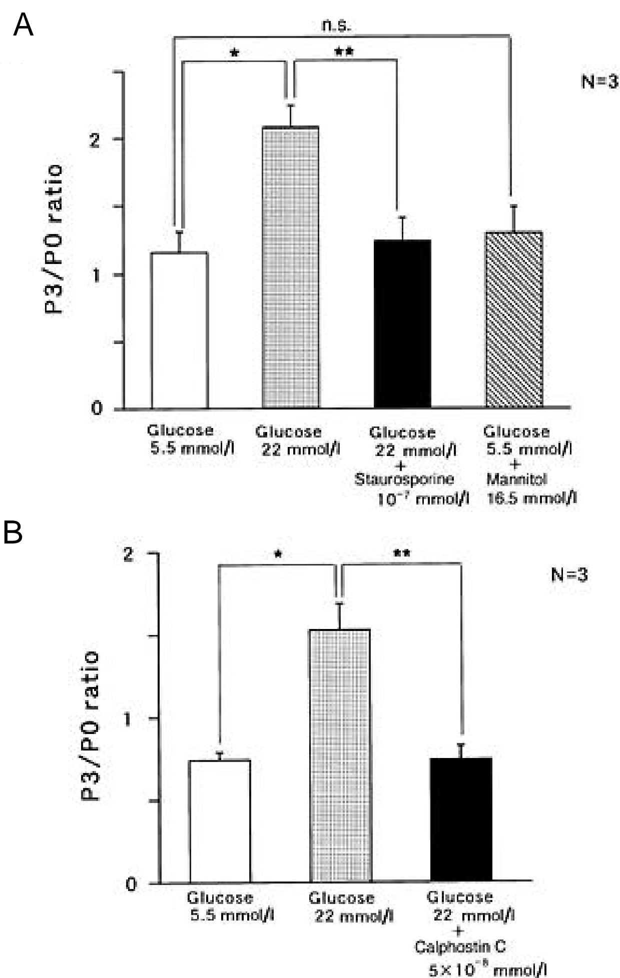

FIG. 8. Effect of staurosporine (*A*) and calphostin C (*B*) on the increased ratio of P3/P0 induced by a high glucose level. Data are means ± SE. *n*, number of experiments. **P* < 0.05; ***P* < 0.01.

explain the migration and proliferation of VSMCs in atherosclerotic processes. GJIC may be intimately involved in the basic processes of cell proliferation and differentiation. This consideration is also supported by evidence that GJIC is highly responsive to a variety of growth signals, including platelet-derived growth factor, epidermal growth factor (19, 20), and tumor promoters as well as viral and cellular Src oncoproteins (42,43). Therefore, cell-to-cell communication via gap junctions as well as the diffusible transport of growth factors and extracellular matrix components may play a role in the stringent growth control of VSMCs. The inhibited GJIC activity induced by a high glucose level could impair the regulation of the proliferation and differentiation of VSMCs. This may account for the acceleration of vascular lesions in diabetic patients.

Regarding the association of gap junction defects and human diseases, the connexin 32 mutation has been reported in cross-linked Charcot-Marie-Tooth disease, demyelinating peripheral neuropathy (44); the mutation in Cx43 has also been reported to underlie various cardiac malformations in visceroatrial heterotaxia syndromes (45). Such evidence appears to unequivocally demonstrate the importance of the gap junction function. However, there have been very few reports concerning the functional alteration of GJIC activity in pathological conditions. Our findings may thus provide new insights into the pathophysiological significance of the gap junction function.

In conclusion, the high glucose–induced inhibition of GJIC in VSMCs may cause homeostatic disorders in the vascular wall, and, as a result, may contribute to the development of the macroangiopathy associated with diabetes.

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