Inhibition of Human Mesangial Cell Proliferation by Calcium Channel Blockers

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Human mesangial cells in culture proliferate in response to platelet-derived growth factor (PDGF) and thrombin. Both of these agents also induce changes in cytosolic calcium that are dependent on both mobilization of intracellular calcium and influx of extracellular calcium. We hypothesized that calcium channel blockers, by preventing influx of extracellular calcium, may inhibit proliferation induced by these mitogens. We found that three different calcium channel blockers, diltiazem, nifedipine, and verapamil, were able to significantly inhibit [3H]thymidine incorporation into human mesangial cells induced by either PDGF or thrombin. The inhibitory effect of these agents was significant at 10^{-7} M. The calcium channel blockers also attenuated the increases in cell number and percentage of labeled nuclei induced by these mitogens. In contrast, dantrolene, an inhibitor of intracellular calcium mobilization, had no significant effect on [3H]thymidine incorporation by PDGF or thrombin. Finally, the calcium channel agonist, Bay K 8644 was found to stimulate [3H]thymidine incorporation into mesangial cells. Although the mechanisms for these effects of calcium channel blockers are not proven, these studies suggest that influx of extracellular calcium is an important signal in mitogen-induced mesangial proliferation and that these agents can be beneficial in preventing or attenuating renal diseases characterized by proliferation of these cells. (Hypertension 1990;15(suppl I): I-76–I-80)

Proliferation of mesangial cells is observed in a variety of immune- and nonimmune-mediated glomerular diseases including hypertensive renal injury. The factors responsible for stimulating this proliferation in vivo are unknown. In vitro studies using mesangial cells in culture, however, have shown that platelet-derived growth factor (PDGF) as well as the coagulation factor, thrombin, are both capable of stimulating DNA synthesis and cell division within these cells. PDGF and thrombin also stimulate increases in phosphoinositide turnover and cytosolic free Ca^{2+} ([Ca^{2+}]_i), which are thought to be critical intracellular signals for their mitogenic effects. The changes in [Ca^{2+}]_i for both of these mitogens show a similar pattern characterized by a rapid increase (as early as 5–10 seconds), a transient peak (at 30–50 seconds), and then a sustained elevation for 10–15 minutes. When extracellular calcium is removed from the media, the rapid transient [Ca^{2+}]_i peak is reduced slightly, but more impressively, the sustained elevation in [Ca^{2+}]_i is obliterated. The importance of these [Ca^{2+}]_i signals in the mitogenic response is unclear, but if influx of extracellular Ca^{2+} is important in the action of PDGF and thrombin, then calcium channel blockers, which inhibit influx of Ca^{2+}, might inhibit the mitogenic effect of these substances.

Calcium channel blockers lower systemic blood pressure and inhibit DNA synthesis in aortic smooth muscle cells in culture and in the aortas of rats with hypertension. They have also been shown to reduce the extent of aortic atherosclerosis in cholesterol-fed rabbits. Within the kidney, calcium channel blockers can vasodilate afferent arterioles and might increase the glomerular ultrafiltration coefficient, which is determined in part by mesangial cell function. In patients with hypertension and renal insufficiency, short-term treatment with calcium channel blockers has been shown to improve glomerular filtration rate. Further, in hypertensive Dahl salt-sensitive rats with subtotal renal ablation, calcium channel blockers reduce histological evidence of glomerular injury despite having little effect on proteinuria. The latter suggests that these agents might have protective effects on hypertensive renal injury apart from their hemodynamic effects.

To help delineate a potential mechanism for the protective effect of calcium channel blockers in renal disease, and in an attempt to determine the role of extracellular Ca^{2+} in the mitogenic signal, we have studied the effect of three chemically different...
calcium channel blockers on PDGF and thrombin-induced mesangial cell proliferation. We find that diltiazem, nifedipine, and verapamil all partially inhibit [H]thymidine incorporation and increases in cell number induced by these mitogens; whereas dantrolene, a blocker of Ca\textsuperscript{2+} release from intracellular stores, had no inhibitory effect. The calcium channel agonist Bay K 8644 was found to stimulate [H]thymidine incorporation into mesangial cells. Thus, we conclude that influx of extracellular calcium is an important signal for the proliferative effect of mesangial cell mitogens and that inhibition of this signal could account for the protective effects of calcium channel blockers in glomerular injury.

**Methods**

Human mesangial cells were cultured, as previously described, from glomeruli isolated from human kidney tissue immediately after surgical nephrectomy or from donor cadaver kidneys judged unsuitable for transplantation. The glomeruli were incubated in Waymouth's media (GIBCO, Grand Island, New York) with 750 units/ml of type IV collagenase (Sigma Chemical Co., St. Louis, Missouri) at 37° C for 60 minutes. Glomerular cores were plated in culture dishes in Waymouth's media supplemented with 15 mM HEPES, pH 7.4, 0.66 units/ml insulin, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, antibiotic antifungal solution, and 17% fetal calf serum (HyClone, Logan, Utah). The dishes were kept at 37° C in 5% CO\textsubscript{2}. After glomeruli attached and cells began to grow, the media was changed two or three times per week. Cells were passed by washing twice with Hank's buffer without Ca\textsuperscript{2+} or Mg\textsuperscript{2+} and then incubating with 0.025% trypsin-0.5 mM EDTA.

Incorporation of [H]thymidine into mesangial cell DNA was assayed in confluent cells grown in 24-well dishes. The cells were made quiescent by incubating with SF media alone and the counts per minute of [H]thymidine incorporated into each of these wells were meaned to determine the basal counts per minute. Additionally, triplicate wells from each experiment were trypsinized and the cells counted in a hemocytometer after trypan blue staining. Cell viability always exceeded 95%. The counts per minute of [H]thymidine incorporated into each of these wells were meant to determine the basal counts per minute incorporated. To further assess the inhibitory effect of calcium channel blockers, the data are expressed as a percentage of the mitogen-induced [H]thymidine incorporation value. Data were analyzed with Student's t test or analysis of variance as appropriate, and p values are as given.

To further assess the inhibitory effect of the calcium channel blockers, cell counting and autoradiography were performed. For autoradiography, mesangial cells were plated on four chamber glass slides (Lab-Tech, Nunc, Inc., Naperville, Illinois) and placed in SF media when subconfluent. The cells were incubated with conditions and [H]thymidine as described above for the [H]thymidine assay. The cells were then washed with methanol-acetic acid (3:1, vol:vol) and exposed to NTB II emulsion (Eastman Kodak, Rochester, New York) for 3 days at 4° C. The slides were then developed, fixed, and stained with Giemsa stain. Over 200 nuclei were counted for each condition, and results are reported as percentage of labeled nuclei.
Results

We have previously found that PDGF purified from platelets (PDGF-AB) and thrombin (both bovine and human) are potent stimulators of \[^3H\]thymidine incorporation into DNA of human mesangial cells. In the present studies using recombinant PDGF-BB, \[^3H\]thymidine incorporation increased from basal levels of 171±22 cpm/10^4 cells in SF wells to 959±253 cpm/10^4 cells in wells incubated with 5 ng/ml PDGF (mean±SEM, n=5, p<0.05). In separate mesangial cell experiments, basal levels of \[^3H\]thymidine incorporation were 270±71 cpm/10^4 cells and increased to 925±355 cpm/10^4 cells after incubation with 5 units/ml thrombin (mean±SEM, n=5, p<0.05). All incubations were performed for a total of 28 hours. The concentrations used in these experiments are those that gave near-maximal effects in dose-response curves.

When the calcium channel blockers, diltiazem, nifedipine, or verapamil (at 10^-5 M), were added to the mesangial cells just before incubation with PDGF, \[^3H\]thymidine incorporation measured 28 hours later was significantly inhibited (Figure 1). This effect was not apparent at lower concentrations of the calcium channel blockers. Nifedipine and verapamil (10^-5 M) also significantly inhibited thrombin-stimulated increases in \[^3H\]thymidine incorporation (Figures 1B and 1C). Diltiazem (at 10^-5 M) had a small inhibitory effect on thrombin stimulation, but because of variability in the effect, this did not prove statistically significant. None of the three calcium channel blockers had any significant effect on basal \[^3H\]thymidine incorporation when no mitogen was present (data not shown). The effect of the calcium channel blockers on \[^3H\]thymidine incorporation into mesangial cells was not because of cell toxicity because cell number per well was not significantly different in wells incubated for 28 hours with SF media alone as compared with SF media plus diltiazem, nifedipine, or verapamil (SF, 1.0±0.3; diltiazem, 1.1±0.1; nifedipine, 0.8±0.2; and verapamil, 0.9±2.2x10^4 cells, n=5). Additionally, cell morphology by phase-contrast microscopy was not altered by any of the calcium channel blockers.

PDGF alone increased mesangial cell number by 83% after 28 hours of incubation. When diltiazem, nifedipine, or verapamil were incubated with PDGF, a marked reduction in this increase in cell number was noted (Table 1). Only a 15% increase in cell number per well was detected after 28 hours of incubation with thrombin (5 units/ml); however, this increase was completely inhibited by each of the calcium channel blockers tested. The results of autoradiography experiments with PDGF or thrombin and the three calcium channel blockers are also shown in Table 1. The percentage of labeled nuclei under basal conditions in our autoradiography experiments was 8.4±0.8 (mean±SEM, n=4 chambers). This increased to 18.5±3.0% and 12.3±0.3% with PDGF and thrombin, respectively (n=3 experiments) (Table 1). Diltiazem, nifedipine, and verapamil all reduced the percentage of labeled nuclei seen in the presence of either PDGF or thrombin.

When dantrolene was included in mesangial cell incubations with PDGF or thrombin and \[^3H\]thymidine incorporation was measured, no significant reduction in stimulated values was detected, even at concentrations as high as 10^-4 M. The percentage of the PDGF-stimulated value was 97±6, 132±46, and 106±17 for dantrolene 10^-6 M, 10^-5 M, and 10^-4 M, respectively, while the percentage of thrombin-stimulated \[^3H\]thymidine was...
120±10, 137±25, and 78±40. The calcium channel agonist Bay K 8644 was also tested for its effect on [3H]thymidine incorporation into human mesangial cells. This agent was found to stimulate incorporation at concentrations between 10^{-7} and 10^{-6} M (Figure 2). At concentrations of 10^{-5} M, however, Bay K 8644 actually reduced [3H]thymidine incorporation below control values.

**Discussion**

We have observed that the [3H]thymidine incorporation and cell proliferation induced by recombinant PDGF-BB and by bovine thrombin can be partially inhibited by calcium channel blockers. Three different types of calcium channel blockers were tested in these studies including dihydroxypridine (nifedipine), diphenylalkylamine (verapamil), and benzothiazepine (diltiazem). All three agents required similar concentrations for the inhibitory effect. The inhibitory effects of the calcium channel blockers on [3H]thymidine incorporation were confirmed with autoradiography studies. Additionally, all three calcium channel blockers inhibited cell proliferation induced by PDGF and thrombin.

Because measurement of cytosolic calcium or phosphoinositide turnover was not performed in these studies, the mechanism whereby the calcium channel blockers exert their effect on mitogen-induced [3H]thymidine incorporation cannot be determined with certainty. It is interesting, however, that three chemically different calcium channel blockers had similar inhibitory effects at similar concentrations. Additionally, dantrolene, an inhibitor of intracellular calcium mobilization, had no effect on mitogen-induced [3H]thymidine incorporation. Bay K 8644, without other mitogens, had a biphasic effect on [3H]thymidine incorporation into mesangial cells, causing stimulation of incorporation at 10^{-7} and 10^{-6} M but an inhibitory effect at 10^{-5} M. Although generally a calcium channel agonist, the racemic mixture of Bay K 8644 has been shown to have calcium channel antagonistic effects at concentrations greater than 10^{-6} M. It is tempting to suggest that these antagonist effects counteract the agonist effects of Bay K 8644 at the highest concentrations. All together, these observations suggest that influx of extracellular Ca^{2+} is an important signal for the mitogenic effects of PDGF and thrombin.

Because high concentrations of the calcium channel blockers were required for the inhibitory effect, however, other possible mechanisms for our observations must be considered. It is possible that the calcium channel blockers could interfere with the binding of the mitogens to their receptors on the cell, or that they might be uncoupling receptor activation from signal transduction pathways within the membrane, as suggested by Block et al. Evidence for these mechanisms comes from their studies with vascular smooth muscle cells, in which they found that calcium channel blockers not only inhibited PDGF-induced mitogenesis but also PDGF-induced vasoconstriction. They demonstrated that the three calcium channel blockers verapamil, nifedipine, and diltiazem all prevented the increases in cytosolic calcium, phosphoinositide turnover, and production of diacylglycerol induced by PDGF-BB and that the first two calcium channel blockers also prevented translocation of protein kinase C to the plasma membrane induced by PDGF-AA. Additional potential mechanisms to be considered are that the calcium channel blockers are inhibiting a secondary Ca^{2+} entry from extrasaccular sources because of inositol 1,3,4,5-tetrakisphosphate or that they are inhibiting mobilization of intracellular Ca^{2+} by binding to receptor sites on the sarcoplasmic reticulum.

Which of these potential mechanisms is operative in these studies is currently under investigation; however, the observation that calcium channel blockers inhibit mitogen-induced mesangial cell proliferation might have implications in renal disease. Mesangial proliferation occurs in response to a variety of injurious stimuli within the glomerulus, and this proliferation can have many consequences for glomerular function. The increase in mesangial cell number could affect transport of macromole-
cules through the mesangium or could affect glomerular filtration by altering the ultrafiltration coefficient. Additionally, increases in the number of mesangial cells can also result in quantitative or qualitative changes in the mesangial matrix proteins that are secreted by these cells and could contribute to glomerulosclerosis. Further studies of calcium channel blockers are needed to determine the effectiveness of these agents in attenuating development of renal injury.

References

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