Nifedipine and Nicardipine Potentiate Glucagon-Stimulated Glycogenolysis in Primary Cultures of Rat Hepatocytes

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The effects of two calcium channel blockers, nifedipine and nicardipine, on glucagon-stimulated glycogenolysis in primary cultures of rat hepatocytes were examined in vitro. When nifedipine and nicardipine (10^-7—10^-6 M) were added to the incubation mixture with various concentrations of glucagon (10^-10—10^-6 M), these dihydropyridine calcium channel blockers significantly potentiated the glycogenolytic action of glucagon by increasing intracellular cAMP levels. 1-Methyl-3-isobutylxanthine (IBMX), caffeine and papaverine, which is known to inhibit cAMP phosphodiesterase, also potentiated the stimulatory effect of glucagon on the glycogenolysis in a dose-dependent manner. Parallel to the potentiation of glycogenolysis, IBMX also increased the glucagon-stimulated intracellular cAMP levels in a dose-dependent manner. These results suggest that the mechanism of potentiation of the glucagon-stimulated glycogenolysis by nifedipine and nicardipine is related to the known inhibition of cAMP phosphodiesterase by these agents.

Keywords calcium channel blocker; glucagon; glycogenolysis; rat hepatocyte primary culture

Glucose production is the most important function of the liver. The glucose is produced as a result of the breakdown of glycogen (i.e., glycogenolysis). When the hepatic glycogen store is exhausted, the glucose supply relies on its synthesis from non-glucidic precursors in the liver (i.e., gluconeogenesis). These metabolic processes are stimulated mainly by glucagon and catecholamines. There is general agreement that the stimulation of hepatic glycogenolysis by glucagon is mediated through changes in the intracellular concentration of its second messenger, cAMP. The cAMP activates a protein kinase, which by catalyzing the phosphorylation of one or more enzymes alters the activity of these enzymes.

In a previous report, we showed that two dihydropyridine calcium channel blockers, nifedipine and nicardipine, but not verapamil and diltiazem, produced mild hyperglycemia in 20-h-starved streptozotocin-diabetic rats in vivo. During the course of studies addressing why nifedipine and nicardipine produce hyperglycemia in streptozotocin-diabetic rats, the author found that these dihydropyridines potentiated glucagon-stimulated glycogenolysis in primary cultures of rat hepatocytes. Although high levels of glucagon, which may occur in diabetes in vivo, increase glucose production by the liver, the mechanism(s) of its potentiation by nifedipine and nicardipine remains to be elucidated.

In the present study, therefore, the author further examined the mechanism(s) of action of these calcium channel blockers on the glucagon-stimulated glycogenolysis in primary cultures of rat hepatocytes.

Materials and Methods

Hepatocyte Isolation and Culture Liver parenchymal cells were isolated from male Wistar rats weighing 200—250 g by a modification of the procedure of Berry and Friend. Briefly, rats were anesthetized with sodium pentobarbital and their livers were first perfused in situ via the portal vein with a calcium-free Hanks' 10 mM Hepes buffer (pH 7.4) at a flow rate of 30 ml/min for 10 min. In the second step, the liver was perfused with the same buffer containing 0.025% collagenase (type II) and 0.075% CaCl_2 at the same flow rate for 10 min. At the end of the perfusion, a cell suspension was formed by gentle disruption of the collagenase-treated livers in Ca^{2+}-free Hank's solution. The cells were washed three times by slow centrifugation (50 g) for 1 min in order to remove cell debris, damaged cells and non-parenchymal cells. Routinely, more than 85% of the cells were intact as monitored by trypan blue exclusion. Hepatocytes were suspended in Williams' medium E containing 10^{-9} M insulin, 10^{-9} M dexamethasone, 5% newborn bovine serum, 100 mg/ml streptomycin, and 100 units/ml penicillin. Inocula of 1 x 10^6 cells were seeded to 35 mm diameter collagen-coated six-well plastic plates in 2 ml of Williams' medium E supplemented with additional glucose (2.5 mg/ml) to allow the accumulation of glycogen in the hepatocytes; the plates were incubated at 37°C under an atmosphere of 5% CO_2 in humidified air. After 3 h at 37°C, non-attached cells were removed by aspiration, and attached cells were washed twice with the culture medium.

Metabolic Experiments After culturing for a further 21 h, the culture media were removed by aspiration. The cells were carefully washed twice with 2 ml of 10 mM Hepes buffer (pH 7.4) and incubated in 1.0 ml of the same buffer. An aliquot of 10 μl of glucagon and/or the calcium channel blockers was simultaneously added to the incubation mixture. Then the hepatocytes were incubated for 30 min at 37°C. The incubation was terminated by cooling the mixture in an ice bath. Glucose in the medium (0.5 ml) was determined by using a commercially available diagnostic kit. Since gluconeogenesis is negligible under these experimental conditions, glucose output in the medium mainly reflects the breakdown of glycogen (i.e., glycogenolysis). Glucose production was linear for over 1 h in the presence of various concentrations of glucagon.

After 30 min of incubation, glycogenolysis was terminated by the addition of 0.1 ml of 1 N HCl solution. The hepatocytes were scraped off with a rubber policeman. The cells and medium were then transferred to test tubes and heated at 100°C for 3 min to obtain deproteinized extracts. The suspension was centrifuged at 1500 x g for 5 min. The supernatant was stored at −70°C and cAMP was determined by the radioimmunoassay procedure of Honna et al. Cellular protein was measured by the method of Lowry et al. using bovine serum albumin as a standard.

Statistics The statistical significance of differences was performed using the unpaired Student's t-test. p values of less than 0.05 were regarded as statistically significant.

Materials The sources of drugs were as follows: collagenase (type II; Worthington Biochemical Co.), Calcium channel blockers (Sigma, St. Louis, MO) and Williams' medium E (Gibco Laboratories, Grand Island, NY). The assay kit for glucose was obtained from Wako Pure Chemical Co. (Osaka, Japan). The assay kit for cAMP was purchased from Yamasa Shoyu Co. (Chiba, Japan). All other reagents were of an analytical grade. Stock solutions of nifedipine and nicardipine were 10^-3 M (in 50% ethanol). These agents were protected from light during use.

Results Figure 1 shows the effects of nifedipine and nicardipine (10^-7—10^-5 M) on glycogenolysis in primary cultures of rat hepatocytes incubated with various concentrations of glucagon. Glucagon alone increased the glucose output from hepatocyte monolayers in a dose-dependent manner up to
Fig. 1. The Effects of Nifedipine and Nicardipine on Glucagon-Stimulated Glycogenolysis in Primary Cultures of Rat Hepatocytes

Hepatocytes were prepared and incubated as described in Materials and Methods. Results were expressed as nmol of glucose formed/30 min/mg of cellular protein. The basal value was 302 ± 15 nmol/30 min/mg protein. Results are the mean ± S.E.M. of 3–4 independent preparations. Values significantly different from the respective control values (glucagon alone) were indicated as a) (p < 0.05). (A): glucagon (Gln, ●), Gln + 10^{-7} M nifedipine (Nif, □), Gln + 10^{-8} M Nif (△), Gln + 10^{-9} M Nif (○). (B): glucagon (Gln, ●), Gln + 10^{-7} M nicardipine (Nic, □), Gln + 10^{-8} M Nic (△), Gln + 10^{-9} M Nic (○).

about 160% of basal glycogenolysis. Treatment of hepatocytes with the dihydropyridine calcium channel blockers (10^{-7}–10^{-5} M) potentiated the same degree of stimulation of glycogenolysis which was achieved by glucagon, except for a significant inhibition by 10^{-5} M of nicardipine. The effect of dihydropyridines was not dose-dependent. Nifedipine and nicardipine at 10^{-8} M had no significant effect on glucagon-stimulated glycogenolysis (not shown). These dihydropyridine calcium channel blockers by themselves did not affect the basal rate of glycogenolysis (not shown).

Glucagon led to dose-dependent increases in the levels of cAMP when hepatocyte monolayers were incubated under glycogenolytic conditions. Nifedipine and nicardipine (10^{-7}, 10^{-6} M) potentiated the glucagon stimulation of cAMP levels in the primary cultures of rat hepatocytes. The potentiations were not dose-dependent. These results correlated well with those of glycogenolytic responses. Nifedipine and nicardipine alone had no significant effect on the basal level of cAMP when applied to the hepatocyte monolayers (Fig. 2A, B).

1-Methyl-3-isobutylxanthine (IBMX, 10^{-6}–10^{-4} M), which is known to inhibit cAMP phosphodiesterase, also potentiated the stimulatory effect of glucagon on glycogenolysis in a dose-dependent manner (Fig. 3A). IBMX alone did not affect the basal rate of glycogenolysis. In addition, caffeine and papaverine (10^{-5}–10^{-4} M) actually potentiated the glucagon-stimulated glycogenolysis in a dose-dependent manner (results not shown).

The effects of IBMX on the glucagon-induced increases in intracellular cAMP levels were examined next when the cells were experiencing glycogenolytic conditions. As shown in Fig. 3B IBMX (10^{-5}–10^{-4} M) potentiated the glucagon-stimulated increases in intracellular levels of cAMP in a dose-dependent manner. IBMX at these concentrations did not affect the basal level of cAMP in the primary cultures of rat hepatocytes. These results were consistent with those of the glycogenolytic responses.

Discussion

Calcium channel blockers, nifedipine, nicardipine, verapamil, and diltiazem, members of a chemically heterogenous group of agents, are widely employed in cardiovascular therapy and are first-line antihypertensive agents.10,11 They share the common property of blocking the entry of calcium into cells by voltage-gated channels in cardiac and smooth muscle.12,13 However, these agents clearly differ both quantitatively and qualitatively in their therapeutic roles: e.g. verapamil and diltiazem can induce significant antiarrhythmic activity, but this is not observed with nifedipine or other dihydropyridines.11 The effects of these agents on the cardiovascular system have been extensively studied in vivo and in vitro, but only a few reports deal with the quantitative and qualitative differences among the calcium channel blockers in relation to hepatic carbohydrate metabolism.

As shown in Figs. 1 and 2, the two dihydropyridine calcium channel blockers, nifedipine and nicardipine, greatly potentiated glucagon-stimulated glycogenolysis by increasing cAMP levels, whereas non-dihydropyridine calcium channel blockers, verapamil and diltiazem (10^{-7}–10^{-5} M) did not potentiate, but actually slightly suppressed the glucagon-stimulated glycogenolysis (data not shown).
The effects of the dihydropyridine calcium channel blockers could not be simply explained by the blockade of Ca\(^{2+}\) entry into hepatocytes in a primary culture, since reduced cytosolic concentrations of Ca may not increase, but rather attenuate, the activity of phosphorylase kinase, which is known to activate glycogen phosphorylase (i.e., the enzyme responsible for glycogenolysis).\(^{21}\)

A possible explanation for these observations is that dihydropyridine calcium channel blockers inhibit cAMP phosphodiesterase activity as reported by Sakamoto et al.,\(^{14}\) Epstein et al.,\(^{15}\) and Norman et al.\(^{16}\) They demonstrated that dihydropyridine Ca\(^{2+}\) entry blockers inhibited both calmodulin-sensitive and -insensitive forms of phosphodiesterase from different tissue sources (e.g., hepatocyte, brain, intestine, etc.) with IC\(_{50}\)s in the \(\mu M\) range. Non-dihydropyridine calcium channel blockers did not exhibit such action. Therefore, it is possible that Ca\(^{2+}\) channel blockers, particularly nifedipine and nicardipine, have a qualitatively different molecular target from the classical voltage-dependent Ca\(^{2+}\) channels at micromolar concentrations. This view was further supported by the observation that glucagon-stimulated glycogenolysis was potentiated by IBMX (Fig. 3), papaverine, and caffeine (10\(^{-5}\) to 10\(^{-4}\) M, not shown) in a dose-dependent manner. In contrast to IBMX, the effects of nifedipine and nicardipine on glucagon-stimulated glycogenolysis and intracellular cAMP levels were not dose-dependent. This discrepancy is difficult to interpret; however, at least two mechanisms may be responsible for the metabolic effects of nifedipine and nicardipine, which include (1) the inhibition of Ca\(^{2+}\) entry into target cells and (2) the inhibition of cAMP phosphodiesterase activity. The observed effects could be the composite of these reciprocal effects of the dihydropyridines.

One unexpected result of this work is the observation that a higher concentration of nicardipine decreased the glucagon-induced glycogenolysis in parallel with cAMP levels. Although this result seems to be difficult to interpret, it may be due to the non-specific inhibition of hepatocytes by a higher concentration of nicardipine.

In connection with the \textit{in vivo} situation, it has been reported that the livers of streptozotocin-diabetic rats contain sufficient glycogen to produce mild hyperglycemia, even after 24 h of starvation.\(^{17}\) Therefore, it is possible that nifedipine and nicardipine at micromolar concentrations effectively potentiate the glucagon-stimulated glycogenolysis by a common mechanism mediated by cAMP in streptozotocin-diabetic rats \textit{in vivo}. Furthermore, it has been reported that nifedipine actually produces hyperglycemia in type II diabetic patients with hypertension,\(^{18}\) and that the effective concentration of nifedipine to decrease diastolic blood pressure in hypertensive patients is 47 ± 20 ng/ml (i.e., 1.3 × 10\(^{-7}\) M).\(^{19}\) These reports suggest that the present experimental findings also have clinical relevance in diabetic patients.

In conclusion, the author found that nifedipine and nicardipine, but not verapamil and diltiazem, potentiated glucagon-stimulated glycogenolysis by increasing the cAMP in primary cultures of rat hepatocytes. The enhanced cAMP levels are likely to be due to the inhibition of cAMP phosphodiesterase activity by the dihydropyridines. These results also support the hypothesis that nifedipine and nicardipine increase plasma glucose levels, at least in part, by potentiating glucagon-stimulated glycogenolysis under certain conditions such as streptozotocin-induced diabetes \textit{in vivo}. Moreover, it should be stressed that together with \textit{in vivo} experiments, the use of primary cultured hepatocytes is a valuable tool for the determination of the role of exogenous and endogenous factors on various hepatic functions.

References

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