Inhibitory Effect of Calcium Channel Blockers on α -Adrenergic Activation of Glycogenolysis and Calcium Efflux in Perfused Rat Liver

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Abstract

In an attempt to verify the importance of calcium ions in mediating α -adrenergic stimulation, the effects of a calcium channel blocker, verapamil, on phenylephrine-induced glycogenolysis and calcium efflux in perfused livers prepared from fed rats were determined.

The blocker inhibited phenylephrine-induced glycogenolysis in a noncompetitive and dose-dependent manner between 50 μ M and 500 μ M. However, it did not affect 2, 4-dinitrophenol-induced glycogenolysis. It had no significant effect on ⁴⁵Ca uptake by the perfused liver, but inhibited basal as well as phenylephrine-induced efflux of ⁴⁵Ca from ⁴⁵Ca-loaded liver. The inhibitory effects on basal ⁴⁵Ca release and phenylephrine-induced glycogenolysis and ⁴⁵Ca release correlated very well. All of the effects of verapamil were reproduced by another calcium channel blocker, diltiazem, suggesting that these effects are common to a variety of calcium channel blockers.

These results indicate that the process of calcium influx and the function of phosphorylase *per se* are not directly involved in the inhibitory action of the blocker. Although it is possible that verapamil interferes with binding of the α -adrenergic agonist to the plasma membrane, the good correlation between the inhibitory effects of verapamil on basal ⁴⁵Ca release and on phenylephrine-induced release of ⁴⁵Ca suggests another mechanism, involving calcium ions. The blocker appears to inhibit the glycogen phosphorylase activity induced by phenylephrine via a cell-membrane mechanism in which calcium ion flux changes are intimately involved.

In the liver, catecholamine stimulates glycogenolysis (Exton *et al.*, 1971) and causes a prompt and transient efflux of preloaded ⁴⁵Ca (Friedmann and Park, 1968). Chen *et al.* (1978) reported that the efflux of calcium induced by epinephrine was a net efflux and suggested the importance of mitochondria as a source of the mobilized calcium. These metabolic effects seem to be elaborated mainly by α -adrenergic action through a process independent of cyclic AMP (Sherline *et al.*, 1972; Hutson *et al.*, 1976; Cherrington *et al.*, 1976;

Birnbaum and Fain, 1977). In addition, the process seems to be calcium dependent (Assimacopoulos-Jeannet *et al.*, 1977; Blackmore *et al.*, 1978; Blackmore *et al.*, 1979a; Garrison *et al.*, 1979).

A hypothesis has therefore been entertained that α -adrenergic stimulation mobilizes calcium ions from mitochondria and increases the cytosolic level of calcium ions, which lead to the activation of phosphorylase b kinase (Brostrom *et al.*, 1971; Ozawa, 1972; Khoo and Steinberg, 1975) and consequently glycogenolysis (Assimacopoulos-Jeannet *et al.*, 1977; Blackmore *et al.*, 1979a). These interpretations are sup-

ported by a recent observation that α -adrenergic stimulation increases the cytosolic free calcium ion concentration two- to three-fold (Murphy *et al.*, 1980).

Recently we showed that the calcium channel blockers, verapamil and diltiazem, markedly inhibited glucagon- and phenylephrine-induced calcium efflux as well as glycogenolysis (Kimura *et al.*, 1978). These observations suggest the possibility that the blockers can be used as a tool to modify the calcium movement induced by hormones, and provide additional information about the relationship between hormone action and calcium ion shift.

In the present communication, the effect of calcium channel blockers on α -adrenergic stimulation was investigated. The results show clearly that verapamil and diltiazem inhibited dramatically both calcium efflux and glycogenolysis induced by α -adrenergic stimulation. Analyses of the inhibitory effects support the concept that calcium ion is involved intimately in mediating the α -adrenergic effect.

Materials and Methods

Animals

Fed, male Wistar rats weighing about 200 g were used. They were raised on regular rat chow (Funabashi Farm, Chiba) and tap water *ad lib*. All experiments were carried out between 08:00 and 10:00 a.m.

Liver Perfusion

The animals were anesthetized with pentobarbital (50 mg/kg, ip). The abdominal wall was incised and the liver was perfused *in situ* by a modification of the method of Mortimore *et al.* (1959). A Krebs-Ringer-Tris (KRT) buffer maintained at 37°C was used as the perfusate after oxygenation. The content of the buffer was the same as that of conventional Krebs-Ringer-bicarbonate buffer except that bicarbonate was replaced by 20 mM Tris-HCl (pH 7.4) and that the concentration of calcium was 0 or 1.0 mM. It was infused constantly into the liver through the portal vein at a speed of 25 ml/min in a flow-through system. The effluent leaving the liver was collected at designated intervals through a canula inserted into

the proximal portion of the inferior vena cava. The time needed for the passage of the buffer from the reservoir to the collecting tube was about 25 sec, and data are presented without correction for the lag.

Phenylephrine or 2,4-dinitrophenol was added to the reservoir of the perfusate 30 min after the beginning of the perfusion. Verapamil or diltiazem was added 10 min before the reagents. The livers were weighed at the end of the experiments. In preliminary experiments, the perfused liver was shown to be well preserved in this system.

⁴⁵Ca efflux study

In experiments in which the movement of ⁴⁵Ca was studied, the livers were perfused with 200 ml of the perfusate containing 50 μ Ci of ⁴⁵CaCl₂ (1 mM) for 45 min in a recirculation system. Then they were perfused in the flow-through system with KRT buffer containing 1 mM CaCl₂ without ⁴⁵CaCl₂ (wash-out perfusion). Verapamil or diltiazem was also included when indicated. Phenylephrine was added 15 min after the switch to the flow-through system. Radioactivity in the effluent was measured with a scintillation spectrometer (Beckman, Model LS-3150P).

Isolation of Mitochondria

The livers were pre-perfused for 45 min with the perfusate containing 1 mM ⁴⁵CaCl₂ in a recirculation system. The system was then switched to the flow-through system with fresh perfusate in which ⁴⁵CaCl₂ was replaced with CaCl₂ and perfused for an additional 3 min (wash-out perfusion) in some experiments. In the other sets of experiment, a wash-out perfusion was carried out for 8 min. In the latter experiments, phenylephrine (5 μ M) was added 5 min before the end of the perfusion. On completion of the perfusion, the livers were removed and mitochondria were isolated as described elsewhere (Kimura and Rasmussen, 1977). Throughout the isolation process, 1.3 mM EGTA (ethyleneglycol-bis-(β -aminoethyl ether) N, N'-tetraacetic acid) was included along with 10 mM Tris-HCl (pH 7.0) in the isotonic sucrose solution to minimize mitochondrial reaccumulation of calcium (Claret-Berthon *et al.*, 1977). Radioactivity in the homogenate, 700 $\times$ g pellet and purified isolated mitochondria was measured with a scintillation spectrometer. Protein was estimated by the method of Lowry *et al.* (1951), in which bovine serum albumin (BSA) served as the standard.

Determination of glucose and glycogen

Glucose concentration in the effluent was measured by the glucose oxidase-peroxidase method (Boehringer-Mannheim Corp). For glycogen assay, a portion of the liver was frozen quickly by means of a pair of precooled aluminum tongs. The frozen tissue was

weighed and digested with 30% KOH in boiling water and glycogen was measured by the anthrone method (Hassid and Abraham, 1957).

Chemicals

Phenylephrine and BSA (Fr. V) were obtained from Sigma Chemical Co. (St. Louis, Missouri). Verapamil and diltiazem were kindly donated by Eisai Co. (Tokyo, Japan), and Tanabe Seiyaku Co. (Osaka, Japan), respectively. All other chemicals were of reagent grade and obtained from local suppliers. Student's *t*-test was used for statistical analyses when needed.

Results

Effects of Verapamil and Diltiazem on Phenylephrine-induced Glycogenolysis and ^{45}Ca Efflux:

Phenylephrine caused a prompt and transient efflux of ^{45}Ca and stimulated the release of glucose (glycogenolysis) from perfused rat liver (Fig. 1). As shown in Fig. 1, verapamil and diltiazem (200 μM) markedly inhibited both of these α -adrenergic effects. In the absence of perfusate calcium, the stimulatory effects of the α -adrenergic agonist and the inhibitory effects of verapamil and diltiazem were both demonstrable (data not shown), indicating that the effects of these calcium channel blockers were not affected by the deletion of extracellular calcium.

To investigate the nature of the inhibitory effects of these calcium channel blockers, various concentrations of verapamil were tested against graded doses of phenylephrine. The net increase in glucose production during the first 10 min after the addition of various doses of phenylephrine is plotted in Fig. 2. Twenty μM verapamil caused a small shift of the curve to the right. However, higher concentrations of verapamil not only shifted the dose-response curve to the right, but also inhibited the maximum response in glucose production. The inhibitory effect of verapamil on the maximum response to phenylephrine in

glucose production and ^{45}Ca release are plotted in Fig. 3. The inhibitory effect of verapamil on phenylephrine-induced efflux of ^{45}Ca closely paralleled the effect on glucose release.

The glycogen content in the liver was 44.6 ± 2.0 mg/g wet tissue (mean $\pm$ SEM, $n=4$) in controls and 39.5 ± 2.1 mg/g wet tissue (mean $\pm$ SEM, $n=4$) in experimental livers where phenylephrine (5 μM) was administered for the last 10 min of the 40-min perfusion. The difference (5.1 mg/g) was close to the amount of glucose mobilized by phenylephrine (4.8 ± 0.4 mg/g, mean $\pm$ SEM, $n=4$, cf Figs. 1, 2). When 200 μM verapamil was included in the perfusate (at 20 min), phenylephrine did not induce a decline in glycogen content; the quantities were 43.1 ± 2.2 mg/g wet tissue and 41.3 ± 2.5 mg/g wet tissue in the absence and presence of phenylephrine (5 μM), respectively.

Effects of Verapamil and Diltiazem on 2, 4-dinitrophenol-induced Glycogenolysis:

Metabolic inhibitors such as iodoacetate or cyanide are known to stimulate hepatic glycogenolysis (Jakob and Diem, 1974). In the present system, 2,4-dinitrophenol, an uncoupler of mitochondrial oxidative phosphorylation, stimulated hepatic glycogenolysis in a dose-dependent manner (Fig. 4). This effect of 2,4-dinitrophenol was not inhibited by verapamil or diltiazem.

Effect of Verapamil on Basal Release of ^{45}Ca from Perfused Rat Liver:

The effect of verapamil on the release of preloaded ^{45}Ca from the liver was studied. The liver was perfused for 45 min with KRT buffer containing 1 mM $^{45}\text{CaCl}_2$ in a recirculation system. Verapamil was added 15 min after the switch to the wash-out perfusion. As shown in Fig. 5, the release of ^{45}Ca was inhibited by 20 to 500 μM verapamil in dose-dependent manner.

Fig. 1. Effects of verapamil and diltiazem on phenylephrine-induced glucose release and ^{45}Ca release from the perfused liver.

A. Livers from fed rats were perfused in a flow-through system with KRT buffer containing 1 mM CaCl_2 . Thirty min after the beginning of perfusion (0 min in the figure), phenylephrine ($5 \mu\text{M}$) was added into the perfusate. Points and vertical bars indicate means and SEMs from four or five independent experiments.

B. Livers were pre-perfused for 45 min in a recirculation system with 1 mM $^{45}\text{CaCl}_2$. Then the system was switched to a flow-through system without $^{45}\text{CaCl}_2$ (wash-out perfusion). The medium for wash-out perfusion contained 1 mM CaCl_2 . Fifteen min after the switch (0 min in the figure), phenylephrine ($5 \mu\text{M}$) was added. Each point represents the mean of two independent experiments.

●—●, phenylephrine only; ○—○, verapamil ($200 \mu\text{M}$) + phenylephrine; △—△, diltiazem ($200 \mu\text{M}$) + phenylephrine; ●...●, no addition (Verapamil or diltiazem was added 10 min before phenylephrine).

Effects of Verapamil and Phenylephrine on Mitochondrial ^{45}Ca :

To determine the effect of verapamil and phenylephrine on the intracellular distribution of ^{45}Ca , studies were performed on subfractions of liver homogenate. First, the effect of verapamil on ^{45}Ca uptake by liver mitochondria was examined. In these experiments, the liver was removed after

the extracellular ^{45}Ca was washed out for a short period of time (3 min), then homogenized. As summarized in Table I, verapamil administered during the loading period did not cause changes in calcium uptake in the liver tissue (homogenate) or in the mitochondria.

Second, the effect of phenylephrine on calcium distribution in the absence of

Fig. 2. Effect of verapamil on the dose-response relationship between phenylephrine and net glucose production.

Livers from fed rats were perfused as described in the legend to Fig. 1A. Phenylephrine-induced increase in glucose production is expressed on the ordinate as a function of phenylephrine concentration.

●—●, no blocker; ○—○, plus 20 μM verapamil; ▲—▲, plus 50 μM verapamil; △—△, plus 100 μM verapamil; ■—■, plus 200 μM verapamil; □—□, plus 500 μM verapamil.

Fig. 3. Effect of verapamil on maximum stimulation of glucose production and ⁴⁵Ca efflux induced by phenylephrine.

For the calculation of the inhibitory rate in glucose production, the maximum response to phenylephrine in the presence or absence of verapamil shown in Fig. 2 was used. In the case of the effect on ⁴⁵Ca, the peak of ⁴⁵Ca release induced by phenylephrine was used.

●—●, ⁴⁵Ca release; ○—○, glucose production.

verapamil was studied. Loading of ⁴⁵Ca was carried out without verapamil, and phenylephrine (5 μM) was added to the perfusate 3 min after the switch to the wash-out perfusion. The α -adrenergic agonist caused a decline in the content of ⁴⁵Ca in the homogenate prepared from the liver

5 min after the phenylephrine administration (Table II), reflecting the efflux of ⁴⁵Ca in the effluent. The content of ⁴⁵Ca in mitochondria isolated from the homogenate was also reduced significantly, indicating the mobilization of ⁴⁵Ca from mitochondria. When verapamil was supplemented in the

Fig. 4. Effect of verapamil and diltiazem on 2,4-dinitrophenol-induced glucose release.

Livers from fed rats were perfused as described in the legend to Fig. 1A, and the data are presented as Fig. 2. 2,4-dinitrophenol was added 30 min after the beginning of perfusion. Verapamil or diltiazem ($500 \mu\text{M}$) was added 10 min before 2,4-dinitrophenol. ●, no blocker; ○, plus verapamil; △, plus diltiazem.

Fig. 5. Effect of verapamil on basal release of ^{45}Ca from perfused liver.

Livers were pre-perfused with KRT buffer containing $1 \text{ mM } ^{45}\text{CaCl}_2$ for 45 min in a recirculation system. Then the system was switched to a flow-through perfusion, in which the perfusion medium contained no $^{45}\text{CaCl}_2$ but 1 mM CaCl_2 . Various concentrations of verapamil were added 15 min after the switch (see insert). Slope *a* minus slope *b* in the insert was plotted on the ordinate as a function of verapamil concentration.

Table I. Effect of verapamil on ^{45}Ca uptake in perfused liver

fraction	^{45}Ca (nmol of medium ^{45}Ca /mg protein)		
	control	verapamil	
homogenate	1.26 ± 0.11	1.06 ± 0.09	ns
700 $\times$ g pellet	0.56 ± 0.07	0.51 ± 0.07	ns
mitochondria	0.53 ± 0.06	0.54 ± 0.08	ns

ns: not significant

Liver was pre-perfused in a recirculation system with KRT buffer containing 1 mM $^{45}\text{CaCl}_2$ for 45 min. Three minutes after the switch to the flow-through system, the liver was excised, homogenized and fractionated as described in the text. Verapamil (500 μM) was included throughout the experiment when indicated. Radioactivity in each fraction was counted in a scintillation spectrometer. The amount of ^{45}Ca was calculated from the specific activity of ^{45}Ca in the KRT buffer used for pre-perfusion. Results are expressed as the mean $\pm$ SEM from four independent experiments.

buffer for wash-out perfusion, the phenylephrine-induced reduction of ^{45}Ca in the homogenate and the mitochondria was completely inhibited.

Discussion

In the present study, glucose released into the perfusate by phenylephrine is assumed to be derived mainly from glycogen through glycogenolysis, because fed rats were used and no substrate was included in the perfusate (Friedmann and Rasmussen, 1970; Friedmann, 1972). Changes in glycogen content in the liver that corresponded to the amount of glucose released into the perfusate lend further support to this assumption.

The results of this study clearly demonstrate that the glycogenolysis and ^{45}Ca efflux induced by phenylephrine, an α -adrenergic agonist, was inhibited by verapamil and diltiazem. These agents are known to inhibit calcium influx in cardiac muscle (Kohlhardt *et al.*, 1972; Saikawa *et al.*, 1977). Verapamil is reported to

Table II. Effect of phenylephrine on the distribution of ^{45}Ca in perfused liver

fraction	^{45}Ca (nmol of medium ^{45}Ca /mg protein)		
	control	phenylephrine	
homogenate	1.08 ± 0.12	0.33 ± 0.07	$P < 0.005$
700 $\times$ g pellet	0.80 ± 0.09	0.24 ± 0.06	$P < 0.005$
mitochondria	0.46 ± 0.07	0.26 ± 0.03	$P < 0.05$

Liver was pre-perfused in a recirculation system with the KRT buffer containing 1 mM $^{45}\text{CaCl}_2$ for 45 min. Three minutes after the switch to the flow-through system, phenylephrine (5 μM) or saline (control) was added to the medium and perfusion was continued for 5 min. At the end of the perfusion, the liver was excised, homogenized and fractionated as described in the text. The results obtained from four independent experiments are presented as in Table 1.

inhibit glucose-induced insulin release from the pancreatic islets (Devis *et al.*, 1975) or potassium-induced ACTH, GH and TSH release from the pituitary (Eto *et al.*, 1974). Under these conditions, verapamil appears to exert its effect by inhibiting the influx of calcium into the cells. The effect of α -adrenergic stimulation is dependent to some extent on extracellular calcium ions (Keppens *et al.*, 1977; Van de Werve *et al.*, 1977; Garrison *et al.*, 1979) and it is reported that catecholamine stimulates calcium influx into hepatocytes (Assimakopoulos-Jeannet *et al.*, 1977; Keppens *et al.*, 1977). Thus, the blockade of calcium influx may inhibit the effect of catecholamine. However, omission of calcium from the perfusate did not modify the inhibitory effect of calcium antagonists. In addition, verapamil did not inhibit the influx of calcium into liver cells under the present conditions. Therefore, the inhibitory effect of verapamil and diltiazem under our conditions seems to be caused by a mechanism other than blocking of the calcium influx.

Following our brief report (Kimura *et al.*, 1978) that verapamil and diltiazem

inhibited α -adrenergic activation of glycogenolysis and ^{45}Ca efflux, Blackmore *et al.* (1979b) reported that verapamil behaved as a competitive binding inhibitor of catecholamines to the α -adrenergic receptor. But this conclusion was not applicable to the effect of verapamil at concentrations higher than $50\ \mu\text{M}$ in the present experiments (Fig 2). Furthermore, it does not explain the fact that verapamil inhibited the effect of glucagon as well (Kimura *et al.*, 1978). Therefore, other possibilities must be entertained. Direct inhibition of phosphorylase by verapamil was ruled out by the experiments with 2,4-dinitrophenol (Fig. 4). In addition, the basal rates of glucose release (glycogenolysis) did not decrease after the addition of verapamil or diltiazem (Fig. 1). These findings indicate that the site of verapamil action may lie at a step (or steps) in or before the activation system of phosphorylase.

The present data confirm that mitochondrial calcium is mobilized by α -adrenergic stimulation (Table II). The stimulation of calcium efflux from mitochondria by phenylephrine may result in an increase in the cytosolic calcium concentration (Murphy *et al.*, 1980) and hence an accelerated release of calcium from the liver. As proposed previously (Assimakopoulos-Jeannet *et al.*, 1977; Blackmore *et al.*, 1979a) an increase in cytosolic calcium will activate phosphorylase kinase and therefore glycogenolysis. Thus, inhibition of calcium mobilization may abolish the glycogenolytic effect of phenylephrine. In fact, in the present study, calcium channel blockers inhibited phenylephrine-induced glucose output when they inhibited calcium efflux from the liver (Fig. 1).

The blockers inhibited not only the α -adrenergic agonist-induced release but also the basal release of calcium from the perfused liver (Fig 5). Moreover, at the concentrations higher than $50\ \mu\text{M}$, the inhibitory effect of verapamil on this basal

calcium release correlated closely with the effect on phenylephrine-induced glucose release and ^{45}Ca release (Figs. 3, 5); the range of effective verapamil concentration and the concentration-effect relationship were identical. Thus, verapamil seems to lower the rate of calcium efflux from intracellular pools induced by α -adrenergic stimulation similarly to the way in which it affects the basal release of calcium. This may be the mechanism by which calcium channel blockers inhibit the glycogenolytic effect of α -adrenergic stimulation. As the effects of verapamil and diltiazem were identical, it is conceivable that the mode of action of the blockers is related to their common property, i. e. the ability to modify the interaction between calcium ion and cell membrane. This interpretation of the mode of action of the blockers seems reasonable, and it is applicable to their inhibitory effects on glucagon-induced glycogenolysis and ^{45}Ca release because phosphorylated phosphorylase can also be stimulated by calcium (Ozawa, 1972).

As already discussed, the possibility that calcium channel blockers directly inhibit the function of phosphorylase or that they inhibit the effect of the α -adrenergic agonist by preventing calcium influx is remote. Although the possibility remains that calcium channel blockers inhibit the binding of the α -adrenergic agonist to its receptor in a noncompetitive way at high concentrations, it is strongly suggested that the inhibition of phenylephrine-induced glycogenolysis by calcium channel blockers is causally related to the inhibition of phenylephrine-induced mobilization of calcium from intracellular calcium pools including mitochondria.

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