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ACTIVATION OF GLYCOGENOLYSIS BY THE REDUCTION IN THE EXTRACELLULAR CALCIUM CONCENTRATION IN VERAPAMIL-PERFUSED RAT LIVER

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Abstract—In an attempt to elucidate the role of Ca^{2+} flux in the initial events of hepatic glycogenolysis, extracellular Ca^{2+} concentration was manipulated in rat liver perfused with Ca^{2+} antagonistic drugs. After the liver had been perfused with a buffer containing verapamil and 1 mM $CaCl_2$, either the addition of ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid to the perfusate or the replacement of the perfusate with Ca^{2+}-free buffer caused a rapid increase in glucose output as well as $^{45}Ca^{2+}$ efflux. Substitution of diltiazem, but not 5–20 mM $LaCl_2$, for verapamil also stimulated glucose output and $^{45}Ca^{2+}$ efflux. However, when Ca^{+}-free buffer was used throughout the experiment, any modes of verapamil or diltiazem perfusion were without significant effects on glucose output or Ca^{2-} efflux. The increases in glucose output and $^{45}Ca^{2+}$ efflux were not affected by either 20 μM phentolamine or 300 μM ouabain, but they were inhibited significantly by 10–100 μM trifluoperazine. These results indicate that rapid decline in the extracellular Ca^{2+} concentration in verapamil- or diltiazem-perfused liver initiates the change in Ca^{2+} equilibirum on or across plasma membrane and activates glycogenolysis through a Ca^{2+}-dependent mechanism.

Alpha-adrenergic activation of glycogenolysis takes place in a cyclic AMP-independent manner, but it is associated with rapid changes in cellular Ca^{2+} flux which are believed to result in the elevation of cytosolic Ca^{2+} concentration [1]. Since the increased Ca^{2+} concentration activates phosphorylase kinase [2–4] and consequently glycogenolysis [5, 6], it has been postulated that glycogenolytic effects of α-adrenergic agonists are mediated through Ca^{2+}-dependent processes [1]. Like α-agonists, glycogenolytic effects of vasopressin and angiotensin II are also associated with the change in Ca^{2+} flux and are thought to be mediated through a cyclic AMP-independent, but Ca^{2+}-dependent, mechanism [7, 8].

In considering the regulatory role of Ca^{2+} in the hormone action, it is important to study the mechanism for the initiation of Ca^{2+} flux changes by hormones. Furthermore, if the hormonal actions can be mimicked by the pharmacological manipulation of Ca^{2+} flux, that will further substantiate the important role of Ca^{2+} in hormone actions.

Verapamil and diltiazem, Ca^{2+} antagonistic drugs, have been used as tools to study the role of Ca^{2+} in various Ca^{2+}-dependent processes because these drugs are known to inhibit Ca^{2+} influx across plasma membrane of several organs [9–12]. The present study was undertaken to explore the role of Ca^{2+} in hepatic glycogenolysis. For this purpose, Ca^{2+} equilibrium in hepatocytes was pharmacologically manipulated with Ca^{2+} antagonistic drugs and by changing the perfusate Ca^{2+} concentration in perfused rat liver. The results demonstrated that a rapid decline in the extracellular Ca^{2+} concentration activates glycogenolysis as well as $^{45}Ca^{2+}$ efflux in a rat liver which has been pre-perfused with verapamil or diltiazem in the presence of extracellular Ca^{2+}. While further study is needed to elucidate the detailed mechanism, it seems that the change in Ca^{2+} equilibrium across/on plasma membrane is the trigger for the present activation of hepatic glycogenolysis.

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MATERIALS AND METHODS

Chemicals. Verapamil hydrochloride and diltiazem hydrochloride were donated by the Eisai Co. (Tokyo, Japan) and the Tanabe Seiyaku Co. (Osaka, Japan) respectively. Trifluoperazine was a gift from the Yoshitomi Seiyaku Co. (Osaka, Japan). Phentolamine, bovine serum albumin (Fr. V), ouabain, and $LaCl_2$ were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). $^{45}CaCl_2$ (18.6 mCi/mg Ca^{2+}) was obtained from the New England Nuclear Corp. (Boston, MA, U.S.A.). All other chemicals were of reagent grade and obtained from local suppliers.

Animals. Male Wistar rats (200–250 g) were obtained from the Shizuoka Experimental Animal Co. (Shizuoka, Japan) and were raised on regular rat chow (Funabashi Farm, Chiba) and tap water *ad lib.*

Liver perfusion. Fed rats were used for all experiments. Under pentobarbital anesthesia (50 mg/kg,

i.p.), livers were perfused *in situ* according to the method described previously [13]. A Krebs–Ringer–Tris buffer (20 mM Tris–HCl, pH 7.4) maintained at 37° was used as the perfusate after oxygenation with 100% O_2. Livers were perfused with chemicals in various modes as indicated in the figure legends. When indicated, the concentration of Ca^{2+} in the perfusate was switched from 1 mM to practically 0 mM by changing the perfusate to a Ca^{2+}-free buffer, or by adding excessive amount of ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA). The effluent was collected for the determination of glucose concentration. Small pieces of liver tissue (30–40 mg) were resected, frozen immediately with dry-ice, and stored at −70° until assayed for cyclic AMP and cyclic GMP. The livers were weighed at the end of perfusion. In all figures for glucose output, 0 min corresponds to 30 min after the initiation of liver perfusion.

$^{45}Ca^{2+}$ efflux study. Livers were preloaded with $^{45}Ca^{2+}$ by perfusing them with 200 ml of Krebs–Ringer–Tris buffer containing 1 mM $CaCl_2$ with 50 μCi of $^{45}Ca^{2+}$ for 30 min in a recirculating system. Then the livers were perfused in a flow-through system with the buffer containing either 1 mM or 0 mM $CaCl_2$. After the perfusion in a flow-through system for 10 min, 500 μM verapamil was added and, 10 min later, 2 mM EGTA was added or the Ca^{2+} concentration of the perfusate was changed from 1 to 0 mM. When indicated, 20 μM phentolamine or 100 μM trifluoperazine was added after $^{45}Ca^{2+}$ preloading. The radioactivity of the effluent was determined with a scintillation spectrometer.

Determination of glucose, cyclic AMP, cyclic GMP and protein. Glucose concentration in the effluent was determined by a glucose oxidase–peroxidase method (Boehringer-Mannheim Co., West Germany). Cyclic AMP and cyclic GMP in liver tissue were extracted with 6% trichloroacetic acid, and the concentrations were determined by the radioimmunoassay of Steiner *et al.* [14] after succinylation. Protein was determined by the method of Lowry *et al.* [15] using bovine serum albumin as a standard.

Data were expressed as means ± S.E., and Student's *t*-test was employed for statistical analyses.

RESULTS

Effect of perfusion of Ca^{2+} antagonistic drugs and EGTA on glucose output. Following pre-perfusion with 0.5 mM verapamil (Fig. 1A) or 0.3 mM diltiazem (Fig. 1C) in the buffer containing 1 mM $CaCl_2$, the addition of 2 mM EGTA elicited a marked increase in glucose output from perfused liver. The increase was significant 2 min after the addition of EGTA, reached a peak at 4 min, and gradually declined thereafter. However, when verapamil or diltiazem was perfused in Ca^{2+}-free buffer, the addition of EGTA did not elicit a significant effect (Fig. 1B and 1D). The addition of verapamil and EGTA in the reverse sequence was without significant effect on glucose output (data not shown). Perfusion with verapamil, diltiazem, or EGTA alone also had no effect on glucose output. When rats that had been fasted for 36 hr were employed instead of fed rats, no significant increase in glucose output was obtained by verapamil and EGTA perfusion (fasted rats, 0.25 ± 0.12 μmole glucose per 15 min per g liver, N = 3; fed rats, 33.08 ± 2.95 μmoles glucose per 15 min per g liver, N = 4, $P < 0.001$).

Dose–response relationship of verapamil and EGTA to net increase in glucose output. As depicted in Fig. 2A, verapamil in concentrations higher than 100 μM markedly increased glucose output in readily saturable fashion, when followed by the simultaneous perfusion of 2 mM EGTA. The effect of EGTA in eliciting the increased glucose output was maximal in a narrow range (0.8 to 2 mM) of concentrations. This suggests that there may be a critical extracellular Ca^{2+} concentration which stimulates glucose output.

Effect of Ca^{2+}-free buffer on the action of Ca^{2+} antagonistic drugs. To study whether the role of EGTA is based on its ability to chelate Ca^{2+}, the $CaCl_2$ concentration of the perfusate was changed from 1 to 0 mM instead of adding EGTA. Changing

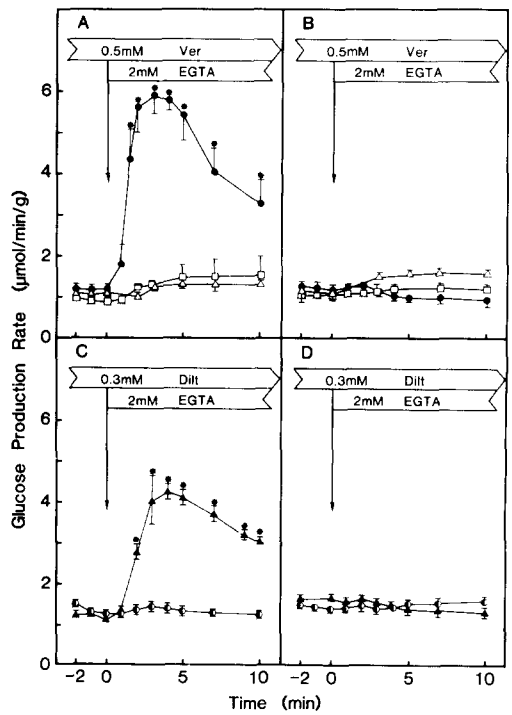

Fig. 1. Effects of verapamil and diltiazem on glucose output. Livers were perfused with 0.5 mM verapamil or 0.3 mM diltiazem, and/or 2 mM EGTA, in various combinations in the presence (A and C) and absence (B and D) of 1 mM $CaCl_2$ in the buffer. In all figures for glucose output, 0 min corresponds to 30 min after the initiation of liver perfusion. Each perfusion was performed as follows: (●——●) verapamil from −10 min and EGTA from 0 min; (▲——▲) diltiazem from −10 min and EGTA from 0 min; (△——△) verapamil from −10 min; (◐——◐) diltiazem from −10 min; and (□——□) EGTA from 0 min to the end of the experiments respectively. Ver and Dilt indicate verapamil and diltiazem respectively. Each point and vertical bar represent the mean and S.E. from three to seven independent experiments. Values which are significantly different from the basal glucose production rate (mean value of those from −2 min to 0 min) are indicated with an asterisk (* at least $P < 0.05$).

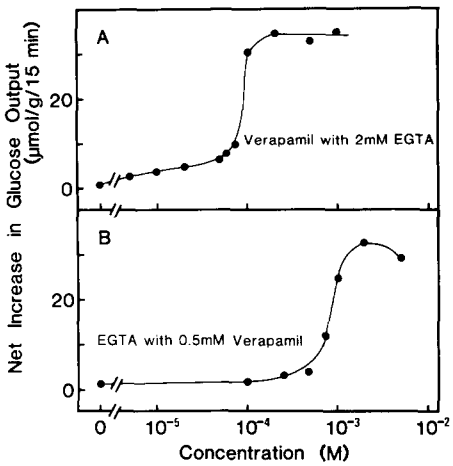

Fig. 2. Dose–response relationship of verapamil and EGTA to the net increase in glucose output. In A, livers were perfused with various concentrations of verapamil from −10 min and with 2 mM EGTA from 0 min to 15 min. In B, livers were perfused with 0.5 mM verapamil from −10 min and with various concentrations of EGTA from 0 min to 15 min. Net increase in glucose output was calculated by using mean glucose production rate determined during 2 min before 0 min as the basal glucose production rate.

to the CA^{2+}-free perfusate (Fig. 3A) during the perfusion with 0.5 mM verapamil or 0.3 mM diltiazem caused a rapid increase in glucose output like that on the addition of EGTA (Fig. 1A and 1C). Moreover, it is obvious that continuous perfusion of verapamil was not required for the present effect (Fig. 3B). In addition, readministration of $CaCl_2$ to the perfusate at 2 min after the perfusion of Ca^{2+}-free buffer acutely inhibited the increase in glucose output (Fig. 3A). The termination of verapamil perfusion without a change in Ca^{2+} concentration had no appreciable effect on glucose output (Fig. 3B).

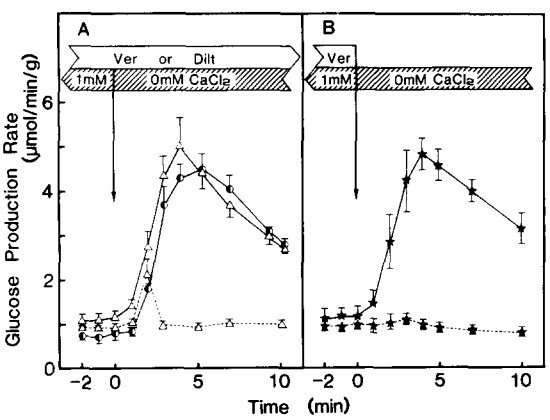

Fig. 3. Effect of Ca^{2+}-free perfusate on the action of verapamil and diltiazem. Livers were perfused with either 0.5 mM verapamil or 0.3 mM diltiazem from −10 min to the end of the experiments (A), or to 0 min (B). The concentration of $CaCl_2$ of the buffer was changed from 1 to 0 mM at 0 min. Panel A: (△——△) verapamil from −10 min; (△- - -△) verapamil from −10 min to the end of experiment with readministration of 1 mM $CaCl_2$ at 2 min; and (◐——◐) diltiazem from −10 min. Panel B: (★——★) verapamil from −10 min to 0 min with a change of $CaCl_2$ concentration; and (★- - -★) verapamil from −10 min to 0 min with 1 mM $CaCl_2$ in the buffer throughout experiments. N = 3–7.

Effect of $LaCl_2$ *and ouabain on glucose output.* To study whether these effects of verapamil and diltiazem are common for other classes of Ca^{2+} influx inhibitors or related to Na^+–K^+ flux, the effects of $LaCl_2$ and ouabain on glucose output were examined. High concentrations of $LaCl_2$ (5–20 mM) did not substitute for verapamil or diltiazem (Fig. 4A). Ouabain (0.5 mM) did not alter the effect of verapamil and EGTA perfusion (Fig. 4B).

Effects of various modes of verapamil perfusion on $^{45}Ca^{2+}$ *efflux.* The effects of various modes of verapamil perfusion on the release of $^{45}Ca^{2+}$ from $^{45}Ca^{2+}$ preloaded liver were examined. Addition of EGTA (Fig. 5A) or the omission of $CaCl_2$ from the perfusate (Fig. 5C) caused a prompt and transient increase in $^{45}Ca^{2+}$ efflux from a liver pre-perfused with verapamil in the perfusate containing 1 mM $CaCl_2$. However, the same mode of verapamil and EGTA perfusion did not stimulate $^{45}Ca^{2+}$ efflux when Ca^{2+}-free buffer was used as the perfusate throughout the experiment (Fig. 5B). The perfusion of verapamil and EGTA in the reverse sequence was without significant effect as in the case of glucose output (data not shown). Verapamil alone (Fig. 5D) or the perfusate alone (Fig. 5A and 5C) did not increase $^{45}Ca^{2+}$ efflux. While EGTA alone was without any appreciable effect on glucose output, it caused a small and gradual increase in $^{45}Ca^{2+}$ release (Fig. 5D). Thus, the change in $^{45}Ca^{2+}$ efflux was essentially parallel to that of glucose output in all modes of verapamil perfusion. Similar results were obtained when diltiazem was substituted for verapamil (data not shown).

Effects of phentolamine and trifluoperazine on the activation of glucose output and $^{45}Ca^{2+}$ *efflux.* Since

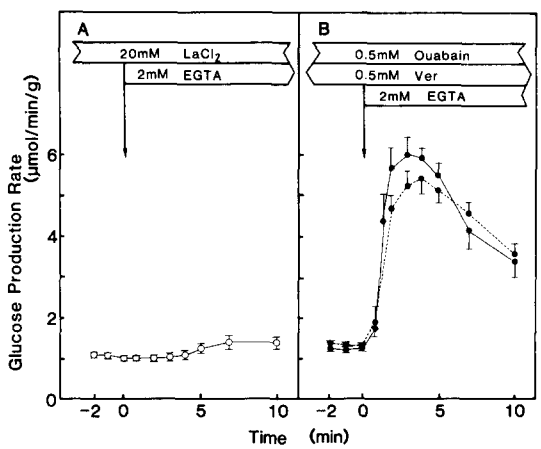

Fig. 4. Effects of $LaCl_2$ (A) and ouabain (B) on glucose output. Livers were perfused in the presence of 1 mM $CaCl_2$ in the buffer as follows: (○——○) 20 mM $LaCl_2$ and 2 mM EGTA from −10 min and 0 min; (●- - -●) 0.5 mM ouabain, 0.5 mM verapamil and 2 mM EGTA from −20, −10, and 0 min respectively; and (●——●) 0.5 mM verapamil and 2 mM EGTA from −10 and 0 min to the end of experiment respectively. N = 3.

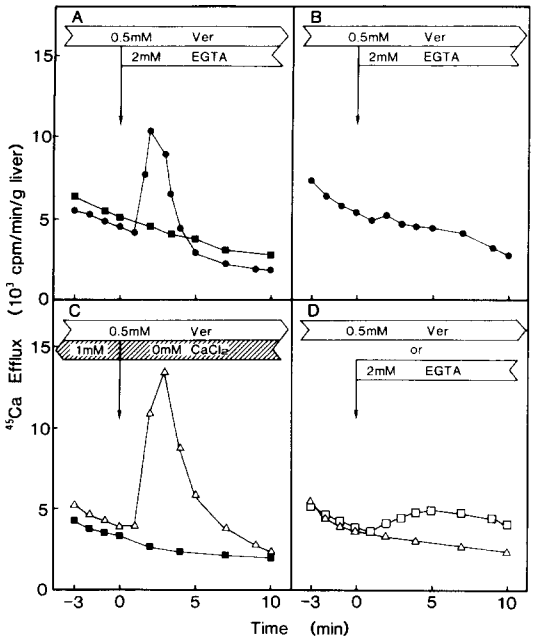

Fig. 5. Effects of various modes of verapamil perfusion on $^{45}Ca^{2+}$ efflux. After loading $^{45}Ca^{2+}$ as described in Materials and Methods, livers were perfused either with the buffer containing 1 mM $CaCl_2$ (A and D) or with Ca^{2+}-free buffer (B), or the $CaCl_2$ concentration was switched at 20 min after $^{45}Ca^{2+}$ loading (0 min in the figure) from 1 to 0 mM (C). Key: (●——●) verapamil from −10 min and EGTA from 0 min; (■——■) perfusate only; (△——△) verapamil from −10 min; and (□——□) EGTA from 0 min to the end of the experiments. Each point represents the mean of two independent experiments.

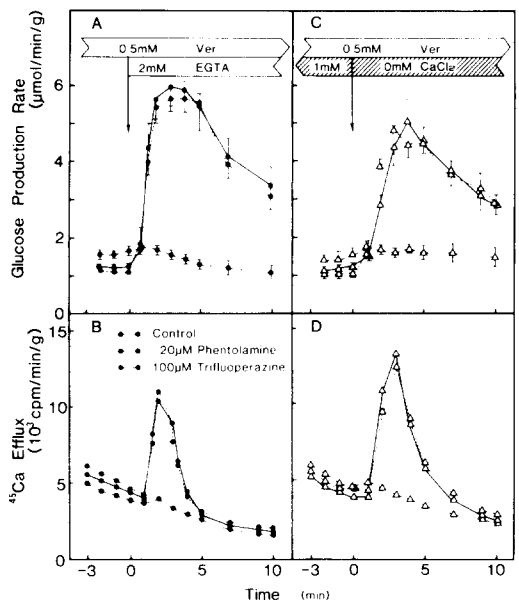

Fig. 6. Effects of phentolamine and trifluoperazine on the activation of glucose output and the $^{45}Ca^{2+}$ efflux elicited by verapamil and Ca^{2+} deprivation. Livers were perfused as in Fig. 1 for glucose output study (A and C), and as in Fig. 5 for $^{45}Ca^{2+}$ efflux study (B and D). The concentration of $CaCl_2$ in the buffer was 1 mM in A and B, and was switched from 1 to 0 mM at 0 min in C and D. Where indicated, 20 μM phentolamine or 100 μM trifluoperazine was added from −20 min to the end of experiments. Symbols with solid lines in A, B, C, and D indicate the controls which are presented in Figs. 1A, 5A, 3A, and 5C, respectively, as experimental values; symbols with broken and hatched lines indicate the results obtained with phentolamine and with trifluoperazine, respectively. The number of experiments for glucose output was three to seven, and that for $^{45}Ca^{2+}$ efflux was two.

Blackmore *et al.* [16] reported that high concentrations of verapamil possess slight α-agonistic activity, the effect of phentolamine, a classic α-antagonist, was examined. A relatively high concentration of phentolamine (20 μM), however, had no inhibitory effects on the increase of either glucose output (Fig. 6A and 6C) or $^{45}Ca^{2+}$ efflux (Fig. 6B and 6D) induced by the removal of extracellular Ca^{2+} in verapamil pre-perfused liver. On the other hand, 100 μM trifluoperazine, a possible inhibitor of calmodulin action [17], completely inhibited the increase in both glucose output (Fig. 6A and 6C) and $^{45}Ca^{2+}$ efflux (Fig. 6B and 6D). Trifluoperazine inhibited glucose output in a dose-dependent manner between 10 and 50 μM (Fig. 7).

Effects of verapamil and trifluoperazine on tissue concentrations of cyclic AMP and cyclic GMP. To study whether the observed effects of verapamil and of trifluoperazine were related to changes in cyclic nucleotide metabolism, tissue concentrations of cyclic AMP and cyclic GMP were determined (Table 1). No significant changes in either cyclic AMP or cyclic GMP concentration were detected by the modes of perfusion that activated glucose output and $^{45}Ca^{2+}$ efflux. Trifluoperazine was also without effect on the concentrations of both cyclic nucleotides.

DISCUSSION

The present study clearly demonstrates that removal of extracellular Ca^{2+} following pre-perfusion with verapamil or diltiazem stimulates glucose output and Ca^{2+} efflux through a cyclic AMP-independent mechanism. The released glucose is assumed to be derived mainly from glycogen through

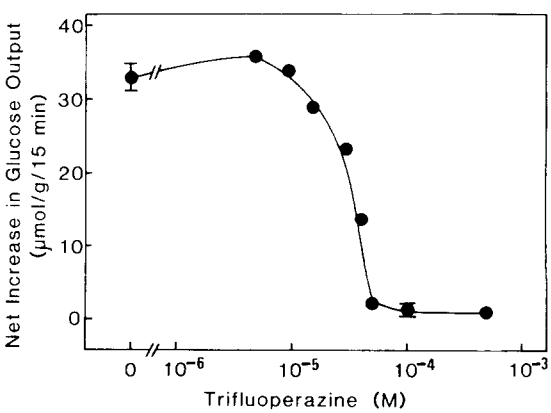

Fig. 7. Dose–response relationship of the inhibitory effect of trifluoperazine on the activation of glucose output by verapamil and EGTA. Livers were perfused with 0.5 mM verapamil and 2 mM EGTA and with various concentrations of trifluoperazine as in Fig. 6A.

Table 1. Effects of verapamil and trifluoperazine on tissue concentrations of cyclic AMP and cyclic GMP*

$CaCl_2$ in buffer	Verapamil (0.5 mM)	EGTA (2 mM)	Trifluoperazine (0.1 mM)	Cyclic AMP (pmoles/mg protein) 0 min	Cyclic AMP (pmoles/mg protein) 5 min	Cyclic GMP (fmoles/mg protein) 0 min	Cyclic GMP (fmoles/mg protein) 5 min
	+	−	−	4.44 ± 0.49	4.23 ± 0.21	111 ± 10	119 ± 12 (3)
	+	+	−	4.62 ± 0.41	3.99 ± 0.39	116 ± 15	100 ± 14 (4)
1 mM	+	+	+	4.83 ± 0.30	4.52 ± 0.25	113 ± 7	102 ± 12 (3)
	−	−	+	4.66 ± 0.39	4.10 ± 0.31	99 ± 8	99 ± 3 (4)
1→0 mM	+	−	−	4.70 ± 0.30	4.51 ± 0.39	100 ± 12	95 ± 10 (5)
	+	−	+	4.74 ± 0.35	5.05 ± 0.36	88 ± 7	104 ± 15 (4)

* Livers were perfused with 0.5 mM verapamil from −10 min with the buffer containing 1 mM $CaCl_2$. At 0 min, either the perfusion of 2 mM EGTA or Ca^{2+}-free buffer was started. In some experiments, 100 μM trifluoperazine was perfused from −10 min. Pieces of liver tissues were resected at 0 and 5 min, and cyclic nucleotide concentrations were determined by radioimmunoassay.

glycogenolysis, because fed rats were used and no substrates for gluconeogenesis were added in the perfusate [18]. Absence of the activation of glucose output in fasted rats further supports this contention.

High concentrations of verapamil, as used in this study (10^{-4} M and above), have been reported to possess slight α-agonistic activity [16]. The present effect of verapamil, however, cannot be ascribed to this property, because verapamil alone did not stimulate glucose output, and high concentrations of phentolamine did not inhibit the effect of verapamil. Furthermore, since the effects of verapamil and diltiazem were identical, it is conceivable that the modes of action of these drugs are related to their common property of modifying cellular Ca^{2+} flux. Calcium-antagonistic drugs have been reported to inhibit Ca^{2+} influx in several tissues by blockade of the so-called slow channels of the cell membrane [9, 19]. This hypothesis, however, has been challenged, at least in muscle, blood vessels [20], and liver [16, 21, 22]. In perfused rat liver, we have reported that these drugs inhibit the activation of glycogenolysis by glucagon [21] and α-agonist [13], without inhibiting Ca^{2+} influx. Failure of verapamil to inhibit transmembrane Ca^{2+} flux was reported also in isolated hepatocytes [16]. In addition, $LaCl_2$, another class of Ca^{2+} influx inhibitor, could not substitute for Ca^{2+} antagonistic drugs. Therefore, the inhibition of Ca^{2+} influx may not be involved in the present effect of these drugs. On the other hand, we have found that these drugs inhibit Ca^{2+} release from intracellular pools induced by glucagon [21] or α-agonist [13]. Similar intracellular actions of verapamil and D-600 were also reported in myocardium [20, 22] and aorta [20]. These results suggest that Ca^{2+} antagonistic drugs affect intracellular handling of Ca^{2+}, and their effects in our system may have been exerted through their actions not only on plasma membrane but also on intracellular Ca^{2+} pools. Nonetheless, the currently available information, on the sites of action and effects of these drugs in liver, is insufficient to draw any conclusions about their roles in the present system.

Although pre-perfusion of Ca^{2+} antagonistic drugs was required, the removal of extracellular Ca^{2+} played a crucial role in the activation of glycogenolysis and $^{45}Ca^{2+}$ efflux. EGTA does not permeate the plasma membrane [23], and simple omission of Ca^{2+} from the buffer may not have any acute direct effects on intracellular processes. Therefore, the site of action of these treatments seems to be on the plasma membrane, and their effects appear to be a change in Ca^{2+} equilibrium across/on plasma membrane by chelation or by relase of plasma membrane-bound Ca^{2+}. At present, neither the initial biochemical event that leads to the activation of glycogenolysis and of changes in Ca^{2+} flux after hormone–receptor binding, nor the nature of the transducing signal from plasma membrane to the intracellular Ca^{2+} pools, is known. In this respect, our results raise an interesting possibility, that release of Ca^{2+} from plasma membrane may somehow generate information leading to the activation of glycogenolysis and Ca^{2+} efflux. This interpretation is compatible with our recent proposal that the release of Ca^{2+} from plasma membrane may be the first step in the activation of glycogenolysis by α-adrenergic agonists [24]. The same hypothesis was also proposed by Althaus-Salzmann *et al.* [25].

In the present study, activation of glycogenolysis was obtained by manipulation of extracellular Ca^{2+} concentration in livers perfused with Ca^{2+} antagonistic drugs. In addition, the effect was associated with a net increase in Ca^{2+} efflux, the time course and magnitude of which were similar to that induced by α-agonists [13, 24, 25] and by vasopressin or angiotensin II [26]. Thus, all of the characteristics indicate that the activation of glycogenolysis in the present system was exerted through a Ca^{2+}-dependent process. In this regard, it is of interest that trifluoperazine completely inhibited the activation of glucose output and $^{45}Ca^{2+}$ efflux. Although trifluoperazine has been reported to act as an α-antagonistic drug in isolated hepatocytes [27, 28], the present stimulation of glycogenolysis induced by Ca^{2+} antagonists and Ca^{2+} manipulation was not inhibited by a classic α-antagonist, phentolamine. Therefore, the inhibitory effect of trifluoperazine in this system was not related to its α-antagonistic activity. This drug has been used as a tool to study the role of calmodulin, a mediator of Ca^{2+} action, in many Ca^{2+}-dependent processes [29]. It has been shown that phosphorylase kinase from skeletal muscle contains calmodulin as its subunit [30, 31], and that its activity is inhibited by trifluoperazine [32]. There is no direct evidence at present, however, for the

involvement of calmodulin in the physiological regulation of hepatic glycogenolysis. One should be cautious, therefore, in suggesting that the present effect of trifluoperazine occurs at the level of intracellular calmodulin-related process. Alternatively, it is possible that trifluoperazine suppressed the elevation of cytosolic Ca^{2+} concentration by inhibiting the mobilization of Ca^{2+}, as reported for pituitary gland [33], and consequently inhibited the present activation of glycogenolysis and of $^{45}Ca^{2+}$ efflux induced, probably, through Ca^{2+}-dependent mechanisms.

Because of the limited information, a detailed schema of the mechanism of activation of glycogenolysis in this system cannot be proposed. Nevertheless, our data strongly indicate that the change in cellular Ca^{2+} equilibrium plays an important role in the regulation of hepatic glycogenolysis. Since the present system mimics the actions of Ca^{2+}-dependent hormones, we believe that this unique experimental condition will provide a new method to elucidate the mechanism of Ca^{2+}-dependent hormone actions.

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