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Inhibition by Calcium Antagonist of Coupling of Insulin Binding and Insulin Action on Glucose Transport in Isolated Fat Cells*)

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ABSTRACT

In the present study we examined the influence of calcium antagonist, nicardipine-HCl, on insulin binding and insulin action on glucose transport in isolated fat cells. The calcium antagonist did not influence the time course of ^{125}I-insulin binding or the insulin concentration required for 50% displacement of ^{125}I-insulin binding. Calcium antagonist prolonged the lag time of insulin action on glucose transport. Calcium antagonist, however, did not influence the insulin concentration required for half maximal response. The prolongation of the lag time was also seen with 2 different calcium antagonists (verapamil and diltiazem-HCl). Calcium antagonists might impair the transmission of signal from insulin receptor by other mechanism(s) rather than the inhibition of transmembrane transport of calcium, because calcium antagonist did not inhibit ^{45}Ca uptake and efflux from preloaded fat cells, and because known calmodulin inhibitors (trifluoperazine and prochlorperazine) prolonged the lag time for insulin action without influence on insulin concentration for the half maximal response.

In conclusion calcium antagonist might act to calmodulin, phospholipids or membrane proteins.

INTRODUCTION

Several drugs with similar pharmacological effects, despite the difference in chemical structure are thought specifically to block Ca^{2+} influx, and grouped together as calcium antagonists[7]. They inhibit contraction of heart and smooth muscle[9], and the release of pancreatic hormones[11,13].

Intracellular free calcium has been considered as a possible mediator of insulin action[6]. In isolated rat adipocytes insulin increases influx of Ca^{2+} [5] or decreases Ca^{+} efflux[10] resulting in increased cytosolic Ca^{2+} concentration. Otherwise, insulin increases Ca^{2+} binding to plasma membrane[14] and endoplasmic reticulum[3], resulting in decreased cytosolic Ca^{2+} concentration.

Recently it became evident that calcium antagonist inhibits the contraction of smooth muscle of vessel, or of heart without influence on calcium influx[2,4] and might act directly on calcium binding proteins such as calmodulin[2].

We studied the effects of newly developed calcium antagonist, nicardipine-HCl, on insulin binding and action on 3-O-methylglucose transport in rat isolated fat cells, and discussed the action mechanism(s) of calcium antagonist on insulin action.

MATERIALS AND METHODS

Reagents

*) 石橋不可止，久保敬二：単離脂肪細胞における カルシウム拮抗剤によるインスリン結合とブドウ糖輸送に対するインスリン作用の coupling に及ぼす阻害作用

3-O-methyl-D-[1-^{3}H]glucose (4 Ci/mM) and $^{45}CaCl_2$ (4.5 mCi/mg Ca) were obtained from Amersham Searle. ^{125}I-insulin (194 μCi/μg) was from Dinabot Lab. (Japan). Bovine serum albumin (Fraction V) was purchased from Sigma Chem. Co.. Collagenase type I was from Worthington Biochem. Co.. Porcine crystalline insulin (lot #615-07J-256) was a generous gift from Eli Lilly Co.. Nicardipine-HCl, verapamil and diltiazem-HCl were obtained from Yamanouchi Pharm. Co., Eizai Pharm. Co., and Tanabe Pharm. Co., respectively.

Fat Cell Preparation

Rat isolated fat cells were prepared according to Rodbell[7] using Wistar rat (200-220 g) fed ad libitum. All incubations were performed in Krebs-Ringer HEPES buffer (pH 7.4) containing 3.0 g/dl crystalline bovine serum albumin.

^{125}I-insulin Binding

For the time course of insulin binding, 2 ml of isolated fat cell suspension (40% v/v) were incubated in a 4 ml polypropylene tube with ^{125}I-insulin (50 pmol/l). The cell suspension was incubated for the indicated periods at 37°C. At given intervals, 100 μl aliquots were transferred into polyethylene centrifuge tube (0.4 ml volume). Separation of cells from incubation medium was performed by centrifugation through dinonylphthalate. The tube was cut at the oil layer and the radioactivity of the cell layer was determined. To measure the non-specific binding an excess of unlabeled insulin (1,000 ng/ml) was added together with the labeled insulin.

For the steady state binding, tracer dosage of ^{125}I-insulin (50 pmol/l) was incubated with fat cells in the presence of the indicated concentrations of cold porcine insulin. Incubation medium contained 50 mg/dl bacitracin in order to prevent extracellular insulin degradation. After 30 minute incubation at 37°C, cells were separated by centrifugation as described above.

3-O-methyl-D-[1-^{3}H]glucose Transport

The uptake of labeled 3-O-methylglucose was measured in round-bottom polypropylene culture tube (11×40 mm) as described by Whitesell and Gliemann[19]. 35 μl albumin free buffer containing 0.05 μCi labeled 3-O-methylglucose was placed in the bottom of tube. 80 μl of cell suspension (40% v/v) were squirted and the reaction was terminated by the addition of 0.4 ml of albumin free buffer containing 0.3 mM phloretin. Separation ot cells from medium by centrifugation was performed within 2 minutes after the addition of phloretin, because 0.3 mM phloretin completely prevented the efflux of labeled 3-O-methylglucose from preloaded fat cells within 5 minutes after the addition of phloretin.

For the study on the effects of calcium antagonist on insulin binding and action, calcium antagonists were added 10 minutes before the addition of insulin. When the effects of calmodulin inhibitors on the kinetics of insulin action were examined, trifluoperazine (0.01-10 μM) or prochlorperazine (20-200 μM) was added 10 minutes before the addition of 10 ng/ml insulin.

^{45}Ca Uptake and ^{45}Ca Efflux in the Fat Cells

For the ^{45}Ca uptake, 4 ml of 40% fat cell suspension were exposed to $^{45}CaCl_2$ (0.5 μCi/ml) and 10 ng/ml insulin simultaneously with or without 10 minute preincubation with 10 μM nicardipine-HCl. At the indicated time 100 μl aliquots were taken for the determination of ^{45}Ca radioactivity trapped in adipocytes. When the ^{45}Ca efflux from preloaded adipocytes were studied, 4 ml fat cell suspension was incubated for 30 minutes with $^{45}CaCl_2$ (0.5 μCi/ml). Then, cells were washed by 2 one-minute centrifugations at 700 g, and resuspended at 40 %. Immediately after resuspension, 10 ng/ml insulin with or without pretreatment by 10 μM nicardipine was added, and 100 μl aliquots were taken for the determination of ^{45}Ca radioactivity remained in fat cells.

Values are mean or mean±SEM.

RESULTS

In order to localize the action site of calcium antagonist the influence of the drug on the time course of ^{125}I-insulin binding and the steady state binding was examined. As shown in Fig. 1, the time course of ^{125}I-insulin binding in the presence or absence of nicardipine was quite similar; the half maximal binding in the presence or absence of calcium antagonist reached in 3 and 3.2 minutes, respectively.

Fig. 2 illustrates the influence of nicardipine on steady state binding as a function of insulin concentration in the medium. The maximum binding and the insulin concentration for 50%

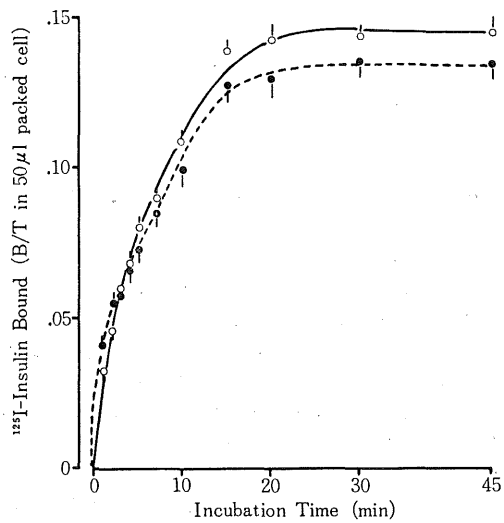

Fig. 1. Time course of specific binding of ^{125}I-insulin in the presence or absence of nicardipine-HCl. 2 ml of 40% fat cell suspension with or without preincubation with 10 μM nicardipine-HCl were incubated with ^{125}I-insulin at 37°C. At given intervals 100 μl aliquots were taken for ^{125}I radioactivity determination. Non-specific bindings were obtained by incubating cells with ^{125}I-insulin in the presence of 1,000 ng/ml cold insulin at given intervals. ○——○; in the presence of 10 μM nicardipine-HCl, ●·····●; without nicardipine-HCl. Values are mean±SEM of 4 experiments.

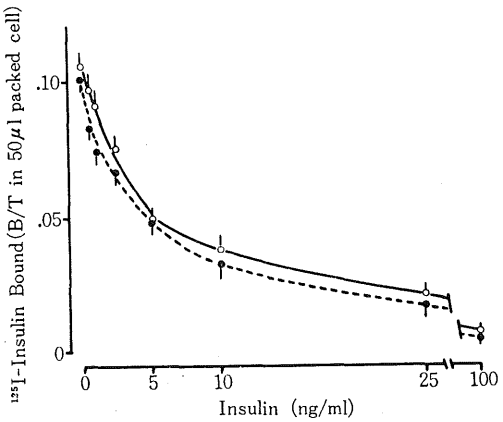

Fig. 2. Effect of nicardipine-HCl on ^{125}I-insulin binding to fat cells in the presence of varying concentrations of cold insulin. ^{125}I-insulin (50 pmol/l) was incubated for 30 min with fat cells with or without nicardipine-HCl (10 μM) pretreatment in the presence of 0.1-100 ng/ml insulin. Non-specific bindings were determined as described above. ○——○; in the presence of 10 μM nicardipine-HCl, ●·····●; without nicardipine-HCl. Values are mean±SEM of 4 experiments.

displacement of ^{125}I-insulin binding were similar in the presence or absence of nicardipine. It is concluded that calcium antagonist does not act at the step of insulin binding to the receptor.

Fig. 3 shows the influence of nicardipine-HCl

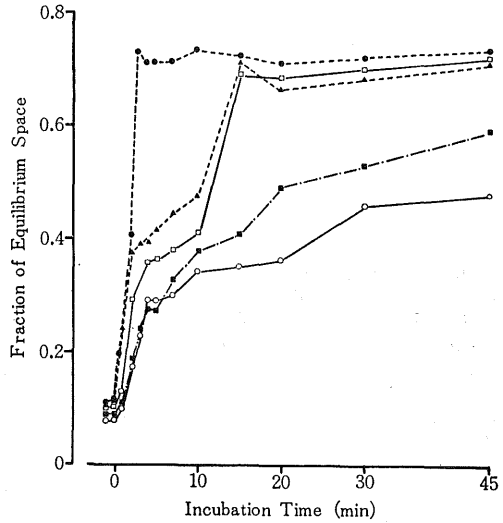

Fig. 3. Effects of varied concentrations of nicardipine-HCl on the time course of insulin action on 3-O-methylglucose uptake in fat cells. 4 ml of fat cell suspension were pretreated for 10 min with 0.01-10 μM nicardipine-HCl. Then, 10 ng/ml insulin was added and at indicated periods 80 μl aliquots were taken out for the determination of 3-O-methylglucose uptake. Non-specific uptake was estimated by the uptake of 3-O-methylglucose in the presence of 0.3 mM phloretin. Results were presented as % occupancy of the equilibrium space of 3-O-methylglucose measured by 10 min pulse with 3-O-methylglucose. ●·····●; without nicardipine-HCl, ▲·····▲; 0.01 μM, □——□; 0.1 μM, ■—·—■; 1 μM, ○——○; 10 μM. Values are mean of 4 experiments, and SEM is less than 8% of Mean.

on the kinetics of the activation of 3-O-methylglucose uptake by insulin. In the absence of nicardipine-HCl, insulin action reached the maximum level in 3 minutes, while varying concentrations of nicardipine-HCl delayed lag time for insulin action. In order to rule out the nonspecific pharmacological effect of this drug on insulin action the influence of two other calcium antagonists on the kinetics of insulin action was examined (Fig. 4). The submaximal concentration of verapamil and diltiazem-HCl delayed lag time for insulin

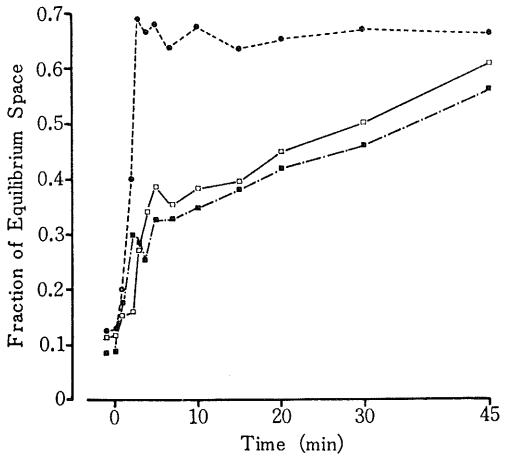

Fig. 4. Influence of verapamil and diltiazem-HCl on the time course of insulin action on 3-O-methylglucose uptake in fat cells. After pretreatment of fat cells for 10 min with 10 μM of verapamil or diltiazem-HCl, the time course of insulin action on 3-O-methylglucose uptake was determined as described in the legend of Fig. 3. ●·····●; without calcium antagonist, ■—·—■; 10 μM verapamil, □——□; 10 μM diltiazem-HCl. Values are mean of 4 experiments, and SEM is less than 7% of mean.

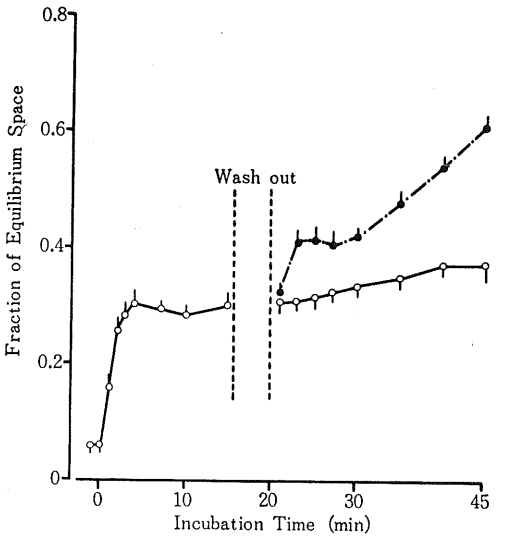

Fig. 5. Reversal of nicardipine-HCl inhibition of insulin action on 3-O-methylglucose uptake in fat cells. 6 ml of 40% fat cell suspension were pretreated for 10 min with 10 μM nicardipine-HCl, and the time course of insulin action was determined as described in Fig. 3. 15 min after the addition of insulin, 2 ml aliquot was taken out and washed by 2 centrifugations at 700 g. Then, cells were resuspended with buffer devoid of nicardipine-HCl at 40%, and 3-O-methylglucose uptake was determined at indicated periods. ●—·—●; with wash, ○——○; without wash. Values are mean±SEM of 6 experiments.

action without influence on the magnitude of insulin action. They did not affect insulin concentration for the half maximal response (data not shown).

Fig. 5 illustrates the reversibility of inhibition by nicardipine-HCl. Ten minute wash by 2 centrifugations reversed the inhibition by nicardipine-HCl.

As shown in Fig. 6, varied concentrations of calcium antagonist did not affect the half maximal insulin concentration for insulin action (control; 0.4 ng/ml vs 0.35–0.50 ng/ml in the presence of 0.1–10 μM nicardipine-HCl).

In order to investigate the action mechanism of nicardipine-HCl on insulin action, the effect of this drug on ^{45}Ca flux was examined. Fig. 7 reveals the effect of nicardipine-HCl on the kinetics of ^{45}Ca uptake in the presence of insulin. Unexpectedly calcium antagonist did not impair ^{45}Ca uptake in isolated fat cells. Nicardipine-HCl did not influence ^{45}Ca efflux from preloaded fat cells in the presence of

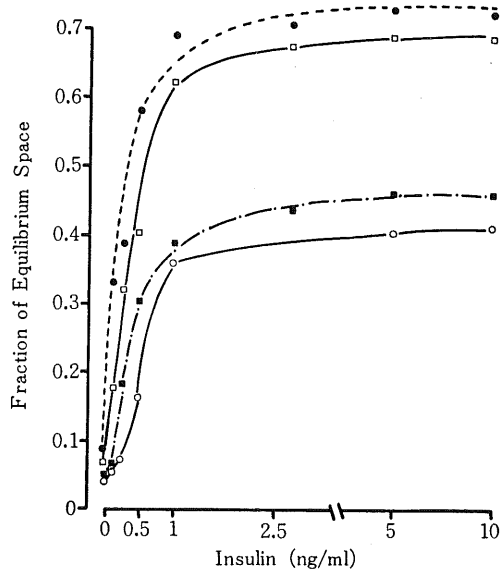

Fig. 6. Effect of varied concentrations of nicardipine-HCl on the concentration dependence of insulin action on 3-O-methylglucose uptake in fat cells. After 10 min pretreatment of fat cells with 0.1-10 μM nicardipine-HCl, fat cells were incubated for 30 min with 0.1-10 ng/ml insulin. Then, 3-O-methylglucose uptake was determined. ●·····●; without nicardipine-HCl, □——□; 0.1 μM., ■—·—■; 1 μM, ○——○; 10 μM. Values are mean of 4 experiments, and SEM is less than 7% of mean.

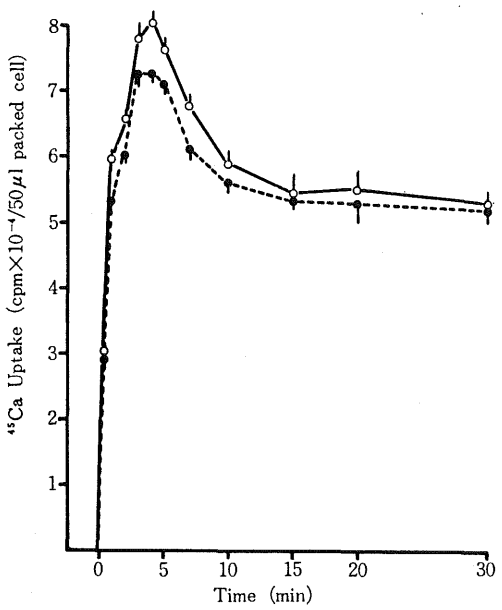

Fig. 7. Influence of nicardipine-HCl on ^{45}Ca uptake in fat cells. 4 ml of 40% fat cell suspension were exposed to $^{45}CaCl_2$ (0.5 μCi/ml) and 10 ng/ml insulin simultaneously with or without 10 min preincubation with 10 μM nicardipine-HCl. At indicated periods, 100 μl aliquots were taken out for the determination of ^{45}Ca radioactivity trapped in fat cells. ●·····●; without nicardipine-HCl, ○——○; 10 μM nicardipine-HCl. Values are mean±SEM of 4 experiments.

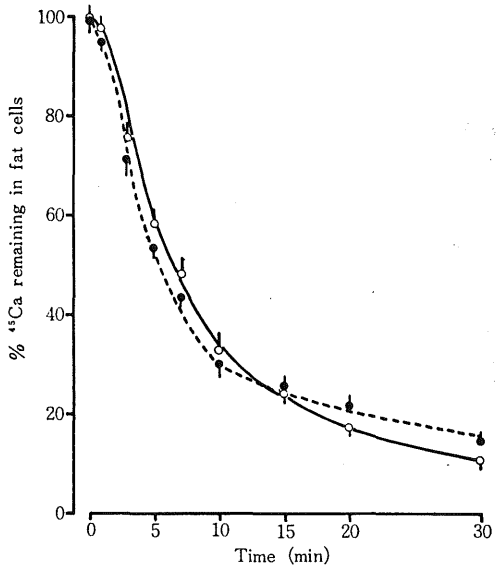

Fig. 8. Effect of nicardipine-HCl on ^{45}Ca efflux from preloaded fat cells. 4 ml of 40% fat cell suspension was incubated for 30 min with $^{45}CaCl_2$ (0.5 μCi/ml). Cells were washed by 2 one-minute centrifugations at 700 g and resuspended at 40%. Immediately after that 10 ng/ml insulin was added with or without pretreatment by 10 μM nicardipine-HCl. 100 μl aliquots were taken out for the determination of ^{45}Ca radioactivity remained in fat cells. ●·····●; without nicardipine-HCl, ○——○; with 10 μM nicardipine-HCl. Values are mean±SEM of 6 experiments.

insulin (Fig. 8). These results suggest that calcium antagonist impairs insulin action by mechanism(s) other than the inhibition of transmembrane calcium flux. Because it was recently suggested that calcium antagonist might inhibit the contraction of vessel wall by directly acting on calmodulin or other Ca dependent proteins[2], the influence of known calmodulin inhibitors on the kinetics of insulin action was studied (Fig. 9). Trifluoperazine as well as prochlorperazine prolonged the lag time for the maximum insulin action (t 1/2: 9.5-40 min and 3.5-14.5 min, respectively). They did not influence the insulin concentration for half maximal response (data not shown).

DISCUSSION

Although calcium antagonists have been used extensively for the treatment of ischemic heart disease and hypertension, little is known about its influence on insulin action.

In the present study newly developed calcium antagonist, nicardipine-HCl, delayed the lag time for insulin action on 3-O-methylglucose uptake beyond the level of insulin receptor. Other two different calcium antagonists also prolonged lag time for the maximum insulin action.

Fleckenstein et al.[7] demonstrated that calcium antagonists inhibit selectively calcium influx into the cell.

Calcium has been considered to be one of the mediators of insulin action. The precise movement of Ca^{2+} in insulin sensitive tissues, however, remained to be clarified. Insulin might act by increasing cytosolic Ca^{2+} concentration by increasing Ca^{2+} influx[5], inhibiting Ca^{2+} effiux[10] or releasing membrane bound Ca^{2+} from mitochondria or microsome[1]. Otherwise, insulin might act by decreasing cytosolic Ca^{2+} concentration by increasing Ca^{2+} uptake by endoplasmic reticulum[18].

It has been believed that calcium antagonists dilate the vessels by inhibiting Ca^{2+} influx without affecting Ca^{2+} efflux and intracellular

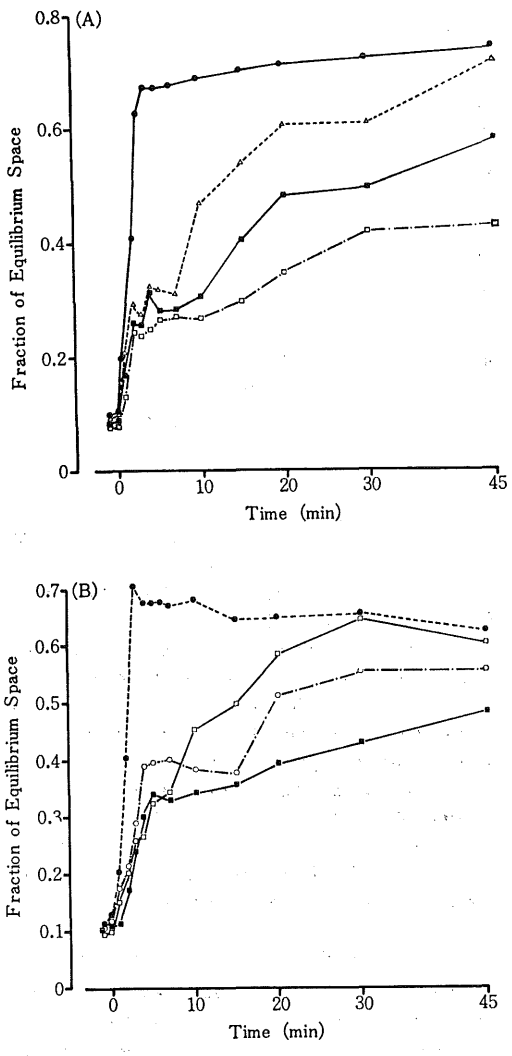

Fig. 9. Influence of varied concentrations of trifluoperazine (A) and prochlorperazine (B) on the time course of insulin action on 3-O-methylglucose uptake. 4 ml of fat cell suspension were pretreated with trifluoperazine (5-50 μM) or prochlorperazine (50-200 μM). Then, the time course of insulin action on 3-O-methylglucose uptake was determined as stated in legend of Fig. 3. (A). ●——●; without trifluoperazine, △·····△; 5 μM, ■——■; 20 μM, □—·—□; 50 μM. (B). ●·····●; without prochlorperazine, □——□; 50 μM, ○—·—○; 100 μM, ■——■; 200 μM. Values are mean, and SEM of mean is less than 7% of mean.

translocation. In the pancreatic islets calcium antagonists inhibit glucose-induced insulin release by interfering calcium influx without influencing calcium efflux[15]. Recent investigations, however, suggested that calcium antagonists act by other mechanisms than the inhibition of transmembrane calcium flux, because calcium antagonists did not inhibit La-resistant calcium uptake in strips of rat portal vein[4]. More recently, Boström et al.[2] suggested that various calcium antagonists interact directly with calcium binding protein such as calmodulin as evidenced by ^{14}C-felodipine binding to calmodulin fraction and the alteration of calcium binding site of calmodulin by felodipine. In the present study as shown in Fig. 8, both calmodulin inhibitors (trifluoperazine and prochlorperazine) delayed the lag time without influencing insulin binding and the half maximal insulin concentration for insulin action on 3-O-methylglucose uptake. Insulin increases phosphodiesterase activity in fat cells[12], and calmodulin, calcium dependent phosphodiesterase, might involved in the action mechanism(s) of insulin[8].

In conclusion, the findings reported here suggest the possible direct interaction between calmodulin and calcium antagonists in insulin sensitive tissues. However, we can not rule out other action mechanisms of calcium antagonists, because calcium antagonists and calmodulin inhibitors have inhibitory effects on membrane phospholipids[16], which might be involved in mediating insulin action[6].

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