Modulation of the L-type Ca-channels by Insulin Treatment in Rat Aorta

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Abstract. Intracellular Ca^{2+} is a major regulator of vascular smooth muscle force generation. Because of this important role, the concentration of Ca^{2+} within the cell is tightly regulated by means of sequestration and extrusion transport processes. Insulin was found to increase the plasmalemma Ca-ATPase expression. In this work we have found that the expression of the L-type calcium channels, which are known to play an important role in the regulation of vasoconstriction and vasodilatation, is affected by insulin.

We have found that single injection of insulin selectively decreased the Ca-channel expression in 150 min. Passive Ca-transport into proteoliposomes of plasma membrane proteins from the rat aorta was also decreased in 150 min, while after a longer resting period the Ca-transport was comparable to that from the control group. When insulin was added *in vitro* directly to a reaction mixture, an increase in the Ca-transport was found. No changes in Na/K-ATPase, which functions as cotransport system with Na/Ca exchanger, were observed.

On the contrary, seven insulin injections and a subsequent 2.5 hour rest did not cause changes in the Ca channel expression. In the animals treated seven times with insulin, the decrease in the Ca-transport was delayed compared to single insulin injection.

Our results suggest that insulin affects L-type calcium channels in the rat aorta by a decrease in the Ca-channel expression.

Key words: Ca-channels — Insulin — Gene expression

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Introduction

Insulin action involves a complex network of molecules at the cellular level, e.g. those that regulate ion homeostasis. Literature data about insulin action on Ca homeostasis are controversial. Insulin may influence vascular smooth muscle function by altering the movement of calcium into muscle cells or by affecting Ca^{2+} mobilization from intracellular stores. Thus, insulin could modulate the contractile properties of vascular smooth muscle and play a role in the regulation of blood pressure (Touyz and Schriffrin 1994). However, most studies have failed to demonstrate insulin-mediated vasoconstriction. This may be partially due to the fact that although insulin raises intracellular calcium concentration $[Ca^{2+}]_i$, this rise may not be high enough to induce vasoconstriction. The mechanism by which insulin modulates intracellular calcium concentrations seems to be through Ca-ATPase, since insulin was found to increase the expression of both, sarcoplasmic reticulum and plasma membrane Ca-ATPases (Kim and Zemel 1993). Insulin has been shown to reduce the influx of Ca^{2+} through receptor operated channels and to decrease the voltage-mediated Ca^{2+} influx (Standley et al. 1991).

In this work, we have focused our interest on the effect of insulin on the voltage-dependent calcium channels of L-type within different time periods after insulin treatment. These channels are known to contribute to the mechanism vasoconstriction/vasodilatation in smooth muscle and therefore they can affect blood pressure.

Materials and Methods

Animals

Male, specific pathogen free Sprague-Dawley rats (280–350 g) were obtained from Charles River Laboratories (Sulzfeld, Germany). Before initiation of the experimental procedures, the animals were housed 3–4 per cage for at least 7 days with lights on from 6 am to 6 pm and a room temperature of 23 ± 2 °C. Food and water were available *ad libitum*. All experiments were done between 8 am and 2 pm.

Insulin treatment

Insulin – Actrapid (NOVO Nordisk, Denmark) diluted by saline (0.1 ml per 100 g body weight) or saline alone was administered intraperitoneally at a dose of 5 IU per kg of body weight. Animals were divided into the groups of seven and each group was injected 1, 6 or 7 times, and sacrificed 2.5, 5, or 24 hours after the drug administration.

Membrane preparation

Plasma membranes were prepared according to Rosemblatt et al. (1981), with some modifications. Each rat aorta was frozen in a liquid nitrogen and homogenized in buffer A (20 $mmol.l^{-1}$ Tris-HCl, pH 7.4, 0.3 $mol.l^{-1}$ sucrose, 1 $mmol.l^{-1}$ iodacetamide, 1 $mmol.l^{-1}$

benzamidine and 1 mmol l^{-1} phenantroline) in a Polytron homogenizer The homogenate was centrifuged at 3000 × *g* for 10 min The sediment obtained was rehomogenized and centrifuged again at 3000 × *g* for 10 min Supernatants from both homogenization and rehomogenization were pooled and centrifuged at 11 000 × *g* for 20 min KCl in a final concentration of 0 5 mol l^{-1} was added to the supernatant The mixture was incubated for 1 hour at 4°C and subsequently centrifuged in a Beckman ultracentrifuge L 8-80 at 150 000 × *g* for 60 min The sediment was resuspended in a small volume (200 µl) of Tris HCl buffer pH 7 4 and used for further studies Proteins were estimated according to Lowry at al (1951)

Protein reconstitution

Five hundred µg of aorta membrane proteins were mixed with 10 mg of azolectin dissolved in 2% 3 [(tri-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) CHAPS was removed by Sephadex G-100 column chromatography Column was equilibrated with 20 mmol l^{-1} HEPES-Tris pH 7 4 and proteoliposomes were formed Unilamellarity of the vesicles was reached by several cycles of freezing and thawing using liquid nitrogen

Calcium transport

Transport of Ca^{2+} was determined as the difference between the radioactivity values obtained under depolarizing (K^+) and normal (Na^+) conditions $^{45}Ca^{2+}$ transport was measured in 50 mmol l^{-1} HEPES NaOH pH 7 4 25 mmol l^{-1} $CaCl_2$ 125 mmol l^{-1} Na^+ and/or K^+ Concentration of the radioactive calcium was 1 µmol l^{-1} After 1 min of incubation the fractions were passed through Sephadex CM columns and washed with 2 ml of buffer mentioned above Radioactivity was measured on a Rackbeta counter (LKB) after addition of Bray s scintillation cocktail

Na/K ATPase activity

Na/K ATPase activity was measured as a difference between total and ouabain-insensitive ATPase activity The Na/K-ATPase procedure was performed as described by Norris and Carey (1981)

Isolation of RNA

RNA was isolated according to the procedure of Chomczynski and Sacchi (1987) Briefly rat aorta was homogenized in solution D (4 mol l^{-1} guanidine isothiocyanate, 25 mmol l^{-1} sodium citrate pH 7 0, 0 5% sarcosyl, 0 1 mol l^{-1} 2 mercaptoethanol) Subsequently, 2 mol l^{-1} sodium acetate pH 4 0, phenol (1 1) and chloroform-isoamylalcohol (24 1) were added The mixture was vortexed precisely after each addition After centrifugation for 20 min at 10 000 × *g*, RNA was precipitated by isopropanol for 1 hour at −20 °C, followed by extraction with phenol-chloroform (1 1) chloroform and precipitation with 96% ethanol RNA was dissolved in redistilled water in a final concentration of 1 µg/µl

Reverse transcription polymerase chain reaction (RT PCR)

Reverse transcription was done by First-Strand cDNA Synthesis Kit (Pharmacia) The reaction was performed using Poly A-primer and Moloney murine leukemia virus reverse transcriptase RNA was dissolved to a concentration of 5 µg per sample Five µl of the first strand mixture were used as a template in PCR, which was performed using CaC1 (5'-AAC AAC AAC TTC CAG ACC TTC-3') and CaC 2 (5'-GGG TCA TAC TCT GCC

CAG AT-3') primers designed according to Yu et al. (1992) in a final volume of 50 μl. The PCR program included 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and polymerization at 72 °C for 1 min. PCR products were determined on 2% agarose gels. Quantification was done by Macintosh II computer – based image analysis system with IMAGE software.

Statistical analyses

Results are presented as means ± S.E.M. Statistical significance of differences was determined by one way analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$.

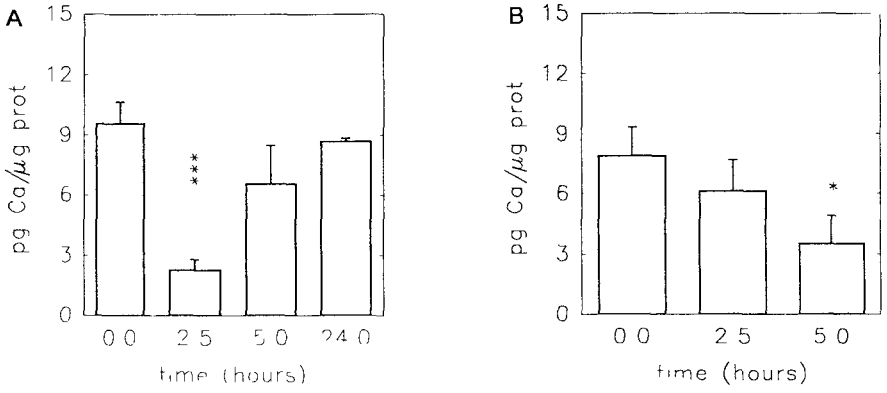

Figure 1. Calcium transport into proteoliposomes of plasma membranes from the rat aorta after one ($n = 7$) (A) and seven ($n = 7$) (B) doses of insulin. After single administration of insulin, calcium transport decreased rapidly in 2.5 hours after insulin application, and returned to control levels thereafter. The decrease of Ca-transport caused by seven applications of insulin was delayed in comparison to single administration of insulin. *** – $p < 0.001$, * $p < 0.05$

Results

Calcium transport into the reconstituted proteoliposomes containing plasma membrane proteins from the rat aorta decreased four times (from 9.57±1.09 to 2.26±0.55 pg Ca/μg prot.) 2.5 hours after a single insulin injection (Fig. 1A). Five hours after insulin application the calcium transport returned to almost normal levels (6.55 ± 1.05 pg Ca/μg prot.), and after 24 hours it reached values not significantly different from the control group. Insulin treatment repeated for six times at 24 hours intervals and a subsequent 24 hours rest (Fig. 1B) did not reveal significant changes in Ca-transport activity as compared to the control animals. After the

seventh administration of insulin, the decrease in the calcium transport was delayed compared to a single administration, and was largest after 5 hours of rest. This decrease could be due to changes in the L-type calcium channels or Na/Ca exchanger. Na/K-ATPase activity measured after a single insulin injection did not show any significant changes compared to the control group (Table 1). Repeated insulin treatment induced a significant increase in Na/K-ATPase activity (2.45 ± 0.59 vs 5.43 ± 0.66 μmols Pi/μg prot.) when measured 2.5 hours after the last injection (Table 1).

Table 1. Na/K-ATPase activity after insulin treatment

Single administration	n	mmol Pi/mg prot.	p
Control	7	2.4 ± 0.6	
I + 2.5 h	7	2.6 ± 1.2	ns
I + 5.0 h	7	1.6 ± 0.3	ns
Repeated administration			
6I + 24 h	7	3.4 ± 0.6	ns
7I + 2.5 h	7	5.4 ± 0.7	0.5

I – insulin, n – number of animals in the group, p – statistical analysis as determined by ANOVA

In vitro application of insulin significantly increased Ca-transport (from 9.6 ± 1.1 pg Ca/μg prot. in control animals to 14.9 ± 2.7 pg Ca/μg prot.). This finding points to a more complex effect than is the direct interaction between L-type calcium channel and insulin.

In order to evaluate possible changes in the L-type calcium channel expression, we performed transcription of mRNA into cDNA and polymerase chain reaction using primers from a conserved region of the Ca-channel mentioned. A typical result of this experiment is shown in Fig. 2*A*. A decrease of the channel expression was observed in all samples from rats, to whom insulin was administered. Quantification of these results is shown in Fig. 2*B*. This analyses confirmed that expression of L-type calcium channels was reduced 2.5 hours after a single administration of insulin and then started to normalize. When dihydropyridine nimodipine was injected to the group of rats *in vivo*, no changes in the Ca-channel expression were observed (215.4 ± 1.7 arbitrary units for the control group vs 214.1 ± 2.3 arbitrary units for the nimodipine treated group), which suggested that nimodipine was unable to modulate the Ca-channel expression.

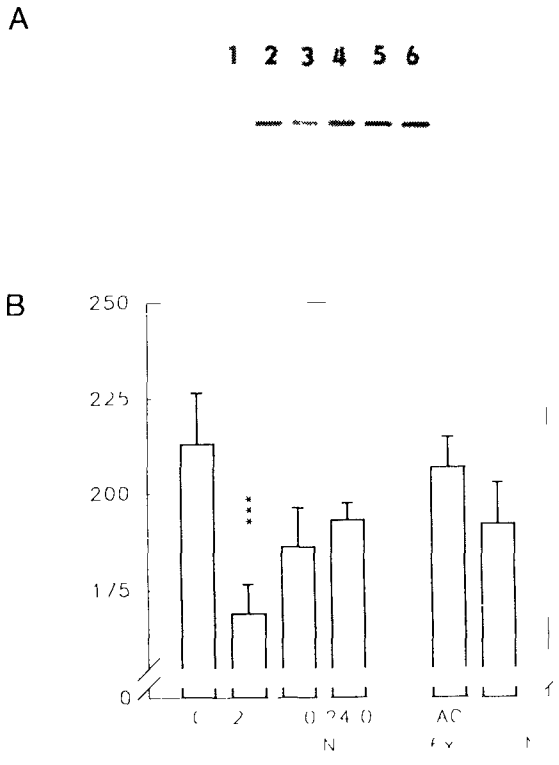

Figure 2. Expression of the calcium channels of L type in the rat aorta from rats treated differently with insulin. Reverse transcription of the mRNA and polymerase chain reaction with appropriate primers was done to determine differences in the Ca channel expression. Products were detected on 2% agarose gels as bands of approximately 330 bp (1). Samples were organized as follows: 1 – 1 × insulin + 2.5 hour; 2 – control (C); 3 – 1 × insulin + 24 hours; 4 – 7 × insulin + 2.5 hours; 5 – 6 × insulin + 24 hours (AC); 6 – 1 × insulin + 5 hours. Each sample was scanned and intensities of the individual bands were compared (*B*). Single insulin administration decreased Ca channel expression significantly in 2.5 hours after treatment. Five hours after insulin administration Ca-channel expression returned almost to control levels. Insulin treatment for seven times did not induce significant decrease in Ca-channels expression. Means ± S.E.M. are from 3 experiments. *** $p < 0.001$

Discussion

Insulin can affect several transmembranous exchange systems and thereby modulate cation metabolism in cells (Moore 1983). Calcium transport is of a special importance since calcium is a second messenger responsible for a variety of metabolic regulations. Zemel et al. (1992) have suggested that insulin may stimulate Ca-ATPase mediated vascular smooth muscle Ca-efflux. The same group demonstrated that insulin incubation stimulated the expression of plasmalemma Ca^{2+} ATPase in A7r5 cultured vascular smooth muscle cells and caused a corresponding increase in Ca^{2+} ATPase mediated Ca^{2+} efflux (Kim and Zemel 1993). Stimulation of the Na/Ca exchanger by insulin was also reported (Mullins 1976). Insulin was also found to reduce Ca^{2+} influx through receptor operated channels

and to decrease voltage-mediated Ca^{2+} influx (Standley et al. 1991). Our results are consistent with this finding, since we observed a decrease of the Ca-transport within 2.5 hours after a single insulin treatment (Fig. 1*A*). These results are in good agreement with a decreased expression of L-type Ca channels (Fig. 2). After multiple (seven) administrations of insulin, a decrease in the Ca-transport occurred after 5 hours of a rest period (Fig. 1*B*), which might point to an adaptation to insulin treatment. Glucose levels in the plasma of insulin-treated groups of rats were also decreased 2.5 hours after injection, and were normalized 5 hours after injection (not shown). Since insulin treatment is a complex process causing changes in a range of metabolic pathways including changes in glucose levels, it is difficult to explain the mechanism of a decrease in the Ca-channel expression. Acute insulin induced rise in cytosolic calcium is thought to be a possible mechanism of the insulin-mediated stimulation of glucose uptake (Draznin et al. 1988). Glucose has been also shown to depress the activity of voltage-sensitive Ca-channels in vascular smooth muscle cells (Lechuga et al. 1990) and might therefore participate in the decrease of Ca-channel expression.

Na/K-ATPase is known to be stimulated by insulin in many tissues (Ferranini et al. 1988; Prakash et al. 1992). In our experiments, no significant increase of Na/K-ATPase activity was observed after single insulin administration (Table 1). However, multiple administrations rapidly increased the Na/K-ATPase activity (Table 1). Therefore, we propose that the Na/Ca exchanger activity is increased only after an augmented treatment with insulin.

To summarize our results, we could show for the first time that in rat aorta, the expression of the L-type calcium channels is downregulated by a single insulin injection 2.5 hours after insulin application.

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