Effects of detergents on $\text{Na}^+ + \text{K}^+$ -dependent ATPase activity in plasma-membrane fractions prepared from frog muscles

Studies of insulin action on Na^+ and K^+ transport

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The increase in Na^+/K^+ transport activity in skeletal muscles exposed to insulin was analysed. Plasma-membrane fractions were prepared from frog (*Rana catesbeiana*) skeletal muscles, and examination of the Na,K -ATPase ($\text{Na}^+ + \text{K}^+$ -dependent ATPase) activity showed that it was insensitive to ouabain. In contrast, plasma-membrane fractions prepared from ouabain-pretreated muscles, by the same procedures, showed extremely low Na,K -ATPase activity. On adding saponin to the membrane suspension, the Na,K -ATPase activity increased, according to the detergent concentration. The maximum activity was about twice the control value, at 0.33 mg of saponin/mg of protein. Thus saponin makes vesicle membranes leaky, allowing ouabain in assay solutions to reach receptors on the inner surface of vesicles. Addition of insulin to saponin-treated membrane suspensions had no effect on the Na,K -ATPase activity, whereas the maximum activity of Na,K -ATPase in whole muscles was stimulated by exposure to insulin. The results show that the stimulation of Na^+/K^+ transport by insulin is not directly due to insulin binding to receptors on the cell surface, but rather support the view that the increase in the Na,K -ATPase induced by insulin requires an alteration of intracellular events.

INTRODUCTION

Besides the well-known effect of insulin on glucose transport in adipocytes (Kono *et al.*, 1982) and muscle cells (Narahara *et al.*, 1960; Morgan *et al.*, 1961; Kitasato & Marunaka, 1985), insulin also stimulates Na^+/K^+ transport in skeletal muscle (Moore, 1973; Erlij & Grinstein, 1976; Kitasato *et al.*, 1980; Resh, 1982; Rosic *et al.*, 1985). However, the mechanism underlying the insulin stimulation of Na^+/K^+ transport is still equivocal. In our previous study (Kanbe & Kitasato, 1986), we reported that the Na,K -ATPase activity of partially purified plasma-membrane fractions, prepared from muscles exposed to insulin, was about twice that of fractions prepared from intact muscles, and that the muscles whose Na,K -ATPase activity had been completely suppressed by ouabain regained the activity after exposure to insulin. The stimulating effect of insulin was blocked by monensin, an inhibitor of intracellular transport of protein (Tartakoff & Vassalli, 1978), whereas actinomycin D failed to block the stimulation. These findings suggested that the insulin stimulation was a consequence of an increased incorporation of membrane components responsible for the activation of Na^+/K^+ transport. Nevertheless, it was difficult to exclude the possibility that insulin binding to receptors may cause activation of latent Na^+/K^+ -transport units via an intramembrane process. We now determine whether insulin stimulated the Na,K -ATPase activity of plasma membranes in systems without intracellular structures. Plasma-membrane vesicles, having Na,K -ATPase activity insensitive to ouabain in the assay solution, were prepared from frog skeletal muscles, and treated with a variety of detergents to make their

membranes permeable to various ligands. It was found that insulin had no effect on the Na,K -ATPase activity in saponin-treated plasma-membrane preparations, whereas the addition of ouabain to the assay solutions caused complete suppression of the activity.

EXPERIMENTAL

Isolation of plasma membranes

The procedure for preparation of crude microsomal fractions is similar to that reported by Seiler & Fleischer (1982). Muscles (40–50 g) dissected from several frogs (*Rana catesbeiana*) were cut into pieces and homogenized for 1 min in 200 ml of KCl solution (300 mM-KCl/1 mM-Tris/HCl, pH 7.4) in a tissue blender (Matsushita MX-910). The homogenate was centrifuged at 3000 rev./min (1500 g) in a Sorvall GS-3 rotor for 5 min, and the supernatant was discarded. The resulting pellet was suspended in 200 ml of sucrose solution (200 mM-sucrose/2 mM-EDTA/2 mM-Tris/HCl, pH 7.4), then homogenized for 1 min and centrifuged at 4500 rev./min (3400 g) for 5 min. The second pellet was resuspended in 150 ml of sucrose solution and homogenized for 1 min. After centrifugation at 8000 rev./min (10000 g) for 15 min, the supernatant was filtered through a gauze cloth, and the filtrate was then centrifuged at 15000 rev./min (27000 g) in a Sorvall SS-34 rotor for 20 min. The fourth supernatant was further centrifuged at 35000 rev./min in a Hitachi RP50T-2 rotor (111000 g_{av}) for 60 min, and then the supernatant was discarded. The pellet was homogenized in 10 ml of sucrose solution with a Teflon homogenizer. The microsomal fraction was divided into 2.5 ml portions and kept at -80°C until use. A 2.5 ml microsomal

Abbreviation used: Na,K -ATPase, $\text{Na}^+ + \text{K}^+$ -dependent ATPase.

Table 1. Yields and activities of marker enzymes in plasma-membrane preparations

'Crude extract' means the filtered supernatant of the third centrifugation (8000 rev./min for 15 min). CON means membrane fractions from intact muscles and INS means those prepared from insulin-exposed muscles. Data are given as means for four experiments; S.E.M. values are shown only for the yields and the activities in plasma-membrane fractions.

		Crude extract	Microsomal fraction	Plasma membrane
Yield (mg of protein/100 g of muscle)	CON	720	59.1	5.8 ± 1.6
	INS	736	59.0	6.4 ± 1.4
5'-Nucleotidase (μmol of adenosine/min per mg of protein)	CON	0.01	0.03	5.6 ± 0.9
	INS	0.01	0.03	5.8 ± 0.2
NADH oxidase (μmol of NAD ⁺ /min per mg of protein)	CON	4.7	24.6	12.9 ± 2.5
	INS	5.0	24.5	12.1 ± 2.1
Galactosyltransferase (nmol of lactosamine/h per mg of protein)	CON	1.0	21.0	7.8 ± 2.2
	INS	1.0	19.0	7.5 ± 1.3
Na,K-ATPase (μmol of P _i /h per mg of protein)	CON	0.7	2.1	9.1 ± 0.5
	INS	1.1	3.3	16.5 ± 0.4

sample was fractionated by discontinuous sucrose-density-gradient (22%, 28%, 37%, 40%) centrifugation in a Hitachi RPS40T rotor for 4 h at 35000 rev./min (152000 *g_{av.}*). A fraction taken at the 22%-/28%-sucrose interface was termed 'plasma-membrane fraction'. All procedures were carried out at 2 °C. 5'-Nucleotidase, UDP-galactose:*N*-acetylglucosamine galactosyltransferase (galactosyltransferase) and NADH oxidase were used as the marker enzymes for the plasma membrane, Golgi apparatus and mitochondrial membrane respectively, to check purity. The yields and activities of marker enzymes in the membrane fractions are summarized in Table 1.

Exposure of muscles to insulin

Pairs of leg muscles were dissected from several frogs. Each pair provided control and test muscles. A group of test muscles was exposed to insulin (100 munits/ml; 3.8 μg/ml) dissolved in Ringer solution for 60 min at 25 °C, whereas a group of control muscles was incubated in normal Ringer solution for the same period and at the same temperature. After incubation, the plasma-membrane fractions were prepared from each group as described above.

Enzyme assays

ATPase activity was measured by a method slightly modified from that previously reported (Kanbe & Kitasato, 1986). A plasma-membrane suspension was added to an assay solution [10 mM-KCl/4 mM-MgCl₂/1.0 mM-histidine/HCl (pH 7.4) and either 100 mM-NaCl or 100 mM-choline chloride], and after 8 min ATP was added to the reaction mixture to give a final concentration of 1.0 mM. In some experiments, the plasma-membrane suspension was added to the assay solution containing a variety of detergents, and the ATP was added after 15 min. The reaction was carried out for 20 min at a temperature of 25 °C. The released P_i was measured by a modification of the method of Martin & Doty (1949). Na,K-ATPase activity was calculated from the difference between the amounts of P_i released in the presence and absence of Na⁺ ions.

Activities of 5'-nucleotidase and NADH oxidase were measured by the method of Avruch & Wallach (1971),

and that of galactosyltransferase was measured by the method of Fleischer (1974). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Chemicals

Bovine insulin, ATP (disodium salt), bovine serum albumin, ouabain, UDP-galactose, *N*-acetyl-D-glucosamine and saponin were obtained from Sigma Chemical Co. NADH and AMP (disodium salts) were obtained from Kohjin (Tokyo, Japan). [¹⁴C]AMP and UDP-[³H]galactose were obtained from New England Nuclear. SDS, Lubrol-PX and other reagents were purchased from Nakarai Chemicals (Kyoto, Japan).

RESULTS

Detergent treatment increases Na,K-ATPase activity in membrane preparations

Previous experiments (Kanbe & Kitasato, 1986) showed that membrane fractions prepared from frog skeletal muscles were insensitive to ouabain, whereas digitoxin, a more hydrophobic cardiac glycoside, completely suppressed the activity. Electron-microscopic observation indicates that all membrane fragments in the preparations are in vesicle form. To make the vesicle membranes permeable to the ligands which were to be examined in assay solution, the membrane preparations were incubated with a variety of detergents. Fig. 1 shows the Na,K-ATPase activity of membrane preparations derived from intact and insulin-exposed muscles at various concentrations of saponin (Fig. 1*a*) and Lubrol-PX (Fig. 1*b*). In the absence of detergent, the specific activity of Na,K-ATPase in plasma-membrane preparations derived from intact muscles was 1.8 times that in preparations from intact muscles, in accord with our previous observations (Kanbe & Kitasato, 1986). The value of the specific activity of preparations derived from intact muscles is almost identical with that reported by other workers (Narahara *et al.*, 1979; Seiler & Fleischer, 1982). Both saponin and Lubrol-PX increased the Na,K-ATPase activity in a dose-dependent manner. At 0.33 mg of saponin/mg of

Fig. 1. Effects of saponin and Lubrol-PX on Na,K-ATPase activity

Plasma-membrane suspensions were added to the assay solutions containing saponin or Lubrol-PX 15 min before the addition of ATP. Assay temperature was 25 °C. ○, Control plasma membrane; ●, plasma membrane prepared from insulin-exposed muscles; △, plasma membrane prepared from insulin-stimulated muscles which had been incubated with 20 μM -ouabain for 60 min before exposure to insulin; ■, the difference of the activity between preparations derived from insulin-exposed and intact muscles. Vertical bar indicates S.E.M. for four experiments.

protein the stimulation was maximal, and further addition of saponin caused an inhibition of the activity. The maximum activity was about twice that in detergent-free assay solution. Lubrol-PX was less effective than saponin, producing a 1.5-fold increase in enzyme activity. Similar effects of detergents were observed on the activity in preparations derived from insulin-exposed muscles. The difference of activity between preparations derived from insulin-exposed and intact muscles is shown in Figs. 1(c) and 1(d). The difference, the insulin-sensitive component of the activity, also increased with increasing saponin. Lubrol-PX was also less effective than saponin for stimulating the insulin-sensitive component.

Addition of insulin to a solution bathing muscles whose Na^+/K^+ -transport activity had been completely suppressed by ouabain has been reported to restore the transport activity (Erlj & Grinstein, 1976; Kitasato *et al.*, 1980). Although membrane fractions prepared from muscles which had been incubated with ouabain (20 μM) for 60 min showed no Na,K-ATPase activity, membrane fractions prepared from muscles which were

Fig. 2. Effect of SDS on Na,K-ATPase activity

Plasma-membrane suspensions were added to the assay solutions containing SDS and preincubated for 15 min at 25 °C before the addition of ATP. ○, Control plasma membrane; ●, plasma membrane prepared from insulin-exposed muscles; △, plasma membrane prepared from insulin-stimulated muscles which had been preincubated with 20 μM -ouabain for 60 min before exposure to insulin. Vertical bar indicates S.E.M. for four experiments.

exposed to insulin after preincubation with ouabain did show Na,K-ATPase activity (Figs. 1c and 1d). The magnitude of this newly developed Na,K-ATPase activity was almost identical with that of the insulin-sensitive component of the Na,K-ATPase activity estimated from the experiments on membrane preparations derived from non-ouabain-treated (intact) muscles shown in Figs. 1(a) and 1(b). The effects of detergents on the newly developed Na,K-ATPase activity in such preparations were also examined, to see whether this newly developed activity is equivalent to the insulin-sensitive component observed in intact muscles. The Na,K-ATPase activities of such plasma-membrane preparations at various concentrations of saponin are shown in Fig. 1(c). Saponin increased the activity in a similar manner to that observed for preparations derived from intact muscles shown in Fig. 1(a). The effect of Lubrol-PX was again much less than that of saponin (Fig. 1d). These observations indicate that the newly developed activity is also located in the vesicle membranes, similarly to that in preparations derived from intact muscles.

SDS is reported to increase the specific activity of Na,K-ATPase in membrane preparations derived from kidney (Jørgensen, 1974) and it has been successfully used for purification of the enzyme (Jørgensen, 1977;

Table 2. Effects of detergents on Na,K-ATPase activity of plasma-membrane vesicles prepared from ouabain-exposed muscles

Muscles were incubated in 20 μ M-ouabain in Ringer solution for 60 min at 25 °C, then plasma membrane was prepared. Concentrations of saponin, Lubrol-PX and SDS were 0.33 mg/mg of protein. Data (μ mol of P_i /h per mg of protein) are given as means $\pm$ S.E.M. for four experiments.

Assay conditions	Na ⁺ -insensitive ATPase activity*	Na,K-ATPase activity†
Without detergent	41.3 $\pm$ 1.9	0.42 $\pm$ 0.16
+ saponin	39.3 $\pm$ 2.4	0.47 $\pm$ 0.20
+ Lubrol-PX	33.6 $\pm$ 0.3	0.46 $\pm$ 0.09
+ SDS	22.9 $\pm$ 1.9	0.17 $\pm$ 0.13

* Assayed in Na⁺-free assay solution.

† Assayed in Na⁺-containing and Na⁺-free assay solutions, and the difference between the amounts of released P_i was calculated.

Karlish & Pick, 1981). However, in our present experiments SDS suppressed Na,K-ATPase activity (Fig. 2).

Detergents have no effect on ATPase activity in preparations derived from ouabain-treated muscles

It is disputed whether detergents may directly act to unmask the latent Na,K-ATPase activity in plasma membrane. Na,K-ATPase that does not bind ouabain owing to latency can, after detergent treatment, provide additional Na,K-ATPase activity blocked by ouabain. To determine whether this is the case, we examined the effect of detergents on the ATPase activity in preparations derived from ouabain-exposed muscles. In these preparations, all enzymes having the active Na,K-ATPase are expected to remain in a suppressed state throughout the experiment; thus any Na,K-ATPase activity detected after addition of detergent can be ascribed to activation of latent enzymes. Results of these experiments are summarized in Table 2. Almost all the ATPase activity in these preparations was insensitive to Na⁺ ions. The detergents examined either had no effect on the

Na⁺-insensitive basal ATPase activity or decreased it. Although there was a small increase in the remaining Na,K-ATPase activity, it was within experimental error for preparations derived from intact muscles. There was no indication of the presence of latent enzymes that were ready to be activated by detergents in preparations derived from muscles exposed to ouabain.

Ouabain inhibits the Na,K-ATPase activity in leaky membrane preparations

It is likely that the populations of inside-out and right-side-out vesicles are similar since saponin increased the Na,K-ATPase activity in membrane preparations by almost 2-fold (Fig. 1a). Saponin makes vesicle membranes sufficiently leaky to permit ATP molecules to reach receptor sites on the inner surface of right-side-out vesicles (i.e. the originally cytosolic surface). If this is the case, the Na,K-ATPase activity in inside-out vesicles would be expected to become ouabain-sensitive with increase in permeability to ouabain. As a test for this, the effect of saponin on the ouabain-sensitivity of Na,K-ATPase activity in membrane preparations was determined. Membrane preparations were incubated in assay solutions containing saponin (0.33 mg/mg of protein) with or without ouabain for 15 min before adding ATP. As summarized in Table 3, ouabain completely inhibited Na,K-ATPase activity in such saponin-treated membrane preparations, but the activity in non-treated membrane preparation was insensitive to ouabain. This finding thus supports the view that the inner surface becomes accessible to ligands in the presence of saponin. Since this treatment may also make membranes permeable to other molecules, the effect of insulin on the Na,K-ATPase activity in saponin-treated membrane preparations was examined. Insulin, however, had no effect on Na,K-ATPase activity even for saponin-treated membrane preparations. This result is consistent with the observations on purified ouabain-sensitive Na,K-ATPase prepared from rat adipocyte plasma membranes (Resh, 1982). The fact that insulin has no effect on the Na,K-ATPase activity in leaky plasma-membrane preparations supports the previous view that the stimulation of Na⁺/K⁺ transport by insulin is mediated through a change of intracellular events, rather than being a direct consequence of insulin binding to receptors on the cell surface.

Characteristics of the Na,K-ATPase activity induced by insulin

Our previous observation that the muscles whose Na,K-ATPase activity had been completely suppressed by ouabain regained Na,K-ATPase activity after exposure to insulin (Kanbe & Kitasato, 1986) indicates that insulin increases measurable enzyme activity in plasma membrane. In connection with this observation, we further studied the characteristics of the Na,K-ATPase induced by insulin. The relationships between the Na,K-ATPase activity and the substrate concentrations for ATP, Na⁺ and K⁺ ions are shown in Figs. 3(a), 3(b) and 3(c) respectively. The Na,K-ATPase activity reached a maximum around a concentration of 1 mM-ATP. It must be stressed that, at any concentrations of substrates examined, the Na,K-ATPase activity was always higher in preparations derived from insulin-treated muscles than in those from the control non-insulin-treated

Table 3. Effects of ouabain and insulin on Na,K-ATPase activity in saponin-treated plasma-membrane preparations

Concentrations of ouabain, insulin and saponin are 20 μ M, 0.33 mg/mg of protein and 100 munits/ml respectively. Data are given as means $\pm$ S.E.M. for four experiments.

Assay conditions	Na,K-ATPase activity (μ mol of P_i /h per mg of protein)	
	Non-treated	Saponin-treated
Normal assay solution	8.6 $\pm$ 1.3	16.8 $\pm$ 1.7
+ ouabain	8.2 $\pm$ 1.10	0.5 $\pm$ 0.3
+ insulin	8.1 $\pm$ 0.5	14.3 $\pm$ 0.9

Fig. 3. Effects of ATP, Na^+ and K^+ on Na,K-ATPase activities

(a) Assay solutions contained 100 mM- Na^+ or choline chloride, 10 mM- K^+ , 4 mM- Mg^{2+} and the various concentrations of ATP. (b) Assay solutions contained the various concentrations of Na^+ or 100 mM-choline chloride, 10 mM- K^+ , 4 mM- Mg^{2+} and 1.0 mM-ATP. (c) Assay solutions contained 100 mM- Na^+ or choline chloride, the various concentrations of K^+ , 4 mM- Mg^{2+} and 1.0 mM-ATP. Solutions of various Na^+ or K^+ concentrations were prepared by replacing NaCl or KCl with choline chloride. Curves were drawn by eye. ○, Control plasma membrane; ●, plasma membrane from insulin-exposed muscles. Vertical bar indicates S.E.M. for four experiments.

muscles. This finding also supports the view that the increase in the Na,K-ATPase activity in preparations derived from insulin-exposed muscles observed under the conditions described in the Experimental section is due to an increase in the number of Na,K-ATPase molecules in the preparations.

The half-saturation concentration for ATP was slightly lower in preparations derived from insulin-treated muscles than that in the control preparations.

The change in the dependencies on the Na^+ and K^+ concentrations was more complex than that observed for the dependency on ATP concentration. The fact that the incubation with insulin induced changes in the dependencies on the substrate concentrations suggests that the Na^+/K^+ -transport units induced by insulin are slightly different in structure from the units that have been already functioning before insulin exerts its effect on Na^+/K^+ transport.

DISCUSSION

Although insulin is known to stimulate Na^+/K^+ transport in skeletal muscles (reviewed by Moore, 1983), the mechanism of the action is still equivocal. It was clear that the effect of insulin in muscles was a selective enhancement of Na,K-ATPase, because no change was shown in the activities of other membrane marker enzymes (Table 1). Monensin, an inhibitor of intracellular transport of protein, blocks stimulation of Na,K-ATPase activity in the whole cell, whereas actinomycin D has no effect on the stimulation by insulin (Kanbe & Kitasato, 1986). These observations suggest that the enhancement of Na,K-ATPase by insulin involves the facilitation of intracellular transport of a membrane component responsible for the increase in Na^+/K^+ -transport activity. However, it has been unclear whether or not insulin binding to receptors on the cell surface could directly stimulate Na^+/K^+ transport through an intramembrane process. The present work is aimed at elucidating the mechanism of the insulin stimulation.

Electron-microscopic observation showed that the plasma-membrane fragments in the preparations used in the present experiments were all in vesicle form. It was also noted that the Na,K-ATPase activity was unaffected by ouabain added to the assay solutions, and only digitoxin, a more hydrophobic cardiac glycoside, could suppress the activity (Kanbe & Kitasato, 1986). Thus it is reasonable to consider that the membranes of vesicles carrying Na,K-ATPase are impermeable to ouabain. As the molecular size of ATP is very similar to that of ouabain, the ATP molecule is not expected to penetrate vesicle membranes. This means that only vesicles having ATP-binding sites on their outer surface, namely inside-out vesicles, will show Na,K-ATPase activity. Insulin molecules cannot reach receptors located on the inner surface of tightly sealed inside-out oriented vesicles. Thus, to examine whether insulin binding to receptors really induces stimulation of Na,K-ATPase activity through an intramembrane pathway, it was necessary to make the vesicle membranes permeable to insulin. Since it is most probable that the population of inside-out vesicles is equal to that of right-side-out vesicles, half of the total Na,K-ATPase activity, corresponding to Na,K-ATPase residing on right-side-out vesicles, is considered to be unavailable, unless such right-side-out vesicle membranes are made leaky. As expected, detergent treatment resulted in an increase in the Na,K-ATPase activity in the vesicle preparations (Fig. 1). Among the detergents examined, saponin was the most effective. Saponin treatment increased the Na,K-ATPase activity in membrane preparations by about 2-fold, suggesting that about half of the vesicle population was in inside-out form. If there is a direct connection between insulin binding and stimulation of

Na,K-ATPase through the intramembrane pathway, addition of insulin to detergent-treated vesicle preparations would bring about an increase in the Na,K-ATPase activity. The vesicles used for these experiments were sufficiently leaky to be permeable to ATP. However, our experiments conducted in cytosol-free systems show that there is no intramembrane pathway directly connecting with insulin binding to stimulation of Na,K-ATPase.

It was surprising that the Na⁺-insensitive ATPase activity, in membrane preparations derived from ouabain-treated muscles, was not affected by detergents. This observation may indicate that the Na⁺-insensitive basal ATPase activity is independent of membrane structure. It is not clear at present what kind of ATPase is responsible for this activity. It was also interesting that saponin treatment of membrane preparations derived from ouabain-treated muscles did not induce any appreciable increase in Na,K-ATPase activity. This finding suggests that not only inside-out vesicles bound ouabain on the inner surface, the originally extracellular surface, but also right-side-out vesicles with ouabain bound to the outer surface have no Na,K-ATPase activity. Thus our results are in accord with the view that ouabain binding to Na,K-ATPase or Na⁺/K⁺-transport units is effectively irreversible (Abeles, 1969; Caswell *et al.*, 1976; Venosa & Horowicz, 1981). Another interesting finding was that the Na,K-ATPase induced by insulin had some different characteristics from that in the control preparations.

Kono *et al.* (1982) have demonstrated that insulin stimulates the translocation of the glucose-transport systems from Golgi-rich fractions to plasma-membrane-rich fractions in fat-cells, and more recently it has been reported that insulin increases the Na⁺-sensitive component of the glucose-transport activity in frog muscle (Kitasato & Marunaka, 1985). Furthermore, insulin has been reported to stimulate the iron uptake in fat-cells by a translocation of transferrin receptors from microsomal fractions to plasma-membrane fractions (Davis *et al.*, 1986). At present, the relationship between the effect of insulin on Na⁺/K⁺ transport and on glucose transport cannot be confirmed, but there are some possibilities that insulin generally stimulates the translocation or the incorporation of membrane proteins.

The present study suggests that a membrane process after the binding of insulin to receptors requires some

soluble substance in cytosol, such as a phosphonucleotide, other than ATP to stimulate Na,K-ATPase activity. The identification of these factors requires further study.

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