Insulin stimulates the translocation of Na^+/K^+ -dependent ATPase molecules from intracellular stores to the plasma membrane in frog skeletal muscle

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The mechanism of the stimulation of Na^+/K^+ transport by insulin in frog skeletal muscle was studied. The ouabain-binding capacity in detergent-treated plasma membranes of insulin-exposed muscles was increased 1.9-fold compared with that of controls. Na^+/K^+ -ATPase activity was found in an intracellular 'light fraction' (fraction II) prepared by using anion-exchange chromatography. Marker enzyme activities for plasma and Golgi membranes were not detected in this fraction. The specific activity of Na^+/K^+ -ATPase in fraction II from insulin-exposed muscles was 58 % of that in an identical fraction from control muscles. No significant difference in the protein yield of the plasma membrane preparation was observed between these two groups. In parallel with the decrease in the Na^+/K^+ -ATPase activity in fraction II from insulin-exposed muscles, the ouabain-binding capacity in this fraction was also decreased. The addition of saponin to fraction II increased both Na^+/K^+ -ATPase activity and ouabain binding, indicating that some of the Na^+/K^+ -ATPase is located in sealed vesicles. These findings support the view that insulin stimulates the translocation of Na^+/K^+ -ATPase molecules from fraction II to the plasma membrane.

INTRODUCTION

Na^+/K^+ -ATPase (EC 3.6.1.37), which is found in a wide variety of tissue cell membranes, is a protein responsible for transporting Na^+ and K^+ across the plasma membrane. The relationship between transport activities and ionic concentrations [1], the inhibition of the activity by cardiac glycoside [2] and its hormonal regulation [3,4] have been studied extensively. In skeletal muscle cells, insulin has been reported to stimulate Na^+/K^+ transport [3–13], as well as having a facilitating effect on glucose and amino acid transport. In the previous studies [11,12], we reported that the Na^+/K^+ -ATPase activity of a plasma membrane fraction prepared from muscles exposed to insulin was 1.8-fold higher than that in the corresponding fraction obtained from control muscles. The addition of insulin to the plasma membrane suspension, however, did not increase the Na^+/K^+ -ATPase activity in the suspension. In the present study, we have confirmed an increase in the number of ouabain-binding sites on plasma membranes prepared from muscles incubated with insulin. On the basis of this finding, efforts were made to determine whether any putative regulatory molecules were generated in the intracellular compartment or incorporated into the plasma membrane. The ouabain-sensitive Na^+/K^+ -ATPase activity was detected in an intracellular fraction which was lighter than the plasma membrane fraction. In this fraction from insulin-exposed muscles, the Na^+/K^+ -ATPase activity was only 58 % of that in the corresponding fraction from control muscles. The ouabain-binding capacity of this fraction from insulin-exposed muscles were also lower than that of the same fraction from control muscles. These findings suggest that insulin stimulates the translocation of Na^+/K^+ pump molecules from intracellular pools to the plasma membrane.

EXPERIMENTAL

Preparation of the plasma membrane fraction

The plasma membrane fraction was prepared by the method described previously [12]. Pairs of leg muscles were dissected

from several frogs (*Rana catesbeiana*). Each pair provided one control and one test muscle. A group of test muscles was exposed to bovine insulin (100 munits/ml) dissolved in Ringer solution (103 mM- NaCl , 2 mM- KCl , 0.9 mM- CaCl_2 and 1 mM-Hepes, pH 7.4) for 60 min at 25 °C, whereas the group of control muscles was incubated in insulin-free Ringer solution for the same period at the same temperature. After the incubation the muscles were homogenized. As a starting step in the preparation of the plasma membrane fraction, the activity of 5'-nucleotidase, a marker enzyme for the plasma membrane, was measured in a crude muscle homogenate (starting homogenate) to allow estimation of the recovery of the plasma membrane. Then microsomal fractions were prepared and stored at –80 °C until use. A portion of microsomal suspension was fractionated using discontinuous sucrose-density-gradient (22 %, 28 %, 37 %, 40 %) centrifugation for 4 h at 152 000 *g*. The fraction at the 22 %/28 %-sucrose interface was termed the plasma-membrane fraction, and this was confirmed by the high activity of 5'-nucleotidase.

Preparation of intracellular fractions

To break down intracellular organelles, muscles were homogenized in a hypo-osmotic solution containing only 1 mM-EDTA and 10 mM-Tris/HCl (pH 7.4) using a tissue blender (Matsushita MX-910). The homogenate was centrifuged at 1500 *g* in a Sorvall GS-3 rotor for 5 min, and the supernatant was centrifuged at 3400 *g* for 5 min. This second supernatant was centrifuged at 10 000 *g* for 15 min. The third supernatant was filtered through a gauze cloth and centrifuged at 27 000 *g* in a Sorvall SS-34 rotor for 20 min. The fourth supernatant was further centrifuged at 111 000 *g*_{av.} (148 000 *g*_{max.}) in a Hitachi RP50T-2 rotor for 60 min. The final (fifth) supernatant was termed the 'starting light fraction' and was stored at –80 °C until use. To fractionate this fraction further, a 10 ml portion was diluted 2-fold with the same volume of a hypo-osmotic Tris buffer (20 mM-Tris/HCl, pH 7.4) and applied to a DEAE-Sephacrose Fast Flow column (1.6 cm × 20 cm; Pharmacia Fine Chemicals) which had already been equilibrated with the same

Abbreviation used: IC₅₀, concn. causing 50 % inhibition of binding.

buffer. After washing the column with 10 ml of the Tris buffer, the solution was switched to a Tris-buffered LiCl solution with a linear concentration gradient from 0 to 0.4 M. The flow rate was 2.0 ml/min. The eluent was fractionated by monitoring the absorbance at 280 nm using a u.v. monitor (UV-1; Pharmacia Fine Chemicals). Proteins remaining in the column were washed out with 1.0 M-NaCl. All procedures were carried out at 4 °C.

Enzyme assays

ATPase activity was measured using the method described previously [12]. A sample fraction was added to an assay solution (10 mM-KCl, 4 mM-MgCl₂, 10 mM-histidine/HCl, pH 7.4, and either 100 mM-NaCl or 100 mM-choline chloride). After preincubation for 8 min in the assay solution, ATP (pH 7.4) was added to the reaction mixture, to the final concentration of 1.0 mM. The reaction was allowed to continue for 20 min at 25 °C. The amount of P_i released was measured using the modified method of Martin & Doty [14]. Na⁺/K⁺-ATPase activity was calculated from the difference between the amounts of P_i released in the presence and the absence of Na⁺.

The activities of 5'-nucleotidase and galactosyltransferase were measured using the methods of Avruch & Wallach [15] and Fleischer [16] respectively. Proteins were measured using the method of Lowry *et al.* [17] with bovine serum albumin as the standard.

[³H]Ouabain binding

The sample fraction was incubated with saponin (0.3 mg/mg of protein) at 37 °C for 15 min to make the vesicle membrane leaky before incubating with [³H]ouabain. A sample fraction was added to a [³H]ouabain-loading solution. The final concentration of each reaction mixture (1 ml) was as follows: 100 mM-NaCl, 4 mM-MgCl₂, 10 mM-Tris/HCl (pH 7.4), 1 mM-ATP, 33 nM-[³H]ouabain (18.5 kBq; 0.5 µCi), with or without unlabelled ouabain, and 0.05–0.1 mg of protein. The concentration of [³H]ouabain was chosen to minimize the non-specific binding. The mixture was incubated for 60 min at 37 °C and then filtered through a cellulose membrane filter (Millipore; pore size 0.45 µm for the saponin-treated plasma membrane fraction and 0.22 µm for the intracellular fraction). The filter was dissolved in ACS-II scintillant (Amersham), and the radioactivity was counted in a liquid scintillation counter. Specific binding was calculated from the difference between values observed in the absence and the presence of 0.5 mM unlabelled ouabain.

Extraction, separation and quantification of phospholipids

Total lipids were extracted from membrane fractions with chloroform/methanol by the method of Bligh & Dyer [18]. Internal light fractions were concentrated by using Pellicon membrane ultrafiltration (Millipore) under the pressure of N₂ gas. The phospholipids were separated by two-dimensional t.l.c. on 250 µm-thick Silica Gel 60 plates (Merck) using chloroform/methanol/acetic acid (13:5:2, by vol.) as the first solvent system and chloroform/methanol/88 % formic acid (13:5:2, by vol.) as the second solvent system. After t.l.c. the spots were revealed with iodine vapour. To quantify the phosphate in each spot, the silica gel was scraped off and digested at 190 °C with HClO₄ [19], and then the phospholipid phosphorus content was measured by the method of Ames & Dubin [20]. Cholesterol content was measured by using the Cholesterol C-Test Wako enzyme kit (Wako Pure Chemical Industries, Osaka, Japan).

Chemicals

Bovine insulin, ATP (Tris salt), bovine serum albumin, ouabain, UDP-galactose and *N*-acetylglucosamine were purchased from Sigma Chemical Co. [¹⁴C]AMP, UDP-[³H]galactose

and [³H]ouabain were purchased from New England Nuclear. All other reagents were obtained from Nacalai Tesque (Kyoto, Japan).

RESULTS

Insulin induces an increase in [³H]ouabain binding to muscle plasma membranes

In our previous study [12], we reported that plasma membranes prepared by the same procedure as that used in the present experiments formed tightly sealed right-side-out and inside-out vesicles in an approximate ratio of 1:1, and that saponin made all of the ouabain-binding sites in vesicles accessible to ouabain molecules in the assay medium. Ouabain binding to saponin-treated plasma membrane preparations was a relatively slow process, and reached an apparent steady state after 40 min of incubation (Fig. 1a). [³H]Ouabain binding was competitively suppressed by unlabelled ouabain (Fig. 1b). The IC₅₀ (concn. causing 50 % inhibition) for [³H]ouabain binding was approx. 0.1 µM unlabelled ouabain, which is similar to the value reported by Venosa & Horowicz [21]. This was the case in both control and insulin-exposed muscles. The amount of ouabain specifically bound to the plasma membrane preparation from insulin-exposed muscles was 1.9 times that bound to the preparation from the control muscles at any concentration of ouabain. There was no significant difference in the amount of non-specific ouabain binding between membrane preparations from insulin-exposed and control muscles. We estimated the recovery of the plasma membrane, on the assumption that the 5'-nucleotidase did not suffer from denaturation during separation process, using the following equation:

$$\text{Recovery} = \frac{[5'\text{-Nucleotidase}]_p \times [\text{Protein}]_p}{[5'\text{-Nucleotidase}]_h \times [\text{Protein}]_h}$$

where [5'-Nucleotidase]_p and [Protein]_p are the specific activity of 5'-nucleotidase and protein yield in the plasma membrane fraction, and [5'-Nucleotidase]_h and [Protein]_h are the specific activity of 5'-nucleotidase and protein yield in the starting homogenate (Table 1). The recovery of plasma membranes into the plasma membrane preparation was estimated to be

Fig. 1. Time course of [³H]ouabain binding and suppression by unlabelled ouabain in detergent-treated plasma membranes

Plasma membrane fractions were incubated with saponin (0.3 mg/mg of protein) at 37 °C for 20 min. A portion of the saponin-treated plasma membrane fraction was then tested for [³H]ouabain binding. (a) Time course of [³H]ouabain binding. Specific binding was calculated by the difference between the values in the absence and presence of 0.5 mM unlabelled ouabain. (b) Inhibition of [³H]ouabain binding by various concentrations of unlabelled ouabain. ●, Plasma membrane fraction prepared from insulin-exposed muscles; ○, control plasma membrane fraction. Vertical bars indicate S.E.M. for four experiments in (a), and for eight experiments in (b).

Table 1. Protein yields and specific activities of marker enzymes in plasma membrane preparations

Plasma membrane and microsomal fractions were prepared by the method described in the Experimental section. 'Starting homogenate' indicates the crude frog muscles homogenate; Ins indicates the fractions prepared from insulin-exposed muscles; Con indicates fractions prepared from control muscles. Data are given as mean $\pm$ S.E.M. for four experiments.

	Protein yield (mg of protein/ g of muscle)		5'-Nucleotidase activity (μ mol of adenosine/ h per mg of protein)		Galactosyltransferase activity (nmol of lactosamine/ h per mg of protein)	
	Ins	Con	Ins	Con	Ins	Con
Starting homogenate	70.16 $\pm$ 8.05	69.53 $\pm$ 6.12	6.6 $\pm$ 0.6	6.6 $\pm$ 0.7	0.40 $\pm$ 0.01	0.40 $\pm$ 0.01
Microsomal fraction	0.59 $\pm$ 0.05	0.59 $\pm$ 0.05	64.5 $\pm$ 7.68	70.7 $\pm$ 14.5	19.0 $\pm$ 0.4	21.0 $\pm$ 0.9
Plasma membrane fraction	0.064 $\pm$ 0.014	0.058 $\pm$ 0.016	348 $\pm$ 12	336 $\pm$ 54	0.008 $\pm$ 0.001	0.008 $\pm$ 0.002

4.5 $\pm$ 0.2% of that in the starting homogenate. There was no statistical difference in the recovery of plasma membranes between insulin-exposed and control muscles. The total activity of Na⁺/K⁺-ATPase in the plasma membranes of 1 g of muscle was calculated by using the values for the specific activity of Na⁺/K⁺-ATPase, protein yield and the recovery of plasma membranes in the plasma membrane fraction. The calculated values will be shown together with the total activity in the intracellular components in Table 4.

Na⁺/K⁺-ATPase activity is observed in the intracellular fraction

Since the Na⁺/K⁺-ATPase activity in the plasma membrane fraction is not directly stimulated by insulin [12], it may be natural to consider either that some putative regulatory substances have unmasked any latent pump units or that the transport molecules themselves have been newly incorporated into the plasma membrane. Conventionally, the location of Na⁺/K⁺-ATPase activity is thought to be limited to the plasma membrane, so much so that it is regarded to be a marker enzyme. We also assumed at first that there were some soluble components in the cytosol mediating the regulation of Na⁺/K⁺ transport activity of the plasma membrane. As a first step we tried to find such putative substances which could unmask latent activities in the plasma membrane. A light fraction (the starting light fraction described in the Experimental section) was prepared, so as to recover all of the soluble components in the intracellular

components into the fraction. Both before and after adding a sample of the light fraction to the plasma membrane suspension, the Na⁺/K⁺-ATPase activity was assayed. A slight increase in the enzymic activity was observed after addition of the light fraction (results not shown). As the next step, we checked whether or not the starting light fraction itself had Na⁺/K⁺-ATPase activity. Against our expectations, Na⁺/K⁺-ATPase activity was present in the starting light fraction, though its specific activity was low (Table 2). The activity in a particular volume corresponded to the observed increase in activity when the same volume of the starting light fraction was added to the plasma membrane fraction.

The starting light fraction was further fractionated using an anion-exchange DEAE-Sepharose column by elution with a linear concentration gradient from 0 to 0.4 M-LiCl. Three peaks were obtained with absorbance at 280 nm (Fig. 2). These peaks were numbered in ascending order according to ionic concentration. Fraction I contained proteins which did not bind to the anion-exchanger. Fractions II and III were eluted at 0.12 M- and 0.2 M-LiCl respectively. The activity of Na⁺/K⁺-ATPase was observed only in fraction II. The peak of Na⁺/K⁺-ATPase activity in fraction II derived from control muscles was higher than that in the identical fraction derived from insulin-exposed muscles, whereas there was no significant difference in the elution patterns.

Despite the fact that the starting light fraction was the final

Table 2. Protein yields and specific activities of marker enzymes in intracellular fractions

Intracellular fractions were prepared as described in the Experimental section. Fractions I, II, and III were defined in Fig. 2. Ins indicates intracellular fractions prepared from insulin-exposed muscles; Con indicates corresponding fractions from control muscles. Data are given as means $\pm$ S.E.M.

Fraction	Protein yield (mg of protein/ g of muscle) (n = 4)		Na ⁺ /K ⁺ -ATPase activity (μ mol of P _i /h per mg of protein) (n = 6)		5'-nucleotidase activity (μ mol of adenosine/h per mg of protein) (n = 4)		Galactosyltransferase activity (nmol of lactosamine/h per mg of protein) (n = 4)	
	Ins	Con	Ins	Con	Ins	Con	Ins	Con
Starting light fraction	11.19 $\pm$ 0.67	10.58 $\pm$ 0.57	0.78 $\pm$ 0.19	1.03 $\pm$ 0.13	2.5 $\pm$ 0.2	2.2 $\pm$ 0.2	0.5 $\pm$ 0.1	0.4 $\pm$ 0.2
Fraction I	0.98 $\pm$ 0.01	0.95 $\pm$ 0.03	0.01 $\pm$ 0.01	0.05 $\pm$ 0.03	0.3 $\pm$ 0.2	0.2 $\pm$ 0.2	0.5 $\pm$ 0.3	0.7 $\pm$ 0.2
Fraction II	3.43 $\pm$ 0.13	3.70 $\pm$ 0.20	1.78 $\pm$ 0.09	3.06 $\pm$ 0.32	3.1 $\pm$ 0.3	2.9 $\pm$ 0.3	0.3 $\pm$ 0.1	0.5 $\pm$ 0.3
Fraction III	2.09 $\pm$ 0.19	2.06 $\pm$ 0.03	0.33 $\pm$ 0.27	0.26 $\pm$ 0.15	4.3 $\pm$ 0.9	4.8 $\pm$ 0.1	0.8 $\pm$ 0.2	0.8 $\pm$ 0.3

Fig. 2. Anion-exchange chromatography on DEAE-Sepharose Fast Flow

The starting light fraction prepared to contain intracellular components was applied to a column (1.6 cm $\times$ 15 cm) which had been equilibrated with 20 mM-Tris/HCl (pH 7.8). The loaded column was eluted with a Tris-buffered LiCl solution of linear concentration gradient from 0 to 0.4 M. Absorbance at 280 nm (—) and Na^+/K^+ -ATPase activity (O) were measured. Elution patterns of the light fraction of control muscles (a), and of insulin-exposed muscles (b) are shown. Peaks were named fraction I, II and III in ascending order of the elution volume.

supernatant after centrifugation at 148000 g_{max} for 60 min, it would still contain a trace amount of plasma membrane or Golgi membrane fragments. To determine the extent of contamination with these membrane fragments, the activities of 5'-nucleotidase (a marker enzyme for the plasma membrane) and of galactosyltransferase (a marker enzyme for Golgi membranes) were investigated. The activities of these marker enzymes are summarized in Table 2. The specific activities of 5'-nucleotidase in the light fractions were negligible compared with those in the plasma membrane fraction. The activities of galactosyltransferase in the light fractions were also very low. From these findings, it is apparent that in these light fractions there is no significant contamination with plasma or Golgi membrane fragments. Hereafter, the light fractions will be referred to as intracellular fractions.

Exposing muscles to insulin causes a decrease in both Na^+/K^+ -ATPase activity and ouabain-binding capacity in fraction II

To characterize the Na^+/K^+ -ATPase found in the intracellular fraction, the effect of saponin on the enzymic activity and its sensitivity to ouabain were examined (Table 3). The direct addition of 1 mM-ouabain to the assay solution inhibited the Na^+/K^+ -ATPase activity in fraction II by about 75 %, whereas ouabain at the same concentration almost completely suppressed activity in saponin-treated fraction II. Furthermore, it was observed that saponin treatment increased the Na^+/K^+ -ATPase activity of fraction II by 1.4–1.55-fold in the absence of ouabain.

Fig. 3. Time course of $[^3\text{H}]$ ouabain binding and suppression by unlabelled ouabain in fraction II

Fraction II was prepared and defined as described in the legend to Fig. 2. A portion of fraction II was used in the $[^3\text{H}]$ ouabain binding assay. (a) Time course of $[^3\text{H}]$ ouabain binding. Specific $[^3\text{H}]$ ouabain binding was calculated by the difference between the values in the absence and presence of 0.5 mM unlabelled ouabain. (b) Inhibition of $[^3\text{H}]$ ouabain binding by various concentrations of unlabelled ouabain. $\blacktriangle$, Fraction II prepared from insulin-exposed muscles; $\triangle$, control fraction II. Vertical bars indicate S.E.M. values for four experiments in (a) and for six experiments in (b).

These observations suggest that about 65–75 % of the enzyme molecules responsible for the observed activity in fraction II may be located in leaky vesicles or membrane fragments, whereas

Table 3. Effect of saponin on Na⁺/K⁺-ATPase activity and ouabain binding in fraction II

Na⁺/K⁺-dependent ATPase activity was calculated from the difference in ATPase activity in the presence and absence of 100 mM-NaCl, and ouabain-sensitive ATPase was calculated from the difference in ATPase activity in the presence and absence of 1 mM-ouabain. Saponin treatment was carried out as described in the legend to Fig. 1. Ins indicates fraction II prepared from insulin-exposed muscles; Con indicates fraction II from control muscles. Data are shown as means $\pm$ S.E.M. for four experiments.

Fraction		ATPase activity (μ mol of P _i /h per mg of protein)		Ouabain binding (pmol of [³ H]ouabain/h per mg of protein)
		Na ⁺ /K-dependent	Ouabain-sensitive	
Non-treated fraction II	Ins	2.28 $\pm$ 0.30	1.70 $\pm$ 0.10	8.09 $\pm$ 1.08
	Con	4.01 $\pm$ 0.63	3.02 $\pm$ 0.61	19.06 $\pm$ 3.07
Saponin-treated fraction II	Ins	3.15 $\pm$ 0.75	3.08 $\pm$ 0.75	10.86 $\pm$ 2.40
	Con	6.23 $\pm$ 1.33	5.96 $\pm$ 1.39	22.13 $\pm$ 4.40
Saponin-treated starting light fraction	Ins	1.05 $\pm$ 0.21		
	Con	1.57 $\pm$ 0.22		
Saponin-treated plasma membrane	Ins	32.57 $\pm$ 2.94		77.54 $\pm$ 10.25
	Con	16.68 $\pm$ 0.78		35.89 $\pm$ 3.90

25–35 % may be embedded in membranes forming tightly sealed small vesicles.

We conducted [³H]ouabain binding experiments with fraction II by a procedure similar to that used for the plasma membrane fraction (see the Experimental section). [³H]Ouabain binding in fraction II reached an apparent steady state after 40 min of incubation (Fig. 3a). Bound [³H]ouabain in fraction II prepared from insulin-exposed muscles were lower than that in the same fraction from the control muscles throughout the incubation period. Unlabelled ouabain suppressed [³H]ouabain binding (Fig. 3b). The IC₅₀ for unlabelled ouabain was 0.1 μ M. Treating fraction II with saponin also increased ouabain binding as well as increasing Na⁺/K⁺-ATPase activity (Table 3). The amount of bound ouabain in fraction II prepared from 1 g of muscle was calculated from the values of specific ouabain binding and protein yield. Similarly, the total activity of Na⁺/K⁺-ATPase in fraction II from 1 g of muscle was calculated. These values are listed, together with the values in the plasma membranes from 1 g of muscle, in Table 4. The Na⁺/K⁺-ATPase activity/ouabain binding ratio was around 4.4 $\times$ 10⁵ mol of P_i/h per mol of [³H]ouabain for the plasma membrane fraction, and 3 $\times$ 10⁵ mol of P_i/h per mol of [³H]ouabain for fraction II. There was no significant difference between these ratios in insulin-exposed and control muscles.

Table 4. Total Na⁺/K⁺-ATPase activity and ouabain-binding in fraction II and the plasma membrane fraction

Total activities were calculated from values of protein yield (Tables 1 and 2) and specific activities in saponin-treated preparations (Table 3). For the calculation of the total activities in the plasma membrane, the recovery of the plasma membranes into the plasma membrane fraction (4.5 %) was used (as described in the text). Ins indicates the preparation derived from insulin-exposed muscles; Con indicates the preparation from control muscles.

Preparation		Total activity	
		Na ⁺ /K ⁺ -ATPase (μ mol of P _i /h per g of muscle)	Ouabain binding (pmol of [³ H]ouabain/g of muscle)
Fraction II	Ins	11 $\pm$ 4	37 $\pm$ 3
	Con	23 $\pm$ 5	82 $\pm$ 9
Plasma membrane	Ins	46 $\pm$ 9	110 $\pm$ 9
	Con	21 $\pm$ 3	46 $\pm$ 6
Starting light fraction	Ins	12 $\pm$ 3	
	Con	15 $\pm$ 3	

Table 5. Lipid composition of intracellular and plasma membrane fractions

Lipid compositions of fraction II and plasma membrane fraction are shown. Light fractions were concentrated before extraction. SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; Tot., total phospholipid. Con indicates fraction prepared from control muscles; Ins indicates the fractions from insulin-exposed muscles.

Fraction		Phospholipid (nmol of P _i /mg of protein)						Cholesterol (μ g/mg of protein)	Cholesterol/ phospholipid (molecular ratio)
		SM	PC	PI	PS	PE	Tot.		
Fraction II	Ins	0.05 $\pm$ 0.02	0.43 $\pm$ 0.06	0.03 $\pm$ 0.01	0.06 $\pm$ 0.01	0.16 $\pm$ 0.01	0.97 $\pm$ 0.01	0.05 $\pm$ 0.01	0.10
	Con	0.07 $\pm$ 0.01	0.41 $\pm$ 0.02	0.04 $\pm$ 0.01	0.07 $\pm$ 0.01	0.17 $\pm$ 0.01	1.01 $\pm$ 0.08	0.06 $\pm$ 0.01	0.12
Starting light fraction	Ins	0.03 $\pm$ 0.01	0.15 $\pm$ 0.01	0.01 $\pm$ 0.01	0.02 $\pm$ 0.01	0.07 $\pm$ 0.01	0.35 $\pm$ 0.04	0.02 $\pm$ 0.01	0.11
	Con	0.04 $\pm$ 0.01	0.17 $\pm$ 0.03	0.02 $\pm$ 0.01	0.03 $\pm$ 0.01	0.08 $\pm$ 0.02	0.40 $\pm$ 0.04	0.02 $\pm$ 0.01	0.10
Plasma membrane	Ins	6.57 $\pm$ 0.37	123.0 $\pm$ 26.4	3.89 $\pm$ 0.62	3.93 $\pm$ 0.45	64.18 $\pm$ 19.83	252.0 $\pm$ 19.5	71.1 $\pm$ 3.4	0.73
	Con	7.11 $\pm$ 0.82	134.8 $\pm$ 33.7	3.95 $\pm$ 0.67	2.92 $\pm$ 0.95	64.64 $\pm$ 16.5	259.3 $\pm$ 14.9	76.4 $\pm$ 8.0	0.76

To obtain further information on the membrane component present in fraction II, we examined the lipid content of the fraction. For comparison, the plasma membrane fraction and the starting light fraction also were analysed (Table 5). The components in fraction II were concentrated by using an ultrafiltration apparatus. The phospholipid content in fraction II was 1.0 ± 0.08 nmol of P_i /mg of protein; this was very low compared with that in the plasma membrane preparation, reflecting that fraction II contained a large quantity of soluble proteins. The cholesterol/phospholipid ratio in fraction II was also much lower than that in the plasma membrane fraction. This low ratio further indicated that the membrane component in fraction II is not due to contamination by plasma membrane fragments.

These experiments showed the presence of Na^+/K^+ -ATPase activity and ouabain-binding capacity in the intracellular fraction which contained phospholipids. Taking the stimulatory effect of saponin on both the enzymic activity and the ouabain-binding capacity into consideration, it may be said that the Na^+/K^+ -ATPase molecules are embedded in a membranous structure of intracellular organelles. The increase in Na^+/K^+ transport across the plasma membrane observed when muscles were exposed to insulin may be accounted for by the translocation of Na^+/K^+ -ATPase molecules from their intracellular stores to the plasma membrane.

DISCUSSION

Insulin is known to stimulate Na^+/K^+ transport in various tissue cells [3,4,5–13,21–24]. However, the mechanisms by which insulin acts upon Na^+/K^+ transport systems have not been established. A number of hypotheses have been reported, such as (1) insulin elevates the affinity of Na^+/K^+ pumps to Na^+ in muscles [5,8,9]; (2) insulin stimulates the unmasking of inactive Na^+/K^+ pump sites in muscles [7]; and (3) insulin stimulates amiloride-sensitive Na^+/H^+ exchange and the increased internal Na^+ concentration activates Na^+/K^+ pumps in cultured myocytes [10] and hepatocytes [22]. The hypothesis that insulin exerts its effect through a rise in the internal Na^+ concentration may be ruled out, since the intracellular Na^+ concentration of skeletal muscle is known to shift to a lower level when the muscle is incubated with insulin [5], and the stimulatory effect of insulin on Na^+/K^+ transport is not suppressed by amiloride in intact rat skeletal muscles [13]. In our previous studies [11,12], we have reported that the addition of insulin to whole muscles increases the specific enzymic activity of Na^+/K^+ -ATPase in the plasma membrane fraction, but this is not due to the stimulation of protein synthesis *de novo*. In contrast with these observations, Resh [23] has reported that there is no increase in the number of phosphorylated intermediates of Na^+/K^+ -ATPase in rat adipocytes exposed to insulin. This inconsistency may be due to the fact that insulin does not stimulate the Na^+/K^+ transport in adipocytes as much as it does in skeletal muscles [24]. The translocation hypothesis may be supported by the following findings. Insulin can enhance ^{22}Na efflux from frog sartorius muscle even after the complete suppression of Na^+/K^+ transport activity by ouabain [7,9]. Furthermore, the Na^+/K^+ -ATPase activity in the plasma membrane fraction prepared from insulin-exposed muscles is higher than that in the preparation from control muscles [11,12]. These findings, however, suggest only an increase in the number of active pump sites in the plasma membrane. The purpose of the present study was to determine whether insulin causes the generation of some putative regulatory substances capable of unmasking latent Na^+/K^+ pumps in the plasma membrane, or whether the hormone stimulates the

translocation of internally stored Na^+/K^+ pumps to the plasma membrane.

To confirm further the increase in active Na^+/K^+ pumps in the plasma membrane, we investigated [3H]ouabain binding in isolated preparations. These experiments showed a decisive increase in ouabain-binding sites in the plasma membrane preparation when muscles were exposed to insulin (Fig. 1). As the next step, we searched for Na^+/K^+ -ATPase activity in an intracellular fraction (Table 1). The starting light fraction was fractionated using DEAE-Sepharose Fast Flow (an anion-exchanger), and Na^+/K^+ -ATPase activity was observed in the second peak (fraction II) eluted by a linear concentration gradient of LiCl (Fig. 2). The recoveries of phospholipid and of Na^+/K^+ -ATPase activity in fraction II from the starting light fraction of control muscles were 88% and 100% and those from insulin-exposed muscles were 85% and 92% respectively (Tables 2 and 5). These observations indicate that almost all of the intracellular Na^+/K^+ pumps and membrane lipid components are recovered in fraction II. The amount of Na^+/K^+ -ATPase activity in the plasma membranes from 1 g of control muscles was calculated from protein yield, specific enzymic activity and recovery of the plasma membranes into the plasma membrane fraction (Table 4). The ratio of the activity of Na^+/K^+ -ATPase in fraction II prepared from 1 g of muscles to that in the plasma membrane fraction prepared from the same weight of muscle was about 1.1 in non-insulin-exposed muscles. In a similar way, the ratio of ouabain binding in fraction II to that in the plasma membrane fraction was calculated to be 1.8. These values are compatible with the morphological estimation of the distribution of Na^+/K^+ -ATPase that has been reported by Wolitzky & Fambrough [25]. According to their report, the immunofluorescent localization of the intracellular sites of Na^+/K^+ -ATPase in cultured chick myogenic cells accounts for approx. 60% of the total Na^+/K^+ -ATPase of the cells. Calculations using the values listed in Table 4 showed that the increase in the activity of Na^+/K^+ -ATPase in the plasma membrane is 25 μ mol of P_i /h per g of muscle, whereas the decrease in the activity in fraction II is 12 μ mol of P_i /h per g of muscle on exposure to insulin. The increase in the enzymic activity in the plasma membrane was not always equal to the decrease in the activity in fraction II, but they were of the same order. Similarly, the increase in ouabain binding in the plasma membrane (64 pmol of [3H]ouabain) was not exactly equal to the decrease in ouabain binding in fraction II (45 pmol of [3H]ouabain). These inconsistencies may come from experimental errors. The ratio of Na^+/K^+ -ATPase activity to ouabain binding in fraction II was smaller than that in the plasma membrane fraction, suggesting that the kinetic properties of Na^+/K^+ pumps in intracellular stores are somewhat different from those of the pumps in the plasma membrane.

The present experiments have shown that almost all of the internal stores of Na^+/K^+ -ATPase seem to be recovered in fraction II, probably in the form of small and but partly sealed vesicles. It is widely accepted that insulin stimulates the translocation of glucose transporters from internal stores to the plasma membrane in adipocytes and muscles [26–31]. Furthermore, the existence of internal stores of Na^+ -selective channels [32] and H^+ -ATPase [33] has been shown. It has also been reported that Na^+/K^+ -ATPase activities in rat lacrimal gland are also found in the internal structures [34]. Besides these findings, insulin has been reported to be capable, in a similar manner, of causing the redistribution of receptors in fat cells for both insulin-like growth factor II [35,36] and transferrin [37]. More recently, Yang *et al.* [38] have demonstrated that protein kinase FA, a multifunctional protein phosphatase in the plasma membrane of human platelets, is activated by being transferred from the plasma membrane to internal sites on exposure of platelets to insulin, suggesting the

possibility that a promotion of the intracellular translocation of membrane proteins is a general feature of insulin action.

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