J. Membrane Biol. 136, 63–73 (1993)

The Journal of
Membrane
Biology
© Springer-Verlag New York Inc. 1993

Quantitation of Na^+/K^+-ATPase and Glucose Transporter Isoforms in Rat Adipocyte Plasma Membrane by Immunogold Labeling

Marianne Voldstedlund[1,2], Jørgen Tranum-Jensen,[2] Jørgen Vinten[1]
[1]Department of Medical Physiology, The Panum Institute, University of Copenhagen, Denmark
[2]Department of Medical Anatomy, The Panum Institute, University of Copenhagen, Denmark

Received: 8 February 1993/Revised: 4 June 1993

Abstract. We have quantitated and studied the topology of isoforms of the Na^+/K^+-ATPase and of the glucose transporter in rat adipocyte plasma membranes.

Adipocytes were incubated with or without insulin for 15 min. Sheets of native plasma membrane, with the cytoplasmic face exposed, were prepared by adsorption to EM grids. Grids were incubated in parallel with monoclonal antibodies against the Na^+/K^+-ATPase isoforms $\alpha1$ and $\alpha2$, and the glucose transporter isoforms GLUT1 and GLUT4, followed by immunogold labeling, negative staining and quantitation by counting of the gold particles in electron micrographs. In addition, the distribution of glucose transporters and Na^+/K^+-ATPase isoforms in subcellular membrane fractions prepared by an established fractionation procedure was monitored by Western blotting.

We found that the Na^+/K^+-ATPases and the glucose transporters were confined to the planar part of the plasma membrane, without association to caveolar invaginations.

The vast majority of the Na^+/K^+-ATPase molecules in the adipocyte plasma membrane were of the $\alpha2$ isoform; GLUT4 was the dominating glucose transporter isoform.

The total number of Na^+/K^+-ATPase molecules labeled in the plasma membrane was 3.5×10^5 per cell, independent of insulin stimulation. Concomitantly, insulin increased GLUT4 labeling sevenfold to a value of 3.5×10^5 per cell.

Key words: Insulin — Immuno-electron microscopy — Caveolae — Na^+/K^+-ATPase — Glucose transporters

Correspondence to: M. Voldstedlund

Introduction

The active transport of Na and K ions across the plasma membrane of mammalian cells is mediated by the Na^+/K^+-ATPase enzyme complex (EC: 3.6.1.3). The smallest functional unit consists of an α, a β and perhaps a γ subunit (Sweadner, 1989; Nørby & Jensen, 1991). The α subunit exists in at least three forms with specific tissue distributions (Emanuel et al., 1987; Herrera et al., 1987; Sweadner, 1989; Lingrel et al., 1990). The $\alpha2$ isoform of the Na^+/K^+-ATPase is thought to be the insulin-sensitive form (Lytton, 1985; Lytton, Lin & Guidotti, 1985; Brodsky, 1990*a*; Hsu & Guidotti, 1991).

The expression of isoforms in different tissues is subject to specific regulation during development (Schmitt & McDonough, 1986; Emanuel et al., 1987; Lingrel et al., 1990). In this respect, the Na^+/K^+-ATPase shows similarities with the glucose transporter, which also exists in different isoforms, among which the GLUT1 is found in most tissues, whereas the GLUT4 is found in insulin-sensitive tissues only (Haney et al., 1991). Data on the expression of Na^+/K^+-ATPase isoforms in genuine fat cells during differentiation are not available, but studies on cation as well as glucose transport in 3T3-fibroblast-adipocyte models have shown the appearance of increased insulin responsiveness during differentiation. While development of insulin-sensitive glucose transport is accompanied by the expression of GLUT4, whether expression of $\alpha2$ is involved in the development of insulin-sensitive cation transport in these models is controversial (Resh, 1982*c*; Russo et al., 1990; Brodsky, 1990*b*; Robinson et al., 1992). The study of Robinson et al. (1992) used a planar membrane prepara-

tion of 3T3-L1 adipocytes adherent to glass, and included immunogold as well as immunofluorescence labeling to assess the number and the topology of GLUT4 and GLUT1; however, the study did not include quantitation of Na^+/K^+-ATPase, nor were isoforms distinguished.

Insulin-induced enhancement of Na^+/K^+ transport in adipocytes and muscle cells is a well-established phenomenon (Clausen & Hansen, 1977; Resh, Nemenoff & Guidotti, 1980; Resh, 1983; Lytton, 1985; Lytton et al., 1985). Likewise, insulin increases glucose transport markedly, and this increase is, at least in adipocytes, concomitant with translocation of the glucose transporter GLUT4 to the plasma membrane (Vinten, Gliemann & Østerlind, 1976; Cushman & Wardzala, 1980; Suzuki & Kono, 1980; James et al., 1988; Smith et al., 1991; Robinson et al., 1992). Although it is the prevailing view that the enhancement of Na^+/K^+ transport is due to an increased activity of the enzyme (*for reviews see e.g.* Resh, 1982*a,b*; Sweadner, 1989), a translocation of the Na^+/K^+-ATPase to the plasma membrane has also been claimed to contribute to this effect of insulin (Erlij & Grinstein, 1976; Omatsu-Kanbe & Kitasato, 1990; Hundal et al., 1992).

A variety of biochemical methods have been applied to estimate the number of Na^+/K^+-pumps in plasma membranes of insulin-responsive and of nonresponsive cells (Clausen & Hansen, 1974; Hootman & Ernst, 1988; Kashgarian & Biemesderfer, 1988; Resh, 1988; Skou, 1988). Commonly applied approaches are binding and activity studies which rely on the conservation of relevant steps in the functional cycle of the enzyme, whereas other rely on immunochemical quantitation of Na^+/K^+ pumps in subcellular fractions and indirect determination of plasma membrane recovery and purity.

To circumvent these difficulties, we have applied a new immuno-electron-microscopical method for quantitation of proteins in pure and structurally defined sheets of plasma membranes from adipocytes, previously described in preliminary reports (Tranum-Jensen, Vinten & Ploug, 1988; Vinten & Tranum-Jensen, 1991). In the present study, we have used this method to obtain a quantitative measure of the insulin effect on the density of the $\alpha 1$ and the $\alpha 2$ isoforms of the Na^+/K^+-ATPase molecule in rat adipocyte membranes. The results are compared to data on glucose transporters (GLUT1 and GLUT4) obtained analogously and in parallel. In addition, data were obtained on the lateral membrane topology of the Na^+/K^+-ATPase and the glucose transporters.

Materials and Methods

Preparation of Adipocytes

Sixteen 180 g male Wistar rats, fed *ad libitum*, were killed by decapitation, two on each of eight experimental days. Adipocytes were prepared as described in Vinten et al. (1976). In short, epididymal fat pads were quickly removed, weighed, and suspended in a 37°C Krebs-Ringer-HEPES buffer (KRH): Na^+, 126 mM; K^+, 6.0 mM; Mg^{2+}, 1.2 mM; Ca^{2+}, 2.5 mM; Cl^-, 128.5 mM; $H_2PO_4^-$, 1.2 mM; SO_4^{2-}, 1.2 mM; HEPES, 10 mM; glucose, 1 mM; pH 7.4 containing 3.5% albumin (Sigma A 4503) and 0.5 mg/ml collagenase (Sigma Type 1 C-0130). Collagenase digestion was carried out at 37°C for 45 min with gentle magnetic stirring. Cells were filtered through cotton gauze and washed five times with 4 ml KRH-buffer containing 1% albumin and 1 mM glucose, to remove debris and collagenase. Two 1-ml aliquots of the cell suspension, diluted to 4% vol/vol, were incubated for 15 min at 37°C with or without insulin (1.67×10^{-4} M, porcine insulin, Novo-Nordisk, Gentofte, DK).

Preparation of Membrane Sheets for EM

All steps were carried out at 4°C. Poly-L-lysine (P-1399 Sigma) coated EM grids (prepared as described below) were rehydrated by floating, with the coated film facing downwards, on the surface of a PBS-G buffer (saline buffered with 10 mM sodium-phosphate, pH 7.4, supplemented with 0.5 mM glucose) contained in a cylindrical 20 ml plastic beaker filled to a fluid level of 40 mm. At the end of the incubation period at 37°C, adipocytes were injected below the floating grids. The cells floated by buoyancy and adhered to the poly-lysine coated grids. The grids were then lifted off the water surface and flushed with a jet from a 20 ml syringe containing a buffer, TK (153 mM KCl; 1.92 mM Tris-HCL buffer, pH 7.4) delivered through an 18 G × 1.5 cannula. The attachment of the cells to the coated grid film was so tenacious that the cells were disrupted leaving a sheet of adsorbed plasma membrane with the cytoplasmic aspect exposed (Jacobson, Cronin & Branton, 1978). The grids were subsequently placed on drops of PBS-G lined up on a dental wax plate.

Immunogold Labeling

All steps were carried out at 20°C. To reduce unspecific binding, a series of grids was incubated for 15 min in TSA 4% (10 mM Tris base; 154 mM NaCl; 4% bovine albumin, pH 7.4). Individual grids were then incubated for 45 min, each with one of the four different primary antibodies listed below. The antibodies were monoclonal mouse IgG, contained in hybridoma culture supernatants, diluted with TSA 4% as described below. One grid in each series was incubated without a primary antibody to determine unspecific gold labeling. In a separate experiment, a mouse monoclonal IgG antibody against a parvo virus antigen was used as a nonsense antibody. The gold particle counts obtained with this antibody were not significantly higher than those obtained when a primary antibody was omitted.

After washing by passage over 5 drops of TSA 4%, all grids were incubated for 45 min on a drop of rabbit anti-mouse antibody, (DAKO, Z412), conjugated to 5 nm colloidal gold particles,

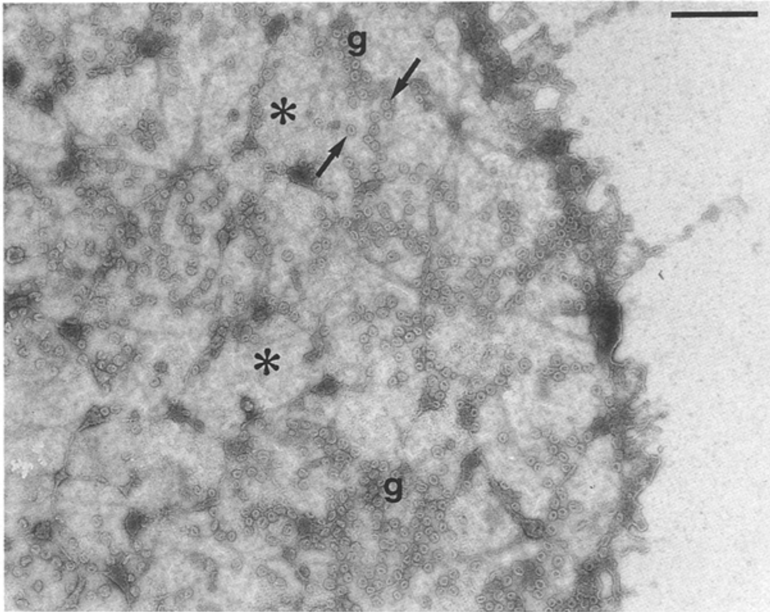

Fig. 1. Low magnification electron micrograph of an adipocyte plasma membrane sheet adsorbed to a poly-lysine-coated EM grid surface, fixed with glutaraldehyde and negatively stained from its cytosolic face. The torn edge of the sheet is seen to the right. The adipocyte membrane is furnished with numerous caveolae (arrows), most of which are grouped (*g*), spaced by larger areas of planar membrane (asterisks). Scale bar indicates 1 μm.

by the method of Slot and Geuze (1985) and diluted to an equivalent of 10–20 μg IgG/ml. Incubation was terminated by a final wash on 3 drops of TSA 4% and 2 drops of PBS-G. To avoid possible dissociation of the immunogold label during negative staining, specimens were fixed for 5 min in 1% glutaraldehyde (GA) before the negative staining procedure. Fixation prior to the antibody incubation was not possible, because the epitope of the α1 Na^+/K^+-ATPase was sensitive to GA (Felsenfeld & Sweadner, 1988). A survey micrograph of an unlabeled membrane sheet is shown in Fig. 1. All labeling procedures applied to membranes from insulin-stimulated and nonstimulated cells were carried out in parallel. The specimens were stained with 2% sodium silicotungstate (Tranum-Jensen, 1988).

Microscope Operation

Micrographs were taken with a JEOL 100CX Transmission Electron Microscope at 80 kV. Magnification was calibrated from micrographs of a Fullam SiO_2 grating replica (cat. no. 6002). Search for membrane sheets was performed at 1,600× magnification. Micrographs were taken at a primary linear magnification of 33,000×. On each grid, plasma membrane sheets of five different cells were photographed. The photographed area of each membrane sheet was randomly chosen, but was not allowed to include the edge of the sheet.

Quantitation of Immunogold Labeling

The total number of gold particles on each micrograph was counted, using a squared grid. Counts were expressed as the mean number of gold particles per μm^2 plasma membrane from five different cells. Each of the five micrographs were equivalent to a membrane area of 4.48 μm^2. Unspecific labeling was counted similarly on grids processed in parallel, omitting the primary antibody, and these "background counts" were subtracted from each of the "mean counts." When expressing the number of gold particles per μm^2 of plasma membrane, all countings were corrected for occasional preparatory or photographic imperfections that made counting uncertain. The ignored area of the micrographs was typically less than 1%.

Membrane Fractionation and Western Blotting

Adipocytes were prepared from twelve 220 g male Wistar rats. The cells were pooled, divided in two aliquots, and incubated with or without 1.67 μM of insulin for 15 min at 37°C. After washing, cells were homogenized and membrane fractions were prepared according to the procedure described by McKeel and Jarret (1970), with the modifications of Cushman and Wardzala (1980), and with the addition of protease inhibitors (N-α-p-tosyl-L-lysine chloro-methyl ketone (TLCK) 0.1 mM; L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK) 0.1 mM; phenyl-methyl-sulfonyl flouride (PMSF) 1 mM). Each of the membrane fractions, i.e. plasma membranes (PM), high- and low-density microsomes (HM and LM), and the mitochondria-rich fraction (Mi) was diluted to the same protein concentration, determined by the method of Lowry (1951) modified by Peterson (1977), and characterized by immunoblotting. Thirty μl sample buffer was added to the 90 μl sample (final concentrations: SDS, 2%; Pyronin G, 0.05%; glycerol, 12.5%; Tris, 12 mM; EDTA, 0.5 mM; dithiothreitol, 50 mM) and incubated for 15 min at 56°C. SDS-PAGE electrophoresis was performed according to Laemmli (1970) in a Biorad electrophoresis apparatus (Protean II), using a stacking gel of 5% and a separating gel of 7.5% acrylamide, and run overnight at 4 mA. Electrotransfer onto nitrocellulose paper and immunostaining was done according to O'Connor and Ashman (1982).

Antibodies

Mouse hybridoma culture supernatants, containing antibodies against the Na^+/K^+-ATPase $\alpha 1$ and $\alpha 2$ subunits (MCK1 and MCB2), were kindly donated by K. J. Sweadner. Detailed characterization of these antibodies is given in Felsenfeld and Sweadner (1988); Urayama and Sweadner (1988); Urayama, Shutt and Sweadner (1989). For immunogold labeling, the supernatants were diluted 1 : 20. For immunoblotting, the MCK1 and MCB2 were diluted to 1 : 40. The monoclonal antibody, F18 (Handberg et al., 1992) against the GLUT1, was used in a protein-A purified form in concentrations of 10 μg/ml for immunogold labeling and 5 μg/ml for immunoblotting. The GLUT4 was detected in immunogold labeling experiments with a monoclonal antibody, F27, directed against the C-terminal peptide described below. The F27 was prepared by Peer N. Jørgensen, Novo-Nordisk, Denmark, and used as a culture supernatant diluted 1 : 10. For immunoblots of the GLUT4 a polyclonal rabbit antiserum (Handberg et al., 1992) was used diluted 1 : 100. This antiserum, as well as the F27, were raised against a commercially synthesized peptide (Kem En Tech, Copenhagen) corresponding to the 14 C-terminal amino acid residues of GLUT4 (Birnbaum, 1989). For detection in immunoblots, an alkaline phosphatase conjugated secondary antibody from DAKO, Denmark (cat. no. D314 DAKO) was used. All antibodies were diluted in TSA-buffer containing 4% albumin for gold labeling experiments and 1% albumin for immunoblotting.

Determination of Fat Cell Size

Equal volumes of a 4% cell suspension in PBS and osmium tetroxide 4% were mixed. After 4 hr–3 days, cells were washed in distilled water and placed in a well on a microscope slide. Cell diameters were measured to the nearest mark of an ocular micrometer with scale divisions equivalent to 3.64 μm at 400× magnification. Cell surface areas were calculated on the assumption that the cells were spherical.

Preparation of Specimen Supports for EM Examination

Carbon-reinforced, thin Formvar films, carried on 400-mesh copper grids were prepared and exposed to plasma glow (Tranum-Jensen, 1988). Following the plasma-glow treatment, a drop of poly-L-lysine (1 mg/ml) in distilled water was placed on the carbon surface, followed by a wash with 10 drops of distilled water. Grids were allowed to dry and could be stored for at least some days without loss of adsorptive properties.

Results

Glucose Transporters

To ascertain that membranes from cells incubated with insulin were in a stimulated state, we used membranes prepared from the same cell pools as those for Na^+/K^+-ATPase labeling to label for the glucose transporter isoforms GLUT4 and GLUT1 with a procedure analogous to that used for labeling of the Na^+/K^+-ATPase. Figure 2 depicts membranes labeled for GLUT4.

In the nonstimulated state (Fig. 2*A*), labeling was scarce overall, but occasional clusters of gold particles were found, centered on spherical or sack-like structures different from the numerous caveolar invaginations. No such clusters were found in the insulin-stimulated state (Fig. 2*B*). Coated pits were rarely seen and did not carry the GLUT4 label. No systematic association of GLUT4 labeling with caveolae was found. The mean-fold increase in gold particle number (including labeling on the sack-like structures) between plasma membranes from non-stimulated and insulin-stimulated adipocytes was about seven (*see* Table 1). Labeling of GLUT1 was much less dense and was significantly different from background only in membranes from insulin-stimulated cells. The insulin-induced increase in GLUT1 labeling was not statistically significant.

Western Blotting

The specificity of the antibodies was assessed by Western blotting of subcellular fractions of adipocyte membranes, prepared by a technique established for the detection of translocation of glucose transporters (Cushman & Wardzala, 1980). Results are shown in Fig. 3. As expected, there was an increase in staining representing GLUT4 in the PM fraction after insulin stimulation of the adipocytes, concomitant with a decrease in the LM fraction. Bands representing the Na^+/K^+-ATPase $\alpha 1$ and the $\alpha 2$ subunit isoforms appeared at a M_r corresponding to about 115 kD. The resolution of the gels did not allow us to observe a reported difference in mobility of the isoforms (Lytton et al., 1985; Sweadner, 1989). The lack of minor bands in the $\alpha 2$, GLUT1 and GLUT4 blots confirmed the high specificity of the antibodies used. We consider the weak band at a position corresponding to 65 kD, observed with the $\alpha 1$ antibody (MCK1) to represent a degradation product, because the intensity of the band varied by addition of different protease inhibitors without which the 115 kD band often disappears entirely. Although both isoforms dominated in the PM fraction, they were detected in the other fractions as well. The intensity of the bands representing $\alpha 1$ and $\alpha 2$ was not influenced by insulin stimulation.

Na^+/K^+-ATPase Immunogold Labeling

Representative micrographs of plasma membranes labeled by the immunogold method for the $\alpha 2$ subunit isoform of the Na^+/K^+-ATPase are shown in

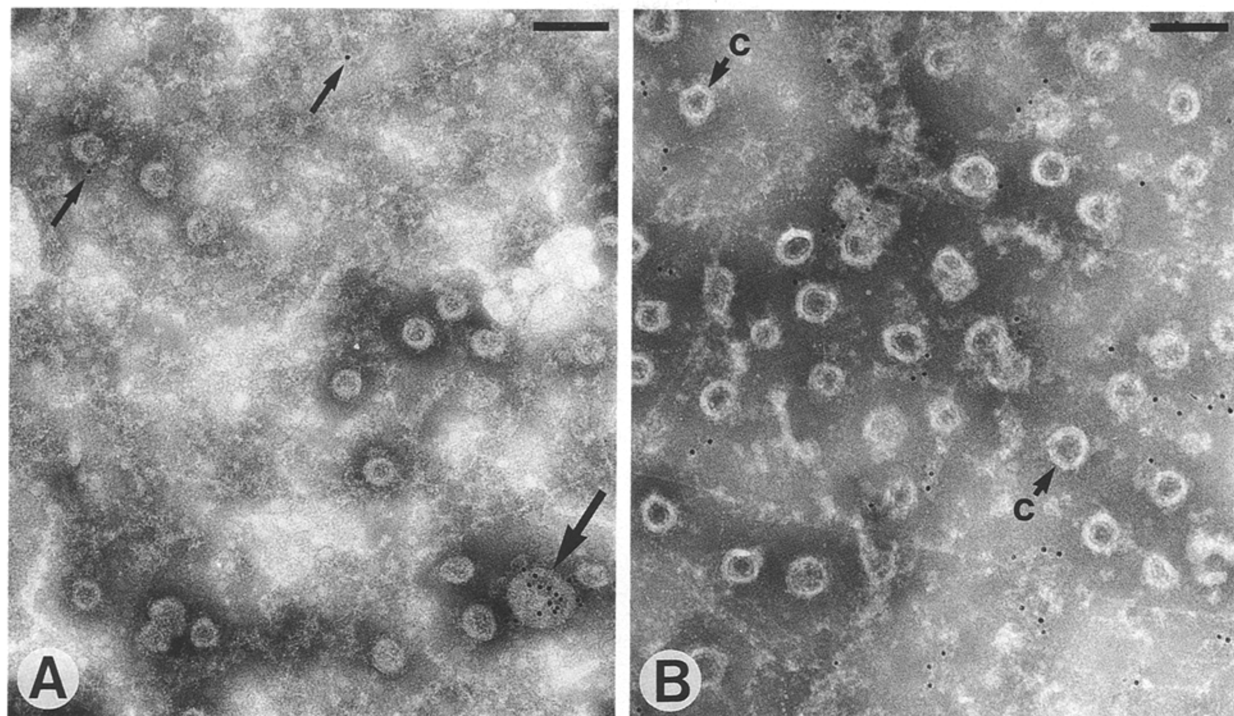

Fig. 2. Electron micrographs of negatively stained sheets of adipocyte plasma membrane, immunogold-labeled from the cytosolic face for GLUT4, without (*A*) and following (*B*) insulin stimulation. In the nonstimulated state (*A*) labeling is scarce over the membrane (small arrows), but high density labeling is found on spherical or sack-like structures associated with the membrane (large arrow). In the stimulated cell (*B*) the density of labeling is markedly increased and appears randomly scattered over the planar areas of the membrane without association to caveolae (*c*). Scale bars indicate 100 nm.

Fig. 4. The membrane sheets were prepared from nonstimulated (*A*) and insulin-stimulated (*B*) adipocytes. In both situations, gold particles were scattered over the planar part of the membranes and not found on the numerous caveolar invaginations.

All of the 75 membrane sheets labeled for the $\alpha 2$ isoform carried label—none of them conspicuously little. Within the individual sheets, the distribution of $\alpha 2$ counts per 2.24 μm^2 (half the full counting frame) was not different from a Poisson distribution as evaluated by a Chi-square test. The coefficient of variation of the $\alpha 2$ counts per 4.48 μm^2 between sheets prepared from the same cell pool was 16% (average of 15 determinations). The variation in gold particle counts between cell pools was not correlated to the (modest) variation in mean adipocyte size (*cf.* Table 1).

Insulin stimulation of the adipocytes prior to membrane preparation had no statistically significant effect on $\alpha 2$ counts ($P = 0.96$, Table 1), nor on the labeling pattern within the membrane sheets.

Immunogold labeling of the $\alpha 1$ isoform was also confined to the planar part of the plasma membrane, but was far less abundant than that of the $\alpha 2$ isoform. The labeling tended to increase upon insulin stimulation, but the increase was not statistically significant ($P = 0.21$, Table 1).

Since insulin had no statistically significant effect on either $\alpha 1$ or $\alpha 2$ labeling, counts were pooled ignoring insulin addition and mean values for each of the two isoforms were calculated. The mean of gold particle counts for $\alpha 2$ labeling was 30 ± 5 (SE) per μm^2 plasma membrane; for $\alpha 1$ the number was 1.5 ± 0.5 (SE) per μm^2. The $\alpha 1$ labeling, although low, was statistically different from zero ($P < 0.01$).

In Figure 5, the results are expressed on a per cell basis by multiplication with the mean cell surfaces in the individual cell pools, determined as described in Materials and Methods, to facilitate comparison with published values.

Discussion

The present results of GLUT4 labeling in plasma membrane sheets showed that this transporter was located to the planar part of the membrane. The occasional, heavily labeled sack-like structures found in nonstimulated membranes could represent, according to the generally accepted translocation

Table 1. Average density of gold particles labeling the indicated membrane proteins, expressed per μm^2 of adipocyte plasma membrane[a]

Insulin	GLUT1 (−)	GLUT1 (+)	GLUT4 (−)	GLUT4 (+)	$\alpha 1$ (−)	$\alpha 1$ (+)	$\alpha 2$ (−)	$\alpha 2$ (+)	Cell size[c] (10^4 μm^2)
Exp. 1			13.10	40.50	1.60	0.70	27.90	52.10	0.8539
Exp. 2			5.60	24.70	0.80	0.00*	33.80	19.00	0.8218
Exp. 3	0.00[b]	2.20	3.50	26.50	1.10	1.60	27.70	24.40	1.2610
Exp. 4			0.00[b]	34.60	2.80	3.80	41.70	26.10	
Exp. 5	0.60	2.80	2.70		0.10	1.40		44.00	1.4463
Exp. 6	3.00	2.20	4.40	9.90	0.00[b]	1.70	21.00	26.50	1.5048
Exp. 7	1.00	1.70	3.60	43.00	0.50	2.10	39.20	22.70	1.3920
Exp. 8			2.02	28.80	1.60	2.70	17.30	23.80	
Mean	1.15	2.23[d]	4.39[d]	29.71[d]	1.06[d]	1.75[d]	29.80[d]	29.83[d]	1.2133
±SE	0.65	0.23	1.38	4.21	0.33	0.41	3.41	4.13	0.1233

[a] Each value is the mean number of gold particles per μm^2 from five different membrane sheets. Unspecific labeling (2.93 ± 0.47, mean ± SE) was determined in each experiment by omission of the primary antibody, and subtracted from the counts obtained with the specific antibodies. +/− indicate the presence *vs.* absence of insulin in the incubation medium prior to membrane isolation.

[b] Zero or a small negative value.

[c] Average cell surface areas expressed in units of 10^4 μm^2. In each experiment, the mean diameter of 100 adipocytes was determined microscopically (with a coefficient of variation of 28%), and used to calculate gold labeling density per cell in each experiment, assuming cells to be spherical.

[d] Values significantly different from background labeling ($P < 0.05$). Abbrevations used: $\alpha 1$, kidney Na/K-ATPase subunit isoform; $\alpha 2$, brain axolemma Na/K-ATPase subunit isoform; GLUT1, erythroid glucose transporter isoform; GLUT4, insulin-regulatable glucose transporter isoform.

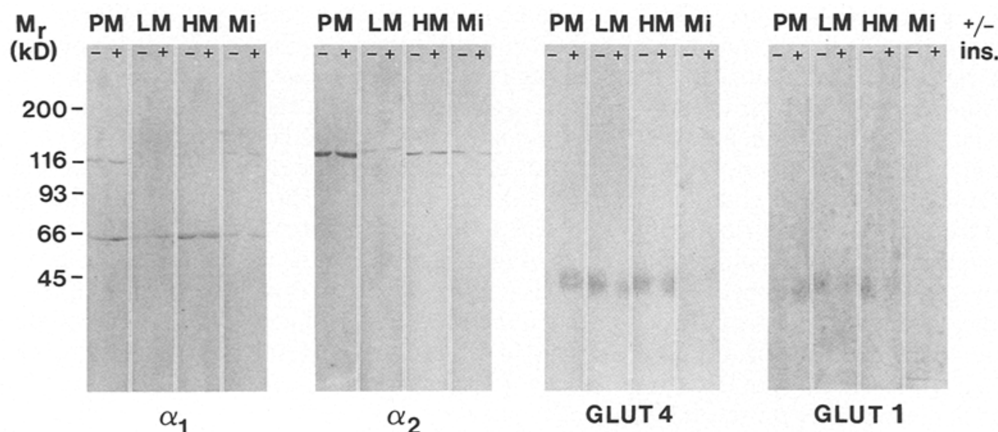

Fig. 3. Western blots of the four subcellular membrane fractions (*PM:* plasma membrane; *LM:* low density microsomes; *HM:* high density microsomes; *Mi:* mitochondria-rich fraction), prepared from rat adipocytes before and after prior stimulation with insulin, indicated by +/− at the top of each pair of lanes. The blots were incubated with antibodies as described in Materials and Methods to detect the membrane proteins indicated below each set of membrane fractions. The positions of molecular weight markers are shown to the left. Note that the translocation of GLUT4 from the LM to the PM fraction in response to insulin stimulation is clearly demonstrated.

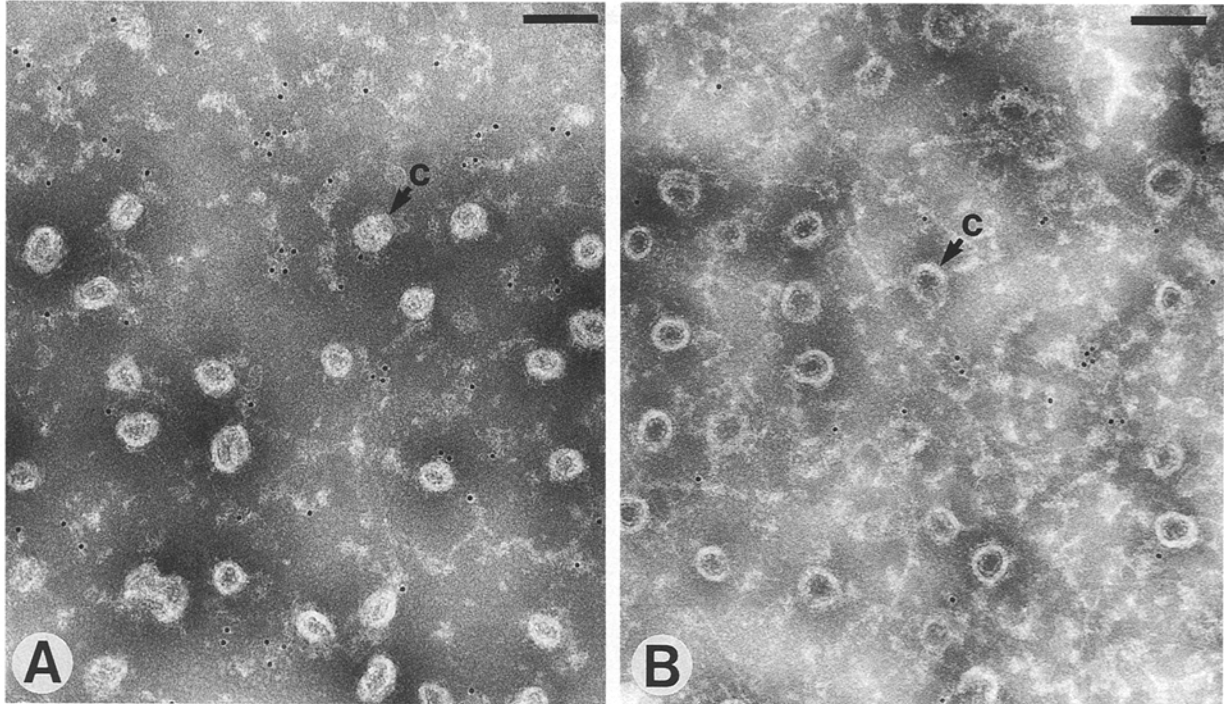

Fig. 4. Electron micrographs of negatively stained sheets of adipocyte plasma membrane labeled from the cytosolic face for the α_2 isoform of the Na^+/K^+-ATPase, before (*A*) and after (*B*) insulin stimulation. In both cases, the gold label is scattered over the planar areas of the membrane without association to caveolae (*c*). Scale bars indicate 100 nm.

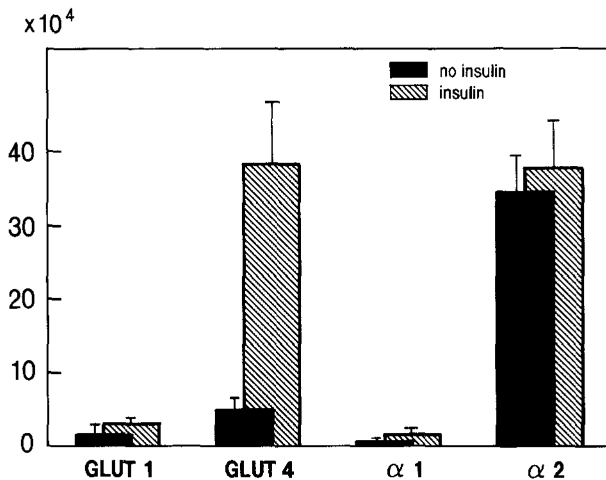

Fig. 5. Columns express numbers of gold particles *per cell*. Bars indicate SE. Hatched columns represent experiments where insulin was added to a final concentration of 1.67 μM in the incubation medium of the cells prior to the preparation of membrane sheets. Abbreviations as in the legend to Table 1.

hypothesis for insulin stimulation of glucose transport (Cushman & Wardzala, 1980), transporter depot vesicles in the process of fusion with or sequestration from the plasma membrane. Stimulation of adipocytes has been reported to increase the rate of mobilization of transporters to the plasma membrane rather than to decrease internalization (Yang et al., 1992). It is therefore tempting to suggest that the occurrence of the heavily labeled structures in noninsulin stimulated membranes results from an abortive stimulation of the translocation mechanism elicited by contact with the grid surface, being only

rudimentarily effective at the low temperature. An earlier immunogold labeling study based on resin-embedded sections of rat adipocytes also pointed to the existence of subplasmalemmal membrane structures rich in GLUT4 that decreased concomitant with an increased plasmalemmal labeling in response to insulin stimulation (Smith et al., 1991). In a recent study of the insulin effect on 3T3-L1 adipocytes (Robinson et al., 1992), gold labeling of GLUT4 was examined in that part of the plasma membrane which had become adherent to the support of the cells during growth. The authors reported that a substantial fraction of the GLUT4 label was associated with coated pits, and that only the GLUT4 label found in the planar part of the membrane increased in proportion to the glucose transport. With respect to the transporters in the planar part of the membrane, these findings agree with the present ones, where the total GLUT4 labeling was increased about sevenfold. However, GLUT4 labeling associated with coated pits was not observed in our preparation. This latter discrepancy is perhaps best explained as a characteristic difference in the properties of the cells in question.

The ultrastructural localization in adipocytes of the Na^+/K^+-ATPase isoforms, to our knowledge, has not been studied previously. Our results show that both the $\alpha 1$ and the $\alpha 2$ Na^+/K^+-ATPase isoforms are randomly distributed in the plasma membrane of adipocytes. No blank membrane sheets, nor sheets with conspicuously low labeling, were found with either of the antibodies, and the distribution of the gold particle densities within individual sheets, as well as between sheets from the same cell pool, was compatible with a Poisson distribution. This excludes the theoretical possibility of the existence of larger (>5 μm^2) plasma membrane domains lacking pumps, as well as subpopulations of adipocytes with aberrant expression of Na^+/K^+-ATPase isoforms (Lytton et al., 1985).

The consistent observation that the caveolar invaginations were devoid of Na^+/K^+-ATPase labeling demonstrates indirectly that this membrane structure is indeed specialized (Anderson et al., 1992), since one protein, otherwise typical of the plasma membrane, is absent from caveolae. This finding (in adipocyte membranes) is at variance with a cytochemical study (Ogawa, Fujimoto & Ogawa, 1986) of ouabain-inhibitable, K^+-activated-phosphatase activity in vascular endothelium and smooth muscle cell plasma membrane, where the cytochemical reaction product was found to be associated with caveolae. It cannot presently be settled whether this discrepancy is due to differences between cell types or whether it is of technical origin (Mayahara & Ogawa, 1988).

It is generally held that immunogold labeling is rather inefficient, i.e., that only a small fraction of the molecules of a particular species are labeled due to inaccessibility and/or to destruction of the epitopes by fixatives (*see e.g.*, Horisberger, 1981; Slot et al., 1989). In the present preparation, classical fixatives were not applied until after the immunogold labeling had been completed, and accessibility is expected to be favorable for labeling of the membrane proteins in question because the epitopes are naturally exposed on the surface of the specimen. Amplification due to multiple binding of secondary antibodies would show up as clusters of closely spaced gold particles. Such clusters could also result from primary clustering of epitopes in oligomers, as suggested for the Na^+/K^+-ATPase (Liang & Winter, 1977; Craig, 1988; Jensen & Nørby, 1988; Buxbaum & Schoner, 1991). Our results do not permit us to distinguish between these two sources of pairs and small clusters of gold particles in the specimens.

The ratio between labeling of the $\alpha 1$ and the $\alpha 2$ isoforms in plasma membrane sheets was about 1 : 20, consistent with the much heavier staining for $\alpha 2$ than for $\alpha 1$ found in immunoblots of the plasma membrane enriched subcellular fraction (*cf.* Fig. 3). Earlier estimates indicate a somewhat lower ratio of $\alpha 2$ to $\alpha 1$. Thus, exploiting the difference in ouabain affinity of the isoforms, it has been estimated from the kinetics of ouabain-stabilized "back-door" phosphorylation (Lytton et al., 1985) that about 25% of the maximum P_i incorporation in Na^+/K^+-ATPase in an adipocyte plasma membrane fraction occurs in the $\alpha 1$ isoform. It is not obvious which of these two estimates is the more reliable.

In Table 2 the number of Na^+/K^+-ATPase molecules per adipocyte, reported earlier in the literature, are given along with the present results. Despite the use of very different methods, these earlier estimates are seen to agree very well, being within a range from 6×10^5 to 2×10^6 per cell. The present estimate of 3.5×10^5 per cell, obtained by immunogold labeling, is thus between 20 and 60% of the previously published figures, and may be taken as an estimate of the method's labeling efficiency.

Insulin has been found to increase the pumping of Na^+/K^+ in skeletal muscle (*see e.g.*, Clausen, 1986, for review), brain synaptosomes (Brodsky, 1990*b*), adipocytes (Resh et al., 1980; Lytton, 1985; Lytton et al., 1985; McGill, 1991; McGill & Guidotti, 1991) and 3T3-L1 adipocytes (Russo et al., 1990).

In adipocytes, Na^+/K^+ pumping in the basal state has been reported to be shared among the $\alpha 1$ and $\alpha 2$ isoforms in the proportion of 2 : 1–4 : 1 (Lytton et al., 1985). By insulin stimulation, the pumping, measured as Rb^+ uptake, increased by 60–75%, and this increase was ascribed to an increase of the $\alpha 2$

Table 2. The number of Na/K-ATPase molecules in rat adipocyte plasma membranes expressed per cell as estimated in this study, compared to estimates given in the literature

Preparation	Technique	Results	Reference
Intact cell	Immunogold	3.5×10^5	(This study)
Intact cell	Ouabain bind.	$7.9 \times 10^{5*}$	(Clausen & Hansen, 1974)
Intact cell	Ouabain bind.	2.0×10^6	(Resh et al., 1980)
Pl. membranes	Ouabain bind.	9.0×10^5	(Resh, 1982*c*)
Pl. membranes	Back-door-P	6.0×10^5	(Resh, 1982*c*)
Pl. membranes	Back-door-P	1.0×10^6	(Lytton et al., 1985)

Abbreviations used: Pl. membranes: experiments based on subcellular fractions enriched in plasma membranes. Immunogold: the electron-microscopic immuno-gold method of the present study. Ouabain bind.: Determination of specific ouabain-binding sites. Back-door-P: ouabain-stabilized back-door phosphorylation.
* The number was calculated for the size of adipocytes used in the present study from the value given in the reference.

activity, to a level where the total activity was shared among the two isoforms in a 1 : 1 ratio (McGill, 1991; McGill & Guidotti, 1991). The present study shows no statistically significant insulin-induced increase in either $\alpha1$ or $\alpha2$ labeling in the plasma membrane, although the actual increase of $\alpha1$ was of a magnitude comparable to that found for the "constitutive" glucose transporter GLUT1. This supports the view that insulin acts by increasing the intrinsic activity of Na^+/K^+-ATPase molecules, without changing the actual number of pumps present in the plasma membrane (Clausen & Hansen, 1977; Resh et al., 1980; Resh, 1982*c*; Lytton, 1985).

In rat skeletal muscle this view has recently been challenged by Hundal et al. (1992), who reported translocation to the plasma membrane of the $\alpha2$ isoform based on Western blot analysis of subcellular membrane fractions. Also in frog skeletal muscle, an insulin-induced translocation of ouabain-binding sites to a plasma membrane enriched subcellular fraction has been reported (Omatsu-Kanbe & Kitasato, 1990). The discrepancy between the report of Hundal et al. (1992) and the present findings, along with the findings of Clausen (1986), might derive from two different tissues being compared (muscle contra fat). A recent study of insulin-responsive cultured 3T3-L1 adipocytes (Robinson et al., 1992) using immuno-labeled membrane sheets (called "lawns") attached to coverslips, and thus comparable to the present method, reports that insulin did not change the content of Na^+/K^+-ATPase in the plasma membrane lawns.

In addition, our immunoblot analysis on subcellular membrane fractions of fat cells did not indicate translocation phenomena relating to the $\alpha1$ or the $\alpha2$ isoform.

In the work of Hundal et al.(1992) mentioned above, the $\alpha1$ isoform was barely detectable in the internal membrane fractions and no increase in staining of the plasma membrane enriched fractions was seen. This is partly in contrast to our Western blot study, in which we were able to detect $\alpha1$ in the internal membrane fraction called heavy microsomes, but the result agrees with the absence of translocation.

Our immunogold labeling method circumvents some of the inherent limitations in traditional methods of membrane preparation. The origin and composition of our adsorbed membrane sheets are defined morphologically, and we regard the sheets as a pure preparation of plasma membrane, with a distribution of proteins and membrane structures close to that in native cells.

The authors wish to thank Ulla Blankensteiner, Jonna Harpøth and Lisette Hansen for their skillful technical assistance and Birgit Risto for excellent work with the photographic prints. The $\alpha1$ and $\alpha2$ antibodies were kindly donated by K.W. Sweadner, Boston, and the F18 and F27 antibodies were granted by Peer N. Jørgensen, Novo-Nordisk, Bagsværd, Denmark. This work was supported by The NOVO Foundation, The Nordisk Insulin Foundation, The Danish Diabetes Foundation, The P. Carl Petersen Foundation and the Foundation of 17-12-81.

References

Anderson, R.G.W., Kamen, B.A., Rothberg, K.G., Lacey, S.W. 1992. Potocytosis: sequestration and transport of small molecules by caveolae. *Science* **255:**410–413

Birnbaum, M.J. 1989. Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell* **57:**305–315

Brodsky, J.L. 1990*a*. Characterization of the (Na^++K^+)-ATPase from 3T3-F442A fibroblasts and adipocytes. *J. Biol. Chem.* **265:**10458–10465

Brodsky, J.L. 1990b. Insulin activation of brain Na-K-ATPase is mediated by $\alpha2$-form enzyme. *Am. J. Physiol.* **258:**C812–C817

Buxbaum, E., Schoner, W. 1991. Phosphate binding and ATP-binding sites coexist in Na^+/K^+-transporting ATPase, as demonstrated by the inactivating $MgPO_4$ complex analogue Co $(NH_3)_4PO_4$. *Eur. J. Biochem.* **195:**407–419

Clausen, T. 1986. Regulation of active Na^+-K^+ transport in skeletal muscle. *Physiol. Rev.* **66:**542–580

Clausen, T., Hansen, O. 1974. Ouabain binding and Na^+-K^+ transport in rat muscle cells and adipocytes. *Biochim. Biophys. Acta* **345:**387–404

Clausen, T., Hansen, O. 1977. Active Na/K transport and the rate of ouabain binding. The effect of insulin and other stimuli on skeletal muscle and adipocytes. *J. Physiol.* **270:**415–430

Craig, W.S. 1988. Determination of quaternary structure of an active enzyme using chemical cross-linking with glutaraldehyde. *In:* Methods in Enzymology. B. Fleischer and S. Fleischer, editors Vol. 156, pp. 333–345. Academic, New York

Cushman, S.W., Wardzala, L.J. 1980. Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. *J. Biol. Chem.* **255:**4758–4762

Emanuel, J.R., Garetz, S., Stone, L., Levenson, R. 1987. Differential expression of Na+,K+-ATPase α- and β-subunit mRNAs in rat tissues and cell lines. *Proc. Natl. Acad. Sci. USA* **84:**9030–9034

Erlij, D., Grinstein, S. 1976. The number of sodium ion pumping sites in skeletal muscle and its modification by insulin. *J. Physiol.* **259:**13–31

Felsenfeld, D.P., Sweadner, K.J. 1988. Fine specificity mapping and topography of an isozyme-specific epitope of the Na,K-ATPase catalytic subunit. *J. Biol. Chem.* **263:**10932–10942

Handberg, A., Kayser, L., Høyer, P.E., Vinten, J. 1992. A substantial part of GLUT-1 in crude membranes from muscle originates from perineurial sheaths. *Am. J. Physiol.* **262:**E721–E727

Haney, P.M., Slot, J.W., Piper, R.C., James, D.E., Mueckler, M. 1991. Intracellular targeting of the insulin-regulatable glucose transporter (GLUT4) is isoform specific and independent of cell type. *J. Cell Biol.* **114:**689–699

Herrera, V.L.M., Emanuel, J.R., Ruiz-Opazo, N., Levenson, R., Nadal-Ginard, B. 1987. Three differentially expressed Na,K-ATPase alpha subunit isoforms: structural and functional implications. *J. Cell Biol.* **105:**1855–1865

Hootman, S.R., Ernst, S.A. 1988. Estimation of Na/K-pump numbers and turnover in intact cells with ^{3}H-ouabain. *In:* Methods in Enzymology. B. Fleischer and S. Fleischer, editors Vol. 156, pp. 213–229. Academic, New York

Horisberger, M. 1981. Colloidal gold: A cytochemical marker for light and fluorescent microscopy and for transmission and scanning electron microscpy. *Scan. Electron Micros.* **2:**9–31

Hsu, Y.-M., Guidotti, G. 1991. Effects of hypokalemia on the properties and expression of the (Na^+,K^+)-ATPase of rat skeletal muscle [*published erratum* appears in *J. Biol. Chem.* 1991, **266:**7962]. *J. Biol. Chem.* **266:**427–433

Hundal, H.S., Marette, A., Mitsumoto, Y., Ramlal, T., Blostein, R., Klip, A. 1992. Insulin induces translocation of the alpha 2 and beta 1 subunits of the Na^+/K^+-ATPase from intracellular compartments to the plasma membrane in mammalian skeletal muscle. *J. Biol. Chem.* **267:**5040–5043

Jacobson, B.S., Cronin, J., Branton, D. 1978. Coupling polylysine to glass beads for plasma membrane isolation. *Biochim. Biophys. Acta* **506:**81–96

James, D.E., Brown, R., Navarro, J., Pilch, P.F. 1988. Insulin regulatable tissues express a unique insulin sensitive glucose transport protein. *Nature* **333:**183–185

Jensen, J., Nørby, J.G. 1988. Membrane-bound Na,K-ATPase: target size and radiation inactivation size of some of its enzymatic reactions. *J. Biol. Chem.* **263:**18063–18070

Kashgarian, M., Biemesderfer, D. 1988. Preparation and use of monoclonal antibodies to Na/K-ATPase. *In:* Methods in Enzymology. B. Fleischer and S. Fleischer, editor Vol. 156, pp. 392–417, Academic, New York

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680–685

Liang, S.-M., Winter, C.G. 1977. Digitonin-induced changes in subunit arrangement in relation to some in vitro activities of the (Na^+,K^+)-ATPase. *J. Biol. Chem.* **252:**8278–8284

Lingrel, J.B., Orlowski, J., Shull, M.M., Price, E.M. 1990. Molecular genetics of Na,K-ATPase. *Prog. Nucleic Acid Res. Mol. Biol.* **38:**37–89

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin reagent. *J. Biol. Chem.* **193:**265–275

Lytton, J. 1985. Insulin affects the sodium affinity of the rat adipocyte (Na^+,K^+)-ATPase. *J. Biol. Chem.* **260:**10075–10080

Lytton, J., Lin, J.C., Guidotti, G. 1985. Identification of two molecular forms of (Na^+,K^+)-ATPase in rat adipocytes. Relation to insulin stimulation of the enzyme. *J. Biol. Chem.* **260:**1177–1184

Mayahara, H., Ogawa, O. 1988. Histochemical localization of Na^+,K^+-ATPase. *In:* Methods in Enzymology. B. Fleischer and S. Fleischer, editors. Vol. 156, pp. 417–430, Academic, New York

McGill, D.L. 1991. Characterization of the adipocyte ghost (Na^+,K^+) pump. Insights into the insulin regulation of the adipocyte (Na^+,K^+) pump. *J. Biol. Chem.* **266:**15817-15823

McGill, D.L., Guidotti, G. 1991. Insulin stimulates both the $\alpha 1$ and the $\alpha 2$ isoforms of the rat adipocyte (Na^+,K^+) ATPase. Two mechanisms of stimulation. *J. Biol. Chem.* **266:**15824–15831

McKeel, D.W., Jarett, L. 1970. Preparation and characterization of a plasma membrane fraction from isolated fat cells. *J. Cell Biol.* **44:**417–432

Nørby, J.G., Jensen, J. 1991. Functional significance of the oligomeric structure of the Na/K-pump from radiation inactivation and ligand binding. *In:* The Sodium Pump: Structure, Mechanism, and Regulation. pp. 174–188. The Rockefeller University, New York

O'Connor, C.G., Ashman, L.K. 1982. Application of the nitrocellulose transfer technique and alkaline phosphatase conjugated anti-immunoglobulin for determination of the specificity of monoclonal antibodies to protein mixtures. *J. Immunol. Methods* **54:**267–273

Ogawa, K.S., Fujimoto, K., Ogawa, K. 1986. Ultracytochemical studies of adenosine nucleotidases in rat aortic endothelial and smooth mucsle cells. *Acta. Histochem. Cytochem.* **19:**601–620

Omatsu-Kanbe, M., Kitasato, H. 1990. Insulin stimulates the translocation of Na^+/K^+-dependent ATPase molecules from intracellular stores to the plasma membrane in frog skeletal muscle. *Biochem. J.* **272:**727–733

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83:**346–356

Resh, M.D. 1982*a*. Insulin action on the (Na^+,K^+)ATPase. Ph.D. Thesis, pp. 451–464. Harvard University, Cambridge, MA

Resh, M.D. 1982*b*. Quantitation and characterization of the (Na^+,K^+)-adenosine triphosphatase in the rat adipocyte plasma membrane. *J. Biol. Chem.* **257:**11946–11952

Resh, M.D. 1982*c*. Development of insulin responsiveness of the glucose transporter and the (Na^+,K^+)-adenosine triphosphatase during in vitro adipocyte differentiation. *J. Biol. Chem.* **257:**6978–6986

Resh, M.D. 1983. Insulin activation of (Na^+,K^+)-adenosinetriphosphatase exhibits a temperature-dependent lag time. Comparison to activation of the glucose transporter. *Biochemistry* **22:**2781–2784

Resh, M.D. 1988. Identification and quantitation of Na,K-ATPase by back-door phosphorylation. *In:* Methods in Enzymology. B. Fleischer and S. Fleischer, editors. Vol. 156, pp. 119–126. Academic, New York

Resh, M.D., Nemenoff, R.A., Guidotti, G. 1980. Insulin stimulation of (Na^+,K^+)-adenosine triphosphatase-dependent $^{86}Rb^+$ uptake in rat adipocytes. *J. Biol. Chem.* **255:**10938–10945

Robinson, L.J., Pang, S., Harris, D.S., Heuser, J., James, D.E. 1992. Translocation of the glucose transporter (GLUT4) to the cell surface in permeabilized 3T3-L1 adipocytes: Effects of ATP, insulin, and GTPγS, and localization of the GLUT4 to clathrin lattices. *J. Cell. Biol.* **117:**1181–1196

Russo, J.J., Manuli, M.A., Ismail-Beigi, F., Sweadner, K.J., Edelman, I.S. 1990. Na^+,K^+-ATPase in adipocyte differentiation in culture. *Am. J. Physiol.* **259:**C968–C977

Schmitt, C.A., McDonough, A.A. 1986. Developmental and thyroid hormone regulation of two molecular forms of Na/K, ATPase in brain. *J. Biol. Chem.* **261:**10439–10444

Skou, J.C. 1988. Overview: The Na/K-pump. *In:* Methods in Enzymology. B. Fleischer and S. Fleischer, editors. Vol. 156, pp. 1–29. Academic, New York

Slot, J.W., Posthuma, G., Chang, L.Y., Crapo, J.D., Geuze, H.J. 1989. Quantitative aspects of immunogold labeling in embedded and in nonembedded sections. *Am. J. Anat.* **185:**271–281

Slot, J.W., Geuze, H.J. 1985. A new method of preparing gold probes for multiple-labeling cytochemistry. *Eur. J. Cell Biol.* **38:**87–93

Smith, R.M., Charron, M.J., Shah, N., Lodish, H.F., Jarett, L. 1991. Immunoelectron microscopic demonstration of insulin-stimulated translocation of glucose transporters to the plasma membrane of isolated rat adipocytes and masking of the carboxyl-terminal epitope of intracellular GLUT4. *Proc. Natl. Acad. Sci. USA* **88:**6893–6897

Suzuki, K., Kono, T. 1980. Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc. Natl. Acad. Sci. USA* **77:**2542–2545

Sweadner, K.J. 1989. Isozymes of the Na^+/K^+-ATPase. *Biochim. Biophys. Acta.* **988:**185–220

Tranum-Jensen, J. 1988. Electron microscopy: Assays involving negative staining. *In:* Methods in Enzymology. S. Harshman, editor. Vol. 165, pp. 357–374. Academic, New York

Tranum-Jensen, J., Vinten, J., Ploug, T. 1988. The anatomy of the cytoplasmic rim and the plasma membrane of rat epididymal fat cells. *J. Ultrastructure Mol. Structure Res.* **100:**293

Urayama, O., Shutt, H., Sweadner, K.J. 1989. Identification of three isozyme proteins of the catalytic subunit of the Na,K-ATPase in rat brain. *J. Biol. Chem.* **264:**8271–8280

Urayama, O., Sweadner, K.J. 1988. Ouabain sensitivity of the $\alpha 3$ isozyme of rat Na,K-ATPase. *Biochem. Biophys. Res. Commun.* **156:**796–800

Vinten, J., Gliemann, J., Østerlind, J. 1976. Exchange of 3-*O*-methylglucose in isolated fat cells. Concentration dependence and effect of insulin. *J. Biol. Chem.* **251:**794–800

Vinten, J., Tranum-Jensen, J. 1991. Immunogold labeling of glucose transporters in adipocyte plasma membranes. *Diabetologia* **34:***A*12 (*Abstr.*)

Yang, J., Clark, A.E., Kozka, I.J., Cushman, S.W., Holman, G.D. 1992. Development of an intracellular pool of glucose transporters in 3T3-L1 cells. *J. Biol. Chem.* **267:**10393–10399.