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Insulin stimulates the Na^+,K^+-ATPase and the $Na^+/K^+/Cl^-$ cotransporter of human fibroblasts

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Abstract

Insulin regulation of K^+ (Rb^+) transport was investigated in cultured human fibroblasts using a non-radioactive method which allows the simultaneous determination of the intracellular concentration of other monovalent cations. Insulin stimulated Rb^+ influx through the Na^+,K^+-ATPase and the $Na^+/K^+/Cl^-$ cotransporter in human fibroblasts. Insulin stimulation was very rapid and maximal effect was observed within 10 min. Insulin stimulation of Rb^+ uptake via the Na^+,K^+-ATPase and the $Na^+/K^+/Cl^-$ cotransporter was dose-dependent, with half-maximal stimulation at 2–3 nM of hormone. Insulin increased the V_{max} of both transporters involved, affecting only minimally their K_m. In other cells, insulin stimulates the Na^+,K^+-pump by increasing Na^+ availability through the Na^+/H^+ exchanger. In human fibroblasts, insulin stimulation of Na^+,K^+-ATPase occurred in the presence of ethyl-isopropyl amiloride, an inhibitor of the Na^+/H^+ exchanger, and without sustained changes in intracellular [Na^+]. By contrast, insulin action on Na^+,K^+-ATPase was impaired by the protein kinase inhibitors staurosporine and genistein. These results indicate that, in human fibroblasts, insulin stimulates both the Na^+,K^+-ATPase and the $Na^+/K^+/Cl^-$ cotransporter, that stimulation of the Na^+,K^+-ATPase occurs in the absence of changes in intracellular [Na^+], and that protein kinase activity is essential for this insulin action.

Keywords: Potassium ion transport; Insulin receptor; Fibroblast; Insulin; Cotransporter, $Na^+/K^+/Cl^-$; ATPase, Na^+/K^+-; (Human)

1. Introduction

The Na^+,K^+-pump and the bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransporter mediate the bulk of K^+ influx in most mammalian cells. The bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransporter mediates an electrically neutral transfer of two cations (Na^+ and K^+) and two anions ($2 \cdot Cl^-$) across the plasma membrane of mammalian cells. This cotransporter does not require chemical energy, although ATP and phosphorylation regulate its activity [1]. A cDNA encoding a bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransporter has been recently cloned from a shark rectal gland cDNA library [2]. The predicted structure of the encoded protein comprises 12 transmembrane spanning domains and several putative phosphorylation sites [2].

The Na^+,K^+-ATPase couples the hydrolysis of ATP to the influx of two K^+ and the efflux of three Na^+ against their electrochemical potential gradients (primary active transport). This enzyme is composed of an α (112 kDa) and a β (35 kDa) subunit. The α subunit contains the binding sites for Na^+, K^+, ATP, and ouabain and is commonly referred to as the catalytic subunit. The β subunit is a glycoprotein whose function, although essential to pump activity, has yet to be determined [3]. There are three isoforms of Na^+,K^+-ATPase α subunits ($\alpha 1$, $\alpha 2$, and $\alpha 3$) and at least two isoforms of Na^+,K^+-ATPase β subunit ($\beta 1$ and $\beta 2$) which have different tissue-specific expression [3,4].

Insulin stimulates the Na^+,K^+-ATPase in a variety of cells and tissues. The mechanism by which this occurs is tissue-specific. In rat hepatocytes [5] and BC3H-1 myocytes [6], insulin stimulates pump activity by increasing the availability of Na^+ in the cytoplasm. Increased Na^+ availability is due to increased Na^+ influx through the amiloride-sensitive Na^+/H^+ exchanger [5,6]. By contrast, in 3T3-F442A fibroblasts and adipocytes insulin stimulates pump activity again by increasing Na^+ entry, but this time through a Na^+-channel, which is not inhibited by amiloride [7]. This channel may correspond to the μ conotoxin-sensitive Na^+ channel recently reported in skeletal muscle [8]. It is not known if increased Na^+ entry raises intracellular

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$[Na^+]$ in 3T3-F442A cells and if this action precedes insulin effect on Na^+,K^+-ATPase [7].

In primary adipocytes, insulin stimulates the Na^+,K^+-pump by increasing its affinity toward intracellular Na^+ [9,10] without increasing the number of Na^+,K^+-ATPases on the plasma membrane [11]. By contrast, in the skeletal muscle, insulin increases the number of Na^+,K^+-ATPases in the membrane [12,13], without affecting the total number of ouabain-binding sites [14]. Immunological studies indicated that insulin specifically recruits preformed $\alpha 2$, but not $\alpha 1$, isoforms from an intracellular pool [12], in analogy with the mechanism of insulin action on the insulin-responsive glucose transporter (GLUT4, Refs. [12,15]. Since adipocytes express the same $\alpha 2$ isoform of Na^+,K^+-ATPase present in the muscle [3], it is not clear why the same isoform of Na^+,K^+-ATPase behaves differently in different tissues and whether the kinase activity of the insulin receptor is required for any of these insulin actions.

In human fibroblasts, the major part of K^+ influx is mediated by the Na^+,K^+-ATPase and the bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransporter [16,17]. Insulin stimulates the Na^+,K^+-ATPase of human fibroblasts [18], but it is unknown if the hormone modifies intracellular Na^+ or K^+ concentration and if the activity of the bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransporter is also affected by insulin.

This paper reports that insulin stimulates both the $Na^+/K^+/Cl^-$ cotransporter and the Na^+,K^+-ATPase in human fibroblasts. Surprisingly, stimulation of Na^+,K^+-ATPase was observed in the absence of changes in intracellular $[Na^+]$ and in the presence of blockers of the Na^+/H^+ exchanger. By contrast, kinase inhibitors blocked insulin stimulation of Na^+,K^+-ATPase, suggesting that activation of ion fluxes was dependent upon the phosphorylation cascade initiated by insulin binding to its receptor.

2. Materials and methods

2.1. Materials

3-*O*-[$U^{14}C$]Methyl-D-glucose (55 mCi/mmol) was from New England Nuclear. Insulin (bovine sodium, 25 U/mg) was from Calbiochem. Ethyl isopropyl amiloride (EIPA) was a gift of Dr. Masayuki Mitsuka, Mitsubishi Casei, Yokohama, Japan. Chemical reagents were ACS grade and were obtained from Sigma or Fisher.

2.2. Experimental techniques

Human fibroblasts were obtained from normal adult skin biopsy as previously described [17] and were propagated in Dulbecco-Vogt medium containing 15% fetal bovine serum (FBS). Cells were seeded in 24-well plates and grown to confluence, renewing the medium every three days and 48 h before each experiment. The day of the experiment, cells were washed twice and incubated for 2 h at 37°C in 0.9 ml of tris(hydroxymethyl)aminomethane (Tris, 26 mM, pH 7.4) buffered Earle's balanced salt solution (EBSS) containing 116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, which was always supplemented with 1% (w/v) bovine serum albumin (RIA grade, Sigma). Bovine serum albumin (BSA) was included in the solution to avoid the perturbation of Na^+ and K^+ gradients which occur in human fibroblasts incubated in the absence of serum [19]. Insulin (100 μl of a tenfold concentration in EBSS) or EBSS (100 μl) were then added to the cells for 5 min, unless otherwise specified. Non-radioactive Rb^+ uptake was then measured for 1–5 min (during which Rb^+ uptake via the Na^+,K^+-ATPase and the $Na^+/K^+/Cl^-$ cotransporter is linear, Ref. [17]) in the presence or absence of insulin and transport inhibitors by incubating cells with a similar solution in which the 5.4 mM KCl was replaced by 5.4 mM RbCl (RbCl-EBSS) [17]. $^{86}Rb^+$ and $^{42}K^+$ are transported similarly in human fibroblasts in the absence or presence of transport inhibitors [16]. Cell monolayers were rapidly washed 4 times with ice-cold 0.1 M $MgCl_2$ (total washing time was below 10 s), using the cluster tray method [20]. Ethanol (0.1 ml) was then added to each well and allowed to dry. 2 ml of 5 mM CsCl in water were added to each well and intracellular Na, Rb, and K contents were determined in each well by emission flame photometry (Perkin Elmer 460 Atomic Absorption Spectrophotometer). Cell monolayers were then dried, solubilized with 200 μl of 0.1% sodium deoxycholate in 1 M NaOH and assayed for protein using a modified Lowry procedure [21].

2.3. Calculations

Intracellular water was evaluated in parallel experiments from the equilibrium distribution of 3-*O*-methyl-D-glucose [22], which is reached within 10 min in cultured human fibroblasts [23]. The mean cell water content was 7.0 ± 0.7 μl/mg of cell proteins in human fibroblasts and was not affected by insulin treatment.

Intracellular ion content was calculated from the total amount of non-radioactive Na, K and Rb (determined by flame photometry) and was then expressed as μmol per ml of cell water, by dividing for the corresponding cell water. Bumetanide- and ouabain-sensitive Rb^+ uptake were determined by subtracting the uptake in the presence of the inhibitor from total Rb^+ uptake [17]. Influx data and intracellular ion concentrations are expressed in the text as means $\pm$S.D. (total influx) or $\pm$S.E., in the case of influx calculated by difference [17]. Statistical comparisons were performed by using analysis of variance.

The analysis of kinetic curves was performed using

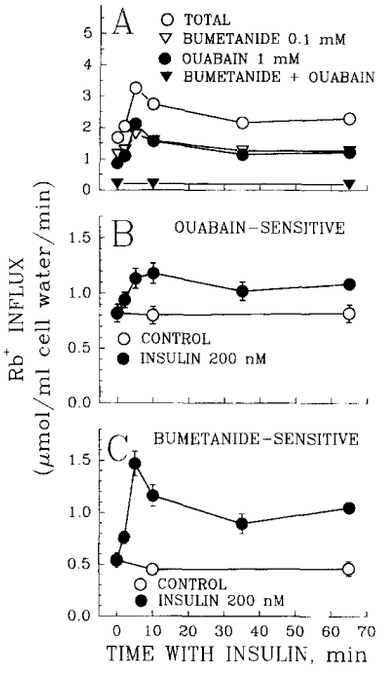

Fig. 1. Time-course for insulin stimulation of Rb^+ influx in human fibroblasts. Panel A: cells were incubated for the time indicated at 37°C in the absence or in the presence of insulin (200 nM). Rb^+ (5.4 mM) influx was measured for the last 2 min without or with ouabain (1 mM) and/or bumetanide (0.1 mM). Ouabain- (panel B) and bumetanide-sensitive (panel C) Rb^+ influx was calculated by difference between total influx and influx in the presence of each inhibitor. Points are means ± S.E. of triplicates. The experiment was repeated three times with similar results.

Marquardt's algorithm for least-squares estimation of nonlinear parameters [24]. The equations used were:

$$v = V_0 + \frac{E_{max} \cdot [\text{Insulin}]}{ED_{50} + [\text{Insulin}]} \quad (1)$$

for analysis of dose-response curves for insulin stimulation of Rb^+ influx, where v is the measured velocity of Rb^+ entry, V_0 is the velocity of Rb^+ entry in the absence of insulin, E_{max} is the maximal stimulation, and ED_{50} the concentration of insulin at which half-maximal stimulation is observed [25]. Influx data were also fitted to a Michaelis-Menten equation for the kinetic analysis of Rb^+ entry [17]. Parameters calculated by nonlinear regression are expressed in the text as means ± S.D. and are statistically compared using 95% or 99% confidence intervals.

3. Results and discussion

3.1. Time-course for insulin stimulation of Rb^+ influx

Fig. 1 shows the time course for insulin stimulation of Rb^+ uptake by human fibroblasts. Cells were incubated for up to 1 h in the presence or absence of insulin and Rb^+ uptake was measured during the last 2 min of incubation. A significant effect of insulin on Rb^+ influx was present at the shortest time point studied (2 min), reached a maximum in 10 min and then declined a little, even though it remained significantly above the control value after 1 h of incubation. By contrast, total Rb^+ influx remained constant in cells incubated in the absence of insulin during the one hour incubation and was (in μmol/ml cell water per min) 1.68 ± 0.04 at 0 time, 1.58 ± 0.10 at 10 min, 1.62 ± 0.08 at 60 min. Similarly, there were no significant variations in ouabain- and bumetanide-sensitive Rb^+ influx in cells incubated in the absence of insulin (Fig. 1B,C). Insulin stimulated both the Na^+,K^+-ATPase (ouabain-sensitive Rb^+ uptake, Fig. 1B) and the $Na^+/K^+/Cl^-$ cotransporter (bumetanide-sensitive Rb^+ uptake, Fig. 1C), and did not affect the portion of Rb^+ influx not inhibited by the simultaneous presence of both inhibitors. In different experiments and in different strains of human fibroblasts, insulin stimulation of Rb^+ influx at 10 min of incubation ranged between 50 and 200% above basal for both the $Na^+/K^+/Cl^-$ cotransporter and the Na^+,K^+-ATPase.

In human fibroblasts, insulin stimulates glucose and amino acid transport [25,26]. These actions of insulin are maximally stimulated only after a significant lag time, which is 30 min for glucose transport and many hours for amino acid transport [25,26]. By contrast, insulin effect on Rb^+ transport was very rapid and could be observed at the shortest time point used (Fig. 1). Insulin stimulated Rb^+ transport by both the $Na^+/K^+/Cl^-$ cotransporter and the Na^+,K^+-ATPase. These effects of insulin preceded stimulation of glucose and amino acid transport.

In these preliminary experiments only a single high dose of insulin (200 nM) was used. To determine if insulin activated ion fluxes by interacting with its own receptor, a dose-response curve for insulin stimulation of Rb^+ transport was performed. Cells were incubated for 10 min in the presence of increasing concentrations of insulin and Rb^+ influx was measured during the last 5 min in the absence and in the presence of insulin and ouabain (1 mM) or bumetanide (0.1 mM). Insulin promoted a dose-dependent increase in the activity of both the Na^+,K^+-pump and the $Na^+/K^+/Cl^-$ cotransporter (Fig. 2). Half-maximal stimulation was observed with 2.5 ± 1.1 and 3.7 ± 1.8 nM of insulin in the case of bumetanide- and ouabain-sensitive Rb^+ influx, respectively. These values are compatible with insulin binding to its own high-affinity receptor [27,28]. Further, the curve for insulin stimulation of Rb^+ transport was similar to that for insulin stimulation of amino acid and glucose transport by normal human fibroblasts [25,27]. This suggests that insulin stimulates Rb^+ transport by interacting with its own receptor.

In many cell types, including human fibroblasts [29], the $Na^+/K^+/Cl^-$ cotransporter is stimulated by serum and growth factors [1,30]. It is surprising that only in few reports the effect of insulin on this cotransporter was tested [30,31]. In Swiss 3T3 fibroblasts, insulin stimulated the

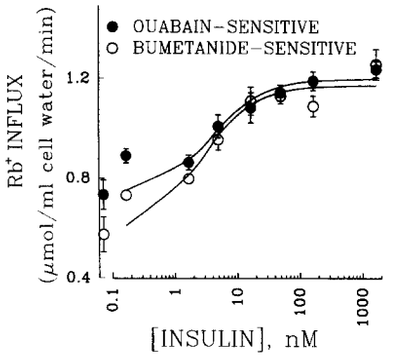

Fig. 2. Dose-response curve for insulin stimulation of Rb^+ influx in cultured human fibroblasts. Fibroblasts were incubated 5 min before and 5 min during influx in the presence of the indicated concentrations of insulin. Rb^+ influx was measured in the absence and presence of ouabain (1 mM) and bumetanide (0.1 mM). Ouabain- and bumetanide-sensitive Rb^+ influx were calculated by difference between total influx and influx in the presence of ouabain and bumetanide, respectively. Each point is the mean ± S.E. of six observations. Curves represent the best fit of data to Eq. 1 in Section 2. The experiment was repeated twice with similar results.

$Na^+/K^+/Cl^-$ cotransporter, but half-maximal stimulation was obtained only with micromolar concentrations of insulin [30], a concentration which is two orders of magnitude higher than that reported here for human fibroblasts (Fig. 2). We do not know if this difference is due to the different methodological approach or to insulin interaction with another less specific receptor in 3T3 fibroblasts. More recently, a stimulatory effect of insulin on the furosemide-sensitive K^+ transporter was reported in BC3H1 myocytes [31]. The transporter stimulated by insulin in these cells, however, probably differs from that reported here in human fibroblasts, since it does not require Na^+ and operates with a much lower K_m of 0.9 mM [31], which is 4–6 times lower of that reported for the $Na^+/K^+/Cl^-$ cotransporter and for the bumetanide-sensitive K^+ transport in human fibroblasts ([17] and Fig. 3). Many of the previous studies focused on the effect of insulin on ouabain-sensitive Rb^+ uptake and it is not known if insulin stimulation of the $Na^+/K^+/Cl^-$ cotransporter reported here in human fibroblasts is also observed in other cell types.

3.2. *Effect of insulin on the kinetic constants for Rb^+ influx*

Insulin may stimulate Rb^+ influx by increasing the affinity of ion transporters toward the substrate or by increasing their number or turnover rate. To address this question, the effect of insulin on the kinetic constants for Rb^+ influx was then evaluated. Fibroblasts were incubated for 10 min in the absence or presence of insulin (1 μM) and Rb^+ (1–20 mM) influx was measured during the last 5 min of incubation. Ouabain- and bumetanide-sensitive Rb^+ influx was calculated at each $[Rb^+]$ by subtracting from total influx the influx in the presence of 1 mM ouabain or 0.1 mM bumetanide, respectively. Corrected data were analyzed by nonlinear regression analysis according to a Michaelis-Menten equation and are reported in Fig. 3 according to an Eadie-Hofstee graphical representation. Insulin increased the V_{max} for ouabain-sensitive Rb^+ uptake from 0.72 ± 0.07 to 1.36 ± 0.05 μmol · ml^{-1} cell water · min^{-1} ($P < 0.01$ using 99% confidence intervals) and did not significantly affect the K_m (from 3.1 ± 0.9 to 2.5 ± 0.3 mM, $P > 0.05$, Fig. 3A). Similarly, insulin increased the V_{max} for bumetanide-sensitive Rb^+ uptake from 0.67 ± 0.06 to 1.61 ± 0.04 μmol · ml^{-1} cell water · min^{-1} ($P < 0.01$ using 99% confidence intervals), without affecting the relative K_m (from 6.9 ± 1.6 to 5.3 ± 0.3 mM, $P > 0.05$, Fig. 3B). In both cases insulin had a major effect on the V_{max} of the transporter, suggesting either that new carriers were recruited or that insulin increased the turnover rate of preexisting transporters.

3.3. *Role of Na^+ and of the Na^+/H^+ exchanger in insulin stimulation of the Na^+,K^+-pump*

In liver cells and BC3H-1 myocytes, insulin stimulates Na^+ entry through the Na^+/H^+ exchanger. The increased amount of intracellular Na^+ available to the pump stimulates its activity [5,6]. In these cells, the effect of insulin on Na^+,K^+-ATPase is blocked by the addition of blockers of the Na^+/H^+ exchanger, such as amiloride. In human fibroblasts, ethyl isopropyl amiloride (EIPA), a specific

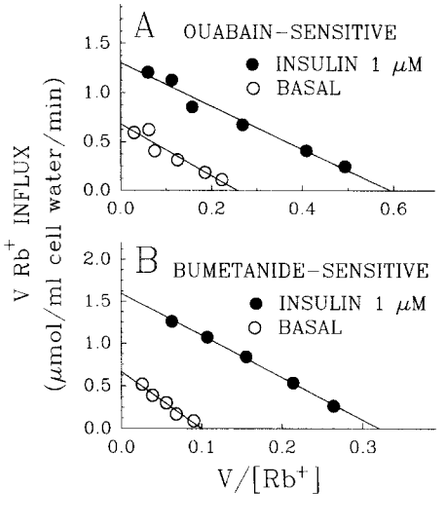

Fig. 3. Effect of insulin on the kinetics of Rb^+ influx through the Na^+,K^+-ATPase and the $Na^+/K^+/Cl^-$ cotransporter of human fibroblasts. Cells were incubated for 5 min at 37°C without or with insulin (1 μM) after which Rb^+ influx was measured for 5 min in the absence or presence of insulin, bumetanide (0.1 mM) or ouabain (1 mM). Ouabain- and bumetanide-sensitive Rb^+ influx was calculated by difference between total influx and influx in the presence of the specific inhibitor. Data are means of triplicates and are reported according to an Eadie-Hofstee graphical representation. Data were analyzed by non-linear regression analysis using a Michaelis-Menten equation and lines represent the best fit of data to such a model. The experiment was repeated twice with similar results.

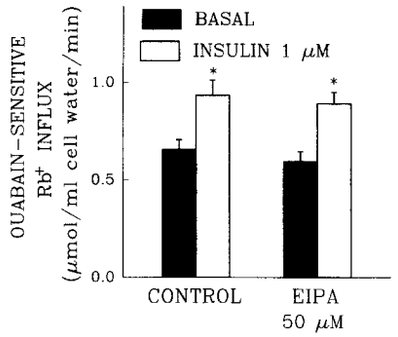

Fig. 4. Effect of ethyl isopropyl amiloride (EIPA) on insulin stimulation of Na^+,K^+-ATPase. Cells were incubated with 50 μM EIPA for 10 min before a 5 min insulin (1 μM) addition. Rb^+ influx was then measured for 5 min at 37°C in the absence or in the presence of insulin, ouabain and EIPA. Data are means ± S.E. of triplicates. In a separate experiment, amiloride (0.5 mM) and bumetanide (0.1 mM) also failed to prevent insulin stimulation of Na^+,K^+-ATPase. * $P < 0.01$ versus basal, using analysis of variance.

inhibitor of the Na^+/H^+ exchanger [32], did not prevent insulin stimulation of the Na^+,K^+-ATPase (Fig. 4). Cells were preincubated with the indicated concentration of inhibitor 10 min before and up to the end of the uptake assay. Inhibition of Na^+ influx through the Na^+/H^+ exchanger did not reduce insulin effect on ouabain-sensitive Rb^+ uptake. These results in human fibroblasts agree with those in the rat soleus muscle, showing that the stimulating effect of insulin on Na^+,K^+-ATPase is resistant to inhibition by amiloride [33]. However, insulin could still increase intracellular $[Na^+]$ availability through other pathways. For this reason, the effect of insulin on intracellular $[Na^+]$ was determined (Fig. 5).

Incubation of human fibroblasts with insulin (1 μM) up to 1 h did not increase intracellular $[Na^+]$, but over time, slightly decreased it (from 20.6 ± 0.9 to 17.4 ± 0.2 mM, $P < 0.01$) while increasing intracellular $[K^+]$ (from 178 ± 2 to 184 ± 3 mM). The decrease in intracellular $[Na^+]$ is more likely to represent an effect of pump activation rather

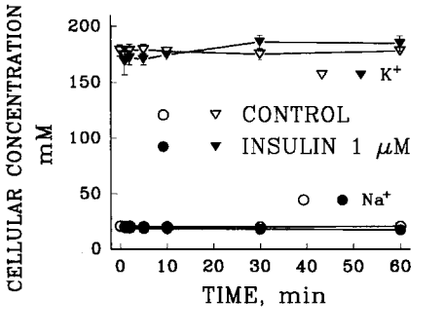

Fig. 5. Effect of insulin on intracellular Na^+ and K^+ concentration. Cells were incubated for the indicated time at 37°C in the presence or absence of insulin (1 μM). Intracellular Na^+ and K^+ content were then determined by flame photometry and corrected for intracellular water, determined in a parallel tray from the equilibrium distribution of OMG. Data are means ± S.D. of triplicates.

Table 1
Effect of insulin and serum on intracellular $[Na^+]$

	$[Na^+]_i$ (mM)	$[K^+]_i$ (mM)
Control	18.7 ± 0.4	150.6 ± 4.1
Insulin 1 μM	18.4 ± 0.6	146.3 ± 4.2
Serum 10%	21.5 ± 0.5 *	146.9 ± 5.2
Control + ouabain	26.0 ± 1.4	157.2 ± 9.4
Insulin + ouabain	24.3 ± 0.8	140.3 ± 6.9
Serum + ouabain	31.1 ± 1.0 *	139.5 ± 5.0

Cells were incubated for 5 min at 37°C in Earle's balanced salt solution (EBSS) ± ouabain 1 mM in the presence of insulin (1 μM) or serum (10%). Cells were washed four times with 0.1 M $MgCl_2$ and intracellular Na and K were measured by flame photometry. Each sample is the mean of triplicates ± S.D. The experiment was repeated twice with similar results.
* $P < 0.01$ vs. paired control.

than its cause and has been observed in other mammalian cells and tissues [34].

The failure to observe an increase in intracellular $[Na^+]$ could be due to the relative insensitivity of the method used in this study. To test this hypothesis, the effect of insulin was compared to that of serum, which increases Na^+ entry through the Na^+/H^+ exchanger and does stimulate Na^+,K^+-ATPase by increasing the availability of intracellular Na^+ [35,36]. Serum (Table 1) increased intracellular $[Na^+]$ in human fibroblasts both in the absence and in the presence of ouabain (used to block the increased Na^+ excretion from the cell). By contrast, insulin did not produce any significant increase in intracellular $[Na^+]$. These results indicate that the method adopted is sufficiently sensitive to detect changes in intracellular $[Na^+]$ and confirm that insulin, unlike serum and other growth factors, does not activate the Na^+/H^+ exchanger of human fibroblasts [35,37]. These data also indicate that the mechanism for insulin stimulation of Na^+,K^+-ATPase activity in human fibroblasts differs from that of serum and that insulin activates the Na^+,K^+-pump without causing a sustained increase in intracellular $[Na^+]$.

These results in human fibroblasts contrast with those in hepatocytes and BC3H-1 myocytes in which insulin increases Na^+ influx. In hepatocytes, however, there was no increase in intracellular $^{22}Na^+$ when measurements were performed in the absence of ouabain [5]. By contrast, in BC3H-1 myocytes, $^{22}Na^+$ accumulation was increased by insulin even in the absence of ouabain [6]. In both cases, however, the total intracellular concentration of Na^+ was not determined either before or after insulin treatment.

3.4. Effect of kinase inhibitors on insulin stimulation of Rb^+ influx

Insulin interacts with specific receptors on the plasma membrane of target cells and stimulates their phosphorylation on tyrosine residues and kinase activity toward endogenous substrates. Other insulin actions, such as stimula-

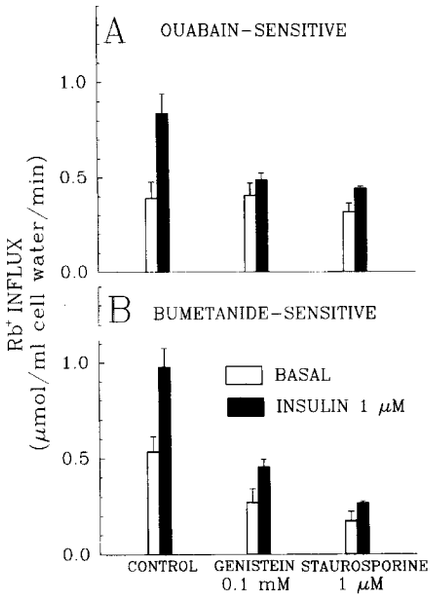

Fig. 6. Effect of kinase inhibitors on insulin stimulation of Rb^+ influx. Fibroblasts were incubated for 5 min in the presence of insulin after which Rb^+ influx was measured for 5 min in the absence or in the presence of insulin with or without transport inhibitors (ouabain 1 mM or bumetanide 0.1 mM). Kinase inhibitors, when present, were added 5 min prior to insulin. Ouabain- and bumetanide-sensitive Rb^+ influx was calculated by difference between total influx and influx in the presence of each inhibitor. Points are means ± S.E. of three observations.

tion of glucose transport, are prevented by the addition of kinase inhibitors [38]. The requirement for the phosphorylation cascade in insulin stimulation of Rb^+ influx was evaluated by preexposing fibroblasts to genistein, an inhibitor of tyrosine-kinases [39] and staurosporine [38], an inhibitor of serine/threonine kinases, which may be secondarily activated by the insulin receptor (Fig. 6). Both inhibitors were added 5 min before insulin addition and were present up to the end of the uptake assay. The concentrations of inhibitors used in this experiment abolish insulin stimulation of glucose transport both in human fibroblasts and in CHO cells (Longo, N. and Griffin, L.D., unpublished data). Both genistein (100 μM) and staurosporine (1 μM) significantly reduced insulin stimulation of Na^+,K^+-ATPase, without affecting significantly basal pump activity (Fig. 6A). These results suggest that the phosphorylation cascade initiated by insulin binding to its own receptor is involved in insulin stimulation of Na^+,K^+-ATPase.

In the case of the $Na^+/K^+/Cl^-$ cotransporter, the effect of kinase inhibitors was more difficult to interpret. In fact, although both inhibitors reduced insulin activation of the $Na^+/K^+/Cl^-$ cotransporter, they also had a marked effect on basal cotransporter activity (Fig. 6B). An effect of kinase inhibitors on basal $Na^+/K^+/Cl^-$ cotransporter activity was not unexpected, since this transporter, although not requiring chemical energy, is strongly modulated by phosphorylation- dephosphorylation [1]. The rapid and marked effect of kinase inhibitors on $Na^+/K^+/Cl^-$ cotransporter activity both in unstimulated and in insulin-stimulated human fibroblasts further supports that this transporter is constantly modulated by a variety of kinases. Intuitively, the insulin receptor may modify the activity of the cotransporter by the same mechanism. Experiments using cells with kinase-defective insulin receptor will be required to confirm this hypothesis in the case of the $Na^+/K^+/Cl^-$ cotransporter.

The relevance of the combined stimulation of the Na^+,K^+-ATPase and the $Na^+/K^+/Cl^-$ cotransporter by insulin remains to be determined. Physiologically, sodium entry through the $Na^+/K^+/Cl^-$ cotransporter may compensate its increased efflux through the Na^+,K^+-ATPase. In this respect, activation of the cotransporter may represent an homeostatic mechanism capable of preventing excessive changes in intracellular $[Na^+]$ and volume following activation of the Na^+,K^+-ATPase by insulin. This is consistent with the data presented in Fig. 5 showing only a minor decrease in intracellular $[Na^+]$ in cells stimulated by insulin. Activation of K^+ transport in human fibroblasts may be relevant to subsequent actions of insulin. Stimulation of the Na^+,K^+-ATPase could hyperpolarize the cell membrane and contribute to enhance the activity of other transport system driven by the electrochemical gradient of Na^+, such as amino acid transport system A [40]. On the other hand, stimulation of the bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransporter is essential for the proliferation of human fibroblasts [41] and it may contribute to the mitogenic action of insulin. It will be of interest to determine if insulin stimulation of the cotransporter or regulation of Na^+/K^+ homeostasis is impaired in fibroblasts with mutant insulin receptor and defective insulin stimulation of cell proliferation [42].

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