Intracellular Ca\textsuperscript{2+} and Mg\textsuperscript{2+} Regulation for Insulin-Stimulated Glucose Uptake into Mouse Diaphragm Muscles

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ABSTRACT—We investigated whether the changed levels of intracellular Ca\textsuperscript{2+} and Mg\textsuperscript{2+} in mouse skeletal muscles affected insulin-stimulated glucose uptake. Insulin alone had no effect on \textsuperscript{45}Ca\textsuperscript{2+} efflux and uptake. A23187 at 20 \mu M increased \textsuperscript{45}Ca\textsuperscript{2+} influx and inhibited insulin-stimulated \textsuperscript{14}C-glucose uptake with normal external concentrations of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} or in the absence of both cations. These results suggest that the increase in Ca\textsuperscript{2+} influx into, and in Mg\textsuperscript{2+} efflux from, skeletal muscles may inhibit insulin-stimulated glucose uptake.

The roles of intracellular Ca\textsuperscript{2+} in insulin-stimulated glucose uptake in skeletal muscle is controversial. Ca\textsuperscript{2+} ion is a mediator of insulin action in skeletal muscle (1), but does not mediate insulin-stimulated 2-deoxyglucose uptake in rat L6 skeletal muscle cell line (2). This discrepancy may depend on intracellular Ca\textsuperscript{2+} concentrations in skeletal muscles because the optimal concentration is required for insulin-stimulated glucose uptake in rat adipocytes (3). Mg\textsuperscript{2+} ion also plays an important role in insulin action because intracellular Mg\textsuperscript{2+} is involved in insulin-stimulated 3-O-methylglucose transport in rat cardiocytes (4). The aim of the present study is to investigate the effect of intracellular Ca\textsuperscript{2+} and Mg\textsuperscript{2+} levels changed by A23187 on insulin-stimulated glucose uptake in mouse diaphragm muscle.

Male ddY mice (weighing 21.0 – 36.0 g, 5 – 9 weeks-old) were fasted for 18 hr and decapitated. Diaphragm muscles were removed from ribs and central tendon; and then they were cut into 8 segments in ice-cold Krebs Ringer bicarbonate (KRB) solution (118.5 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl\textsubscript{2}, 1.3 mM KH\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgSO\textsubscript{4} and 25.0 mM NaHCO\textsubscript{3}, pH 7.3) which contained 2.8 mM glucose and was saturated with 95% O\textsubscript{2} and 5% CO\textsubscript{2}.

Diaphragm muscles were preloaded for 10 min at 37\textdegree C with 14.8 kBq/ml \textsuperscript{45}CaCl\textsubscript{2} (0.37 – 1.5 GBq/mg calcium, Amersham, Tokyo, Japan) in KRB solution containing 0.5% bovine serum albumin (BSA, Sigma, St. Louis, MO, U.S.A.) and 8.3 mM glucose. Then the muscles were washed, incubated for 10 min, 30 min or 1 hr at 37\textdegree C in KRB solution containing 0.5% BSA and 8.3 mM glucose with or without 1 \mu g/ml insulin (Shimizu Pharmaceutical), chilled, washed 4 times with ice-cold KRB solution, weighed, and dissolved in Protosol (New England Nuclear, MA, U.S.A.). The radioactivity was determined with a liquid scintillation spectrometer (Packard, U.S.A.). \textsuperscript{45}Ca\textsuperscript{2+} efflux from dia-

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Phragm muscle segments was also determined with the spectrometer. The percentage of $^{45}\text{Ca}^{2+}$ efflux was expressed as (dpm $^{45}\text{Ca}^{2+}$ efflux)/(dpm residual in muscles + dpm $^{45}\text{Ca}^{2+}$ efflux) $\times$ 100.

Diaphragm muscle segments were incubated for 1 hr at 37°C with 14.8 kBq/ml $^{45}\text{CaCl}_2$ or 14.8 kBq/ml $^{14}\text{C}$-glucose (9.99 MBq/$\mu$mol, Amersham Japan) in KRB solution containing 0.5% BSA, 8.3 mM glucose in the presence or absence of 20 $\mu$M A23187 (Sigma), or 100 ng/ml insulin inducing maximal glucose uptake. A23187 was dissolved in ethanol and then diluted to 0.5% ethanol solution with KRB solution. The statistical significance was assayed by the unpaired Student's t-test at $P < 0.01$ or 0.05.

Insulin stimulates Ca$^{2+}$ efflux from rat skeletal muscles (5), where a high concentration (4 $\mu$g/ml) of insulin is used for long incubation time (3 hr). In the present study, 64.3% of the preloaded $^{45}\text{Ca}^{2+}$ (1581 dpm/mg wet wt.) was effluxed from diaphragm muscles in the absence of insulin. One $\mu$g/ml insulin did not increase $^{45}\text{Ca}^{2+}$ efflux for 10, 30 min (data not shown) or 1 hr (Fig. 1a). A23187 at 20 $\mu$M significantly increased, but insulin at 100 ng/ml did not affect, $^{45}\text{Ca}^{2+}$ uptake into diaphragm muscles (Fig. 1b).

Insulin at 100 ng/ml without A23187 stimulated $^{14}\text{C}$-glucose uptake into diaphragm muscles in normal (2.5 mM Ca$^{2+}$) solution and 5 mM Ca$^{2+}$-KRB solution (data not shown). Twenty micromolar A23187, though having no effect alone, inhibited insulin-stimulated glu-
cose uptake by 89% (Fig. 2a). Insulin at 100 ng/ml also significantly stimulated 14C-glucose uptake without external Ca2+, independently of the presence or absence of 20 μM A23187 (Fig. 2a). However, insulin did not stimulate 14C-glucose uptake with normal external Ca2+ (2.5 mM) in the presence of A23187. These results suggested that the inhibition of insulin action by A23187 is due to Ca2+ uptake into diaphragm muscles.

Insulin at 100 ng/ml stimulated 14C-glucose uptake either without external Mg2+ or without both Mg2+ and Ca2+ (Fig. 2b). In the presence of A23187 without extracellular Mg2+ and Ca2+, the basal level of glucose uptake was suppressed, and insulin could not stimulate the glucose uptake to any extent (Fig. 2b). The effect of A23187 on insulin-stimulated glucose uptake was not investigated with 2.5 mM Ca2+ and 0 mM Mg2+ because it may be expected that the uptake would also be suppressed under these conditions. These evidence suggest that the insulin effect on glucose uptake is dependent on intracellular Mg2+.

The present study demonstrated that Ca2+ efflux or Ca2+ uptake was not changed by insulin, suggesting no linkage to insulin signaling for stimulating glucose uptake in skeletal muscles as reported by Klip et al. (2). These results are not consistent with those by Bihler et al. (6), suggesting that A23187 may release Ca2+ from intracellular storage and binding sites on the sarcoplasmic reticulum in rat diaphragm muscles (6). The complete inhibition by A23187 of insulin-stimulated glucose uptake at 2.5 mM Ca2+ were not due to Ca2+ from the sarcoplasmic reticulum but due to extracellular Ca2+, because A23187 did not inhibit insulin-stimulated glucose uptake in the absence of external Ca2+.

Insulin-stimulated glucose uptake depends on activation of glucose transporters. The glucose transporter in skeletal muscles is the same type of GLUT4 as in adipose tissue (7). Insulin-stimulated glucose uptake is due to the activation of insulin receptor tyrosine kinase in Chinese hamster ovary cells (8). Insulin receptor interacts with protein kinase C. Protein kinase C phosphorylates insulin receptor and decreases tyrosine-specific protein kinase activity in rat hepatoma cells (9). However, increase in intracellular Ca2+ in adipocytes decrease insulin receptor kinase activity and insulin-stimulated 2-deoxyglucose uptake, independently of protein kinase C activation (10). An abnormal increase in Ca2+ is observed in diabetic skeletal muscles (11), where Ca2+-activated neutral protease activity is also increased (12). Increased cytosolic Ca2+ in obesity or hyperglycemia inhibits insulin-stimulated 2-deoxyglucose uptake in rat adipocytes (13). Atomic absorption spectrometry demonstrated that A23187, an ionophore for Mg2+, in addition to Ca2+, increases Mg2+ efflux from isolated soleus muscles without extracellular Mg2+ and Ca2+ (14). Mg2+ is also involved in the phosphorylation reaction, and insulin stimulates the phosphorylation of tyrosine-specific protein kinase on the insulin receptor with Mg2+ in rat liver (15). The decrease in intracellular Mg2+ level may impair insulin receptor kinase activity, and the increase in intracellular Ca2+ level may prevent insulin receptor kinase from playing its functional role.

In conclusion, the increase in intracellular Ca2+ level and the decrease in intracellular Mg2+ level inhibit insulin-stimulated glucose uptake in mouse diaphragm muscles.

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