Cardiotonic Steroids Stimulate Glycogen Synthesis in Human Skeletal Muscle Cells via a Src- and ERK1/2-dependent Mechanism*

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The cardiotonic steroid, ouabain, a specific inhibitor of Na+,K+-ATPase, initiates protein-protein interactions that lead to an increase in growth and proliferation in different cell types. We explored the effects of ouabain on glucose metabolism in human skeletal muscle cells (HSMC) and clarified the mechanisms of ouabain signal transduction. In HSMC, ouabain increased glycogen synthesis in a concentration-dependent manner reaching the maximum at 100 nM. The effect of ouabain was additive to the effect of insulin and was independent of phosphatidylinositol 3-kinase concentration, similarly to 100 nM ouabain. In conclusion, ouabain treatment decreased the surface abundance of α2-subunit, whereas abundance of the α1-subunit was unchanged. Marinobufagenin, an endogenous vertebrate bufadienolide cardiotonic steroid, increased glycogen synthesis in HSMC at 10 nM concentration, similarly to 100 nM ouabain. In conclusion, ouabain and marinobufagenin stimulate glycogen synthesis in skeletal muscle. This effect is mediated by activation of a Src-, ERK1/2-, p90rsk- and GSK3-dependent signaling pathway.

For almost 200 years cardiotonic steroids (CTS)2 extracted from Digitalis purpurea were successfully used to treat patients with heart failure. Ouabain is one of the cardiac glycosides (obtained from the seeds of Strophanthus gratus), which specifically binds to and inhibits the activity of Na+,K+-ATPase, a plasma membrane cation pump, which is essential for maintenance of intracellular and extracellular sodium and potassium concentrations, cell volume, osmotic balance, and electrochemical gradients (1, 2). In addition to the cardiac glycosides of plant origin, the endogenous CTS were recently described as a new class of steroid hormones, endogenously produced in mammalian adrenal glands and central nervous system (3). Endogenous ouabain (EO) has been isolated as a stereoisomer of ouabain and identified as a constituent of human blood, bovine adrenal glands, and hypothalamus (3, 4) and circulates in elevated concentrations in the blood of 50% of Caucasians with high blood pressure (5). Bovine adrenal cortical cells in tissue culture release EO in response to norepinephrine, corticotropin, and angiotensin II (6, 7). Later, another mammalian endogenous CTS, a bufadienolide marinobufagenin (MBG), was found in human urine and plasma (8, 9). The plasma levels of MBG become elevated in several volume-expanded hypertensive states (10, 11). This hormone exhibits in vivo vasoconstrictor and natriuretic effects (10, 11). Importantly, exercise and stress cause an acute rise in the circulating levels of EO (12). Recent evidence from genetically engineered mice with modified cardiac glycoside binding affinity of the α1- and α2-subunits isoforms of the Na+,K+-ATPase indicate that the cardiac glycoside-binding site, which mediates the pharmacological effects of digitalis, is also the receptor for endogenous CTS (13).

Digitalis drugs appear to promote cardiac hypertrophy (14, 15). Similarly to other hypertrophic stimuli, ouabain regulates transcription of several hypertrophic marker genes in cardiac myocytes (16, 17). Recent evidence shows that in addition to a role in ion transport function, Na+,K+-ATPase can sense low concentrations of ouabain and play an important role as a signal transducer (18). Binding of Src to Na+,K+-ATPase forms a functional signaling complex (19). In cardiac myocytes, interaction of ouabain with the Na-pump causes activation of a Src, Ras/Raf, p42/44 MAPK signaling pathway, increases [Ca2+]i, generates reactive oxygen species in mitochondria, and acti-

PKC, protein kinase C; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; CaMK, calmodulin kinase; AMPK, AMP-activated protein kinase.

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‡ The abbreviations used are: CTS, cardiotonic steroid(s); EO, endogenous ouabain; HSMC, human skeletal muscle cells(s); GSK, glycogen synthase kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MBG, marinobufagenin; PI, phosphatidylinositol;
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Ouabain, generates slow Ca2+

sitol 1,4,5-trisphosphate receptor, which in the presence of

under physiological conditions. Human

much higher than concentrations of endogenous CTS observed

of ouabain used in experiments with rat muscles (21) were

form is resistant to ouabain (23). Therefore, the concentrations

p90rsk was a kind gift from Dr. D. Alessi (University of Dundee,

nyl-4H-1-benzopyran-4-one) were from Calbiochem. Rabbit

and PI 3-kinase inhibitor LY294002 (2-(4-morpholinyl)-8-phe-

5-[4-chlorophenyl]-7-[t-butyl] pyrazolo[3,4-d]-pyrimidine),

antibodies to phospho-ERK1/2 (Tyr204) and phospho-Akt/PKB

naling Technology, Inc. (Beverly, MA). Rabbit polyclonal

was from BD Transduction Laboratories. Mouse monoclonal

Rabbit polyclonal antibody to c-Src was from Santa Cruz Bio-

(Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phos-

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EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Ouabain was obtained from Sigma. Marinobufagenin was purified from Bufo marinus toad venom, as described previously (26). Insulin (Actrapid) was from Novo Nordisk (Denmark). MEK1/2 inhibitor PD98059 (2'-amino-3'-methoxystilavone), c-Src inhibitor PP2 (4-amino-5-[4-cholorophenyl]-7-[t-butyl] pyrazolo[3,4-d]-pyrimidine), and PI 3-kinase inhibitor LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) were from Calbiochem. Rabbit polyclonal antibodies to phospho-GSK3α/β (Ser21 of GSK3α and Ser9 of GSK3β), phospho-c-Src (Tyr416), phospho-p90rsk (Thr387), phospho-PKA/β (Thr286/287), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-AMPKα (Thr172), phospho-CaMKII (Thr286), and monoclonal antibody to phospho-Thr-Pro motif were from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit polyclonal antibodies to phospho-ERK1/2 (Thr202 and phospho-Akt/PKB (Ser473) were from New England Biolabs Inc. (Beverly, MA). Rabbit polyclonal antibody to c-Src was from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibody to phospho-Test was from BD Transduction Laboratories. Mouse monoclonal and rabbit polyclonal antibodies to α1-subunit of Na+,K+-ATPase were a kind gift from Dr. M. Caplan (Yale University, New Haven, CT). Mouse monoclonal antibody to α1-subunit of Na+,K+-ATPase was a kind gift from Dr. K. Sewardner (Massachusetts General Hospital, Charlestown, MA), rabbit polyclonal antibody to α2-subunit of Na+,K+-ATPase was the kind gift from Dr. T. Pressley (Texas Tech University Health Sciences Center, Lubbock, TX). Rabbit polyclonal antibody to p90rsk was a kind gift from Dr. D. Alessi (University of Dundee, Dundee, Scotland). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G was obtained from Bio-Rad. Protein A-Sepharose CL-4B and horseradish peroxidase-linked protein A were from Amersham Biosciences. Protein-L-agarose and protein G-Sepharose were from Sigma. Reagents for enhanced chemiluminescence were obtained from Amersham Biosciences. Streptavidin-agarose beads and EZ-link Sulfo-NHS-SS-biotin were from Pierce. Cross-tide (Gly-Arg-Pro-Arg-Thr-Ser-Ser-Phe-Ala-Glu-Gly) was from Sigma-Aldrich. Cell culture media and reagents were from Invitrogen. Dimethyl sulfoxide (Calbiochem) was used as a sol-

vent for protein kinases inhibitors. All other reagents were of

analytical grade (Sigma).

Cell Culture—Human skeletal muscle satellite cells were iso-

lated from muscle biopsies and cultured, as previously described (27). The experiments were performed on passages 3 and 4. To initiate differentiation into myotubes, Ham's F-10 medium with 20% FBS was removed from cells, and DMEM containing 1% PeSt (100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen)) and 4% FBS were added for 48 h. The medium was changed to DMEM containing 1% PeSt and 2% FBS. Fusion and multinucleation of the cells was observed at day 3 after initiation of the differentiation protocol. Glucose uptake in HSMC was measured, as previously described (28).

Glucose Incorporation into Glycogen—Myoblasts were seeded 1500 cells/well in 6-well plates and differentiated at 70 – 80% confluence. Differentiated myotubes (5–7 days) were serum-starved (DMEM + 0% FBS) overnight prior to the experiment to reduce the basal level of insulin- and cytokine-dependent kinase activity. Cells were preincubated with 1) 22 µM PD98059, 20 µM PP2, 10 µM LY294002, or Me2SO for 20 min, then incubated with 100 nM ouabain for 10 min, and finally stimulated with 120 nM insulin for 20 min at 37 °C in 950 µl of serum-free DMEM; 2) cells were incubated with ouabain or marinobufagenin for 30 min; or 3) cells were preincubated with 22 µM PD98059, 20 µM PP2, 10 µM LY294002, or Me2SO for 20 min, and then ouabain, marinobufagenin, insulin or Me2SO were added. Thereafter, 50 µl of the isotope solution (p-[U-14C] glucose with 1 µCi/ml; final specific activity, 0.18 µCi/µmol in DMEM) was added, and the plates were incubated for 30 min. The reactions were terminated by placing the plates on ice. The medium was aspirated, and the wells were washed with ice-cold PBS three or four times. The plates were frozen directly after at −80 °C, or myotubes were solubilized with 1 ml 0.03% SDS for 1 h at room temperature. Aliquots (0.85 ml) of the suspension was transferred to 10-ml tubes, and 100 µl (2 mg) of carrier glycogen was added. The remained suspension was used for protein concentration determination. The samples were boiled for 30 min. A 3-ml solution of 98% ethanol was added to precipitate glycogen. Tubes were incubated overnight at 4 °C with slight agitation and centrifuged at 5000 × g for 35 min at 4 °C. Pellet was washed once with 70% ethanol, samples were centrifuged at 5000 × g for 10 min, and ethanol was aspirated off. The pellet was solubilized in 200 µl of distilled H2O and transferred to 4-ml scintillation vials. Samples and aliquots of the media were counted in a liquid scintillation counter (1214 Rackbeta, Wallac, Turku, Finland).
Measurement of Ouabain-sensitive $^{86}\text{Rb}^+$ Uptake—The initial rate of ouabain- or marinobufagenin-sensitive $^{86}\text{Rb}^+$ uptake through Na$^+$,K$^+$-ATPase of HSMC was measured as previously described (29). Uptake that was inhibited by 100 $\mu$M ouabain was taken as the maximal rate of active uptake.

Western Blot Analysis—HSMC were lysed in 500 $\mu$l of homogenization buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 20 mM Tris, pH 8.0, 1% Triton X-100, 10% (v/v) glycerol, 10 mM NaF, 0.5 mM Na$_3$VO$_4$, 5 $\mu$g/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 5 $\mu$g/ml aprotinin, and 1 $\mu$M microcystin) on the rotation wheel at 4 $^\circ$C during 1 h. Then samples were centrifuged at 12,000 $\times g$ for 10 min, 4 $^\circ$C. The supernatants were collected, and the protein concentration was measured, using BCA™ protein assay kit (Pierce). The samples were prepared for SDS-PAGE (7.5 or 10% resolving gel). The proteins were transferred to polyvinylidene difluoride membranes and blocked with 7.5% nonfat milk in Tris-buffered saline with Tween 20. The membranes were incubated with primary antibodies overnight at 4 $^\circ$C on a shaking platform. The membranes were washed with Tris-buffered saline with Tween 20 and incubated with anti-rabbit or anti-mouse secondary antibody or protein A conjugate with horseradish peroxidase. The proteins were visualized by ECL and quantified by densitometry.

Immunoprecipitation—Cell lysates (500 $\mu$l) were incubated with 40 $\mu$l of protein A-Sepharose beads, with rotation for 30 min at 4 $^\circ$C. After brief centrifugation, the supernatants were collected and immunoprecipitated with 1) antibodies to c-Src overnight at 4 $^\circ$C and 2) antibodies to p–Tyr overnight at 4 $^\circ$C. The immunoprecipitates were collected on protein A-Sepharose beads for 2 h at 4 $^\circ$C. HSM myotubes lysates (300 $\mu$g of protein) were immunoprecipitated for 2.5 h at 4 $^\circ$C with anti-phospho-Thr-Pro mouse IgM. Immunoprecipitates were collected on protein–L–agarose beads. For immunoprecipitations, the beads were washed three times in homogenization buffer and twice in ice-cold PBS. The pellets were resuspended in Laemmli sample buffer.

Cell Surface Biotinylation—Myotubes (6 day) were preincubated in PBS in the absence or presence of 100 $\mu$M ouabain for 1 h and thereafter exposed to EZ-link Sulfo-NHS-SS-biotin at a final concentration of 1.5 mg/ml in PBS at 4 $^\circ$C for 60 min with gentle shaking. Cell surface biotinylation was performed as described (27). After streptavidin precipitation, the samples were analyzed by SDS-PAGE with subsequent Western blotting with appropriate antibodies.

p90rsk Activity Assay—Cultured myotubes treated with 100 $\mu$M ouabain for 2 h in the presence or absence of inhibitors PD98059 and PP2 were analyzed. Myotube lysates (100 $\mu$g of protein/sample) were immunoprecipitated at 4 $^\circ$C overnight with anti-p90rsk antibody, previously equilibrated with protein G-Sepharose in homogenization buffer. Immunoprecipitates were washed three times in homogenization buffer, containing 0.5 M NaCl and twice in buffer B (50 mM Tris–HCl, pH 7.5, 0.03% Brij-35, 0.1 mM EGTA, 0.1% β-mercaptoethanol). The samples were resuspended in 30 $\mu$l of kinase buffer (50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 17 $\mu$M cAMP, 1 mM ATP, and 1 mM [γ-32P]ATP) and incubated at 30 $^\circ$C for 10 min. The reactions were terminated on ice by the addition of sample buffer (125 mM Tris, 6 M urea, pH 6.8). Reaction products were resolved on a 40% acrylamide gel, and 32P incorporation into peptide substrate was analyzed by exposing gels to a phosphorimaging device (Fuji BAS-1800II).

Statistics—The data are presented as the means $\pm$ S.E. Comparisons between groups were performed using Student’s t test. Significance was established at $p < 0.05$.

RESULTS

Effect of Ouabain on Glycogen Synthesis and Cell Signaling—HSMC were incubated with different concentrations of ouabain (10 nm, 100 nm, and 1 $\mu$m) for 30 min. [U-14C]Glucose incorporation into glycogen was measured as described under “Experimental Procedures.” The values are the means $\pm$ S.E. (n = 6). *, $p < 0.05$ versus basal. B, dose-dependent inhibition of total $^{86}\text{Rb}^+$ uptake by ouabain. The assay was performed as described under “Experimental Procedures.” The results are the means $\pm$ S.E. for six independent experiments performed in duplicate. *, $p < 0.05$ versus control.

C, phosphorylation of the protein kinases c-Src, Akt/PKB, PKCα/β, c-JNK, p38 MAPK, ERK1/2, p90rsk, GSK3α/β, AMPK, and CaMKII in response to ouabain in HSMC. Representative blots from four independent experiments are shown.

Measurement of Ouabain-sensitive $^{86}\text{Rb}^+$ Uptake

Western Blot Analysis

Immunoprecipitation

Cell Surface Biotinylation

p90rsk Activity Assay

Figure 1. Dose-dependent response of HSMC to ouabain. Human skeletal muscle cells were incubated with increasing concentrations of ouabain (10 nm, 100 nm, and 1 $\mu$m) for 30 min. A, [U-14C]Glucose incorporation into glycogen was measured as described under “Experimental Procedures.” The values are the means $\pm$ S.E. (n = 6). *, $p < 0.05$ versus basal. B, dose-dependent inhibition of total $^{86}\text{Rb}^+$ uptake by ouabain. The assay was performed as described under “Experimental Procedures.” The results are the means $\pm$ S.E. for six independent experiments performed in duplicate. *, $p < 0.05$ versus control. C, phosphorylation of the protein kinases c-Src, Akt/PKB, PKCα/β, c-JNK, p38 MAPK, ERK1/2, p90rsk, GSK3α/β, AMPK, and CaMKII in response to ouabain in HSMC. Representative blots from four independent experiments are shown.
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Figure 2. Na\(^{+}\),K\(^{+}\)-ATPase \(\alpha\)-subunits tyrosine phosphorylation and interaction with Src in response to ouabain. HSMC were incubated in the presence or absence of 100 nm ouabain and inhibitors PD98059 (22 \(\mu\)M) and PP2 (20 \(\mu\)M) under basal (□) or insulin-stimulated (●) (120 nm) conditions. The cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies and blotted with antibodies against \(\alpha\)- and \(\alpha\)-subunit of Na\(^{+}\),K\(^{+}\)-ATPase as described under "Experimental Procedures." Representative Western blots (upper panels) and quantitative data (means ± S.E.) (lower panels) for \(\alpha\)-subunit (A) and \(\alpha\)-subunit (B) are shown (n = 5). Na\(^{+}\),K\(^{+}\)-ATPase \(\alpha\)-subunits co-immunoprecipitate with anti-Src antibodies. The cell lysates were precipitated with anti-Src antibodies and blotted with antibodies against \(\alpha\)- and \(\alpha\)-subunit of Na\(^{+}\),K\(^{+}\)-ATPase as described under "Experimental Procedures." Representative Western blots (upper panels) and quantitative data (means ± S.E.) (lower panels) for \(\alpha\)-subunit (C) and \(\alpha\)-subunit (D) are shown (n = 5). *p < 0.05 versus basal without ouabain; †, p < 0.05 versus insulin-stimulated without ouabain; #, p < 0.05 versus ouabain without the inhibitor.

Kinases previously implicated in ouabain-induced signal transduction (18) and regulation of glycogen synthesis (30). Phosphorylation of Src, ERK1/2, p90rsk, and GSK3α/β increased in a dose-dependent manner in response to ouabain, reaching the maximum at 100 nm after a 30-min incubation, whereas phosphorylation of the stress-activated MAPKs, c-JNK and p38 was unchanged. Ouabain (100 nm) stimulates Src, ERK1/2, p90rsk, and GSK3 phosphorylation already after 15 min of incubation (data not shown). Notably, phosphorylation of Akt/PKB was unaffected by ouabain. PKCα/β phosphorylation was slightly decreased in the presence of 100 nm or higher concentration of ouabain (Fig. 1C). Because ouabain inhibition of the Na\(^{+}\),K\(^{+}\)-ATPase may lead to local changes in ATP concentrations and an increase in [Ca\(^{2+}\)], we assessed also phosphorylation of AMPK and CaMKII in response to ouabain. AMPK and CaMKII phosphorylation was unchanged in response to ouabain. Thus, in HSMC ouabain, in concentrations that are unable to significantly decrease Na-pump activity, stimulates glycogen synthesis, activates c-Src, ERK1/2-p90rsk, and leads to phosphorylation of GSK3α/β. Based on the results, ouabain at a concentration of 100 nm was used for stimulation of human muscle cells studying subsequent experiments.

Ouabain Stimulates Tyrosine Phosphorylation of Na\(^{+}\),K\(^{+}\)-ATPase and Enhances Src Interaction with Na\(^{+}\),K\(^{+}\)-ATPase \(\alpha\)-Subunits—To further investigate whether Src interacts with Na\(^{+}\),K\(^{+}\)-ATPase to form a signaling complex, whether Src activation by ouabain promotes phosphorylation of Na\(^{+}\),K\(^{+}\)-ATPase, and whether the phosphorylation by Src or ERK1/2 is important for the complex formation, we incubated HSMC with 100 nm ouabain in the absence and presence of 120 nm insulin and inhibitors of Src (PP2) and MEK1/2 (PD98059). We assessed tyrosine phosphorylation of Na-pump \(\alpha\)-subunits by immunoprecipitation with anti-phosphotyrosine antibody and performed subsequent Western blot analysis with antibodies against \(\alpha\)- and \(\alpha\)-subunits. As expected, insulin stimulates tyrosine phosphorylation of \(\alpha\)- and \(\alpha\)-subunits (Fig. 2, A and B). However, tyrosine phosphorylation of the \(\alpha\)- and \(\alpha\)-subunits was dramatically increased by ouabain, additively to the effect of insulin. Tyrosine phosphorylation of Na\(^{+}\),K\(^{+}\)-ATPase \(\alpha\)-subunits was completely blocked by the inhibition of Src (PP2) and was unaffected by inhibition of MEK1/2 (PD98059) (Fig. 2, A and B).

To assess interaction of Src with Na\(^{+}\),K\(^{+}\)-ATPase, we immunoprecipitated Src from cell lysates and analyzed co-immunoprecipitated Na\(^{+}\),K\(^{+}\)-ATPase \(\alpha\)-subunits by Western blot. The amount of immunoprecipitated Src was unaffected by conditions during cell stimulation (data not shown). \(\alpha\)- and \(\alpha\)-subunits were co-immunoprecipitated with c-Src at the basal, unstimulated condition. Insulin increased the amount of \(\alpha\)-subunits co-immunoprecipitated with Src (Fig. 2, C and D). Co-immunoprecipitation of the \(\alpha\)- and \(\alpha\)-subunits of the Na-pump with Src was markedly increased after stimulation with ouabain, in the absence or presence of insulin. This effect of ouabain was diminished by PP2 and was unaffected by PD98059. Taken together, these findings suggest that ouabain stimulates Na\(^{+}\),K\(^{+}\)-ATPase \(\alpha\)-subunit phosphorylation on tyrosine residues and facilitates complex formation of Na\(^{+}\),K\(^{+}\)-ATPase with Src. The Na\(^{+}\),K\(^{+}\)-ATPase-
Ouabain-stimulated Signal Transduction toward Glycogen Synthesis—Stimulation of HSMC with ouabain leads to an increase in the phosphorylation of GSK3 (Ser\(^{21}\) of GSK3\(\alpha\) and Ser\(^{9}\) of GSK3\(\beta\)) (Fig. 1A). The inhibitory phosphorylation of GSK3 promotes the dephosphorylation and activation of glycogen synthase (31). Intriguingly, ouabain stimulates glycogen synthesis in HSMC without stimulation of Akt, a known kinase for GSK3 in skeletal muscle (Fig. 1, A and D). The PI 3-kinase-Akt/PKB signaling pathway is the major pathway regulating glycogen synthesis in skeletal muscle. To test the involvement of the PI 3-kinase signaling pathway, we studied the effect of ouabain on glycogen synthesis in the presence or absence of the PI 3-kinase inhibitor, LY294002. As expected, LY294002 decreased basal and completely blocked insulin-stimulated [\(^{14}\)C]glucose incorporation into glycogen (Fig. 3A). Incubation with ouabain stimulated glycogen synthesis additively to insulin. Importantly, LY294002 inhibited only the insulin-stimulated component of this additive effect (Fig. 3A). Basal and insulin-stimulated Akt/PKB (Ser\(^{473}\)) phosphorylation was unaffected by ouabain (Fig. 3B). Incubation of HSMC with 100 nM ouabain did not affect glucose uptake under basal (7.03 ± 0.83 \text{ pmol} \times \text{mg of protein}\(^{-1} \times \text{min}\(^{-1}\)) or insulin-stimulated (13.61 ± 1.08 \text{ pmol} \times \text{mg of protein}\(^{-1} \times \text{min}\(^{-1}\)) conditions. These findings suggest that the PI 3-kinase signaling pathway is not involved in ouabain-induced stimulation of glycogen synthesis in HSMC.

GSK3 can also be phosphorylated by a downstream kinase of classical MAPK cascade, p90rsk (MAPKAP-K1) (30), which is phosphorylated by ERK1/2. In HSMC, insulin increases ERK1/2 phosphorylation and p90rsk activity. The effect of insulin was abolished by the MEK1/2 inhibitor PD98059 and was unaffected by the Src inhibitor PP2 (Fig. 4, A and B). Additional stimulation with ouabain leads to an increase in basal and insulin-stimulated ERK1/2 phosphorylation and p90rsk activity (Fig. 4, A and B). The effect of ouabain on MAPK activation was additive to the effect of insulin. Preincubation of insulin- and/or ouabain-stimulated myotubes with PD98059 abolished all stimulatory effects, whereas incubation with PP2 restored ERK1/2 phosphorylation and p90rsk activity to levels achieved without ouabain. These results indicate that in HSMC, p90rsk can be activated by insulin or, independently and additively to insulin, by ouabain via c-Src stimulation.

As expected, insulin stimulates GSK3 phosphorylation and increases [\(^{14}\)C]glucose incorporation into glycogen. PD98059 and PP2 had no effect on GSK3 phosphorylation and glycogen synthesis under basal and insulin-stimulated conditions (Fig. 4, C and D). Ouabain (100 nM) profoundly stimulated GSK3 phosphorylation and increased [\(^{14}\)C]glucose incorporation into glycogen under basal and insulin-stimulated conditions, and this effect was additive to the effect of insulin. The effect of ouabain on GSK3 phosphorylation and glycogen synthesis was abolished by PD98059 and PP2 (Fig. 4D).

Our findings provide evidence to suggest that in human differentiated myotubes, glycogen synthesis can be activated by insulin via PI 3-kinase-Akt pathway or independently to insulin action by ouabain via PI 3-kinase-independent, Src-activated MAPK signaling cascade.

Effect of Marinobufagenin on HSMC—To evaluate the physiological significance of ouabain-induced stimulation of glycogen synthesis in HSMC, it was of interest to compare the effect of ouabain with an effect of an endogenous CTS. Human myotubes were incubated with an endogenous bufadienolide, MBG. MBG (1 nM) insignificantly (17% above control) increased glycogen synthesis, whereas 10 nM MBG stimulated [\(^{14}\)C]glucose incorporation into glycogen similarly to 100 nM ouabain (Fig. 5A). Increased concentrations of MBG did not further increase glycogen synthesis (data not shown). MBG at 1 and 10 nM inhibited total ouabain-sensitive \(^{86}\)Rb\(^{+}\) uptake 15 and 21%, respectively (Fig. 5B). This inhibition was similar to that of 100 nM ouabain (Fig. 1B). Western blot analysis of lysates prepared from HSMC incubated for 30 min with 10 nM MBG and 100 nM ouabain revealed a similar increase in the phosphorylation of c-Src, ERK1/2, p90rsk, and GSK3a/b (Fig. 5C). Akt/PKB phosphorylation was unaffected by MBG. In contrast to ouabain, PKCa\(/\beta\) phosphorylation was unaltered in the presence of 10 nM MBG. Thus, in human myotubes, the endogenous bufadienolide CTS, MBG, exhibits an effect similar to that of ouabain, but at lower range of concentrations.

Cell Surface Abundance of Na-pump Subunits in Response to Ouabain—In our previous study (29), insulin increased the phosphorylation of the \(\alpha\)-subunits of Na-pump in an ERK1/2-dependent manner. Because ouabain signal transduction involves activation of the MAPK pathway, we determined whether the human Na-pump is phosphorylated by ERK1/2. HSMC lysates were immunoprecipitated with an antibody against phospho-Thr-Pro motif, which is an ERK phosphorylation site, and thereafter the samples were blotted with antibodies against \(\alpha_{1}\)- and \(\alpha_{2}\)-subunits of Na\(^{+},K\(^{+}\)-ATPase. Phosphorylation of the \(\alpha\)-subunits was significantly increased in response to 100 nM ouabain (Fig. 6A).
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ERK1/2 phosphorylation of Na⁺,K⁺-ATPase α-subunits regulates Na-pump cell surface abundance (29, 32). We utilized a biotinylation technique to assess the cell surface abundance of Na-pump α-subunits in response to ouabain. We have shown that after 1 h of incubation of cells with ouabain, the cell surface content of the α₂-subunit is significantly decreased, whereas the cell surface abundance of the α₁-subunit was unaffected (Fig. 6B). Inhibition of MEK1/2 by PD98059 decreased the Na-pump α₂-subunit cell surface abundance under basal conditions and enhanced the ouabain-stimulated disappearance of the α₂-subunit from the plasma membrane (Fig. 6B), suggesting that ERK1/2-mediated signaling is important for Na-pump endocytosis.

DISCUSSION

Ouabain is a cardiac glycoside and a specific inhibitor of Na⁺,K⁺-ATPase. A role for digitalis glycosides to improve cardiac contractility is well established, because inhibition of Na-pump leads to an increase in [Na⁺], and an elevation of [Ca²⁺], because of the activation of the Na⁺-Ca²⁺ exchanger (33, 34). Recent reports (16, 20, 35–38) provide evidence for a novel functionally different from the well characterized “low affinity” inhibitory binding site (40).

A biotinylation technique to assess the cell surface abundance of the Na-pump 2-subunit from the plasma membrane (Fig. 6B). Inhibition of MEK1/2 by PD98059 decreased the Na-pump α₂-subunit cell surface abundance under basal conditions and enhanced the ouabain-stimulated disappearance of the α₂-subunit from the plasma membrane (Fig. 6B), suggesting that ERK1/2-mediated signaling is important for Na-pump endocytosis.

FIGURE 4. ERK1/2 phosphorylation (A), p90rsk activity (B), GSK3 α/β phosphorylation (C), and glycogen synthesis (D) in HSMC under basal (■) or insulin-stimulated ( ) (120 nm) conditions in the presence or absence of 100 nm ouabain and inhibitors PD98059 and PP2. HSMC were incubated in the absence or presence of 100 nm ouabain and 22 μM PD98059 and 20 μM PP2 under basal or insulin-stimulated (120 nm) conditions. Western blot analysis of cell lysates, p90rsk activity assay, and [U-14C]glucose incorporation into glycogen were performed as described under “Experimental Procedures.” Representative blots and autoradiogram (upper panels) and quantitative data (means ± S.E.) (lower panels) are shown (n = 6). *p < 0.05 versus basal without ouabain; †p < 0.05 versus basal with ouabain

C-Src has been implicated as a key molecule in the ouabain signaling pathway (35, 41, 42). Co-immunoprecipitation of α₁- and α₂-subunits of Na⁺,K⁺-ATPase with Src is markedly increased in response to ouabain. Concomitantly, ouabain stimulates Src-dependent phosphorylation of α₁- and α₂-subunits on tyrosine residues. We hypothesize that tyrosine phosphorylation of the α-subunits facilitates the interaction between Na⁺,K⁺-ATPase and the photophosphorylase binding SH2 domain of Src, thereby leading to the formation of a signaling complex similar to a receptor-kinase complex of cytokine receptors. An interaction between Na⁺,K⁺-ATPase with Src homology domain-containing proteins has been reported (43, 44). In a pulldown assay, Na⁺,K⁺-ATPase preferably binds glutathione S-transferase fusion protein that contains the SH2 domain from Src (19). Abolishing Na⁺,K⁺-ATPase α-subunit tyrosine phosphorylation with PP2 eliminates the ouabain-stimulated Na⁺,K⁺-ATPase-Src association; however, some association remains equivalent in magnitude to control.
Ouabain Stimulates Glycogen Synthesis via ERK1/2

**FIGURE 5.** The effect of marinobufagenin on HSMC. Human skeletal muscle cells were incubated with 100 nM ouabain or 10 nM MBG. A, [U-14C]glucose incorporation into glycogen was measured as described under "Experimental Procedures." The values are the means ± S.E. (n = 5). *, p < 0.05 versus control. B, dose-dependent inhibition of total 86Rb uptake by MBG. The assay was performed as described under "Experimental Procedures." The results are the means ± S.E. for nine independent experiments performed in duplicate. *, p < 0.05 versus control. C, phosphorylation of the array of proteins: c-Src, Akt/PKB, PKCα/β, ERK1/2, p90rsk, GSK3α/β in response to MBG or ouabain in HSMC. Representative blots from four independent experiments are shown.

levels. This suggests that another yet unidentified sequence motif(s) or protein(s) is involved in the Na\(^{+},K^{+}\)-ATPase-Src signaling complex formation.

The lack of an effect of PD98059 on the ouabain-induced Src interaction with the α-subunits indicates that MAPK activation in response to ouabain is downstream from the signaling complex formation. ERK1/2 phosphorylates α-subunits on a Thr-Pro motif (29, 32) located next to the polyproline motif. The polyproline motif has been implicated to interact with the Src homology SH3 domain of PI 3-kinase p85α subunit (45). The Thr-Pro motif phosphorylation in response to ouabain (Fig. 6A) could be involved in the Na\(^{+},K^{+}\)-ATPase- Src interaction; however, the observed lack of effect of PD98059 on ouabain-induced Src interaction with the α-subunits provides evidence against such a hypothesis. Notably, the Na\(^{+},K^{+}\)-ATPase does not interact with a glutathione S-transferase fusion protein that contains the SH3 domain from Src (19). Thus, ouabain binding to plasma membrane Na-pump triggers a Na\(^{+},K^{+}\)-ATPase tyrosine phosphorylation and interaction of the pump with Src. This interaction constitutes one of the first signaling events in response to ouabain (18, 46). Differentiated human myotubes express α\(_{1}\)- and mostly α\(_{2}\)-subunits of Na-pump (28). Our data suggest that in HSMC, α-subunits can bind Src and serve as a receptors for ouabain.

The Src inhibitor PP2 blocks insulin- and ouabain-stimulated tyrosine phosphorylation of the pump α-subunits. Tyrosine phosphorylation of Na\(^{+},K^{+}\)-ATPase α\(_{1}\)- and α\(_{2}\)-subunits in response to insulin by an unidentified kinase has been previously reported (47, 48). Src phosphorylates the Na\(^{+},K^{+}\)-ATPase α-subunit in vitro (40, 49). Thus, the tyrosine kinase that phosphorylates the Na\(^{+},K^{+}\)-ATPase α-subunits in response to insulin can be Src. Interestingly, insulin-stimulated tyrosine phosphorylation of the Na\(^{+},K^{+}\)-ATPase α-subunit leads to an increase in the enzyme affinity to Na\(^{+}\) and increases pump activity. Therefore, tyrosine phosphorylation of α-subunit could explain the paradoxical increase in the pump activity in response to subnanomolar concentration of ouabain or endogenous CTS (40).

Activation of Src increases MAPK signaling (18). Src catalyzes the phosphorylation and activation of c-Raf, which leads to an activation of p42/p44 MAPK (50). In cardiac myocytes, ouabain increases MAPK signaling and thereby affects gene transcription and translation (18). Ouabain also acts as the potent promoter of growth via ERK1/2 activation in rat kidney epithelial cells (36). Ouabain stimulates ERK1/2 phosphorylation under basal and insulin-stimulated conditions in human skeletal muscle cells. ERK1/2 activation leads to activation of p90rsk, a downstream effector of MAPK (51). Similarly in HSMC, ouabain stimulates p90rsk activity and phosphorylation.

Serine residue near the amino terminus of GSK3 (Ser\(^{21}\) of GSK3α and Ser\(^{27}\) of GSK3β) are the main target of PKB/Akt. Active GSK3 (dephosphorylated) inhibits glycogen synthase. Phosphorylation and inhibition of GSK3 promotes dephosphorylation and activation of glycogen synthase and promotes glycogen synthesis. In addition to Akt/PKB, p70 ribosomal S6 kinase, and p90rsk phosphorylates GSK3α/β on Ser\(^{21,27}\) and inhibits GSK3 (52, 53). In Swiss 3T3 cells, phorbol myristate acetate and epididymal growth factor leads to phosphorylation and inhibition of GSK3 via activation of MAPK signaling pathway and p90rsk (54). Because ouabain-stimulated glycogen synthesis was unaffected by inhibition
of PI 3-kinase-Akt/PKB signaling pathway, with a profound increase in p90rsk activity, we hypothesize that in HSMC, ouabain can stimulate GSK3 phosphorylation and glycogen synthesis via p90rsk.

In human skeletal muscle cells, ouabain causes a dose-dependent increase in GSK3 phosphorylation on the Ser21/9 phosphorylation site at basal and insulin-stimulated conditions, concomitant with an increase in glycogen synthesis. Ouabain-stimulated GSK3 phosphorylation and glycogen synthesis was inhibited in the presence of the MEK1/2 inhibitor PD98059 and the Src inhibitor PP2. PKB/Akt phosphorylation was unaffected in response to ouabain, under basal and insulin-stimulated conditions. Thus, the present study extends the signaling paradigm of ouabain to include the p90rsk and GSK3 kinases. Moreover, in HSMC, ouabain-induced phosphorylation of GSK3, caused by activation of MAPK-p90rsk-signaling cascade, promotes glycogen synthesis (Fig. 7).

Exercise and stress cause an acute rise in EO circulating levels (55). EO release from the adrenal cortical cells is stimulated by angiotensin II and catecholamines. In humans, EO peaked immediately after a 15-min cycling exercise and return to basal level 1 h thereafter (6). In rats subjected to acute stress by swimming, the circulating levels of EO peaked within 40 min after stress and returned to basal level at 70 min. Therefore, the stimulatory effect of EO on glycogen synthesis in skeletal muscle may have physiological relevance and may constitute a mechanism for the adaptive response to exercise. Heightened EO levels may activate glycogen synthesis immediately after exercise, when insulin levels are low.

In humans, exercise raises the EO level up to 100 nmol liter⁻¹ range (6). Animal studies indicate an increase in endogenous CTS concentrations in response to different stimuli within a modest low nanomolar range (13, 56). The assays for EO and other endogenous CTS are usually based on custom made antibodies and may vary in different laboratories. Additionally, immunoassays for endogenous CTS are often nonselective to different Na⁺,K⁺-ATPase inhibitors. Thus, in HSMC, we tested a purified CTS of animal origin, i.e. MBG. It was discovered in the amphibians and was recently isolated from human urine and plasma (8). MBG differs in action from ouabain and exhibits a greater affinity for rodent ouabain-resistant α₁-subunit of Na⁺,K⁺-ATPase (57). In HSMC, MBG promotes Src phosphorylation, glycogen synthesis, and MAPK signaling in a manner analogous to ouabain; however, the effects were observed at lower concentrations. In contrast to ouabain, MBG did not affect PKCα/β phosphorylation. In the present study, MBG inhibited the Na-pump at much lower concentrations compared with ouabain (Figs. 5B and 6).
endocytosis and increasing the plasma membrane proteins, and clathrin, thereby preventing Na appearance from the cell surface under basal conditions and abundance caused by constitutive exocytosis. Indeed, inhibition of hypothesizing that in skeletal muscle, the Na–K ATPase is phosphorylated by Thr-Pro motif. After ouabain exposure the subunit cell surface abundance was unaltered, whereas the content was decreased (Fig. 6B). This observation is consistent with the hypothesis that in skeletal muscle, the subunit is subjected to tighter regulation in response to different stimuli compared with α1. Taking into account a relatively high expression of α2 in HSMC, this observation may point out a possible mechanism of desensitization of ouabain signaling.

Phosphorylation of Na+/K+-ATPase α-subunits by ERK1/2 can provide a positive feedback mechanism on pump activity in skeletal muscle (Fig. 7). This may be especially important when the Na-pump is partially inhibited by cardioactive steroids. We hypothesize that phosphorylation of Na+/K+-ATPase α-subunit by ERK1/2 arrests the formation of an endocytic complex consisting of Na+/K+-ATPase, adaptor proteins, and clathrin, thereby preventing Na+/K+-ATPase endocytosis and increasing the plasma membrane α-subunit abundance caused by constitutive exocytosis. Indeed, inhibition of the ERK1/2 signaling pathway promotes α-subunit disappearance from the cell surface under basal conditions and augments an ouabain-stimulated decrease in α2-subunit cell surface abundance (Fig. 6B). Interaction of Na+/K+-ATPase α-subunit and clathrin adaptor protein 2 is disturbed, whereas α-subunit is phosphorylated by ERK1/2.3

In conclusion, our studies provide evidence that the cardioactive steroids ouabain and MBG increase glycogen synthesis, additively to insulin in skeletal muscle. This effect is mediated by activation of a Src-, ERK1/2-, p90rsk-, and GSK3-dependent signaling pathway and may constitute a physiologically relevant feedback mechanism of adaptation of skeletal muscle to exercise. We also propose that digitalis drugs may have a beneficial side effect to enhance insulin action in skeletal muscle. Elucidation of the signal transducer function of the Na+/K+-ATPase in skeletal muscle may have important clinical implications for delineating the mechanisms involved in the development of muscle fatigue, cardiovascular diseases, and complications of diabetes mellitus.

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