Formation of New High Density Glycogen-Microtubule Structures Is Induced by Cardiac Steroids

Eleonora Fridman†, David Lichtstein§, and Haim Rosen†

From the Departments of †Microbiology and Molecular Genetics and §Medical Neurobiology, Institute for Medical Research Israel-Canada, the Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

Background: Cardiac steroid (CS)-Na\(^+\),K\(^+\)-ATPase interactions regulate numerous physiological functions.

Results: CS, low extracellular K\(^+\), and hypoxia induce formation of glycogen-microtubule clusters adjacent to the nucleus.

Conclusion: Formation of these clusters is mediated by Na\(^+\),K\(^+\)-ATPase, ERK1/2 signaling, and an additional unknown factor.

Significance: These novel CS-induced structural changes may be part of a new type of cellular stress response.

Cardiac steroids (CS), an important class of naturally occurring compounds, are synthesized in plants and animals. The only established receptor for CS is the ubiquitous Na\(^+\),K\(^+\)-ATPase, a major plasma membrane transporter. The binding of CS to Na\(^+\),K\(^+\)-ATPase causes the inhibition of Na\(^+\) and K\(^+\) transport and elicits cell-specific activation of several intracellular signaling mechanisms. It is well documented that the interaction of CS with Na\(^+\),K\(^+\)-ATPase is responsible for numerous changes in basic cellular physiological properties, such as electrical plasma membrane potential, cell volume, intracellular [Ca\(^{2+}\)] and pH, endocytosed membrane traffic, and the transport of other solutes. In the present study we show that CS induces the formation of dark structures adjacent to the nucleus in human NT2 and ACHN cells. These structures, which are not surrounded by membranes, are clusters of glycogen and a distorted microtubule network. Formation of these clusters results from a relocation of glycogen and microtubules in the cells, two processes that are independent of one another. The molecular mechanisms underlying the formation of the clusters are mediated by the Na\(^+\),K\(^+\)-ATPase, ERK1/2 signaling pathway, and an additional unknown factor. Similar glycogen clusters are induced by hypoxia, suggesting that the CS-induced structural change, described in this study, may be part of a new type of cellular stress response.

The plasma membrane Na\(^+\),K\(^+\)-ATPase hydrolizes ATP and uses the free energy thus generated to drive potassium into the cell and sodium out of the cell, against their electrochemical gradients. Consequently, this enzyme plays an important role in regulating cell volume, the electric potential of the plasma membrane, and cytoplasmic pH and Ca\(^{2+}\) levels, through the Na\(^+\)/H\(^+\) and Na\(^+\)/Ca\(^{2+}\) exchangers, respectively, and several secondary transport systems of organic molecules (for review, see Refs. 1, 2). The Na\(^+\),K\(^+\)-ATPase is a heteromeric protein composed of an α catalytic subunit that binds Na\(^+\),K\(^+\), and ATP, and β and FXYD subunits that can modulate substrate affinity (3, 4). Specific steroids originally identified in plants and amphibians, (e.g. digitalis, cardiacenolides, bufadienolides) here collectively termed cardiac steroids (CS), bind to a specific site on the α subunit and inhibit ATP hydrolysis and ion transport (5, 6). These compounds have been used in Western and Eastern medicine for many centuries to treat cardiac insufficiency and heart arrhythmia, as well as for other indications (7). In the past decade, CS were identified in animal and human tissues and were shown to be present at nanomolar concentrations in the circulation (8, 9). These steroids are synthesized in and released from the adrenal gland and are considered to function as hormones involved in salt and water homeostasis and in the regulation of blood pressure, cell viability, and growth (10, 11). The Na\(^+\),K\(^+\)-ATPase/CS system has also been shown to be involved in several pathological states, including hypertension, cancer, neurological hereditary diseases, and depressive disorders (12–14).

The interaction of CS with Na\(^+\),K\(^+\)-ATPase was shown to affect cell function via several molecular pathways. These include the inhibition of Na\(^+\) and K\(^+\) transport across the plasma membrane, activation of intracellular signal transduction mechanisms (15), activation of cytoplasmic Ca\(^{2+}\) oscillation (16), induction of apoptosis (17), and inhibition of endocytosed membrane traffic (18). The notion that Na\(^+\),K\(^+\)-ATPase functions as a receptor for CS, thereby activating an intracellular phosphorylation cascade, was originally based on the observation that the addition of CS to neonatal rat cardiac myocytes induces the activation of Ras and the p42/44 mitogen-activated protein kinase (MAPK) pathway (19). This concept was confirmed by numerous studies in various cells and experimental systems (11). The Na\(^+\),K\(^+\)-ATPase molecules, which mediate signal transduction, were defined as “nonpumping” ATPases located in defined functional units in the plasma membrane termed “signalosomes” (20, 21). Signaling through the CS-Na\(^+\),K\(^+\)-ATPase interaction was shown to be involved in several biological processes, including heart muscle contractility, cell growth, and glycogen synthesis (22–24).

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1 To whom correspondence should be addressed: the Kuvin Center for the Study of Infectious and Tropical Diseases, Institute for Medical Research Israel-Canada, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel. Tel.: 927-2-675-8409; Fax: 927-2-675-8092; E-mail: haimr@ekmd.huji.ac.il.

2 The abbreviations used are: CS, cardiac steroid(s); IF, immunofluorescence; PAS, periodic acid-Schiff.
Although all CS have a similar chemical structure (cardiotoxic steroids are composed of three major components: a steroid core, a 5-membered or 6-membered lactone ring (cardenolides or bufadienolides), and, in most cases a sugar moiety), their effects are highly diverse (25). The pharmacological profile of the effect of the most studied compounds (ouabain, digoxin, bufalin) was attributed to their ability to bind to and inhibit Na+,K+-ATPase. However, numerous recent studies revealed CS-induced phenomena that do not correlate with Na+ and K+ pumping per se (20, 26).

We recently showed that CS affect intracellular membrane traffic and that this effect is accompanied by numerous changes in subcellular structures: treatment of NT2 cells with CS elicited the appearance of large vesicles and dark structures adjacent to the nucleus (18). The nature of the vesicles was studied, and the mechanisms of their production were elucidated (27). In the present investigation we focus on the dark structures emerging following CS treatment and show that these structures are novel, high density glycogen-microtubule granules. The mechanism underlying the formation of the granules involves activation of the MAPK signaling pathway following CS-Na+,K+-ATPase interaction.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Bufalin, digoxin, ouabain, digoxigenin, ouabagenin, nocodazole, periodic acid-Schiff (PAS) reagent, and monoclonal anti-α-tubulin clone B-51-2 antibody were purchased from Sigma-Aldrich. Rabbit monoclonal anti-glycogen synthase (15B1) antibody, Mouse monoclonal anti-phospho-p44/42 MAPK (Thr202/Tyr204) E10 antibody, and U0126 were purchased from Cell Signaling Technology. All fluorescence probes were purchased from Molecular Probes. Polyclonal Cy5-conjugated AffiniPure F(ab)2 fragment goat anti-rabbit IgG, polyclonal Cy5-conjugated AffiniPure F(ab)2 fragment goat anti-mouse IgG, and peroxidase-conjugated F(ab)2 fragment goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories. The SV total RNA isolation system, the reverse transcriptional system, GoTaq DNA polymerase, pCineo, and the psiSTRIKE U6 cloning system were acquired from Promega. ECL kits were obtained from Pierce. The FuGENE 6 transfection reagent was provided by Roche Diagnostics. Serum, cell culture medium, and antibiotics were provided by Biological Industries.

**Cell Culture**—NT2 cells (human neuronal precursor cells) and ACHN cells (human renal cell adenocarcinoma) were purchased from ATCC. The cells were cultured in T25 tissue culture flasks in DMEM-F12 medium supplemented with 2 mM glutamine, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 5% CO2 in 37 °C, as described previously (18). For fluorescence microscopy analysis, the cells were grown on glass coverslips mounted with neutral contact glue on a 1.4 × 2.4-cm aperture in the center of 35-mm dishes, 24–48 h before the experiments. For transfection, NT2 cells were plated in 35-mm tissue culture plates at 105 cells/plate and transfected with 1 μg of plasmid DNA using the FuGENE 6 transfection reagent (reagent:DNA ratio 3:1), according to the manufacturer’s instructions.

**Western Blot Analysis**—Cells were plated in 100-mm dishes to 80% confluence and then incubated in medium containing 1% serum for 18 h. The medium was then replaced with fresh medium (1% serum) and CS for 5, 10, and 15 min at 37 °C. Then the cells were washed with ice-cold PBS, incubated with 500 μl of 1.5 × SDS loading buffer (93.5 mm Tris-HCl, pH 6.8, at 25 °C), 3% (w/v) SDS, 15% glycerol, and 0.015% (w/v) bromophenol blue, 62.5 mm dithiothreitol (DTT)), immediately scraped off the plates, and transferred to a microcentrifuge tube at 4 °C. The lysate was sonicated for 30 s, heated to 95–100 °C for 5 min, cooled on ice, and microcentrifuged for 2 min. The supernatant was loaded onto a SDS-polyacrylamide gel and subjected to Western blot analysis (14).

**Fluorescence Microscopy**—Fluorescence microscopy experiments were performed as previously described (27). In brief, microscopy was performed using an Axisvert 200 microscope fitted with a 63XLD Achroplan 0.75NA Korr Ph2, X63 or an X100 1.3NA Neofluar Ph3 objective (Carl Zeiss). An HBO 100 mercury lamp with a short pass excitation filter was used at 488 nm for GFP, at 530 nm for Cy3, and a long pass filter was used at 630 nm for Cy5. Images were acquired with a cooled SensiCAM charge-coupled device camera and analyzed using IP Plus 4.1 version (Signal Analytics) software. Unlabeled cells were used to determine autofluorescence. The image background was corrected as follows: two or three regions were selected from cell-free areas in each field, and the average intensity of these...
Cardiac Steroid-induced Cluster Formation

regions was considered the background value for that field. This value was then subtracted from each pixel in the field. Images were saved in TIFF format and transferred to Adobe Photoshop, version CS3 software, for printing.

Electron Microscopy—Electron microscopy analysis was performed as described previously (28) with several modifications: NT2 cells were grown on ACLAR embedding film in 35-mm plates for 2 days. They were then incubated for 4.5 h with 20 nM bufalin and fixed in 2.5% glutaraldehyde plus 2% formaldehyde in 0.1 M sodium cacodylate, pH 7.4, and 13% sucrose for 1 h at room temperature. Samples were washed four times (10 min each) in 0.1 M sodium cacodylate, pH 7.4, and incubated in the same buffer overnight at 4 °C. Subsequently, the cells were incubated for 1 h at room temperature in osmium tetroxide solution (1% OsO₄, 1.5% K₃Fe(CN)₆, 0.1 M sodium cacodylate, pH 7.4). The samples were then washed four times (10 min each) in sodium cacodylate, pH 7.4, and dehydrated by sequential incubation in 30, 50, 70, 90, and 95% ethanol (10 min each), followed by three washes (30 min each) in 100% ethanol. The dehydrated samples were then incubated overnight at 4 °C in 50% agar 100 resin in ethanol, transferred to room temperature, and incubated in open jars for 8 h to evaporate the ethanol. The samples were polymerized for 2 days at 60 °C in a dry oven, and then 70–90 nm thin sections were made, using a LKB ultra microtome. Sections were collected, picked up on 200-mesh thin bar copper grids, stained with uranyl acetate and lead citrate, and observed under an electron microscope (Philips Tecnai 12 TEM) equipped with a Mega-View II CCD camera, and analySIS version 3.0 software (Soft Imaging System).

Construction and Transient Transfection of shRNA Expression Vectors—α1 Na⁺⁺,K⁺⁺-ATPase siRNA-specific sequences were determined using the siRNA Target Designer program (Promega). The sequences A4-ATP1A1, 5'-GGTTGCTGTCT-GATCTTTGATA-3'; scrambled A4-ATP1A1, 5'-GGCA-CATATGTTGGTTCTT-3'; and A5-ATP1A1, 5'-GCAAGC-TGTACCTGGGTGTGGTGCTA-3' were synthesized, annealed to their complementary oligonucleotides, and then cloned into the psiSTRIKE U6 vector, according to the manufacturer’s instructions. Positive clones were confirmed by nucleotide sequencing.

Reverse transcription in a 20-μl reaction mix. The amplification profile involved denaturation at 93 °C for 1 min, primer annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. This cycle was repeated 30 times. The PCR products were separated on agarose gel (27).

RESULTS

CS-induced Morphological Changes in NT2 and ACHN Cells—In the course of investigating the physiological and pharmacological effects of CS, we observed, using phase-contrast light microscopy, that the addition of CS to human neuronal progenitor NT2 cells induced the appearance of large vesicles (depicted as white spots), adjacent to the nucleus (Fig. 1A). We characterized these vesicles as the recycling late endosomes and explored the mechanisms involved in their assembly (18, 27). The experiments also revealed that in addition to these vesicles, CS caused the generation of dark, large and asymmetrical structures that too are adjacent to the nucleus. An example of the effect of 20 nm bufalin is shown in Fig. 1B. Such structures are not seen in the control cells (Fig. 1A).

The effect of bufalin, tested following a 4.5-h incubation on the formation of the dark structures, depends on the concentration of the steroid (Fig. 2A). Under these conditions, dark structures were already evident at 5 nm steroid, and the maxi-
mal number of cells containing dark structures was reached at 20 nM. The bufalin-induced effect was time-dependent (Fig. 2B). At 20 nM, a significant number of cells containing dark structures was observed after a 1.5-h incubation, plateauing at 4.5 h. At 5 nM bufalin, a similar maximal effect and plateau were reached after 24 h of incubation (data not shown). The presence of the dark structures depends on the continuous presence of the steroid. As shown in Fig. 2C, 2 h following removal of the steroid, >90% of the dark structures disappeared, indicating the reversibility of the process.

A comparison between different CS in their ability to induce the formation of the dark structures is shown in Fig. 2D. The potency of the various steroids was examined following incubation of the cells with the steroid for 4.5 h. Bufadienolide bufalin was found to be the most potent inducer of the dark structures (maximal effect at 20 nM). The two cardenolides, ouabain and digoxin, showed a similar efficiency, which is about 10 times lower than that of bufalin. Surprisingly, the ouabain aglycone, ouabagenin did not induce the formation of the dark structures, even at 1 µM (Fig. 2D). The absence of the ouabagenin effect was evident even when the cells were incubated for 24 h with the steroid (data not shown). The digoxin aglycogen, digoxigenin, however, did induce dark structure formation, with a maximal effect at 500 nM (Fig. 2D). These results show that the ability of different CS to induce the formation of the dark structure does not correlate with their ability to inhibit Na⁺,K⁺-ATPase activity.

The formation of dark structures by CS is not restricted to NT2 cells. Similar structures were observed in human renal carcinoma ACHN cells (Fig. 1D). However, CS failed to induce the formation of such dark structures in human neuroblastoma SH-SY5Y and HeLa cells. Bufalin-induced Dark Structures Are Composed of Glycogen, Which Is Redistributed in Cell—We previously established that bufalin affects endocytosed membrane traffic (18). Hence, the direct working hypothesis was that the CS-induced dark structures are related to this process and are derived from membrane compartments. We therefore tested the possible presence of specific marker of different membrane components in the CS-induced dark structures. Our results unequivocally nullify this hypothesis. Markers of Golgi apparatus and endoplasmic reticulum are not present in the dark structures (see supplemental Figs. S1 and S2). Similar results were obtained with markers for components of lysosome and the plasma membrane (data not shown).

To elucidate the nature of the CS-induced dark structures we resorted to basic electron microscopy (EM) analysis of 20 nM bufalin-treated cells. The nucleus, nuclear membrane, mitochondria, and ribosomes are clearly evident in the control cells (Fig. 3, A and C). The bufalin-treated cells (Fig. 3, B and D–F)
Cardiac Steroid-induced Cluster Formation

contain membrane-free clusters of large number of symmetrical granules, adjacent to the nucleus membrane. Importantly, the observed structures are typical of glycogen granules. As seen in Fig. 3, E and F, high magnification analysis revealed bright fiber-like structures embedded in the glycogen clusters.

To verify that the structures are composed of glycogen, sugar-specific PAS histological staining was performed. As shown in Fig. 4, A and B, the addition of 20 nM bufalin to NT2 cells induced a marked increase in PAS staining, all of which was concentrated inside the dark structures, confirming the glycogen nature of the granules. Immunofluorescence (IF) analysis of glycogen synthase distribution in the control and bufalin-treated cells confirmed this conclusion. As seen in Fig. 4, C–H, in the control cells glycogen synthase, known to be tightly bound to glycogen, is spread throughout the cytoplasm, whereas in the bufalin-treated cells almost all of the enzyme is concentrated in the dark structures. Similarly, IF staining for glycogen synthase of the bufalin-induced dark structures in ACHN cells also revealed their glycogen nature (see supplemental Fig. S4).

A conceivable possibility is that the CS-induced formation of the glycogen granules is associated with changes in glycogen metabolism. Accordingly, changes in the rate of glycogen biosynthesis and degradation in NT2 cells were measured in the presence and absence of bufalin. This was performed using an established methodology based on the incorporation of 14C-labeled glucose into glycogen. The experiments showed that bufalin did not affect glycogen synthesis and degradation (see supplemental Fig. S4), suggesting that the glycogen clusters result from redistribution of the cytoplasmic glycogen granules and not from de novo synthesis of glycogen.

Bufalin-induced Dark Structures Are also Composed of Distorted Microtubules—The dramatic changes in glycogen redistribution in the cells and the crucial role of the cytoskeleton in shaping overall cellular structure, prompted us to examine possible changes in the microtubule network following CS treatment. This was analyzed by IF, using specific anti-tubulin antibodies. As shown in Fig. 5, E and F, the addition of 20 nM bufalin to NT2 cells caused a substantial distortion of the microtubule network, resulting in the condensation of a significant portion of the IF signal in the glycogen-containing clusters.

Co-localization of the CS-induced glycogen granules with the distorted microtubules suggested a common mechanism or dependence in their relocation in the cells. To explore this issue, we examined the effect of bufalin on microtubule distortion in cells depleted of glycogen and on the formation of glycogen clusters in cells with a depolymerized microtubule net-
work. Glycogen depletion was achieved by growing the cells for 5 generations at low (0.3 mM) glucose concentrations. This treatment dramatically reduced the glycogen levels in the cells (Fig. 6, A and B). Importantly, however, the microtubule network was not affected by this growth condition (Fig. 6D). Under these conditions, as expected, the addition of bufalin did not induce the formation of glycogen clusters (Fig. 6B). Nevertheless, the microtubule network was severely distorted throughout the cell (Fig. 6E). These results clearly indicate the independence of the effect of bufalin on microtubules from that on glycogen redistribution. A simultaneously conducted positive control Fig. 6, C and F, shows typical bufalin-induced increases in PAS staining and tubulin accumulation. The effect of changes in microtubule integrity on the bufalin-induced effect on glycogen clustering was examined using the antineoplastic drug nocodazole, which is known to interfere with the polymerization of microtubules and causes their distortion. The addition of 500 nM nocodazole to NT2 cells resulted in the disappearance of the microtubule network within 1.5 h and the appearance of scattered microtubule staining throughout the cytoplasm (Fig. 7B). The addition of bufalin following nocodazole treatment caused the appearance of the dark structures (Fig. 7D) and the accumulation of the distorted tubulin in the glycogen clusters (Fig. 7E). These results indicate that the
Cardiac Steroid-induced Cluster Formation

FIGURE 7. **Effect of nocodazole on bufalin-induced tubulin distortion.** NT2 cells were grown as described in the legend to Fig. 1. Immunostaining of tubulin was performed as described in the legend to Fig. 5. The cell medium was replaced with medium containing nocodazole (500 nM) and 1.5 h later 20 nM bufalin (D–F) with or without the steroid (A–C) was added for 4.5 h. The cells were fixed with 1.5% glutaraldehyde and stained with anti-α-tubulin monoclonal antibodies, and images were acquired. A and D, phase microscopy; B and E, fluorescence microscopy; C and F, merged phase contrast and fluorescence images. Scale bar, 20 μm.

bufalin-induced relocation of glycogen is independent of the integrity of the tubulin network.

**CS-Na⁺,K⁺-ATPase-induced ERK1/2 Phosphorylation Is Essential but Not Sufficient for CS-induced Formation of Glycogen-Microtubule Clusters.**—The only established receptor for CS is the Na⁺,K⁺-ATPase (2, 6). Hence, it was expected that the relative potency of the different CS in inducing the formation of the glycogen-microtubule clusters would correlate with the ability of the steroids to bind to the Na⁺,K⁺-ATPase. Surprisingly, however, as shown in Fig. 2D, dramatic differences were revealed in the ability of different CS to induce the glycogen-microtubule clusters (see above). This raised the question as to the participation of Na⁺,K⁺-ATPase in CS-induced cluster formation. To test this issue, we utilized a genetic approach, down-regulating Na⁺,K⁺-ATPase mRNA, using shRNA. These experiments are depicted in Fig. 8. Transfection of NT2 cells with specific shRNA, designated A4 (29), caused a specific down-regulation of α1 Na⁺,K⁺-ATPase mRNA (Fig. 8, A and B). This transfection caused a significant increase in the extent of the bufalin-induced formation of the glycogen-microtubule clusters (Fig. 8C): whereas 5 nM bufalin treatment for 4.5 h induced a marginal increase in the number of dark structures (see also Fig. 2), it induced a >50% effect in the A4-transfected cells. Similar results were obtained using shRNA against different sequence of the α1 within the open reading frame (designated A5), demonstrating the specificity of the effect. Control experiments using shRNA against the A4 scrambled sequence, pCineo empty vector, and mock transfection (Fig. 8C) did not have a significant effect on the CS-induced formation of the glycogen-microtubule clusters. These experiments attest to the involvement of Na⁺,K⁺-ATPase in mediating the CS-induced granule formation.

As described in the Introduction, CS-induced activation of intracellular signaling pathways has been shown to be involved in CS-induced biological responses. We therefore examined the possibility that similar mechanisms are involved in the CS-induced cluster formation. To this end, we first tested the effect of a specific inhibitor of ERK1/2 activity, U0126, on the CS-induced formation of the glycogen-microtubule clusters. As shown in Fig. 9A, pretreatment of NT2 cells with the inhibitor attenuated, in a dose-dependent manner, the appearance of the CS-induced clusters. At 50 μM, the inhibitor completely abolished their appearance. These results support the involvement of the ERK1/2 signaling pathway in the newly described phenomenon. Direct measurements of CS-induced ERK1/2 phosphorylation also support this notion. As shown in Fig. 9B, the addition of bufalin to NT2 cells induced ERK1/2 phosphorylation, both rapidly and transiently. CS-induced phosphorylation of ERK1/2 was detected within 5 min of incubation, peaking after 10 min (Fig. 9B). Similar results were obtained with other CS such as digoxin and ouabain (Fig. 9C). Digoxigenin and ouabagenin, which did not induce the formation of the glycogen-microtubule clusters at 200 nM, caused marked ERK1/2 phosphorylation at this concentration (Fig. 9D), indicating a complex relationship between ERK1/2 phosphorylation and glycogen-microtubule cluster formation (see “Discussion”).

The molecular nature of CS-induced protein phosphorylation is not completely understood. It was suggested that Na⁺,K⁺-ATPase at the E2 conformational state is responsible for the activation of molecular cascades by CS (30). This hypothesis prompted us to examine the effect of low [K⁺], a perturbation that fixes Na⁺,K⁺-ATPase in the E2 state, on the formation of the glycogen-microtubule clusters. These experiments are depicted in Fig. 10. Incubation of NT2 cells in media containing low (1 mM) or no K⁺ caused the appearance of the glycogen-microtubule clusters within 1 h. Furthermore, the specific ERK1/2 inhibitor U0126 significantly inhibited this
phenomenon. In addition, in agreement with previous study (30), exposure of the cells to low K+/H11001 caused, within several minutes, a marked increase in the phosphorylation of ERK1/2 (Fig. 10).

Hypoxia-induced Glycogen Clusters in NT2 Cells—The data, presented above pertaining to the induction of glycogen clusters by CS and low K, suggested that this phenomenon may be related to a more general cellular stress response. If so, we predicted that other stress stimuli would induce a similar effect. To test this hypothesis, we exposed NT2 cells to a low media O2 concentration (hypoxia) of 1% O2 and 5% CO2 at 37 °C for 24 h. As shown in Fig. 11, such treatment induced the appearance of glycogen clusters, as seen under phase-contrast light microscopy, as well as by PAS histological staining.

DISCUSSION

Besides increasing the force of contraction of heart muscle, CS exert numerous pleiotropic effects in various tissues. These compounds were shown to affect viability, growth, and differentiation of several cell types, sodium excretion by the kidney, lung liquid clearance, regulation of systemic blood pressure, and behavior (for review, see Refs. 8, 9, 11, 31). At the molecular level, these effects can be demonstrated at the molecular level. For example, exposure to hypoxia induces the expression of genes involved in glucose metabolism, such as the genes encoding for glucose transporters and glycogen synthase. This can be demonstrated by using techniques such as RT-PCR or microarray analysis.
level, in addition to inhibiting the activity of the Na\(^+\),K\(^+\)-ATPase, CS-Na\(^+\),K\(^+\)-ATPase interactions were shown to induce the activation of several signal transduction pathways (15, 26), suppression of cell differentiation by antagonizing RORyt activity (32), inhibition of interferon-\(\beta\) gene expression (33), regulation of the sperm EGF receptor and initiation of the acrosome reaction (34), up-regulation of nuclear Bcl2 (35), inhibition of hypoxia-inducing factor-1\(\alpha\) synthesis (36), and modulation of alternative splicing (37). In the present study we identified a novel CS-induced effect at the cellular level, the formation of new cellular structures. These large dark structures are observed in living cells only under phase microscopy, indicating a composition of organized particles.

A thorough characterization of the CS-induced structures, performed in this study, identified glycogen as their major component (Figs. 3, 4). Glycogen, a carbohydrate consisting of a large number of glucose units joined together by glycosidic bonds, is one of the main readily accessible energy storage compounds in many cell types. Although it is stored in large amounts mainly in liver and skeletal muscle, glycogen synthesis and degradation are central to the metabolism of most living cells (38–40). Cellular storage of these molecules consists of granules that contain not only glycogen but also enzymes involved in its metabolism, such as glycogen synthase and glycogen phosphorylase (40). A large number of glycogen granules have been shown to assemble into larger molecular complexes.
Consisting of clusters or glycogen particles (39). In our study we show that in the presence of CS these granules are relocated to form a cluster of glycogen-protein complexes. The clusters are not surrounded by membranes and are different from most glycogen complexes described in association with the inherited disorders of glycogen metabolism known as glycogen storage diseases. The CS-induced glycogen clusters resemble, however, structures characteristic of diabetic glycogen nephrosis or Armanni-Ebstein lesions. The lesions are characterized by the accumulation of glycogen particles in the cytoplasm of epithelial cells and can be prevented by insulin treatment (41). Similar glycogen deposits were described also in Tako-Tsubo cardiomyopathy or “transient LV apical ballooning” (42). In this later report, PAS staining revealed large intracytoplasmic areas filled with glycogen in the acute phase of the disease, which were markedly reduced following recovery and completely absent from healthy individuals. Glycogen clusters more similar to those described in our study were demonstrated by PAS staining coupled with ultrastructure analysis in growing colonies of human embryonic stem cells. The structures were detectable throughout colony growth (43). Importantly, none of these reports suggested a function or a mechanism for the induction of these glycogen deposits. In view of previous reports on the effect of CS on glycogen metabolism (22), we tested the possibility that changes in glycogen synthesis and/or breakdown are involved in CS-induced cluster formation. Our results, however, preclude these options (supplemental Fig. S4), indicating that only glycogen redistribution is involved.

The other main component of the CS-induced clusters is distorted microtubules (Fig. 5). Microtubules serve as structural components within cells and are involved in many cellular processes, including mitosis, cytokinesis, and vesicular transport. Although microtubule disassembly was described following pharmacological treatment of cells with microtubule-stabilizing drugs such as nocodazole (44) or sulforaphane (45), CS-induced distortion of these filaments, as observed in our study, has, to the best of our knowledge, never been described before.

In our investigation, CS-induced glycogen cluster formation (supplemental Fig. S3) and tubulin distortion (data not shown) were also seen in human kidney-derived ACHN cells. Other cells such as human renal cell carcinoma 786-O and human ovary cell carcinoma TOV-21G, which also derive from cells rich in glycogen, did not respond in this fashion to CS (data not shown). Thus, a high level of glycogen in the cell is not the cause of glycogen cluster formation.

Our results show unequivocally that CS induction of glycogen-microtubule clusters is mediated by Na\(^+\),K\(^+\)-ATPase, as α1 shRNA clearly influenced this effect (Fig. 8). Furthermore, the partial silencing of the Na\(^+\),K\(^+\)-ATPase (A4- and A5-transfected cells) amplified the response to bufalin. This condition may have caused a state in which the inhibition of even a small fraction of the remaining pumps affects the ionic balance. This suggests that the inhibition of transport and alteration of intracellular ion composition are essential factors in the CS-induced formation of glycogen-microtubule clusters.

A comparison between the effect of different CS revealed that the ouabain aglycone ouabagenin does not induce the formation of the glycogen-microtubule cluster, even at 1 μM (Fig. 2D). Ouabagenin, which binds to Na\(^+\),K\(^+\)-ATPase at very low affinity, is a potent inhibitor of Na\(^+\),K\(^+\)-ATPase activity (46). Hence, the ability of different CS to induce the formation of the glycogen-microtubule clusters correlates with their relative binding affinity but not with their ability to inhibit Na\(^+\),K\(^+\)-ATPase activity. The uniqueness of ouabagenin from other CS was repeatedly documented. For example, Paula et al. have conducted a thorough characterization of CS relative binding and relative inhibitory potencies on lamb kidney isolated Na\(^+\),K\(^+\)-ATPase. This study has demonstrated that although ouabagenin has a reduced binding affinity of >300-fold relative to ouabain, the enzyme inhibition potency was reduced only by approximately 2-fold (46). In addition, the inhibition of Na\(^+\),K\(^+\)-ATPase activity by ouabagenin was found to be the most sensitive to changes in pH compared with other CS (47). Similarly, ouabagenin significantly differed from ouabain in terms of both Na\(^+\),K\(^+\)-ATPase inhibition and cell signaling activation in Madin-Darby canine kidney cells (48). Furthermore, recently, Cornelius et al. characterized the inhibition of Na\(^+\),K\(^+\)-ATPase activity by various metal fluorides and subsequent reactivation by high Na\(^+\). They have found that ouabain, but not ouabagenin, prevented this high Na\(^+\) reactivation, demonstrating the pivotal role of the sugar moiety in closing the extracellular cation pathway of the enzyme (49). These studies point to a fundamental difference in ouabagenin-Na\(^+\),K\(^+\)-ATPase interactions in regard to binding, inhibition of ion pumping, and activation of signaling pathway which presumably result in the failure of this steroid to induce glycogen-microtubule clusters formation. Our results demonstrate that CS induce a rapid increase in ERK1/2 phosphorylation in NT2

**FIGURE 11.** Effect of hypoxia on the formation of dark structures. NT2 cells were grown in DMEM-F12 medium containing 10% FCS at 5% CO\(_2\) and 20% (A and C) or 1% (B and D) O\(_2\) at 37 °C. Images were acquired as described under “Experimental Procedures” without staining (A and B) or after PAS histological staining (C and D) 24 h after introduction to hypoxic condition. White arrowheads point to the dark structures. Scale bar, 20 μm.
Cardiac Steroid-induced Cluster Formation

cells. In addition, the highly specific ERK1/2 inhibitor U0126 completely abolished CS-induced formation of the glycogen-microtubule clusters (Fig. 9). Cumulatively, these findings suggest that the activation of the ERK1/2 signal transduction pathway is obligatory for CS-induced formation of the glycogen-microtubule clusters. However, similar activation of ERK1/2 phosphorylation was observed by treatment of NT2 cells with ouabagenin and digoxigenin at concentrations that did not induce the formation of the glycogen-microtubule clusters (Fig. 2). Hence, it may be concluded that although the activation of ERK1/2 signaling is obligatory, it is not sufficient to produce the clusters.

Na\textsuperscript{+},K\textsuperscript{+}-ATPase is the main transporter of Na\textsuperscript{+} and K\textsuperscript{+} ions, and its activity establishes the concentration gradients of these ions across the plasma membrane of all mammalian cells. The activity of Na\textsuperscript{+},K\textsuperscript{+}-ATPase was described by reaction cycles based on the generally accepted Post-Albers cycle model (50, 51). Na\textsuperscript{+} ions from the cell interior bind to a high affinity site in the ATPase E1 state. Ion binding triggers phosphorylation of the enzyme by Mg\textsuperscript{2+}-ATP, leading to the phosphorylated E1-P state. In the phosphorylated E2-P state, the Na\textsuperscript{+},K\textsuperscript{+}-ATPase is unable to phosphorylate ADP, and has a reduced affinity for Na\textsuperscript{+}, which is released to the exterior. K\textsuperscript{+} ions bind from the outside and, on hydrolysis of the phosphorylated Asp, the enzyme releases K\textsuperscript{+} to the interior and rebinds Na\textsuperscript{+}. A significant conclusion that may be drawn from the present study is that Na\textsuperscript{+},K\textsuperscript{+}-ATPase in the E2-P state is the activator of the signaling pathway responsible for glycogen-microtubule cluster formation. This conclusion was drawn from the observation that a reduction in extracellular K\textsuperscript{+} induced the appearance of the clusters in the absence of CS (Fig. 10). Furthermore, this induction was dependent on the activation of ERK1/2 (Fig. 10). These findings are in accord with the well established notion that CS reduce the rate of dephosphorylation of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, thereby increasing the accumulation of the E2-P state in the membrane. Recently, Ye et al. demonstrated that changes in E1/E2 Na/K-ATPase obtained by lowering extracellular K\textsuperscript{+} induce the activation of ERK1/2 (30). Our results not only confirm this observation, but show that the E2-P-induced activation of the signaling pathway has significant cellular consequences, manifested by the formation of glycogen-microtubule clusters. It is tempting to speculate other perturbations that affect the plasma membrane, causing an increase in the E2 state of the enzyme and resulting in the activation of signaling through the Na\textsuperscript{+},K\textsuperscript{+}-ATPase.

The CS-induced formation of the glycogen-microtubule granules is a complex process that requires the participation of different signaling pathways. Our results show that ouabagenin does not induce the formation of the clusters, despite its ability to inhibit Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity (increased E2-P) and increase ERK1/2 phosphorylation (Figs. 2 and 9, respectively). This indicates that these two events are indeed necessary but not sufficient to cause CS-induced formation of the glycogen-microtubule clusters. The identity of the additional molecular participant in this mechanism is not known. In view of the pharmacological profile of the CS-induced glycogen-microtubule cluster formation and the well known diversity of CS actions at the cellular and systemic levels (25), the possibility of the existence of an additional receptor for CS, which is involved in the phenomena described in this study, should also be considered.

In a search for the physiological/pathophysiological relevance of the CS-induced formation of glycogen-microtubule clusters and in view of the reports on such clusters in diabetic glycogen nephrosis, Tako-Tsubo cardiomyopathy, and human embryonic stem cells, we reasoned that the clusters may represent a more general consequence of cellular stress. To test this possibility we investigated the effects of hypoxia, a fundamental stimulus in biology and medicine, on the formation of the glycogen clusters. We indeed discovered that exposure of NT2 cells to hypoxic conditions resulted in the formation of these clusters. Hypoxia changes the expression of about 400 genes, which collectively serve to allow the cell and the tissue in which it resides to adapt to the low oxygen environment (52). Therefore, the elucidation of the molecular mechanisms governing hypoxia-induced glycogen cluster formation presents a complex problem and is now under investigation in our laboratory. Interestingly, it was demonstrated that hypoxia triggers a significant reduction in plasma membrane Na\textsuperscript{+},K\textsuperscript{+}-ATPase (53) as well as an increase in the release of endogenous CS from the brain and adrenal (54, 55). Furthermore, in the present study we demonstrate that a reduction in Na\textsuperscript{+},K\textsuperscript{+}-ATPase potentiates CS-induced glycogen formation (Fig. 8). In view of all of these findings, it is tempting to suggest that these changes in the Na\textsuperscript{+},K\textsuperscript{+}-ATPase/CS system are involved in the hypoxia-induced formation of glycogen clusters.

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6528 JOURNAL OF BIOLOGICAL CHEMISTRY

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