Cardiotonic Steroids Differentially Affect Intracellular Na\(^+\)/[K\(^+\)]\(_i\)-independent Signaling in C7-MDCK Cells

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Recently, we reported that ouabain kills renal epithelial and vascular endothelial cells independently of elevation of the [Na\(^+\)]\(_i\)/[K\(^+\)]\(_i\) ratio. These observations raised the possibility of finding cardiotonic steroids (CTS) that inhibit the Na\(^+\),K\(^+\) pump without attenuating cell survival and vice versa. To test this hypothesis, we compared CTS action on Na\(^+\),K\(^+\) pump, [Na\(^+\)]\(_i\), content, and survival of Madin-Darby canine kidney cells. At a concentration of 1 \(\mu\)M ouabain and other tested cardenolides, as well as bufadienolides such as bufalin, cinobufagin, cinobufotalin, and telobufotoxin, led to 10-fold inhibition of the Na\(^+\),K\(^+\) pump, a 2-3-fold decrease in staining with dimethylthiazol-diphenyltetrazolium (MTT), and massive death indicated by detachment of ~80% of cells and caspase-3 activation. In contrast, Na\(^+\),K\(^+\) pump inhibition and elevation of [Na\(^+\)]\(_i\), seen in the presence of 3 \(\mu\)M marinobufagenin (MBG) and marinobufotoxin did not affect MTT staining and cell survival. Inhibition of the Na\(^+\),Rb\(^+\) pump in K\(^+\)-free medium was not accompanied by a decline of MTT staining and cell detachment but increased sensitivity to CTS. In K\(^+\)-free medium, half-maximal inhibition of 36Rb influx was observed in the presence of 0.04 \(\mu\)M ouabain and 0.1 \(\mu\)M MBG, whereas half-maximal detachment and decline of MTT staining were detected at 0.03 and 0.004 \(\mu\)M of ouabain versus 10 and 3 \(\mu\)M of MBG, respectively. Both ouabain binding and ouabain-induced [Na\(^+\)]\(_i\)/[K\(^+\)]\(_i\)-independent signaling were suppressed in the presence of MBG. Thus, our results show that CTS exhibit distinctly different potency in Na\(^+\),K\(^+\) pump inhibition and triggering of [Na\(^+\)]\(_i\)/[K\(^+\)]\(_i\)-independent signaling, including cell death.

Na\(^+\)/K\(^+\)\(-\)ATPase is an ubiquitous plasma membrane heterodimer detected in all types of animal cells. Its larger \(\alpha\)-subunit (~110 kDa) provides ATP hydrolysis coupled to the transport of three Na\(^+\) and two K\(^+\) ions against their electrochemical gradients, whereas its \(\beta\)-subunit (~35 kDa) is mainly involved in plasma membrane delivery and assembly of the enzyme (1, 2). In addition to its unique enzymatic properties, the Na\(^+\)/K\(^+\)\(-\)ATPase \(\alpha\)-subunit is the only known target of cardiotonic steroids (CTS) initially extracted from the leaves of Digitalis purpurea and Digitalis lanata plants and termed as digitals. Since the report of Sir William Withering in 1789, glycosides have played a prominent role in the treatment of congestive heart failure (3). Later on, ouabain was extracted from Strophanthus gratus, and because of much higher water solubility, it was employed in an overwhelming number of animal and in vitro studies (for more details, see Ref. 4). Side-by-side with plant-derived cardenolides, other members of the CTS superfamily, bufadienolides, have been isolated from amphibians (5). Moreover, augmented levels of CTS indistinguishable from cardenolides (ouabain and digoxin) and bufadienolides (marinobufagenin (MBG)) have been found in the plasma of hypertensive subjects and animals with volume-expanded hypertension (6–8).

In myocytes, inhibition of the electrogenic Na\(^+\)/K\(^+\) pump by CTS leads to depolarization, elevation of [Na\(^+\)]\(_i\), activation of electrogenic Ca\(^2+\)\(-\)/Na\(^+\)\(_i\) exchanger, and the opening of voltage-gated Ca\(^2+\) channels that results in elevation of Ca\(^2+\)\(_i\), contraction of cardiomyocytes, and augments the sensitivity of vascular smooth muscle cells to endogenous constrictors (9, 10). Recently, several research teams proposed that CTS are also involved in Na\(^+\)-independent signaling. This hypothesis was based on data showing that at lower concentrations CTS augment cell proliferation (11, 12), DNA synthesis (11), mitogen-activated protein kinase activity (11, 13, 14), and the production of reactive oxygen species (15, 16) without significant inhibition of the Na\(^+\),K\(^+\) pump and elevation of [Na\(^+\)]\(_i\).

More direct evidence of [Na\(^+\)]\(_i\)-independent signaling was obtained in a study of epithelial cells from the Madin-Darby canine kidney (MDCK). In these cells, long term exposure to ouabain at concentrations sufficient to completely inhibit Na\(^+\)-K\(^+\)\(-\)ATPase resulted in attenuated staining with dimethylthiazol-diphenyltetrazolium (MTT) (17), commonly used to estimate the relative number of alive cells and their redox state (18), and massive detachment of dead cells (19). The death of ouabain-treated C7-MDCK cells shares markers with apoptosis (caspase-3 activation) and necrosis (cell swelling and smear pattern of DNA degradation) and, in contrast to “classic” apoptosis, is resistant to the pan-caspase inhibitor N-benzyloxy-carbonyl-Val-Ala-Asp (17). Importantly, both a decline of MTT staining and death were triggered by ouabain in the absence of

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§§ The abbreviations used are: CTS, cardiotonic steroids; MDCK, Madin-Darby canine kidney; MTT, dimethylthiazol-diphenyltetrazolium; MBG, marinobufagenin; DMEM, Dulbecco’s modified Eagle’s medium.
FIG. 1. Chemical structures of CTS and other steroids used in the present study.

FIG. 2. Kinetics of modulation of $^{22}$Na uptake (A) and MTT staining and cell attachment (B) in C7-MDCK cells by ouabain. A, the cells were incubated in DMEM containing 2 μCi/ml $^{22}$Na and in the absence (line 1) or in the presence (line 2) of 1 μM ouabain. B, cells were incubated in DMEM containing 1 μM ouabain. MTT assay (line 1), and measurement of protein content in attached cells (line 2) was performed as described under "Materials and Methods." The means ± S.E. from experiments performed in triplicate ($^{22}$Na uptake), quadruplicate (protein content) or octaplicate (MTT staining) are given.
a transmembrane gradient of monovalent cations (17). Similar results were obtained under analysis of the ouabain action on primary cultures of endothelial cells from the porcine aorta (20). This intriguing discovery raises the possibility of finding CTS that trigger \([Na]/\)[K]-independent signaling without significant modulation of intracellular ion homeostasis and vice versa. Here, we confirm the hypothesis and demonstrate that MBG and marinobufotoxin do not affect MTT staining and survival of C7-MDCK cells at concentrations sufficient to inhibit Na\(^+\),K\(^+\)-ATPase and augment the Na\(^+\)/K\(^+\) ratio to the same extent as ouabain and other CTS under investigation.

### MATERIALS AND METHODS

**Cell Culture**—C7-MDCK cells resembling principal cells from collecting ducts (21) were kindly provided by Dr. Michel Gekle (University of Warzburg, Warzburg, Germany) and maintained in culture as described previously (17). Before the experiments, the cells were seeded in 12-, 24-, or 96-well plates and grown until confluency in the presence of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (100 units/ml), streptomycin (100 µg/ml), and penicillin (100 units/ml). Cell morphology was evaluated by phase contrast microscopy at ×100 magnification without preliminary fixation. To estimate cell attachment, cells growing in 24-well plates in the absence or presence of CTS were washed four times with medium W containing 100 mM MgCl\(_2\) and 10 mM HEPES-Tris (pH 7.4), and the protein content of attached cells was measured by the modified Lowry method (22). Staining with MTT was performed in cells seeded in 96-well microplates and grown to confluency as described in detail previously (17). The optical density of the resulting solution was measured at 590 nm. Caspase-3 activity in cells growing in 6-well plates was measured as the rate of the caspase-3 inhibitor (Ac-DEVD-CHO)-sensitive component of caspase-3 fluorescent substrate (DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-AMC) hydrolysis according to a previously described protocol (23).

**Na\(^+\),K\(^+\)-ATPase Activity**—Na\(^+\),K\(^+\)-ATPase activity was studied as a CTS-sensitive component of the rate of 86Rb influx. To measure maximal Na\(^+\),K\(^+\) pump activity, the cells were preloaded with Na\(^+\) in accordance with the protocol described previously (17). Briefly, C7-MDCK cells seeded in 24-well plates were washed twice with 2-ml aliquots of phosphate-buffered saline and incubated for 2 h at 37 °C in K\(^+\)-free medium containing 140 mM NaCl, 1 mM MgCl\(_2\), 1 mM MgSO\(_4\), 29.8 mM NaHCO\(_3\), 9.0 mM NaH\(_2\)PO\(_4\), 5 mM glucose, and vitamins and amino acids at concentrations indicated in the DMEM recipe. In the absence of CTS, the cells were seeded in DMEM-like medium containing 2 mM KCl and 1 mM MgCl\(_2\). In the presence of CTS, the cells were seeded in DMEM-like medium containing 2 mM KCl and 1 mM MgCl\(_2\), where [K\(^+\)]/Na\(^+\)-free medium in the presence of \([3H]\)ouabain at concentrations <0.5 µCi/ml and other compounds mentioned in the table and figure legends. Then the cells were transferred on ice, washed four times with 2 ml of ice-cold medium W, and lysed with 0.5% SDS, 4% EDTA mixture. Radioactivity of the incubation medium and cell lysate was measured with a liquid scintillation analyzer, and the rate of \(^{3}H\)influx was calculated as \(V = A/(V_{0}t)\), where \(A\) was the radioactivity of the samples (cpm), \(V_{0}\) was the specific activity of \(^{3}H\)ouabain calculated from the total concentration of K (up to 80 mM) and Rb (up to 10 mM) (cpm/mmol), \(m\) was the protein content (mg), and \(t\) was incubation time with the isotope.

**Intracellular Content of Exchangeable Na\(^{+}\)**—Intracellular content of exchangeable Na\(^{+}\) was measured as the steady-state distribution of extracellular and intracellular \(^{22}Na\) (17). In these experiments, the cells were incubated for 5 h in physiologically balanced medium containing 2 mM KCl and 2 µCi/ml \(^{22}Na\)Cl with or without CTS. Then the cells were transferred to ice, washed four times with 2 ml of ice-cold medium W, and lysed with SDS/EDTA mixture. The radioactivity of the incubation medium and cell lysate was measured, and intracellular cation content was calculated as \(A/(V_{0}t)\), where \(A\) was the radioactivity of the samples (cpm), \(a\) was the specific radioactivity of Na\(^{+}\) in the medium (cpm/mmol), and \(m\) was the protein content (mg).

**[3H]Ouabain Binding**—Cells grown in 24-well plates were incubated for 3 h in complete or K\(^{-}\)-free DMEM-like medium in the presence of \([3H]\)ouabain at concentrations 0.5–100 µCi/ml and other compounds mentioned in the table and figure legends. Then the cells were transferred on ice, washed four times with 2 ml of ice-cold medium W, and lysed for the measurement of radioactivity as indicated above. At a concentration of 3 mM, \([3H]\)ouabain binding was not affected by K\(^{-}\), elevation from 0 to 140 mM, indicating nonspecific (Na\(^{+}\),K\(^{+}\)-ATPase-independent) binding. These values (dpm/mg protein) were subtracted from \([3H]\)ouabain binding measured at concentrations below 1 µM.

**Chemicals**—Ouabain was from ICN (Irvine, CA); digoxin, digitoxin, digoxigenin, digoxigenin, digitoxigenin, strophanthidin, bufalin, andacetylandrosol were synthesized at the Institute of Organic Chemistry, Bucharest, Bucharest, Romania. The remaining chemicals were from Sigma. For caspase-3 inhibitor (Ac-DEVD-CHO) and DEVD-AMC were obtained from Bachem. Warburg, Warburg, Germany) and maintained in culture as described in detail otherwise (24).

**TABLE 1**

| Compounds                  | Na\(^{+}\),K\(^{+}\) pump activity | Cell attachment | MTT staining |
|----------------------------|---------------------------------|----------------|-------------|
|                           | %                               | %              | %           |
| None (control)             | 100                             | 100            | 100         |
| Cardenolides               |                                 |                |             |
| Ouabain (1 µM)             | 45.2 ± 1.1                      | 32.4 ± 2.1     | 46.3 ± 1.5  |
| Digoxin (1 µM)             | 57.3 ± 0.8                      | 45.0 ± 0.7     | 58.9 ± 3.6  |
| Digitoxin (1 µM)           | 33.5 ± 1.0                      | 30.3 ± 3.6     | 40.1 ± 5.0  |
| Digoxigenin (1 µM)         | 33.4 ± 0.6                      | 27.3 ± 3.3     | 43.5 ± 0.9  |
| Digitoxigenin (10 µM)      | 24.1 ± 0.3                      | 20.8 ± 1.1     | 42.0 ± 1.8  |
| Strophanthidin (1 µM)      | 26.0 ± 0.1                      | 23.1 ± 1.7     | 37.8 ± 2.2  |
| Bufadienolides             |                                 |                |             |
| Bufalin (1 µM)             | 10.5 ± 1.1                      | 10.0 ± 6.0     | 17.3 ± 1.8  |
| Cinobufagin (1 µM)         | 10.3 ± 0.4                      | 20.3 ± 2.8     | 54.7 ± 1.8  |
| Cinobufotalin (1 µM)       | 53.5 ± 1.5                      | 29.5 ± 1.7     | 51.2 ± 4.4  |
| Telobufotoxin (1 µM)       | 74.3 ± 18.3                     | 35.6 ± 3.0     | 58.1 ± 4.7  |
| MBG (1 µM)                 | 41.9 ± 0.4                      | 92.4 ± 3.3     | 91.2 ± 11.8 |
| Marinobufogenin (1 µM)     | 51.1 ± 2.0                      | 89.6 ± 5.1     | 107 ± 14    |

**FIG. 3.** Phase contrast microscopy of C7-MDCK cells after 24-h incubation in DMEM containing 1 µM ouabain, MBG, and marinobufotoxin.
The means ± S.E. from experiments performed in triplicate (A and B), octaplicate (C), or quadruplicate (D) are given.

**TABLE II**

Effect of ouabain, MBG, and marinobufotoxin on caspase-3 activity in C7-MDCK cells

| Incubation medium Addition of CTS | Caspase-3 activity nmol (mg protein)⁻¹ h⁻¹ |
|----------------------------------|------------------------------------------|
| DMEM-like None (control)          | 0.37 ± 0.04                              |
| DMEM-like Ouabain (1 μM)          | 0.03 ± 0.03                              |
| DMEM-like Marinobufotoxin (1 μM) | 0.34 ± 0.03                              |
| DMEM-like Marinobufotoxin (3 μM) | 0.69 ± 0.11b                             |
| DMEM-like MBG (1 μM)              | 0.41 ± 0.08                              |
| DMEM-like MBG (3 μM)              | 0.38 ± 0.04                              |
| DMEM-like MBG (10 μM)             | 0.87 ± 0.18b                             |
| K⁺-free None                      | 0.33 ± 0.07                              |

a p < 0.001 compared with control.  
b p < 0.05 compared with control.

PerkinElmer Life Sciences. [21,22-3H]Ouabain (specific activity, 17 Ci/mmol) was purchased from Amersham Biosciences, and the remaining chemicals were from Sigma, Invitrogen, and AnaChemia (Montreal, Canada). Stock solutions of steroids (5 mM of each) were prepared in water (ouabain), ethanol (digoxin), ethanol:chloroform (1:1, v/v) (digitoxin), methanol (digitoxigenin and digitozigenin), or dimethylsulfoxide (other compounds) and kept at -20 °C.

**RESULTS**

**Screening of CTS**—The addition of cardenolides at a concentration of 1 μM resulted in 2-4-fold inhibition of maximal Na⁺,K⁺-ATPase pump activity measured as the rate of bumetanide-resistant ⁸⁶⁸Rb influx in Na⁺-loaded cells (Table I). About the same Na⁺,K⁺-pump inhibition was detected in cells treated with 1 μM of marinobufotoxin, MBG, cinobufotalin or telobufo- 

toxin, whereas bufalin and cinobufagin suppressed its activity by 10-fold (Table I).

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Fig. 2A shows that in the presence of 1 μM ouabain accumulation of ²²Na reached a plateau in 2 h of ouabain addition, whereas the death of C7-MDCK cells, indicated by detachment and the appearance of rounded, floating cells (Fig. 3), was detected after 10 h of ouabain addition. In contrast to the delayed kinetics of cell detachment seen, significant attenuation of MTT staining was observed in 30 min with a 2-3-fold decrease of optical density in 6 h (Fig. 2B). The second phase of MTT staining decline, detected in 10 h of ouabain addition, was in proportion to the reduced number of attached cells. Based on these results, we compared the potency of CTS as modulators of MTT staining and triggers of cell detachment after 6 and 24 h, respectively.

Similarly to ouabain, the addition of other cardenolides as well as bufalin, cinobufagin, cinobufotalin, and telobufotoxin led to a 2-3-fold decrease of MTT staining and cell detachment (Table I). In contrast, neither MBG nor marinobufotoxin affected MTT staining and attachment of C7-MDCK cells (Table I and Fig. 3).

**Dose Dependences of Ouabain and MBG**—To examine mechanism(s) of the low sensitivity of MTT staining and cell attachment to two bufadienolides compared with other CTS under investigation, we focused on a comparison of MBG and ouabain. At [K⁺]₀ = 5 mM, ouabain inhibited base-line activity of the Na⁺,K⁺ pump, increased intracellular Na content, attenuated MTT staining, and detached cells with ID₅₀ values of ~0.1, 0.3, and 0.3 μM, respectively. The half-maximal action of MBG on ⁸⁶⁸Rb influx and [Na⁺]₀ content was detected at 0.5 and 1 μM, respectively (Fig. 4A and B). Neither MTT staining nor cell survival was significantly affected by MBG at concentrations under 3 μM (Figs. 3 and 4, C and D). At 10 μM, MBG decreased MTT staining and cell attachment by 20–25% only (Fig. 4, C and D). The low potency of MBG and marinobufotoxin in the triggering of death signal was further confirmed by measurement of caspase-3 activity (Table II).

**Effect of Extracellular K⁺**—In K⁺-free medium containing 4.1 μM Rb, the rate of ouabain-sensitive ⁸⁶⁸Rb influx was decreased by ~30-fold compared with medium containing 5 mM KCl (Fig. 5A). Na⁺,K⁺ pump inhibition seen in K⁺-free medium resulted in ~10-fold elevation of [Na⁺]₀ content (Fig. 5B). About the same [Na⁺]₀ elevation was observed in cells treated...
with 1 μM ouabain in the presence of [K\(^+\)]. However, in contrast to ouabain, Na\(^+\),K\(^+\) pump inhibition in K\(^+\)-free medium did not affect cell survival estimated by phase contrast microscopy (Fig. 6), cell attachment (Fig. 5D), caspase-3 activity (Table II), and slightly augmented rather than attenuated MTT staining (Fig. 5C).
It is well documented that affinity of the \( \text{Na}^+\text{K}^-\text{ATPase} \) pump for ouabain is increased in \( \text{K}^- \)-depleted medium (25–27). Indeed, in \( \text{K}^- \)-free medium, ouabain and MBG inhibited \(^{86}\text{Rb} \) influx with \( ID_{50} \) values of 0.04 and 0.2 \( \mu \text{M} \), respectively (Fig. 7A). In this medium, a half-maximal decline of MTT staining and cell attachment were revealed at ouabain concentrations of \(-0.004 \) and \( 0.03 \mu \text{M} \), respectively (Fig. 7, B and C). Elevation of MBG in the range from \( 0.3 \) to \( 10 \mu \text{M} \) resulted in a modest reduction of MTT staining (Fig. 7B) and sharp cell detachment (Fig. 7C) compared with \( \text{K}^- \)-containing medium (Fig. 4D). The distinct effect of \( 10 \mu \text{M} \) MBG on attachment of cells in control and \( \text{K}^- \)-free medium was confirmed by phase contrast microscopy (Fig. 6).

Effect of MBG on Ouabain Binding and Signaling—Scatchard plot analysis showed that at \([\text{K}^+]_o = 5 \text{ mM} \), ouabain binding was fit by a single component of hyperbolic saturation with \( K_d \) and \( B_{max} \) values of \(-40 \text{ nM} \) and \( 3.5 \text{ pmol/mg protein} \), respectively (Fig. 8B). Incubation in \( \text{K}^- \)-free medium increased ouabain affinity \( (K_d = -4 \text{ nM}) \) but did not affect the number of binding sites (Fig. 8).

Recently, Ward et al. (28) reported that side by side with “classic” extracellular \( \text{K}^- \)-inhibited sites, bovine adrenocortical cells exhibit high affinity ouabain binding sites in the presence of \( 20 \text{ mM KCl} \), i.e. under conditions when its binding with \( \text{Na}^+\text{K}^-\text{ATPase} \) is negligible. Keeping these data in mind, we examined the effect of \( [\text{K}^+]_o \), on ouabain binding in the range from \( 0.6 \) to \( 10 \text{ mM} \) (Fig. 9). We observed that \([\text{K}^+]_o \) elevation up to \( 20 \text{ mM} \) led to further suppression of ouabain binding compared with control and \( \text{K}^- \)-free medium. These results are in contrast to the presence of high affinity ouabain-binding sites distinct from the \( \text{Na}^+\text{K}^-\text{ATPase} \alpha \)-subunit in \( \text{C7-MDCK} \) cells.

MBG dose-dependently inhibited ouabain binding (Fig. 10). The inhibitory action of MBG was in inverse proportion to ouabain concentration, indicating their competition for the same binding sites. Indeed, binding of 0.1 \( \mu \text{M} \) ouabain was suppressed by \( 10 \mu \text{M} \) MBG by \(-10 \)-fold, whereas at \( 1 \mu \text{M} \) ouabain MBG attenuated its binding by only \( 2 \)-fold (Fig. 10B). In contrast to CTS, we observed slight inhibition of ouabain binding and \(^{86}\text{Rb} \) influx by \( 100 \mu \text{M} \) acetylansdrosl, whereas the other tested steroids were completely inactive (Table III).

In the presence of \( 5 \text{ mM} \) \( \text{K}^- \), ouabain triggered a full scale decline of MTT staining and cell detachment at concentrations of \( 0.1 \) and \( 1 \mu \text{M} \), respectively (Fig. 4). Table IV shows that both ouabain-induced signals were attenuated in the presence of \( 10 \mu \text{M} \) MBG. Neither base-line MTT staining and cell attachment nor the decline of these parameters triggered by ouabain was affected by steroids distinct of CTS and listed in Table II (data not shown).

**DISCUSSION**

The data obtained in the present study reveal that, similarly to ouabain, the addition of other tested cardenolides as well as bufalin, cinobufagin, cinobufotalin, and telobufotoxin resulted in decreased MTT staining and death of \( \text{C7-MDCK} \) cells. Both the decline of MTT staining and cell detachment of CTS-treated cells were independent of the inhibition of \( \text{Na}^+\text{K}^-\text{ATPase} \) pump-mediated ion fluxes and elevation of the \( [\text{Na}^+]_i/[\text{K}^+]_i \) ratio. [\( \text{Na}^+]_i[\text{K}^+]_i \)-independent signaling triggered by CTS is probably mediated by their interaction with the \( \text{Na}^+\text{K}^-\text{ATPase} \) pump.
ATPase α-subunit. However, the potency of CTS in triggering of [Na\(^+\)], [K\(^+\)],-independent signaling is not proportional to their potency in inhibiting the Na\(^+\),K\(^+\)-pump.

[Na\(^+\)], [K\(^+\)],-independent mechanisms of decreased staining with MTT and death of CTS-treated cells are supported by several observations. First, in K\(^-\)-free medium, Na\(^+\),K\(^+\)-pump activity measured as the rate of ouabain-sensitive \(^{86}\)Rb influx was ~2 orders of magnitude lower than in control medium containing 5 mM KCl (Fig. 5A). Similarly to ouabain, 5 h of incubation in K\(^-\)-free medium resulted in ~6-fold elevation of [Na\(^+\)]\(_{i}\) content (Fig. 5B) and massive loss of intracellular K\(^+\) (20). However, neither decreased MTT staining nor cell detachment was detected in K\(^-\)-free medium (Fig. 5, C and D). Second, at [K\(^+\)]\(_{i}\) = 5 mM, maximal decrement of MTT staining was seen at 0.1 \(\mu\)M ouabain (Fig. 4C). At this concentration, ouabain did not significantly affect [Na\(^+\)]\(_{i}\) content (Fig. 4B). Third, MBG and marinobufotoxin caused about the same Na\(^+\),K\(^+\)-pump inhibition as was detected with other tested CTS (Table I and Fig. 4A). At a concentration of 3 \(\mu\)M, MBG led to ~6-fold elevation of [Na\(^+\)]\(_{i}\) content; the same effect was observed with 1 \(\mu\)M ouabain. (Fig. 4B). However, in contrast to cardenolides and other bufadienolides, MBG and marinobufotoxin did not decrease MTT staining and only slightly affected cell attachment (Table I and Figs. 3 and 4, C and D).

During the last two decades, the MTT assay was widely employed to assess the redox state as a marker of mitochondrial function and/or cell viability (18). Keeping these data in mind, decreased MTT staining in CTS-treated cells might be considered as an early marker of induction of the cell death machinery. It should be underlined, however, that in control as well as in K\(^-\)-free medium, maximal decrement of MTT staining occurred at lower concentrations of ouabain than triggering of cell detachment (Figs. 4 and 7). In contrast, in K\(^-\)-free medium, 3 \(\mu\)M marinobufagenin evoked detachment of cells without significant modulation of MTT staining (Fig. 7). Thus, additional experiments should be performed to study the mechanism of reduced MTT staining in CTS-treated cells and its relevance to the triggering of death signal.
It may be proposed that [Na\(^+\)],[K\(^-\)]-independent signaling triggered by CTS is mediated by a target distinct from the Na\(^+\),K\(^-\)-ATPase α-subunit. This hypothesis contradicts several observations. First, Scatchard analyses revealed the single class of [\(^3\)H]ouabain-binding sites (Fig. 8). Second, both ouabain binding (Fig. 8) and Na\(^+\),K\(^-\) pump inhibition by ouabain (Figs. 4A and 7A) were suppressed under [K\(^-\)]\(_o\) elevation, which is consistent with previously reported data (25–27). The same left-hand shift was detected in the effect of ouabain and MBG on cell detachment and MTT staining (Figs. 4, 6, and 7). Third, neither cell attachment nor MTT staining was affected by steroids distinct from cardenolides and bufadienolides. This observation is consistent with the negligible effect of these compounds on ouabain binding and \(^{86}\)Rb influx (Table III).

Our data show that being potent inhibitors of Na\(^+\),K\(^-\)-ATPase, MBG, and marinobufotoxin are much less potent inducers of [Na\(^+\)],[K\(^-\)]-independent cell death machinery and reduced MTT staining than the other CTS tested. Previously, it was shown that substitution of the amino by the hydroxy group at the 14β position of the ouabain analogue, compound LDN-623, sharply increased its ability to inhibit Na\(^+\),K\(^-\)-ATPase but attenuated its cytotoxic action (29). Daniel et al. (30) reported that incorporation of the 14,15-epoxy group in bufadienolide derivatives reduced their cytotoxicity. This observation is consistent with our data disclosing that the presence of the 14,15-epoxy group in the absence of the 16-acetoxy group (Fig. 1) determines the attenuated cytotoxicity of MBG and marinobufotoxin.

Several crucial questions should be answered to clarify the novel function of Na\(^+\),K\(^-\)-ATPase as a receptor differentially affected by CTS. We do not know whether or not [Na\(^+\)],[K\(^-\)]-independent actions of CTS are limited to their interaction with the α1 Na\(^+\),K\(^-\)-ATPase subunit, i.e. the only isoform detected in the renal epithelium, or whether the cell death signal can be also generated by cell type-specific α2-α4 subunits. Moreover, we do not know whether or not sharp differences in the efficiency of CTS, as triggers of these signaling pathways revealed in C7-MDCK cells, are applicable to other cell types abundant with α1 Na\(^+\),K\(^-\)-ATPase. Indeed, the cell type-specific effect of CTS on Na\(^+\),K\(^-\) pump activity and cell survival has been detected in comparison of wild type and mutated cell lines (31). Apart from cell type- and tissue-specific differences, CTS interaction with the Na\(^+\),K\(^-\) pump is affected by hypertension-induced cardiovascular remodeling (32) and extracellular stimuli, such as dietary salt (33) and modulators of protein kinase C activity (33, 34). The role of these stimuli in the regulation of the [Na\(^+\)],[K\(^-\)]-mediated -independent signaling triggered by CTS should be examined further.

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