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Drug Resistance Induced by Ouabain via the Stimulation of *MDR1* Gene Expression in Human Carcinomatous Pulmonary Cells[1]

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ABSTRACT

The inhibition of the Na^+/K^+-ATPase by cardiotonic drugs like ouabain deeply perturbs both the properties of the cell membrane and the ionic composition of the cytoplasm and hence alters fundamental cell reactions. These three types of reactions may be involved in the stimulation of multidrug resistance 1 (*MDR-1*) gene expression and the synthesis of permeability glycoprotein [P-glycoprotein (P-gp)]. We have determined whether ouabain, which binds to an extracellular motif of the Na^+/K^+-ATPase, stimulates *MDR-1* gene expression by measuring both mRNA and protein and whether the resulting P-gp extrudes hydrophobic compounds and causes resistance to antimitotic agents. The experiments were performed on Calu-3 cells, a human cell line from a pulmonary carcinoma. Northern blotting showed that treating the cells with submicromolar concentrations of ouabain stimulated *MDR-1* gene expression within 24 h. The ouabain-induced stimulation of *MDR-1* expression was not restricted to Calu-3 cells but also occurred in human carcinomatous colon (T-84 and HT-29) and hepatic (H7V3) cells. However, it is not ubiquitous because it was not found in HeLa cells. The stimulation was reproduced by other Na^+/K^+-ATPase inhibitors and occurred via enhanced gene transcription, apparently due to the increased cytosolic calcium concentration. Ouabain also increased the membrane content of P-gp, as detected by immunoblotting and immunohistology. We have developed a microvideo assay based on the properties of acetoxymethyl ester calcein and calcein to show that this P-gp extruded the hydrophobic acetoxymethyl ester calcein. Ouabain also caused the Calu-3 cells to become resistant to doxorubicin and vinblastine. Thus, although ouabain acts extracellularly, it may stimulate *MDR-1* gene expression and P-gp synthesis and make cells resistant to hydrophobic cytotoxic compounds.

INTRODUCTION

The development of multidrug resistance is a major obstacle to the success of chemotherapy of neoplastic diseases. P-gp[3] is a transmembrane protein that acts as an energy-dependent efflux pump to remove natural drugs from cells. It is encoded by the *MDR-1* gene. P-gp is normally present in the apical membrane of some epithelial cells in renal proximal tubules, hepatic bile ducts, and colon villi, but its function in these cells is not clear (1). Its clinical deleterious role arises from the multidrug resistance caused by its production triggered by carcinogenesis and antimitotic treatments (2). The overproduction of P-gp is usually due to enhanced transcription of the *MDR-1* gene, but the molecular mechanisms involved in the reaction are not clear. Various agents, such as differentiating compounds or environmental stresses (UV radiation and acid external pH), stimulate *MDR-1* gene transcription, and many transcription factors can bind to and activate the gene promoter (3). Recent studies demonstrate that the stimulation of *MDR-1* gene transcription by UV irradiation is due to the combined actions of several transcription factors and histone modification (4, 5). Several molecular processes may also be involved in the multidrug resistance produced by P-gp. P-gp is a multifunctional protein that not only acts as an efflux pump for various hydrophobic compounds but also regulates membrane composition via its flippase properties and ion transport by modulating the activity of the cell swelling-stimulated Cl^- conductance (6, 7). The relationship between its various properties is not clear. The multidrug cell resistance produced by P-gp may occur because the protein alters intracellular ion concentrations (8).

We have therefore examined the question of whether inhibiting Na^+/K^+-ATPase, a major membrane protein responsible for maintaining the membrane electrical potential and cell homeostasis, modulates *MDR-1* gene expression. Na^+/K^+-ATPase is specifically inhibited by cardiotonics, the best known of which is ouabain. Ouabain also inhibits the cytotoxicity of various anticancer agents (9, 10), but this quick-acting effect of ouabain is not related to P-gp activity, even when it acts against doxorubicin, a P-gp substrate (9). The effect of prolonged administration of ouabain has not been studied in terms of *MDR-1* mRNA and P-gp. Ouabain, which can induce gene transcription (11), may exert such an action, and the phenomenon might have practical implications because cardiotonics are often administered to prevent cardiac damage during anticancer treatment. We have treated human pulmonary cancerous Calu-3 cells (12) with ouabain and monitored *MDR-1* gene expression and P-gp concentration. We have also explored P-gp function by monitoring the transport of CAL-AM, a P-gp substrate, and cell resistance to doxorubicin and vinblastine toxicity.

MATERIALS AND METHODS

Cell Culture and Treatment

Calu-3 cells were obtained from the ATCC and cultured in DMEM containing 1 mM sodium pyruvate, nonessential amino acids, and 10% FCS at 37°C in 5% CO_2-enriched atmosphere. Cells were cultured on plastic for RNA and protein analysis and on glass slides for measuring the P-gp function. They were incubated with freshly prepared ouabain (Sigma-Aldrich) for the indicated time (generally, 24 h for RNA analysis and 48 h for protein detection and functional studies). Ouabain toxicity was assessed by treating the confluent cell cultures with various concentrations of the drug for 48 h and then counting the living cells (trypan blue exclusion). The capacity of ouabain to modulate the *MDR-1* mRNAs in other human cell lines was also studied. The cells were T-84 and HT-29 cells derived from colon carcinoma (obtained from the ATCC), HeLa cells (obtained from the ATCC), and hepatic carcinomatous HuH7 cells (13). The T-84 cells were cultured in DMEM/Ham's F-12; the HT-29, HeLa, and HuH7 cells were cultured in DMEM.

RNA Extraction and Analysis

MDR-1 mRNAs were measured in confluent cells placed in serum-free medium for 24 h before being treated with ouabain in the absence of FCS. Total RNAs were isolated with phenol/chloroform using the Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions, fractionated on 0.9% agarose gels (15 μg/well), and transferred to nylon membranes (Promega) (14). The membranes were hybridized with ^{32}P-labeled cDNA probes (specific activity $> 10^9$ cpm/μg) with the Quik Hyb protocol provided by Stratagene. The MDR-1 probe was the 1.5-kb *Eco*RI-*Eco*RI

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[3] The abbreviations used are: P-gp, P-glycoprotein; CAL-AM, acetoxymethyl ester calcein; CAL, calcein; ATCC, American Type Culture Collection; BAPTA, [1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraaceticacid].

fragment of human MDR-1 cDNA probe (generously supplied by Dr. J. P. Marie; Institut National de la Santé et de la Recherche Médicale E.9912, Paris, France), and the human β-actin cDNA probe was purchased from Oncogene Science. The mRNAs were quantified by densitometry using an ImageMaster VSD (Pharmacia-Biotech-Amersham, Orsay, France), and the amounts of *MDR-1* mRNA were normalized to those of β-actin. All experiments were repeated at least four times.

Protein Analysis

Western Blot Analysis. Newly synthesized P-gp was detected in membranes extracted from cells treated for 48 h with ouabain by Western blotting (15) using the monoclonal antibody C-219 against P-gp (Valbiotech). The 100,000 g membrane extracts were prepared from cells lysed in hypotonic buffer enriched with a classical antiprotease mixture. The extract was then electrophoresed in a denaturing 6% SDS-polyacrylamide gel run at 15–25 mV, with 30 μg membrane protein/lane. The separated proteins were transferred to a nitrocellulose membrane (0.45 μm; Bio-Rad), which was probed with the anti-Pgp monoclonal antibody C-219. The secondary antibody was a horseradish peroxidase-conjugated antimouse IgG. The blots were developed with the enhanced chemiluminescence reagent (ECL kit; Amersham) and exposed to Hyperfilm (Amersham).

Immunocytochemistry. Cells grown on glass slides under basal conditions or after treatment with 0.2 μM ouabain for 48 h were fixed in PBS/3% formaldehyde. They were then washed with PBS and incubated for 2 h at room temperature with a monoclonal antibody directed against an extracellular epitope of human P-gp, mAb MRK-16 (Valbiotech; diluted 1:10). The secondary FITC-conjugated antibody (Nordic Immunology; diluted 1:500) was used to detect mAb MRK-16. The slides were washed in PBS, mounted on a horizontal stage, and imaged using a Zeiss confocal microscope. Images were collected using a ×40 plan-apochromatic oil immersion objective. Serial scans were collected using a 495 nm laser line to excite the Nordic fluorochrome.

Functional Assays

P-gp-mediated Transport of CAL-AM. We monitored the function of newly synthesized P-gp by microvideo imaging using the properties of CAL-AM and CAL. The nonfluorescent hydrophobic CAL-AM enters the cells by diffusion and is de-esterified to form fluorescent CAL in the cytoplasm (16). CAL fluorescence is insensitive to ion concentration, such as $[Ca^{2+}]_i$ or pH_i, which may vary during many cell stimulation (17). Therefore, the increase in fluorescence of cells incubated with CAL-AM represents CAL accumulation. P-gp in the membrane extrudes a portion of CAL-AM before it is de-esterified to fluorescent CAL, so that cell fluorescence increases more slowly ($\Delta F/\Delta t$ is smaller than in controls). The assay was done using cells subcultured on glass slides with or without ouabain (0.2 μM) for 2 days. The cells were then placed in a perfusion chamber on the stage of an inverted microscope (Diaphot, Nikon, France) and perfused with physiological saline at 37°C containing 0.25 μM CAL acetylmethyl ester (Molecular Probes), the membrane-permeable nonfluorescent form of the dye. The CAL fluorescence (excitation, 390 nm; emission, 410 nm) was measured in single cells with a digital imaging system and a CDD camera (Photonic Sci, Millham, United Kingdom). The results were analyzed using Imstar software (Paris, France), which calculates the mean fluorescence of the 10–12 cells present in the field at a given time (every 30 or 60 s). The slopes of the curves ($\Delta F/\Delta t$) reflecting the rate of formation of intracellular CAL were determined using Microsoft Excel software. CAL-AM efflux by P-gp was antagonized by adding mAb MRK-16 (10 μg/ml), which inhibits P-gp function (2), to the superfusing medium.

Analysis of the Cell Sensitivity to Doxorubicin and Vinblastine. Confluent Calu-3 cells were cultured under control conditions or with 0.2 μM ouabain for 2 days and then cultured for 4 h with doxorubicin and vinblastine (0.5 or 1 μM) alone or with the P-gp inhibitor verapamil (50 μM), or in normal medium with or without verapamil (18). The cells were washed twice with culture medium, trypsinized, seeded at $10^5/cm^2$ in normal culture medium, and allowed to grow for 3 days. The toxicity of doxorubicin and vinblastine was then assessed by counting the living cells (trypan blue test). For each experimental condition (pretreatment or no pretreatment with ouabain; the presence or absence of verapamil), the number of living cells found in the cultures of doxorubicin- or vinblastine-treated cells was expressed as a percentage of the number found in the cultures of cells that were not incubated with the anticancer agent.

RESULTS

MDR-1 mRNA

Serum-deprived Calu-3 cells treated with ouabain for 24 h showed dose-dependent increases in *MDR-1* mRNA from 0.05 to 0.5 μM ouabain. There was very little *MDR-1* mRNA in control cells, and 0.1 μM ouabain produced a significant increase (Fig. 1). The ouabain effect was time dependent; it was maximal at 24 h and half-maximal after 12 h (Fig. 2, *A1* and *B*). The effect of ouabain was reversible (Fig. 2*A2*); cells treated for 24 h with 0.2 μM ouabain that were rinsed and allowed to recover for 24 h in normal medium had the same *MDR-1* mRNA content as untreated cells.

High concentrations of ouabain also decreased the *β-actin* mRNA in Calu-3 cells (Figs. 1 and 2), and this decrease was also reversed by washing out the ouabain. Despite their opposing changes, the increase in *MDR-1* mRNA and the decrease in *β-actin* transcripts triggered by ouabain may both be due to stress caused by the drug. There were significantly fewer living cells in cultures treated with 0.5 or 1 μM ouabain for 48 h than in controls (Table 1). Therefore, all subsequent experiments were performed on cells treated with 0.2 μM ouabain.

Ouabain also stimulated *MDR-1* gene expression in human colic

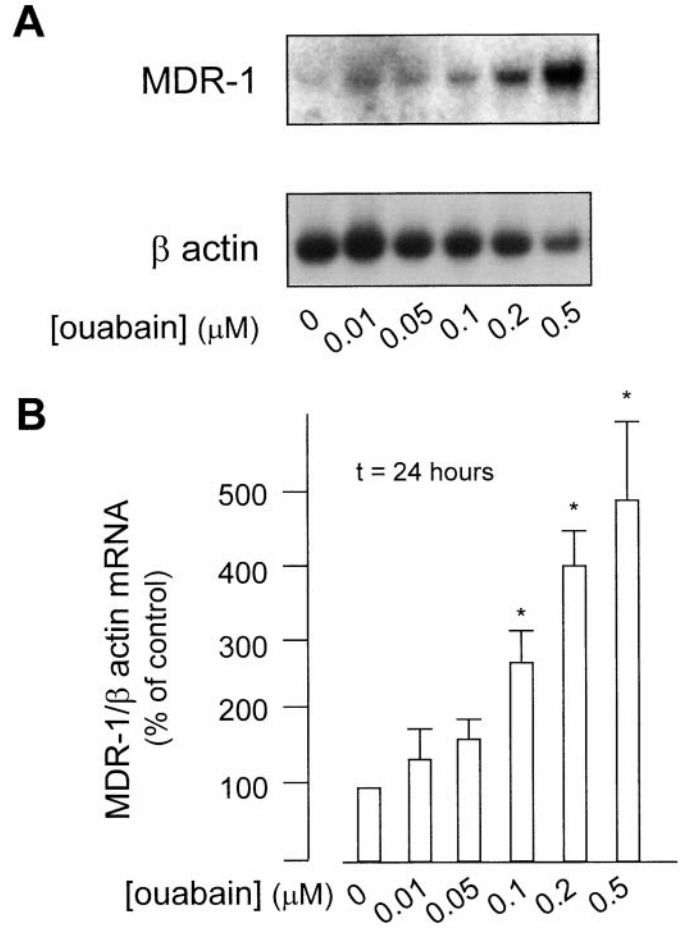

Fig. 1. Concentration dependence of the ouabain effect. Serum-deprived Calu-3 cells were incubated in serum-free culture medium containing various amounts of ouabain for 24 h. *A*, typical Northern blot obtained with 15 μg of total RNAs extracted from cells incubated with the indicated amounts of ouabain. *B*, Northern blot data expressed by the experimental MDR-1:β-actin mRNA ratios expressed as percentages of the mean ratio found in untreated cells. Each value is the mean ± SE of six experiments. *, $P < 0.05$.

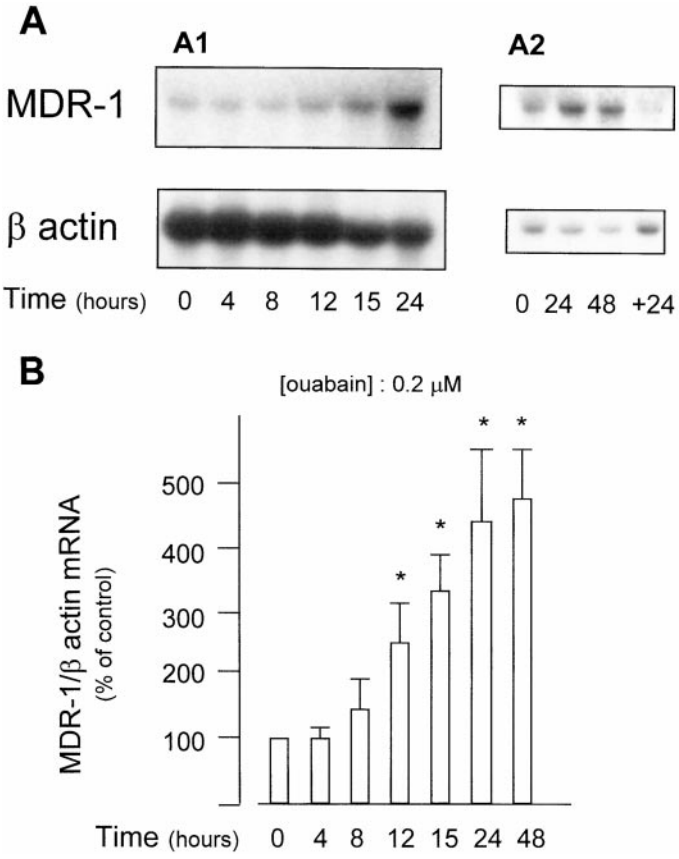

Fig. 2. Time course of the ouabain-induced increase in MDR-1 mRNA. The results were obtained and expressed as described in the Fig. 1 legend. *A1*, typical Northern blot obtained after incubating the cells with 0.2 μM ouabain for the indicated times. *A2*, on this Northern blot, the signal *+24* was obtained after a 24-h incubation followed by 24 h of recovery without ouabain. *B*, each MDR-1:β-actin ratio is the mean ± SE of six experiments. *, $P < 0.05$.

Table 1 *Ouabain-induced cell death*

Confluent cells were treated for 48 h with ouabain, washed twice, trypsinized, centrifuged, and resuspended in trypan blue in physiological saline. The living cells were counted on a Malassez cell. Three different wells were treated in each experiment, and each of them was counted three times. The values are the means ± SE of six experiments. Each experimental value was compared with that of the control untreated culture, using Student's *t* test.

Ouabain (μM)	No. of living cells ($\times 10^3$/cm^2)
0	603 ± 48 (100)
0.05	610 ± 47 (101) NS[a]
0.1	576 ± 45 (101) NS
0.2	518 ± 47 (86) NS
0.5	489 ± 49 (81) $P < 0.05$
1	387 ± 43 (64) $P < 0.01$

[a] NS, nonsignificant.

T-84 and HT-29 cells and in hepatic HuH7 cells, but not in HeLa cells (Fig. 3).

Digoxin and palitoxin, two other Na^+/K^+-ATPase inhibitors chemically distinct from ouabain (15), had the same the effect as ouabain on Calu-3 cells (Fig. 4*A*). Increased *MDR-1* gene transcripts thus appeared to be triggered by inhibiting the Na^+/K^+ pump. The ouabain-induced increase in *MDR-1* mRNA still occurred in the presence of cycloheximide (6 μM; Fig. 4*B*), indicating that the phenomenon does not require protein synthesis. The inhibition of the ouabain effect by actinomycin D (5 μg/ml added to the medium 30 min before ouabain) suggests that ouabain stimulates *MDR-1* gene transcription (Fig. 4*B*). The stimulation of *MDR-1* gene transcription by ouabain was enhanced by thapsigargin, which increases cytosolic calcium concentration, and suppressed when intracellular calcium was chelated by BAPTA (Fig. 4*C*). These results indicate that the increase in cytosolic calcium concentration produced by inhibiting the Na^+/K^+-ATPase participates in the stimulation of *MDR-1* gene transcription.

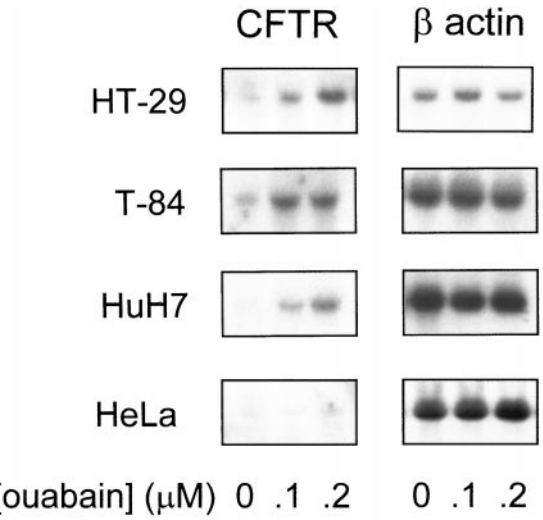

Fig. 3. Effect of ouabain on MDR-1 mRNA in four different cell lines. Like the Calu-3 cells, the T-84, HT-29, HuH7, and HeLa cells were grown to confluence, deprived of serum for 24 h, and then treated with ouabain (0.1 or 0.2 μM) for 24 h. The Northern blots were performed on 15 μg of total RNA. Each value is representative of four experiments.

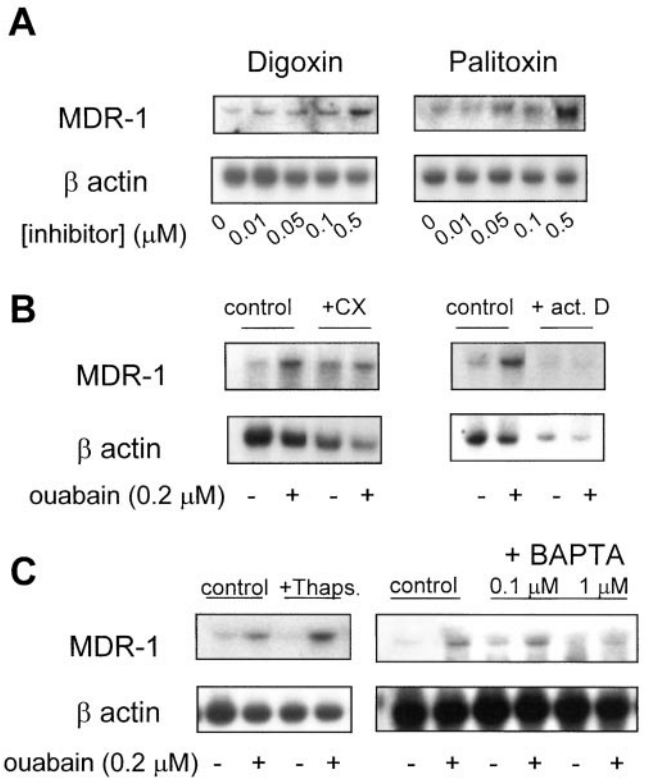

Fig. 4. Properties of the ouabain-induced increase in MDR-1 mRNA. All of the Northern blots were obtained with 15 μg of total RNAs extracted from serum-deprived cells treated for 24 h as indicated. *A*, concentration dependence of the effects of digoxin and palitoxin (the figure is representative of four experiments). *B*, cycloheximide (*CX*; 6 μM) and actinomycin D (*act. D*; 5 μg/ml) were added to the cultures 30 min before ouabain (0.2 μM) and remained in the medium during the ouabain treatment (the figure is representative of three experiments). *C*, thapsigargine (*Thaps.*; 2 μM) and BAPTA-AM at the indicated concentrations were added to the medium 15 min before ouabain (0.2 μM) for the incubation time.

P-gp Synthesis

Western blot analysis with mAb C-219 showed a single immunoreactive band of about M_r 170,000, compatible with P-gp, in the microsomes from Calu-3 cells. It was very faint in control membranes and dose-dependently increased by ouabain (0.05 to 0.2 μM; Fig. 5).

Immunocytochemistry (Fig. 6) also revealed a surface immunoreactivity to mAb MRK-16; it was very faint in control cells (Fig. 6*B1*) and enhanced by treating the cells with 0.2 μM ouabain for 48 h (Fig. 6*C1*). Labeling was specific because it did not exist when the cells were incubated with the secondary antibody directly (Fig. 6*A1*). Ouabain thus stimulates *MDR-1* gene transcription and P-gp synthesis.

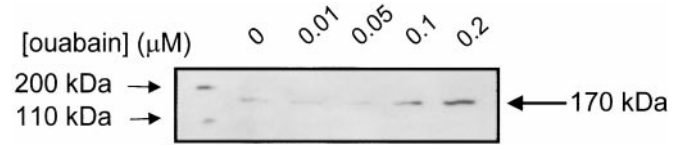

Fig. 5. Changes in membrane P-gp. The Western blots were performed as indicated in "Materials and Methods" on membranes extracted from cells incubated for 48 h with the indicated concentrations of ouabain. The proteins were separated by electrophoresis (30 μg/lane) and transferred to the membrane. They were detected by incubation with mAb C-219 directed against human P-gp. The figure is representative of three experiments.

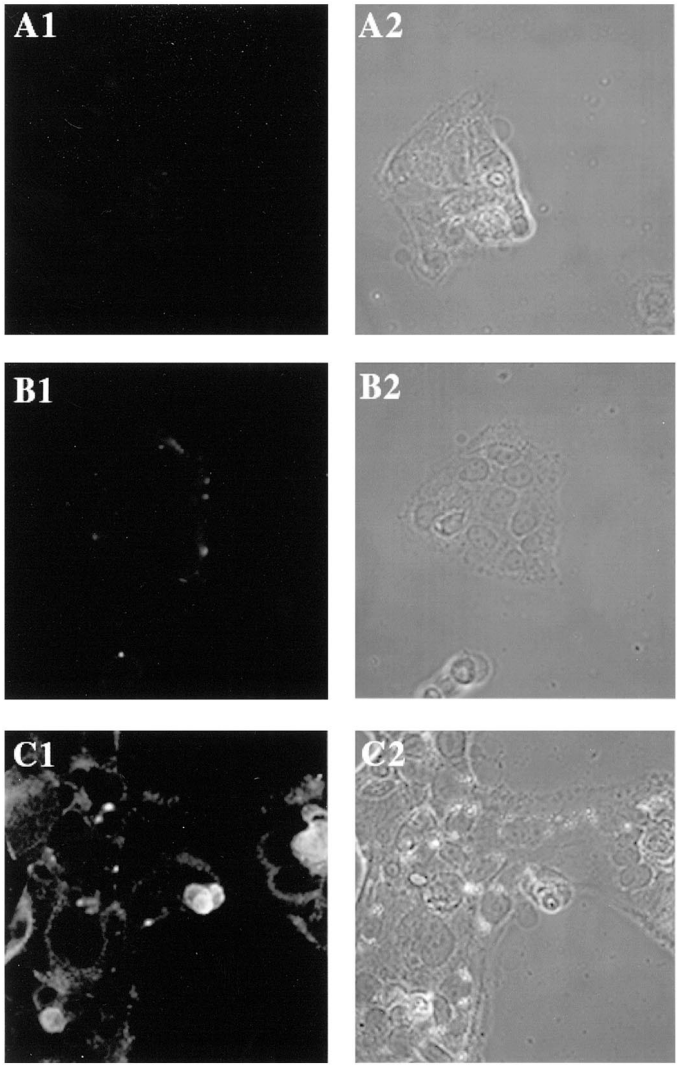

Fig. 6. Immunocytochemistry. The pictures on the *left* show the immunological staining with mAb MRK-16. The cells, either under basal conditions (*A* and *B*) or after incubation for 48 h with 0.2 μM ouabain (*C*), were incubated with mAb MRK-16 for 3 h (*B* and *C*). *A*, cells incubated with the second antibody directly. The pictures on the *right* show the same cells observed under a phase-contrast microscope.

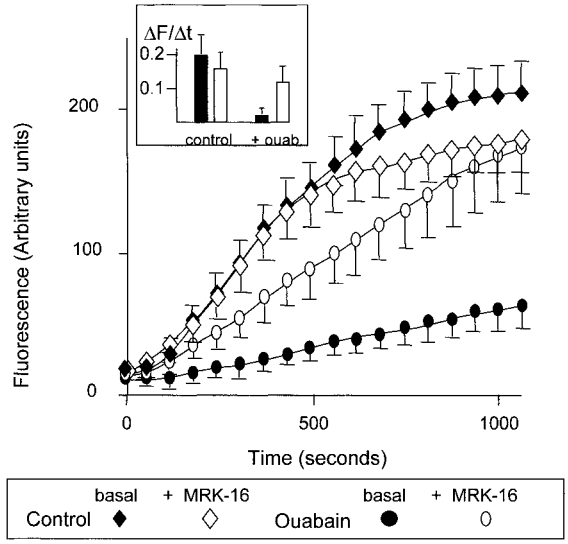

Fig. 7. P-gp-mediated CAL-AM efflux. Representative experiment showing the increase in the intracellular fluorescence of CAL, expressed in arbitrary units, on control cells (*diamonds*) and on cells treated for 48 h with 0.2 μM ouabain (*circles*) incubated with CAL-AM (0.25 μM, *black symbols*) alone or with mAb MRK-16 (0.25 μM) plus CAL-AM (*empty symbols*). Fluorescence intensity and SD were calculated from the Imstar software integrating the values found in each cell of the slide. *Inset*, means ± SE of the $\Delta F:\Delta t$ ratios calculated on 14 experiments in each experimental condition. $n = 14$; *, $P < 0.001$.

P-gp Function

CAL Efflux. When control Calu-3 cells were incubated with CAL-AM (0.25 μM), the intracellular de-esterification of the dye led to increased cell CAL fluorescence [$(\Delta F/\Delta t)_{basal} = 0.198 \pm 0.056$ ($n = 14$)] (Fig. 7). Pretreating the cells with 0.2 μM ouabain considerably reduced the rate of increase of fluorescence [$(\Delta f/\Delta t)_{ouab} = 0.066 \pm 0.022$ ($n = 14$)], which went on for 30 min (data not shown). Incubation with the specific anti-P-gp mAb MRK-16 (10 μg/ml added 15 min before CAL-AM) significantly reduced the difference between the treated and untreated cells. The rate of increase of fluorescence in the ouabain-treated cells in the presence of MRK-16 [$(\Delta F/\Delta t)_{MRK16\text{-}ouab} = 0.125 \pm 0.037$ ($n = 14$)] was very close to that of controls [$(\Delta F/\Delta t)_{MRK16\text{-}control} = 0.145 \pm 0.066$ ($n = 14$)], and the maximal fluorescence was the same in both cases. This indicates that the low fluorescence of the ouabain-treated cells not incubated with MRK-16 could be due to a rapid extrusion (before its de-esterification) of the CAL-AM from these treated cells. Verapamil (50 μM) reproduced the effect of mAb MRK-16 and increased the rate of fluorescence of the ouabain-treated cells (results not shown), but it also decreased the responses of the control cells at this concentration. These results show that the ouabain-induced P-gp extrudes hydrophobic compounds.

Resistance to Doxorubicin and Vinblastine. The toxicity of doxorubicin and vinblastine, estimated as indicated in "Materials and Methods" 3 days after treating the cells with the anticancer drugs, showed that treating the cells with ouabain (0.2 μM for 48 h) made them resistant to a 4-h exposure with 0.5 μM of both drugs (Fig. 8). Three days after the exposure, control cells treated with 0.5 μM doxorubicin or vinblastine were less numerous [$-38 \pm 8\%$ ($n = 6$) and $-42 \pm 6\%$ ($n = 4$), respectively] than those that were not incubated with the anticancer agents; verapamil had no significant effect. However, ouabain-treated cells were significantly resistant to doxorubicin, which decreased their numbers by only 18 ± 10% ($n = 6$), and to vinblastine, which decreased their numbers by

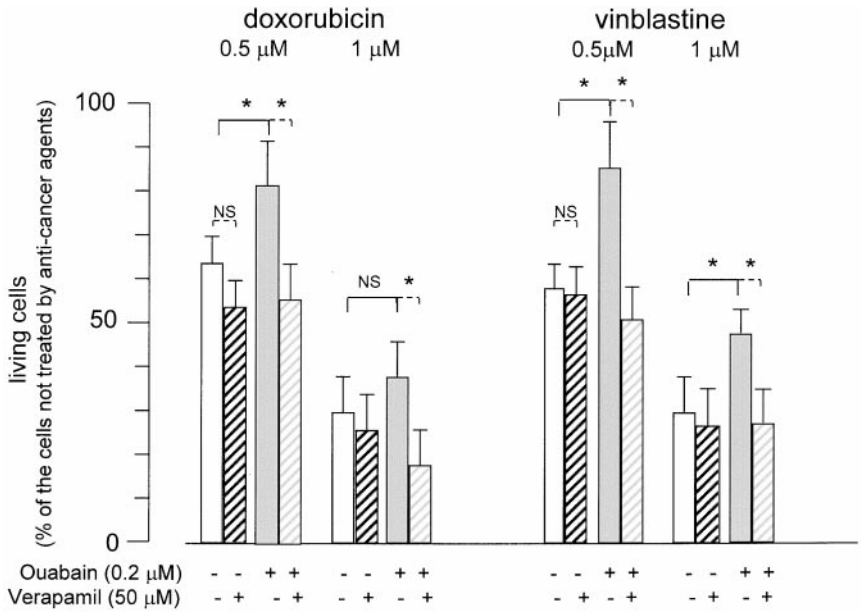

Fig. 8. Ouabain-induced resistance of the cells to doxorubicin and vinblastine. Control cells (□) and cells treated with ouabain (0.2 μM; 48 h; ▨) were incubated for 4 h with doxorubicin or vinblastine (0.5 and 1 μM) with (*hatched columns*) or without verapamil (50 μM), washed, counted, and incubated for an additional 3 days. They were then counted, and the results for doxorubicin- and vinblastine-treated cells were expressed as percentages of those for controls. Each figure is the mean ± SE of four (vinblastine) or six (doxorubicin) experiments. The means were compared using Student's unpaired *t* test. *, $P < 0.05$.

19 ± 9% ($n = 4$). In both cases, the resistance was inhibited by verapamil; with verapamil, doxorubicin and vinblastine decreased the number of living ouabain-pretreated cells by 47 ± 9% ($n = 6$) and 56 ± 9% ($n = 4$), respectively. The ouabain treatment did not significantly modify the effect of 1 μM doxorubicin (however, verapamil significantly increased doxorubicin-induced cell death in ouabain-treated cells), but it still protected the cells against 1 μM vinblastine.

DISCUSSION

We have shown that submicromolar concentrations of ouabain stimulate *MDR-1* gene expression and P-gp synthesis in human tumoral cells. We believe that this is the first demonstration of a P-gp-mediated drug efflux that is pharmacologically induced by a hydrophylic glycoside that acts on an extracellular site of a transmembrane protein, the Na^+/K^+-ATPase. The microscopic CAL-AM/CAL assay developed to demonstrate this P-gp function at the cellular level may be suitable for clinical use on small tissue samples.

The concentration dependence of the increase in *MDR-1* transcripts caused by ouabain corresponds to the parameters of the Na^+/K^+-ATPase inhibition. The changes in *MDR-1* mRNA occurred at 0.1–0.5 μM ouabain, whereas the drug binds to the Na^+/K^+ pump with an affinity constant of about 0.01 or 0.1 μM, depending on the form of the α protein subunit. Half-maximal inhibition of the human Na^+/K^+ pump occurs at 0.1 μM in nonmyocardial human cells (19). The fact that both digoxin, another Na^+/K^+-ATPase inhibitor that is a lipid compound and acts intracellularly, and palitoxin, which decreases the pump activity by a different mechanism (19), also increase *MDR-1* transcripts further demonstrates that inhibition of Na^+/K^+-ATPase activity triggers *MDR-1* gene expression in our experimental model. This *MDR-1* gene overexpression is not limited to Calu-3 cells because it also occurred in T-84 and HT-29 colon cells, which differ in differentiation, and in HuH7 carcinomatous hepatic cells. However, the fact that it was not found in HeLa cells demonstrates that it is not a ubiquitous cell response to the cardiotonic.

The inhibition of the Na^+/K^+-ATPase by ouabain is immediate, leading to gradual changes in cytosolic ion concentrations. Various ion transports are activated during the cell adaptation that follows these changes. The time dependence of the reversible ouabain-induced increase in *MDR-1* gene expression agrees with the participation of P-gp in the adaptation of the cell to the Na^+/K^+ pump blockade. The increase in *MDR-1* transcripts (and the decrease in β-actin mRNA) in Calu-3 cells may be due to ouabain-induced changes in cell ion concentrations. It appears to start with enhanced transcription and does not depend on the synthesis of any intermediate protein. The contrast between the stimulation of *MDR-1* gene expression triggered by low concentration of ouabain in Calu-3, T-84, HT-29, and HuH7 cells and the absence of response of HeLa cells may result from cell-to-cell differences in either cytoplasmic or nuclear reactions. Further work is needed to define these cell characteristics, which may be responsible for the differing sensitivities of cancerous cells to multidrug resistance. The elevation in cytosolic calcium concentration that follows the increase in intracellular Na^+ caused directly by the Na^+/K^+-ATPase blockade (20) may be involved in the phenomenon. For example, ouabain stimulates early-activated gene (c-*fos* and c-*jun*) transcription via increased cytosolic calcium concentration in both rat cardiac myocytes and various human cell lines (10, 21, 22). The reaction (which is triggered in HeLa cells by micromolar concentrations of ouabain; Ref. 22) does not require protein synthesis but results from the stimulation of mitogen-activated protein kinases (21), which control various gene expressions in several cell types (23). In Calu-3 cells, an increased intracellular calcium concentration also appears to be the link between Na^+/K^+-ATPase inhibition and the increase in *MDR-1* mRNA stimulated by ouabain because the ouabain effect was reinforced by thapsigargin and suppressed by the calcium chelator BAPTA. Because it was detected with low concentrations of ouabain (0.1 μM), it cannot be a consequence of the cell damage caused by higher concentrations of ouabain via elevated cytosolic calcium and activation of kinases. Ouabain-induced cell detachment, which is due to phosphorylation of proteins devoted to cell support or cell-cell contacts and which leads to cell death (24), may occur in our model with 0.5 and 1 μM ouabain, but it is preceded by *MDR-1* gene overexpression, which is maximally induced by 0.2 μM ouabain.

Enhancement of *MDR-1* gene expression by ouabain led to synthesis of P-gp, which was immunodetected in the cell membranes. The efflux of hydrophobic drugs is the major function of P-gp, and this was why we looked for it in the ouabain-treated Calu-3 cells. Because P-gp extrudes the nonfluorescent hydrophobic CAL-AM before its

de-esterification to fluorescent CAL, we measured the rate of increase of fluorescence in cells incubated with CAL-AM to detect functional P-gp in the cell membrane. This technique was first developed to assess the P-gp-related multidrug resistance in large numbers of tumoral cells by flow cytometry (16). These results show that the rate of increase of the fluorescence was slower in cells from patients who had multidrug resistance or in cultured cells treated for a long time with high concentrations of drugs such as doxorubicin or colchicine that induce *MDR-1* gene expression. However, this method can only be used with large numbers of cells producing large amounts of P-gp. We detected activities of P-gp in isolated cells or clusters of cells (50 cells) in our experiments using low CAL-AM concentrations (up to 0.25 μM) and a sensitive videomicroscopy system. The difference between control and treated cells was very significant under these conditions. Although CAL fluorescence is insensitive to variation in ionic concentration (17), this difference could be due to some alteration in the cytoplasm induced by ouabain. Its inhibition by mAb MRK-16, which allows the maximal fluorescence of ouabain-treated cells to reach control level, clearly indicates that the inhibition of CAL accumulation in ouabain-treated cells in the absence of the P-gp antagonist is linked to P-gp function. These results agree with our data on the resistance of ouabain-treated cells to doxorubicin and vinblastine cytotoxicity. The cell resistance was not complete and was seen only with low concentrations of the anticancer drugs. However, it was significant, especially *versus* vinblastine, and was inhibited by verapamil. Hence, our results show that inhibiting Na^+/K^+-ATPase may cause multidrug resistance by stimulating *MDR-1* gene expression and drug efflux mediated by P-gp.

The stimulation by ouabain of the synthesis of P-gp able to extrude hydrophobic compounds appears to result from disturbed cell ion concentrations. The data show that cells do not need to be exposed to cytotoxic agents to make P-gp able to extrude such drugs, but the cell damage caused by ouabain via the ionic disorders that stimulate *MDR-1* gene expression cannot be ruled out. The P-gp-mediated CAL-AM efflux and the resistance of the ouabain-treated Calu-3 cells to both doxorubicin and vinblastine are not very large. Low resistance to chemotherapeutic drugs was shown to be a characteristic of cells transfected with *MDR-1*, and the alterations in pHi and membrane potential caused by the induced P-gp were sufficient to trigger this reduced multidrug resistance (8). Additional studies are now required to determine whether the ouabain-treated cells behave as a pharmacological equivalent of these "pure transfects." This would mean that P-gp is essential for the integrated control of cell ion concentrations. The ouabain-induced multidrug cell resistance might also be important in clinical situations because cardiotonics may be prescribed together with certain anticancer drugs.

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