

# Ouabain exerts biphasic effects on connexin functionality and expression in vascular smooth muscle cells

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- 1 We have compared the effects of ouabain on the maintenance of gap junctional communication in rat aortic A7r5 smooth muscle cells, monkey COS-1 fibroblasts and human HeLa epithelial cells.
- 2 Ouabain (1 mM) interrupted dye coupling between confluent A7r5 cells within ~1 h, and high concentrations of ouabain were similarly required to reduce coupling between COS-1 cells selected to express the rat  $\alpha_1$  Na<sup>+</sup>/K<sup>+</sup>-ATPase subunit, which is ouabain resistant. By contrast, low concentrations of ouabain (1–10  $\mu$ M) attenuated dye transfer in wild-type COS-1 and HeLa cells, whose endogenous  $\alpha_1$  subunits possess relatively high affinity for the glycoside ( $K_i$  ~0.3 vs ~100  $\mu$ M). Ouabain-induced reductions in dye transfer therefore correlated with the ability of the glycoside to bind to the Na<sup>+</sup>/K<sup>+</sup>-ATPase isoenzymes expressed in these different cell lines.
- 3 No consistent relationship between inhibition of intercellular dye transfer and secondary changes in [Ca<sup>2+</sup>]<sub>i</sub> or pH<sub>i</sub> could be identified following incubation with ouabain.
- 4 In separate experiments, the effects of ouabain on real-time trafficking of connexin protein were monitored by time-lapse microscopy of A7r5 cells transfected to express a fluorescent Cx43-green fluorescent protein (GFP) and the ability of the glycoside to modulate endogenous expression of connexins (Cx) 40 and 43 evaluated in A7r5 cells by immunochemical and Western blot analysis.
- 5 Ouabain (1 mM) depressed vesicular trafficking of Cx43-GFP after ~1 h, and caused a time-dependent loss of endogenous Cx40 and Cx43 protein that was first evident at 2 h and almost complete after 4 h. These effects of ouabain on Cx expression were reversed ~90 min following washout of the glycoside.
- 6 We conclude that ouabain exerts biphasic effects on the intercellular communication that involve an initial decrease in gap junctional permeability followed by a global reduction in the expression of Cx protein. Further studies are necessary to establish to what extent these actions of ouabain reflect inversion of the normal [Na<sup>+</sup>]<sub>i</sub>/[K<sup>+</sup>]<sub>i</sub> ratio and/or conversion of the Na<sup>+</sup>/K<sup>+</sup>-ATPase into a general signal transducer that regulates downstream protein synthesis.

*British Journal of Pharmacology* (2003) **140**, 1261–1271. doi:10.1038/sj.bjp.0705556

**Keywords:** Cell communication; gap junctions; EDHF

**Abbreviations:** BCECF, 2',7', -bis(2-carboxyethyl)-5(6)-carboxyfluorescein; Cx, connexin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; dtt, dithiothreitol; GFP, green fluorescent protein; PBS, phosphate-buffered saline; HBS, HEPES-buffered saline; fura-2 AM, 5-oxazolecarboxylic acid, 2-[6-[bis[2-acetyloxy]-methoxy]-2-oxoethyl]amino]-5-[2-[2-[bis[2-(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]-2-benzofuranyl]-, (acetyloxy)methyl ester

## Introduction

The glycoside ouabain, which binds to the membrane Na<sup>+</sup>/K<sup>+</sup>-ATPase, affects cell function by modulating ionic homeostasis and intracellular signal transduction cascades, ultimately leading to changes in the expression of a wide range of intracellular proteins (Blanco & Mercer, 1998; Haas *et al.*, 2002; Taurin *et al.*, 2002). Since the Na<sup>+</sup>/K<sup>+</sup>-ATPase is electrogenic, in vascular smooth muscle ouabain may promote an increase in contractile tone by causing membrane depolarization and thereby increasing Ca<sup>2+</sup> influx *via* voltage-

dependent Ca<sup>2+</sup> channels (Sato & Aoki, 1991). The loss of ionic pump activity that follows binding of ouabain also contributes to elevations in tone by elevating [Na<sup>+</sup>]<sub>i</sub> and causing a secondary increase in [Ca<sup>2+</sup>]<sub>i</sub> *via* Na<sup>+</sup>–Ca<sup>2+</sup> exchange (Sato & Aoki, 1991; Borin *et al.*, 1994). The glycoside may additionally modulate vascular function by attenuating agonist-induced relaxations that are mediated *via* the endothelium, but are independent of nitric oxide (NO) and vasodilator prostanoids. Among the mechanisms postulated to contribute to such responses is an extracellular release of K<sup>+</sup> associated with activation of endothelial K<sub>Ca</sub> channels. In this scenario, K<sup>+</sup> serves as a freely transferable endothelium-derived hyperpolarizing factor (EDHF) and ouabain has been hypothesized to attenuate relaxation by blocking the increase

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in  $\text{Na}^+/\text{K}^+$ -ATPase activity that results from elevated  $[\text{K}^+]_o$  (Edwards *et al.*, 1998). Alternatively, ouabain might attenuate NO/prostanoid-independent relaxations by attenuating the electrotonic spread of agonist-induced endothelial hyperpolarization into the vascular wall (Griffith *et al.*, 2002; Sandow *et al.*, 2002), since the glycoside is also able to block signalling *via* gap junctions (Schirrmacher *et al.*, 1996; Harris *et al.*, 2000). These intercellular communication channels allow direct transfer of ions and small polar molecules  $<1$  kDa in size, thereby conferring electrical continuity between coupled cells, and are constructed from connexins (Cx), a highly conserved group of at least 20 distinct proteins, of which the Cx37, 40, 43 and 45 subtypes (designated according to molecular mass in kDa) are found in the vasculature (Chaytor *et al.*, 2001; Ko *et al.*, 2001; Kruger *et al.*, 2002; Berman *et al.*, 2002; Hill *et al.*, 2002; Ujiie *et al.*, 2003). Since the ability of ouabain to interrupt direct intercellular signalling has not previously been investigated in vascular cells, in the present study we have examined the role of the  $\text{Na}^+/\text{K}^+$ -ATPase in maintaining gap junctional communication in rat aortic A7r5 myocytes. These cells are highly coupled and exhibit abundant expression of Cx43 and Cx40 in gap junction plaques at points of cell–cell contact (Chaytor *et al.*, 2001).

The minimal functional unit of the  $\text{Na}^+/\text{K}^+$ -ATPase is a heterodimer consisting of a catalytic  $\alpha$ -subunit and a regulatory  $\beta$ -subunit (Blanco & Mercer, 1998). Four  $\alpha$ - and three  $\beta$ -isoforms have been characterized and are expressed as various combinations in a cell- and tissue-specific manner (Juhászova & Blaustein, 1997; Blanco & Mercer, 1998). All such isoenzymes are inhibited by ouabain, although its potency may vary. Many cells express an  $\alpha_1$  isoform that is highly sensitive to ouabain, but mutations in the binding site render the rodent  $\alpha_1$  subunit relatively ouabain-resistant (Vilsen, 1999). In the present study, we have exploited the ability of the rat  $\alpha_1$  subunit to heterodimerize with  $\beta$  subunits in COS-1 cells to construct a model cell system that expresses the ouabain-resistant rodent pump (Belusa *et al.*, 2002; Kristensen *et al.*, 2003a). Comparison of the effects of ouabain on dye transfer of Lucifer yellow in A7r5 cells, wild-type (wt) and ouabain-resistant (oubR) COS-1 cells was then used to confirm the specific role of the  $\text{Na}^+/\text{K}^+$ -ATPase in the maintenance of gap junctional communication. Analogous experiments were also performed in HeLa cells, rendered communication competent by selection to express a chimeric Cx43-green fluorescent protein (GFP), and in which the  $\alpha_1$  subunit is ouabain sensitive (Martin *et al.*, 2001; Berman *et al.*, 2002). In osteoblastic cells, the ability of ouabain to impair intercellular communication has been hypothesized to result from the increase in  $[\text{Ca}^{2+}]_i$  that follows inhibition of the  $\text{Na}^+/\text{K}^+$ -ATPase (Schirrmacher *et al.*, 1996). However, it is also conceivable that secondary changes in intracellular pH, which may follow loss of the ionic activity of the pump in some cell types (Arisaka *et al.*, 1988; Souza *et al.*, 2000), modulate gap junction permeability, since intracellular acidification can reduce the permeability of gap junctions (Morley *et al.*, 1996). We therefore investigated if the effects of ouabain on dye transfer in A7r5 cells, wtCOS cells and oubRCOS cells could be linked to changes in  $[\text{Ca}^{2+}]_i$  and  $\text{pH}_i$  that might follow blockade of the  $\text{Na}^+/\text{K}^+$ -ATPase. Finally, given the ability of ouabain to modulate protein expression (Taurin *et al.*, 2002), and the short half-life of Cx proteins, for example, 1–2 h for Cx43 (Darrow *et al.*, 1995; Saffitz *et al.*, 2000), we

examined the effects of the glycoside on real-time trafficking of Cx protein in A7r5 cells transfected to express Cx43-GFP (Martin *et al.*, 2001) and the effects of longer-term incubation (2–4 h) on the expression of endogenous Cx40 and Cx43 by immunocytochemical and Western blot analysis of control A7r5 cells. Taken together, the findings demonstrate the existence of a previously unrecognized biphasic effect of ouabain on gap junction functionality and Cx expression in vascular smooth muscle.

## Methods

### Cell culture and transfection

The rat aortic smooth muscle A7r5 cell line, the human HeLa Ohio epithelial cell line and the monkey kidney fibroblast COS-1 cell line were maintained in complete DMEM (Chaytor *et al.*, 2001; Martin *et al.*, 2001). For HeLa cells selected to express Cx43-GFP, the medium was supplemented with geneticin sulphate (G418-sulphate,  $4 \text{ mg ml}^{-1}$ ) (Martin *et al.*, 2001; Berman *et al.*, 2002). COS-1 cells resistant to ouabain were generated by transfection with rat  $\alpha_1$   $\text{Na}^+/\text{K}^+$ -ATPase cDNA and were maintained in complete DMEM containing  $10 \mu\text{M}$  ouabain as previously described (Kristensen *et al.*, 2003a). For dye transfer studies, cells were grown on 60 mm tissue culture dishes. A7r5 cells were transiently transfected with  $0.25 \mu\text{g}$  Cx43-GFP cDNA using Lipofectamine 2000. For immunocytochemistry, timelapse microscopy, and  $\text{Ca}^{2+}$  and pH measurements cells were cultured in  $24 \text{ mm}^2$  coverglass chambers.

### Gap junction functionality

The role of the  $\text{Na}^+/\text{K}^+$ -ATPase in maintaining cell to cell coupling was assessed by studying the effects of 60 min incubation with ouabain ( $1 \mu\text{M}$  –  $1 \text{ mM}$ ) on dye transfer of Lucifer yellow CH (charge  $-2$ , MW 457 Da). At 15 min following intracytoplasmic microinjection of individual cells within confluent monolayers, the cultures were fixed in 4% w/v paraformaldehyde and the percentage of injections resulting in dye transfer to different numbers of neighbouring cells assessed on a Zeiss Axiovert fluorescence microscope (Martin *et al.*, 2001; Berman *et al.*, 2002).

### Intracellular calcium and pH levels

For intracellular  $\text{Ca}^{2+}$  measurements A7r5, COS-1 and HeLa cells were loaded with Fura-2 AM ( $2 \mu\text{g ml}^{-1}$ ) and for pH measurements A7r5 and COS-1 cells were loaded with BCECF AM ( $1.25 \mu\text{g ml}^{-1}$ ) by incubation for 20 min at  $37^\circ\text{C}$  in HBS ( $133 \text{ mM NaCl}$ ,  $4.2 \text{ mM KCl}$ ,  $1 \text{ mM MgCl}_2$ ,  $5.8 \text{ mM glucose}$ ,  $10 \text{ mM HEPES}$ ,  $0.1\% \text{ w/v BSA}$  pH 7.5) supplemented with  $1.3 \text{ mM CaCl}_2$ . Cells were viewed on a fluorescent microscope and images collected every 10 s for 30 min on a heated stage maintained at  $37^\circ\text{C}$ . Ouabain ( $100 \mu\text{M}$ – $1 \text{ mM}$ ) was added at 30 s. The fluorescence intensities resulting from excitation at 340 and 380 nm (for  $\text{Ca}^{2+}$  measurements) and from excitation at 480 and 440 nm (for pH measurements) were recorded using Kinetic Imaging software. The data were exported to a Microsoft Excel spreadsheet and the 340:380 and 480:440 nm ratios calculated to determine changes in  $[\text{Ca}^{2+}]_i$  and  $\text{pH}_i$ .

### Immunocytochemistry

The effects of ouabain on endogenous Cx43 expression in A7r5 cells were assessed with a monoclonal antibody against the carboxyl tail of Cx43 (1:250 dilution) and a secondary goat anti-mouse antibody conjugated to Alexa 488 (1:700 dilution) (Chaytor *et al.*, 2001; Ujji *et al.*, 2003). Images were acquired with an Axiovert 100 microscope linked to a BIORAD MRC 1024MP laser scanning system, typically under a  $\times 40$  lens, with a gain of 930 and Iris 4 and a Kalman filter (16 scans). Quantification of the fluorescence distribution before and after treatment with ouabain was assessed by analysing up to 10 regions of equal area along the plasma membrane per field of view and recording their fluorescent intensity. To obtain a mean pixel intensity, four to five images were analysed in this way for each experimental group and similarly defined areas selected to estimate background fluorescence which was subtracted from the mean pixel intensity of membrane fluorescence to derive a relative fluorescent index for each set of treatments.

### Western blot analysis

Confluent A7r5 cells were incubated for up to 4 h with ouabain (100  $\mu\text{M}$ –1 mM) and harvested in 100  $\mu\text{l}$  of ice-cold lysis buffer (1% w/v SDS, 1 mM DTT, 1 mM  $\text{NaVO}_4$ , 4 ng  $\text{ml}^{-1}$  leupeptin, 4 ng  $\text{ml}^{-1}$  aprotinin and 1 mM phenylmethylsulfonyl fluoride in PBS), followed by sonication. Protein content was measured using the BIORAD assay kit and equal amounts of protein (50  $\mu\text{g}$ ) were analysed on SDS–PAGE (10%) followed by transfer to nitrocellulose in transfer buffer (20 mM  $\text{Na}_2\text{CO}_3$ ) for 3 h at 300 mA. The transfer efficiency was assessed by Ponceau S staining prior to further probing with a polyclonal antibody to Cx43 that recognizes multiple phosphorylated/nonphosphorylated isoforms of Cx43 (1:4000 dilution) or an antibody to Cx40 (1:1000 dilution), and a secondary goat anti-rabbit horseradish peroxidase antibody. To standardize protein expression, the blots were also probed with a monoclonal antibody against  $\alpha$ -tubulin (1:5000 dilution) and a secondary goat anti-mouse horseradish peroxidase antibody. Blots were developed by enhanced chemiluminescence (ECL) and analysed using a BIORAD 700 densitometer.

### Time lapse microscopy

At 30 h post-transfection of A7r5 cells with Cx43-GFP, cells were washed twice in PBS and the medium replaced with 1 ml of HBS supplemented with 1.3 mM  $\text{CaCl}_2$  or HBS containing only trace  $\text{Ca}^{2+}$ . Time-lapse sequences were acquired using a  $\times 40$  1.3 oil immersion lens to provide image sequences in 15 min batches (60 images of 15 s length), which enabled Cx trafficking to be monitored 0–15, 30–45, 60–75 min under control conditions and following treatment with ouabain. The BIORAD Lasersharp software package was used to analyse Cx43-GFP trafficking events using an approach analogous to that used to measure spatial co-localization (Manders *et al.*, 1993). Time points 15 s ( $t_1$ ), 7.5 min ( $t_2$ ) and 15 min ( $t_3$ ) from each batch of images were assigned the colour channels red, green and blue, respectively (see Figure 5). Superposition of these coloured images provided visual and quantifiable information about the dynamics of Cx43-GFP trafficking, with the relative location of red, green and blue pixels

indicating the direction of trafficking towards, away from or parallel to the plasma membrane, and static vesicular Cx protein giving a merged image with white pixels. Comparison of time points 1 and 3 from each data set permitted the extraction of a temporal colocalization coefficient ( $C_E$ ), which quantifies the number of red pixels at time point 1 that superimpose with blue pixels at time point 3 (Martin *et al.*, 2001). As the value of  $C_E$  tends towards 0 the extent of overlap is small as a result of Cx43-GFP intensities changing position. By contrast, if the value approaches 1 then the extent of overlap was large indicating no movement. The extent of temporal overlap can also be represented on a pixel-by-pixel basis by a scatter plot showing the distribution at  $t_1$  (red axis) compared with  $t_3$  (blue axis). Complete colocalization is shown along the line of identity (Martin *et al.*, 2001).

### Cell viability

Cell viability was assessed by loading cells with 2.5  $\mu\text{M}$  calcein AM for 15 min in complete medium at 37°C, followed by three washes in HBS containing 1.3 mM  $\text{Ca}^{2+}$ . Cells were also incubated with 5  $\mu\text{g ml}^{-1}$  propidium iodide for 5 min and viewed on a confocal laser-scanning microscope with dual excitation spectra of 488 and 567 nm and emissions of 530/540 and 580 nm LP for calcein and propidium iodide, respectively.

### Materials

All cell lines used were obtained from ECACC, Wiltshire, U.K. DMEM, supplements for cell growth and Lipofectamine 2000 were supplied by Invitrogen (Glasgow, U.K.). G418-sulphate was purchased from Promega (Southampton, U.K.) and Lucifer yellow, Fura2 AM, BCECF, calcein AM and goat anti-rabbit Alexa488 were obtained from Molecular Probes (Leiden, Netherlands). The Cx43 monoclonal antibody used for immunofluorescence and the  $\alpha$ -tubulin antibody were supplied by Chemicon (Chandlers Ford, U.K.). Other antibodies for Western blotting of Cx43 and Cx40 were obtained from Zymed (Cambridge, U.K.) and Alpha Diagnostics (San Antonio, U.S.A.), respectively. The goat anti-mouse horseradish peroxidase was supplied by BioRad (Hemel Hempstead, U.K.) and the ECL system from Pierce (Tattenhall, U.K.). All other reagents were supplied by Sigma (Poole, U.K.). Ouabain was dissolved initially in DMSO to give a stock solution of 0.1 M. In control experiments, DMSO was inactive at the final concentrations employed. Other agents were dissolved directly in buffer.

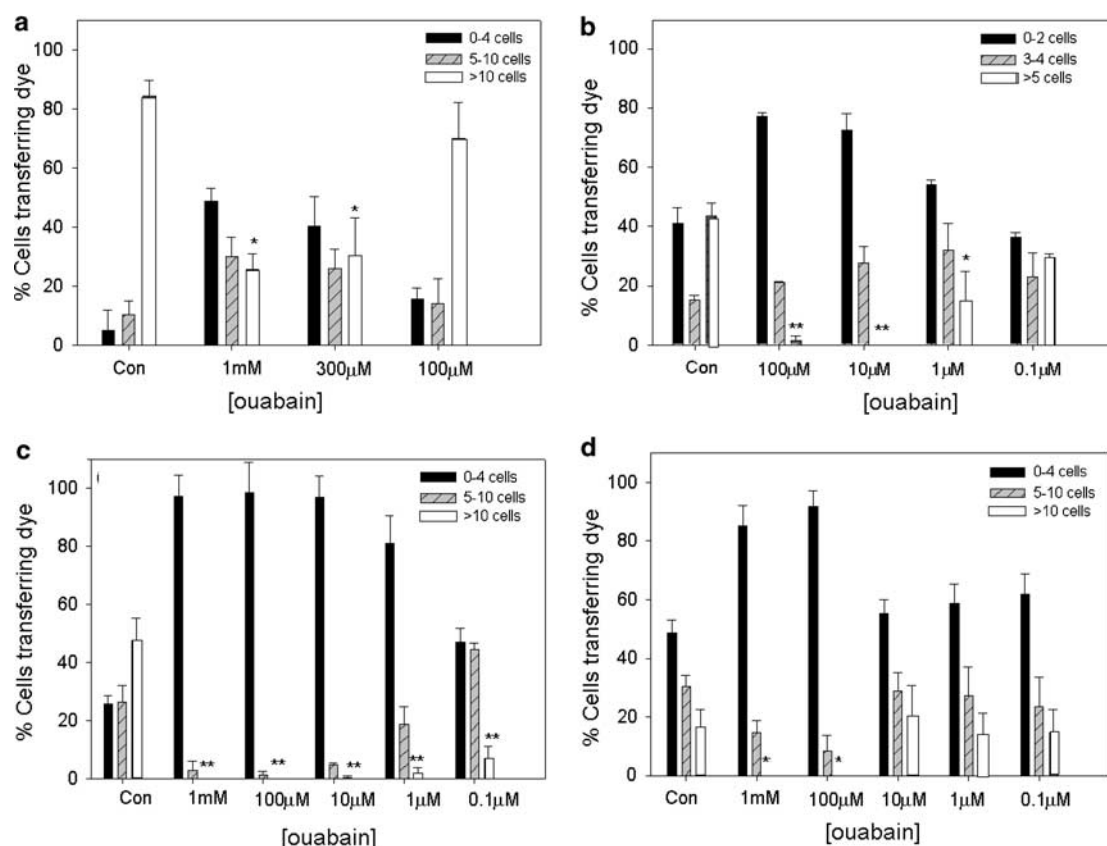
### Statistics

Data were evaluated by ANOVA followed by Dunnett's multiple comparison test, with  $P < 0.05$ , being considered significant.

## Results

### Gap junction functionality

Under control conditions more than 80% of A7r5 cells transferred dye to  $>10$  neighbours. Ouabain at 300  $\mu\text{M}$  and 1 mM attenuated coupling to  $>10$  neighbours by  $\sim 70\%$



**Figure 1** Inhibitory effects of ouabain on dye coupling in A7r5 cells, HeLa cells expressing Cx43-GFP, wild-type COS-1 cells and ouabain-resistant COS-1 cells. Results are expressed as the percentage of cells transferring Lucifer yellow to 0–4, 5–10 and >10 neighbours (for A7r5 and COS cultures) and to 0–2, 3–4 and >5 neighbours in HeLa cultures (because of lower dye coupling). Experiments were performed in triplicate with >50 injections per plate, and this protocol repeated three times. Data are shown as mean  $\pm$  s.e.m. Asterisks indicate significant effects of ouabain on the highest level of coupling compared to control \* $P$  < 0.05, \*\*  $P$  < 0.005.

( $P$  < 0.01), whereas incubation with 100  $\mu$ M was without effect (Figure 1). Under control conditions ~50% of HeLa cells selected to express Cx43-GFP transferred dye to >5 neighbours and ouabain attenuated coupling by ~90% at 100 and 10  $\mu$ M ( $P$  < 0.01), by ~70% at 1  $\mu$ M ( $P$  < 0.01), but was without effect at 0.1  $\mu$ M (Figure 1).

Under control conditions wt and oubrCOS-1 cells both transferred dye to >10 neighbours (Figure 1). Treatment of either population with 1 mM or 100  $\mu$ M ouabain attenuated dye transfer to >10 cells by ~90% ( $P$  < 0.05). Ouabain at concentrations of 0.1, 1 and 10  $\mu$ M significantly attenuated dye transfer in wt cells, but was without effect in oubr cells (Figure 1). This difference is to be expected, as although the abundance ratio of the endogenous and rat  $\alpha_1$  Na<sup>+</sup>/K<sup>+</sup>-ATPase is close to unity in oubrCOS cells (Kristensen *et al.*, 2003a), they were continuously maintained in 10  $\mu$ M ouabain. For reasons that are unclear, under baseline conditions, the percentage of oubr cells transferring dye at the highest level (to >10 neighbours) was only ~30% of that of wt cells.

#### Intracellular Ca<sup>2+</sup> and pH levels

Under control conditions, A7r5 cells maintained a steady basal [Ca<sup>2+</sup>]<sub>i</sub> with a 340:380 ratio of ~1. Treatment with 100  $\mu$ M ouabain induced a transient increase in [Ca<sup>2+</sup>]<sub>i</sub> that peaked at ~20 min before declining, whereas 1 mM ouabain induced a

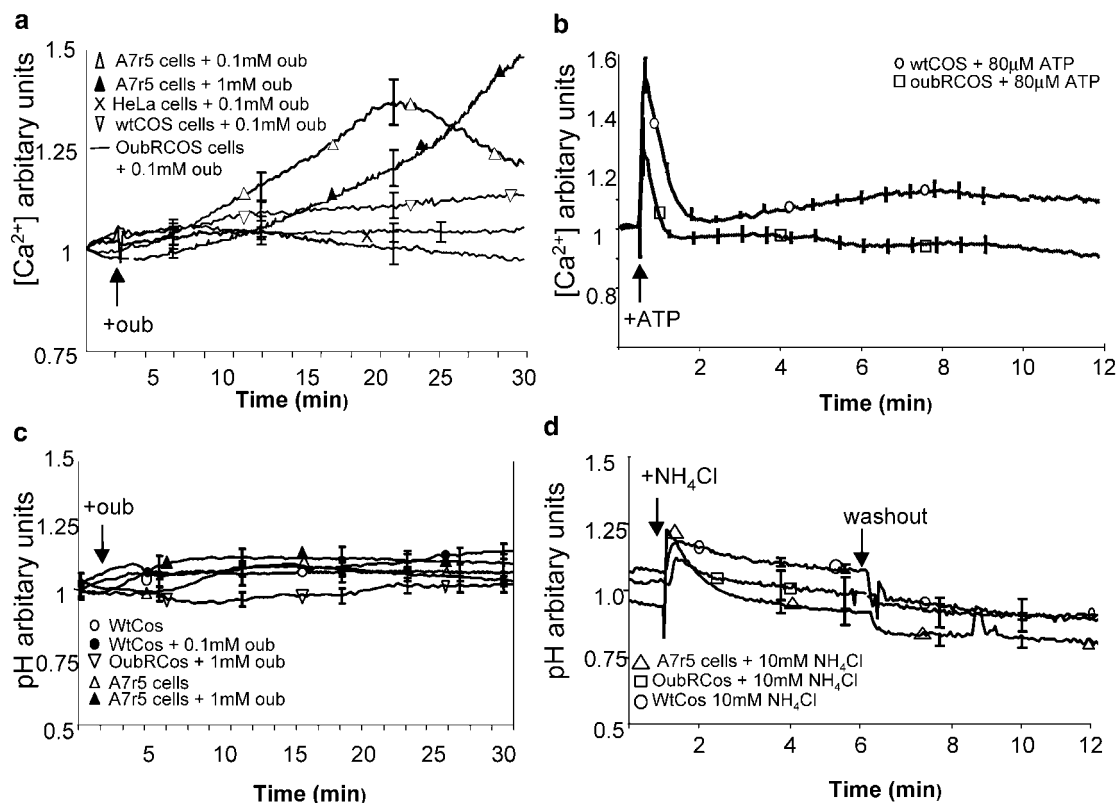
monotonic increase in [Ca<sup>2+</sup>]<sub>i</sub> over 30 min (Figure 2a). By contrast, 100  $\mu$ M ouabain had no effect on basal [Ca<sup>2+</sup>]<sub>i</sub> in HeLa cells expressing Cx43-GFP or oubrCOS-1 cells, whereas a small increase in [Ca<sup>2+</sup>]<sub>i</sub> was observed in wtCOS-1 cells (Figure 2a). ATP (80  $\mu$ M) induced Ca<sup>2+</sup> transients in both COS cell lines (Figure 2b).

Under control conditions, A7r5 and wt and oubrCOS cells loaded with BCEFC AM maintained a steady basal pH<sub>i</sub> with a 480:440 nm ratio of ~1 (Figure 2c). Following treatment with 1 mM or 100  $\mu$ M ouabain, pH<sub>i</sub> remained constant (Figure 2c), whereas 10 mM NH<sub>4</sub>Cl induced a rapid initial alkalinization followed by over-acidification on washout (Roos & Boron, 1981) (Figure 2d).

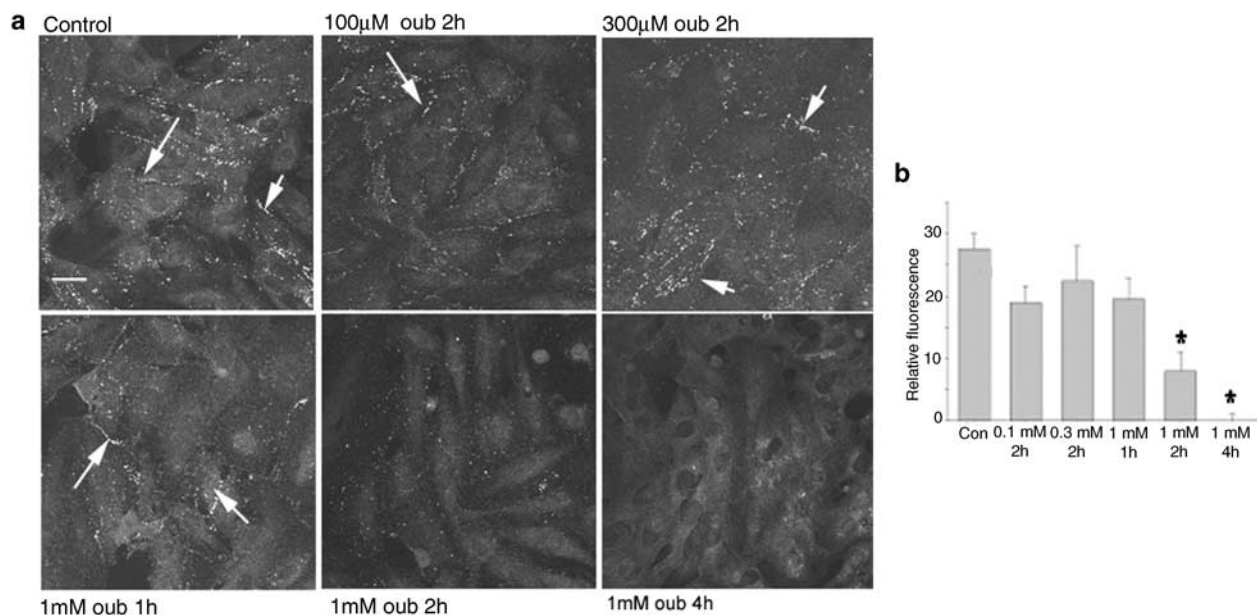
#### Endogenous connexin expression

Treatment of A7r5 cells with 100  $\mu$ M or 300  $\mu$ M ouabain for 2 h had no effect on the incidence of plaques at regions of cell-to-cell contact (Figure 3). After 1 h incubation with 1 mM ouabain, plaques in the plasma membrane similarly remained intact with levels of fluorescence not differing from control, whereas after 2 h there was a reduction in membrane Cx43 staining of up to 50%, and by 4 h all Cx43 staining was lost (Figure 3).

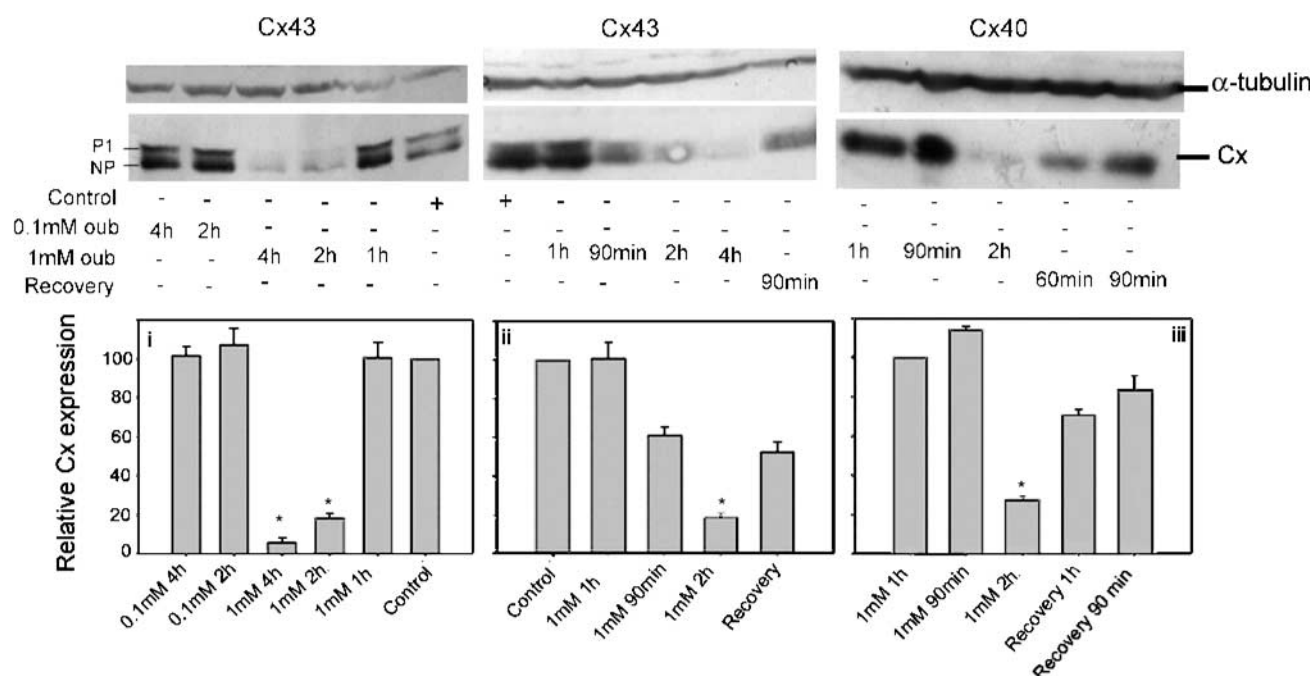
Western analysis of extracts of A7r5 cells indicated that these cells expressed Cx43 in nonphosphorylated (NP) and P1



**Figure 2** Effects of ouabain on intracellular  $[Ca^{2+}]$  and pH. (a) Fura 2 fluorescence before and during exposure of HeLa, wtCOS, oubRCOS and A7r5 cells to 100 μM ouabain. A7r5 cells were also treated with 1 mM ouabain. (b) Fura 2 fluorescence in wtCOS and oubRCOS cells treated with 80 μM ATP. (c) BCECF fluorescence in wtCOS, oubRCOS and A7r5 cells under control conditions and during exposure to 1 mM ouabain. (d) BCECF fluorescence in wtCOS, oubRCOS and A7r5 cells treated with 10 mM  $NH_4Cl$  for 5 min followed by washout and a further 5 min monitoring. Results are expressed as the 340 : 380 nm ratio for  $Ca^{2+}$  measurements and 480 : 440 nm ratios for pH measurements. In all, 10 cells were analysed per field of view and results averaged ( $n = 3$  cultures for each experimental group).



**Figure 3** (a) Integrity of gap junction plaques in A7r5 cells fixed and stained for Cx43 following incubation with 100 μM–1 mM ouabain for 1–4 h as indicated. Bar = 10 μm; arrows indicate plaques at points of cell–cell contact. (b) Plaque integrity quantified by analysis of Cx43 fluorescence at the plasma membrane following the various treatments subtracted from background fluorescence. Results are given as mean relative fluorescence  $\pm$  s.e.m. Asterisk indicates a significant difference from control ( $n = 3$ ,  $P < 0.01$ ).



**Figure 4** Western blot analysis of Cx43 and Cx40 expression profiles in A7r5 cell lysates following treatment with 100  $\mu$ M or 1 mM ouabain for 1–4 h and recovery of expression after 2 h treatment. Equivalent amounts of protein (50  $\mu$ g) were loaded onto the gels and detected using a primary monoclonal antibody to  $\alpha$ -tubulin as internal standard (top panels) and a polyclonal antibody against Cx43 or Cx40 (middle panels) as indicated. (i–iii): Densitometric analysis of the mean Cx expression  $\pm$  s.e.m. Asterisk indicates a significant difference from control ( $n = 3$ ,  $P < 0.05$ ). Each blot was standardized as a percentage of the control signal.

phosphorylated states (Figure 4). Treatment with ouabain for up to 4 h did not alter Cx43 expression at concentrations of 100  $\mu$ M (Figure 4) or 300  $\mu$ M (not shown). Treatment with 1 mM ouabain, however, caused a time-dependent decrease in both Cx43 and Cx40 expression without affecting the relative NP/P1 phosphorylation status of Cx43. At 1 h expression levels remained similar to those in nontreated cells, whereas by 2 h the total amount of Cx43 or Cx40 expressed was reduced by  $\sim 80\%$ , but was substantially restored 90 min following drug washout. Expression of Cx43 was further decreased by 4 h incubation with 1 mM ouabain to levels  $< 95\%$  of control (Figure 4).

Cell viability was preserved, even at late time points following treatment with 1 mM ouabain. Under control conditions and following treatment with 1 mM ouabain for periods up to 4 h, the cells were able to process calcein AM and  $> 95\%$  of the cells excluded propidium iodide.

#### Effects of ouabain on Cx43-GFP trafficking in A7r5 cells

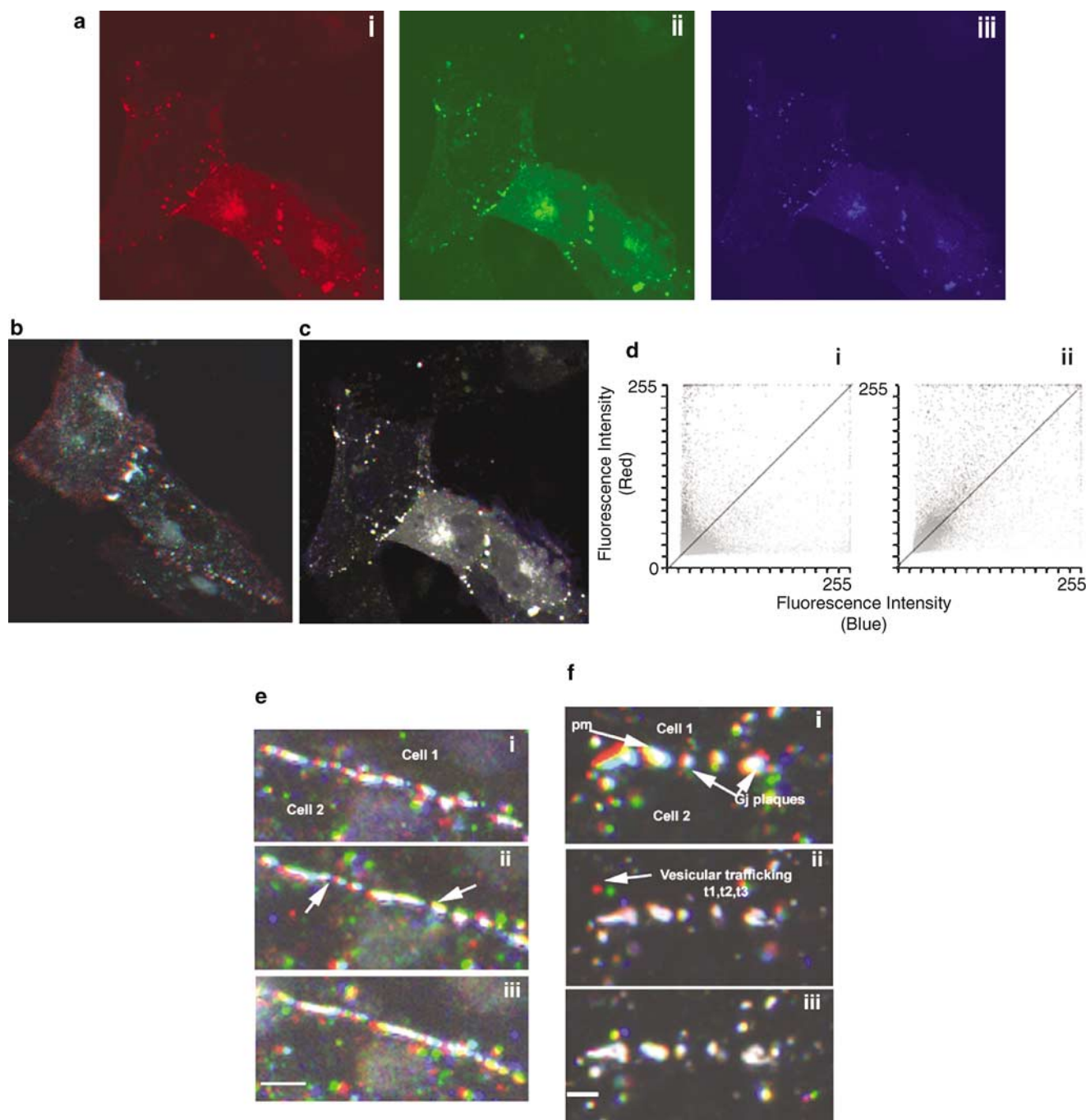
Control experiments were performed in HBS containing 1.3 mM  $\text{Ca}^{2+}$ . Cx43-GFP was localised in gap junction plaques and in intracellular particles, which exhibited movement within the cytosol. Superimposed on this vesicular trafficking was movement attributable to the spontaneous contractile activity of this smooth muscle cell line (Figure 5b and supplementary on-line movie). Both types of movement are reflected by the scatter graph showing that many pixel intensities were uniquely red or blue and lie on the x- and y-axes, giving a colocalization coefficient ( $C_E$ ) of  $\sim 0.5$  (Figure 5di). Following 45 min incubation with 1 mM ouabain, contractile activity and intracellular vesicular Cx trafficking

were both depressed (supplementary movie). This was reflected by greater temporal colocalization of the images (Figures 5c and dii) and an increase in  $C_E$  to  $\sim 0.9$  with only limited intracellular trafficking of Cx protein then being apparent in merged images (Figure 5c). Figures 5e and f illustrate vesicular trafficking of Cx43-GFP protein in A7r5 cells in trace  $\text{Ca}^{2+}$  medium, which suppressed spontaneous contractile activity so that the net apparent movement of Cx protein was depressed (Table 1). Under control conditions, the appearance of colour in these merged images nevertheless still revealed substantial trafficking of vesicular connexin protein in the vicinity of the plasma membrane with a  $C_E$  of  $\sim 0.7$ . Addition of 1 mM ouabain resulted in a time-dependent reduction in trafficking that was evident after 30–45 min and greater after 60–75 min when  $C_E$  approached 1 (Table 1).

## Discussion

We have evaluated the effects of ouabain on gap junctional communication in rat A7r5 aortic myocytes and model COS-1 and HeLa cell systems. The salient findings are that the glycoside attenuates dye coupling within  $\sim 1$  h in each of these cell types and progressively reduces endogenous expression of Cx40 and Cx43 over a period of 4 h in A7r5 cells.

Intercellular transfer of Lucifer yellow between A7r5 aortic myocytes, which express the ouabain-resistant rodent  $\alpha_1$  subunit, was significantly attenuated by ouabain at concentrations  $\geq 300$  M, consistent with the known  $K_i$  ( $\sim 100$   $\mu$ M) of the  $\text{Na}^+/\text{K}^+$ -ATPase in this cell line (Vilsen, 1999; Haas *et al.*, 2002). Ouabain concentrations  $\geq 100$   $\mu$ M were required to attenuate dye coupling in oubRCOS cells selected to express



**Figure 5** (a) Cx43-GFP trafficking events in A7r5 cells in the presence of external  $\text{Ca}^{2+}$  (1.3 mM). Images were recorded every 15 s over a 15 min time period. To analyse the data, time points 1 (15 s,  $t_1$ ), 30 (7.5 min,  $t_2$ ) and 60 (15 min,  $t_3$ ) were assigned the colours red (i), green (ii) and blue (iii), respectively. Merging these three images allows the assessment of vesicular trafficking and movement of the cell (b) under control conditions or (c) following treatment for 45 min with 1 mM ouabain. (d) Scatter plots of the extent of colocalization of the red and blue channels in the absence (i) and presence (ii) of ouabain, where the line of identity is indicated. (e) Cx43-GFP trafficking events in A7r5 cells in the vicinity of the plasma membrane observed in  $\text{Ca}^{2+}$ -free medium: (i) merged image for time 1–15 min, (ii) 20–35 min and (iii) 45–60 min. These findings indicate that imaging of the same cell for prolonged periods did not result in significant photobleaching and that intracellular trafficking was maintained, thus excluding secondary effects from laser damage and focus movement. Bar = 10  $\mu\text{m}$ . (f) Effects of 1 mM ouabain on Cx43-GFP trafficking in  $\text{Ca}^{2+}$ -free medium, with merged images illustrating trafficking (i) before treatment, (ii) 35–45 min following addition of ouabain and (iii) 60–75 min following addition of ouabain. Bar = 5  $\mu\text{m}$ .

the rat  $\alpha_1$  subunit, whereas in wtCOS cells, in which the  $K_d$  of the endogenous  $\alpha_1$  subunit is  $\sim 0.3 \mu\text{M}$  (Belusa *et al.*, 2002; Kristensen *et al.*, 2003a), attenuation of dye transfer was

evident at  $0.1 \mu\text{M}$  and maximal at  $10 \mu\text{M}$ . Dye transfer was similarly depressed by low micromolar concentrations of ouabain in HeLa cells transfected to express Cx43-GFP.



**Table 1** Effects of 1 mM ouabain on Cx43-GFP movement in A7r5 cells

| Treatment                           | Mean $\pm$ s.e.m. |
|-------------------------------------|-------------------|
| Control + Ca <sup>2+</sup> (1.3 mM) | 0.52 $\pm$ 0.05   |
| Ouabain + Ca <sup>2+</sup> (1.3 mM) | 0.92 $\pm$ 0.03   |
| Control (trace Ca <sup>2+</sup> )   | 0.69 $\pm$ 0.07   |
| Ouabain (trace Ca <sup>2+</sup> )   | 0.94 $\pm$ 0.09   |

Colocalization coefficients reflecting net movement of the protein were calculated over 15 min periods (60–75 min after treatment with ouabain) from three experimental sets under the various treatments.

Although the rodent  $\alpha_1$  subunit of A7r5 and oubRCOS cells has the same  $K_i$  for ouabain (Vilsen, 1999), the apparently slightly lower efficacy of the glycoside as an inhibitor of dye transfer in A7r5 cells may simply reflect the ability of this smooth muscle cell line to express high levels of Cx protein, whereas COS fibroblasts express limited amounts of endogenous Cx43 detectable only by RT-PCR (George *et al.*, 1999; Chaytor *et al.*, 2001). Taken together, these observations indicate that the ability of ouabain to attenuate dye coupling in the four cell lines investigated closely matches the affinity of their respective Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha_1$  subunits for the glycoside, thus suggesting that specific binding to the enzyme underpins the modulation of downstream events involved in the maintenance of intercellular communication. Na<sup>+</sup>/K<sup>+</sup>-ATPases containing  $\alpha_2$  and  $\alpha_3$  subunits are unlikely to contribute to the findings as HeLa cells express only the  $\alpha_1$  subunit, COS-1 cells derive from the kidney which expresses the  $\alpha_1$  subunit almost exclusively (>99%), and the dominant  $\alpha$  subtype found in A7r5 cells is the ouabain-resistant rodent  $\alpha_1$  subunit (Tirupattur *et al.*, 1993; Lucking *et al.*, 1996; Zahler *et al.*, 1997). Although small amounts of  $\alpha_2$  and  $\alpha_3$  protein may also be detected in cultured rat aortic myocytes (Tirupattur *et al.*, 1993; Songu-Mize *et al.*, 1996), Na<sup>+</sup>/K<sup>+</sup>-ATPases containing these subunits are inhibited by nanomolar concentrations of ouabain (Blanco & Mercer, 1998), which did not affect transfer of Lucifer yellow between A7r5 cells.

Maximal inhibition of dye coupling was observed at ouabain concentrations known to cause >95% inhibition of the ionic activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase in intact cells, that is, 1 mM for A7r5 cells and 1–10  $\mu$ M for HeLa and wtCOS-1 cells (Zahler *et al.*, 1997; Vilsen, 1999; Belusa *et al.*, 2002; Kristensen *et al.*, 2003a). By increasing intracellular [Na<sup>+</sup>]<sub>i</sub>, blockade of the pump will alter the transmembrane Na<sup>+</sup> gradient for Na<sup>+</sup>/Ca<sup>2+</sup> and Na<sup>+</sup>/H<sup>+</sup> exchange, and thus in theory modulate cell–cell coupling, because increases in [Ca<sup>2+</sup>]<sub>i</sub> or decreases in pH<sub>i</sub> may both reduce the permeability of gap junctions (Schirrmacher *et al.*, 1996; Morley *et al.*, 1996). However, in the A7r5 and COS cells employed in the present study, ouabain failed to evoke changes in intracellular acidification, thus excluding a secondary role for pH<sub>i</sub> in the attenuation of dye transfer in smooth muscle cells. Furthermore, ouabain-induced changes in [Ca<sup>2+</sup>]<sub>i</sub> did not correlate with impairment of intercellular coupling. In A7r5 cells, 100  $\mu$ M ouabain caused a transient increase in [Ca<sup>2+</sup>]<sub>i</sub>, consistent with partial blockade of the Na<sup>+</sup>/K<sup>+</sup>-ATPase, but exerted negligible effects on dye transfer, whereas in oubRCOS cells, 100  $\mu$ M ouabain attenuated dye coupling without affecting [Ca<sup>2+</sup>]<sub>i</sub>. The observation that 100  $\mu$ M ouabain induced a small rise in Ca<sup>2+</sup> levels in wtCOS, but

not oubRCOS cells, is consistent with the higher affinity of the wtCOS  $\alpha_1$  subunit for the glycoside, and is unlikely to reflect nonspecific effects of cell selection because both cell lines were capable of elevating [Ca<sup>2+</sup>]<sub>i</sub> in response to ATP. Although ouabain-induced increases in [Ca<sup>2+</sup>]<sub>i</sub> have been hypothesized to attenuate dye coupling in osteoblasts (Schirrmacher *et al.*, 1996), their apparent dissociation in the present study provides no evidence that these events can be universally regarded as causally related. Indeed, in HeLa cells expressing Cx43-GFP, ouabain attenuated dye transfer at concentrations as low as 1  $\mu$ M, but even at 100  $\mu$ M failed to elevate [Ca<sup>2+</sup>]<sub>i</sub>, presumably because this cell line lacks the Na<sup>+</sup>/Ca<sup>2+</sup> exchange mechanism and voltage-gated Ca<sup>2+</sup> channels (Li *et al.*, 1992; Furman *et al.*, 1995).

We also investigated longer-term effects of ouabain on Cx expression in A7r5 cells, which express high endogenous levels of Cx40 and Cx43 (Chaytor *et al.*, 2001). Immunostaining demonstrated that ouabain caused a progressive concentration- and time-dependent loss of gap junction plaques containing Cx43, first observed 2 h after incubation with 1 mM ouabain, with almost total disappearance of membrane staining being apparent by 4 h. In contrast, lower concentrations of ouabain had no effect on Cx expression, even after prolonged exposure. Western blot analysis of cell extracts confirmed parallel time-dependent loss of Cx40 and Cx43 following incubation with 1 mM ouabain, thus indicating that ouabain-induced reductions in Cx expression were a global phenomenon. Cx43 can exist as a nonphosphorylated (42 kDa) isoform and two principal phosphorylated isoforms, P1 (44 kDa) and P2 (46 kDa), and in some cell systems connexin phosphorylation modulates cell–cell coupling (Lampe & Lau, 2000; van Rijen *et al.*, 2000). However, observations that ouabain did not affect the relative expression profile of the NP and P1 isoforms that predominate in A7r5 cells provide no evidence that its effects on Cx43 functionality and expression can be attributed to altered Cx phosphorylation in vascular smooth muscle. Diminished recruitment of Cx protein to the cell membrane could, at least in part, contribute to the time-dependent loss of membrane plaques as real-time imaging of A7r5 cells transfected to express Cx43-GFP demonstrated that 1 mM ouabain depressed Cx trafficking, as evidenced by a decrease in the vesicular movement of Cx43-GFP in the cytosol of cells incubated with the glycoside in the absence of extracellular Ca<sup>2+</sup> (a strategy used to suppress the intrinsic rhythmic activity of A7r5 cells). However, the almost total loss of Cx40 and Cx43 content observed after incubation with 1 mM ouabain suggests that the glycoside ultimately modulates mechanisms linked to *de novo* Cx synthesis in A7r5 cells. These previously unrecognized effects of ouabain on Cx trafficking and expression were not secondary to nonspecific cytotoxic actions of the glycoside, as the cells remained able to process calcein AM, and reductions in Cx content were substantially reversed on washout of the glycoside after ~90 min. Although inhibition of the Na<sup>+</sup>/K<sup>+</sup>-ATPase may in theory cause osmotic cell swelling, since three Na<sup>+</sup> ions are expelled by the pump for every two K<sup>+</sup> ions that enter, efflux of ions and solutes (e.g. taurine) *via* other transport systems may compensate or overcompensate. Thus, in mouse neurones a biphasic response is observed with a 10% increase in cell volume being evident after 1–2 h, but a reduction in cell volume becoming apparent after 3–5 h (Xiao *et al.*, 2002), and in the COS-1 cells employed in the present study a



40% decrease in  $\text{Na}^+/\text{K}^+$ -ATPase activity causes a 20% reduction in cell section after 45 min (Kristensen *et al.*, 2003b). Although we did not formally measure cell volume in the present experiments, no obvious ouabain-induced changes in the contour of A7r5 cells were evident (see Figure 4), and to our knowledge there are no previous reports causally linking changes in cell volume to alterations in Cx expression.

The effects of ouabain observed in the present study closely mimic the action of glycyrrhetic acid and its derivatives, which have been widely employed as inhibitors of gap junctional communication and, like ouabain, are steroidal in structure and bind to the  $\text{Na}^+/\text{K}^+$ -ATPase, albeit with lower affinity (Terasawa *et al.*, 1992). In epithelial cells, the initial interruption of cell–cell coupling by 18 $\alpha$ -glycyrrhetic acid is rapid (within 30–60 min), not associated with changes in the integrity of gap junction plaques or Cx43 phosphorylation, and is followed by a delayed, concentration-dependent reduction in the expression of Cx43 (Guo *et al.*, 1999). There is good evidence that glycyrrhetic acid derivatives inhibit endothelium-dependent 'EDHF-type' relaxations by impairing the electrotonic spread of endothelial hyperpolarization into the vascular media *via* gap junctions (Taylor *et al.*, 1998; Edwards *et al.*, 1999; Yamamoto *et al.*, 1999; Chaytor *et al.*, 2000; Doughty *et al.*, 2000; Kenny *et al.*, 2002), so that the present findings suggest that the ability of ouabain to attenuate such responses might similarly involve diminished gap junction functionality and/or Cx expression. In rat mesenteric, gastric, renal and femoral arteries, for example, the glycoside attenuates or abolishes EDHF-type relaxations only at high concentrations in the range 100 nM–1 mM, consistent with the involvement of the ouabain-resistant rodent  $\alpha_1$  subunit (Doughty *et al.*, 2000; Van de Voorde & Vanheel, 2000; Jiang & Dusting, 2001; Savage *et al.*, 2003). By contrast, hyperpolarizations evoked by exogenous  $\text{K}^+$  ions in endothelium-denuded rat arteries are inhibited by much lower ouabain concentrations (100 nM), and this smooth muscle electrical response to  $\text{K}^+$  is likely to be mediated *via*  $\text{Na}^+/\text{K}^+$ -ATPases containing ouabain-sensitive  $\alpha_2$  and  $\alpha_3$  subunits, since  $\alpha_1$  containing isoenzymes may be almost fully activated at normal levels of  $[\text{K}^+]_o$  (Blanco & Mercer, 1998; Weston *et al.*, 2002). Further studies are necessary to determine the mechanisms through which low concentrations of ouabain (100 nM–1  $\mu\text{M}$ ) abolish EDHF-type relaxations in other species. For example, porcine arteries express  $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$  subunits that will be sensitive to the glycoside, but impairment of relaxation is time

dependent with full inhibition requiring prolonged (>90 min) incubation (Beny & Schaad, 2000; Büssemaker *et al.*, 2002; Nelli *et al.*, 2003). In this species, it therefore remains conceivable that low concentrations of ouabain modulate gap junction conductance and Cx expression, as well as  $\text{K}^+$ -induced smooth muscle hyperpolarization.

Further studies are also necessary to determine if the inhibitory effects of ouabain against intercellular coupling and Cx protein trafficking and expression in A7r5 cells reflect the loss of normal cellular  $\text{Na}^+/\text{K}^+$  homeostasis and inversion of the normal  $[\text{Na}^+]_i/[\text{K}^+]_i$  ratio associated with administration of the glycoside, and/or conversion of the  $\text{Na}^+/\text{K}^+$ -ATPase into a general signal transducer that acts independently of the ionic activity of the enzyme. Ouabain-induced increases in  $[\text{Na}^+]_i$  have been directly linked to disruption of cytoskeletal components that are known to play a key role in the assembly of gap junctions and are thought to be causally related to decreases in the plasma localization of cell adhesion proteins such as ZO-1 and occludin, which associate with Cx to form multiprotein complexes involved in cell communication and attachment (Giepmans *et al.*, 2001; Martin *et al.*, 2001; Rajasekaran *et al.*, 2001; Toyofuku *et al.*, 2001). However, ouabain also promotes Src-mediated inter-receptor crosstalk between the  $\text{Na}^+/\text{K}^+$ -ATPase and EGFR in A7r5 cells, thereby resulting in the activation of downstream signal transduction pathways (e.g. Ras and p42/44 MAPKs) that effect alterations in cell function and gene expression that are independent of changes in membrane potential and  $[\text{Na}^+]_i$  (Haas *et al.*, 2002).

In conclusion, we have shown that ouabain modulates gap junctional communication in A7r5 vascular smooth muscle cells, COS-1 and HeLa cells through an initial action that correlates with its ability to bind to the  $\alpha_1$  subunits of the  $\text{Na}^+/\text{K}^+$ -ATPase isoenzymes expressed in these cells. In confluent A7r5 monolayers, prolonged exposure to ouabain also depresses Cx trafficking and ultimately abolishes expression of Cx40 and Cx43. Although proteome analysis has identified changes in the expression of over 200 vascular smooth muscle proteins following incubation with ouabain (Taurin *et al.*, 2002), its effects on Cx expression have not previously been appreciated. Our observations therefore add Cxs to the increasing number of cellular proteins whose function and expression can be modulated by this glycoside.

This work was supported by the MRC.

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(Received July 22, 2003

Revised September 8, 2003

Accepted September 20, 2003)