Effect of angiotensin II and ethanol on the expression of connexin 43 in WB rat liver epithelial cells

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The turnover of connexin 43 (Cx43) is very rapid in many cells and involves both the lysosomal and proteasomal protease pathways. Here we show that Ca^{2+}-mobilizing agonists such as angiotensin II (Ang II) can up-regulate the expression of Cx43 in WB rat liver epithelial cells. Vasopressin had the same effect in A7R5 smooth-muscle cells. The effect of Ang II was not prevented by pretreatment with proteasomal or lysosomal inhibitors and was associated with an enhanced biosynthesis of Cx43 as measured by metabolic labelling experiments. The accumulation of Cx43 occurred in intracellular compartments and at the cell surface, as determined by confocal immunofluorescence studies and by immunoblotting of fractions soluble and insoluble in Triton X-100. Chronic treatment of WB cells with ethanol inhibited Cx43 expression; this was associated with decreased biosynthesis of Cx43. Neither treatment with Ang II nor treatment with ethanol altered the levels of Cx43 mRNA. Incubation of WB cells with Ang II did not alter gap-junctional communication as judged by a dye-coupling assay. However, treatment with ethanol markedly decreased gap-junctional communication and this effect was diminished in Ang-II-treated cells, demonstrating that gap-junctional communication is linked to the level of Cx43 expression. We conclude that Cx43 biosynthesis is regulated by Ca^{2+}-mobilizing agonists and ethanol in WB cells. The changes in Cx43 expression might have a role in modifying the conduction of metabolites and second messengers between cells.

Key words: Ca^{2+} waves, ethanol, gap-junctional communication, inositol trisphosphate.

INTRODUCTION

Connexins are gap-junction proteins that function as intercellular protein channels for the diffusional exchange between cells of small molecules and ions, including Ins(1,4,5)P_3 and Ca^{2+} [1,2]. Gap-junction channels are formed from two hexameric assemblies of connexins (connexons) that are embedded in apposing plasma membranes. Several connexin proteins have been identified with molecular masses in the range 26–56 kDa. In the present study we focused on connexin 43 (Cx43), which is expressed in many different cells and is the predominant connexin in cardiomyocytes and many epithelial cells [3].

The turnover of Cx43 is very rapid and involves both the lysosomal and proteasomal pathways [4–7]. This permits considerable flexibility in the expression pattern of the protein, which can be developmentally regulated or altered by several agonists. For example, increased Cx43 expression is observed in several cell types in response to cAMP agonists [8,9], prostaglandin E2 [10], parathyroid hormone [10], epidermal growth factor (EGF) [11], thyroid hormones [12] and retinoic acid [13]. Prolonged treatment with phorbol esters has been shown to decrease the expression of Cx43 [14]. The mechanisms underlying these changes are poorly understood, although transcriptional [10,15] and post-transcriptional mechanisms [11,13] have been proposed.

Gap junctions have a major role in the propagation of Ca^{2+} waves [16–18], and Ca^{2+} itself inhibits gap-junction conductance [19,20]. However, few studies have specifically examined the role of Ca^{2+}-mobilizing agonists in modulating Cx43 expression. We have previously reported that Cx43 levels are up-regulated by treatment with angiotensin II (Ang II) in WB rat liver epithelial cells [21]. Under the same conditions, Ins(1,4,5)P_3 receptor protein was down-regulated via activation of the ubiquitin/proteasome degradation pathway [21]. In contrast, Ins(1,4,5)P_3 receptor expression was enhanced by chronic treatment of WB cells with ethanol [22]. Although long-chain alcohols are well established as inhibitors of gap-junctional communication, the effect of chronic treatment with ethanol on connexin expression has not previously been examined. We show here that chronic treatment with ethanol inhibits Cx43 expression in WB cells. The present study was initially undertaken to use Ang II and ethanol as perturbing agents to explore the possibility of a reciprocal relationship in the expression of Ins(1,4,5)P_3 receptor and Cx43, two key proteins involved in Ca^{2+} signalling. Although no evidence for such a relationship was found, the present results provide insights into the mechanism of the effects of Ang II and chronic treatment with ethanol on Cx43 expression and gap-junctional communication in WB cells.

MATERIALS AND METHODS

Materials

Ang II, bradykinin, vasopressin (VP), EGF, PMA, PMSF, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN), *N*-acetyl-L-leucinyl-L-leucinyl-L-methioninal, cycloheximide, probenecid and chloroquine were purchased from Sigma (St Louis, MO, U.S.A.). Lactacystin was purchased from Dr E. J. Corey (Harvard University, Cambridge, MA, U.S.A.). Calcein acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR, U.S.A.). Stabilized acrylamide solution (Protogel) for the preparation of SDS gels was obtained from National Diagnostics (Atlanta, GA, U.S.A.). Richter's modified minimal essential medium was from Irvine Scientific Co. (Santa Ana, CA, U.S.A.).

Abbreviations used: ALLN, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; Ang II, angiotensin II; Cx43, connexin 43; EGF, epidermal growth factor; RIPA buffer, radioimmunoprecipitation assay buffer; VP, vasopressin.

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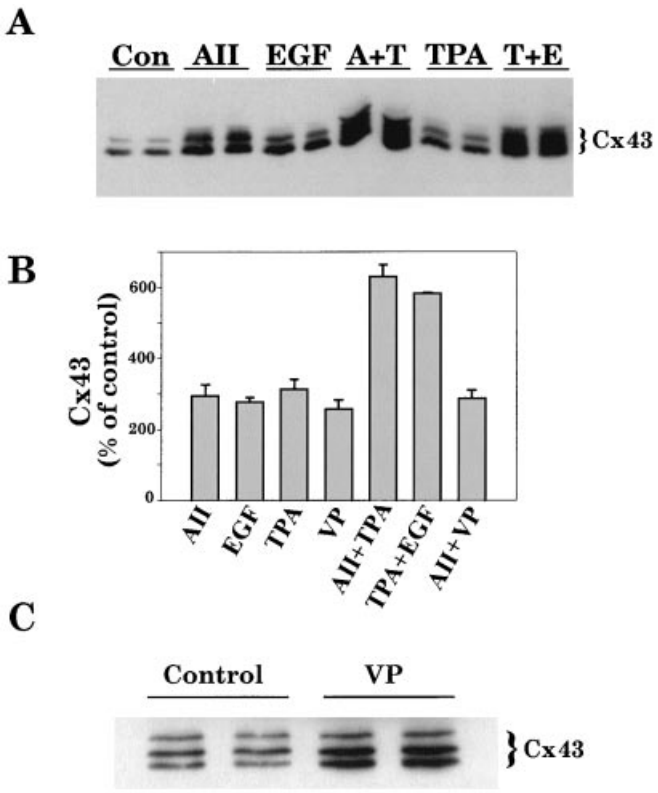

Figure 1 Effect of Ang II and other agonists on Cx43 in WB liver epithelial cells and A7R5 smooth-muscle cells

WB cells were serum-deprived for 24 h and stimulated for 6 h with 100 nM Ang II (AII), 10 nM EGF, 50 nM PMA (TPA) or 100 nM VP. In some cases Ang II (A + T) and EGF (T + E) were combined with PMA as indicated. Lysates were processed in duplicate by SDS/PAGE [10% (w/v) gel] and immunoblotted with Cx43 antibody. (**A**) Representative blot of some of the treatment conditions. Abbreviation: Con, control. (**B**) Densitometric quantification of multiple experiments in which immunoreactive protein is expressed relative to untreated cells. Results are means ± S.E.M. for three different experiments. (**C**) A7R5 cells were treated with 3 μM VP for 5 h and the lysates were immunoblotted with Cx43 antibody.

Rabbit polyclonal Cx43 antibody was a gift from Dr Alan Lau (Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI, U.S.A.). Anti-Cx43 monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Tran^{35}S-label was purchased from ICN Radiochemicals (Irvine, CA, U.S.A.).

Cell culture and pretreatment

Early-passage WB cells were a gift from Dr Robert Whitson (Beckman Research Institute of the City of Hope, Duarte, CA, U.S.A.) and were used between passages 14 and 28. The cells were grown in 100 mm dishes in Richter's minimal essential medium. At approx. 70% confluence, the cells were serum-deprived and stimulated with 100 nM Ang II or treated in the presence or absence of ethanol for 24 h. At the end of the treatment period the medium was aspirated and the plates were washed twice in ice-cold PBS. The cells were scraped into 350 μl of a buffer containing 150 mM NaCl, 50 mM Tris/HCl, pH 7.8, 1% (w/v) Triton X-100, 1 mM EDTA, 0.5 mM PMSF and 5 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor and 5 μg/ml leupeptin (WB solubilization buffer). Insoluble material was removed by centrifugation for 10 min at 25000 ***g***. When Cx43 in the Triton X-100-insoluble fractions was analysed, the pellets were solubilized in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% (w/v) deoxycholate, 1% (w/v) Triton X-100, 0.5 mM PMSF and 5 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor and 5 μg/ml leupeptin. Protein (25 μg) was subjected in duplicate to SDS/PAGE [10% (w/v) gel] and the separated polypeptides were transferred to nitrocellulose for immunoblotting with Cx43 antibody.

Immunofluorescent labelling of cultured cells

WB cells were grown on glass coverslips and stimulated with 100 nM Ang II for 6 h or treated with 150 mM ethanol for 24 h. Cells were washed twice with PBS and fixed in methanol for 20 min at −20 °C, then permeabilized in PBS containing 0.1% saponin. The cells were washed with PBS and blocked in PBS containing 5% (v/v) fetal bovine serum and 1% (w/v) BSA. The cells were incubated for 60 min with anti-Cx43 monoclonal antibody diluted 1:250 in blocking buffer, followed by horseradish peroxidase conjugated goat anti-mouse antibody (dilution 1:500), with three intervening washes in buffer consisting of 0.1 M Tris/HCl, pH 7.5, 0.15 M NaCl and 0.05% (v/v) Tween 20. The cells were then incubated for 5 min in fluorescein tyramide (dilution 1:50) and mounted with Vectashield. The cells were viewed by confocal microscopy (Bio-Rad) on an Olympus microscope with a 60× objective.

Metabolic labelling and immunoprecipitation of WB cell extracts

WB cells were grown to confluence in 60 mm dishes and stimulated with 100 nm Ang II for 5 h or treated with 150 mM ethanol for 24 h. Richter's medium was replaced by methionine-free Dulbecco's modified Eagle's medium containing 125 μCi/ml Tran^{35}S-label and incubated for a further 10 or 30 min. The medium was removed and the cells were washed twice with PBS, then lysed in WB solubilization buffer. The lysates were pre-cleared with 10 μl of 50% (v/v) slurry of *Staphylococcus aureus* cell wall and immunoprecipitated overnight with anti-Cx43 monoclonal antibody. The immune complexes were washed, processed on 10% (w/v) gel and transferred to a nitrocellulose blot, which was then autoradiographed.

Gap-junctional intracellular communication assay

Intercellular communication in WB cells was measured by the 'parachute' assay, in which the transfer of calcein from a donor cell to adjacent recipient cells is quantified [23]. Donor cells were prepared from WB cells trypsinized from a 50% confluent 100 mm dish. The cells were centrifuged and loaded with 1.5 μM calcein acetoxymethyl ester for 20 min at 37 °C in the presence of 2.5 mM probenecid, an inhibitor of anion transport that minimizes the leakage of calcein from the cells. The cells were then washed with medium containing 2.5 mM probenecid and incubated for a further 15 min at 37 °C. Recipient cells were grown on coverslips and treated with 100 nM Ang II or ethanol as described above. Calcein-loaded donor cells were layered on the recipient cells at a ratio of 1:500, calculated on the basis of the initial seeding density of the recipient cells. The coverslips were incubated for 90 min at 37 °C, washed with Hepes buffer [10 mM Hepes/120 mM NaCl/4.7 mM KCl/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/5 mM $NaHCO_3$/10 mM glucose (pH 7.4)/0.25% BSA] and mounted on the stage of an inverted microscope (Olympus 1X70) maintained thermostatically at 37 °C. Fluorescence images were collected at a wavelength of

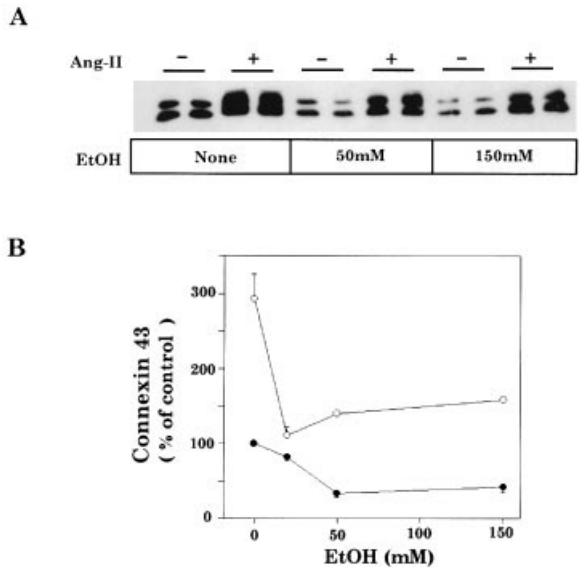

Figure 2 Effect of chronic exposure to ethanol on Cx43 levels

(**A**) WB cells were treated with 20, 50 or 150 mM ethanol (EtOH) for 24 h and then incubated in the presence (+) or absence (−) of 100 nM Ang II for 6 h. The lysates were immunoblotted with Cx43 antibody. (**B**) Cumulative data from three independent experiments. Symbols: ●, control; ○, Ang II.

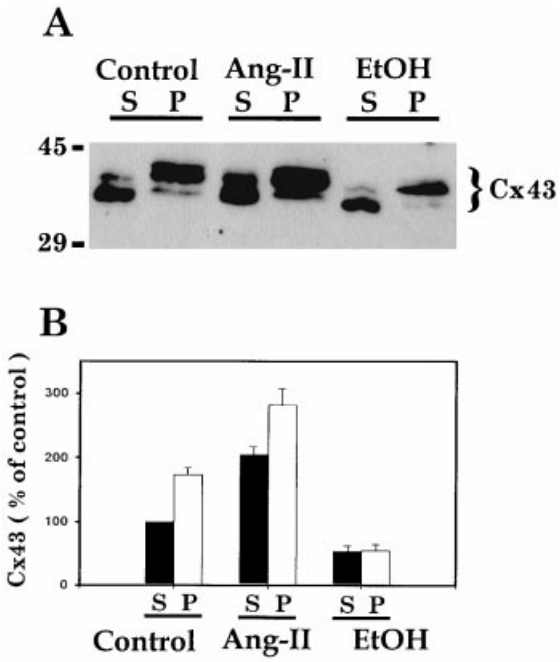

Figure 3 Distribution of Cx43 in fractions soluble (S) and insoluble (P) in Triton X-100

Triton X-100 lysates were prepared from control, Ang II-treated (100 nM for 6 h) and ethanol (EtOH)-treated (150 mM for 24 h) WB cells by using 300 μl of WB solubilization buffer. After centrifugation at 10000 ***g*** for 10 min, the Triton X-100-insoluble pellet was resuspended in 300 μl RIPA buffer, sonicated briefly and recentrifuged. Samples of the Triton X-100-soluble and Triton X-100-insoluble lysates were estimated for protein, and equal protein loads were subjected to SDS/PAGE and immunoblotted with Cx43 antibody. (**A**) Representative blot. (**B**) Pooled data from the densitometric analysis of three experiments (means ± S.E.M.). Values are expressed as percentages of the Cx43 expression observed in the Triton X-100-soluble fraction of control cells. There were no differences in the recovery of protein in the Triton X-100-soluble (610 ± 30 μg) and Triton X-100-insoluble (312 ± 9 μg) fractions under the different treatment conditions.

495 nm and the number of fluorescent neighbouring cells was counted.

Northern analysis of Cx43 mRNA

WB cells were treated with Ang II and ethanol as described above and total RNA was prepared with Trizol reagent (Gibco-BRL, Grand Island, NY, U.S.A.). Equal amounts of RNA (5 μg) from control, Ang-II-treated and ethanol-treated cells were separated by agarose-gel electrophoresis and transferred to nylon membrane (0.2 μm pore size; Schleicher and Schuell, Keene, NH, U.S.A.). Cx43 mRNA was measured by hybridization with a radioactively labelled *Eco*RI-linearized plasmid containing the entire coding sequence of rat Cx43 (kindly provided by Dr Mike Koval, Department of Physiology, University of Pennsylvania, Philadelphia, PA, U.S.A.). β-Actin mRNA was measured with a radioactively labelled 500 bp PCR product. The content of Cx43 mRNA was normalized to β-actin mRNA after densitometric analysis of the data.

RESULTS

Effect of agonists on Cx43 expression.

Cx43 is a phosphoprotein and at least three separate bands have been identified in cell lysates, which correspond to one non-phosphorylated and two different phosphorylated species [24]. Under our extraction and electrophoresis conditions, only two bands were clearly evident in unstimulated WB cells, of which the non-phosphorylated (rapidly migrating) band was the most prominent (Figure 1A). In agreement with our previous observations [21], Figure 1(A) shows that treatment of WB cells for 5 h with Ang II induces a marked increase in the levels of both Cx43 bands as determined by immunoblotting with Cx43-specific antibodies. To determine whether the up-regulation of Cx43 exhibited any specificity, we also stimulated WB cells with 10 nM EGF, 50 nM PMA and 100 nM VP for 5 h. The densitometric analysis of the changes in the Cx43 bands from multiple experiments is shown in Figure 1(B). All three agents increased Cx43 expression (Figures 1A and 1B). The average increase in Cx43 level induced by the agents added alone was 3-fold. A combination of Ang II and PMA or PMA and EGF resulted in an additive increase (Figures 1A and 1B) and under these conditions a triplet of Cx43 bands was apparent (Figure 1A). The addition of two Ca^{2+}-mobilizing stimuli together (Ang II and VP) did not yield an additive increase in Cx43 levels (Figure 1B), indicating that the mechanism involved in the up-regulation of Cx43 by Ca^{2+}-mobilizing agonists is different from those used by PMA or EGF. We have previously observed that the down-regulation of Ins(1,4,5)P_3 receptors in WB cells was selective for Ang II and did not occur with PMA or less potent Ca^{2+}-mobilizing agonists such as VP, EGF or bradykinin [21]. In contrast, the changes observed in Cx43 expression are clearly not specific to Ang II.

VP is known to down-regulate Ins(1,4,5)P_3 receptors in A7R5 vascular smooth-muscle cells [25] and Cx43 is the primary connexin present in this cell type [26]. To determine whether a Ca^{2+}-mobilizing stimulus also increases Cx43 in this cell line, we stimulated A7R5 cells with 3 μM VP for 5 h and determined immunoreactive Cx43 levels (Figure 1C). A triplet of Cx43 bands was resolved in lysates prepared from control A7R5 cells and all three bands were increased after treatment with VP.

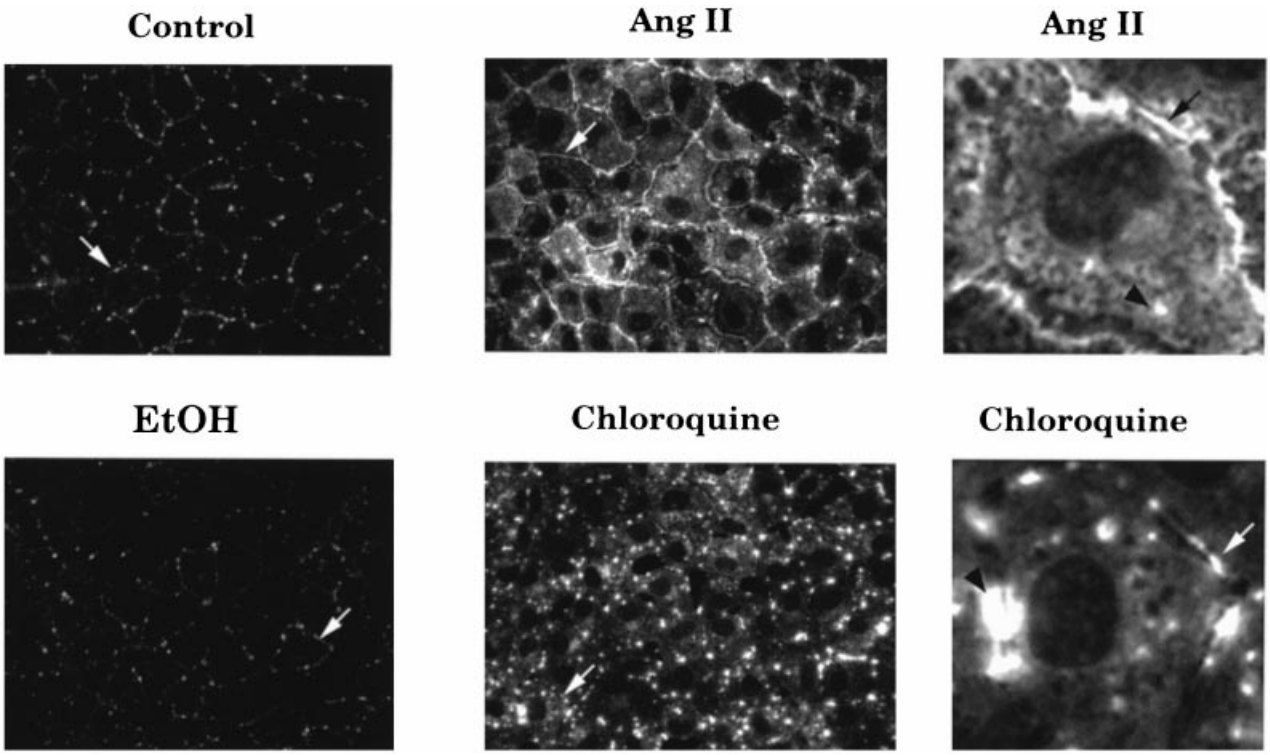

Figure 4 Immunofluorescence of Cx43 in WB cells

WB cells were treated with 100 nM Ang II for 5 h, 150 mM ethanol (EtOH) for 24 h or 200 μM chloroquine for 5 h and then processed for immunofluorescence as described in the Materials and methods section. Confocal images were obtained with a BioRad laser scanning microscope. A single cell from the field of view in Ang II-treated and chloroquine-treated cells is shown in the right-hand panels. The arrows and arrowheads indicate membrane and intracellular Cx43 staining respectively.

Effect of ethanol on Cx43 expression

We have reported an increase in Ins(1,4,5)P_3 receptor protein in WB cells chronically treated with ethanol [22]. Figure 2 shows Cx43 levels in WB cells treated with various concentrations of ethanol for 24 h (Figure 2A). Ethanol treatment caused a 65% decrease in Cx43 levels; maximal effects were observed at 50 mM ethanol (Figure 2B). When the ethanol-treated cells were subsequently stimulated for 6 h with 100 nM Ang II, an enhancement of Cx43 expression was still observed but the magnitude of the Ang II effect was smaller than that for untreated cells (Figure 2B).

Distribution of Cx43 in fractions soluble and insoluble in Triton X-100

It is known that aggregation of gap-junction channels into junctional plaques results in Cx43 becoming resistant to extraction in Triton X-100, a property that is particularly evident for the most highly phosphorylated form of Cx43 [24]. The changes in Cx43 expression shown in Figures 1 and 2 were observed in WB cell lysates prepared with Triton X-100. To exclude the possibility that the observed changes reflected a redistribution of Cx43 between fractions soluble and insoluble in Triton X-100, we examined the effects of Ang II and ethanol treatments in the Triton X-100-insoluble fraction after solubilization in RIPA buffer (Figure 3). The immunoblots suggest that the major portion of Cx43 resided in the Triton X-100 insoluble fraction and, as observed previously, was predominantly enriched in the more highly phosphorylated species [24]. Treatment with Ang II elevated Cx43 levels by about the same extent in both the Triton X-100-soluble and Triton X-100-insoluble fractions (Figure 3B). Similarly, Cx43 was decreased in both fractions in ethanol-treated cells; a proportionately larger effect of ethanol was observed in the Triton X-100-insoluble fraction. We conclude that the changes in Cx43 expression induced by Ang II and ethanol are not due simply to a redistribution of the protein.

Immunofluorescence of Cx43 in WB cells

To verify the observations made from immunoblotting experiments we also performed immunofluorescence measurements on WB cells treated with Ang II or ethanol (Figure 4). Untreated cells showed punctate staining at the boundary of adjoining cells, with relatively little cytoplasmic Cx43 immunoreactivity. In agreement with the results in Figure 3, immunostaining of Ang-II-treated cells revealed an increase in both plasma-membrane-localized gap junctional plaques and cytoplasmic fluorescence in Ang-II-treated cells. Ethanol-treated cells showed a diminished number of gap junctional plaques and the overall level of Cx43 staining was decreased. In these experiments we also tested the effects of blocking the lysosomal degradation of Cx43 by chloroquine treatment. This drug enhanced both membrane and cytoplasmic Cx43 staining in a manner similar to that of Ang II, although the staining patterns were not identical. A more

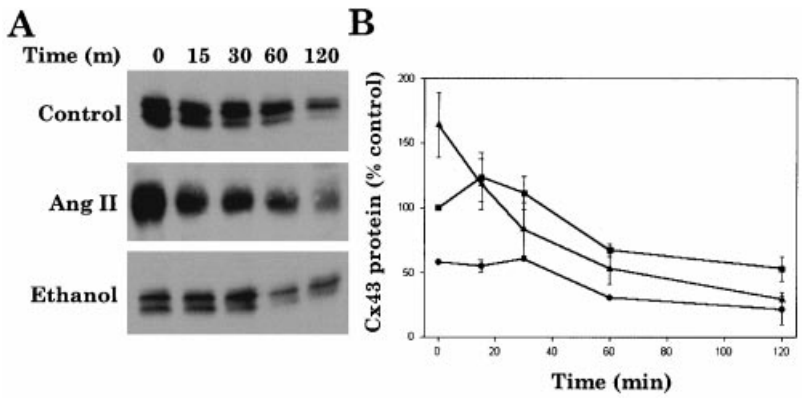

Figure 5 Rate of degradation of Cx43 in control, Ang II-treated and ethanol-treated WB cells

WB cells were stimulated with Ang II for 5 h or treated with 150 mM ethanol for 24 h, then incubated with 100 μg/ml cycloheximide for 0, 15, 30, 60 or 120 min. (**A**) Lysates were obtained after solubilization in RIPA buffer for 10 min and analysed for Cx43 by immunoblotting. (**B**) Densitometric quantification of the results. Results are means $\pm$ S.E.M. for three different experiments. Symbols: ■, control; ▲, Ang II; ●, ethanol.

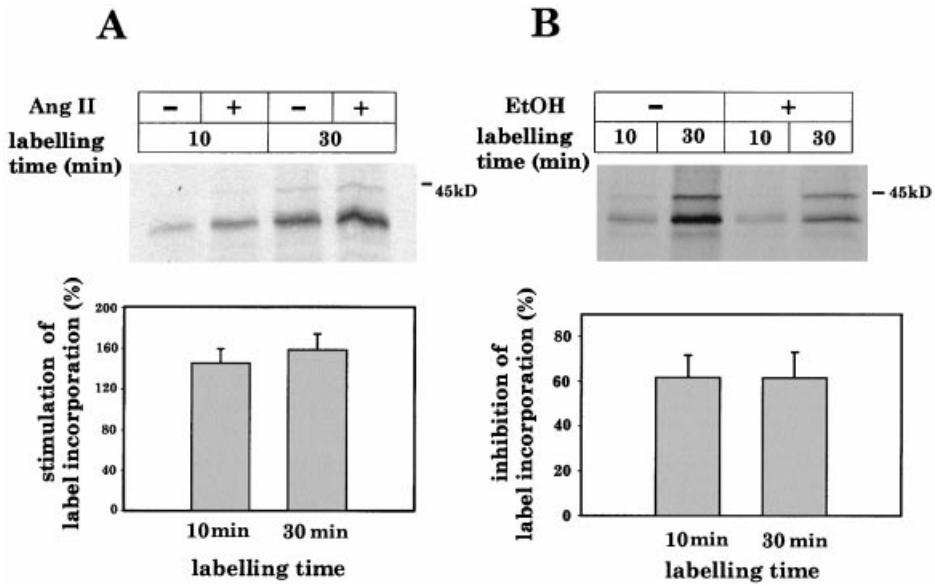

Figure 6 Effect of Ang II and ethanol on the biosynthesis of Cx43

WB cells were pulse-labelled with Tran^{35}S-label for 10 or 30 min after 5 h of treatment with 100 nM Ang II (**A**) or 24 h of treatment with 150 mM ethanol (EtOH) (**B**). Cell lysates were immunoprecipitated with Cx43 antibody and analysed as described in the Materials and methods section. Upper panels: autoradiographs of a representative experiment. Lower panels: densitometric quantification of three different experiments (means $\pm$ S.E.M.).

condensed and predominantly intracellular labelling was observed with chloroquine in comparison with regions of almost continuous staining of plasma membranes obtained with Ang II (Figure 4).

Rate of Cx43 degradation

To determine whether altered rates of Cx43 degradation underlie the observed changes in Cx43 levels, we treated cells with Ang II or ethanol and monitored the disappearance of Cx43 when the biosynthesis of Cx43 was halted with cycloheximide [27]. Whole-cell extracts were prepared with RIPA buffer to include all the pools of Cx43. In untreated WB cells, Cx43 declined rapidly with a half-life of between 1 and 2 h (Figure 5), which is in agreement with measurements made in other cultured cells [28]. Surprisingly, treatment with Ang II stimulated the degradation of Cx43 by decreasing its half-life to 35 min. Thus the increase in Cx43 protein by Ang II cannot be accounted for by an inhibition of degradation. Although treatment with ethanol decreased Cx43 levels, there was no significant change in the half-life of Cx43 degradation (Figure 5).

Effect of protease inhibitors on Cx43 levels

We also tested the effects of pretreating WB cells for 90 min with lysosomal protease inhibitors (5 mM NH_4Cl or 200 μM chloroquine), 50 μM ALLN (which inhibits lysosomal and proteasomal proteases) and 10 μM lactacystin (a specific proteasomal inhibitor). The addition of all three classes of inhibitor increased the basal levels of Cx43 (results not shown). This is in agreement

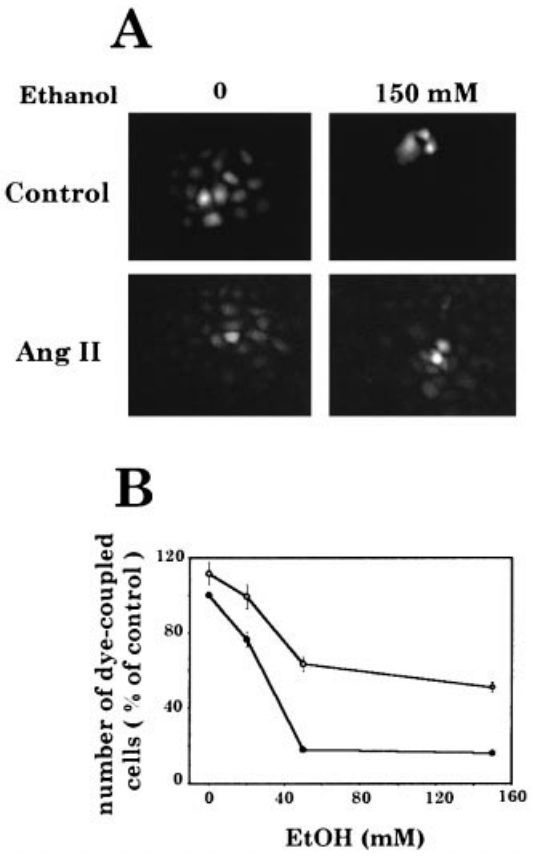

Figure 7 Effect of Ang II and ethanol on gap-junctional communication

WB cells were grown on coverslips and treated with the indicated concentrations of ethanol (EtOH) for 24 h. One set of coverslips was also treated with 100 nM Ang II for the last 5 h of the ethanol treatment. Gap-junctional intracellular communication was measured after overlay with donor cells loaded with calcein acetoxymethyl ester as described in the Materials and methods section. (**A**) Images from a representative experiment. (**B**) Data from three separate experiments (means $\pm$ S.E.M.) in which the number of fluorescent cells after each treatment was counted and expressed as a percentage of the untreated cells. Symbols: ●, control; ○, Ang II.

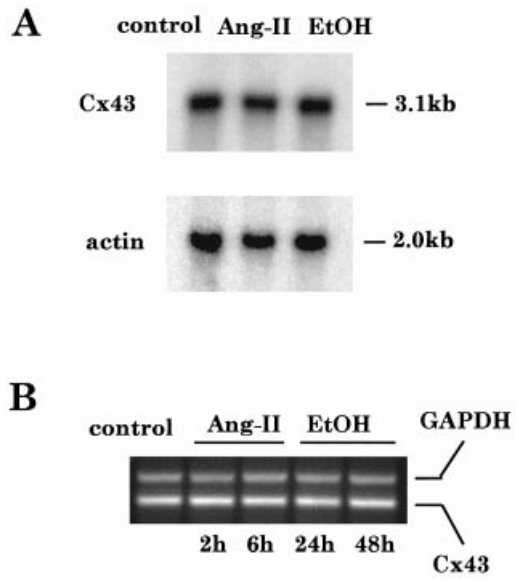

Figure 8 Analysis of Cx43 mRNA

(**A**) Total RNA was isolated from WB cells treated with 100 nM Ang II for 6 h or with 150 mM ethanol (EtOH) for 24 h. The samples were run on formaldehyde/agarose gels, transferred to nylon membranes and incubated with ^{32}P-labelled probes for Cx43 and β-actin. The results shown are representative of three separate experiments. Exposure times were limited to ensure that the film response was not saturated. Normalization of the Cx43 signal to β-actin in all three experiments showed no significant differences between treatment conditions. (**B**) Total mRNA was isolated from Ang II-treated and ethanol-treated WB cells after the indicated times and analysed by reverse-transcriptase-mediated PCR with primers specific for Cx43 (292 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (430 bp). The results are representative of two separate experiments.

with previous conclusions that Cx43 turnover involves both lysosomal and proteasomal pathways in other cell types [5]. None of the inhibitors affected the ability of Ang II to increase Cx43 levels (mean percentage increase by Ang II in the absence of inhibitors, 274 ± 49; with lysosomal inhibitors, 267 ± 14; with ALLN, 257 ± 15; with lactacystin, 250 ± 18). These results and those in Figure 5 suggest that the increase of Cx43 induced by Ang II might be the result of enhanced biosynthesis rather than an effect on degradation.

Effect of Ang II and ethanol on the biosynthesis of Cx43

To measure the biosynthesis of Cx43, WB cells were pulse-labelled for 10 or 30 min with [^{35}S]methionine as described in the Materials and methods section and the lysates were immunoprecipitated with Cx43 antibody. Autoradiographs of the immunoprecipitates are shown in the upper panels of Figure 6. A doublet of radioactive bands was observed on the autoradiographs, with the lower band being more intensely labelled. Both bands were aligned precisely with two Cx43 bands observed when the same nitrocellulose blot was immunoblotted with Cx43 antibody (results not shown). Cells pretreated with Ang II for 5 h showed an enhanced incorporation of ^{35}S label into both Cx43 bands, suggesting that Ang II stimulated the biosynthesis of Cx43 (Figure 6A). This result was not related to the larger pool size of Cx43 in Ang-II-treated cells because no enhancement of ^{35}S label incorporation into Cx43 was observed in the presence of NH_4Cl (results not shown). Figure 6(B) shows that pretreating the cells with ethanol for 24 h had the opposite effect and decreased the incorporation of ^{35}S label into Cx43 by approx. 50 %, suggesting that chronic treatment with ethanol inhibited the biosynthesis of Cx43.

Effect of Ang II and ethanol on gap-junctional communication

To determine whether the changes in Cx43 expression produced by treatment with Ang II and ethanol are associated with functional changes, we used the 'parachute assay' to measure gap-junctional communication [23]. This assay measures the transfer of calcein from donor cells overlaid on cells that have been subjected to various treatment conditions. Figure 7(A) shows the distribution of calcein from a central donor cell to surrounding cells over a 90 min period. No distribution of dye was observed when the donor cells were overlaid on WB-ab1 cells, a mutant cell line with defective gap-junctional communication ([29]; kindly donated by Dr James Trosko, Department of Pediatrics and Human Development, Michigan State University, East Lansing, MI, U.S.A.) (results not shown). Dye transfer was not significantly enhanced when the recipient cells were pretreated with Ang II for 5 h (Figure 7A). By contrast, the cells incubated with 150 mM ethanol for 24 h showed a marked inhibition of dye transfer (Figure 7A). The

results in Figure 7(B) show the progressive decline in dye transfer that occurred in WB cells treated with increasing concentrations of ethanol over a 24 h period. Inhibition was observed even at low (20 mM) concentrations of ethanol (Figure 7B). Although Ang II did not stimulate dye transfer in control cells, a stimulation was observed when Ang II was added to cells in which Cx43 expression and gap-junction permeability had been suppressed by previous treatment with ethanol (Figure 7).

Measurement of Cx43 mRNA

Figure 8(A) shows a Northern analysis of mRNA isolated from cells treated with Ang II for 6 h or with ethanol for 24 h. When normalized to the levels of β-actin mRNA there were no significant changes in Cx43 mRNA under either of the treatment conditions. This finding was confirmed by reverse-transcriptase-mediated PCR analysis, which also showed no changes in Cx43 mRNA at earlier time periods of treatment with Ang II or ethanol (Figure 8B).

DISCUSSION

Both proteasomal and lysosomal degradation pathways are believed to be involved in the pathway of degradation of Ins(1,4,5)P_3 receptors and Cx43 [5,21,30]. The present study was initiated to determine whether there was a causal relationship between the reciprocal changes in Ins(1,4,5)P_3 receptor and Cx43 expression observed after treatment of WB rat liver epithelial cells with Ang II or ethanol [21,22]. Our findings that several stimuli can enhance Cx43 expression, whereas only Ang II can down-regulate Ins(1,4,5)P_3 receptors in WB cells, suggest that the expression of these two proteins is not linked. Previous studies in WB cells have shown that PMA [31], EGF [32] and lysophosphatidic acid [33] can disrupt gap-junctional communication by mechanisms involving enhanced phosphorylation of Cx43. Such changes were monitored over short durations (less than 10 min) and no changes in total Cx43 were noted in these studies. Our measurements over a longer period show that Cx43 expression can be increased by Ca^{2+}-mobilizing agonists that act through G-protein-coupled receptors (vasopressin, Ang II and lysophosphatidic acid) (results not shown), through tyrosine kinase receptors (EGF) and by the pharmacological activation of protein kinase C with PMA (Figure 1). In the present study we have not attempted a detailed analysis of the signalling pathways involved in the actions of each of these classes of stimulus. However, the finding that the effects of Ang II and EGF are additive with PMA suggests that the effects of Ca^{2+}-mobilizing agonists are unlikely to be mediated solely through the activation of protein kinase C and might involve the stimulation of multiple signalling pathways that regulate Cx43 expression.

The only other previous study to examine the effect of Ca^{2+}-mobilizing agonists on Cx43 expression showed that Ang II causes a 2-fold increase in Cx43 protein over a 24 h period and increased metabolic labelling of Cx43 in neonatal rat ventricular myocytes [34]. The effect of Ang II is thought to be important in ventricular myocytes in the remodelling of gap junctions after myocardial infarction [34]. Our findings in the present study with rat liver epithelial cells and A7R5 smooth-muscle cells are similar to those in ventricular myocytes, suggesting that a basic mechanism to up-regulate Cx43 expression in response to prolonged exposure to a Ca^{2+}-mobilizing stimulus is present in many cell types.

The results of our experiments with protease inhibitors and metabolic labelling indicate that the biosynthesis of Cx43 was increased by treatment with Ang II. Under the same conditions, measurements of the rate of Cx43 degradation indicated that this process was also enhanced, implying that the overall turnover of Cx43 was stimulated by treatment with Ang II. The elevation of the steady-state level of Cx43 protein promoted by Ang II indicates that the effect on biosynthesis is dominant.

Enhanced biosynthesis in the presence of Ang II could be due to transcriptional activation of the Cx43 gene. Examples of such a mechanism for increasing Cx43 expression have been observed in WB cells stimulated with thyroid hormones or retinoic acid [12]. However, treatment with Ang II did not change the levels of Cx43 mRNA. This suggests that the increased expression of Cx43 protein might result from an enhanced translation efficiency of Cx43 mRNA. The long 5′ untranslated region of Cx43 forms stable secondary structures and contains an internal ribosome entry site that is required for efficient translation in transfected cells [35]. Increased translation efficiency of Cx43 mRNA could involve Ang-II-mediated regulation of translation initiation factors such as eIF-4E or the phosphorylation of ribosomal protein S6. Further work will be required to test this model of regulation of Cx43 expression.

It is well established that long-chain alcohols such as octanol and heptanol rapidly inhibit gap junction channels by a mechanism thought to involve changes in membrane fluidity [36]. However, these effects are not mimicked by ethanol [37,38]. It has been reported [39,40] that the acute addition of 20 mM ethanol can block gap-junctional communication in cultured rat hepatocytes, which contain predominantly connexins 32 and 26. This effect was shown to require ethanol metabolism and not to be related to changes in membrane fluidity. In the present study we show that chronic exposure of WB cells to ethanol decreases Cx43 expression and inhibits gap-junctional communication. Treatment with ethanol inhibited the metabolic labelling of Cx43 without altering Cx43 mRNA levels or affecting Cx43 degradation. We conclude that treatment with ethanol inhibits Cx43 biosynthesis, possibly by decreasing the efficiency of translation of Cx43 mRNA.

Whereas the decrease in Cx43 expression by treatment with ethanol inhibited gap-junctional communication, the up-regulation of Cx43 expression by Ang II did not have a stimulatory effect. There are several factors that might have contributed to this result. First, unstimulated WB cells already have a high gap-junction conductance [41,42] and further increases induced by Ang II might not have been detected by the dye-coupling assay employed in the present study. Secondly, a substantial portion of the Ang-II-induced increase in Cx43 occurs within intracellular compartments of the cell. Finally, it is known that individual connexons form large plaques at locations of cell–cell contact and only a small fraction of the channels in each plaque are functional [43]. Hence a precise correlation between Cx43 expression and gap-junctional communication is not to be expected. Despite this, the suppression of Cx43 expression by ethanol does inhibit gap-junctional communication and this inhibitory effect is diminished when Cx43 levels are elevated by treatment with Ang II. This suggests that the increased Cx43 expression induced by Ca^{2+}-mobilizing agonists might serve to make the cells less sensitive to other agents that act to disrupt gap-junctional communication.

Many cell types respond to hormonal, neurotransmitter, mechanical or electrical stimulation with waves of cytoplasmic Ca^{2+} increase [16–18]. Although several cell-specific mechanisms exist for intracellular and intercellular wave propagation, it is clear that Ins(1,4,5)P_3 receptors and gap-junction channels have a crucial role in these processes. Different models of wave propagation have invoked either Ins(1,4,5)P_3 [44,45] or Ca^{2+} [46] as the messenger transferred between adjacent cells. Recent

studies suggest that different connexins have different permeabilities to Ins(1,4,5)P_3 [47]. In addition, the inhibition of Cx-32-containing gap junctions by Ca^{2+} has been attributed to the direct interaction of calmodulin with an N-terminal calmodulin-binding sequence that is highly conserved in other members of the connexin family [20]. Three-dimensional reconstruction of the location of Cx43 labelled with green fluorescent protein indicates that junctional plaques might be in close apposition to elements of the endoplasmic reticulum [48]. Thus Ins(1,4,5)P_3 receptors and gap-junction channels might be in close proximity and both proteins might be subject to co-ordinate regulation by factors such as Ca^{2+}. The physiological consequences of changes in the expression of connexin and/or Ins(1,4,5)P_3 receptors in the propagation of intercellular Ca^{2+} waves remains to be explored.

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