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Journal of Cellular Biochemistry 113:1915–1925 (2012)

Journal of Cellular Biochemistry

Effects of Medium Calcium, and Agents Affecting Cytoskeletal Function, on Cellular Volume and Morphology in Liver Tissue In Vitro

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ABSTRACT

The possible role of an exocytotic, vesicular mechanism in cellular volume regulation under iso-osmotic conditions has been studied in slices of rat liver. The effects of incubation conditions and agents affecting the actin cytoskeleton were examined for changes of water, ionic composition, and ultrastructure. Slices were pre-incubated at 1°C in an iso-osmotic buffered medium to induce swelling. Upon restoration to 37°C in the same medium, tissue lost water. The Na^+–K^+ adenosine triphosphatase (ATPase) inhibitor ouabain inhibited water extrusion of about 50%, an effect that was accompanied by the formation of characteristic vesicles in the cytoplasmic region between the Golgi apparatus and the bile canaliculi. Water extrusion in the presence of ouabain was partially inhibited by trifluoroperazine and completely inhibited when the medium was free of Ca^{2+}. In the presence of ouabain, brefeldin A caused a small reduction of water extrusion, whereas phalloidin and cytochalasins A, D, or E caused a marked inhibition. In these conditions there was a marked increase in size and number of cytoplasmic vesicles and a more widespread distribution of them within the cells, lacking the more specific orientation to the Golgi and canalicular regions that was seen in the presence of ouabain alone. Water extrusion was inhibited by phalloidin and cytochalasins in the absence of ouabain. In conclusion, our results are consistent with the hypothesis that iso-osmotic expulsion of water from hepatocytes can proceed partly through an accumulation of water in cytoplasmic vesicles, followed by exocytosis. This mechanism does not depend on Na^+–K^+ ATPase activity. J. Cell. Biochem. 113: 1915–1925, 2012.

KEY WORDS: CELL VOLUME; CYTOSKELETAL MORPHOLOGY; HEPATOCYTE FUNCTION

When incubated in vitro in an iso-osmolar, saline medium many mammalian cells accumulate water, largely as a consequence of the high intracellular concentrations of charged macromolecules and the associated Donnan effect [Opie, 1949; McCance and Robinson, 1950; Macknight and Leaf, 1977; Civan and Macknight, 2004]. When cellular metabolism is active, the swelling is limited, an effect attributable largely to the 3 Na^+/2 K^+ stoichiometry of the Na^+–K^+-adenosine triphosphatase (ATPase, EC 3.6.3.9) which provides a net driving force for water expulsion [Macknight et al., 1974; Garfield and Daniel, 1977; Russo et al., 1977; Russo et al., 1994], The extrusion of Cl^- under these conditions provides electrical balance as well as contributing to water expulsion.

However, it has been shown with some tissues that inhibition of the Na^+/K^+ ATPase by its specific inhibitor, ouabain, or by the omission of medium K^+, still permits a substantial degree of water expulsion from previously swollen cells [van Rossum and Russo, 1981, 1984; Russo et al., 1994]. We have proposed that the persisting, ouabain-resistant regulation of cell volume is brought about by an accumulation of water in cytoplasmic vesicles which pass via the Golgi system and expel their contents by exocytosis [Farber et al., 1989]. The driving force for the accumulation of water in the vesicles could be provided by the proton-transporting chloride-coupled vacuolar ATPase of the vesicular membranes [van Rossum et al., 1987].

The vesicular motion and exocytosis responsible for such mechanism would require activity of cytoskeleton. Preliminary results indicated that cytochalasin B, an inhibitor of actin microfilament function, significantly reduced the ouabain-resistant extrusion of water from rat liver slices in the presence of ouabain.

Additional supporting information may be found in the online version of this article.

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Manuscript Received: 28 July 2011; Manuscript Accepted: 5 January 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 10 January 2012

DOI 10.1002/jcb.24060 • © 2012 Wiley Periodicals, Inc.

We now describe the effects that several agents, in the presence and absence of ouabain in vitro, have on cellular water extrusion, and, through TEM analysis, on cellular architecture. The results are consistent with the exocytotic model proposed for ouabain-resistant water expulsion and, for the first time, suggest that a cytoskeleton-related process can be involved in the extrusion of water taking place independently from Na^+/K^+ ATPase activity.

MATERIALS AND METHODS

ANIMALS AND TREATMENTS

Male albino rats (150–250 g) of a Wistar strain were used, as approved by Temple University's Animal Care and Use Committee. The experimental procedures were those previously described in detail [Russo et al., 1977, 1985, 1994]. Briefly, liver was removed immediately postmortem and slices (approximately 200-μm thick) were cut with a blade guided by a glass slide. The slices were immediately pooled in 50 ml of ice-cold Ringer medium (see below for composition) for the first 10 min of pre-incubation at 1°C and then transferred to fresh medium twice at 10-min intervals to remove K^+ and other materials leaking from the cells. Batches (4–6 slices) were then transferred to Erlenmeyer flasks (25 ml capacity) each holding 3 ml aliquots of medium containing the agents to be studied, as noted in Results Section. Duplicate or triplicate flasks were used for each treatment. Flasks were gassed with 100% O_2 and pre-incubation at 1°C continued to a total of 90 min, at which time the contents of three flasks were collected for determination of the composition of the swollen tissue. Most of the flasks were then tightly stoppered and transferred to a shaking water bath (110 rpm) at 37°C and the tissue slices collected for analysis at the times indicated in Results Section. The basic incubation medium contained (mM): Na^+ 146; K^+ 5.0; Ca^{2+} 1.2; Mg^{2+}1.0; Cl^- 164; SO_4^{2-} 1.0; (tris)-hydroxymethyl amino methane (Tris) 10.0, pH 7.4; inulin 0.5% (w/v). In the case of experiments with trifluoroperazine (TFP), the medium pH was 7.25 for all flasks. For calcium-free media, $CaCl_2$ was replaced by ethyleneglycol tetracetic acid (0.2 mM EGTA), this being added to chelate residual Ca^{2+}. Ouabain was used at 2 or 5 mM; the latter only in the experiments with cytochalasin A; these high concentrations are required to ensure maximal inhibition of the net movements of Na^+ and K^+ because of the low sensitivity of rat tissues to ouabain [Russo et al., 1977; Farber et al., 1989]. Phalloidin, cytochalasins, and TFP were added as stock solutions in dimethylsulfoxide (DMSO) while the stock solution of brefeldin A was made in methanol; in the experiments studying these agents the same final concentration of solvent vehicle was added to all control and treatment. The slices were collected for analysis by tipping the entire contents of a flask onto a disc of filter paper (Whatman no. 54; Whatman Inc., Piscataway, NJ) held under suction on a sintered glass filter; the slices were then briefly rinsed on the filter with 2 ml of ice-cold 150 mM NaCl. After gentle blotting, the slices from each flask were divided into two lots which were transferred separately to tared, stoppered weighing bottles, and the tissue wet wt. determined. The wet tissue in one of each pair of weighing bottles was taken for an assessment of *extracellular* water as determined by inulin content (see below). The tissue in the second of each pair was dried overnight at 110°C for gravimetric determination of the tissue dry weight and total water content. Ions were then extracted from the dry tissue with 0.1 N HNO_3. Na^+ and K^+ were determined by emission flame spectrometry, Ca^{2+} by atomic absorption spectrometry and Cl^- by electrometric titration with Ag (Amico-Cotlove Titrator; American Instruments Co., Hartland, WI).

INTRACELLULAR AND EXTRACELLULAR WATER

The volume of distribution of inulin (MW approximately 5,000) was taken as the measure of extracellular water; intracellular water was determined by difference from the total water. The choice of inulin was based on previous comparisons of possible markers, both ionic and non-ionic, with molecular weights ranging from 99 to 25,000 [Parsons and van Rossum, 1962; Mariani et al., 1991]. Inulin was extracted overnight from the wet tissue in the first weighing bottle of each pair (see above), using 1% (w/v) trichloroacetic acid, and was assayed colorimetrically [Kulka, 1956] and corrected for values obtained from inulin-free tissue samples. Medium samples were also collected from the incubation vessels, after removal of the tissue slices, and assayed for inulin. We assume that inulin provides at least a semi-quantitative estimate of changes of extracellular water in response to the effects of the agents tested. However, an extra factor is the occurrence of histologically detected areas of cellular necrosis in some conditions. As discussed below, it is not clear whether such areas necessarily form part of the extracellular (inulin-containing) compartment. Accordingly, in the text changes of inulin distribution are referred to as *apparent extracellular* and *intracellular* water.

STATISTICAL ANALYSIS

Assay results are expressed relative to the tissue dry weight and are presented as mean ± standard error of the mean (number of observations). Tests for statistical significance of differences between samples used paired *t*-test.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

For TEM, sample slices were removed directly from the incubation flasks prior to collection of the remaining slices for chemical analysis. Fixation, embedding, staining, observation, and morphometric evaluation were as described previously [Russo et al., 1977].

RESULTS

TISSUE SWELLING AND RECOVERY: EFFECTS OF OUABAIN AND CALCIUM ON VESICLE FORMATION AND TRAFFIC

Pre-incubation of liver slices at 1°C in the iso-osmotic medium causes loss of K^+ and increases of water, Na^+, Cl^-, and Ca^{2+} when compared to fresh tissue. Subsequent warming and oxygenation of slices in medium containing Ca^{2+} and lacking ouabain reversed these changes (Fig. 1A). Ouabain completely inhibited the re-accumulation of K^+ at 37°C, but nevertheless only partially inhibited the extrusion of water, Na^+ and Cl^-, suggesting the presence of an independent ouabain-resistant mechanism of volume control. Incubation in the Ca^{2+}-free medium caused no decrease of Ca^{2+} content below that of the fresh, unincubated tissue (i.e., 4.8 + 0.3 mmol/kg dry wt), but volume recovery (i.e., extrusion of water) was partially inhibited. However, all recovery was absent

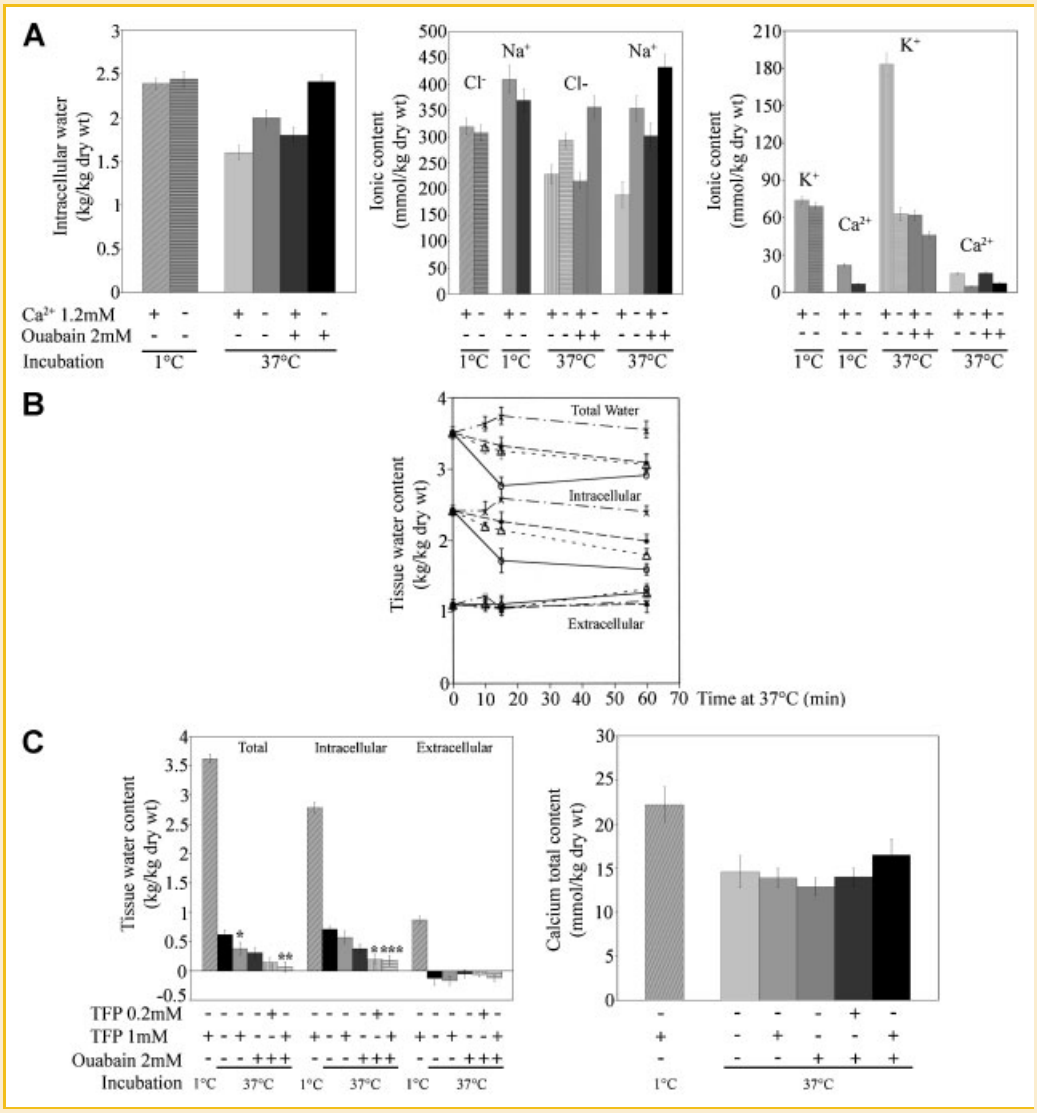

Fig. 1. Effects of ouabain, TFP and the absence of Ca^{2+} on the time-course of changes of tissue water and ionic contents in liver slices. A: *From left to right.* Intracellular water and ionic contents of liver slices were measured in the presence or absence of Ca^{2+} at the end of pre-incubation at 1°C. Alternatively, intracellular water and ionic contents were measured during the subsequent 60-min incubation at 37°C in the presence or absence of ouabain. The Ca-free medium contained 0.2 mM EGTA. Values given are mean ± SEM of 16 observations. Fresh, unicubated slices had a Ca content, assessed postmortem, of 4.8 ± 0.3 mmol/kg. B: Procedures were as in Materials and Methods Section. Values on the ordinate are the net changes of intracellular and extracellular water following restoration of cold pre-incubated slices to 37°C. Values at $t = 0$ are those obtained after 90 min at 1°C. The symbols represent the mean ± SEM of the following numbers of observations: at 0 min, n = 16; 10 min, n = 10; at 15 min, n = 4; at 60 min, n = 18. ○ Control; △ Ouabain (2 mM); ● Ca^{2+}-free medium + 0.2 mM EGTA; X Ca^{2+}-free medium + EGTA and 2 mM ouabain. C: *From left to right.* Changes in intracellular and extracellular water and in total calcium content in liver slices induced to swell during 90 min at 1°C before being transferred to oxygenated medium at 37°C for 15 min further incubation. Ouabain and TFP were present in the incubation vessels during the last 30 min at 1°C and throughout incubation at 37°C. Values given at 1°C are the mean contents of the slices after the 90-min pre-incubation. Other values are the net changes of tissue composition during the subsequent 15 min at 37°C; positive values are a net extrusion of water and Ca^{2+}.

from Ca^{2+}-free medium when ouabain was present. Ouabain itself had no effect on the partial extrusion of excess Ca^{2+} at 37°C.

The net extrusion of total and intracellular water from control slices (i.e., in medium containing Ca^{2+} but without ouabain) was well advanced by 15 min at 37°C (Fig. 1B). When the Ca^{2+}-free medium contained ouabain, there was an initial retention which was evident during the first 10 min at 37°C. Thus these two conditions appear to have an additive effect on the mechanism(s) of water extrusion.

To study if the inhibition of volume regulation upon calcium depletion was due to the relatively massive reduction of total tissue Ca^{2+} recorded in Figure 1A or whether in a subfraction of the total slice Ca^{2+} was the crucial factor. We used TFP, an inhibitor of the regulatory calcium-binding protein, calmodulin. The calcium-transporting ATPase of erythrocyte plasma membranes is inhibited by TFP with I_{50} in the range 10–150 μM [Ansah et al., 1984]. In liver slices incubated in medium with Ca^{2+} (1.2 mM) and ouabain (2 mM), the presence of 200 μM TFP reduced the extrusion of both total and

intracellular water after 15 min at 37°C, with more marked effects at 1 mM (Fig. 1C). TFP (1 mM) also reduced the extrusion of both total and intracellular water from liver slices in the absence of ouabain. By contrast, the net extrusion of Ca^{2+} at 37°C was not significantly affected by TFP. Thus, the principal effect of TFP appears directed towards a smaller pool of Ca^{2+} apparently involved in membrane trafficking events and including an ouabain-resistant mechanism of volume control.

Swelling at 1°C led to drastic modifications of the slice ultrastructure but, in the control Ringer medium, structural recovery was initiated within the first 10 min at 37°C [see also Russo et al., 1977; van Rossum and Russo, 1984] and by 60 min the changes seen during swelling had been almost completely reversed (Fig. 2A). The recovery included an increased electron density of the cytosol, restoration of a well-defined cell periphery, including the bile canaliculi and spaces of Disse (Fig. 2A). During the recovery in the presence of ouabain, we observed the appearance of a large number of rounded vesicles, of various sizes, close to the bile canaliculi, and extending towards the Golgi region (Fig. 2B). This appearance was quite different from the vesiculation resulting from incubation with inhibitors of cellular energy metabolism [Russo et al., 1977; van Rossum and Russo, 1984]. Slices incubated in the Ca^{2+}-free medium, either in the absence (Fig. 2C) or presence of ouabain (Fig. 2D), showed a large number of rounded vesicles widely distributed throughout the cell. At greater magnification the shape and content of the vesicles, and the appearance of bile canaliculi and junctional

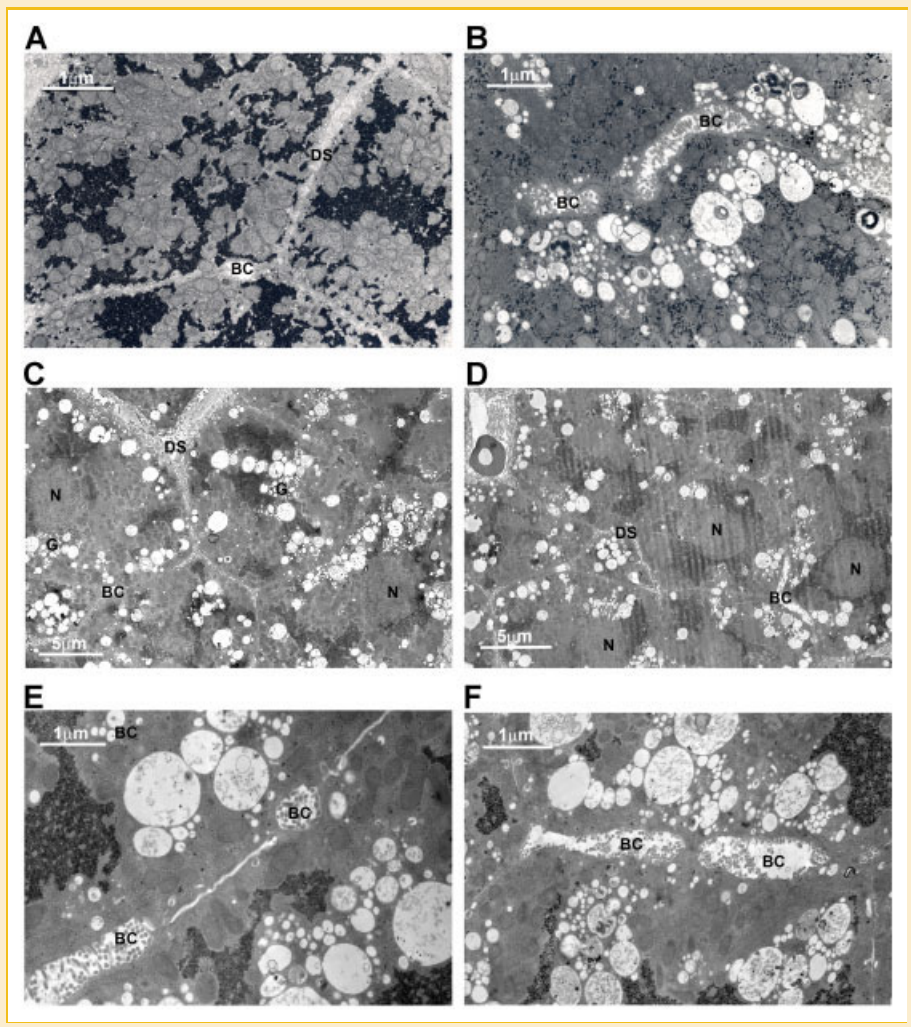

Fig. 2. Morphology of control, oubain, and oubain plus calcium-depleted liver slices. A: The ultrastructure has recovered well from the alterations in the cold pre-incubation. The ultrastructure appears to have a normal electron density. The overall cellular architecture is very similar to the fresh normal unincubated liver. B: Liver slice after 90 min at 1°C followed by 60 min at 37°C in the presence of 2 mM ouabain. A large number of rounded, clear vesicles is present, mainly localized around the bile canaliculi and towards the Golgi region. The ground substance and other sub-cellular components are similar to the control in A. C: Calcium-depleted liver slice incubated for 90 min at 1°C and 60 min at 37°C in the absence of ouabain. Many vesicles are visible at this low magnification. They are mostly scattered in groups throughout the cells without a precise or polarized distribution. D: Calcium-depleted liver slice incubated in the presence of 2 mM ouabain. Their appearance is very similar to that of the calcium-depleted slice in C. E: Detail of a calcium-depleted slice without ouabain seen in C. Vesicles shown are around a bile canaliculus. They have an appearance, sizes, and content very similar to that of the control slice incubated with ouabain (B). F: Detail of a calcium-depleted slices incubated with ouabain in the medium (conditions as in D). The structural details are virtually identical to those seen in E, despite the difference of the water and ionic content under the two conditions. BC, bile canaliculi; DS, Disse space; G, Golgi region; N, nucleus.

complexes were all very similar with (Fig. 2E) and without Ca^{2+} (Fig. 2F) despite the reduced water extrusion in its absence (Fig. 1A). These observations suggest: (a) that Ca^{2+} is not required for the increased formation and expansion of the vesicles characteristic of the presence of ouabain; (b) but that Ca^{2+} is needed for the vesicles to follow their normal path of distribution in the Golgi and canalicular region and/or to undergo exocytosis.

EFFECTS OF BREFELDIN A ON WATER, IONS, AND MORPHOLOGY

Brefeldin A disrupts the structure and function of the Golgi apparatus and, in the concentration range 18–72 μM, interferes with cellular membrane traffic [Lippincott-Schwartz et al., 1991; Patterson et al., 2008]. Its effect on liver slices recovering at 37°C from prior swelling was tested over the concentration range of 36–216 μM. Brefeldin A had no significant effect on either the water content or the re-uptake of K^+ in the absence of ouabain. For example, the final K^+ contents were 237 ± 10 mmol/kg in the absence of brefeldin and 227 ± 8 in its presence (n = 6 in each case). By contrast, in the presence of ouabain a range of brefeldin concentrations from 36 to 108 μM partially inhibited the extrusion of intracellular and total water without affecting the *extracellular* water (Fig. 3A).

Tissue slices were examined after recovery for 60 min at 37°C in the presence of ouabain *plus* brefeldin A (108 μM). Compared to slices with ouabain alone (Fig. 2B) the addition of brefeldin markedly reduced the number and size of the ouabain-related vesicles (Fig. 3B). However, some large, clear vesicles were still present in the Golgi and peri-canalicular regions. At a higher magnification (Fig. 3B, right side) a considerable number of smaller, rounded vesicles was seen in the region of the endoplasmic reticulum and Golgi apparatus; these appear similar to those observed by Lippincott-Schwartz et al. [1991]. This suggests that the paucity of larger vesicles resulted from inhibition of the fusion of the smaller vesicles.

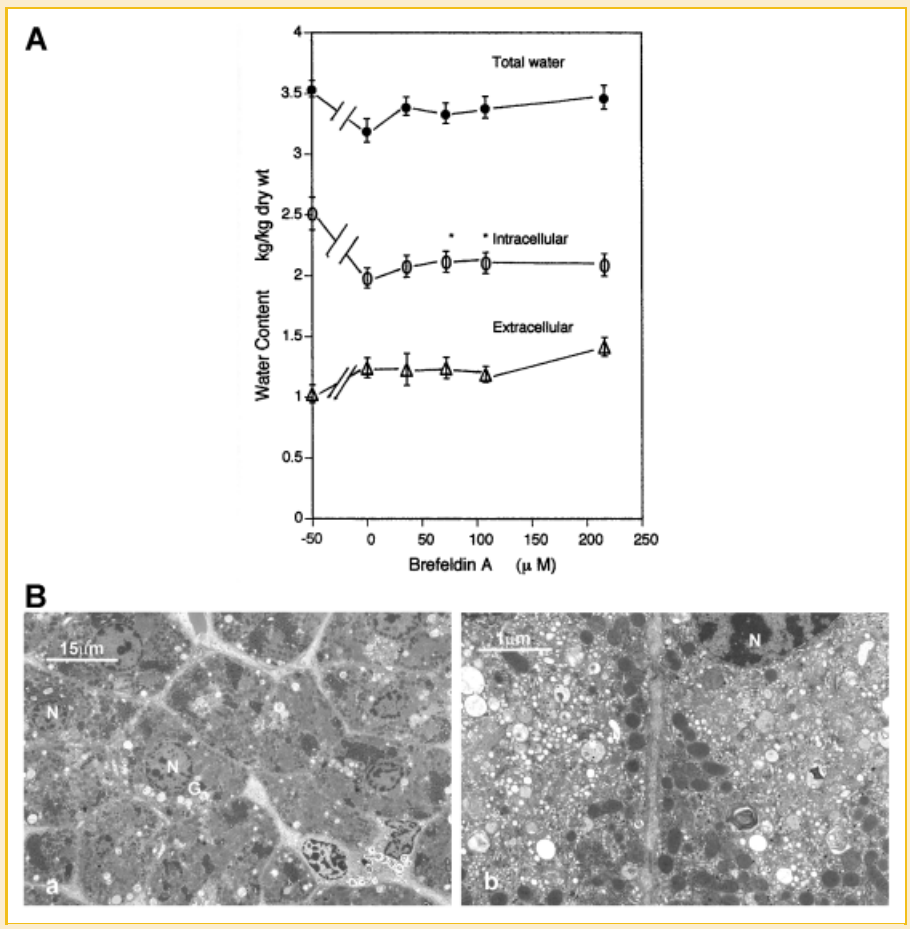

Fig. 3. Effects of the different concentrations of brefeldin A on water content of liver slices in the presence of ouabain (2 mM). A: Slices were pre-incubated for 90 min at 1°C followed by 60 min at 37°C in the presence of the indicated concentrations of brefeldin A. The contents of the water compartments after the pre-incubation (i.e., at the onset of incubation at 37°C) were as follows (kg/kg dry wt): total water 3.65 ± 0.06; intracellular water 2.75 ± 0.11; extracellular water 1.12 ± 0.07. The points are the mean ± SEM of 6–12 observations. ● Total tissue water; ○ intracellular water; △ extracellular water. B: Incubation in the presence of brefeldin A (108 μM) and ouabain (left side). When compared to the slices with ouabain alone the number and size of the clear vesicles appear markedly reduced. The vesicles are still localized in the region between the nucleus and the canaliculi with some tendency to spread through the cell. Incubation with brefeldin A and ouabain, seen at a higher magnification (right side). A number of small, rounded vesicles are associated with the endoplasmic reticulum close to the Golgi region. Only a few, rather large vesicles remain associated with the canaliculus. G, Golgi region; N, nucleus.

EFFECTS OF PHALLOIDIN ON WATER, IONS, AND MORPHOLOGY

Phalloidin binds to polymerized α-actin and so stabilizes microfilaments, reducing their function [Rebello and Ludescher, 1998]. Its effect on liver-slice volume and morphology were studied in the presence of ouabain. After 10 min at 37°C, phalloidin (12.5 μM) markedly inhibited the ouabain-resistant secretion of total and intracellular water (Fig. 4A, left side). The effect of phalloidin on water content was closely paralleled by retention of intracellular Na^+ and, less closely, of Cl^- (Fig. 4A, right side). Similar results were given by 30 μM phalloidin (not shown).

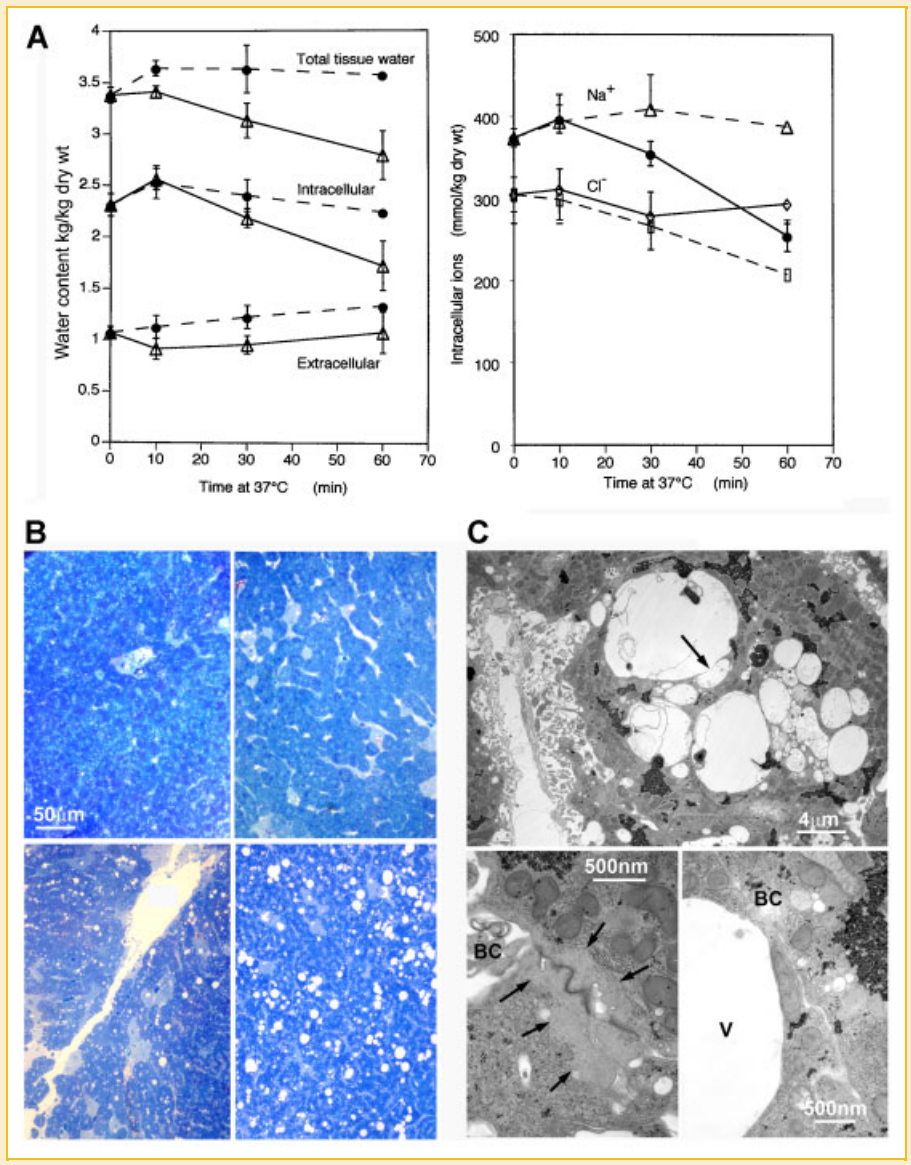

Fig. 4. Effect of phalloidin (12.5 μM) in the presence of ouabain (2 mM) in liver slices. A: Time-course of the changes of composition of liver slices during return to 37°C after 90 min at 1°C. Tissue water compartments. Values at $t = 0$ are the water contents after the incubation at 1°C. Points represent mean ± SEM ($n = 6$). *Left side.* △ ouabain alone; ● phalloidin + ouabain. Intracellular contents of Na^+ and Cl^- as indicated on right side plot. ● Na^+ content in presence of ouabain; Δ Na^+ content with ouabain *plus* phalloidin; □ Cl^- content with ouabain alone; ◇ Cl^- content with ouabain *plus* phalloidin. B: *Upper left.* Optical micrograph of liver slice incubated for 90 min at 1°C followed by 10 min at 37°C, with 10 μM phalloidin *plus* 2 mM ouabain. Phalloidin was added 60 min before the end of the cold incubation. The tissue appears unchanged compared to slices incubated for similar times with ouabain alone. *Upper right.* Optical micrograph of slice after exposure to ouabain alone during cold incubation and 60 min at 37°C. The general tissue structure is well preserved, despite the large number of vesicles around the canaliculi, characteristic of ouabain treatment they appear small at this magnification but are similar to those seen with ouabain alone in the electron microscope. *Lower left.* Slice treated with 10 μM phalloidin and ouabain for 60 min. This plate shows an increase in the size and number of the canalicular-related vesicles during this treatment. *Lower right.* Slice treated with 25 μM phalloidin and ouabain for 60 min. The higher concentration of phalloidin shows a further increase of vesicle number and size compared to the same time at the lower concentration. C: *Upper panel.* The vesicles show a rather irregular outline, and probably result from the fusion of smaller vesicles (arrow). The vesicular contents are clear, and similar to that seen with ouabain alone. *Lower left panel.* Detail of homogeneous sub-membrane zone (arrows) typical of the stabilization effect of phalloidin on cortical actin. *Lower right panel.* Detail of the membrane of a large vesicle (V) lacking cortical actin as instead seen in the left panel.

Sample slices taken from the experiments of Figure 4A were examined by both light and TEM. For comparison, an optical micrograph of a tissue slice incubated for 60 min at 37°C with ouabain alone is shown (Fig. 4B, upper left). After 10 min at 37°C with 12.5 μM Phalloidin plus 2 mM ouabain, there was little difference from the slices incubated with ouabain alone for 60 min (compare Fig. 4B, upper left and upper right). In each case, the general structure was well preserved with vesicles in the regions of the Golgi apparatus and bile canaliculi. Moreover, the size and number of vesicles had increased substantially after 60 min (compare Fig. 4B, upper right and lower left). In addition, larger numbers of vesicles were observed after 60 min in the presence of 30 μM phalloidin (compare Fig. 4B, lower left and lower right). TEM of slices incubated for 60 min with ouabain *plus* 12.5 μM phalloidin shows vesicles of various sizes and contents, several of which were larger than any seen with ouabain alone (compare Figs. 2B and 4C, upper panel). Some of the vesicles also had a less regular outline and contained membranous material (Fig. 4C) suggesting they originated from the fusion of smaller vesicles. Further, the action of phalloidin as a stabilizer of microfilaments is apparent from the enlarged sub-membrane zone (*cortical actin*) of stabilized filaments (Fig. 4C, lower panel).

EFFECTS OF CYTOCHALASINS ON WATER, IONS, AND MORPHOLOGY

Cytochalasins prevent elongation of actin microfilaments and thus inhibit cellular functions depending on the actin cytoskeleton [Schliwa, 1982; Yahara et al., 1982]. The effects of cytochalasins A, D, and E on liver slices were examined at concentrations of 66 and 200 μM in the presence and (except for cytochalasin A) absence of ouabain.

Cytochalasin D. In the absence of ouabain and cytochalasin (Fig. 5A, left side) we observed, after 60 min recovery at 37°C, a net extrusion of 1.21 ± 0.07 kg total water/kg dry wt. The presence of cytochalasin D at 66 and 200 mM reduced the net extrusion to 0.77 ± 0.08 and 0.64 ± 0.10 kg/kg, respectively. The *intracellular* water compartment responded to the cytochalasin by retaining water in parallel with total water, while the extracellular compartment was not significantly affected. The contents of Na^+ and Cl^- in the total and intracellular compartments increased in parallel with the water contents but, unexpectedly, the net uptake of tissue K^+ was not significantly reduced: in the presence of 0, 66, and 200 μM cytochalasin D the final K^+ contents were 270 ± 11, 257 ± 13, and 277 ± 17 mmol/kg dry wt, respectively. Thus, the Na^+/K^+ ATPase appears to be fully active despite the reduction of water extrusion, apparently providing a further example of the partial independence of volume control from Na^+/K^+ ATPase activity. In the presence of ouabain, the net extrusion of total water was reduced to 0.80 ± 0.10 kg/kg (a final content of 3.03 ± 0.08 kg/kg dry wt) and the presence of cytochalasin D (200 μM) further reduced this to a net extrusion of 0.25 ± 0.07 kg/kg (Fig. 5A, right side). Electron microscopy in the presence of 66 μM cytochalasin D showed less than 10% of necrotic tissue (see below).

Cytochalasins E and A. Without ouabain, cytochalasin E inhibited the extrusion of total water to an extent similar to the effect of cytochalasin D (Fig. 5B, left side). Again there was no effect of cytochalasin E on the accumulation of K^+. The final K^+ contents were 225 ± 10, 235 ± 8, and 230 ± 13 mmol/kg dry wt for 0, 66, and 200 μM cytochalasin E, so that a normal uptake of K^+ occurred despite increased tissue swelling. This suggests that the increase of tissue water was not a consequence of cellular necrosis. In the presence of ouabain, cytochalasin E inhibited the total water extrusion to approximately the same extent as cytochalasin D (Fig. 5B, right side). Cytochalasin A, in the presence of 5 mM ouabain, caused nearly complete inhibition of total water extrusion (Fig. 5C).

Figure S1 shows the ultrastructural effects of cytochalasin D (at 66 μM) in the presence of ouabain; the effects of the other cytochalasins were basically similar. Features include numerous, rounded vesicles, mostly with clear contents which were widely distributed in the cells, with not obvious polarization towards Golgi or canalicular regions (Fig. S1A). Canaliculi seen in cross-section were rounded, lacked microvilli, and were surrounded by a very narrow or unapparent, sub-membranous layer of cortical actin. The higher magnification of a typical bile canaliculus (Fig. S1B) shows small vesicles and particles closely associated with the canalicular membrane.

DISCUSSION

These experiments strengthen our previous evidence that a component of iso-osmotic cell volume regulation in rat liver is partially independent of the Na^+–K^+ ATPase and is reduced under conditions inhibiting the activity of the actin components of the cytoskeleton. In addition, for the first time we give evidence that a cytoskeleton-dependent component can contributes to volume regulation even when the Na^+–K^+ ATPase is active.

The experimental model adopted for this work, namely pre-incubation of tissue slices at 1°C followed by recovery at 37°C in the presence of various agents, has been well characterized previously [e.g., Macknight et al., 1974; Russo et al., 1977; Cittadini and van Rossum, 1978; van Rossum et al., 1987]. The qualitative characteristics and quantitative balance of the ionic and water exchanges taking place with and without ouabain (Fig. 1) are closely similar to the previous results. Ouabain (2 mM) completely inhibited the net re-uptake of K^+ at 37°C suggesting that this concentration effectively inhibited the Na^+–K^+ ATPase. Thus, increasing ouabain to 5 mM caused no further effect, while omitting K^+ from the medium produced effects closely similar to those of ouabain [see also van Rossum and Russo, 1984]. Further, the uni-directional influx of ^{86}Rb (an analog of K^+) was maximally inhibited by 2 mM ouabain [Farber et al., 1989]. We conclude that the expulsion of water, Na^+, and Cl^- continuing in the presence of ouabain is driven by an energy-providing mechanism other than the Na^+/K^+ ATPase. We have proposed that this mechanism is highly sensitive to the decrease of ATP and involves the entry of water and ions into cytoplasmic vesicles that expand in the presence of ouabain followed by expulsion of the vesicular contents by exocytosis into the canaliculus (Figs. 1 and 2) [Russo et al., 1977; van Rossum and Russo, 1984; van Rossum et al., 1987].

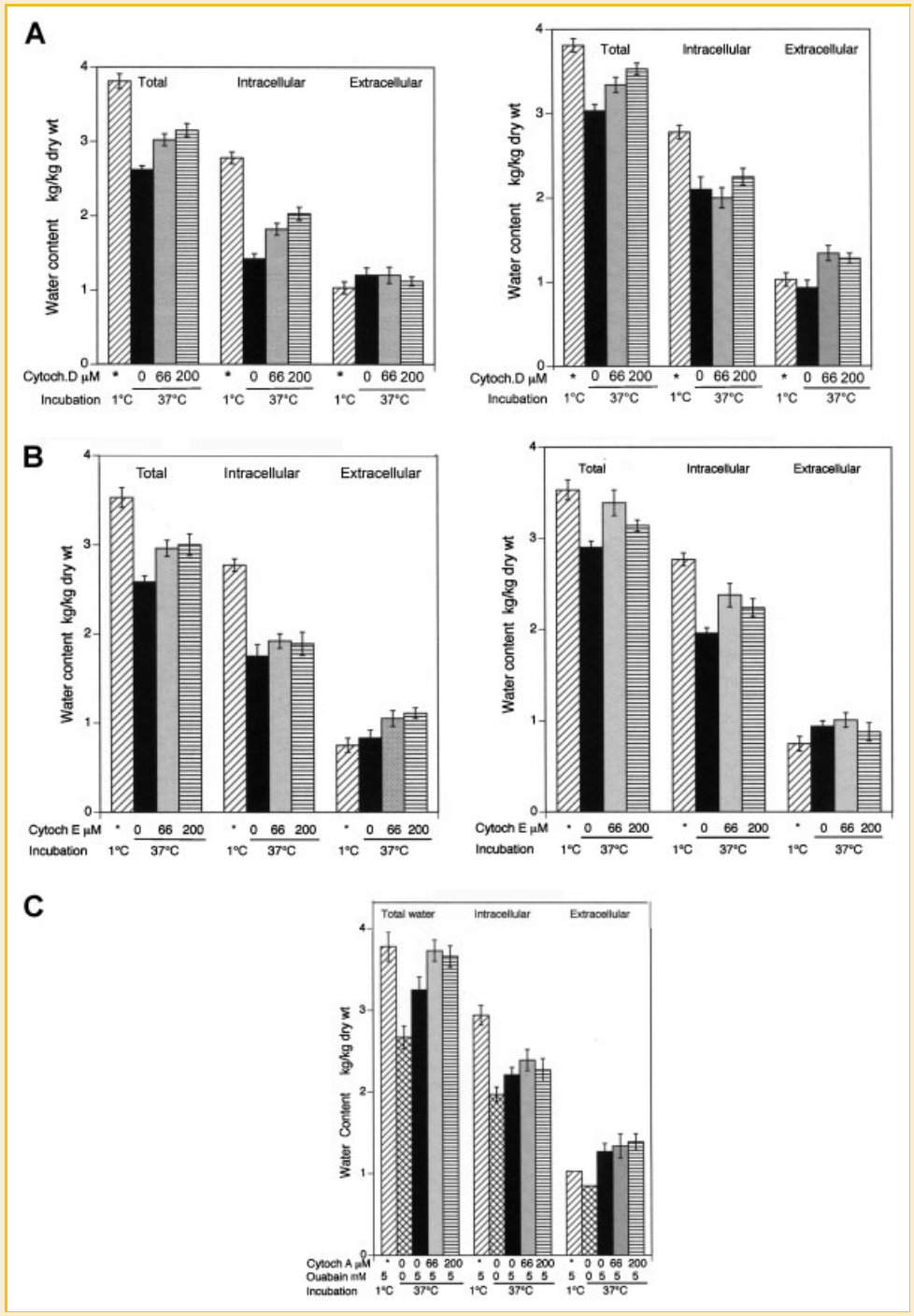

Fig. 5. Effects of cytochalasins on recovery of tissue water compartments. A: *Left side.* Content of tissue water compartments after incubation without ouabain for 90 min at 1°C and further incubation at 37°C for 60 min. Concentrations of cytochalasin D were 0, 66, and 200 μM, as indicated. Bars and error bars represent mean ± SEM for 12 or (at 200 μM cytochalasin) 6 observations. *Right side.* Net changes of tissue water compartments after incubation with cytochalasin D in the presence of 2 mM ouabain. B: *Left side.* Content of tissue water compartments after incubation without ouabain for 90 min at 1°C and further incubation at 37°C for 60 min. Concentrations of cytochalasin E were 0, 66, and 200 μM, as indicated. Bars and error bars represent mean ± SEM for 18 or (at 200 μM cytochalasin) 12 observations. *Right side.* Net changes of tissue water compartments after incubation with cytochalasin E in the presence of 2 mM ouabain. C: Content of tissue water compartments after incubation with ouabain for 90 min at 1°C and further incubation at 37°C for 60 min. Concentrations of cytochalasin A were 0, 66, and 200 μM, as indicated. Bars and error bars represent mean ± SEM for 4 observations.

TISSUE WATER COMPARTMENTS

Total tissue water was assayed gravimetrically (Materials and Methods Section) but for interpretation of the changes it has also been important to evaluate changes at other levels, using both morphological and ion and water compartment analysis. To distinguish between total tissue water and intracellular water we used inulin as a quantitative marker of extracellular water, determining intracellular water by difference. From a morphological standpoint the number and size of vesicles can increase either because they accumulate water prior to exocytosis or because of an inability to expel their contents by exocytosis. Chemically, using inulin as a marker for extracellular water, it would be expected that water accumulated intracellularly during swelling at 1°C would be largely inulin-free and that reactivation of volume control at 37°C would, consequently, result predominantly in expulsion of inulin-free water. This appeared to be true of the inhibition caused by all agents in the absence of ouabain. In many cases, it was also true in the presence of ouabain, namely for the effects of Ca^{2+} depletion, TFP (at 15 min), brefeldin A (at low concentrations), phalloidin (at low concentrations), and cytochalasin E.

Presumably, during recovery from cold pre-incubation all the lost water would initially be actively extruded from the swollen cells. Inhibition of the recovery would then result predominantly in retention of water within the *intracellular* (inulin-free) water compartment. However, in all cases, both with and without ouabain, the net extrusion of slice water was accompanied by some expansion of the inulin containing water space. In time-course experiments, the increase of inulin-containing *extracellular* water appeared early after transfer to 37°C and continued thereafter (Figs. 1 and 4a). We suggest that this effect arises from the equilibrium of newly expelled, inulin-free water, with the inulin-containing water of the medium.

ROLE OF CALCIUM

In addition to preventing K^+ re-accumulation at 37°C, omission of Ca^{2+} from the incubation medium caused a partial inhibition of volume recovery in the absence of ouabain and totally inhibited the ouabain-resistant portion. This was so despite the total slice content of Ca^{2+} in the calcium-free medium with EGTA remaining similar to that in the un-incubated tissue (Fig. 1). Reduction of medium Ca^{2+} below 1 mM causes decreased electrical resistance and increased ion fluxes across biological membranes (e.g., Frankenhaeuser and Hodgkin, 1957; Dumont et al., 1960; Herrera and Curran, 1963) and artificial phospholipid membranes [Nash and Tobias, 1964; McLaughlin et al., 1971]. In our experiments the apparent concentration of Ca^{2+} in the total slice water was approximately 2 mM [Conway, 1957; Borle, 1967]. Further, localized depletion of cell Ca^{2+} is likely to have occurred in the liver slices at 1°C, leading to a generalized increase of permeability of plasma membranes and to reduction of the ability to maintain transmembrane ionic gradients and to induction of swelling.

An alternative or complementary effect of Ca^{2+} depletion could arise from a disturbance of calmodulin-regulated, or other Ca^{2+}-dependent functions. We find preliminary evidence for a role of calmodulin in the regulation of cell volume in the inhibitory effect of TFP on cell volume control, especially in the presence of ouabain. Low levels of Ca^{2+} are required for the formation and contractile functions of the cytoskeleton, and hence in the traffic of vesicles through the cytoplasm [Dedman et al., 1979; Moore and Pastan, 1979; Ji et al., 2006]. In general, the observed inhibition of water extrusion and the associated accumulation of cytoplasmic vesicles upon Ca^{2+} depletion, suggests a role for the Ca^{2+}-dependent cytoskeletal function in volume control in the presence of ouabain.

BREFELDIN A

The morphological effects of brefeldin A seen in the liver slices were similar to those reported by others, including disruption of vesicles, and, thus inhibition of intracellular transport and exocytosis [Oda et al., 1990; Lippincott-Schwartz et al., 1991; Dinter and Berger, 1998]. Brefeldin A alone did not affect the recovery of volume and caused only partial inhibition of the ouabain-resistant water expulsion. However, the number of large, clear cytoplasmic vesicles normally seen with ouabain was reduced. In this case, the partial inhibition of volume control could have arisen from the inhibition by brefeldin A of the formation of the vesicles required for exocytotic expulsion of water. This contrasts with the effects of other agents (see below) which appear to permit accumulation of water in vesicles but to inhibit its expulsion.

PHALLOIDIN AND CYTOCHALASINS

Cytoskeleton involved in the hepatocyte exocytosis includes two main structures, microtubules [Orci et al., 1973] and actin microfilaments. In a previous work, the effects of colchicine and other anti-microtubular agents on the ouabain-resistant cell volume control have been explored. At concentrations normally used to disorganize microtubules (1 μM), colchicine was apparently ineffective, while showing some inhibition of vesicle-dependent mechanism when higher concentrations (10 μM) were used. However, these latter concentrations depresses ATP synthesis [Van Rossum, 1972] and appeared to be quite toxic as evidenced by large areas of necrosis seen especially in the presence of ouabain [van Rossum and Russo, 1981].

The role of actin filaments have been explored by using different strategies. Phalloidin and cytochalasins inhibit actin-dependent processes by respectively stabilizing actin filaments (phalloidin) or preventing their elongation (cytochalasins). Each of these agents we studied reduced the extrusion of water, Na^+, and Cl^- in the presence, and, to a lesser extent in the absence, of ouabain without affecting the re-accumulation of K^+. Furthermore, these agents alone also induced the type of vesiculation characteristic of the presence of ouabain. These findings appear to be strong evidence in favor of a volume-regulating mechanism which is independent of the Na^+/K^+ ATPase and also suggest that the vesicular mechanism proposed to occur in the presence of ouabain is potentially active in its absence.

In the presence of ouabain, these results with phalloidin and cytochalasins A, D, and E were all rather similar to the more limited observations made previously with cytochalasin B alone [van Rossum and Russo, 1981]. All these agents inhibited the ouabain-resistant extrusion of total tissue water to varying degrees and those studied morphologically (phalloidin and cytochalasin D) caused an increase in the number, size, and area of distribution beyond that seen with ouabain alone. With phalloidin, time- and concentration-

dependence of the occurrence of vesiculation was also apparent. Further, the vesicles lacked the orientation towards the Golgi and excretory pole as seen with ouabain alone. This distribution suggests that the vesicles could be formed, but were unable to move on their normal path and/or to extrude their content at the canalicular side. The reduced extrusion of total and intracellular water was, in each case, associated with an approximately equimolar reduction of the extrusions of Na^+ and Cl^-.

As a tentative basis for comparing the relative effect of these agents against ouabain-resistant water expulsion we can use the maximal percent inhibition of the extrusion of total water volume. Maximal inhibitions in the presence of ouabain were: phalloidin 67%; cytochalasins: E 71%; D 69%; B 66%; and A 43%. However, whereas the maximal effect of phalloidin was given at a concentration of 12.5 μM, the maximal activities of the cytochalasins required either 66 or 200 μM.

The above findings are all consistent with the concept of a second mechanism, involving exocytotic water expulsion, which can maintain a measure of volume regulation when the Na^+/K^+-ATPase is reduced in activity by a toxin, such as ouabain. However, several of our results with phalloidin and cytochalasins, suggest that such a system may also act simultaneously with the volume-controlling aspect of the Na^+/K^+ ATPase when the latter is also active.

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