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Vasopressin-induced morphological changes in polarized rat hepatocyte multiplets: dual calcium-dependent effects

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ABSTRACT (200 words)

Calcium-mobilizing hormones and neurotransmitters are known to affect cell morphology and function including cell differentiation or division. In this study, we examined vasopressin (AVP)-induced morphological changes in a polarized system of rat hepatocytes. Light and electron microscope observations showed that AVP induced microvilli formation and a remodeling of the isolated hepatocyte F-actin submembrane cytoskeleton, these two events being correlated. We showed that these effects were rapid, reversible, observed at nanomolar AVP concentration and mediated by the V_{1a} receptor. On polarized multicellular systems of hepatocytes, we observed a rapid reduction of the bile canaliculi lumen at the apical pole and microvilli formation at the basolateral domain with an enlarged F-actin cytoskeleton. Neither activation of protein kinase C nor A via phorbol ester or dibutyryl cAMP induced such rapid morphological changes, at variance with ionomycin, suggesting that AVP-induced intracellular calcium rise play a crucial role in those effects. By using spectrofluorimetry and cytochemistry, we showed that calcium release from intracellular stores was involved in bile canaliculus contraction, while calcium entry from the extracellular space controlled microvilli formation. Taken together, AVP and calcium-mobilizing agonists differentially regulate physiological hepatocyte plasma membrane events at the basal and the apical domains via topographically specialized calcium-dependent mechanisms.

INTRODUCTION

AVP has been reported to regulate blood pressure [1], water reabsorption in the kidney [2], adrenocorticotropin release [3] and more recently cognitive functions [4]. In the liver, AVP is involved in ureogenesis [5], glycogenolysis and neoglucogenesis [6], in the regulation of bile flow [7, 8] and liver regeneration in the rat [9, 10]. These effects are mediated by the V_{1a} receptor (V_{1a} -R) which is highly expressed in hepatocytes. V_{1a} -R are coupled to phospholipase $C\beta$ via α_q/α_{11} G proteins and their stimulation leads to the selective hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate ($InsP_3$) which binds to specific receptors ($InsP_3$ -R) located on the endoplasmic reticulum membrane. Upon binding of $InsP_3$ on its receptor, Ca^{2+} from endoplasmic internal stores is released [11]. In rat hepatocytes, V_{1a} -R activation also stimulates Ca^{2+} influx from the external medium by opening plasma membrane Ca^{2+} channels [12]. Both AVP-stimulated Ca^{2+} release and Ca^{2+} influx induce strong and rapid elevations of $[Ca^{2+}]_i$ which pattern is complex in time and space [13]. In hepatocytes, many agonists including AVP generate $[Ca^{2+}]_i$ oscillations [13] which can propagate as intracellular and intercellular Ca^{2+} waves in cell multiplets [14-17] and in the intact perfused liver [18,19]. Mechanisms that generate Ca^{2+} waves in hepatocytes involve the heterogeneous distribution of signaling molecules at the intracellular and at the tissue (lobular) scale [20, 21]. For example, type 2 $InsP_3$ -R, the main intracellular Ca^{2+} channel in hepatocytes, is concentrated at the pericanalicular region, from which intracellular Ca^{2+} waves start [22]. In the intact liver, although literature is controversial, there are evidence suggesting that AVP-induced intercellular Ca^{2+} waves begin at the lobule centre (perivenous hepatocytes), an area where V_{1a} -R are more abundant [17, 23]. Those waves, propagating towards the periportal zone, have been proposed to regulate bile flow through the stimulation of canalicular peristaltism [8, 18].

In a number of cell types, raise in $[Ca^{2+}]_i$ is known to be associated with morphological modifications. Membrane ruffling in human neutrophils [24] and cell surface microvilli formation in HIT cells [25] involve a remodeling of the F-actin cytoskeleton, which has been reported to be functionally important, not only for the maintenance of plasma membrane integrity, cell locomotion and contraction, but also for cell differentiation, exocytosis and secretion processes [26]. In WRK1 cells and in isolated rat neurohypophysial nerve terminals, exocytotic events induced by extracellular ATP could be triggered by a Ca^{2+} -dependent disorganization of cytoskeleton structures [27]. The role of intracellular calcium movements has been widely recognized in secretion processes [28]. For example, it has been shown on bovine chromaffin cells that Ca^{2+} -dependent disorganization of the submembrane F-actin cytoskeleton may contribute to catecholamine secretion [29].

In isolated single hepatocytes AVP is known to induce Ca^{2+} -dependent formation of microvilli and blebs [30, 31] although it has been assessed only in experimental conditions using high concentrations of agonist over a long period of stimulation. Yet, it remains unknown to what extent such AVP effects may occur in more complex multicellular systems of rat hepatocytes (2-5 hepatocytes), which constitute isolated parts of hepatocyte plates [17, 32]. Within multiplets, hepatocytes conserve a structural and functional polarity similar to that found in liver tissues with three plasma membrane domains well defined (basal, lateral and apical or canalicular). In this experimental model, Ca^{2+} mobilizing agonists like ATP induce canalicular contraction via a putative remodeling of the pericanalicular F-actin cytoskeleton [33, 34], an observation that may in part explain the choleric action of calcium-mobilizing hormones [7-10].

In this study, we explored the effects of nanomolar concentration of AVP on the morphology of freshly isolated rat hepatocyte multiplets and demonstrated the crucial role of Ca^{2+} movements in these effects.

MATERIALS AND METHODS

Abbreviations:

AVP: arginine vasopressin; ACTH: adrenocorticotropin; V_{1a} -R: V_{1a} receptor; $InsP_3$: inositol 1,4,5-trisphosphate; $InsP_3$ -R: $InsP_3$ receptors; $[Ca^{2+}]_i$: cytosolic calcium concentration; $[Ca^{2+}]_e$: external calcium concentration; AM: acetoxymethyl ester; Manning compound: $d(CH_2)_5[Tyr(Me)^2]AVP$; FA: formaldehyde; SSH: saline solution supplemented with 20 mM Heps; BC: bile canaliculus; S.E.M: standard error of the mean.

Chemicals

Unless otherwise stated most standard chemicals were obtained from Sigma Chemical Co (St Louis, MO). Williams' medium E was from Gibco (Life Technologies, Paisley, UK). Quin2-acetoxymethyl ester (AM) was from Molecular Probes (Leiden, The Netherland). $d(CH_2)_5[Tyr(Me)^2]AVP$ (Manning compound) was generously provided by Dr. M. Manning (School of Medicine, Ohio University, USA).

Preparation of hepatocytes

Adult female Wistar rats weighing 200-250 g were obtained from CERJ (Le Genest St-Isle, France). Experiments were performed according to the European legislations (directives for animal experimentation, decree 2001-131; 'J.O.'06/02/01). Hepatocytes were isolated by collagenase perfusion as previously described [23]. Cell viability, assessed by trypan blue exclusion, was greater than 96%. For F-actin fluorescence staining, cells were plated on collagen-coated glass coverslips in a Williams' medium E containing glutamine and antibiotics for 90 min at 37°C under a controlled atmosphere (5% CO_2 and 95% air). They were then, rinsed and equilibrated for 15 min at 37°C with saline solution supplemented with

20 mM Hepes , pH 7.4 and stimulated during 90 s with or without (control) the agents to be tested.

Morphological study

Control or AVP-stimulated hepatocytes (4 min at 37°C) were fixed 2 hours at room temperature with 2,5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After rinsing in the same buffer, they were either 1) observed under a light microscope after staining with toluidine blue (1% in borate sodium buffer), or 2) treated for electron microscope observation. For this they were postfixed 1 hour at room temperature in 1% osmium tetroxide in cacodylate buffer, dehydrated in graded concentrations of ethanol and embedded in araldite. After removing the glass coverslips in liquid nitrogen, punches of 1.5 mm in diameter were cut through the different cell cultures and mounted on araldite blocks. After being cut into ultrathin sections, they were observed in an Hitachi H 7100 electron microscope after slight staining with lead citrate and uranyl acetate.

F-actin fluorescence studies

Hepatocytes were fixed 15 min at room temperature with 2% formaldehyde (FA) and then stored in 0.4% FA at 4°C until use. After staining F-actin with TRITC-phalloidin, cells were observed and imaged with an Axioskop epifluorescence photomicroscope (Carl Zeiss, France) equipped with a sensitive CCD camera (Nikon DXm 1200) or with a confocal microscope (Eclipse TE-2000-Nikon-C1, France) equipped with an air-cooled argon laser for 488 nm and a He-Ne laser for 543 nm. An optical section at the cell equatorial level was recorded with constant settings of laser intensity and pinhole opening. In some experiments, hepatocyte F-actin labeling was represented as a z-series projection of all optical sections through the entire cell. Images were processed using Photoshop. Negative controls were carried out systematically by avoiding the TRITC-phalloidin in the incubation medium.

Fluorescence intensity profile analysis

This analysis was performed as previously described [35] with minor modifications. Briefly, pseudocolor digitized confocal images from TRITC-phalloidin-labeled control or AVP-treated hepatocytes were used to generate radial line fluorescence intensity profiles from the basal membrane to the nucleus using the MATROX Magic Software. On the Y axis, fluorescence intensity (arbitrary units) was expressed as a percentage of the maximal fluorescence (peak) obtained from corresponding control cells (normalized fluorescence intensity profile). The X-axis represented the distance in pixels (1 pixel = 0.25 μ m) from the cell membrane towards the nucleus. The first pixel which exhibited a F-actin fluorescence value distinctly different from background (at least 2 fold) was considered as pixel 1. To estimate the thickness of the membrane labeling we measured the width of the fluorescence intensity peak at half of its height. This value was expressed in pixels.

In control or AVP-stimulated multicellular systems of hepatocytes the percentage of multiplets exhibiting microvilli and dilated or contracted bile canaliculus (BC) were determined with an epifluorescence photomicroscope. More than 150 cells per condition, originating from 3 distinct preparations were generally used for such determinations.

[Ca²⁺]_i measurements

[Ca²⁺]_i measurements were performed in cell suspensions (10⁶ cells /ml) as previously described [23]. In some experiments, an aliquot of 40 μ l was withdrawn from the spectrofluorimeter cuvette and fixed with FA as described above to analyze both intracellular calcium and F-actin labeling on the same groups of cells.

Data analysis

Unless otherwise stated, the data presented here are the mean of triplicate determinations and representative of at least 3 distinct experiments. Results are expressed as mean \pm standard error of the mean (S.E.M.). Statistical analysis of the data was performed using one-way ANOVA. Homogeneity of variance was assessed by Bartlett's test and P values were obtained from Dunnett's tables.

RESULTS

AVP induces microvilli formation and F-actin redistribution in rat hepatocytes.

When isolated hepatocytes (single cells and multipler) were incubated with various concentrations of AVP (3-10 nM), numerous protrusions rapidly appeared at the cell surface (Fig. 1 A-C). The electron microscope observations indicated that, as compared with controls, AVP-treated hepatocytes exhibited typical microvilli extending from the cell surface that appeared more numerous and elongated than in control cells (Fig. 1 D and E). The visualization of the F-actin cytoskeleton by TRITC-phalloidin also indicated a strong effect of AVP (compare upper and lower left panels in Fig. 2A). In unstimulated hepatocytes, a thin labelling of the basolateral domain was observed on equatorial sections. After application of 3nM AVP during 90 s, the F-actin labelling at the basolateral domain became significantly thicker as compared to those of un-stimulated cells (for comparison see upper and lower right panels in Fig. 2A). Radial fluorescence intensity profiles from the plasma membrane towards the nucleus were established from confocal images like those presented in Fig. 2A (upper and lower right panels). As shown in Fig. 2B, upon AVP stimulation, the brightness of F-actin labeling peak (plasma membrane level) increased by $50 \pm 0.5\%$ as compared to control cells. Similarly, the thickness of the F-actin labeling also increased by 62%, (2.9 ± 0.3 pixels and 4.7 ± 0.6 pixels for control and AVP-stimulated cells respectively, $p < 0.05\%$), while cell diameter did not change significantly (20.0 ± 0.3 and 20.5 ± 0.3 μm in control and AVP-treated cells respectively). A strong correlation between the percentage of cells with microvilli and the thickness of F-actin labeling was observed (Fig. 2C). Thus, in the following experiments, only measuring the percent of cells exhibiting microvilli will monitor the influence of AVP on hepatocyte morphology.

To delineate the mechanisms by which AVP induced microvilli formation, we investigated the pharmacological characteristics of AVP effects. As illustrated in Fig. 3, the effect of 1nM AVP was rapid, the maximum microvilli formation being observed 2 min after hormone addition (Fig. 3A), and saturable, the maximal microvilli formation being obtained for 3 nM VP ($EC_{50} = 0.5 \pm 0.3$ nM) (Fig. 3B). The V_{1a}-R subtype mediated this effect since when hepatocytes were pre-incubated 5 min with 100 nM of a selective V_{1a} antagonist (Manning compound), the effect of AVP on microvilli formation was completely suppressed (Fig. 3B). Moreover, this effect was reversible upon extensive washing as illustrated in Fig. 3C. Similar experiments were also performed on hepatocyte multiplets and the same morphological effects, both on microvilli formation and F-actin redistribution, were observed (Fig. 2 and data not shown).

Mechanisms of AVP-stimulated microvilli formation

As stated above, the V_{1a} receptor is coupled in hepatocytes to $[Ca^{2+}]_i$ mobilization. We thus analyzed the involvement of this transduction pathway in microvilli formation. As summarized in table 1, other Ca^{2+} -mobilizing agonists such as angiotensin II, ATP and noradrenaline, also rapidly stimulated microvilli formation. By contrast, glucagon which is principally known to activate adenylyl cyclase and to a lesser extent $[Ca^{2+}]_i$ mobilization [13], weakly stimulated microvilli formation under similar experimental conditions. To further understand the mechanisms involved in these F-actin reorganization processes, we tested the effects of other compounds that are able to mimic hormonal responses. As illustrated in Fig. 4, short incubation with 1 μ M ionomycin, a ionophore known to induce calcium entry when used at this concentration, induced rapid microvilli formation. In the same experimental conditions, neither PMA (which stimulates protein kinase C activity), nor dibutyryl cAMP

(which stimulates protein kinase A activity) exhibited any effect on microvilli formation. As already reported and further verified in this study, long incubation periods (15 min) with these compounds or with AVP, induced the formation of blebs but not of microvilli (data not shown) [31].

To study the source of Ca^{2+} accounting for hepatocyte morphological modifications upon AVP stimulation, we performed two sets of experiments. When hepatocytes were incubated in a high external Ca^{2+} medium (free $[\text{Ca}^{2+}]_e = 1.8 \text{ mM}$), both AVP (3 nM) and ionomycin (1 μM) rapidly induced a sustained and very high increase of $[\text{Ca}^{2+}]_i$ (Fig 4A). When similar experiments were performed in a low external Ca^{2+} medium (free $[\text{Ca}^{2+}]_e = 100 \text{ nM}$), only AVP and highest (5 μM) concentrations of ionomycin were able to significantly increase the $[\text{Ca}^{2+}]_i$. Moreover, this effect was transient and of low amplitude as compared to experiments done in a high $[\text{Ca}^{2+}]_e$ medium. These experiments confirmed that AVP in low external Ca^{2+} stimulated the increase of $[\text{Ca}^{2+}]_i$ via the mobilization of calcium from intracellular InsP_3 sensitive pools, whereas ionomycin in high external Ca^{2+} acted via Ca^{2+} entry from the extracellular medium. As already reported, high ionomycin concentrations were able to release Ca^{2+} from internal stores. To correlate Ca^{2+} movements from different sources and hepatocyte morphology modifications, aliquots of hepatocytes subjected to the experimental conditions described above were fixed and stained for F-actin. As shown in Fig. 5B and 5C, AVP and ionomycin (either 1 or 5 μM) induced microvilli formation only if experiments were performed in a high $[\text{Ca}^{2+}]_e$ medium. Only a small and non significant increase of hepatocytes exhibiting microvilli was observed if stimulation was done in a low $[\text{Ca}^{2+}]_e$ medium. These results suggest that Ca^{2+} mainly from extracellular source is involved in agonist-induced microvilli formation in hepatocytes.

AVP induces BC contraction in rat hepatocyte couplets.

For this study, only hepatocyte multiplets with detectable bile canaliculi (based on phalloidin F-actin staining) were examined. Upon AVP stimulation, the morphology of the apical domain was modified. As shown in Fig. 5A and 5B, most of the control hepatocyte doublets and multiplets exhibited dilated BC. After AVP stimulation, both the number of dilated BC (Fig. 6B) and the BC lumen diameter (Fig. 6C) were significantly reduced. This effect was dose-dependent (data not shown) and mediated via the activation of the V_{1a} receptor, since it was completely blocked by incubation with a V_{1a} receptor antagonist (Fig 5B). As observed for the basolateral domain of single hepatocytes, AVP also induced a strong reorganization of the pericanalicular F-actin cytoskeleton. Both the intensity and the width of the BC lips labeling were significantly increased as compared to those of control BC (Fig 5C). In basal conditions, BC were mainly dilated whatever the $[Ca^{2+}]_e$. AVP 3nM induced a contraction of around 60 % and 50 % of the dilated BC, respectively in high and low $[Ca^{2+}]_e$ medium (Fig 5 D). Under the same experimental conditions, 1 μ M ionomycin was without significant effect on BC contraction whatever the $[Ca^{2+}]_e$, while 5 μ M ionomycin led to similar BC contraction as compared with AVP.

DISCUSSION

Previous studies performed on rat hepatocytes reported plasma membrane modifications (blebs and microvilli) upon AVP treatment [30, 31]. Yet these experiments have been conducted on single hepatocytes which have lost their cellular polarity and which have been stimulated either with very high AVP concentration (1 μM) or for prolonged incubation periods (15 min). In this study, we explored the effect of nanomolar concentration of AVP on the cell morphology of polarized hepatocyte couplets and demonstrated different hormonal action depending on the plasma membrane domain observed.

Observations at the light and electron microscope levels indicated that AVP rapidly stimulated the extension of numerous typical microvilli along hepatocyte surface, with very scarce occurrence of blebs, even for the strongest AVP stimulations used (10 nM AVP, 15 min). Under a short period of incubation with nanomolar AVP concentrations, a strong reorganization of the sub membrane F-actin cytoskeleton occurred, associated with an earlier described increase in cell surface microvilli [30]. We showed that these effects were receptor-mediated and reversible. Moreover, increasing the duration of stimulation or the dose of AVP induced blebs formation, probably because of non-physiological over-stimulation of the $V_{1a}\text{-R}$ ([36] and data not shown). Such F-actin cytoskeleton remodeling observed upon calcium mobilizing agents stimulation has been described in various biological models. It has been shown in a monocytic cell line that ionomycin-induced $[\text{Ca}^{2+}]_i$ rise led to a rapid actin microfilament redistribution from cortical areas towards the membrane interior and to changes in cell shape [37]. Triffaro et al. [29] also showed that ATP induced a rapid disorganization of the F-actin perimembrane cytoskeleton of bovine chromaffine cells. Similarly, in rat glomerulosa and WRK1 cells, AVP induced a rapid disorganization of the F actin network

[38, 39]. Altogether these data suggest a general role for AVP and V_{1a}-R in controlling plasma membrane morphology.

The observed hepatocyte microvilli formation or elongation upon AVP challenge may result from an actin-dependent membrane remodeling during which calcium-dependent exocytotic processes may occur and contribute to regulate ion and water absorption [40]. As already suggested in kidney and bladder epithelial cells, microvilli formation or elongation may be related to these processes, allowing ion and water transport [41, 42]. Interestingly, the ability of AVP and other calcium-mobilizing agonists to interfere with exocytosis has been previously reported not only in kidney epithelial cells [43] but also in hepatocytes [40]. Altogether these data suggest that vasopressin-induced microvilli elongation or formation, increasing the exchange surface at the sinusoidal pole of the hepatocyte, possibly through exocytosis, would favor ion and solute transfer to and from the Disse space and the plasma. The extent to which such a putative hormonal regulation of exchange surface at the sinusoidal membrane domain could occur *in vivo*, in physiological or pathological conditions, remains unknown.

Finally, the effects of AVP on hepatocyte BC contraction were analyzed. Hepatocytes possess a fully developed cytoskeleton particularly composed of F-actin filaments, several actin-associated protein and myosin II. These proteins are associated with the canalicular membrane as well as with gap and tight junctions, which form a cohesive interconnected network around the BC. Functional integrity of these structures is necessary for hepatocyte secretion and bile formation [21]. BC contraction has previously been reported as a calcium-dependent process in isolated rat hepatocyte couplets and triplets [34, 44]. The importance of BC contraction in bile secretion is still matter of debate but cytoskeleton alterations (cytochalasin B or phalloidin) have been reported to be associated with cholestasis [33, 45]. Also, *in vivo* experiments in the living rat showed that BC contraction propagated along

hepatocyte plates from pericentral to periportal areas of the hepatic lobule and had an impact on bile flow regulation [33]. Here we further suggest that AVP-induced BC contraction is dependant upon calcium mobilization from intracellular stores. These results are in agreement with anterior studies stated that AVP-induced intra and intercellular calcium signals contributed to bile flow regulation, at least in part through the modulation of canalicular peristaltism [7-10].

Taken together, our data suggest that intracellular mechanisms triggering AVP-induced F-actin cytoskeleton remodeling and correlated morphological alterations differ according to the hepatocyte plasma membrane domain considered, basolateral (sinusoidal) or canalicular (biliary). Microvilli formation occurring at the sinusoidal pole required mainly extracellular calcium entry, although canalicular contraction needed principally internal calcium stores (Fig.7). These data fit with previous reports indicating that type II InsP3 receptor, the main hepatocyte intracellular calcium channel, was located in the pericanalicular region [22], an area where calcium release appeared to be functionally important for BC contraction. Moreover, our study reinforces the view that rat hepatocyte calcium signaling is polarized, suggesting that a single hormonal stimulation may induce distinct cell morphological modifications, regulating distinct cellular functions at different cellular loci.

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Figure legends

Table 1. Influence of drugs or calcium mobilizing agents on hepatocyte microvilli formation. Rat hepatocytes were stimulated 90 s at 37°C with or without (control) various drugs or calcium mobilizing agents, fixed immediately with FA and F-actin labeled as described under methods. The percentage of cell with microvilli was then determined. Results are the mean \pm sem of more than 150 cells per condition originating from 3 distinct cell preparations.

Ca ²⁺ -mobilizing-Agonists	Cells with microvilli (%)
Control	3 \pm 1
Vasopressin (10 nM)	75 \pm 5
Angiotensin II (10 nM)	67 \pm 7
Noradrenaline (10 μ M)	73 \pm 7
ATP (10 μ M)	78 \pm 5
Glucagon (100 nM)	22 \pm 5

Figure legends

Figure 1. AVP-induced morphological modifications in isolated hepatocytes.

A-C: light microscope images of hepatocytes either control (A) or stimulated for 4 min at 37°C with 1nM (B) or 10 nM (C) vasopressin, fixed and stained with toluidine blue. As compared with control hepatocytes (A), AVP stimulated hepatocytes exhibit numerous protrusions at their membrane surface (B and C).

D-E: electron microscope images of hepatocytes either control (D) or stimulated 4 min at 37°C with 10 nM vasopressin. The AVP stimulated hepatocyte exhibits typical microvilli that appear more numerous and more elongated than in the control (A). N: nucleus; ld: lipid droplet;

Figure 2. Effects of AVP on hepatocyte F-actin organization

A. Confocal images of equatorial sections (right panels) and 3D projections (Z series, left panels) of hepatocytes stimulated for 90 s at 37°C with (+VP) or without (-VP) 3nM vasopressin, fixed and F-actin-stained. Photographs are representative of more than 90 cells originating from 6 different hepatocyte preparations.

B. F-actin radial fluorescence intensity profiles of hepatocytes stimulated for 90 s at 37°C with (+AVP) or without (-AVP) 3nM vasopressin, were obtained as described in methods. Profiles illustrated are the mean \pm sem of at least 90 individual cell profiles originating from at least 6 hepatocyte preparations.

C. Hepatocytes were incubated with 3 nM AVP during 0, 30, 60, or 120 s at 37°C. The percentage of cells exhibiting microvilli was determined and plotted against the thickness of the cell membrane F-actin labelling. Results are the mean \pm sem of 3 experiments for which at least 60 distinct cells per condition were studied.

Figure 3. Pharmacological properties of AVP-induced microvilli formation

Panel A. Hepatocytes were incubated for different periods of time at 37°C with 1 nM AVP, fixed and stained for F-actin. The % of cells exhibiting microvilli was determined and plotted against the time of stimulation. Results were the mean \pm sem of more than 150 cells per condition, originating from 3 experiments.

Panel B. Hepatocytes were stimulated with increasing amounts of AVP, fixed and stained for F-actin. A 5 min preincubation at 37°C was performed with or without (control) 100 nM of a specific V1a antagonist (Manning compound) and the % of cells exhibiting microvilli determined.

Panel C. Hepatocytes were incubated 90 s at 37 °C with (+AVP) or without 3 nM AVP (-AVP). Some samples were fixed and stained for F-actin, other were further rinsed twice with saline buffer, fixed and stained for F-actin (rinse). The percentage of cells exhibiting microvilli was then determined as in panel A.

Figure 4. Increase in $[Ca^{2+}]_i$ triggers microvilli formation .

Hepatocytes were stimulated 90 s at 37°C by various drugs or vehicle (control) as described in Table 1, fixed and stained for F-actin. Panel A. Confocal images of hepatocyte equatorial section. Panel B. % of cells exhibiting microvilli in response to various treatments. Results illustrated in this figure were representative of at least 150 cells per conditions, originating from 3 hepatocyte preparations

Figure 5. Ca^{2+} influx is crucial for hepatocyte F-actin cytoskeleton reorganization during AVP stimulation.

Hepatocytes were incubated various time at 37°C in SSH medium containing either 1.8 mM or 100 nM free Ca^{2+} without AVP (-AVP), with 3 nM AVP (+AVP) or with 1 or 5 μ M ionomycin (iono). Panel A. $[Ca^{2+}]_i$ was monitored in each condition as described in Methods. $[Ca^{2+}]_i$ values, expressed in nM, are the mean \pm sem of 3 experiments . Panel B. Aliquots of each hepatocyte preparation were fixed and stained for F-actin and confocal images of hepatocyte equatorial sections illustrated. Panel C. Percent of cells exhibiting

microvilli. Results are the mean +/- sem of more than 150 hepatocytes per condition, from 3 experiments.

Figure 6. AVP-induced bile canaliculi contraction is dependent on intracellular Ca^{2+} stores.

Panel A. Hepatocyte multipllets were pre-incubated 5 min at 37°C with or without (control) 100 nM AVP antagonist and further incubated 90 s with (+AVP) or without 3nM AVP (-AVP), in 1.8 mM free $[\text{Ca}^{2+}]_{\text{ext}}$ medium. Hepatocytes were then fixed and stained for F-actin. The bile canaliculi (BC) were identified by arrows on confocal image of equatorial section of hepatocyte

Panel B. The number of dilated BC (BC which exhibited lumen in negra) were determined and expressed as percent of total BC observed in cell preparation.

Panel C. Representative examples of fluorescence F-actin intensity profiles through BC are given for one dilated control and one AVP (3 nM)-stimulated BC.

Panel D. Hepatocytes were incubated 90 s without AVP (-AVP), with 3 nM AVP (+AVP) or with 1 or 5 μM ionomycin (iono) in 1.8 mM or 100 nM free $[\text{Ca}^{2+}]_{\text{ext}}$ media. The number of dilated BC, expressed in percent of total BC observed, was determined. Results are the mean +/- sem of at least 60 observed BC per condition from 3 experiments.

Figure 7. Dual effects of agonist-induced changes in $[\text{Ca}^{2+}]_i$ on polarized hepatocyte couplets.

Schematic representation of mechanisms by which Ca^{2+} -mobilizing agonist-induced F-actin cytoskeleton remodeling. According to the hepatocyte plasma membrane domain

considered, basal (sinusoidal) or canalicular (biliary) Ca^{2+} -mobilizing agonist-induced different morphological alterations. Microvilli formation occurring at the sinusoidal pole required mainly calcium entry from the extracellular space (white arrows), although BC contraction needed principally mobilization of internal Ca^{2+} stores (black arrows).