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Receptor-oriented intercellular calcium waves evoked by vasopressin in rat hepatocytes

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Agonist-induced intracellular calcium signals may propagate as intercellular Ca^{2+} waves in multicellular systems as well as in intact organs. The mechanisms initiating intercellular Ca^{2+} waves in one cell and determining their direction are unknown. We investigated these mechanisms directly on fura2-loaded multicellular systems of rat hepatocytes and on cell populations issued from peripheral (periportal) and central (perivenous) parts of the hepatic lobule. There was a gradient in vasopressin sensitivity along connected cells as demonstrated by low vasopressin concentration challenge. Interestingly, the intercellular sensitivity gradient was abolished either when D-myo-inositol 1,4,5-trisphosphate ($InsP_3$) receptor was directly stimulated after flash photolysis of caged $InsP_3$ or when G proteins were directly stimulated with AlF^{4-}. The gradient in vasopressin sensitivity in multiplets was correlated with a heterogeneity of vasopressin sensitivity in the hepatic lobule. There were more vasopressin-binding sites, vasopressin-induced $InsP_3$ production and V1a vasopressin receptor mRNAs in perivenous than in periportal cells. Therefore, we propose that hormone receptor density determines the cellular sensitivity gradient from the peripheral to the central zones of the liver cell plate, thus the starting cell and the direction of intercellular Ca^{2+} waves, leading to directional activation of Ca^{2+}-dependent processes.

Keywords: intercellular calcium waves/oriented/vasopressin receptor

Introduction

Hormone-induced intracellular signals may propagate in connected cells via intercellular waves of second messenger increases (for reviews, see Kasai and Petersen, 1994; Sanderson *et al*., 1994). Intercellular Ca^{2+} waves are thought to spread in certain epithelial cells via the putative intercellular diffusion of D-myo-inositol 1,4,5-trisphosphate ($InsP_3$) and/or Ca^{2+} across gap junctions (Sanderson *et al*., 1994; Sneyd *et al*., 1995), and there may also be paracrine involvement (Schlosser *et al*., 1996; Frame and De Feijter, 1997). However, mechanisms of cell to cell propagation of Ca^{2+} waves are not completely elucidated.

In the liver cell plate, a cord one cell thick and ~20 hepatocytes long between the portal and centrolobular veins, cells are connected extensively by large aggregates of connexins 32 and/or 26 through which hormone-induced intercellular Ca^{2+} waves are thought to propagate (Saez *et al*., 1989; Nathanson and Burgsthaler, 1992). We have described reproducible sequences of hormone-induced intracellular calcium concentration ($[Ca^{2+}]_i$) increases resulting in apparent unidirectional intercellular Ca^{2+} waves in multicellular rat hepatocyte systems or multiplets (Combettes *et al*., 1994). In noradrenaline-induced $[Ca^{2+}]_i$ increases in multiplets, gap junction coupling is crucial for coordinating Ca^{2+} transients between cells, but agonist–receptor interactions are also required at the surface of each hepatocyte, ensuring that the cells are in the required excitable state for Ca^{2+} responses (Tordjmann *et al*., 1997a). Similar directional intercellular Ca^{2+} waves running through hepatocyte cords have also been described in the intact liver (Nathanson *et al*., 1995; Robbgaspers and Thomas, 1995). However, the factors determining the cell from which the wave begins and the direction of the Ca^{2+} waves in hepatocyte multiplets or cords are unknown. Generally, agonist-induced signaling in epithelial cells is thought to spread from cell to cell starting from an initial stimulated cell, then spreading throughout the tissue in all directions. Only in excitable cells, such as neurons and heart cells, are action potentials propagated in one direction, along a specific intercellular circuit. In neurons, this is due to asymmetrical chemical synapses and clustering of neurotransmitter receptors and ion channels (Jessel and Kandel, 1993). In cardiac pacemaker cells, signals are propagated unidirectionally due to tissue micro-architecture and the distribution of gap junctions and ion channels (Anumonwo and Jalife, 1995). In the liver, there are morphological and functional differences between hepatocytes, mostly occurring in gradients between the periphery and the center of the lobules (for a review, see Jungermann and Kietzmann, 1996). Zonal differences in vasopressin sensitivity have been reported between periportal (i.e. 'peripheral', PP) and perivenous (i.e. 'central', PV) hepatocyte populations (Tordjmann *et al*., 1996). For hepatocyte pairs and more complex groups of freshly isolated connected cells, it is assumed that the relative *in situ* position of each hepatocyte in the lobule is retained with orientation along the liver cell plate axis. Thus, it could be hypothesized that, in a freshly isolated multicellular system representing a small part of a hepatocyte plate, there is a gradient of subtle and gradual differences in vasopressin sensitivity between cells. Because hormonal latency is reflecting the sensitivity of hepatocytes to agonists (Thomas *et al*., 1996), the reproducible sequence of Ca^{2+} responses in multiplets may result from a gradient of vasopressin sensitivity in the connected cells.

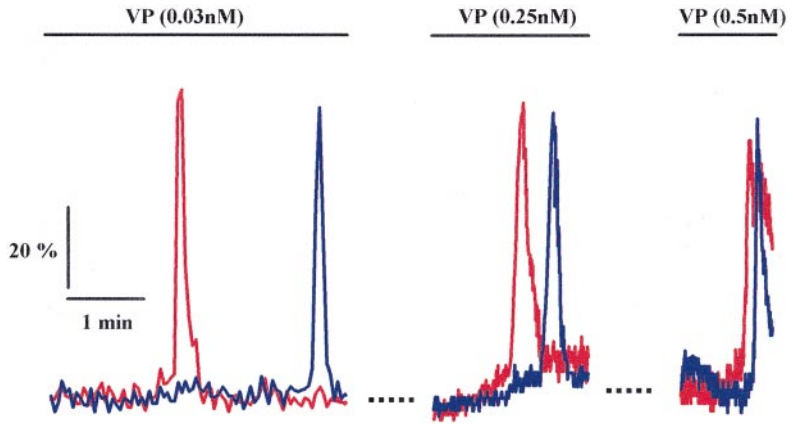

Fig. 1. Effect of low vasopressin concentrations on $[Ca^{2+}]_i$ transients in hepatocyte doublets. Hepatocyte doublets were microinjected with fura2, then stimulated with vasopressin by global perfusion. The same cell doublet was challenged successively with increasing doses of vasopressin. Cells were washed with saline for 5–10 min between each vasopressin challenge. Left panel: 0.03 nM vasopressin elicited a $[Ca^{2+}]_i$ rise in a first cell after a 5.5 min latency. The second responding cell of the doublet exhibited a $[Ca^{2+}]_i$ rise after a 7.5 min latency period. Frame ratioing: 1 image/3 s. The trace begins 4 min after agonist addition. Middle panel: 0.25 nM vasopressin elicited a $[Ca^{2+}]_i$ rise in the same first cell, after a 1.7 min latency period. The second responding cell of the doublet exhibited a $[Ca^{2+}]_i$ rise after a 24 s intercellular delay: 1 image/500 ms. Right panel: 0.50 nM vasopressin elicited a $[Ca^{2+}]_i$ rise in the same first cell, after a 50 s latency period. The second responding cell of the doublet exhibited a $[Ca^{2+}]_i$ rise after an 8 s intercellular delay: 1 image/500 ms. The order in which the cells responded was constant throughout the experiment. These traces are representative of those obtained with six doublets and four triplets in three independent experiments.

In this work, we combined single-cell studies with experiments on cell populations isolated from the PP and PV zones of the liver cell plate. We obtained strong evidence that the sequential pattern of Ca^{2+} responses to vasopressin in multicellular rat hepatocyte systems was due to a cell to cell gradient of sensitivity for the hormone, with maximal sensitivity in the first responding hepatocyte and minimal sensitivity in the last. This gradient of sensitivity was consistent with the zonal differences in the number of hormone receptors in the liver cell plate, leading to a gradient in $InsP_3$ production. Cellular sensitivity gradients may impose an orientation on intercellular Ca^{2+} waves in liver cells. Such a pacemaker-like mechanism may play a key physiological role in regulating intercellular communication in the liver and other epithelial cells.

Results and discussion

A gradient in vasopressin sensitivity exists in fura2-loaded multicellular rat hepatocyte systems

Each hepatocyte may have its own particular sensitivity to vasopressin, reflected by its proper delay and $[Ca^{2+}]_i$ oscillation frequency after hormonal stimulation (Ogden *et al*., 1990; Chiavaroli *et al*., 1994; Thomas *et al*., 1996). If there is a cell to cell gradient in vasopressin sensitivity in connected cells of multicellular systems, there should be very low concentrations of this agonist for which, within a minute range period of time, one cell in the multiplet can respond whereas the others cannot. Latency is inversely related to the concentration of agonist applied for single hepatocytes (Ogden *et al*., 1990; Chiavaroli *et al*., 1994; Thomas *et al*., 1996), so it is easier to detect differences in latency between cells using very low hormone concentrations. Thus, we used 0.03 nM vasopressin to stimulate fura2-injected pairs of connected hepatocytes. Cell to cell diffusion of microinjected fura2 ensured that the cells were coupled efficiently by gap junctions. At these low levels of vasopressin concentrations, only a small percentage of cells exhibited changes in $[Ca^{2+}]_i$ (Woods *et al*., 1986; Thomas *et al*., 1996). Upon global vasopressin perfusion challenge (0.03 nM), $[Ca^{2+}]_i$ increased after a latency period of 5.5 min for one cell of a doublet, whereas the other cell responded 7.5 min after vasopressin stimulation (Figure 1, left panel). When the vasopressin concentration was increased to 0.25 nM, for the same doublet, $[Ca^{2+}]_i$ increased sequentially in the two cells, with a 24 s intercellular time lag (Figure 1, middle panel). The cell that responded first to 0.25 nM vasopressin stimulation (1.7 min latency) was the one that appeared most sensitive to very low hormone doses (0.03 nM) (Figure 1, left and middle panels). The same sequential response was observed with 0.50 nM vasopressin; the latency of the first cell was 50 s, and the time lag between cells was shorter (8 s) (Figure 1, right panel). The order in which cells responded was constant throughout the experiment. Only the rate of intercellular Ca^{2+} wave was accelerated as vasopressin concentration rose. This last feature has been observed previously on hepatocytes (Combettes *et al*., 1994; Nathanson *et al*., 1995; Robbgaspers and Thomas, 1995). In contrast, the rate of the intracellular Ca^{2+} wave does not depend on the applied agonist concentration (Thomas *et al*., 1996). Similar results were obtained with six doublets and four triplets in four cell preparations. It is thus clear that the two connected cells of a hepatocyte doublet have different sensitivities to vasopressin, and that these differences may account for the ordered sequence of $[Ca^{2+}]_i$ increases.

Mechanism generating the sensitivity gradient for vasopressin in multicellular hepatocyte systems and in the hepatocyte plate

We determined which factors in the transduction pathway were responsible for the differences in sensitivity to vasopressin between adjacent connected hepatocytes by analyzing the $InsP_3$–$InsP_3$ receptor reaction and the $InsP_3$ production machinery. We performed this analysis for

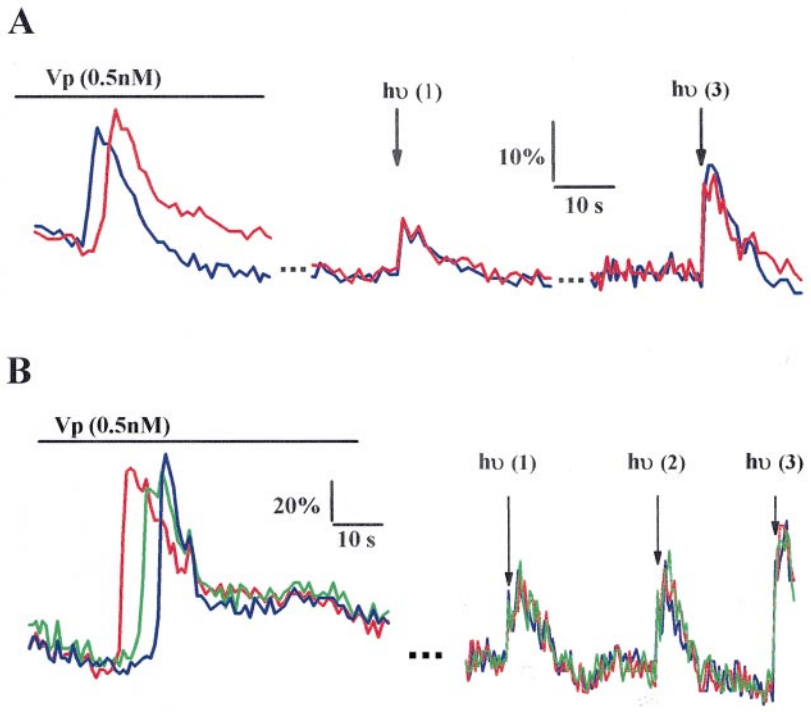

Fig. 2. Effect on intercellular calcium waves in rat hepatocyte multiplets of photolytic release of $InsP_3$ from caged $InsP_3$. (**A**) Left panel: an hepatocyte doublet loaded with fluo3 was challenged with vasopressin (0.5 nM) for the time indicated by the horizontal bar. A sequential $[Ca^{2+}]_i$ rise was observed in the two cells. Right panel: the same doublet was subjected to flash photolysis, at the time indicated by vertical arrows. Immediate and simultaneous $[Ca^{2+}]_i$ rises were observed in the two connected cells. (**B**) Left panel: an hepatocyte triplet loaded with fluo3 was challenged with vasopressin (0.5 nM) for the time shown by the horizontal bar. A sequential $[Ca^{2+}]_i$ rise was observed in the three hepatocytes. Right panel: the same triplet was subjected to flash photolysis, at the time indicated by vertical arrows. Immediate and simultaneous $[Ca^{2+}]_i$ rises were observed in the three connected cells. Ca^{2+}-associated changes in fluorescence were recorded with 1 image/≤500 ms. Flashlamp intensity was 100 mV (×1), 200 mV (×2) or 300 mV (×3). Note that the first spike in flash photolysis traces [hν (1) in A and B] results from the optical artifact arising from the UV pulse. These traces are representative of those obtained with seven doublets and five triplets in three independent experiments.

adjacent connected cells, and by studying PP and PV hepatocyte populations from the liver cell plate.

$InsP_3$ receptor sensitivity to $InsP_3$. Caged $InsP_3$ (500 μM in the pipet) was microinjected into one cell of fluo3-loaded doublets and triplets of hepatocytes (see Materials and methods). The cells were washed for at least 10 min with saline, and then were challenged homogeneously with vasopressin perfusion (0.5 nM). As shown in Figure 2 (representative of seven doublets and five triplets in three experiments), vasopressin induced typical sequential $[Ca^{2+}]_i$ increases in the connected cells of one doublet (Figure 2A, left panel) and one triplet (Figure 2B, left panel). Then cells were washed and $InsP_3$ was released from caged $InsP_3$ by flash photolysis (see Materials and methods), and the same doublet and triplet were analyzed for $[Ca^{2+}]_i$ changes. Upon flash photolysis, there were immediate and simultaneous $[Ca^{2+}]_i$ rises in the connected cells of the doublet and triplet, with no detectable time lag between the responses of each cell (Figure 2A and B, middle and right panels). When small photolytic liberation of $InsP_3$ was performed, a calcium response with a slower time course occurred, but $[Ca^{2+}]_i$ still rose concomitantly in the connected cells (first flash, Figure 2A and B). Thus, the simultaneous release of $InsP_3$ in connected cells caused simultaneous Ca^{2+} responses, whereas uniform vasopressin stimulation induced sequential $[Ca^{2+}]_i$ transients. In line with these results, the latency of Ca^{2+} mobilization is determined mostly by the time required for the ligand-bound receptor to activate its G protein and for phospholipase C-β (PLC-β) to produce the threshold concentration of $InsP_3$ required to release Ca^{2+} (Thomas *et al.*, 1996), whereas $InsP_3$ interacts with its receptor within a fraction of a second in rat hepatocytes (Champeil *et al.*, 1989; for a review, see Ogden *et al.*, 1990).

The differences in sensitivity to vasopressin identified in our previous study of PP and PV cell populations were also thought to be due to one or more of the upstream steps in the transduction pathway, because similar Ca^{2+} responses to $InsP_3$ were obtained with permeabilized PP and PV hepatocyte populations (Tordjmann *et al.*, 1996). In the present work, we have shown using a more physiological approach that even between adjacent cells, putative differences in $InsP_3$ receptor sensitivity cannot account for the cell to cell differences in vasopressin sensitivity.

$InsP_3$ accumulation. Our data suggest that $InsP_3$ accumulation in response to vasopressin may be the key factor responsible for cell to cell differences in cellular sensitivity to the hormone in multicellular systems, and for zonal heterogeneity of vasopressin sensitivity in the hepatocyte plate. $InsP_3$ cannot be measured in single cells, but $InsP_3$ mass was measured in populations of PP and PV cells isolated from the same liver. Resting values were similar for the two cell populations [1.1 ± 0.4 and 1.0 ± 0.5 pmol $InsP_3$/mg protein, respectively in PP (n = 10) and PV cells (n = 10)] (Figure 3), and were consistent with published results for hepatocytes (Palmer *et al.*, 1989). Vasopressin (10 nM) treatment resulted in significantly

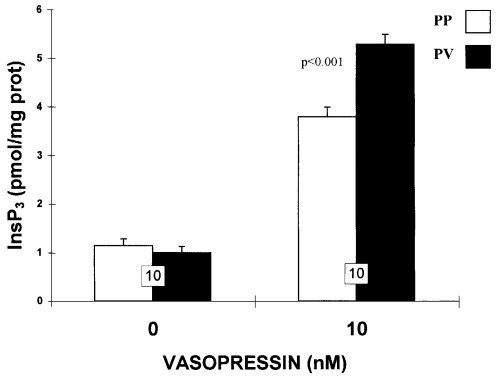

Fig. 3. $InsP_3$ accumulation in PP and PV hepatocyte populations. Vasopressin (10 nM)-induced $InsP_3$ accumulation in PP and PV cells. $InsP_3$ resting values were 1.1 ± 0.4 and 1.0 ± 0.5 pmol $InsP_3$/mg protein in PP and PV cells respectively. Data are expressed as means ± SEM from *n* (number in bar) separate experiments for each cell population.

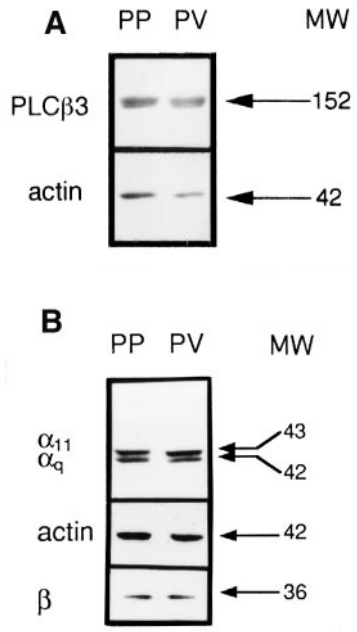

Fig. 4. PLC-β and G protein immunobloting in PP and PV hepatocytes. (**A**) Relative amounts of PLC-β3 in PP and PV hepatocyte membranes. Membrane proteins (50 μg) from PP and PV hepatocytes were resolved by SDS–PAGE (7.5% acrylamide) and immunoblotted using anti-PLC-β3 and anti-actin antibodies. The result of one experiment representative of four performed in quadruplicate, is presented. (**B**) Relative amounts of αq/α11 and β G protein subunits in PP and PV hepatocyte membranes. Membrane proteins (30 μg) from PP and PV hepatocytes were resolved by SDS–PAGE (13% acrylamide) and immunoblotted using anti-$G_{\alpha q}/G_{\alpha 11}$, anti-$G_\beta$ or anti-actin antibodies. PV cells contained 101 ± 8% of the amount of αq/α11 G protein subunits of PP cells, and 99 ± 13% of the amount of β G protein subunits of PP cells. The result of one experiment, representative of six performed in duplicate, is presented.

higher levels of $InsP_3$ in PV than in PP hepatocytes (Figure 3). The paired ratio of vasopressin-induced $InsP_3$ accumulation in PV over that in PP cells from the same liver was 1.40 ± 0.04 (n = 10).

The differences described above in vasopressin-induced $InsP_3$ accumulation between PP and PV hepatocytes may be due to differences in $InsP_3$ degradation or from differences in $InsP_3$ production *per se*. $InsP_3$ 5-phosphatase activity, which has been reported to control primarily the levels of intracellular $InsP_3$ (De Smedt *et al*., 1997; Dupont and Erneux, 1997), has been measured in the two cell populations (see Materials and methods). The activity of the enzyme was not found to be significantly different in PP and PV cells, or in control (i.e. conventionally isolated) hepatocytes (2.28 ± 0.40, 1.61 ± 0.16 and 2.37 ± 0.27 nmol/min/mg protein respectively; n = 4), and was in agreement with previously reported data in hepatocyte homogenates (Shears, 1989). Differences in the $InsP_3$ production *per se* may result from differences in agonist–receptor interaction, guanidine nucleotide-binding proteins (G proteins) or PLC-β activation. PLC-β3, the member of the PLC-β subfamily most sensitive to stimulation by the Gq α and βγ subunits of G proteins (Blank, 1996), was studied in PP and PV hepatocyte populations by immunoblotting. There was no significant difference in the relative amount of PLC-β3 in PP and PV membranes (Figure 4A). The signal obtained for PV membranes represented 99 ± 2% of the signal found for PP membranes (results from four distinct PP and PV membrane preparations, each performed in quadruplicate). The α and β subunits of the G_q and G_{11} G proteins, which are coupled to the V1a vasopressin receptor (Taylor *et al*., 1991), were also studied in PP and PV hepatocyte membranes by the same technique. The relative amounts of the subunits were similar for PP and PV membranes (Figure 4B). PV cells represented 101 ± 8% of the amount of αq/α11 G protein subunits of PP cells, and 99 ± 13% of the amount of β G protein subunits of PP cells (results from six distinct PP and PV membrane preparations, each performed in duplicate). We checked that these results for G proteins and PLC-β expression were functionally relevant by directly stimulating G proteins with AlF^{4-} and analyzing the subsequent Ca^{2+} responses, in connected adjacent cells, and in PP and PV hepatocyte populations. Direct stimulation of G proteins with AlF^{4-} mimics $[Ca^{2+}]_i$ oscillations (Woods *et al*, 1990) and intracellular Ca^{2+} waves (Rooney *et al*., 1990) elicited by agonists in rat hepatocytes. We compared the effects of vasopressin and AlF^{4-} (10 μM $AlCl_3$, 20 mM NaF) on fura2-loaded rat hepatocyte multiplets. As shown in Figure 5A (left panel), 0.1 nM vasopressin induced a $[Ca^{2+}]_i$ increase which occurred as ordered transients, with the two cells being activated sequentially at each spike. Once the cells were washed, AlF^{4-} treatment elicited increases in $[Ca^{2+}]_i$ in both cells after a 6 min latency, with no ordered sequence (Figure 5A, middle panel), suggesting random responses in the connected cells. The cells were washed after AlF^{4-} treatment, and vasopressin challenge resulted in $[Ca^{2+}]_i$ rises in the same cell order within the doublet as was observed before AlF^{4-} treatment (Figure 5A, right panel). Quin2-loaded PP and PV hepatocytes were studied by spectrofluorimetry to measure basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ after treatment with AlF^{4-} (10 mM NaF, 5 μM $AlCl_3$). PP and PV hepatocytes responded similarly to these treatments, although there were significant differences between the two populations under vasopressin stimulation, PV cells being more responsive than PP cells (Figure 5B). Therefore, both cell to cell differences in a multiplet and between the PP and PV zones in the liver lobule in vasopressin-induced Ca^{2+} signals do not persist if the

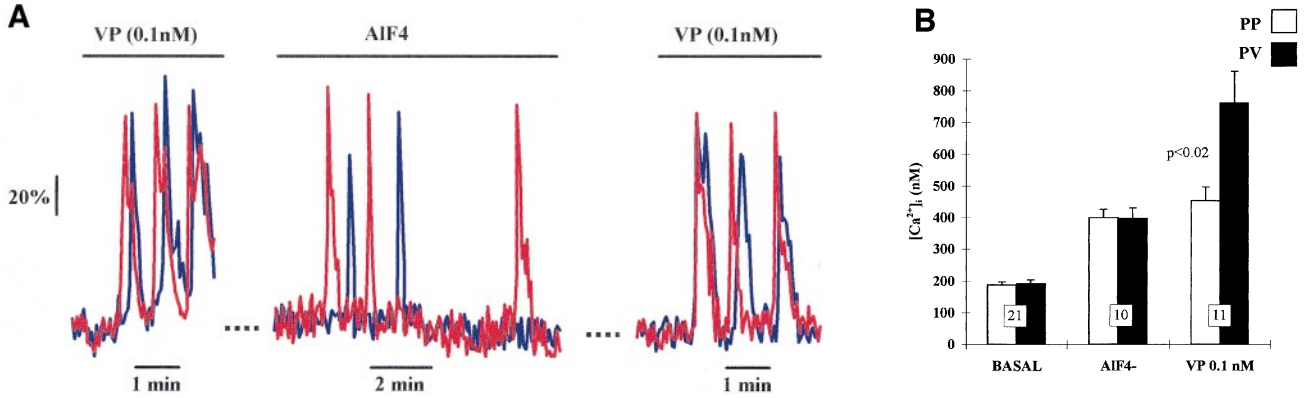

Fig. 5. $[Ca^{2+}]_i$ increase induced by direct stimulation of G proteins. (**A**) Vasopressin- and AlF^{4-}-induced $[Ca^{2+}]_i$ changes in hepatocyte multiplets, studied by videomicroscopy. A fura2-loaded hepatocyte doublet was challenged with vasopressin (0.1 nM) for the time indicated by the horizontal bar. Sequential $[Ca^{2+}]_i$ rises were observed in the two connected cells (left panel). The doublet was washed with saline for 10 min, then treated with AlF^{4-} (10 μM $AlCl_3$, 20 mM NaF), superfused into the incubating medium for the time indicated by the open box. After a 6 min latency, $[Ca^{2+}]_i$ increased in a disorganized manner in both cells (middle panel). The cells were washed again and sequential $[Ca^{2+}]_i$ rises were again induced in the doublet by vasopressin (right panel). Ca^{2+}-associated fluorescence changes were recorded with a frame ratioing of 1 image/3 s. This experiment is one representative of five similar traces obtained in four experiments. For technical convenience, traces were interrupted (the gap represents 5 min). Note that a $[Ca^{2+}]_i$ rise induced by AlF^{4-} treatment was observed in only 30% of the cells, with a latency always >5 min. (**B**) Vasopressin- and AlF^{4-}-induced $[Ca^{2+}]_i$ changes in PP and PV hepatocyte populations, studied by spectrofluorimetry. Basal $[Ca^{2+}]_i$ was 188 ± 10 nM in PP (n = 21) and 192 ± 12 nM in PV (n = 21) hepatocytes. Vasopressin-induced $[Ca^{2+}]_i$ changes in hepatocytes from the left lateral and median liver lobes were not significantly different (data not shown).

transduction pathway is stimulated immediately downstream from the hormone receptors.

Heterogeneity of V1a vasopressin receptors in PP and PV hepatocytes

Hormone receptors cannot be quantified accurately for single cells, but we investigated V1a vasopressin receptor expression in PP and PV hepatocytes by binding experiments and RNase protection assays. The number and affinity of [^{125}I]phenylacetyl-D-Tyr^1 (Mc)^2-Phe^3-Gln^4-Asn^5-Arg^6-Pro^7-Arg^8-NH_2 (HO-LVA)-binding sites in PP and PV membranes were assessed. B_{max} values were higher for PV than for PP membranes (Figure 6A). The mean B_{max} value was 260 ± 81 (n = 7) for PP and 412 ± 137 (n = 7) for PV hepatocyte membranes. [^{125}I]HO-LVA B_{max} values differ between animals, so only paired ratios (PV/PP) of zonal-enriched membranes issued from the same liver can highlight a statistically significant acinar heterogeneity. The mean PV/PP was 1.43 ± 0.06 (n = 7) (P <0.001). This result demonstrated a significantly greater number of [^{125}I]HO-LVA-binding sites in the PV than in the PP area and confirms that comparisons between PP and PV hepatocytes are more accurate when both cell populations are isolated from a single liver (Tordjmann *et al*., 1997b). The affinity of [^{125}I]HO-LVA-binding sites for vasopressin (10–30 pM) appeared to be similar in PP and PV cells (PV/PP, 0.99 ± 0.17, n = 7). Moreover, there was no significant difference in B_{max} or affinity of [^{125}I]HO-LVA-binding sites between total populations of hepatocytes issued from median and left lateral lobes (data not shown). As a control, we checked that pharmacological profiles of the [^{125}I]HO-LVA-binding sites were similar in PP and PV hepatocyte membranes (data not shown). In both preparations, [^{125}I] HO-LVA-binding sites exhibited the same affinity for vasopressin analogs generally used to characterize vasopressin-binding sites (Grazzini *et al*., 1996). These differences in the number of vasopressin-binding sites were in agreement with *in situ* autohistoradiography performed with [^{125}I]HO-LVA on liver frozen sections, showing that binding sites were predominant near the central veins (personal observations). Such a patchy distribution of vasopressin V1a receptors has been noted previously (Barberis *et al*., 1995).

An RNase protection assay was performed on guanidine thiocyanate (GuSCN) hepatocyte lysates with a V1a and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cRNA probe. A 231 nucleotide band, corresponding to the V1a mRNA, was protected (Figure 6B), whereas hybridization with GAPDH mRNA gave a 100 nucleotide protected fragment. Direct counting of the gel with Instant Imager and analysis of the signal ratios gave a PV/PP ratio of 1.42 ± 0.17 (P = 0.01, mean ± SEM from seven separate determinations on six animals) for V1a mRNA. As a control, cell lysates from total hepatocyte populations of median and left lateral lobes were also hybridized with V1a probe, and direct counting gave an observed median lobe/lateral lobe ratio of 0.94 ± 0.08, expressed as the mean ± SEM of triplicate determinations for four animals.

The density of receptors for peptide hormones in the different zones of the liver acinus is unknown, although it has been suggested that the V1a vasopressin receptor is not distributed evenly throughout the lobule *in situ* (Ostrowski *et al*., 1993; Nathanson *et al*., 1995). Our results, suggesting that there is a gradient of hormone receptor density between the PP and PV zones, are consistent with the observed direction of vasopressin-induced intercellular Ca^{2+} waves in the intact liver, moving from PV to PP areas (Nathanson *et al*., 1995; but see also Robbgaspers and Thomas, 1995).

Cell excisions

We further investigated the mechanisms involved in cell to cell propagation of vasopressin-induced directional Ca^{2+} waves, using an experimental approach similar to that used in studies of pacemaker activity in cardiac sinoatrial cell pairs (Anumonwo and Jalife, 1995). We

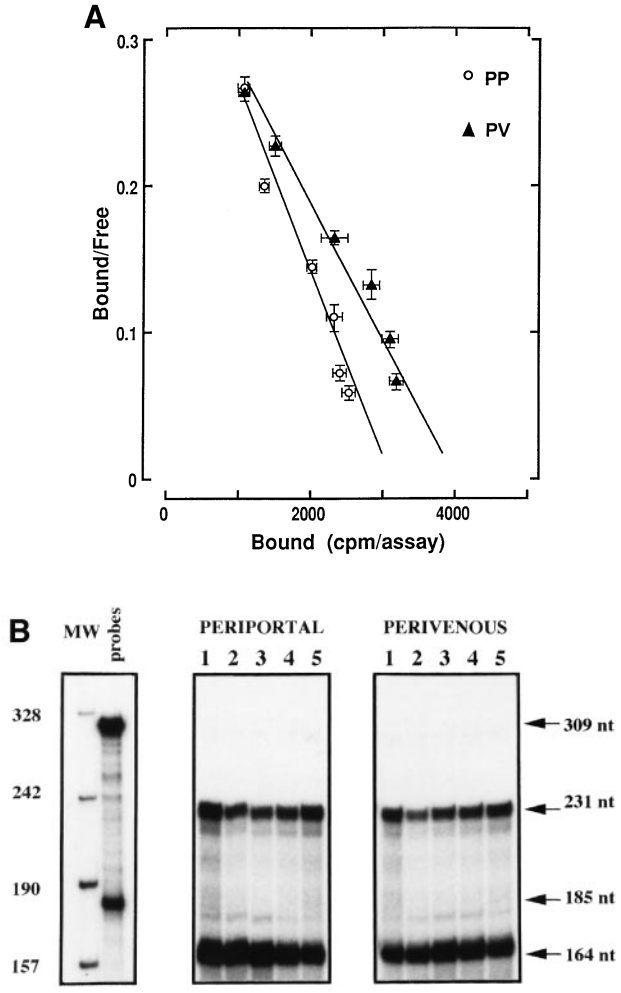

Fig. 6. Distribution of [^{125}I]HO-LVA-binding sites and of the V1a vasopressin receptor mRNA in rat PP and PV hepatocytes. (**A**) Specific HO-LVA-binding sites from rat PP and PV hepatocyte membranes. Crude membranes (2.4 μg per assay) from rat PP (○) or PV (▲) hepatocytes were incubated for 1 h at 37°C in the presence (non-specific binding) or absence (total binding) of 1 μM unlabeled AVP with increasing amounts of [^{125}I]HO-LVA (free). Specific binding (bound), calculated as the difference between total and non-specific binding, was determined as described in Materials and methods, and was plotted against the ratio bound/free. Results are the mean of triplicate determinations from a single experiment representative of six. (**B**) Detection of V1a mRNA in PP and PV hepatocyte lysates. Hepatocytes solubilized in GuSCN (20 μl containing equivalent amounts of DNA, i.e. 60 ng) were hybridized with 10^5 c.p.m. of labeled V1a cRNA probe. DNA molecular weight markers (mol. wt in bp) are DNA fragments from *Msp*I-digested pBluescript II SK+ phagemid labeled with [^{32}P]dCTP using the Klenow fragment of *Escherichia coli* DNA polymerase I. RNase protection assay and polyacrylamide gel electrophoresis were performed as described in Materials and methods.

excised the first then the second responding cells of hepatocyte triplets in succession, and compared the cellular latencies of remaining cells with 0.5 nM vasopressin stimulation, before and after excision. We studied only the remaining cells that were undamaged, as indicated by the similar fluorimetric ratio (0.75 ± 0.06 and 0.76 ± 0.07; $n = 18$) before and after excision (nine triplets). When the first cell was excised, the second cell and the third cell responded with a 17 ± 7% and 17 ± 6% longer latency ($n = 9$), respectively, than before excision. When the first and second responding cells were excised, the third cell responded with a 60 ± 37% longer latency ($n = 3$) than before excision. Consistent with previous reports, repeated hormonal challenges did not lead to significantly longer latencies (Combettes *et al*., 1994; and data not shown). It is noteworthy that the latency of the first responding cell in a triplet stimulated with vasopressin before and after excision of the second or third cell was not significantly modified and was 2.0 ± 3.3% ($n = 6$) longer than before excision. These results support the notion of a gradient in vasopressin sensitivity in the connected hepatocytes of freshly isolated multiplets, and suggest that the diffusion of an intercellular messenger may sensitize adjacent cells, accelerating their response (Tordjmann *et al*., 1997a). Such a configuration in which the most responsive hepatocytes drive the response of the less sensitive cells is similar to the cell to cell triggering of cardiac pacemaker cells (Anumonwo and Jalife, 1995). This kind of response may be due to the very specialized organization of the liver cell plate *in situ* (Gumucio *et al.*, 1994).

Our results suggest a model for the sequentiality of vasopressin-induced Ca^{2+} signals in freshly isolated multicellular rat hepatocyte systems and in the liver cell plate. After the rapid distribution of vasopressin at each cell surface, the cell with the most agonist-binding sites is the first to respond. $InsP_3$ is then produced rapidly in this cell, leading to Ca^{2+} release from internal stores. The second cell to respond, which has slightly fewer hormone receptors, produces $InsP_3$ more slowly, and reaches the threshold for Ca^{2+} release more slowly than the first cell (Figure 7). The sequential succession of $[Ca^{2+}]_i$ increases results in an apparent unidirectional intercellular Ca^{2+} wave. Cell to cell coupling via gap junctions should allow the diffusion of an intercellular messenger, the nature of which is unknown, favoring the coordination and probably leading to an acceleration of cellular responses within the system (Saez *et al*., 1989; Nathanson and Burgstahler, 1992; Combettes *et al*., 1994; Tordjmann *et al*., 1997a). The estimated magnitude of cell to cell differences in the number of vasopressin-binding sites ranges from 5 to 10% (i.e. ~10 000 sites) between adjacent hepatocytes. This range of values is, from a theoretical point of view, compatible with observed cell to cell differences in latency during sequential Ca^{2+} responses in multiplets (G.Dupont, T.Tordjmann, C.Clair, M.Claret and L.Combettes, in preparation). Such receptor-oriented intercellular Ca^{2+} waves have never been reported and may be compared with directional intracellular Ca^{2+} waves driven by an $InsP_3$ receptor sensitivity gradient from the basal to the apical cell domain, which have been described in pancreatic acinar cells (Kasai *et al*., 1993; Thorn *et al*., 1993). Directional intercellular Ca^{2+} waves may be of key physiological importance, transforming a total signal input to numerous cells into an organized, oriented intercellular information. In the liver, in which multiple functions—especially bile secretion and canalicular contraction—are performed along the axis of the hepatocyte plate, the orientating of intercellular communication by cellular sensitivity gradients may be fundamental. Cell to cell communication in other epithelia may also be regulated finely by receptor-oriented intercellular Ca^{2+} waves.

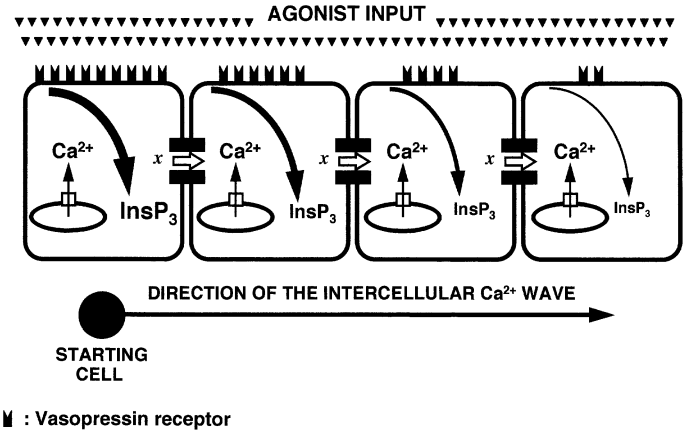

Fig. 7. A model for the sequence of agonist-induced $[Ca^{2+}]_i$ rises in multicellular rat hepatocyte systems. A gradient in hormone receptor density results in a sensitivity gradient along the connected cells. The cell with the most agonist receptors produces $InsP_3$ fastest, resulting in Ca^{2+} release from internal stores with the shortest delay. The adjacent cell, with slightly fewer hormone receptors, produces $InsP_3$ slightly more slowly, resulting in a delayed Ca^{2+} release as compared with the first responding cell. Applied to the whole system, this model leads to the creation of an apparent intercellular Ca^{2+} wave, the starting point and orientation of which are imposed by the agonist sensitivity gradient. Cell to cell coupling via gap junctions should allow the diffusion of an intercellular messenger (*x*) whose nature is unknown, possibly $InsP_3$, leading to the coordination and probably the acceleration of cellular responses within the system of connected cells (Combettes *et al*., 1994; Tordjmann *et al*., 1997a).

Materials and methods

Isolation of cells

Collagenase perfusion. Total liver parenchymal cells were isolated by the two-step collagenase perfusion technique (Seglen, 1976), from fed adult female Wistar rats (CERJ, Le Genest, France) weighing 200–250 g, as previously described (Combettes *et al*., 1994). Cell viability, assessed by trypan blue exclusion, was consistently >96%.

Isolation of PP and PV cells from a single rat liver. The initial steps of the experimental procedure were essentially as for the conventional digitonin–collagenase technique (Quistorff, 1985; Tordjmann *et al*., 1996). Major modifications were made to the method such that PP and PV cell populations could be isolated from a single rat liver, as recently described (Tordjmann *et al*., 1997b). Briefly, after clamping of the median lobe vessels, a digitonin solution (4 mg/ml) was infused via the portal vein at a rate of 10 ml/min at room temperature until a regularly scattered PP pattern of decoloration was obtained. Digitonin was washed out by perfusion with saline continuously gassed with O_2/CO_2 (19:1), in the retrograde direction (through the caudal vena cava) at a rate of 20 ml/min for 2 min at room temperature. The clamp was removed then placed on the left lateral lobe vessels, and digitonin was infused via the caudal vena cava at a rate of 10 ml/min until a regularly scattered PV discoloration pattern was observed. Digitonin was then washed out by perfusion with a Ca^{2+}-free solution, continuously gassed with O_2/CO_2 (19:1) at pH 7.4, in the antegrade direction via the portal vein at a rate of 20 ml/min for 10 min at 37°C. The subsequent steps of the isolation procedure were identical to those for the conventional dispersion of rat liver cells (Seglen, 1976), except that median and left lateral lobes were separated after collagenase perfusion, and then processed concurrently. Cell viability, assessed by trypan blue exclusion, was consistently >96%.

Enzyme assays

The enrichment of cell suspensions in PP and PV hepatocytes was monitored by measurements of glutamine synthetase (GS) (PV marker) and alanine aminotransferase (ALT) (PP marker). GS activity was determined as previously described (Rowe *et al*., 1986), and ALT was assessed using a commercial kit (Sigma Chemical Corporation, St Louis, MO) as described by the manufacturer. The enzyme activities were consistent with the published data and showed a significant enrichment of each cell population with either PP or PV hepatocytes (data not shown), as reported previously (Jungermann and Kietzmann, 1996; Tordjmann *et al*., 1997b). $InsP_3$ 5-phosphatase activity was determined as previously described (De Smedt *et al*., 1997).

Determination of $[Ca^{2+}]_i$ changes in hepatocytes

$[Ca^{2+}]_i$ imaging on dye-loaded hepatocyte multiplets. Loading of hepatocytes with fura2 or fluo3, short primary cultures and Ca^{2+} imaging were essentially as described by Combettes *et al*. (1994). The cells were dye-loaded on coverslips, then placed on the stage of an Axiovert 35 epifluorescence microscope and superfused with continually renewed modified Eagle's medium. Perfusing solutions (saline and agonists) converged on the chamber by inlet tubes, at 34°C. Microinjection of fura2 was performed using an Eppendorf microinjector (5242), as described previously (Tordjmann *et al*., 1997a).

Flash photolysis of microinjected caged $InsP_3$. Inactive photolabile 'caged' $InsP_3$, the P-4 or the P-5 1-(2-nitrophenyl) ethyl esters of $InsP_3$ (Walker *et al*., 1989) were introduced into fluo3-loaded multiplets using an Eppendorf microinjector (5242). Fura2 (5 mM in the pipet) was microinjected together with caged $InsP_3$. Cells were then allowed to recover for at least 10 min during which fura2 (and caged $InsP_3$, Carter *et al*., 1996) diffused from the microinjected cell to the other connected hepatocytes. $InsP_3$ was released by photolysis from caged $InsP_3$, by a 1 ms pulse from a short arc xenon flashlamp focused to produce an image 2–3 mm across at the cell as described previously (Ogden *et al*., 1990). The spot of light produced by the flashlamp thus released $InsP_3$ in all the hepatocytes into which caged $InsP_3$ could diffuse via gap junctions (Carter *et al*., 1996). The subsequent changes in fluo 3 fluorescence (λ_{ex} = 480 nm, λ_{em} = 520 nm) due to intracellular calcium mobilization were recorded, as previously described (Combettes *et al*., 1994).

Spectrofluorimetry on quin2-loaded hepatocyte suspensions. Calcium movements in PP and PV hepatocyte populations were measured as previously described (Tordjmann *et al*., 1997b). Changes in the fluorescence of quin2-loaded hepatocytes (λ_{ex} = 340 nm, λ_{em} =492 nm) were calibrated for Ca^{2+} as previously described (Tordjmann *et al*., 1996).

$InsP_3$ mass measurements

An aliquot of 10^6 cells (PP or PV) was incubated in 1 ml of saline at 37°C. At 10 and 20 s after treatment with the agonist (10 nM vasopressin) or with H_2O (control samples), cells were mixed immediately with an ice-cold perchloric acid solution and incubated on ice for $InsP_3$ extraction as described by Chaliss (1995). $InsP_3$ was determined in the neutralized extracts by the radioreceptor binding assay (Amersham, Biotrack™ assay system). In each experiment, $InsP_3$ accumulation reached a maximum 10 or 20 s after agonist stimulation. Paired ratios between PP and PV cells were calculated for each animal. Proteins were determined according to Lowry *et al.* (1951) for each sample.

Membrane preparation

Crude membranes were obtained from PP and PV cells, as described for total hepatocyte populations (Guillon *et al*., 1986). Cells were washed twice in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} at pH 7.4, then suspended in an ice-cold hypotonic homogenization buffer containing 10 mM Tris–HCl pH 7.4, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (2×10^7 cells/ml). The cells were incubated in the buffer for 30 min, then disrupted at 4°C by 15 strokes in a Potter homogenizer (Teflon/glass, tight piston) and centrifuged (10 min at 4°C at 100 *g*). The supernatant was collected and centrifuged

for 15 min at 21 000 *g* at 4°C. The final pellet was suspended at a protein concentration of 3–5 mg/ml in the homogenization buffer. Membranes were stored at –20°C and thawed just before use.

Immunoblotting

The $G_{\alpha q}$, $G_{\alpha 11}$ and G_{β} G protein subunits and PLC-β3 present in PP and PV cell membranes were resolved by electrophoresis as described elsewhere (Ibarondo *et al*., 1995), and were quantified by immunoblotting. The antibodies directed against $G_{\alpha q}$ and $G_{\alpha 11}$, and against G_{β} were specific and their properties have been described previously (Ibarondo *et al*., 1995). Polyclonal antibodies directed against PLC-β3 were purchased from Santa Cruz Biotechnology Inc. (California). Monoclonal antibodies directed against actin were purchased from Amersham. Peroxidase-conjugated secondary antibodies were from Pierce Immunological Laboratories. Proteins (30–50 μg) in crude membrane preparations from PP or PV hepatocytes were solubilized and resolved by SDS–PAGE and transferred onto nitrocellulose. A chemiluminescence kit from Amersham was used for antibody detection. The relative intensities of the αq/α11, β and PLC-β3 bands were normalized using the signal obtained for actin, for the same sample.

[^{125}I]HO-LVA binding assays

[^{125}I]HO-LVA (2000 Ci/mmol) was prepared as previously described (Barberis *et al*., 1995). This iodinated vasopressin analog labeled rat liver V1a vasopressin receptor with a high affinity (K_d = 8 pM). The binding protocol used has been described elsewhere (Grazzini *et al*., 1996). Briefly, hepatocyte membranes (1–2 μg protein per assay) were incubated in a medium containing 50 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 1 mg/ml bovine serum albumin (BSA), 0.5 mg/ml bacitracin, 0.01 mg/ml leupeptin, 10^{-4} M PMSF and 5–200 pM [^{125}I]HO-LVA (total binding). The reaction was initiated by adding the membranes which were then incubated in the solution for 60 min at 37°C. The reaction was stopped by adding 3 ml of ice-cold filtration buffer (10 mM Tris–HCl pH 7.4, 1 mM $MgCl_2$) and immediately filtering through GF/C Whatman glass filters pre-soaked for at least 2 h in 10 mg/ml BSA. Filters were then washed three times with 3 ml of filtration buffer and counted for radioactivity. All determinations were performed in triplicate. Non-specific and specific binding were determined as described previously (Grazzini *et al*., 1996). For competition experiments, the membranes were incubated with 20 pM [^{125}I]HO-LVA in the presence or absence (control) of increasing amounts of unlabeled vasopressin analogs as described previously (Grazzini *et al*., 1996).

RNase protection assays

We carried out direct solution hybridization of GuSCN-solubilized hepatocytes, as previously described (Kaabache *et al*., 1995). The V1a riboprobe was prepared from the full-length V1a rat cDNA (Morel *et al*., 1992). A *Kpn*I–*Pvu*II fragment from the V1a rat cDNA (bases 891–1286) was subcloned into pBluescript II SK+ phagemid (Stratagene, USA). The GAPDH riboprobe was prepared from an 85 bp *Xba*I–*Apa*I fragment from rat GAPDH cDNA (Fort *et al*., 1985). Equivalent amounts of PP and PV hepatocyte lysates, as assessed by DNA content determined as described by Labarca and Paigen (1980), were hybridized with the labeled V1a vasopressin receptor cRNA probe and, as a control, with the GAPDH cRNA probe. The samples were treated with RNase, extracted and precipitated. They were then suspended in loading buffer and subjected to a denaturing polyacrylamide gel electrophoresis to separate the protected RNA fragments. The radioactive protected bands were quantified by direct counting of the gel with Instant Imager (Packard, Groningen, The Netherlands), and the signal ratio between protected fragments (V1a mRNA/GAPDH mRNA) was calculated.

Statistical analysis

Student's *t*-test was used to compare sample means with paired controls. Results are expressed as means ± SEM. *P*-values of $\leqslant 0.05$ were considered to be statistically significant.

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