Strongly decreased gap junctional permeability to inositol 1,4,5-trisphosphate in connexin32 deficient hepatocytes

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Abstract Livers from connexin32 (Cx32) deficient mice have been shown to be defective in hormonally induced glucose mobilization. In order to determine whether this effect is due to decreased diffusion of the second messenger inositol 1,4,5-trisphosphate (IP_3) between hepatocytes, we injected iontophoretically different amounts of IP_3 in Fura-2 loaded hepatocyte doublets (i.e. cell pairs) from wild type or Cx32 deficient mice. Whereas 84% of wild type hepatocytes showed an intercellular Ca^{2+} wave spreading from the injected cell to the neighboring cell, only 25% of Cx32 deficient hepatocyte doublets did so. The amount of IP_3 necessary to induce an intercellular Ca^{2+} wave in Cx32 deficient hepatocyte doublets was estimated to be about 25-fold higher than in wild type doublets. This confirms the notion that the low hormonally or electrically induced glucose mobilization found in Cx32 deficient livers relative to wild type livers is due to largely hindered diffusion of IP_3 between Cx32 deficient hepatocytes.

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Key words: Gap junction channel; Connexin26; Intercellular communication; Hepatocyte doublet; Connexin null mutation; Transgenic mouse

1. Introduction

The cells of secretory exocrine and endocrine glands are known to be extensively coupled via gap junction proteins [1]. Liver parenchymal cells express the gap junction subunit proteins connexin32 (Cx32) and connexin26 (Cx26), which assemble into homotypic Cx32 and Cx26 gap junction channels, as well as heterotypic Cx26/Cx32 channels [2,3]. The analysis of Cx32 deficient mice showed that Cx32 containing gap junction channels are required for efficient mobilization of glucose from hepatic glycogen stores triggered by electrical stimulation of sympathetic liver nerves [4]. More recently, Stümpel et al. [5] found that even at non-saturating concentrations of the glycogenolytic hormone norepinephrine, used for perfusion of whole livers, less glucose was mobilized from glycogen stores of Cx32 deficient mice compared to wild type mice. In liver parenchymal cells, norepinephrine is known to stimulate glycogenolysis via the synthesis of inositol 1,4,5-trisphosphate (IP_3) and subsequent release of intracellular Ca^{2+} [6]. The Ca^{2+} response to hormones acting on the inositol phosphate pathway has been shown to propagate in a wave-like fashion from the periportal to the perivenous site of the liver lobule [7]. The mechanism of this wave propagation could not be determined in detail, although it was shown by reversed perfusion that no paracrine mechanism appeared to be involved. Previously, diffusion of IP_3 through hepatic gap junctions had been demonstrated [8]. Thus, it is likely that gap junctional coupling contributes significantly to the propagation and coordination of a hepatic Ca^{2+} wave.

Furthermore, the threshold concentration for Ca^{2+} mobilization in hepatocytes was shown to be dependent on the location of the cell in the liver lobule [9]. Intercellular IP_3 diffusion through gap junctions could contribute to homeostasis of hormone sensitivity and therefore allow more effectively the mobilization of glucose from hepatic stores at low stimulus concentrations.

In order to elucidate to what extent hepatocytes from Cx32 deficient mice display decreased gap junctional permeability to IP_3, we performed IP_3 injections into hepatocyte doublets (i.e. hepatocyte pairs) acutely isolated from wild type or Cx32 deficient mice. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored during injection using the fluorescent Ca^{2+} indicator dye Fura-2 [10].

Our results show that an IP_3 triggered Ca^{2+} wave can spread between wild type mouse hepatocytes at a 25-fold lower IP_3 concentration than between Cx32 deficient hepatocytes. Thus, Cx32 containing hepatic gap junction channels are of high importance for the transmission of the second messenger IP3 between parenchymal liver cells.

2. Materials and methods

Hepatocyte doublets were prepared following the method described by Seglen [11] and Graf et al. [12]. Briefly, male and female wild type (F1 generation of C57BL/6 and Sv129 strains) and Cx32 deficient mice [4] were anesthetized, wiped off with 70% ethanol and placed on the back under a surgical microscope. The abdominal cavity was opened and the gut was fixed outside the abdomen, in order to expose the portal vein. A thin polyethylene tubing was placed near the portal vein while slowly pouring prewarmed perfusion buffer containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM EGTA, 10 mM glucose, pH 7.4. After the portal vein had been clamped distally, the tubing was inserted into the vein and fixed with two ligatures. The vena cava inferior was opened to let the perfusion buffer pour into the abdominal cavity. Afterwards, the heart was exposed and the right ventricle was cut to enable outflow of the perfusion buffer to the cardiac site of the liver. After 5 min, the perfusion buffer was changed to digestive buffer containing 0.1% collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA), 140 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, pH 7.4. Digestion was allowed to proceed during 6–8 min with a flow rate of 5 ml/min, then the liver lobules were excised, chopped up with scissors and transferred into 4°C cold RPMI medium (Sigma, Deisenhofen, Germany) supplemented with 10% fetal calf serum (Life Technologies Inc., Rockville, MD, USA). Cells were suspended by gentle shaking, filtered through a 100 μm mesh and centrifuged twice at 4°C with 200 rpm for 10 min. Cells were plated onto coverslips coated with collagen

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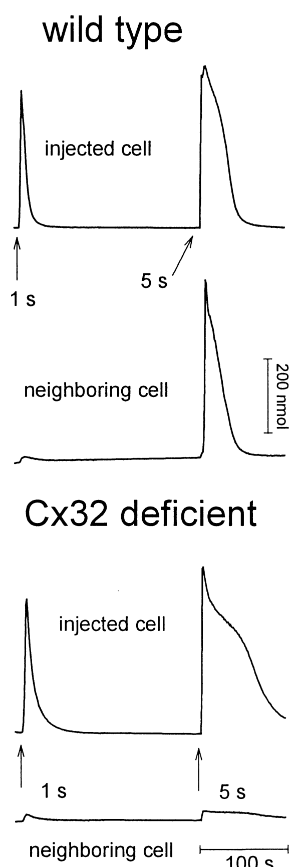

Fig. 1. IP_3 injection into Fura-2 loaded wild type or Cx32 deficient hepatocyte doublets. First and second traces: IP_3 was injected for 1 s with 1 nA injection current. The injected cell (first trace) showed a transient Ca^{2+} release, which only very slightly affected the neighboring cell (second trace). The second injection of 5 s duration resulted in a stronger and longer lasting Ca^{2+} release in the injected cell and a transient increase in intracellular Ca^{2+} was also observed in the neighboring cell. IP_3 injection into Cx32 deficient hepatocyte doublets (traces 3 and 4) showed the same pattern in the injected cell, but only minor Ca^{2+} elevation was observed for both injections in the neighboring cell. IP_3 injections were carried out in Ca^{2+} free extracellular medium. Pipette concentration of IP_3 was 1 mM.

gel, which had been glued on the bottom of drilled culture dishes. Cells were incubated for at least 90 min at 37°C under a humid atmosphere of 10% CO_2 before they were equilibrated with 5 μM Fura-2 (Molecular Probes, Eugene, OR, USA) in HEPES buffered solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose for 1 h at 37°C. After loading, the cells were washed twice with Ca^{2+} free HEPES buffered salt solution and placed onto the stage of an inverse microscope (Zeiss, Oberkochen, Germany). All experiments were performed at room temperature.

Only hepatocyte doublets with extended contact area were used for injections. Intracellular Ca^{2+} concentration was monitored with a frequency of 1 Hz during 99 s by recording fluorescent frames at alternating 340 and 380 nm light excitation (Ca-Imager System by T.I.L.L. Photonics, Planegg, Germany). IP_3 was injected iontophoretically, 5 s after the beginning of the measurement, with a sharp microelectrode filled with 100 mM KCl and 10 mM HEPES, pH 7.2. The amount of IP_3 injected was increased using higher IP_3 pipette concentrations and higher injection currents indicated in the table in Fig. 3. Cells were injected only once, except when noted otherwise. Five to six injections were performed on each coverslip and the time of one experiment never exceeded 45 min. During this time cells remained attached to the collagen gel and no spontaneous change in intracellular Ca^{2+} concentration was observed. All experiments were performed within 8 h after plating. Ca^{2+} waves were considered to be transmitted between cells when the intracellular Ca^{2+} concentration in the neighboring cell reached at least 10% of the peak Ca^{2+} concentration in the injected cell.

3. Results and discussion

Fig. 1 shows a representative example of injections of different amounts of IP_3 into one cell of a wild type or Cx32 deficient hepatocyte doublet. The first injection, performed with a pipette concentration of 1 mM IP_3 and 1 nA current over a period of 1 s, triggered a Ca^{2+} release in the injected hepatocyte from wild type and Cx32 deficient mice but did not result in Ca^{2+} wave propagation to the neighboring hepatocyte. The subsequent injection for 5 s elicited a longer lasting Ca^{2+} release which was immediately followed by a transient Ca^{2+} elevation in the neighboring cell of the wild type hepatocyte doublet but not of the Cx32 deficient cell pair.

In order to investigate whether IP_3 or Ca^{2+} ions are responsible for Ca^{2+} wave propagation into the neighboring cell in wild type hepatocyte doublets, we first induced an intercellular Ca^{2+} wave by IP_3 injection (see the table in Fig. 3, parameter C) to ensure that cells were well coupled (Fig. 2). Then we discharged the Ca^{2+} stores of the injected cell by inducing several times a non-propagating Ca^{2+} release with short injections of 1 s duration. Since injections were performed in Ca^{2+} free extracellular buffer, reloading of the Ca^{2+} stores was prevented. Therefore, subsequent injection of a higher amount of IP_3 only resulted in a minor elevation of $[Ca^{2+}]_i$ in the injected cell. In contrast, the neighboring cell showed a gradually increasing $[Ca^{2+}]_i$ during the first injections followed by a peak, in response to the last and stronger IP_3 injection. The peak concentration of Ca^{2+} in the neighboring cell clearly exceeded the $[Ca^{2+}]_i$ in the injected cell, which indicates that the Ca^{2+} increase in the neighboring cell was not the result of a Ca^{2+} diffusion. We conclude that Ca^{2+} diffusion is only of minor influence on intercellular propaga-

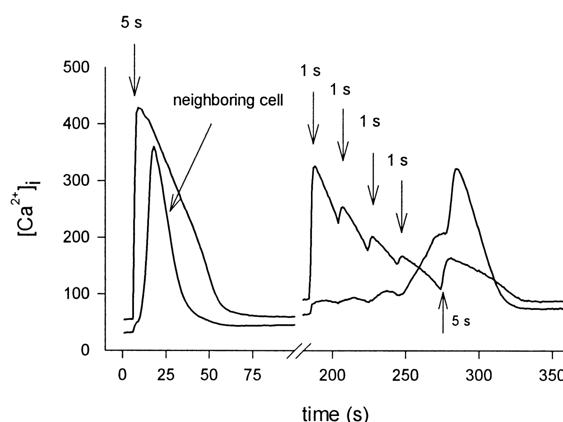

Fig. 2. IP_3 was injected for 5 s into one cell of a wild type hepatocyte doublet in order to induce an intercellular Ca^{2+} wave (0–100 s). Subsequently, four IP_3 injections of 1 s duration were performed on the same cell, followed again by a 5 s injection. The response of the injected cell to the short injections successively decreased, whereas the intracellular Ca^{2+} concentration in the neighboring cell increased. The following injection of 5 s resulted in only a minor Ca^{2+} increase in the injected cell, which was about 3–4-fold exceeded by the Ca^{2+} increase in the neighboring cell. Injections were carried out with 1 nA injection current and 1 mM IP_3 pipette concentration in Ca^{2+} free extracellular medium.

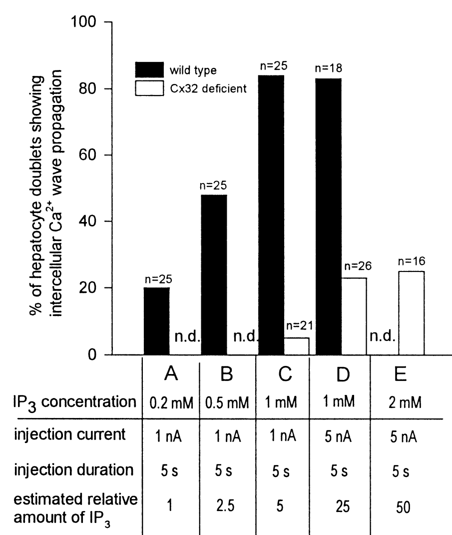

Fig. 3. Percentage of wild type or Cx32 deficient hepatocyte doublets showing intercellular Ca^{2+} wave propagation in response to injections of different amounts of IP_3. The relative amount of IP_3 injected was varied by increasing the injection current and the pipette concentration of IP_3 as indicated in the table. Black bars, wild type hepatocyte doublets; white bars, Cx32 deficient hepatocyte doublets. n: number of hepatocyte doublets probed with that specific injection parameter. n.d.: not determined.

tion of the Ca^{2+} wave and that the major source of the Ca^{2+} elevation in the neighboring cell was its own Ca^{2+} stores. Hence, the intercellular propagation of the Ca^{2+} wave is mediated by IP_3.

In order to evaluate the difference in cell-to-cell coupling between wild type and Cx32 deficient hepatocyte doublets, we varied the amount of IP_3 injected by changing the IP_3 pipette concentration and the injection current (see the table in Fig. 3). The results of these IP_3 injections are summarized in Fig. 3. The black bars represent injections in wild type hepatocyte doublets, the white bars represent the results obtained with Cx32 deficient hepatocyte doublets.

Injections into wild type hepatocyte doublets with the injection parameters C and D (table in Fig. 3) resulted in the propagation of an intercellular Ca^{2+} wave in 21 of 25 (84%) and in 15 of 18 cases (83%) respectively. In contrast, in Cx32 deficient hepatocyte doublets, intercellular Ca^{2+} wave propagation could only be observed in one of 21 injections with parameter C (5%) and in five of 26 injections (23%) with parameter D (see the table in Fig. 3). The percentage of Cx32 deficient hepatocyte doublets showing Ca^{2+} wave propagation could not be augmented even when the IP_3 pipette concentration was increased to 2 mM (table in Fig. 3, parameter E, four of 16 (25%) hepatocyte doublets). A further augmentation of the IP_3 concentration in the pipette was not possible due to artefacts in the injected cell caused by IP_3 leaking out of the injection pipette.

In order to determine the threshold concentration for Ca^{2+} wave propagation for wild type hepatocyte doublets, we lowered the pipette concentration of IP_3 to 0.5 and 0.2 mM (see table in Fig. 3, parameters B and A, respectively). Twelve of 25 injections (48%) with parameter B and five of 25 injections (20%) with parameter A led to intercellular transfer of a Ca^{2+} wave. Assuming a linear relationship between the amount of IP_3 injected and the IP_3 pipette concentration as well as the injection current, we conclude that the amount of IP_3 necessary to trigger an intercellular Ca^{2+} wave in Cx32 deficient hepatocyte doublets is about 25 times higher than in wild type hepatocyte doublets.

4. Conclusions

We have shown that in about 75% of Cx32 deficient hepatocyte doublets gap junctional cell coupling is not sufficient to trigger a IP_3 based intercellular Ca^{2+} wave even at very high IP_3 concentrations, which are very unlikely to be reached in hepatocytes responding to hormonal stimulation in the intact liver (omitted in corrected version). This result is consistent with the recent finding of Valiunas et al. [3], who demonstrated with double whole cell patch clamp recordings 10-fold lower gap junctional coupling in Cx32 deficient hepatocyte doublets than in wild type doublets under the same conditions as used in this work. These authors determined in Cx32 deficient hepatocytes a mean gap junctional conductivity of 1.7 nS, which may be too low to allow detectable intercellular diffusion of IP_3. Our results confirm the notion that the decreased glucose mobilization found in Cx32 deficient livers relative to wild type livers is due to obstructed IP_3 diffusion between hepatocytes.

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