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Hydrogen Peroxide Inhibits Gap Junctional Coupling and Modulates Intracellular Free Calcium in Cochlear Hensen Cells

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Abstract. The double whole-cell patch-clamp configuration was applied to analyze gap junctional conductance (G_j) of isolated pairs of cochlear supporting Hensen cells of guinea pig under control conditions and in the presence of hydrogen peroxide (H_2O_2). Under control conditions, the dependence of G_j on transjunctional voltage (V_j) appeared to vary between different cell pairs with a maximum value of about 40 nS at V_j close to 0 mV. The voltage dependence and the maximum amplitude of G_j stayed constant for at least 2 hr. Addition of H_2O_2 to the bath at concentrations above 0.08 mM caused a significant decrease of G_j, but the membrane potential of about −30 mV was not affected. In parallel, intracellular free calcium ($[Ca^{2+}]_i$) was followed using fura-2. At 0.8 mM H_2O_2, a sustained increase of $[Ca^{2+}]_i$ was observed, while 0.08 mM H_2O_2 evoked an oscillating-like behavior of $[Ca^{2+}]_i$. We propose that the H_2O_2-evoked inhibition of gap junctional coupling of Hensen cells is closely related to pathophysiological conditions such as noise-induced hearing loss, aminoglycoside-related ototoxicity and presbycusis, which are known to be associated with production of free radicals.

Key words: Cochlea — Hensen cells — Ototoxicity — Free radicals — Gap junctions — Double whole-cell patch-clamp

Introduction

Direct intercellular communication is mediated by gap junctions that are composed of cell-to-cell channels between adjoining animal cells. One cell-to-cell channel consists of two connexons (hemichannels) that are formed by six subunits (connexins) each. So far, 13 different connexins have been cloned in rodents and classified according to their theoretical molecular weight (cf. Bruzzone et al., 1996). In most cell systems, more than one gene of connexin (Cx) is expressed. This raises, besides homomeric connexons and homotypic gap junction channels, the possibility of different associations of connexins to heteromeric connexons and heterotypic gap junctional channels composed of more than one type of connexin (Sosinsky, 1995). The physiological functions of gap junctions have been described, which include ionic homeostasis, cellular growth and differentiation, but they are not well-established for specific cell systems (Bruzzone et al., 1996).

Intense gap junctional coupling between Hensen cells in the organ of Corti has been documented by electron microscopical studies (Iurato et al., 1976), by dye coupling (Santos-Sacchi, 1986a), on the basis of capacity measurements (Santos-Sacchi, 1991), dual voltage-clamp (Zhao & Santos-Sacchi, 2000) and by immunohistochemical studies (Kikuchi et al., 1995; Forge, Becker & Evans, 1997; Lautermann et al., 1998). Cx26/Cx30 to a large extent and Cx32/Cx43 to a lesser extent (Kikuchi et al., 1995; Forge et al., 1997; Lautermann et al., 1998) have been localized between cochlear supporting cells.

The proven, significant gap junctional coupling of supporting cochlear cells has been related to their proposed glia-like function, i.e., to providing the cellular basis for ionic homeostasis of sensory cells (Santos-Sacchi, 1985; Oesterle & Dallos, 1990; Santos-Sacchi, 2000). The neuronal activity of outer hair cells (OHC) requires an efficient potassium buffering to enable a fast repolarization in the range of milliseconds after post-

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stimulatory potassium release (Johnstone et al., 1989). This so-called "K-sink-function" has been attributed to supporting cells (Oesterle & Dallos, 1990; Santos-Sacchi, 2000). In Hensen cells, gap junctional coupling has been studied as a function of mechanical stress (Zhao & Santos-Sacchi, 1998), external pH, Ca^{2+} (Sato & Santos-Sacchi, 1994; Sato et al., 1998), CO_2, octanol (Santos-Sacchi, 1991), and temperature (Santos-Sacchi, 1986b).

The present paper explores the role of reactive oxygen species on gap junctional coupling. The effects of reactive oxygen species and free radicals in particular on cellular structures and biochemical pathways are variable. They are known to cause multiple cellular damages, i.e., lipid peroxidation, DNA damage, direct protein interaction, dysfunction of ATP synthesis and increase of intracellular Ca^{2+} (Halliwell & Gutteridge, 1984, cf. Halliwell, 1992, Lander, 1997). Studies of the effect of the model substance hydrogen peroxide (H_2O_2) on gap junctional coupling appeared to yield controversial results. In a Syrian hamster embryonic cell line, an increase of gap junctional coupling has been observed (Mikalsen & Sanner, 1994), whereas in hepatocytes gap junctional uncoupling followed application of free radicals (Guo, Ohno & Takanaka, 1993).

The generation of oxidizing agents and free radicals in the inner ear are related to pathophysiological events such as noise-induced hearing loss (NIHL) (Yamane et al., 1995), ototoxic drugs (Takayama et al., 1997) and presbycusis (Lautermann et al., 1997; Ohlemiller, Wright & Dugan, 1999). Moreover, externally applied hydrogen peroxide induces morphological changes and bleb formation in OHC in vitro (Clerici, DiMartino & Prasad, 1995).

So far, the effect of oxidative stress on gap junctional coupling of cochlear supporting (Hensen) cells has not yet been studied in detail. In the present study, a hydrogen peroxide (H_2O_2) concentration-dependent decrease of gap junctional conductance in isolated pairs of Hensen cells will be revealed using the double whole-cell patch-clamp technique. In addition, the responsiveness of intracellular free calcium to H_2O_2 was recorded. These results may contribute to a better understanding of the mechanisms of OHC cellular damage under oxidative stress and may thus provide deeper insight into the regulation of gap junctional coupling in the organ of Corti.

Materials and Methods

Cell Preparation

Pigmented guinea pigs, weighing 250–400g and with a positive Preyer's reflex, were killed by a lethal dose of phenobarbital. The bullae tympanica were removed and prepared as described previously (Ernst et al., 1994). The bony shell of the cochlea was gently removed, the organ of Corti was dissected and incubated in Hanks' balanced salt solution (HBSS). Hensen cells were separated from other cells on the basis of their characteristic lipid inclusions and settled on Cell Tak-coated coverslips (Becton Dickinson). The cell suspension was digested for 30 min with Collagenase II 500 U/ml in nominally Ca^{2+}-free HBSS. The cell suspension was washed with HBSS, transferred into the perfusion chamber and allowed to settle for at least 45 min. The perfusion chamber was mounted on an Axiovert 35 (Zeiss) microscope.

Solutions

HBSS (Sigma, St. Louis, MO) was used as extracellular solution. The control pipette filling solution contained in mM: 135 K-gluconat, 5 NaCl, 5 Na_2ATP, 5 EGTA, 10 HEPES, 0.1 cAMP, 5 $MgCl_2$: (free Mg^{2+} about 1 mM), 3 $CaCl_2$ (free Ca^{2+} about 0.1 μM). For spectrofluorometric studies of cytoplasmic free calcium the pipette filling solution contained in mM: 120 KCl, 10 glucose, 5 Na_2ATP, 0.5 EGTA, 10 HEPES, 0.1 cAMP, 1.5 $MgCl_2$, 0.58 $CaCI_2$ and 200 μM fura-2 pentapotassium salt (Molecular Probes, Eugene, OR). Pipette solutions were adjusted to pH 7.2 using KOH and to an osmotic pressure of 295–305 mOsmol. Hydrogen peroxide was purchased from Sigma-Aldrich. All experiments were carried out at room temperature (20–22°C).

Pipettes

Pipettes were pulled with a two-stage puller (Narashige) using borosilicate glass. The pipettes had resistances of 2–5 MΩ when filled with the pipette filling solutions.

Electrophysiological Recording

The double whole-cell patch clamp configuration was established according to Neyton and Trautmann (1984) (cf. Ngezahayo & Kolb, 1994). Series resistances of 10–20 MΩ were measured and the cell membrane resistance varied between 200 MΩ and 2 GΩ. The series and membrane resistance were derived from the corresponding current measurements by application of simultaneous pulses of −20 to +20 mV to each pipette in double whole-cell mode. The resistances were determined at the beginning of the experiment. They were measured several times during the recording and at the end of the experiment. For determination of the time course of G_j, one cell of a cell pair was voltage-clamped to a holding potential of −40 mV and test pulses of 50 msec were applied to −20 mV and −60 mV, respectively. For determination of the voltage dependence of G_j transjunctional potentials of 200 msec from −120 mV to 120 mV were applied from a holding potential of −40 mV. A stationary conductance was obtained about 10–15 min after starting the double whole-cell recording. H_2O_2-containing solutions were superfused thereafter. For calculation of G_j we used the formula of Donaldson et al. (1995). If cell 1 was voltage-pulsed and cell 2 held at a constant potential, G_j can be derived from the equation:

$$G_j = \frac{-\Delta I_{p2} \cdot \left(1 + \frac{R_{a2}}{R_{m2}}\right)}{\Delta V_{p1} - R_{a1} \cdot \Delta I_{p1} + R_{a2} \cdot \Delta I_{p2}}.$$

$R_{a1,2}$, input resistance of pipette *1* and *2;* R_{m2}, resistance of non-junctional membrane of cell *2;* ΔV_{P1}, the potential pulse applied to cell *1;* $I_{P1,2}$ the corresponding current recorded from cell *1* and *2*, respectively.

The data were amplified by two EPC 7 (List Medical, Darmstadt, Germany), the output signal was fed into an interface ITC 16 (Instru-

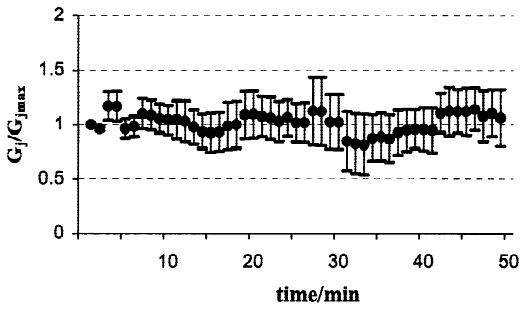

Fig. 1. Time dependence of gap junctional conductance G_j of pairs of isolated cochlear Hensen cells in double whole-cell configuration. For clearer presentation the data were normalized to the mean of G_j obtained during the first 5 min after establishing the double whole-cell configuration denoted as G_{jmax}. Control bath and pipette filling were used. Mean and SEM values of 5 different cell pairs are shown.

tech, MN) and analyzed with a Macintosh computer. For data evaluation Pulse Pulsefit 8.09 (HEKA Electronics, Lamprecht, Germany) was used. The data are given as mean ± SEM for n independent cell pairs.

Measurement of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was performed as previously described (Ngezahayo & Kolb, 1993). Isolated cell pairs in the whole-cell configuration were loaded with 200 μM fura-2-containing pipette filling solution. $[Ca^{2+}]_i$ was monitored with a photomultiplier-based system (Luigs & Neumann, Ratingen, Germany) and calculated from the fluorescence ratio (360/390 nm) as described by Grynkiewicz et al., 1985.

Results

Voltage Dependence of Gap Junctional Conductance

Hensen cells were identified within the isolated cell suspension according to their characteristic lipid vacuoles as described previously (Todt et al., 1999a). Using morphologically intact Hensen cell pairs, we established the double whole-cell patch clamp configuration. At control bath and pipette solution and a constantly applied holding potential of −40 mV, a gap junctional conductance G_j = 29.9 ± 2.6 nS (n = 31) was found, which did not significantly change during recording times of up to two hr (*see* Fig. 1 and also Todt et al., 1999a).

G_j varied differently with transjunctional voltage (V_j) for different cell pairs investigated. Fig. 2*a–d* shows typical examples of the different functional relationships of $G_j(V_j)$ observed within 14 analyzed cell pairs. In Fig. 2*a*, a near symmetrical, but less pronounced dependence of G_j on V_j is observed, with a maximum conductance at about V_j = 0 mV. As Fig. 2*a* indicates, G_j decreased by 15.3 ± 1.1% (n = 7) for a change of V_j from 0 mV to ±120 mV. In contrast, in Fig. 2*b* a rectifying behavior of G_j was found with a near constant value at V_j of 0 to −60 mV. From the maximum value, G_j decreased by 45.7 ± 4.3% at positive V_j (n = 4). In addition to these two most frequently observed voltage dependencies of G_j, we found further patterns as described in Fig. 2*c* and *d*, respectively. Comparison of Fig. 2*c* and *b* indicates that the polarity of the voltage sensitivity of G_j appears to be inversely related. From Fig. 2*c*, a monophasic decrease of G_j by about 60% can be observed with V_j changing from −120 mV to 120 mV. Whereas in Fig. 2*d*, G_j increased as V_j changed from −120 mV to 120 mV. The difference between the maximum and minimum conductance was about 35%.The observed voltage dependence was not correlated with the actual membrane potential. The latter was found to be −30.0 ± 1.2 mV (n = 108), independently of the observed voltage sensitivities.

Effect of H_2O_2 on Gap Junctional Conductance

The time-course of G_j was recorded in the absence (Fig. 1) and presence of H_2O_2 in the bath. Fig. 3*a* shows the mean time course of G_j at five different incubation conditions, respectively. After addition of H_2O_2, G_j decreased with increasing incubation time in a dose-dependent manner. For concentrations of H_2O_2 above 0.08 mM, G_j decreased significantly to the limit of resolution (*see* Material and Methods). The lag phase for a significant effect of H_2O_2 on G_j decreased from 20 min at 0.4 mM to less than 1 to 2 min at 8 mM H_2O_2, but the slope of the H_2O_2-induced decline showed no significant dependence within the applied concentration range of 0.4–8 mM H_2O_2. The effect of H_2O_2 on G_j appeared to be irreversible and was not mimicked by a corresponding change of nonjunctional membrane conductance. As Fig. 3*b* indicates, the current-voltage relationship of the non-junctional membrane current was not affected by application of H_2O_2.

H_2O_2-evoked Changes of Cytoplasmic Free Calcium

The effect of H_2O_2 on cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) was measured after loading the two cells of a cell pair with fura-2. In the absence of H_2O_2, $[Ca^{2+}]_i$ was found to be in the range of 100 to 200 nM as indicated by the time course of $[Ca^{2+}]_i$ prior to addition of H_2O_2 in Fig. 4. Superfusion of the cells with a bath solution containing H_2O_2 evoked a time- and concentration-dependent change of $[Ca^{2+}]_i$. Representative results are given in Fig. 4. At 0.08 mM H_2O_2, an oscillatory pattern of $[Ca^{2+}]_i$ was recorded from a single cell, with a periodicity of about 20 min (Fig. 4*a*). In parallel, gap junctional coupling was not significantly affected within recording times of up to one hr (Fig. 3*a*). Addition of 0.8 mM H_2O_2 to the bath induced a sustained increase of $[Ca^{2+}]_i$ reaching a maximum 25 min after addition of H_2O_2 (Fig. 4*b*), and in parallel, gap junctional coupling is inhibited (*compare* Fig. 3*a*). At each H_2O_2 concentration, four independent experiments have been performed

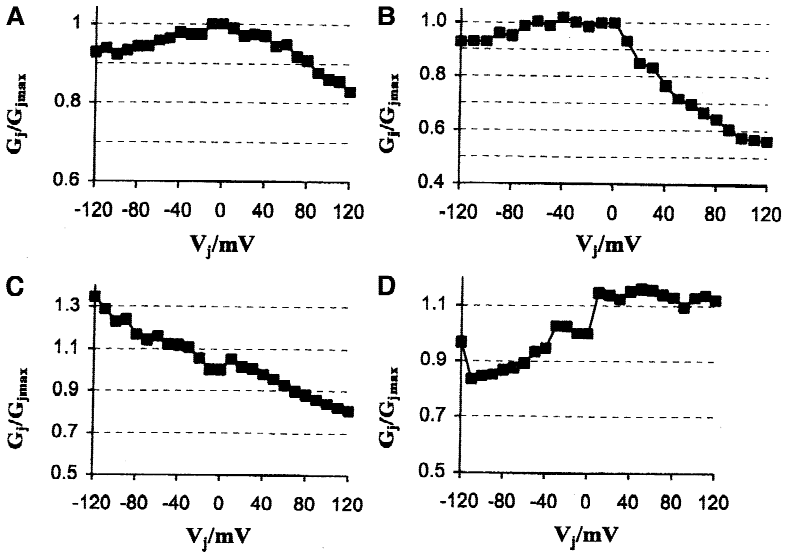

Fig. 2. Voltage dependence of gap junctional conductance G_j of Hensen cell pairs. *a, b, c, d* present the most commonly observed dependencies of G_j on transjunctional voltage V_j. Control bath- and pipette filling were used. For further explanation *see* text.

showing similar results as given in Fig. 4*a* and *b*, respectively.

Discussion

It is well established that K^+-ions are released from outer hair cells (OHC) of the cochlea into the surrounding extracellular space upon acoustic stimulation (Johnstone et al., 1989). This has led to the hypothesis that supporting cells around cochlear OHC play a role in the auditory neuroepithelium by taking up these K^+-ions released from stimulated OHC (Santos-Sacchi, 1985; Oesterle & Dallos, 1990; Santos-Sacchi, 2000). Moreover, they are considered to be responsible for K^+-sinking and metabolic cooperation whereby the gap junctional coupling enables the supporting cells to act as a functional syncytium of well-coupled cells (Santos-Sacchi, 1986a, 1991; Zhao & Santos-Sacchi, 2000; *see also* Kikuchi et al., 1995). However, direct experimental evidence for an involvement of cochlear supporting cells in potassium buffering is not available so far. Therefore, the physiological function of supporting cells as well as of their intense gap junctional coupling by cell-to-cell channels has not yet been fully elucidated. However, it was recently demonstrated by molecular biological and epidemiological studies that the subunits of cell-to-cell channels, the connexins, in their wildtype form are essential for normal cochlear function and, thus, for normal hearing (Kelsell et al., 1997; Denoyelle et al., 1998; Estivill et al., 1998; Xia et al., 1998, Grifa et al., 1999).

In a previous study we analyzed gap junctional coupling of cochlear Hensen cells after superfusion with the well-known ototoxic aminoglycoside gentamicin. We concluded that the aminoglycoside-induced ototoxicity could be related to an inhibition of gap junctional coupling of Hensen cells (Todt et al., 1999a). This finding might become important for a better understanding of the ototoxic mechanisms of gentamicin in the organ of Corti beyond the well-known effects on OHC (Ernst et al. 1984).

In the present paper, we investigated the influence of reactive oxygen species (ROS) and free radicals on gap junctional coupling of Hensen cells. Free radicals are known to be involved in various cochlear pathophysiological conditions, e.g., drug-related ototoxicity (Hirose, Hockenbery & Rubel, 1997; Takayama et al., 1997), noise-induced hearing loss (NIHL) (Ohlemiller et al., 1999; Yamane et al., 1995) and presbycusis (Lautermann et al., 1997). The inhibition of gap junctional coupling by free radicals was already proposed in hepatocytes (Spray et al., 1994). In contrast, an increase of gap junctional coupling was observed in Syrian hamster embryo cells (Mikalsen et al., 1994). In order to study the mechanism of ROS and free radical-induced changes of gap junctional coupling, isolated Hensen cells were exposed to one of the most common reactive oxygen species generated in vivo, i.e., hydrogen peroxide (H_2O_2). H_2O_2, a reactive oxygen, which by itself is poorly reactive, serves as a precursor of highly oxidizing tissue-damaging radicals. Besides that, H_2O_2 diffuses easily through lipid membranes so that it can affect cells distant from the place where it has been produced (Halliwell & Gutheridge, 1984; Halliwell, 1992). At concentrations of H_2O_2 above 0.08 mM the gap junctional conductance is reduced in a concentration-dependent manner. This concentration dependence is reflected by the sensitivity of the lag phase to H_2O_2 prior to a significant change of G_j. The lag phase decreased from 20 min at 0.4 mM H_2O_2 to less than 2 min at 8 mM H_2O_2. Surprisingly, the slope of H_2O_2-induced inhibition of gap junctional conductance appeared to be dose-independent (Fig. 3*a*). Living cells of aerobe organisms are confronted with endogenous and extracellular signal-induced pro-

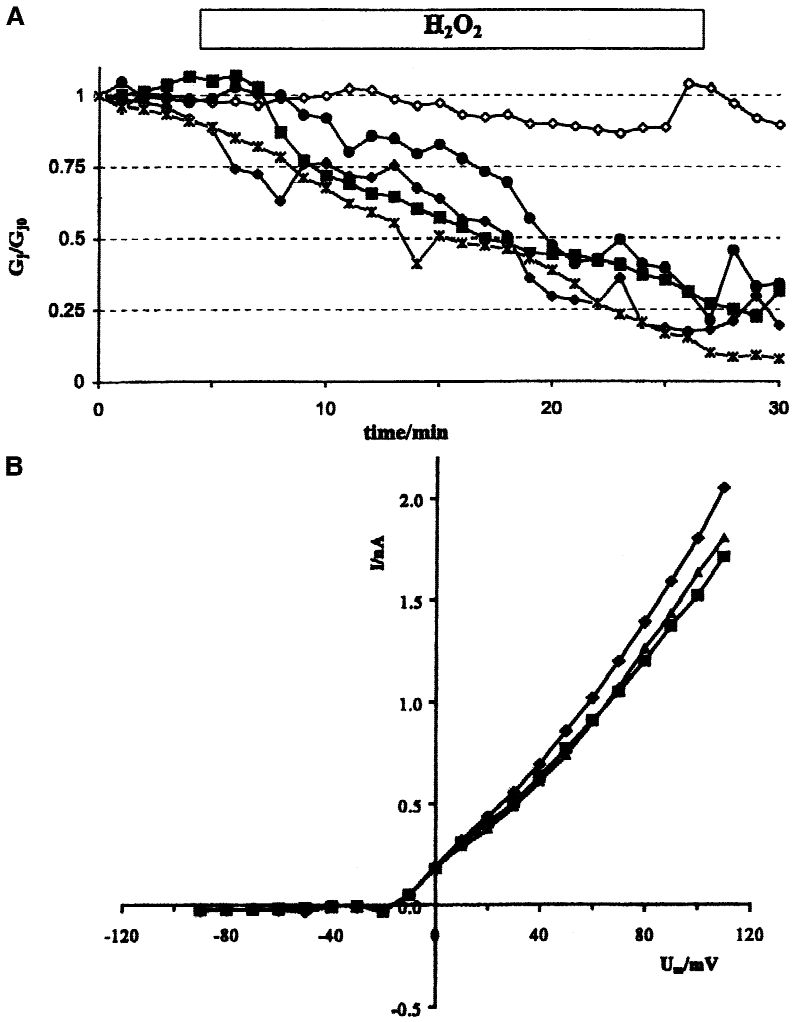

Fig. 3. Effect of H_2O_2 on the time course of gap junctional conductance (*a*) and the corresponding nonjunctional membrane current-voltage relationship (*b*). (*a*) Response of gap junctional conductance G_j of isolated cell pairs after addition of H_2O_2 to the bath. H_2O_2 was added to the bath 15 min after establishing the double whole-cell configuration at various concentrations (in mM): 0.08 (◇, $n = 4$); 0.4 (●, $n = 4$); 0.8 (◆, $n = 5$); 4.0 (∗, $n = 5$); 8.0 (■, $n = 6$). Data of each experiment were normalized to the mean of G_j obtained during the first 5 min after establishing the double whole-cell configuration in control bath denoted as G_{jo}. Mean values are presented, respectively. *n* denotes the number of independent cell pairs. (*b*) Nonjunctional membrane current versus membrane voltage of isolated Hensen cells at control bath (◆, $n = 4$), and 30 min after addition of 0.8 mM H_2O_2 (▲, $n = 3$) and 8 mM H_2O_2 (■, $n = 3$) to the bath. Control pipette filling was used throughout.

duction of ROS like H_2O_2, which can either protect the cells against pathological agents or damage the cells in different ways; yet, the initial target proteins of ROS remain largely unidentified (Gutteridge & Halliwell, 1984; Halliwell, 1992). To survive, the cells have developed efficient mechanisms to adapt to increased ROS or to reduce ROS. If these mechanisms are not overruled by sustained ROS production, cellular reactions induced by ROS can not be observed. In the opposite case the ROS are found to act in a quasi concentration-independent manner on cellular functions (Davies, 1999). Since we did not observe a dependence of the slope conductance of H_2O_2-induced inhibition of gap junctional coupling, we suggest that gap junctional uncoupling depends on the capacity of H_2O_2-removal in Hensen cells to suppress the action of ROS. As soon as this capacity is significantly reduced, uncoupling mechanisms are activated that cause a reduction of gap junctional coupling with a time course independent of the applied dose.

In the range of 0.05 mM to 20 mM H_2O_2 causes significant structural damage of OHC in vitro (Clerici et al., 1995). More recently, apoptotic changes of OHC have also been described upon exposure to hydrogen peroxide (Huang et al., 2000). Surprisingly, no such cellular deformities were found in Hensen cells in our experimental series for concentrations of 0.08 mM to 8 mM H_2O_2 for at least 2 hr. This observation is in line with previous findings in the presence of H_2O_2 (Dehne et al., 1999) or other free radical-inducing agents (Richardson & Russell, 1991). In addition, we found no effect of hydrogen peroxide on membrane potential and nonjunctional membrane current/conductance (Fig. 3*b*) at the above concentration range. At 0.08 mM H_2O_2 no significant change of gap junctional coupling was found. It must be stressed that micromolar concentrations of H_2O_2 reflect physiological conditions (Mueller, Riedel & Stremmel, 1997), but 0.08 mM H_2O_2 already induced a slow oscillatory change of cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) with a periodicity of about 10 min (Fig. 4*a*). Oscillations of $[Ca^{2+}]_i$ have been related to numerous intracellular events, i.e., gene expression, ATP synthesis in mitochondria, cell growth and differentiation (Nakahara et al., 1998) as well as secretion (Ngezahayo

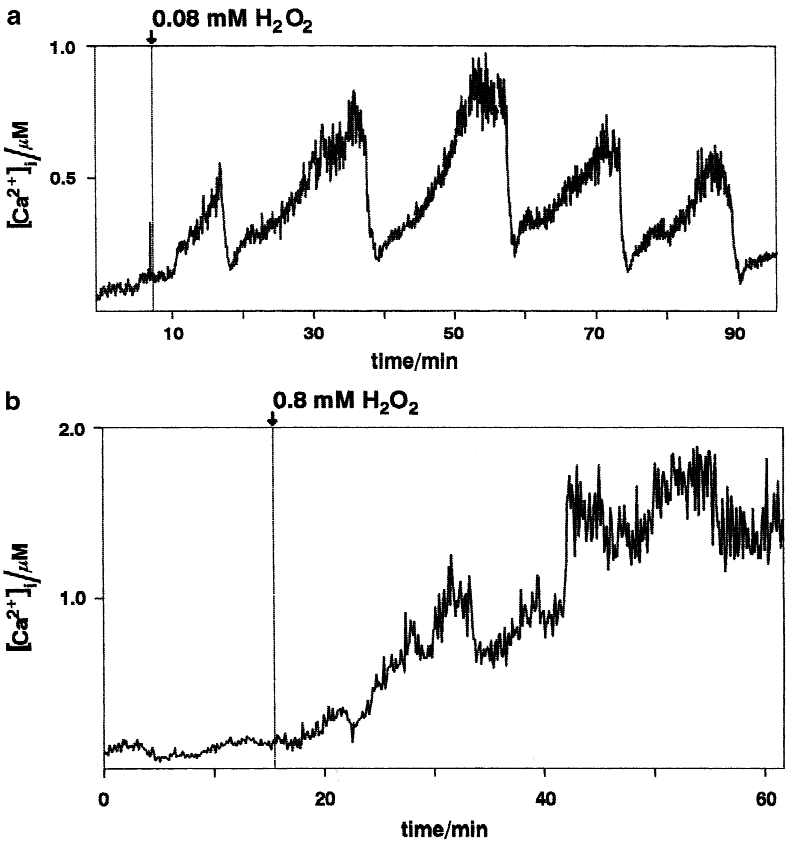

Fig. 4. Temporal pattern of cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) elicited in different cell pairs by H_2O_2. (*a*) 0.08 mM H_2O_2; (*b*) 0.8 mM H_2O_2. H_2O_2 was added to the control bath 15 min after establishing the double whole-cell configuration. A cell pair was loaded with 0.2 mM fura-2 containing pipette solution (*see* Materials and Methods).

& Kolb, 1993). Furthermore, it has been shown that free radicals mediate a calcium response by ryanodine receptors (Favero, Zable & Abramson, 1995; Burlando et al., 1997) and can induce oscillations of $[Ca^{2+}]_i$ (Kannan et al., 1997). Ryanodine-sensitive stores have also been documented for Hensen cells (Sato et al., 1998), which are known to be involved in the oscillatory Ca^{2+}-response by a fast Ca^{2+}-sequestration mechanism (Hernández-Cruz, Escobar & Jimenez, 1997). Therefore, it is proposed that 0.08 mM H_2O_2 activates a ryanodine-sensitive calcium store (Favero et al., 1995) causing the observed oscillations of $[Ca^{2+}]_i$. At higher concentrations of H_2O_2, a decrease of gap junctional conductance (Fig. 3*a*) in parallel with a sustained increase of $[Ca^{2+}]_i$ is observed (Fig. 4*b*). It is generally accepted that the cytoplasmic electrolyte in whole-cell configuration is determined by the pipette filling solution. $[Ca^{2+}]$ in the pipette solution is buffered to 100 nM by addition of 5 mM EGTA. It can be excluded that there is a significant H_2O_2-induced increase of $[Ca^{2+}]_i$ in the whole cytoplasm, but it is known that even in the presence of high concentrations of EGTA, an increase of $[Ca^{2+}]_i$ can occur in regions close to the cell membrane (You, Pelzer & Pelzer, 1997), which could represent the observed response of $[Ca^{2+}]_i$ given in Fig. 4*b*. The observed sustained increase of $[Ca^{2+}]_i$ by addition of H_2O_2 could be involved in the corresponding gap junctional uncoupling (*see* Fig. 3*a*). $[Ca^{2+}]_i$-induced uncoupling is well known and has been related to a binding of Ca^{2+} to gap junctional channels (cf. Somogyi & Kolb, 1991). For supporting cells in the organ of Corti it has been shown that elevation of $[Ca^{2+}]_i$ to millimolar concentration uncouples the cells within 2–3 min (Sato & Santos-Sacchi, 1994). In many cellular systems a comparable increase of $[Ca^{2+}]_i$ to the millimolar level (*compare to* Fig. 4) has been related to pathophysiological conditions (Trump & Berezesky, 1995). It can be speculated that H_2O_2 does not solely act on gap junctional channels via an increase of $[Ca^{2+}]_i$, but the observed H_2O_2-evoked elevation of $[Ca^{2+}]_i$ (Fig. 4) may modify intracellular reactions, e.g., those involving G-proteins, yielding to activation/inhibition of proteinkinases (Nishida et al., 2000) and finally to the observed reduction of gap junctional conductance (Fig. 3*a*).

The present data indicate that the previously reported dose-dependent effect of gentamicin on gap junctional coupling can be mimicked by a dose-dependent influence of H_2O_2 on gap junctional coupling. In both experimental conditions, oxidative changes seem to be responsible for gap junctional uncoupling. This corresponds to our finding that catalase is highly effective in suppressing a gentamicin-induced gap junctional uncoupling (Todt et al., 1999). Thus, the inhibition of gap junctional coupling in cochlear supporting cells appears to be one key step of oxidative-stress related ototoxicity (Huang et al., 2000).

The effect of H_2O_2 on gap junctional coupling was not related to the observed voltage-dependence of gap junctional conductance. For cell pairs in double whole-cell configuration, a diverse pattern for the dependence of gap junctional conductance (G_j) on transjunctional voltage (V_j) was observed. In addition to the reported symmetrical dependence of G_j on V_j as well as the absence of a voltage dependence (Zhao & Sacchi, 2000), we observed also an asymmetrical dependence on V_j (Fig. 2). It has been speculated that this diversity is due to heterotypic or heteromeric gap junctional channels (Zhao & Sacchi, 2000) formed by the connexins Cx26, Cx30, Cx32 and Cx43 (Kikuchi et al., 1995; Forge et al., 1997; Lautemann et al., 1998) which have been found in the cochlea. In experiments with *Xenopus* oocytes, such asymmetrical voltage-dependency was observed by pairing oocytes expressing Cx26 with those expressing Cx32 or Cx30 (Barrio et al., 1991; Dahl et al., 1996). Further experiments with connexins expressed in oocytes are, however, required to be able to attribute the observed dependence of G_j on V_j to an expression of the specific connexins found in cochlear supporting cells.

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