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ORIGINAL ARTICLE

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Nitric oxide uncouples gap junctions of supporting Deiters cells from Corti's organ

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Abstract Supporting cells of Corti's organ are electrically coupled via gap junctions. They probably serve to maintain the unique cochlear environment that is required for normal sensory function. In this study we used input capacitance measurements under whole-cell voltage-clamp conditions to evaluate the effects of nitric oxide on gap junctional communication between pairs of isolated supporting Deiters cells. We show that the nitric oxide (NO) donor sodium nitroprusside causes the uncoupling of Deiters cells, and that an NO synthase inhibitor blocks the effect. The cGMP analogue 8-bromo-cGMP also uncouples Deiters cells. With either treatment, the input capacitance of pairs of Deiters cells drops to single-cell levels within minutes of application, indicative of electrical uncoupling. We surmise that the NO/cGMP pathway may serve to modulate normal cochlear homeostasis and possibly plays a role in ototoxic mechanisms.

Key words Calcium · cGMP · Gap junctions · Membrane capacitance · Supporting cells

Introduction

The mammalian organ of Corti is comprised of sensory (inner and outer hair cells) and supporting cells, e.g., Hensen, Deiters, and Pillar cells. The supporting cells are structurally and electrically coupled together by gap junctions, which permit the direct cellular exchange of ions, dyes, and metabolites [5]. Cochlear homeostasis is believed to rely on intercellular coupling [4]. Coupling in the organ can be modulated by changes in intracellular pH and Ca^{2+}, temperature, membrane voltage (junctional and non-junctional), and membrane tension (e.g., [8]). Recently, free radicals have been shown to uncouple Henson cells [7].

An understanding of nitric oxide's (NO) role in inner ear function is presently limited, but evolving (see [1] for review). The Ca^{2+}-dependent constitutive isoforms of nitric oxide (NO) synthase (NOS), neuronal bNOS (NOS I) and endothelial eNOS (NOS III) are found in the inner ear [2]. More specifically, Hess et al. [2] showed that bNOS is present in Deiters cells and in efferent nerve fibers, while eNOS is found in afferent nerve fibers and synaptic endings. The inducible Ca^{2+}-independent iNOS (NOS II) is a third isoform that has not been found in the cochlea under normal conditions but can be stimulated by bacterial lipopolysaccharides (LPS) and tumor necrosis factor α (TNF-α) [2]. In the present study we investigate the effects of NO on the electrical coupling between supporting cells. We show that the generation of NO can uncouple gap junctions of Deiters cells, and that the effect probably occurs through the NO/cGMP pathway.

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Materials and methods

Guinea pigs were killed by decapitation. The organ of Corti was dissected from the temporal bone in Ca^{2+}-free Leibovitz medium (NaCl 142.2 mM, KCl 5.37 mM, $MgCl_2$ 1.48 mM, HEPES 10.0 mM, dextrose 5.0 mM, pH 7.2, 300 mosmol/l). Pairs of Deiters cells were obtained by agitating the organ of Corti in Ca^{2+}-free Leibovitz medium containing 1 mg/ml trypsin for 10 min. The cell-enriched supernatant was then transferred to a 700-µl perfusion chamber, and the cells were allowed to settle and attach to the untreated glass bottom of the chamber at room temperature for 15 min. An ionic blocking solution [tetraethylammonium (TEA) 20.0 mM, CsCl 20.0 mM, $CoCl_2$ 2.0 mM, $MgCl_2$ 1.47 mM, HEPES 10.0 mM, NaCl 99.2 mM, 2 mM $CaCl_2 \cdot 2H_2O$, pH 7.2, 300 mosmol/l] was used as the normal extracellular perfusate in order to block outward (K) and inward (K, Ca) currents. The use of ionic blocking solutions has no effect on gap junctional coupling in supporting cells [4, 8], and is simply used so that capacitive currents can be evaluated in isolation. Better voltage control is an additional benefit. $CaCl_2$ was included to facilitate pipette seal formation and cell attachment to the chamber bottom. Before starting an experi-

ment, enzyme and cell debris were removed by perfusing for 15 min. The bath perfusion was continued for the whole of each experiment. Patch electrodes were made from borosilicate glass capillaries. The initial resistance was 3–5 MΩ, corresponding to tip sizes of 1–2 µm in diameter. The pipette solution was composed of 140 mM CsCl, 1 mM EGTA, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.2, 300 mosmol/l. In some experiments, 10 mM BAPTA replaced EGTA to more efficiently limit intracellular Ca^{2+}activity. Electrical coupling between isolated pairs of Deiters cells was evaluated by monitoring the input capacitance obtained by the whole-cell voltage clamp of one cell in the pair. This method is a sensitive indicator of junctional coupling [4, 8]. All data collection and analysis were performed with the software program jClamp (www.med.yale.edu/surgery/otolar/ santos/jclamp.html).

A 1 mM sodium nitroprusside (SNP) solution (Sigma, St. Louis, Mo., USA) and a 1 mM 8-bromo-guanosine 3′, 5′-cyclic monophophate (cGMP) solution (Calbiochem, La Jolla, Calif., USA) were freshly prepared in the normal medium prior to each experiment. Each solution was locally delivered to the cells via Y-tube microperfusion while the normal bath perfusion continued. This was done to limit the treatment effect to the cell pair under study. NOS activity was blocked by a 500-µM solution of *S*-methyl-L-thiocitrulline (MTC, Sigma), a competitive NOS inhibitor.

Results and discussion

Deiters cells are intimately associated with outer hair cells, providing a cellular buffer between the outer hair cell and the basilar membrane. Additionally, via their apical processes, they contribute to the formation of the reticular lamina, a boundary between endolymph and perilymph. These cells are strategically placed to support the normal function of the outer hair cell, which serves as the basis of the "cochlear amplifier". Pairs of Deiters cells are readily isolated from Corti's organ, and remain electrically well coupled (Fig. 1). This coupling effectively provides contiguity of the plasma membrane among Deiters cells, as evidenced by measurements of input capacitance. Figure 1A shows the input capacitance of a pair of Deiters cells where octanol is used reversibly to uncouple the cells. Input capacitance decreases to single-cell values during uncoupling and increases during washout with normal solution. Isolated pairs of Hensen cells show similar responses [4], and indeed this supporting cell type has been used to confirm the utility of input capacitance measures in coupling studies. For example, visually confirmed single cells, pairs and triplets have average input capacitances of 31.0, 64.8, and 104 pF, respectively [8]. In the present study, the input capacitance of single Deiters cells was 42.4±7.4 pF (*n*=12).

In order to evaluate the effects of NO on the coupling of Deiters cells, we perfused solutions containing the NO donor SNP (1 mM) during electrical recording. Figure 2A illustrates that SNP reduces the input capacitance of cell pairs to single-cell levels, indicating that gap junctional communication was disrupted. This type of result was obtained in 11 of 13 cell pairs. For those pairs that uncoupled, the initial input capacitance averaged 101.3±9.9 pF (mean ±SE). The time course of effects was variable, but, on average, input capacitance was reduced to 75% after 493±97 s following the start of SNP perfusion. The minimum input capacitance ultimately

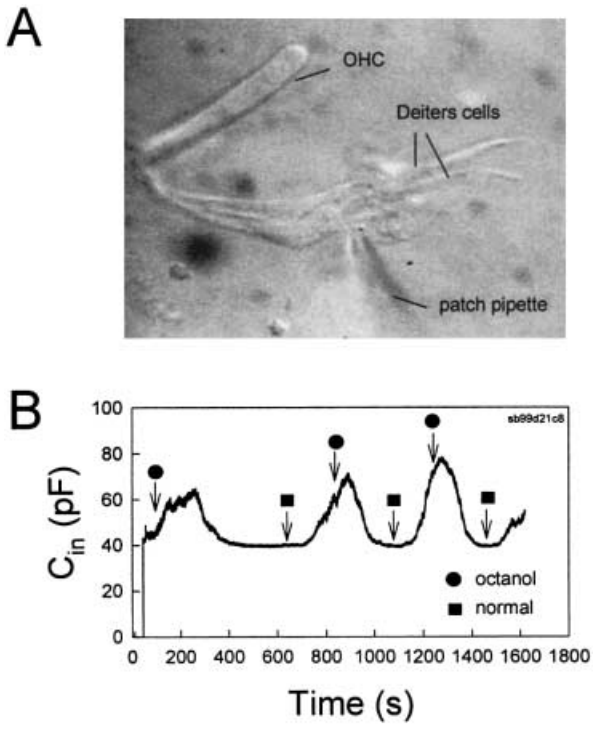

Fig. 1 **A** Digitally captured image of an isolated pair of Deiters cells, one of which is whole-cell voltage clamped. An outer hair cell (*OHC*) remains attached to the apical processes. **B** Effect of octanol on the input capacitance (C_{in}) of a pair of Deiters cells. After about 30 s, the pipette-membrane seal was ruptured and C_{in} jumped to 43 pF; initially, coupling was slight, but increased over the course of a few minutes, i.e., as C_{in} increased. The application of octanol (≅1 mM) via Y-tube perfusion uncoupled the cells as indicated by a drop of capacitance to single-cell levels. After washout of octanol, re-coupling occurred. The effects were repeatable. The absence of an immediate effect upon the initial application of octanol is probably due to perfusion blockage, as subsequently, and in previous studies (e.g. [4]), octanol uncouples supporting cells within tens of seconds

reached was 50.6±7.4 pF. In some cells, re-coupling occurred following the washout of SNP. In an additional ten pairs, the NOS inhibitor MTC (0.5 mM) was perfused continuously during SNP delivery (Fig. 2B). In eight of ten cells no reduction of input capacitance to single-cell levels was observed; in two cases where input capacitance dropped to single-cell levels, the quality of the cells may have been low since the recordings were made at the end of an experimental session.

Many of the effects of NO are mediated by activation of soluble guanylate cyclase (sGC) and the subsequent action of cyclic guanosine monophosphate (cGMP) [3]. sGC is found in supporting cells, including Deiters cells [1]. We tested the possibility that NO acts via the cGMP pathway by treating coupled Deiters cells with a membrane-permeant form of cGMP (1 mM). Fig. 2C illustrates that 8-bromo cGMP causes Deiters cells to uncouple. As with SNP treatment, input capacitance drops to single-cell levels following treatment. All six cell pairs tested evidenced uncoupling. The initial input capacitance averaged 112.6±18.2 pF (mean ±SE). Similar to

Table 1 The input capacitance of a pair of Deiters cells and the effects of altering gap junctional communication between them. (*cGMP* 8-Bromo-guanosine 3′, 5′-cyclic monophophate, *MTC* *S*-methyl-L-thiocitrulline, *SNP* sodium nitroprusside.) Single-cell values were 42.4±7.4 pF (*n*=12; mean ±SD)

Treatment	Initial C_{in} (pF)	Time (s) to 75% C_{in}	Ultimate C_{in} (pF)	% decrease
SNP (*n*=11)	101±9.9	493±97	50.6±7.4	50
SNP + MTC (*n*=7*)	90.6±20.4	–	79.3±13.4	88
cGMP (*n*=6)	112±18	908±163	46.2±13.6	41

*Only seven of the eight pairs that did not reduce to single-cell levels were perfused with MTC throughout the recording period. In the other pair, MTC was washed out and subsequently SNP did reduce C_{in} to single-cell levels

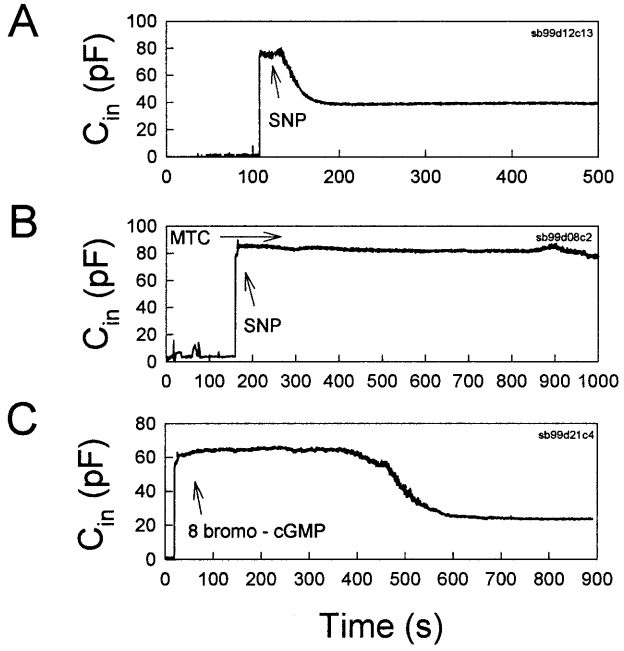

Fig. 2 **A** After about 110 s, the whole-cell configuration was established, indicated by the jump in capacitance (C_{in}). Perfusion of 1 mM sodium nitroprusside (*SNP*) caused the cell pair to rapidly uncouple, indicated by the drop in capacitance to a single-cell value of 40 pF. **B** Perfusion of the nitric oxide synthase (*NOS*) inhibitor MTC (0.5 mM) started before the whole-cell configuration was established, and continued during application of SNP. C_{in} remained fairly constant throughout the recording period. **C** Treatment with 8-bromo cGMP (1 mM) uncoupled this pair of Deiters cells after about 7 min

SNP, the time course of effects was variable, but, on average, input capacitance was reduced to 75% after 908±163 s following the start of 8-bromo-cGMP perfusion. The minimum input capacitance ultimately reached was 46.2±13.6 pF. Our results are summarized in Table 1.

The susceptibility of supporting cell gap junctions to intracellular calcium is well documented (see [5]), and, indeed, second messenger mechanisms that elevate intracellular calcium levels, e.g., inositol 1,4,5-trisphosphate or IP_3, can uncouple supporting cells [6]. In preliminary tests, we found that replacing the patch pipette solution Ca^{2+} chelator EGTA (1 mM) with the more effective chelator BAPTA (10 mM) blocked the effect of SNP on supporting cell coupling (six cell pairs). The enzyme probably responsible for NO generation is one of the Ca^{2+}-dependent constitutive ones, because of the rapidity of the uncoupling effects. Consequently, it might be that Ca^{2+} sequestration interferes with NO generation. Alternatively, it is conceivable that NO is generated within the adjacent un-patched cell, or other cells within the perfusion area, and that it is the buffering of Ca^{2+} within the immediate vicinity of gap junctions that maintains coupling. BAPTA, based on molecular weight, can traverse gap junctions into adjacent Deiters cells so as to maintain low Ca^{2+} levels within the vicinity of gap junctions. Further studies are required to resolve the mechanism of BAPTA's effects.

Among other possibilities, we have suggested that coupling between supporting cells provides for the spatial buffering of potassium ions within the organ of Corti, which otherwise would compromise the normal activity of hair cell and neural elements [4]. The present results may underscore a mechanism for the physiological control of ionic buffering within the organ of Corti. Additionally, the potential for damage caused by free radicals subsequent to NO generation may have a bearing on the detrimental affects of bacterial toxins [2] and ototoxic drugs [7] on cochlear function.

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