Electrical Coupling in Sustentacular Cells of the Mouse Olfactory Epithelium

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Vogalis, Fivos, Colleen C. Hegg, and Mary T. Lucero. Electrical coupling in sustentacular cells of the mouse olfactory epithelium. J Neurophysiol 94: 1001–1012, 2005. First published March 23, 2005; doi:10.1152/jn.01299.2004. Sustentacular cells (SCs) line the apical surface of the olfactory epithelium (OE) and provide trophic, metabolic, and mechanical support for olfactory receptor neurons. Morphological studies have suggested that SCs possess gap junctions, although physiological evidence for gap junctional communication in mammalian SCs is lacking. In the present study we investigated whether coupling exists between SCs situated in tissue slices of OE from neonatal (P0–P4) mice. Under whole cell recording conditions, Alexa 488 added to the pipette solution failed to reveal dye coupling between SCs. Electrical coupling was deduced from the biexponential decay of capacitive currents recorded from SCs and from the bell-shaped voltage dependency of a P2Y-receptor-activated current, both of which were abolished by 18β-glycyrrhetinic acid (20–50 μM), a blocker of gap junctions. These data provide strong evidence for functional coupling between SCs, the physiological importance of which is discussed.

INTRODUCTION

Diffusion of cytoplasmic solutes between neighboring cells occurs through gap junctions. These aqueous intercellular tunnels are formed by 2 extracellularly linked hemichannels, or connexons, embedded in the membranes of adjoining cells (Bennett and Verselis 1992; Bennett et al. 1991, 2003; Spray and Bennett 1985). Each hemichannel consists of 6 subunits, or connexins, that vary in their primary sequences, leading to the formation of gap junctions composed entirely of the same type of connexin (homotypic) or of various permitted combinations (heterotypic). Thus gap junctions may have widely varying properties with regard to conductance, permeability, dependency on transjunctional voltage, and regulation by second messengers (Valiunas et al. 2002). Gap junctions not only allow cytoplasmic solutes to diffuse between neighboring cells to dissipate concentration gradients (Rose and Ransom 1997) but they also enable intercellular spread of electrical current and messenger molecules to coordinate the activity of cells, as in hepatocytes (Tordjmann et al. 1997).

In a recent study, we characterized the electrical properties of sustentacular cells (SCs) in slices of olfactory epithelium (OE) of neonatal (P0–P4) mice (Vogalis et al. 2005). Sustentacular cells form a barrier on the apical surface of the OE, extend processes across the width of the epithelium to the basement membrane, and may function like glial cells in the brain to provide metabolic, structural, and trophic support for olfactory receptor neurons (Getchell and Getchell 1992). A striking property of SCs in tissue slices of murine OE was the presence of a comparatively large resting “leak” conductance (gL) (Vogalis et al. 2005) that was permeable to cations and anions and that showed outward rectification. A conductance of a similar ionic nature but with a 10-fold smaller magnitude has been described in dissociated SCs from the vomeronasal organ (VNO) of adult mice (Ghiaroni et al. 2003). In olfactory SCs in slices, gL was largely inhibited by 18β-glycyrrhetinic acid (18β-GA), a blocker of gap junctions (Davidson and Baumgarten 1988), which is also thought to block hemichannels (Contreras et al. 2003; Saez et al. 2003), suggesting that gL is mediated by the opening of unopposed gap junction channels. It is also possible that the decrease in the input resistance of SCs after treatment with 18β-GA may have been a result of blockade of gap junction channels between coupled SCs, or between SCs and other cell types in the OE. This was not addressed in the previous study because when either Lucifer yellow or Alexa 488 was perfused internally into SCs by the patch pipette, we failed to detect any dye-coupling that would suggest the presence of gap junctions. However, because the duration of a typical recording from a SC lasted about 30 min, this period of time may not have been sufficiently long to permit transfer of a detectable amount of dye across gap junctions into the coupled cells, even though SCs may be electrically coupled. In the frog OE, for example, gap junctions are thought to electrically couple SCs (Trotier 1998), although there is little evidence of dye-coupling. Thus an absence of dye-coupling does not rule out the possibility that SCs possess functional gap junctions that permit current flow (Goldberg et al. 2004).

Studies in the mouse OE using antibodies raised against connexin 43 (Cx43) have shown immunoreactivity at discrete points on the membranes of adjacent SCs (Miragall et al. 1992) and LacZ reporter gene staining driven by the Cx43 promoter has been demonstrated in SCs of adult murine OE (Zhang et al. 2000). In addition, there is evidence for the presence of Cx45 subunits in the mouse OE in regions containing SCs (Zhang and Restrepo 2002). As a rule, gap junctions are thought to be largely impermeable to molecules with molecular weights exceeding 1 kDa. However, the rate at which molecules smaller than 1 kDa, including fluorescent dyes, diffuse across gap junctions depends on the connexin composition and the macroscopic gap junctional conductance. As shown in electrically coupled pairs of Xenopus oocytes, dye transfer across homotypic gap junctions composed of Cx43 or Cx45 may require hours for equilibrium to be reached (Weber et al. 2004). Thus an absence of discernible dye coupling may underestimate the extent of electrical coupling between cells. In the absence of discernible dye coupling in tissue slices, we failed to detect any dye-coupled SCs.

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present study, we investigated the functionality of gap junctions between SCs in OE slices taken from neonatal mice (P0–P4) using electrophysiological recordings from SCs and by measuring changes in cell capacitance and input resistance after application of 18β-GA. Our results indicate that despite a lack of dye-coupling, SCs are likely to be electrically coupled, suggesting that they possess functional intercellular gap junctions.

METHODS

Preparation of OE slices for patch-clamp recording

Coronal slices of olfactory epithelium (250 μm thick) were prepared from neonatal Swiss Webster mice (P0–P4), as described previously (Hegg and Lucero 2001). Mouse pups were killed by decapitation, as approved by the University of Utah Institutional Animal Care and Use Committee. Slices were pinned out onto a 12-mm coverslip coated with elastomer (Sylgard, Dow Corning) and placed in a tissue perfusion chamber (about 0.25 ml in volume), on an upright fluorescence microscope (Nikon). The slice was perfused (about 1 ml/min) with Ringer solution at room temperature for 1 h before recording.

Patch pipettes were made from borosilicate thick-walled glass tubing (ID 0.87 mm, OD 1.15 mm; Sutter) and pulled to have resistances of 5–8 MΩ when filled with internal pipette-filling solutions. The bath was grounded with a 3M KCl-agar bridge and a Ag/AgCl wire. To minimize pipette capacitance artifacts, depth of the tip was reduced by 100 μm in the glass with a 3 M KCl-agar bridge (Vogalis et al. 2005).

Drugs and other chemicals

All the reagents used for making bathing solutions and internal pipette solutions were purchased from Sigma (St. Louis, MO) unless indicated otherwise. 18β-Glycyrrehitin acid (18β-GA) was dissolved in ethanol as a 0.1 M stock (final [ethanol], 0.15 mM; 0.015%). Suramin (100 μM) was directly dissolved in Ringer solution and tetraethylammonium chloride (TEA) was made up as a 1 M stock solution, added to the perfusate as required.

Statistical tests and data handling

The mean values of measurements reported here represent averages computed from “n” number of cells. Where measurements were performed on the same cells, before and after a particular treatment, we used the paired Student’s t-test. Otherwise we used Student’s t-test for unpaired observations. In both cases, statistical significance was set at P < 0.05, or else the P value is given. Curve fitting of the capacitive current (Ic) and construction of I–V relationships and G–V relationships was performed using Igor 4.0 (WaveMetrics). For patch recordings (cell-attached and outside-out patches), single-channel current amplitude (i) was determined by measuring the current of 10–20 openings and taking the average. All-points histograms were constructed from 30-s segments of recordings using Clampfit (Axon) and fitted with Gaussian curves. From these curves, the open probability of channel opening was determined by summing of the integrated area of each Gaussian curve × the channel level, and dividing this value by the summed area under all the Gaussian curves. Linear regression lines were fitted to data points of I versus pipette potential (Vp) to determine unit conductance and reversal potential.

RESULTS

Absence of dye coupling between SCs in the olfactory epithelium

Within minutes of whole cell break-in, SCs rapidly filled with fluorescent dye (Alexa 488, 100 μM) contained in the pipette-filling solution. Fluorescence intensity was strongest in the cell body and relatively weak in the processes. However, there was little evidence for accumulation of dye in what looked like neighboring SCs ≤25 min after whole cell access (Fig. 1Ai). Not surprisingly, a similar pattern of fluorescence was seen in SCs that had been treated with 18β-GA (20 μM) to block gap junction channels (Fig. 1Bi). Similar observations were made in >30 cells under both conditions and suggested that SCs in the OE of neonatal mice are either not significantly dye-coupled or else the concentration of dye used or the time allowed for dye transfer may not have been sufficient.

Despite the absence of detectable dye-coupling, the normalized capacitive current (Ic norm) recorded from the dye-filled cell depicted in Fig. 1Ai decayed nonmonotonically when plotted on a logarithmic scale (Fig. 1Aii). In contrast, the Ic norm of the 18β-GA–treated cell (Fig. 1Bii) decayed for the most part (>99% of peak current) with a single exponential (Fig. 1Bii).

Perfusion solutions and internal pipette-filling solutions

The standard Ringer solution used to perfuse slices consisted of (in mM): NaCl, 140; KCl, 5; MgCl2, 1; CaCl2, 2; HEPES, 10; glucose, 10; pH 7.4, 330 mOsm. The standard internal pipette-filling solutions (KF) had the following composition (in mM): KF, 125; KCl, 15; MgCl2, 3; HEPES, 10; EGTA, 11; pH 7.2 with KOH. To minimize current flow through K+ channels, a CsF-based internal solution was also used that consisted of (in mM): CsF, 125; CsCl, 15; MgCl2, 1; CaCl2, 1; EGTA, 2.5; HEPES, 10; ATPK7, 2; GTPLi, 0.2; pH 7.2 with CsOH. Liquid junction potentials (LJP) were calculated using JPCalc in Clampex (Barry and Diamond 1970) and equaled 8 mV for KF internal solution and 9 mV for CsF internal solution and were subtracted from the relevant membrane potential measurements.

Measurement of the input cell capacitance (Ccell), cell membrane resistance (Rmem), series resistance (Rs), and input resistance (Rin) was performed off-line, from recordings of capacitive current (Ic)

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As described below, these effects of 18β-GA on the time course of $I_{C_{norm}}$ suggest that SCs are electrically coupled.

**Effect of 18β-GA on the input capacitance and membrane resistance of SCs**

When 2 cells are electrically coupled by a finite junctional resistance ($R_j$), the whole cell capacitance measured in the patched cell will include a contribution from the capacitance of the coupled cell. It then follows that the magnitude of the “apparent” cell capacitance ($C_{cell}$) will decrease if the junctional conductance ($G_j = 1/R_j$) is blocked. We found in the pooled results that $C_{cell}$ derived from the time integral of $I_C$ recorded from SCs bathed in Ringer solution averaged 18.5 ± 0.5 pF ($n = 191$), whereas in SCs treated with 18β-GA, $C_{cell}$ was significantly smaller ($P < 0.05$, unpaired $t$-test) at 16.3 ± 0.6 pF ($n = 106$), a difference of 12%. An even larger difference in $C_{cell}$ was seen in matched recordings from 8 SCs, before and after treatment with 18β-GA. Here $C_{cell}$ was decreased from 22.1 ± 1.9 to 16.6 ± 2.3 pF after treatment, a 25% reduction ($P < 0.05$, paired $t$-test) (Fig. 2, Ai and Bi). The larger difference in $C_{cell}$ seen in the matched recordings was likely attributable to the fact that the majority of cells that were selected for treatment with 18β-GA had a clearly discernible slowly decaying component of $I_C$ that, as described below, is an indication of electrical coupling (Fig. 2, Bi and C).

Application of 18β-GA also elicited a large increase in the input resistance ($R_{in}$) of SCs, as we have reported previously (Vogalis et al. 2005). $R_{in}$ averaged 187 ± 10 MΩ ($n = 191$) in SCs bathed in Ringer’s solution and 656 ± 37 MΩ ($n = 106$) in SCs pretreated with 18β-GA, a 3.5-fold difference. A similar difference was seen in the matched recordings from 8 SCs in which $R_{in}$ was increased from 149 ± 32 to 485 ± 114 MΩ by 18β-GA (Fig. 2, Aii and Bii).

**FIG. 1.** Lack of dye-coupling between sustentacular cells (SCs) in the olfactory epithelium of neonatal mice. A, i: fluorescence photomicrograph of SC filled with Alexa 488 (100 μM), taken about 20 min after break-in. Fluorescence is seen in the cell body and processes but not in neighboring cells. ap, apical surface; bas, basal lamina. ii: time course of the normalized capacitive current ($I_{C_{norm}}$) recorded in the same cell showing nonmonotonic decay composed of 2 exponential terms, indicated by straight lines fitted by eye. B, i: fluorescence photomicrograph of a dye-filled SC in a different slice, bathed in 18β-glycyrrhetinic acid (18β-GA; 20 μM), taken about 20 min after break-in. ii: $I_{C_{norm}}$ recorded in the 18β-GA-treated cell showing monoexponential decay, complete within 1.5 ms.

**FIG. 2.** Reduction of cell input capacitance ($C_{cell}$) and increase in input resistance ($R_{in}$) of SCs by 18β-GA. A: time course of the change in (i) $C_{cell}$, (ii) $R_{in}$, and (iii) series resistance ($R_s$) in SC, with wash-in of 18β-GA (20 μM). Dashed line represents a gap of 4 min. B, i: application of 18β-GA significantly decreased $C_{cell}$ in 8 SCs; ii: conversely, 18β-GA significantly increased (by nearly 4-fold) the $R_{in}$ of the same 8 SCs. C, i: superimposed recordings of capacitive current ($I_C$) evoked by a 10-mV hyperpolarizing step in the absence and presence of 18β-GA. Note decrease of the steady-state current after 18β-GA. ii: time course of $I_{C_{norm}}$ recorded in Ringer solution and after application of 18β-GA. Note biexponential decay of the current in Ringer’s and marked reduction in the magnitude of the slow component of $I_C$ ($I_{C_{slow}}$) in the presence of 18β-GA. Solid lines were fitted by eye.
Estimation of $R_j$ from the capacitive current ($I_C$)

The increase in $R_m$ after 18β-GA treatment could be explained by blockade of gap junctions or unopposed gap junctions (hemichannels) or a combination of the two, assuming that 18β-GA at 20 μM specifically targets connexins. However, because the number of cells to which a given SC is coupled in the OE slice is not known, it is difficult if not impossible to estimate the values of $R_j$ from changes in the input $R_m$ and $C_{cell}$, as has been performed, for example, in patch recordings from one of a pair of taste bud cells (Bigiani and Roper 1995). Another indication of electrical coupling between cells is provided by the nonmonotonic decay of $I_C$ recorded from the patched cell. This is because $R_j$ will be in series with the capacitance of the coupled cells and will therefore contribute a component to the series with the capacitance of the coupled cells and will represent charging of part of the membrane capacitance of one or more of the coupled cells (Mosser 1998). Therefore the peak of $I_{Cslow}$ will be proportional to the sum of the junctional conductances in the patched cell.

As shown in Figs. 1Ai and 2Ci, $I_C$ or $I_{Cnorm}$, recorded from SCs bathed in Ringer solution invariably decayed with 2 exponential time constants ($\tau_{fast}$ and $\tau_{slow}$) corresponding to $I_{Cfast}$ and $I_{Cslow}$ over the initial 5–10 ms after the current peak (Fig. 2Ci). This biexponential time course was manifest as 2 nearly linear current components when $I_{Cnorm}$ was plotted on a logarithmic scale (Figs. 1Ai and 2Ci). In matched recordings from 8 SCs, $\tau_{fast}$ was unchanged ($P > 0.05$) after 18β-GA application (0.21 ± 0.04 ms before vs. 0.34 ± 0.08 ms after) and in 5 of these cells, $\tau_{slow}$ was increased from 1.4 ± 0.3 to 2.5 ± 0.8 ms, whereas $I_{Cslow}$ was abolished in the other 3 after 18β-GA treatment (Fig. 2Ci). Overall the proportion of $I_{Cslow}$ in these 8 SCs was decreased significantly ($P < 0.05$) by 18β-GA, from 11 ± 3 to 2 ± 1% of peak $I_C$, indicating that 9% of the peak current was attributable to charging of the membrane capacitance of cells to which the patched cell was coupled.

If one assumes that $I_{Cslow}$ represents mainly current flow across gap junctions into neighboring coupled cells, then the decrease in $I_{Cslow}$ after blockade of these gap junctions by 18β-GA can be used to estimate $R_j$ because the difference in the peak $I_{Cslow}$ before and after treatment with 18β-GA will be equal to the junctional current ($I_j$). $R_j$ is then equal to $R_m$, corrected for $R_{cell}$, multiplied by 10 mV (i.e., the depolarizing step that elicited $I_C$). In the 8 SCs examined $R_j$ averaged 247 ± 84 MΩ ($n = 8$).

A similar analysis of the pooled data also revealed differences in $I_{Cslow}$ between SCs treated with 18β-GA and those bathed in Ringer solution. As illustrated in Fig. 3Ai, the mean $I_C$, derived by trace-averaging the capacitive currents recorded from 118 SCs bathed in Ringer solution, had a larger steady-state current component at −78 mV than the corresponding mean $I_C$ derived from 91 SCs treated with 18β-GA, reflecting the lower $R_m$ of the untreated cells. The 2 traces of mean $I_C$ were then normalized to the respective peak mean current after subtraction of the steady-state component to obtain the respective mean $I_{Cnorm}$ traces. When plotted on a logarithmic scale, these traces revealed that the $I_{Cnorm}$ of untreated cells contained both a fast and a slow component and could be fitted with the sum of 2 exponential functions (Fig. 3Aii, Ringer’s), whereas...
the slowly decaying component of the mean $I_{C_{\text{norm}}}$ of the treated cells was decreased in amplitude (Fig. 3Ai, 18β-GA). The proportion of the peak $I_C$ attributable to $I_{C_{\text{slow}}}$ in the untreated cells was 9% and $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ were 0.3 and 2.4 ms, respectively. The proportion of the mean peak $I_{C_{\text{norm}}}$ attributable to $I_{C_{\text{slow}}}$ in the 18β-GA–treated cells, however, was only 3% (Fig. 3Aii) and had a time constant ($\tau_{\text{slow}}$) of 1.7 ms, whereas $\tau_{\text{fast}}$ was 0.3 ms. These results indicate that in a larger random sample of SCs, about 6% of $I_C$ is attributable, on average, to charging of the capacitance of one or more coupled cells. Because the peak $I_C$ averaged 505 pA in the untreated SCs (after correction for $R_s$), the mean $I_t$ was equal to 29 pA, yielding a $R_s$ value of 341 MΩ (10 mV/29 pA).

The specificity of 18β-GA on the slower component of $I_C$ is also illustrated in Fig. 3Bi, which shows the normalized cumulative distribution of SCs plotted as a function of the 2 time constants, $\tau_{\text{fast}}$ (triangles) and $\tau_{\text{slow}}$ (circles), of the biexponential functions fitted to the $I_C$ in SCs treated (filled symbols) and untreated (open symbols) with 18β-GA. The larger shift in the distribution representing $\tau_{\text{slow}}$ after 18β-GA shows that $I_{C_{\text{slow}}}$ is associated with current flow across gap junctions, whereas the shift in the distribution representing $\tau_{\text{fast}}$ suggests that 18β-GA may also have nonspecific effects on membrane properties. In a large proportion of untreated SCs (36/118 or 31%), $I_{C_{\text{slow}}}$ accounted for 12–50% of $I_C$ (Fig. 3Bi) and suggests that the strength of electrical coupling between SCs in OE is variable.

As shown in Fig. 4, carbenoxolone (100 μM), another inhibitor of gap junctions, had an effect similar to that of 18β-GA, in that $I_t$ was substantially reduced (Fig. 4A, i and ii). The rate at which this occurred was much slower and required >40 min of continuous perfusion for $R_{in}$ to increase 2-fold (Fig. 4C). The increase in $R_{in}$ was accompanied by a decrease in the $I_{C_{\text{slow}}}$ component, as illustrated by the plot of $I_{C_{\text{norm}}}$ on a logarithmic scale (Fig. 4B). These results indicate that carbenoxolone, like 18β-GA, decreases $I_{C_{\text{slow}}}$, an effect that is likely attributable to the blockade of gap junctions.

### Evidence for electrical coupling by the presence of multiple $I_Na$ current transients

Sustentacular cells in the OE of neonatal mice generate voltage-gated Na$^+$ channel currents ($I_{Na}$) (Vogalis et al. 2005). We found that peak $I_{Na}$ at $-30$ mV was decreased ($P < 0.05$, unpaired $t$-test) by 18β-GA (Ringer’s: 827 ± 15 pA, $n = 13$; 18β-GA: 679 ± 8 pA, $n = 14$) but that the voltage of half-maximal activation ($V_{act}$) of the underlying conductance was also shifted some 10 mV positively from $-60$ to $-51$ mV, whereas the slopes of the respective Boltzmann curves fitted to the data were 5 and 6 mV. This suggests that 18β-GA may also affect the voltage-sensitivity of Na$^+$ channels.

We noted in 33/118 SCs that $I_{Na}$ evoked at potentials between $-50$ and $+30$ mV consisted of an initial transient current component that was followed, after a variable latency (1–5 ms), by one or more smaller inward current transients. A phenomenon similar to this has been reported in electrically coupled pairs of taste bud cells (Bigiani and Roper 1995), indicating that the SCs from which these additional current transients were recorded were electrically coupled to other cells. Moreover these additional inward current transients were absent in recordings from SCs that had been treated with 18β-GA. The 33 SCs had a significantly larger $C_{cell}$ of 25.1 ± 2.1 pF than the overall mean of SCs ($P < 0.05$, unpaired $t$-test) and their $R_s$ determined from the amplitude of $I_{C_{\text{slow}}}$ averaged 367 ± 76 MΩ. Recordings from one such SC, before and after treatment with 18β-GA, are reproduced in Fig. 5. They show that the characteristic inward leak current ($I_L$) activated at potentials between $-18$ and $-148$ mV was largely inhibited by 18β-GA (20 μM) (Fig. 5A, i and ii). In the same cell, the $I_{Na}$ evoked in Ringer solution at $-18$ mV consisted of one major current transient that was followed by smaller secondary and tertiary transients that disappeared after 18β-GA treatment (Fig. 5Aiii). The corresponding recordings of $I_C$ in the absence and presence of 18β-GA showed that the slow component of $I_C$ ($I_{C_{\text{slow}}}$) was abolished by the gap-junction inhibitor (Fig. 5B, i and ii), which also increased $R_{in}$ (Fig. 5Bi). $R_j$ in this cell was estimated to be 164 MΩ.

### Effects of ATP on SCs reveal electrical coupling between SCs

Stimulation of P2 receptors in SCs triggers robust Ca$^{2+}$-transients (Hegg et al. 2003), the effects of which on Ca$^{2+}$-dependent ionic conductances have not been studied. One target of such cytoplasmic Ca$^{2+}$-transients is likely to be the large-conductance Ca$^{2+}$-activated K$^+$ (BK) channels that are expressed in abundance by SCs that hyperpolarize when BK channels are activated (Vogalis et al. 2005). In an electrical syncytium, this hyperpolarization will induce current flow...
Although ATP increased the $P_o$ of BK channels in cell-attached patches $\times$10-fold, there was no correspondingly large increase in the net outward current that was recorded from SCs in whole cell mode, attributed to the fact that the internal solution for these recordings contained 11 mM EGTA to buffer internal Ca$^{2+}$ to nanomolar levels. Application of ATP (20–50 $\mu$M), however, did elicit a comparatively small outward current (10–20 pA) at a holding potential of $-78$ mV under these conditions. To determine the voltage dependency of this ATP-induced current $I_{\text{ATP}}$ SCs were ramp-depolarized between $-128$ and $+42$ mV over 2 s, before and during the peak of the response to ATP (Fig. 7Ai). The reversal potential ($E_{\text{rev}}$) of the ramp current recorded in Ringer solution was shifted significantly negatively from $-41 \pm 3$ to $-56 \pm 3$ mV ($n = 13$) in the presence of ATP ($P < 0.05$) (Fig. 7Ai), whereas the ramp-difference current (i.e., $I_{\text{ATP}}$) reversed at $-95 \pm 6$ mV (Fig. 7Aii). An examination of the $I-V$ relationship of $I_{\text{ATP}}$ revealed a quasi-linear region between $-130$ and $-20$ mV and a pronounced inward rectification at potentials positive to 0 mV (Fig. 7Aii). The slope of $I_{\text{ATP}}$ between $-130$ and $-20$ mV ($E_{\text{rev}}$) averaged 1.9 $\pm$ 0.3 nS. In 4 SCs tested, 2–5 mM external TEA decreased $I_{\text{ATP}}$ and the residual $E_{\text{rev}}$ averaged only 0.2 $\pm$ 0.2 nS ($P < 0.05$, TEA vs. control, unpaired t-test).

Similarly in 4 SCs pretreated with suramin (100 $\mu$M), a P2Y receptor antagonist, $g_{\text{ATP}}$ averaged only 0.3 $\pm$ 0.2 nS ($n = 4$; $P < 0.05$ vs. control, unpaired t-test), suggesting that $I_{\text{ATP}}$ was generated by the opening of BK channels after stimulation of P2Y receptors on the coupled cells.

To determine whether the $I-V$ relationship of $I_{\text{ATP}}$ was consistent with current flow across a junctional conductance ($G_j$), we fitted the ramp-difference currents elicited by ATP with an equation incorporating the symmetrical “bell-shaped” dependency shown by many types of homotypic gap junction conductances on junctional voltage ($V_j$) (Moreno 2004). As shown in Fig. 7Aiii, $I_{\text{ATP}}$ was well fitted by a curve generated by this equation (the gray solid curve in Fig. 7Aiii), in which it was assumed that in response to ATP, $R_m$ of the coupled cells decreased from 200 to 50 M$\Omega$ as they hyperpolarized from $-50$ to $-85$ mV, whereas the $R_m$ and resting potential of the patched cell were unchanged. As shown in Fig. 7Aiii, the fitted curve adequately described $I_{\text{ATP}}$ and peak $G_j$ was 3.8 nS ($R_j$ =...
FIG. 7. Whole cell recordings from SCs using KF internal solution containing 11 mM EGTA. A: ramp currents elicited by ramp depolarizations as indicated, before and during application of ATP to the slice. Inset: time course of the increase in the outward current at a time point during the ramp depolarization corresponding to −10 mV. ii: ATP-induced difference current (I_{ATP}) obtained by subtraction of the 2 traces shown in i. Difference current was curve-fitted with Eq. 1, which was derived by first summing 2 Boltzmann functions to generate a symmetrical bell-shaped curve as a function of voltage to describe the voltage dependency of the normalized junctional conductance. This curve was multiplied by the junctional voltage predicted between the patched cell and the coupled cell at rest (Eq. 2) assuming that, at rest, R_j in the coupled cell and patched cell are 200 MΩ and their resting potentials are −50 mV. Similarly, the bell-shaped normalized G−V curve was multiplied with the junctional voltage that would exist during ATP application (Eq. 3), where it was assumed that R_j in the coupled cell decreased to 50 MΩ and its resting potential increased to −85 mV. Thus by subtracting the junctional current during ATP from the junctional current at rest, one can obtain a voltage dependency of the junctional conductance by solving for R_j. Thus

\[ I = V_p/R_j \left( 1/(1 + \exp(V_h + V_s - V_j)) \right) - V_p/R_j \left( 1/(1 + \exp(V_h - V_j)) \right) \]  

(Eq. 1)

where V_h is the transjunctional voltage in the absence of ATP and is equal to

\[ V_h = \left[ V_p - ( - 50) \right] R_j/R_p + 200 - 50 \]  

(Eq. 2) and V_s is the transjunctional voltage in the presence of ATP and is equal to

\[ V_s = \left[ V_p - ( - 85) \right] R_j/R_p + 50 - 85 \]  

(Eq. 3)

where V_p in Eqs. 4 and 5 is the pipette potential in the patched cell. Curve-fitting the ramp difference-current with Eq. 1 yielded a bell-shaped conductance–voltage curve (inset), which had a V_e of 38 mV and a slope factor (V_s) of 16 mV. R_j in this cell equaled 265 MΩ. B: i: in another cell pretreated with 18β-GA, ATP failed to elicit a response with ramp depolarization; ii: difference current over the voltage range tested was essentially zero, indicating that ATP, after blockade of gap junctions, failed to elicit a response.

265 MΩ) and had a half-maximal activation/deactivation potential (V_t) of ±38 mV and a slope factor (V_s) of 16 mV (inset in Fig. 7Aii). A similar analysis of I_{ATP} in 8 SCs yielded an average V_t of ±41 ± 6 mV and a V_s of 17 ± 2 mV, whereas peak G_j averaged 2.2 ± 0.3 nS, which is equivalent to a R_j of 454 MΩ.

To confirm that I_{ATP} was a junctional current, OE slices were pretreated with 18β-GA before ATP was applied. Under these conditions, ATP failed to activate a significant outward ramp current (Fig. 7Bi). In 9 K⁺-filled SCs, E_{rev} of the ramp current averaged −41 ± 3 mV before and −44 ± 4 mV (P > 0.05, paired t-test) during the application of ATP, whereas g_{ATP} averaged 0.2 ± 0.1 nS (P < 0.05, vs. control, unpaired t-test) (Fig. 7Biii). Although these results strongly support the likelihood that activation of I_{ATP} is dependent on the presence of conducting gap junctions, it is possible that 18β-GA may also block purinergic receptors. To investigate this possibility, we obtained cell-attached patch recordings from SCs that were pretreated with 18β-GA (20 μM) before application of ATP. We found that BK channels were active at the resting potential of SCs (Fig. 8A) and that application of ATP elicited robust increases in BK channel activity in 3 cells tested (Fig. 8, A and B). These results indicate that 18β-GA does not block activation of P2Y receptors by ATP.

Consistent with the idea that I_{ATP} is a junctional conductance, ATP also activated an outward current in cells that were filled with Cs⁺ (Fig. 9Ai). As in the K⁺-filled cells, the E_{rev} of the ramp current was shifted negatively by about 13 mV by ATP, from −46 ± 12 to −60 ± 10 mV (Fig. 9Ai), whereas I_{ATP} reversed at −91 ± 3 mV and g_{ATP} for the 4 Cs⁺-filled
cells averaged 1.3 ± 0.2 nS (Fig. 9Ai). As in the K\textsuperscript{+}-filled cells, pretreatment with 18β-GA blocked I\textsubscript{ATP} in these cells (Fig. 9B, i and ii). Curve fitting of the I\textsubscript{ATP} recorded in a Cs\textsuperscript{+}-filled cell (solid trace in Fig. 9Aii) also generated a bell-shaped G\textsubscript{i}-V\textsubscript{j} relationship (Fig. 9Aii, inset), which had a V\textsubscript{h} of −38 mV, a V\textsubscript{s} of 20 mV, and a maximal G\textsubscript{j} of 2.2 nS, corresponding to a R\textsubscript{j} of 452 MΩ. These results indicate that I\textsubscript{ATP} is likely to be generated indirectly after the opening of BK channels in the coupled cells and suggest that the amount of Cs\textsuperscript{+} diffusing from the patched cell to the coupled cells is insufficient to inhibit outward current flow through BK channels in the coupled cells.

Simulation of the effect of changes in R\textsubscript{j} and R\textsubscript{m} on the capacitive current

To illustrate the individual and combined effects of G\textsubscript{j} and G\textsubscript{m} on the time course of I\textsubscript{C}, we performed a series of simulations of capacitive currents (I\textsubscript{Csim}) in 2 model circuits, one consisting of 2 identical cells (Fig. 10Ai) and the other consisting of one patched cell coupled to 4 others (Fig. 10Bi). In the 2-cell model, cells were connected by a R\textsubscript{j} with a value of 300 MΩ, or a G\textsubscript{j} of 3.3 nS, whereas R\textsubscript{j} was set to 800 MΩ in the five-cell model to yield a summed G\textsubscript{j} of 5 nS between the patched cell and the 4 cells to which it was coupled. Experimentally derived values of R\textsubscript{m1}, R\textsubscript{j}, R\textsubscript{s}, and C\textsubscript{cell} were used in both models. When R\textsubscript{m1} and R\textsubscript{j} were both set to equal 300 MΩ in the 2-cell model, to simulate the resting state, R\textsubscript{m} in the patched cell (cell 1) was 219 MΩ (Fig. 10Aii, trace a) and I\textsubscript{Csim} had a distinct nonmonotonic decay (Fig. 10Aii, trace a). When R\textsubscript{j} and R\textsubscript{m1} were both increased to 1 GΩ, to simulate blockage of both G\textsubscript{j} and G\textsubscript{m} by 18β-GA, the R\textsubscript{m} was increased over 4-fold to 988 MΩ (Fig. 10Aii, trace c), whereas I\textsubscript{Csim} now decayed with a single exponential (Fig. 10Aiii, trace c). Blocking G\textsubscript{j} alone abolished the slowly decaying component of I\textsubscript{Csim} (Fig. 10Aiii, trace b) but increased R\textsubscript{m} by only 26% (Fig. 10Aiii, trace b).

In the extended 5-cell model consisting of a patched cell coupled to 4 others, a similar nonmonotonic decay in I\textsubscript{Csim} was seen under resting conditions (Fig. 10Biii, trace d) in which R\textsubscript{m}
was equal to 161 ΜΩ (Fig. 10Bii, trace d). Increasing Rj1–Rj4 by 10-fold abolished the slow component of I_{C_{sim}} (Fig. 10Bii, trace e) and increased R_{in} to 285 ΜΩ (Fig. 10Bii, traces d and e). However, a 10-fold increase of both Rj1–Rj4 and R_{in} resulted in an increase of R_{in} to 1.4 GΩ (Fig. 10Bii, trace f) and removed the slow component of I_{C_{sim}} (Fig. 10Bii, trace f). These simulations illustrate that the slow component of I_{C} is likely to reflect electrical coupling of the patched cell to one or more neighboring cells with similar properties. In addition, they demonstrate that the membrane conductance of the patched cell also influences the overall R_{in} and that blockade of both G_m and G_j is required for R_{in} to be increased to levels that we observed experimentally (>600 ΜΩ) after treatment with 18β-GA.

DISCUSSION

Sustentacular cells in the olfactory epithelium of neonatal mice show electrical coupling

In the present study, we have demonstrated that SCs in the olfactory epithelium of neonatal mice are electrically coupled. On average, the magnitude of the junctional resistance (R_j) was in the region of 300 ΜΩ, although it ranged from 150 to >500 ΜΩ. This range of values is considerably larger than the R_j measured between pairs of cardiac myocytes where R_j may be as low as 2–5 ΜΩ (Sugiura et al. 1990; Weingart and Maurer 1987), but is similar to the value of R_j measured in chromaffin cells in tissue slices of rat adrenal gland (<1 GΩ) (Moser 1998). Just as we found in SCs, chromaffin cells in situ also failed to show dye-coupling (Moser 1998), suggesting that R_j needs to be in the tens of ΜΩ before dyes with molecular weights of several hundred Dalton can diffuse across gap junctions at a sufficient rate to accumulate in neighboring cells, assuming the connexins involved are nonselective and form homotypic channels. The range of deduced R_j values in SCs suggests that electrical coupling between SCs is not uniform, which may reflect, in part, ongoing dynamic control of electrical coupling by intracellular and extracellular factors (Conteras et al. 2002). Generally speaking, our results indicate that the apical surface of the OE behaves like a functional syn-cytium in which chemical mediators and electrical current can flow between SCs by gap junctions.

The extent to which our findings in OE slices from neonatal mice are applicable to SCs in the adult OE is unclear. Immunohistochemical studies in the OE of mice have shown that Cx43 immunoreactivity in newborn mice (P0) is similar to that in adult mice (Miragall et al. 1992). A more recent study in which FRIL (freeze-fracture replica immunogold labeling) was performed on SCs in the OE of adult mice showed that the majority of the 20 or so gap junctions found on each cell were composed of Cx43 subunits (J. Rash, personal communication). It should also be mentioned that expression of Cx45 has...
also been detected in the subapical region of the mouse OE where the cell bodies of SCs reside (Zhang and Restrepo 2002). This suggests that gap junctions in SCs of the mouse of OE may be formed from either Cx43 or Cx45 subunits or a combination of both these connexins (Martinez et al. 2002). If gap junctions on SCs are composed exclusively of Cx43 subunits, then we estimate that there would be nearly 30 gap junctions per cell, given the Rj value of 300 MΩ, which is equivalent to a Gj of 3.3 nS, and a unitary conductance of homotypic Cx43 gap junctions of 110 pS (Bennett et al. 2003; Conteras et al. 2003). This number is in close agreement with estimates from the FRIL study (J. Rash, personal communication) and confirms the relatively weak degree of electrical coupling, compared with cardiac myocytes where Gj can be as high as 200 nS (Sugiura et al. 1990; Weingart and Maurer 1987). If gap junctions between SCs were composed of Cx45, however, then there would be a 3-fold larger number of channels predicted to exist because the unitary conductance of homotypic Cx45 channels is 30 pS, whereas heterotypic channels would have conductances of 50–60 pS (Moreno 2004). This is still a relatively small number of gap junctions in SCs and may not be sufficient to permit significant dye transfer over the course of a typical recording (30 min), especially because large molecular weight fluorescent dyes such as Lucifer yellow are ≤35-fold less permeant than K through Cx43 gap junctions (Goldberg et al. 2004; Valiunas et al. 2002), whereas Cx45 gap junctions are thought to be impermeable to negatively charged dye molecules (Moreno 2004). In pairs of Xenopus oocytes expressing Cx43 channels, for example, transfer of Alexa 488 across a 10,000-fold larger Gj than what we have deduced for SCs, was barely detectable up to 0.5 h after intracellular injection (Weber et al. 2004).

Electrical coupling and resting leak conductance

In a previous study, we showed that SCs have a resting leak conductance (gL) that is permeable to cations and anions and can be substantially decreased by substituting Na for K in the bathing solution with a larger, less-permeable cation, that is, NMDG (Vogalis et al. 2005). Because the bulk of this conductance (about 7 nS) was inhibited by 18β-GA (20 μM), we attributed it to the opening of hemichannels that are permeable to both cations and anions (Valiunas et al. 1997), although this does not rule out the possibility that other types of “leak” channels may be responsible for this conductance. In light of our present results, and the fact that 18β-GA is likely to block hemichannels and gap junction channels without discrimination, it is likely that a large proportion of gL is attributable to Gj. Because the magnitude of Gj calculated from the amplitude of ICslow (Moser 1998) was estimated to be 4 nS for the matched recordings and 3 nS from the pooled data, then the membrane conductance (Gm) of a typical SC would be 3–4 nS. This means that up to half of gL that was inhibited by 18β-GA is likely to block a large proportion of gL attributable to Gj. Therefore based on a value of 161 MΩ that we reported previously as the apparent Rmem of the SC, which did not take into account Rj (Vogalis et al. 2005), the actual Rmem would be 305 MΩ and Rj would be 341 MΩ. From this value of Rj and the Rm value of 187 MΩ in Ringer solution, we estimate the coupling coefficient (CC) of SCs to be 0.39, where CC = (Rm/Rj)[1 + (Rm/Rj)] (Galarreta et al. 2004).

To illustrate the influence of electrical coupling on the time course of IC, we performed a series of simulations based on the experimentally derived values of Rj, Rmem, Ccell, and Rj. The results of these simulations indicated that the magnitude of the slow component of IC reflects the presence of junctional conductances between cells, whether a given cell is coupled to one or many. Despite not knowing the coupling stoichiometry of SCs, these simulations support our general conclusion that SCs are electrically coupled and that a portion of the Rm is attributable to a resting membrane conductance that may be generated by the opening of hemichannels. It should be mentioned that a portion of the resting conductance may be generated by a Cl-selective conductance because we previously found that 9-anthracene carboxylic acid (9-AC), albeit at a high concentration (0.5 mM), also decreased IC by about 40% (Vogalis et al. 2005). A more precise estimate of the relative magnitudes of Rj and Rmem between SCs and the relative contributions of individual ionic conductances may be obtained by recordings from pairs of SCs, before and after blockade of gap junctions, as has been performed in taste bud cells (Bigiani and Roper 1995).

Electrical coupling and presence of multiple-peak INa transients

In about a third of SCs, we noted that the INa triggered by a depolarizing step consisted of more than a single current peak. The additional current transients were absent, however, in SCs that were treated with 18β-GA, indicating that the smaller transients were likely to be generated in the coupled cells. This phenomenon can be explained as follows. If the resting potential of SCs in the OE slice is −50 mV (Vogalis et al. 2005) and Rj and Rmem have the same value and assumed to equal 300 MΩ, then in a simple 2-cell model, hyperpolarization of the patched cell to a holding potential of −110 mV will result in hyperpolarization of the coupled cell to −80 mV, at which 20–30% of INa would be available for activation. Thus when the patched cell is depolarized to a suprathreshold potential (e.g., 0 mV), the membrane potential of the coupled cell will be depolarized to −25 mV where the deinnected Na channels will be activated to generate an inward current. However, this will occur after a considerable latency because the change in membrane potential in the coupled cell will occur more slowly than in the patched cell, due to Rj, resulting in the activation of a delayed and distorted inward current. This is similar to what we observed experimentally and strongly suggests that SCs are electrically coupled.

Role of electrical coupling in purinergic receptor-activated conductances

Stimulation of P2 receptors in SCs increased the open probability of BK channels in cell-attached patches over 10-fold. We took advantage of this response, and the fact that in SCs dialyzed internally with 11 mM EGTA, no commensurate increase in IK was seen with ATP application, to further investigate the coupling between SCs. We found that the current that was activated by ATP in whole cell recordings from SCs filled with EGTA could be fitted with an equation for an underlying conductance that had a bell-shaped voltage dependency and was symmetrical about 0 mV. These proper-
ties are similar to those of gap junction conductances with respect to junctional voltage (Moreno 2004). The fact that this ATP-induced current was blocked by pretreatment with 18β-GA further supports the likelihood that it was generated by current flow across gap junctions. Interestingly, the peak $G_j$ estimated from these curve fits matched the values estimated from the magnitudes of $I_{\text{G慢}}$ (2–4 nS). The fact that a similar ATP-induced current was recorded from SCs filled with Cs$^+$ suggests that the rate of diffusion of Cs$^+$ from the patched cell to the coupled cell is not fast enough to accumulate in the latter and block BK channels. This situation would be analogous to trying to fill a cell with Cs$^+$-rich internal solution through a 300- to 500-MΩ sharp electrode.

Although the $I-V$ relationships of the junctional currents could be curve-fitted with equations describing gap junctional conductances quite adequately, the obvious assumptions about the change in membrane resistance of the coupled cells and the lack of action of ATP on other ionic conductances means that these fits are unlikely to accurately reflect the voltage dependency of the junctional conductances of SCs. Notwithstanding these shortcomings, the voltage of half-maximal activation and the slope factors of the $G_j-V$ curves suggest that the gap junctions in SCs may be formed by a mixture of Cx43 and Cx45 subunits (Moreno 2004), both of which have been detected in the mouse OE (Zhang and Restrepo 2002; Zhang et al. 2000).

**Functional significance of electrical coupling in SCs**

In tissues where the constituent cells function as a syncytium, gap junctions provide a means by which cellular activity can be coordinated. Gap junctions allow intracellular ions and messenger molecules to diffuse between cells under the relevant chemical and voltage gradients. In addition, gap junctions allow electrical current to flow between cells, although the importance of this function in SCs is unclear because under resting conditions, at room temperature, the resting potential of SCs (~50 mV) prevents them from generating action potentials (Vogalis et al. 2005). It is possible, however, that after suppression of the resting leak conductance, which may be generated by unopposed gap junction channels, SCs may hyperpolarize to a level that deinactivates Na$^+$ channels, allowing action potentials to be generated. By analogy with Müller cells in the retina (Reichenbach et al. 1986), SCs in the OE may possess electrogenic Na-K-ATPase that could generate sufficient outward current at higher temperatures to achieve this level of hyperpolarization, which would in turn deactivate the resting $g_{\text{leak}}$ (Vogalis et al. 2005). This would then increase the availability of Na$^+$ channels and allow SCs to fire action potentials that may propagate across gap junctions.

We have observed spontaneously generated Ca$^{2+}$ waves in OE slices (Hegg et al. 2005) but their dependency on intact gap junctions and hemichannels has not been tested. Ca$^{2+}$ waves could serve as a “housekeeping” mechanism to maintain a functional OE. One of the consequences of an increase in cytoplasmic Ca$^{2+}$ in SCs is the increased opening of BK channels. If BK channels are preferentially distributed on the apical surface of the SCs, then efflux of K$^+$ will be accompanied by efflux of water, which may assist in maintaining the surface of the OE semiaqueous, as well as providing a mechanism, in conjunction with Na-K-ATPase, for clearance of K$^+$ that accumulates in the OE. Gap junctions may assist in the redistribution of K$^+$ in the epithelium between regions of unequal concentration.

In summary, our results indicate that SCs in the OE are electrically coupled, despite the absence of dye-coupling. The magnitude of the junctional conductance is roughly similar to a resting membrane “leak” conductance, both of which are blocked by inhibitors of gap junctions. Further experiments will investigate the role of these membrane ion channel proteins in the propagation of Ca$^{2+}$ waves and related phenomena.

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