Electrical Coupling in Sustentacular Cells of the Mouse Olfactory Epithelium

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Abstract

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 or not coupling exists between SCs situated in tissue slices of OE from neonatal (P0-P4) mice. Using whole-cell and cell-attached patch recordings from SCs, we demonstrate that SCs are electrically coupled via junctional resistances on the order of 300 MΩ. 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 bi-exponential decay of capacitive currents recorded from SCs and from the bell-shaped voltage-dependence 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 two extracellularly linked hemichannels, or connexons, embedded in the membranes of adjoining cells (Spray and Bennett 1985; Bennett et al. 1991; Bennett and Verselis 1992; Bennett et al. 2003). Each hemichannel consists of six subunits, or connexins, which 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, dependence on trans-junctional 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 ($g_L$) (Vogalis et al. 2005) that was permeable to cations and anions and which showed outward rectification. A conductance of a similar ionic nature but with a
ten-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, $g_L$ 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 $g_L$ is mediated by the opening of unopposed gap junction channels. It is also possible that the decrease in the input resistance of SCs following treatment with 18β-GA may have been due to block 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 via the patch pipette, we failed to detect any dye-coupling that would suggest the presence of gap junctions. However, since 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) but 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 1kDa. However the rate at which molecules smaller than 1kDa, 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 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 following 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.

**Materials and Methods**

*Preparation of OE slices for patch clamp recording*

Coronal slices of olfactory epithelium, 250 µm in thickness, 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 on to a 12-mm cover slip coated with elastomer (Sylgard, Dow Corning) and placed in a tissue perfusion chamber (ca. 0.25 ml in volume), on an upright fluorescence microscope.
The slice was perfused (~1 ml/min) with Ringer’s solution at room temperature for 1 hr 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 bath was kept to a minimum. Currents were recorded and voltage command potentials were applied to cells using an Axopatch 200A amplifier (Axon Instruments, USA), driven by Clampex 8 (Axon Instruments), running on a dedicated PC. Capacitive currents were subtracted online by applying a P/4 subtraction protocol. Voltage-gated currents were low-pass filtered at 10 KHz and sampled at 20 KHz, while “leak” currents were sampled at 1 KHz. Patch recordings were low-pass filtered at 1 KHz and sampled at 5 KHz. Cells were filled with Alexa Fluor 488, hydrazine salt, dissolved in the pipette solution at 100 µM and were visualized using a B2A filter block (450–490-nm excitation and 520-nm barrier filters). Fluorescence images of cells and bright field images of the slices were captured using a monochrome digital camera (Microfire, Olympus USA) attached to the microscope and accompanying PictureFrame software. Images were processed using PictureFrame, Photoshop 6.0 and Illustrator 9 (Adobe).

SCs were patched by positioning the tip of the pipette adjacent to the apical surface of the OE using a micromanipulator (MP-225, Sutter) and then slowly advancing the electrode towards the surface. For consistency, recordings were obtained from the region corresponding to the dorsal nasal cavity. Positive pressure was applied until contact with the outermost cell layer and a high-resistance seal (0.5 - 2 GΩ) was allowed
to form before the membrane was ruptured to establish the whole-cell recording configuration.

Measurement of the input cell capacitance ($C_{\text{cell}}$), cell membrane resistance ($R_{\text{mem}}$), series resistance ($R_s$) and input resistance ($R_{\text{in}}$) was performed off-line, from recordings of capacitive current ($I_C$) elicited by 10 ms, 10 mV step hyperpolarizations from a holding potential of -78 mV, using an Igor Procedure file (Vogalis et al. 2005). SCs could be distinguished from olfactory receptor neurons (ORNs) by having a larger $C_{\text{cell}}$ (> 10 pF) and lower values of $R_{\text{in}}$ (Vogalis et al. 2005).

**Perfusion solutions and internal pipette-filling Solutions**

The standard Ringer’s solution used to perfuse slices consisted of (in mM): NaCl, 140; KCl, 5; MgCl$_2$, 1; CaCl$_2$, 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; MgCl$_2$, 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 which consisted of (in mM): CsF, 125; CsCl, 15; MgCl$_2$, 1; CaCl$_2$, 1; EGTA, 2.5; HEPES, 10; ATPK$_4$, 2; GTPLi, 0.2; pH 7.2 with CsOH. Liquid junction potentials (LJPs) 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 have been subtracted from the relevant membrane potential measurements.

**Drugs and other chemicals**
All the reagents used for making bathing solutions and internal pipette solutions were purchased from Sigma (St Louis, USA) unless indicated otherwise. 18β-glycyrrhetinic 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’s solution and tetraethylammonium chloride (TEA) was made up as a 1M stock solution which was 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 (I_C) 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 times 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 (V_p) to determine unit conductance and reversal potential.
Results

Absence of dye coupling between sustentacular cells (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 up to 25 min following whole-cell access (Fig. 1A(i)). 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. 1B(i)). 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 (I_Cnorm) recorded from the dye-filled cell depicted in Fig. 1A(i) decayed non-monotonically when plotted on a logarithmic scale (Fig. 1A(ii)). In contrast, the I_Cnorm of the 18β-GA-treated cell (Fig. 1B(i)) decayed for the most part (> 99% of peak current) with a single exponential (Fig. 1B(ii)). As described below, these effects of 18β-GA on the time course of I_Cnorm suggest that SCs are electrically coupled.

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

When two cells are electrically coupled via 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 \( C_{\text{cell}} \) will decrease if the junctional conductance \( G_j = 1/R_j \) is blocked. We found in the pooled results that \( C_{\text{cell}} \) derived from the time integral of \( I_C \) recorded from SCs bathed in Ringer’s solution averaged 18.5 ± 0.5 pF (n=191), while in SCs treated with 18β-GA, \( C_{\text{cell}} \) 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 \( C_{\text{cell}} \) was seen in matched recordings from 8 SCs, before and after treatment with 18β-GA. Here \( C_{\text{cell}} \) was decreased from 22.1 ± 1.9 pF to 16.6 ± 2.3 pF following treatment, a 25% reduction (p < 0.05, paired \( t \)-test) (Fig. 2 A(i), B(i)). The larger difference in \( C_{\text{cell}} \) seen in the matched recordings was likely due to the fact that the majority of these cells that were selected for treatment with 18β-GA had a clearly discernible slowly decaying component of \( I_C \) which, as described below, is an indication of electrical coupling (Fig. 2B(i), C).

Application of 18β-GA also elicited a large increase in the input resistance \( R_{\text{in}} \) of SCs, as we have reported previously (Vogalis et al. 2005). \( R_{\text{in}} \) 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 \( R_{\text{in}} \) was increased from 149 ± 32 MΩ to 485 ± 114 MΩ by 18β-GA (Fig. 2A(ii), B(ii)).

Estimation of \( R_j \) from the capacitive current \( I_C \)

The increase in \( R_{\text{in}} \) following 18β-GA treatment could be due to block 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 Rj from changes in the input Rin and
Ccell, 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 non-monotonic decay of IC recorded from the patched cell. This
is because Rj will be in series with the capacitance of the coupled cells and will therefore
contribute a component to the IC recorded in the patched cell. If IC decays with the sum
of two exponentials, then the dominant fast component (ICfast) will reflect charging of the
capacitance of the patched cell while the minor slow component (ICslow) will represent
charging of part of the membrane capacitance of one or more of the coupled cells (Moser
1998). Therefore the peak of ICslow will be proportional to the sum the the junctional
conductances in the patched cell.

As shown in Fig. 1A(ii) and Fig. 2C(ii), IC, or ICnorm, recorded from SCs bathed
in Ringer’s solution invariably decayed with two exponential time constants (τfast and
τslow) corresponding to ICfast and ICslow over the initial 5-10 ms following the current peak
(Fig. 2C(i)). This bi-exponential time course was manifest as two approximately linear
current components when ICnorm was plotted on a logarithmic scale (Fig.1A(ii) and Fig.
2C(ii)). In matched recordings from 8 SCs, τfast was unchanged (p > 0.05) following
18β–GA application (0.21 ± 0.04 ms before vs. 0.34 ± 0.08 ms after) and in 5 of these
cells, τslow was increased from 1.4 ± 0.3 to 2.5 ± 0.8 ms while ICslow was abolished in the
other three following 18β–GA treatment (Fig. 2C(ii)). Overall the proportion of ICslow 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 $I_{C_{slow}}$ represents mainly current flow across gap junctions into neighboring coupled cells, then the decrease in $I_{C_{slow}}$ following block of these gap junctions by 18β-GA can be used to estimate $R_j$ because the difference in the peak $I_{C_{slow}}$ before and after treatment with 18β-GA will be equal to the junctional current ($I_j$). $R_j$ is then equal to $I_j$, corrected for $R_s$, multiplied by 10 mV (i.e. the depolarizing step that elicited $I_C$). In the 8 SCs examined $R_j$ averaged $247 \pm 84$ MΩ ($n=8$).

A similar analysis of the pooled data also revealed differences in $I_{C_{slow}}$ between SCs treated with 18β-GA and those bathed in Ringer’s solution. As illustrated in Fig. 3A(i), the mean $I_C$ that was derived by trace-averaging the capacitive currents recorded from 118 SCs bathed in Ringer’s 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 $R_{in}$ of the untreated cells. The two traces of mean $I_C$ were then normalized to the respective peak mean current following subtraction of the steady-state component to obtain the respective mean $I_{C_{norm}}$ traces. When plotted on a logarithmic scale, these traces revealed that the $I_{C_{norm}}$ of untreated cells contained a fast and slow component and could be fitted with the sum of two exponential functions (Fig. 3A(ii), Ringer’s) while the slowly decaying component of the mean $I_{C_{norm}}$ of the treated cells was decreased in amplitude (Fig. 3A(ii), 18β-GA). The proportion of the peak $I_C$ attributable to $I_{C_{slow}}$ in the untreated cells was 9% and $\tau_{fast}$ and $\tau_{slow}$ were 0.3 ms and 2.4 ms respectively. The proportion of the mean peak $I_{C_{norm}}$ attributable to $I_{C_{slow}}$ in the 18β-GA-treated cells, however, was only 3% (Fig. 3A(ii)) and had a time constant ($\tau_{slow}$) of
1.7 ms while $\tau_{fast}$ 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. Given that peak $I_C$ averaged 505 pA in the untreated SCs (after correction for $R_s$), the mean $I_j$ was equal to 29 pA, yielding a $R_j$ of 341 M$\Omega$ (10 mV/29 pA).

The specificity of 18$\beta$-GA on the slower component of $I_C$ is also illustrated in Fig. 3B(i) which shows the normalized cumulative distribution of SCs plotted as a function of the two time constants, $\tau_{fast}$ (triangles) and $\tau_{slow}$ (circles), of the bi-exponential functions fitted to the $I_C$ in SCs treated (filled symbols) and untreated (open symbols) with 18$\beta$-GA. The larger shift in the distribution representing $\tau_{slow}$ following 18$\beta$-GA shows that $I_{C_{slow}}$ is associated with current flow across gap junctions, while the shift in the distribution representing $\tau_{fast}$ suggests that 18$\beta$-GA may also have non-specific affects on membrane properties. In a large proportion of untreated SCs (36/118 or 31%), $I_{C_{slow}}$ accounted for 12-50% of $I_C$ (Fig. 3B(ii)) 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 a similar effect to 18$\beta$-GA, in that $I_L$ was substantially reduced (Fig. 4A(i) & (ii)). The rate at which this occurred was much slower and required > 40 min of continuous perfusion for $R_{in}$ to increase two-fold (Fig. 4C). The increase in $R_{in}$ was accompanied by a decrease in $I_{C_{slow}}$ component as illustrated by the plot of $I_{C_{norm}}$ on a logarithmic scale (Fig. 4 B). These results indicate that carbenoxolone, like 18$\beta$-GA, decreases $I_{C_{slow}}$, an effect which is likely to be attributable to block 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 positive from -60 mV to –51 mV, while the slopes of the respective Boltzmann curves fitted to the data were 5 mV 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 $R_j$, determined from the amplitude of $I_{Cslow}$, 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) & (ii)). In the same cell, the $I_{Na}$ evoked in Ringer’s solution at -18 mV consisted of one major current transient that was followed by smaller secondary and tertiary transients that disappeared following 18β–GA treatment (Fig. 5A(iii)). 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) & (ii)), which also increased R_{in} (Fig. 5B(i)). R_{j} in this cell was estimated to be 164 MΩ.

**Effects of ATP on SCs reveals 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 likely target of such cytoplasmic Ca^{2+} transients are 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 a electrical syncytium, this hyperpolarization will induce current flow across R_{j}, if the membrane potential of the patched cell is clamped and activation of BK channels by Ca^{2+} is prevented, thereby allowing R_{j} to be estimated. To demonstrate that BK channels in SCs can be activated by purinergic receptor stimulation, ATP (40 µM) was peri-perfused on to the OE slice while recording channel activity from a cell-attached patch of a SC which resulted in rapid activation of BK channels (Fig. 6A, B(i), (i i)). In 5 patches from different SCs, ATP application increased their open probability (P_{o}) from 0.03 ± 0.02 to 0.38 ± 0.12 (p < 0.05, paired t-test) at a pipette potential (V_{p}) of 0 mV (Fig. 6B(iii), (iv)).

Although ATP increased the P_{o} of BK channels in cell-attached patches greater than ten-fold, there was no correspondingly large increase in the net outward current that was recorded from SCs in whole-cell mode, due to the fact that the internal solution for these recordings contained 11 mM EGTA to buffer internal Ca^{2+} to nM levels. Application of ATP (20 – 50 µM), 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-dependence of this ATP-induced current (I_{ATP}) SCs were ramp-
depolarized between -128 mV to + 42 mV over 2 s, before and during the peak of the
response to ATP (Fig. 7A(i)). The reversal potential (E_{rev}) of the ramp current recorded
in Ringer’s solution was shifted significantly negative from −41 ± 3 mV to −56 ± 3 mV
(n=13) in the presence of ATP (p < 0.05) (Fig. 7A(i)) while the ramp difference-current
(i.e. I_{ATP}) reversed at −95 ± 6 mV (Fig. 7A(ii)). An examination of the I-V relationship
of I_{ATP} revealed a quasi-linear region between −130 mV and −20 mV and a pronounced
inward rectification at potentials positive to 0 mV (Fig. 7A(ii)). The slope of I_{ATP}
between -130 and -20 mV (g_{ATP}) averaged 1.9 ± 0.3 nS. In 4 SCs tested, 2-5 mM
external TEA decreased I_{ATP} and the residual g_{ATP} averaged only 0.2 ± 0.2 nS (p < 0.05,
TEA vs. control, unpaired t-test). Similarly in 4 SCs pretreated with suramin (100 µM), a
P2Y receptor antagonist, g_{ATP} averaged only 0.3 ± 0.2 nS (n=4; p < 0.05 vs. control,
unpaired t-test), suggesting that I_{ATP} was generated by the opening of BK channels
following stimulation of P2Y receptors on the coupled cells.

To determine whether the I-V relationship of I_{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” dependence
shown by many types of homotypic gap junction conductances on junctional voltage (V_j)
(Moreno 2004). As shown in Fig. 7A(ii), I_{ATP} was well fitted by a curve generated by
this equation (the grey solid curve in Fig. 7A(ii)), in which it was assumed that in
response to ATP, R_{in} of the coupled cells decreased from 200 MΩ to 50 MΩ as they
hyperpolarized from −50 mV to −85 mV, while the R_{in} and resting potential of the
patched cell were unchanged. As shown in Fig. 7A(ii), the fitted curve adequately described $I_{\text{ATP}}$ and peak $G_j$ was 3.8 nS ($R_j = 265 \text{ M}\Omega$) and had a half-maximal activation/deactivation potential ($V_h$) of $+/−38 \text{ mV}$ and a slope factor ($V_s$) of 16 mV (inset in Fig. 7A(ii)). A similar analysis of $I_{\text{ATP}}$ in 8 SCs yielded an average $V_h$ of $+/−41 ± 6 \text{ mV}$ and a $V_s$ of $17 ± 2 \text{ mV}$, while peak $G_j$ averaged $2.2 ± 0.3 \text{ nS}$, which is equivalent to a $R_j$ of $454 \text{ M}\Omega$.

To confirm that $I_{\text{ATP}}$ was a junctional current, OE slices were pre-treated with 18β−GA before ATP was applied. Under these conditions, ATP failed to activate a significant outward ramp current (Fig. 7B(i)). In 9 K⁺-filled SCs, $E_{\text{rev}}$ of the ramp current averaged $−41 ± 3 \text{ mV}$ before and $−44 ± 4 \text{ mV}$ ($p > 0.05$, paired $t$-test) during the application of ATP, while $g_{\text{ATP}}$ averaged $0.2 ± 0.1 \text{ nS}$ ($p < 0.05$, vs. control, unpaired $t$-test) (Fig. 7B(ii)). Although these results strongly support the likelihood that activation of $I_{\text{ATP}}$ is dependent on the presence of conducting gap junctions, it is possible that that 18β-GA may also block purinergic receptors. To investigate this possibility, we obtained cell-attached patch recordings from SCs that were pre-treated 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 three cells tested (Fig. 8A,B). These results indicate that 18β-GA does not block activation of P2Y receptors by ATP.

Consistent with the idea that $I_{\text{ATP}}$ is a junctional conductance, ATP also activated an outward current in cells that were filled with Cs⁺ (Fig. 9A(i)). As in the K⁺-filled cells, the $E_{\text{rev}}$ of the ramp current was shifted negative by about 13 mV by ATP, from $−46 ± 12 \text{ mV}$ to $−60 ± 10 \text{ mV}$ (Fig. 9A(i)) while $I_{\text{ATP}}$ reversed at $−91 ± 3 \text{ mV}$ and $g_{\text{ATP}}$ for
the 4 Cs\(^+\)-filled cells averaged 1.3 ± 0.2 nS (Fig. 9A(ii)). As in the K\(^+\)-filled cells, pre-treatment with 18β-GA blocked \(I_{ATP}\) in these cells (Fig. 9B(i)&(ii)). Curve fitting of the \(I_{ATP}\) recorded in a Cs\(^+\)-filled cell (solid trace in Fig. 9A(ii)), also generated a bell-shaped \(G_j-V_j\) relationship (Fig. 9A(ii), inset) which had a \(V_h\) of –38 mV, a \(V_s\) of 20 mV and a maximal \(G_j\) of 2.2 ns corresponding to a \(R_j\) of 452 M\(\Omega\). These results indicate that \(I_{ATP}\) is likely to be generated indirectly following the opening of BK channels in the coupled cells and suggest that the amount of Cs\(^+\) 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 \(R_j\) and \(R_m\) on the capacitice current

To illustrate the individual and combined effects of \(G_j\) and \(G_m\) on the time course of \(I_C\), we performed a series of simulations of capacitive currents (\(I_{Csim}\)) in two model circuits, one consisting of two identical cells (Fig. 10A(i)) and the other comprised of one patched cell coupled to four others (Fig. 10B(i)). In the two-cell model, cells were connected by a \(R_j\) with a value of 300 M\(\Omega\), or a \(G_j\) of 3.3 nS, while \(R_j\) was set to 800 M\(\Omega\) in the 5-cell model to yield a summed \(G_j\) of 5 nS between the patched cell and the four cells to which it was coupled. Experimentally derived values of \(R_{mem}, R_j, R_s\) and \(C_{cell}\) were used in both models. When \(R_{m1}\) and \(R_j\) were both set to equal 300 M\(\Omega\) in the two-cell model, to simulate the resting state, \(R_{in}\) in the patched cell (cell 1) was 219 M\(\Omega\) (Fig. 10A(ii) trace a) and \(I_{Csim}\) had a distinct non-monotonic decay (Fig. 10A(iii), trace a). When \(R_j\) and \(R_{m1}\) were both increased to 1 G\(\Omega\), to simulate block of both \(G_j\) and \(G_m\) by 18β-GA, the \(R_{in}\) was increased over four-fold to 988 M\(\Omega\) (Fig. 10A(ii), trace c) while
ICsim now decayed with a single exponential (Fig. 10A(iii), trace c). Blocking Gj alone abolished the slowly decaying component of ICsim (Fig. 10A(iii), trace b) but increased Rm by only 26% (Fig. 10A(ii), trace b).

In the extended 5-cell model consisting of a patched cell coupled to 4 others, a similar non-monotonic decay in ICsim was seen under resting conditions (Fig. 10B(iii), trace d) in which Rm was equal to 161 MΩ (Fig. 10B(ii), trace d). Increasing Rj1 – Rj4 ten-fold abolished the slow component of ICsim (Fig. 10B(iii), trace e) and increased Rm to 285 MΩ (Fig. 10B(ii), traces d, e). However, increasing both Rj1 – Rj4 and Rm1 ten-fold resulted in an increase in Rm to 1.4 GΩ (Fig. 10B(ii), trace f) and removed the slow component of ICsim (Fig. 10B(iii), trace f). These simulations illustrate that the slow component of IC 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 Rm and that block of both Gm and Gj is required for Rm to be increased to levels that we observed experimentally (> 600 MΩ) following 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 (Rj) was in the region of 300 MΩ, although it ranged from < 150 MΩ to > 500 MΩ. This range of values is considerably larger than the Rj measured between pairs of cardiac myocytes where Rj may as low as 2-5 MΩ (Weingart and Maurer 1987; Sugiura
et al. 1990), but is similar to the value of $R_j$ measured in chromaffin cells in tissue slices of rat adrenal gland ($< 1 \, \text{G} \Omega$) (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 $\text{M} \Omega$ 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 non-selective 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, on going dynamic control of electrical coupling by intracellular and extracellular factors (Contreras et al. 2002). Generally speaking, our results indicate that the apical surface of the OE behaves like a functional syncytium in which chemicals mediators and electrical current can flow between SCs via 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 is similar in newborn mice (P0) as 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 sub-apical 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 approximately 30 gap junctions per cell, given an $R_j$ of 300 MΩ, which is equivalent to a $G_j$ of 3.3 nS, and a unitary conductance of homotypic Cx43 gap junctions of 110 pS (Bennett et al. 2003; Contreras 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 $G_j$ can be as high as 200 nS (Weingart and Maurer 1987; Sugiura et al. 1990). If gap junctions between SCs were composed of Cx45, however, then there would be a three-fold larger number of channels predicted to exist, since the unitary conductance of homotypic Cx45 channels is 30 pS, while 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 since large molecular weight fluorescent dyes such as Lucifer Yellow are up to 35-fold less permeant than $K^+$ through Cx43 gap junctions (Valiunas et al. 2002; Goldberg et al. 2004) while 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 $G_j$ than what we have deduced for SCs, was barely detectable up to half an hour following 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 ($g_L$) which is permeable to cations and anions and can be substantially decreased by
substituting Na\(^+\) in the bathing solution with a larger less permeable cation, namely NMDG (Vogalis et al. 2005). Because the bulk of this conductance (~ 7 nS) was inhibited by 18-βGA (20 µM), we attributed it to the opening of hemichannels that are permeable to both cations and anions (Valiunas et al. 1997) but 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 \(g_L\) is attributable to \(G_j\). Given that the magnitude of \(G_j\) calculated from the amplitude of \(I_{C_{\text{slow}}}\) (Moser 1998) was estimated to be 4 nS for the matched recordings and 3 nS from the pooled data, then the membrane conductance \((G_m)\) of a typical SC would be 3-4 nS. This means that up to half of \(g_L\) that was inhibited by 18-βGA is attributable to \(G_j\).

Therefore, based on a value of 161 MΩ that we reported previously as the apparent \(R_{\text{mem}}\) of a SC, which did not take into account \(R_j\) (Vogalis et al. 2005), the actual \(R_{\text{mem}}\) would be 305 MΩ and \(R_j\) will be 341 MΩ. From this value of \(R_j\) and an \(R_{in}\) of 187 MΩ in Ringer’s solution, we estimate the coupling coefficient \((CC)\) of SCs to be 0.39, where \(CC = (R_{in}/R_j)/(1 + R_{in}/R_j)\) (Galarreta et al. 2004).

To illustrate the influence of electrical coupling on the time course of \(I_C\), we performed a series of simulations based on the experimentally derived values of \(R_{sv}\), \(R_{\text{mem}}\), \(C_{\text{cell}}\) and \(R_j\). The results of these simulations indicated that the magnitude of the slow component of \(I_C\) 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 \(R_{in}\) is attributable to a resting membrane...
conductance that may be generated by the opening of hemichannels. It should be mentioned that a component of the resting conductance may be generated by a Cl- selective conductance because we found previously that 9-anthracene carboxylic acid (9-AC), albeit at a high concentration (0.5 mM), also decreased I_L by about 40 % (Vogalis et al. 2005). A more precise estimate of the relative magnitudes of R_j and R_mem 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 I_{Na} transients

In about a third of SCs, we noted that the I_{Na} 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 R_j and R_mem have the same value and assumed to equal 300 MΩ, then in a simple two-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 I_{Na} 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 de-inactivated 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 $R_j$, 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 ten-fold. We took advantage of this response, and the fact that in SCs dialysed internally with 11 mM EGTA, no commensurate increase in $I_K$ 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 dependence and was symmetrical about zero mV. These properties 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 pre-treatment 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 $I_{Cs,\text{slow}}$ (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 300-500 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-dependence of the junctional conductances of SCs. Notwithstanding these shortcomings, the voltage of half-maximal activation and the slope-factors of the $G_J-V_J$ 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 et al. 2000; Zhang and Restrepo 2002).

**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 following suppression of the resting leak conductance, which may be generated by unopposed gap junction channels, SCs may hyperpolarize to a level that de-inactivates Na$^+$ channels, allowing action potentials to be generated. By analogy with Muller 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 $g_L$ (Vogalis et al. 2005). This would then increase the availability of \( \text{Na}^+ \) channels and allow SCs to fire action potentials that may propagate across gap junctions.

We have observed spontaneously generated \( \text{Ca}^{2+} \) waves in OE slices (Hegg et al. 2005) but their dependence on intact gap junctions and hemichannels has not been tested. \( \text{Ca}^{2+} \) waves could serve as a “housekeeping” mechanism to maintain a functional OE. One of the consequences of an increase in cytoplasmic \( \text{Ca}^{2+} \) 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 \( \text{K}^+ \) will be accompanied by efflux of water, which may assist in maintaining the surface of the OE semi-aqueous, as well as providing a mechanism, in conjunction with Na-K ATPase, for clearance of \( \text{K}^+ \) that accumulates in the OE. Gap junctions may assist in the re-distribution of \( \text{K}^+ \) 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 \( \text{Ca}^{2+} \) waves and related phenomena.
Figure Legends

Fig. 1. Lack of dye-coupling between sustentacular cells (SCs) in the olfactory epithelium of neonatal mice. A, (i) Fluorescence photomicrograph of a SC filled with Alexa 488 (100 µM), taken approximately 20 min following 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_{Cnorm}) recorded in the same cell showing non-monotonic 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-GA (20 µM), taken approximately 20 min following break-in. (ii) I_{Cnorm} recorded in the 18β-GA-treated cell showing mono-exponential 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 a 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 nearly four-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_{Cnorm} recorded in Ringer’s solution and following application of 18β-GA. Note bi-exponential 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 $18\beta$-GA. Solid lines were fitted by eye.

**Fig. 3.** Sustentacular cells (SCs) pretreated with $18\beta$-GA have larger $R_{in}$ and lower $C_{cell}$ values than SCs bathed in Ringer’s. A, (i) Trace-averaged mean $I_C$ (inverted) obtained by averaging the capacitive currents recorded from 118 SCs bathed in Ringer’s solution and 91 SCs treated with $18\beta$-GA. $I_C$ was evoked by a 10 mV hyperpolarizing step. The grey region about each trace represents ± SEM. Note approximately four-fold larger $R_{in}$ in the $18\beta$-GA-treated cells. (ii) Time-averaged mean $I_{C_{norm}}$ for each population of cells, plotted on a semi-log scale. The current traces have each been fitted with a sum of two exponential functions and the fitted curves are superimposed on the data. Note the decrease in the relative amplitude of the slow component of decay of $I_{C_{norm}}$ ($I_{C_{slow}}$) in the $18\beta$-GA-treated cells, which was reduced from 9% to 3% of the peak current. B, (i) Cumulative distribution of SCs plotted as a function of 2 time constants fitted to the $I_C$ in each cell. $\tau_{fast}$, triangles; $\tau_{slow}$, circles. Open symbols represent recordings in Ringer’s solution and filled circles represent recordings in the presence of $18\beta$-GA. (ii) Histogram distribution of the relative amplitude of $I_{C_{slow}}$ in the untreated cells. In 31% of cells, $I_{C_{slow}}$ was > 12% of the peak current.

**Fig. 4** Suppression of leak current and reduction of $I_{C_{slow}}$ by carbenoxolone. A, (i) Whole-cell recording from a SC using a KF internal solution, showing the negatively deactivating leak current ($I_L$) in response to step hyperpolarization of the cell bathed in Ringer’s solution. (ii) Marked suppression of $I_L$ by carbenoxolone (100 µM) after one
hour of continuous perfusion. B, Reduction of the $I_{C_{slow}}$ component of $I_C$ by carbenoxolone. Arrow points to time course of $I_{C_{slow}}$ in Ringer’s solution. C, Slow time course of increase in $R_{in}$ by carbenoxolone. $R_{in}$ was determined from the current deflections elicited by 10 ms, 10 mV step hyperpolarizations of the cell at 2 Hz from a holding potential of -78 mV. Note failure of $R_{in}$ to recover following 20 min wash out of carbenoxolone (*).

**Fig. 5.** Electrical coupling is associated with multiple peaks in $I_{Na}$ recorded from sustentacular cells. A, (i) “Leak” current ($I_L$) recorded from a SC bathed in Ringer’s solution, in response to the voltage steps as indicated (upper traces). (ii) Following application of 18β-GA (20 µM), $I_L$ was substantially decreased. (iii) In Ringer’s solution, $I_{Na}$ evoked by a test depolarization to -18 mV had multiple peaks (arrow) indicating activation of $I_{Na}$ in neighboring cells. Superimposed is $I_{Na}$ evoked by the same test depolarization in the presence of 18β-GA, showing abolition of the additional multiple peaks. B, (i) Recordings of $I_C$ when the cell was bathed in Ringer’s solution and following application of 18β-GA, as indicated, showing decrease in $R_{in}$ in the presence of 18β-GA. Dashed lines indicate the baseline current level. (ii) Plots of $I_{C_{norm}}$ recorded from the same cell, before and after treatment with 18β-GA. Note the abolition of the slow exponential term by the gap junction inhibitor, indicating that current flow between the patched cell and the coupled cells was blocked.

**Fig. 6.** Cell-attached patch recordings from a sustentacular cell bathed in Ringer’s solution. Pipette was filled with KF internal solution containing 11 mM EGTA. A,
Continuous recording with the pipette potential ($V_p$) maintained at 0 mV. Peri-perfusion of ATP (bar below trace) increased the openings of large-conductance $K^+$ (BK) channels. Basal activity was restored following termination of ATP perfusion. B, (i) Expanded portion of the trace in panel A, showing the onset of increased BK channel activity in the patch, which contained 2 channels. Openings are upward and indicated by dashed lines; "c" represents closed state. (ii) Unitary current of the BK channel at different $V_p$. Regression line fitted through the data points yielded a unitary conductance of 247 pS. (iii) & (iv) All-points histograms constructed from 10-s portions of the recording in panel A, before (iii) and during application of ATP (iv), showing >10-fold increase in open probability ($P_o$) (see Methods).

Fig. 7. Whole-cell recordings from SCs using KF internal solution containing 11 mM EGTA. A, (i) Ramp currents elicited by ramp depolarizations as indicated, before and during application of ATP to the slice. Inset shows the 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 two traces shown in (i). The difference current was curve-fitted with equation 3. Equation 3 was derived by firstly summing two Boltzmann functions to generate a symmetrical bell-shaped curve as a function of voltage to describe the voltage dependence 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. 4) assuming that at rest, $R_{\text{in}}$ 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. 5), where it was assumed that $R_{in}$ 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 dependence of the junctional conductance by solving for $R_j$. Thus:

$$I_j = \frac{V_{jc}}{R_j} \left[ \frac{1}{1+\exp((V_{jc}+V_h)/-V_s)} + \frac{1}{1+\exp((V_{jc}-V_h)/V_s)} -1 \right]$$

$$- \frac{V_{ja}}{R_j} \left[ \frac{1}{1+\exp(V_{ja}+V_h)/-V_s}) + \frac{1}{1+\exp(V_{ja}-V_h)/V_s}) -1 \right]$$

(eq. 3)

Where $V_{jc}$ is the transjunctional voltage in the absence of ATP and is equal to:

$$V_{jc} = \frac{(V_p - (-50)) \times R_j}{(R_j + 200)} - 50$$

(eq. 4)

and $V_{ja}$ is the transjunctional voltage in the presence of ATP and is equal to:

$$V_{ja} = \frac{(V_p - (-85)) \times R_j}{(R_j + 50)} - 85$$

(eq. 5)

where $V_p$ in eq. 4 & 5 is the pipette potential in the patched cell. Curve-fitting the ramp difference-current with eq. 3 yielded a bell-shaped conductance-voltage curve (inset) which had a $V_h$ of 38 mV, 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) The difference current over the voltage range tested was essentially zero, indicating that ATP, following block of gap junctions, failed to elicit a response.

Fig. 8  Cell-attached patch recordings from a sustentacular cell bathed in Ringer’s solution containing 18β-GA (20 µM). Pipette was filled with KF internal solution containing 11 mM EGTA. A, Continuous recording with the pipette potential (V_p) maintained at +10 mV. “c” represents closed channel level. Perfusion of ATP (50 µM) from a pipette adjacent to the slice caused a large increase in the frequency of channel opening and the number of channels open. B, All-points histograms constructed from 20-s portions of the trace in panel A. In the absence of ATP, 2 BK channel openings are evident. During ATP application, up to 7 BK channels are open simultaneously indicating that 18β-GA does not affect stimulation of purinergic receptors by ATP and subsequent activation of BK channels.

Fig. 9. Whole-cell recordings from SCs using CsF internal solution containing 2.5 mM EGTA. A, (i) Ramp currents elicited by ramp depolarizations as indicated, before and during application of ATP to the slice. Inset shows the time course of the reversible increase in the outward current at a time-point during the ramp depolarization corresponding to -10 mV. (ii) Instantaneous I-V relationship of I_{ATP} in the Cs-filled cell showing a linear region between -20 mV and -130 mV. The superimposed trace represents the curve fit of the data using eq. 3 and the inset shows the voltage-dependence of the relative G_j in this cell. V_h was 38 mV, V_s was 20 mV and R_j was
equal to 452 MΩ. B, Ramp currents elicited as in panel A, in another SCs filled with CsF, and treated with 18β-GA. (ii) There was little or no $I_{ATP}$ activated as indicated by the I-V relationship, suggesting induction of $I_{ATP}$ is dependent on gap junctional communication.

**Fig. 10.** Simulations of capacitive currents in a model circuits. A, (i) Model consisting of 2 cells connected by a junctional resistance ($R_j$). Simulations were performed using Topspice Demo Version (http://www.penzar.com). $R_s$ (the series resistance) is 20 MΩ, $C_1$ and $C_2$ represent the capacitance of the cells, both equal to 16 pF, and $R_{m1}$ and $R_{m2}$ are the membrane resistances of the cells and initially equaled 300 MΩ, as with $R_j$, the junctional resistance. (ii) Simulated $I_C$ ($I_{Csim}$) (trace “a”) evoked by a 10 mV voltage step with initial values of $R_{m1}$ and $R_j$. The input resistance ($R_{in}$) under these conditions was 219 MΩ. Trace “b” represents $I_{Csim}$ when $R_j$ is increased to 1 GΩ, resulting in an increase of $R_{in}$ to 295 MΩ. Trace “c” represents $I_{Csim}$ when $R_j$ and $R_{m1}$ were increased to 1 GΩ, to simulate block of both hemichannels and gap junctions. This raised $R_{in}$ to 988 MΩ. (iii) Semi-log plot of normalized $I_{Csim}$ traces as indicated, showing characteristic bi-exponential decay of the $I_{Cnorm}$ when the cells were connected by a $R_j$ of 300 MΩ (trace “a”), and abolition of the slow decaying component when $R_j$ was increased to 1 GΩ, to simulate gap junction block by 18β-GA (trace “b”). Setting $R_{m1}$ to 1 GΩ had no additional effect on rate of decay $I_{Cnorm}$ (trace “c”). B,(i) Simulations in a 5 cell model. Parameter values are the same as in panel A with the exception that the junctional resistances ($R_j1$-$R_j4$) were initially 800 MΩ. (ii) $I_{Csim}$ in cell 1 under initial conditions (trace d). Increasing $R_j1$-$R_j4$ to 8 GΩ to simulate gap junction blockade
increased $R_{in}$ from 161 to 285 MΩ (trace e). Further block of the resting membrane conductance of cell 1 ($R_{m1}$) to 3 GΩ increased $R_{in}$ to 1.4 GΩ (trace f). (iii) Traces of $I_{Cnrm}$ of the corresponding traces shown in (i), showing that an $I_{Cslow}$ component is discernible in a model consisting of one cell coupled to four other via a junctional conductance.
References


Fig. 1
Fig. 2
Fig. 3
(i) Ringer's

(ii) carbenoxolone 100 µM (1 hr)

Fig. 4
Fig. 5
Fig. 6

A TP 40 µM

Vp = 0 mV

200 ms

10 pA

\[ \gamma = 247 \text{ pS} \]

Vp (mV)

P0 = 0.014

0.8

0.12

P0 = 0.6

Fig. 6
Fig. 7
Fig. 8

A

V_p = +10 mV

ATP 50 µM

18β-GA 20 µM

20 pA

10 s

B

 counts x 10^3

-ATP

+ATP

0 20 40 60 80 100 pA

0 20 40 60 80 100 pA

counts

400

200

100

50

0

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Fig. 9
Fig. 10