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ATP and Nitric Oxide Modulate Intracellular Calcium in Isolated Pillar Cells of the Guinea Pig Cochlea

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ABSTRACT

Supporting cells in the mammalian cochlea have recently received attention as potential targets of neurotransmitters, neuromodulators, and neurohumoral agents. Calcium homeostasis in Deiters' and Hensen's cells, for example, is regulated by ATP and nitric oxide. We studied the intracellular calcium concentration $[Ca^{2+}]_i$ in isolated pillar cells of the guinea pig cochlea in response to extracellular ATP and nitric oxide using the fluorescent indicator fluo-3. $[Ca^{2+}]_i$ increased rapidly and significantly throughout the pillar cell in response to a bolus of ATP or 2-methylthio ATP while α, β-methylene ATP was ineffective. The response to ATP was inhibited by suramin and Cibacron Blue but not by pyridoxal phosphate 6-azopheny1-2′,4′-disulfonic acid. This pharmacological profile is consistent with a $[Ca^{2+}]_i$ increase largely mediated by P2Y receptors. In Ca^{2+}-free medium supplemented with EGTA, the response to extracellular ATP was reduced by 33%, suggesting a contribution of calcium influx to the overall effect. The ATP-induced increase of $[Ca^{2+}]_i$ was attenuated by NO donors (sodium nitroprusside or diethylamine NONOate), and this attenuation was reversed by KT5823, an antagonist to protein kinase G. The results indicate the involvement of purinergic mechanisms and the nitric oxide/cyclic GMP/protein kinase G pathway in the regulation of $[Ca^{2+}]_i$ in cochlear pillar cells.

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INTRODUCTION

The so-called supporting cells of the mammalian cochlea, primarily Deiters', Hensen's, and pillar cells, have historically been considered a structural support for the anatomical organization of the organ of Corti. However, recent evidence has shown that these cells may influence auditory transduction in a more active fashion. Deiters' and Hensen's cells are innervated (Burgess et al. 1997; Fechner et al. 1998) and contain receptors for neurotransmitters and neuromodulators such as ATP and acetylcholine (Dulon et al. 1991, 1993; Sugasawa et al. 1996; Matsunobu and Schacht 2001). Furthermore, intracellular calcium $[Ca^{2+}]_i$ in Deiters' and Hensen's cells is modulated by purinergic receptors and the nitric oxide/cyclic GMP/protein kinase G (NO/cGMP/cGK) pathway (Matsunobu and Schacht 2000). The fact that such mechanisms are widespread in the cochlea reinforces the idea that supporting cells are under neuronal and humoral control and participate in homeostatic regulation or feedback signaling in the organ of Corti in both normal and pathological states (Thorne and Housley 1996; Fridberger et al. 1998; Flock et al. 1999).

Pillar cells form a tight junction with the hair cells and maintain the space of Nuel, transmitting stimulus-induced motion between the basilar membrane, the reticular lamina, and the hair cells (Slepecky 1996). Their structural role is emphasized by a large number of organized microfilaments and microtubules in their cell bodies, providing tensile strength. Pillar cells are not innervated but are responsive to neurohumoral

agents. ATP can induce an inward current by activation of P2X receptors (Chen et al. 1998), and the presence of soluble guanylate cyclase and protein kinase G (Fessenden and Schacht 1997; Tian et al. 1999; Michel et al. 1999) provides for the possible involvement of NO in the regulation of Ca^{2+} homeostasis. In this study, we investigate $[Ca^{2+}]_i$ in isolated pillar cells from the guinea pig cochlea in response to purinergic stimulation and NO.

MATERIALS AND METHODS

Cell preparation

Pigmented adult male guinea pigs (200–250 g) were obtained from Elm Hill Breeding Laboratory (Chelmsford, MA). The experimental protocols were in compliance with the guidelines of the National Institutes of Health and the Declaration of Helsinki and were approved by the University of Michigan's Committee on Use and Care of Animals. Animal care was under the supervision of the University of Michigan's Unit for Laboratory Animal Medicine.

Animals were anesthetized with CO_2 and decapitated. The cochlea was removed and immersed in Hank's Balanced Salt Solution (HBSS; pH 7.4, 300 ± 2 mOsm). The bony wall was immediately removed and the stria vascularis and the tectorial membrane were detached. The organ of Corti was then separated from the spiral lamina using a fine metal probe, and pillar cells were dissociated essentially as described for the isolation of outer hair cells (Zenner et al. 1985; Zajic and Schacht 1987). Dissected strips of the organ of Corti were transferred into a 50-μL droplet of HBSS. A 50-μL aliquot of collagenase (Sigma type IV; 1 mg/mL in HBSS) was added, and the strips were incubated for 20 min at room temperature. They were then transferred into a 50-μL droplet of fresh HBSS, and gentle trituration of the tissue pieces through a 50-μL Hamilton syringe completed the dissociation procedure. The isolated cells were allowed to settle onto the coverslip for 20 min prior to the experiments.

Calcium imaging

Changes in $[Ca^{2+}]_i$ were determined with fluo-3, a fluorescent calcium indicator that increases its fluorescence emission upon binding to nanomolar concentrations of calcium (Minta et al. 1989). One-millimolar stock solutions of the cell-permeant acetoxymethyl ester derivative of fluo-3 (fluo-3/AM) were prepared in dry dimethyl sulfoxide (DMSO) and stored at −20°C. For dye loading, cells were incubated for 30 min at room temperature with fluo-3/AM diluted in HBSS to a final concentration of 5 μM. The cells were maintained in a 50-μL droplet of HBSS on a glass coverslip in a humid chamber. After loading, they were carefully rinsed with fresh HBSS.

Cells loaded with fluo-3 were observed with an inverted microscope (Leitz Fluovert FS) fitted with an epifluorescence system (ArcLamp HBO 100 W, a 450–490-nm bandpass exciter filter, 510-nm dichroic mirror, and a 520-nm barrier filter for excitation and emission, respectively) and Nikon objectives (Plan A 60/1.4 oil, Fluor 100/1.3 oil). The excitation irradiance was reduced by two neutral density filters, attenuating the passage of light by 93.5% in order to prevent photobleaching. No significant photobleaching was noted with illumination of up to 5 min, the maximal continuous exposure time in this study.

Cell fluorescence was monitored via a Silicon Intensified Target camera (SIT 66, Dage-MTI Inc., Michigan City, IN) and the recorded images were analyzed with an Axon Imaging system using Workbench 2.1 software (Axon Instruments Inc., Foster City, CA). Thirty-two sequential frames (within 1.066 s) from the camera were averaged and the fluorescence intensity of the cell images was measured by averaging the pixel radiance value of the cell or segments of the cell.

The amplitude of an evoked response is defined according to Neher and Augustine (1992) as the ratio of the relative fluorescence under test conditions to the fluorescence at time zero of the experiment (F/F_0). Absolute values for $[Ca^{2+}]_i$ were estimated by a combined *in vitro* and *in vivo* calibration (Kao et al. 1989; Dulon et al. 1990). $[Ca^{2+}]_i$ was calculated by using the equation $[Ca^{2+}]_{free} = K_d(F - F_{min})/(F_{max} - F)$. K_d is the dissociation constant for the fluo-3/calcium complex and F is the observed *in situ* fluorescence in arbitrary units of intact fluo-3-loaded cells. F_{max} is the maximum fluorescence and is calculated by using the equation $F_{max} = (F_{Mn} - F_{bkg})/0.2 + F_{bkg}$, where F_{Mn} is the fluorescence obtained after treatment of the cells with ionomycin (10 μM for 60 s) and $MnCl_2$ (2 mM for 3–5 min) to saturate the intracellular indicator (Vandenberghe and Ceuppens 1990), and F_{bkg} is the fluorescent background signal remaining after lysis of the cells with 100 μg saponin/mL. The factor 0.2 is the ratio of the fluorescence of Mn^{2+}-saturated fluo-3 to Ca^{2+}-saturated fluo-3. The fluorescence minimum F_{min} was obtained from the equation $F_{min} = (F_{max} - F_{bkg})/40 + F_{bkg}$, where 1/40 is the ratio of the fluorescence of metal-free fluo-3 to its Ca^{2+} complex (Harkins et al. 1993). *In vitro* fluorescence F values for $[Ca^{2+}]_{free}$ were obtained at different calcium concentrations using the calcium calibration buffer kit (Molecular Probes Inc., Eugene, OR). The data obtained from *in situ* and *in vitro* calibration were plotted as log $[Ca^{2+}]_{free}$ vs. log $\{(F - F_{min}) / (F_{max} - F)\}$. From the x-intercept, a K_d of 316 nM was calculated,

a value similar to that found in other reports (Minta et al. 1989; Kao et al. 1989).

Application of purinergic agonists

The cells were stimulated by a 10-s bolus of ATP, 2-methylthio ATP (2MeSATP), or α, β-methylene ATP (α, β-meATP; each in HBSS, pH 7.4, 298 mOsm) applied extracellularly through a glass pipette (Sterile Femtotips, Eppendorf, Hamburg, Germany) connected to a pressure ejector (PLI-100, Medical Systems Corp., Greenvale, NY). The pipette (opening = 0.5 ± 0.2 μm) was placed about 50 μm away from the cell. A negative holding pressure was applied after a bolus delivery to prevent diffusion of the solution out of the pipette.

Concentrations of all agents applied in this manner are stated as the concentration in the ejected solution, and the actual concentration reaching the cells was approximated as follows. A solution of 1 mM fluorescein was ejected from the pipette into 50 μL of distilled water. By comparison of the resultant fluorescence in the water to a standard curve of fluorescein, the dilution of the injected solution was determined. From the dilution factor, a volume of 32 ± 5 pL per bolus was calculated. Since the pipette was located 50 μm away from the cell, the bolus will diffuse—under the simplest assumption—into a sphere with a radius of 50 μm and a volume of >500 pL before reaching the cell. The agents are thus diluted 10- to 20-fold from their original concentration when they reach the cell, and this concentration will rapidly decrease as the bolus further spreads around the cell.

Application of other agents

Purinergic antagonists, NO donors, and inhibitors were preincubated with the cells for 5 or 20 min (see the Results section for details) prior to the application of ATP or other agonists. The agents were added to the 50-μL droplet containing the cells as 1-μl aliquots of 50× stock solutions. Suramin, PPADS, and Cibacron Blue 3GA were each dissolved in HBSS (pH 7.4) and the osmolality was adjusted to 300 ± 2 mOsm. Sodium nitroprusside (SNP) and diethylamine NONOate (DEA-NO) were also dissolved in HBSS (pH 7.4). KT5823 was dissolved in DMSO and diluted in HBSS to obtain the desired concentration (final concentration of DMSO in the bath was 0.06%). During the preincubations, cells were not illuminated in order to avoid photobleaching.

Chemical reagents

Hank's Balanced Salt Solution (HBSS: 1.25 mM $CaCl_2$, 5.55 mM glucose, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 136.9 mM NaCl, 0.34 mM Na_2HPO_4, 5.4 mM KCl, 5.0 mM sodium HEPES, with osmolality adjusted to 300 ± 2 mOsm with NaCl; pH 7.4) and Ca^{2+}-free HBSS were purchased from Gibco (Grand Island, NY). Fluo-3/AM, fluo-3 pentapotassium salt, and calcium-buffered standard solutions were obtained from Molecular Probes Inc. (Eugene, OR), DEA-NO from Cayman Chemical (Ann Arbor, MI), and KT5823 from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

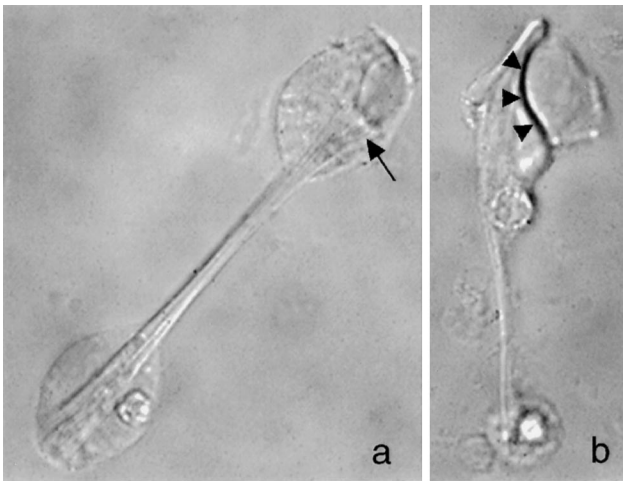

FIG. 1. Isolated outer (a) and inner (b) pillar cells. Cells show distinct microtubules, a clearly delineated cell membrane, and nucleus. Microtubules fan out at the apical portion of the outer pillar cells (arrow). In the inner pillar cell, the microtubules bend toward the site of the outer pillar and outer hair cell, and the apical portion forms a cuplike structure for contact with the outer pillar cell (arrowheads).

Data analysis

All values are presented as mean ± SEM. Differences between mean values were evaluated using one-way ANOVA. A p value of <0.05 was considered significant.

RESULTS

Identification and selection of pillar cells

The pillar cells maintained a distinct shape after isolation (Fig. 1) reminiscent of their morphology *in vivo*. The cell body of outer pillar cells displayed a round to elliptical shape and contained straight bundles of microtubules spreading at the apex (Tolomeo and Holley 1997). Inner pillar cells were distinguished by their curved microtubule bundles (Tucker et al. 1993). Only cells with a clearly delineated cell membrane, intact microtubules, and nucleus were used.

After isolation, dye loading, and washing with fresh

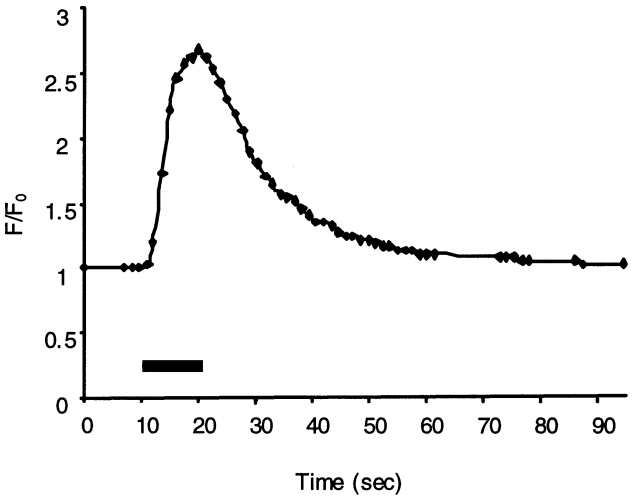

FIG. 2. Response of intracellular calcium to an extracellular bolus of ATP. ATP (100 μM) was delivered for 10 s (bar) to an outer pillar cell. The fluorescence intensity F/F_0 increased rapidly and returned exponentially to basal levels. Because of the spread of the bolus, the ATP concentration was diluted at least 10- to 20-fold before reaching the cell (see Methods section).

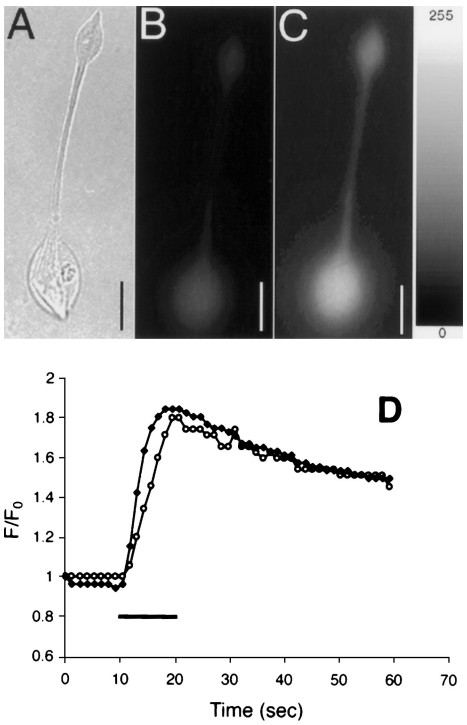

FIG. 3. Base/apex distribution of calcium responses. **A.** Bright field photomicrograph of an outer pillar cell. **B, C.** Fluorescence images of the cell at rest (**B**) and at the maximum of $[Ca^{2+}]_i$ following ATP stimulation (**C**). Scale bar = 10 μm. The calibration bar on the right shows the fluorescence intensity in a gray scale. **D.** Representative tracing of changes in $[Ca^{2+}]_i$ in the basal and apical portion of an outer pillar cell following a 10-s application of ATP (bar). Closed symbols, base of the cell; open symbols, apex.

HBSS, two cochlea yielded 6–10 inner and outer pillars with outer pillars representing the majority of cells. The initial fluo-3 fluorescence in HBSS indicated resting levels of $[Ca^{2+}]_i$ from 30 nM upward. Approximately 25% of the isolated cells were rejected because of an unstable fluorescence baseline or a $[Ca^{2+}]_i$ exceeding 100 nM. Cells included in the studies averaged 49 ± 3 nM (mean ± SEM; $n = 53$) and resting levels were similar in all experimental groups.

$[Ca^{2+}]_i$ response to ATP

Fluorescence was significantly increased in all cells ($n = 27$) by the application of a bolus of 100 μM ATP. Relative fluorescence (F) increased from 42 ± 3 (F_0) to 115 ± 9 (F_{max}), yielding a ratio of $F_{max}/F_0 = 2.7 \pm 0.1$. Inner and outer pillar cells responded similarly. Of the 27 cells tested, 23 were outer pillar cells giving a response of $F_{max}/F_0 = 2.78 \pm 0.15$; four inner pillars responded with $F_{max}/F_0 = 2.80 \pm 0.29$. The agonist 2-methylthio ATP (see below) elicited similar responses in 12 outer pillars ($F_{max}/F_0 = 2.90 \pm 0.24$) and 3 inner pillars ($F_{max}/F_0 = 2.95 \pm 0.61$).

Fluorescence began to increase almost immediately after starting the ATP application and attained a maximum (F_{max}) at around 9 s (Fig. 2). Fluorescence then gradually decreased, reaching a plateau after 2–3 min. A response to ATP could be obtained repeatedly in the same cell but the F_{max} values were successively decreasing ($n = 5$). A bolus application of HBSS alone did not change the fluorescence ($F_{max}/F_0 = 1.00 \pm 0.01$; $n = 5$).

The calcium signal elicited by ATP appeared to be a global response of the cell. There were no obvious local foci nor were there any differences in the kinetics between the basal and apical portion of the cells (Fig. 3). Of five cells studied, two showed a simultaneous rise of fluorescence in base and apex, in one cell the basal increase preceded the apex, and in two the apex preceded the base (Fig. 3D). Likewise, the magnitude of the signal did not differ between base and apex. In two cells the basal and apical maxima were within ±5%, in two cells the apex was higher, in one cell the base was higher yielding an average F_{max}/F_0 of 2.68 ± 0.43 in the apex and 2.51 ± 0.40 in the base.

$[Ca^{2+}]_i$ response to ATP in Ca^{2+}-free HBSS

In Ca^{2+}-free HBSS, resting $[Ca^{2+}]_i$ was 50 ± 8 nM. Fluorescence increased in 8 out of 10 cells when ATP was applied within 5 min after adding 1 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) to Ca^{2+}-free HBSS. In responding cells, the F_{max}/F_0 ratio was 1.8 ± 0.3.

After preincubation of the cells with 1 mM EGTA for 20 min, resting levels of $[Ca^{2+}]_i$ were significantly reduced to 33 ± 7 nM ($n = 13$) and ATP no longer

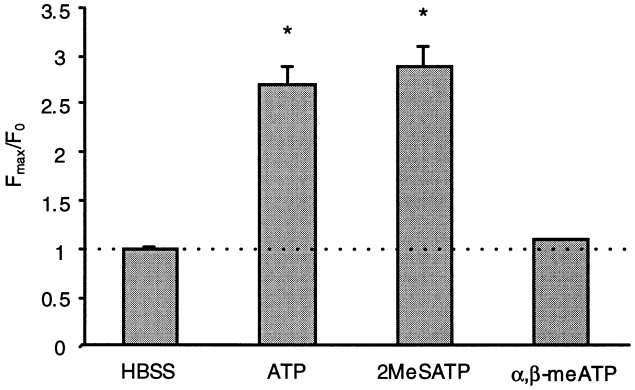

FIG. 4. Calcium response to purinergic agonists. ATP or other agonists were applied as described in the Methods section. The concentrations were 100 μM each in the delivery pipette and the bolus was diluted at least 10- to 20-fold before reaching the cell (see the Methods section). The relative maximal fluorescence intensity (F_{max}/F_0) is shown as mean ± SEM, and the dashed line indicates the resting fluorescence (F/F_0 = 1). Asterisks indicate that responses to ATP (n = 27) and to 2MeSATP (n = 15) differ from HBSS controls (n = 5; $p < 0.05$). Responses to α, β-meATP (n = 6) do not differ significantly from HBSS controls.

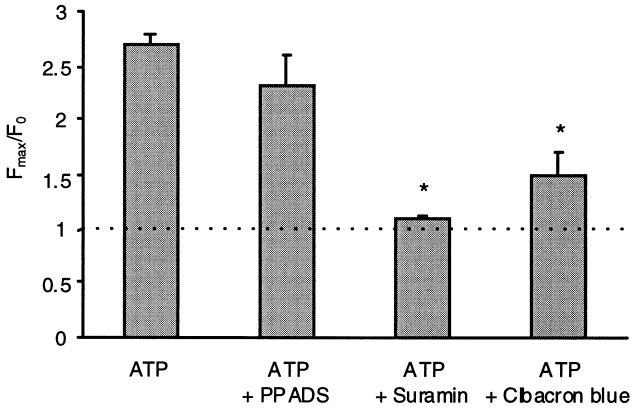

FIG. 5. Calcium response in the presence of antagonists. ATP (100 μM) was applied to cells preincubated with purinergic antagonists as described in the Methods section, and the relative maximal fluorescence (F_{max}/F_0) is given as mean ± SEM. The dashed line indicates the resting fluorescence (F/F_0 = 1). Asterisks indicate that responses to ATP in the presence of 500 μM suramin (n = 6) or 100 μM Cibacron Blue (n = 7) are significantly different from the response to ATP alone (n = 27; $p < 0.05$). Ten μM PPADS (n = 8) has no significant effect on the responses to ATP.

induced a change of fluorescence. When normal HBSS was restituted, an ATP bolus 5 min after restitution elicited a response (F_{max}/F_0 = 1.8 ± 0.2, n = 3).

$[Ca^{2+}]_i$ response to other purinergic agonists

2-Methylthio ATP (2MeSATP) is a P2 receptor agonist that can induce G-protein-linked intracellular Ca^{2+} mobilization via P2Y receptors (Burnstock and Kennedy 1985; Fredholm et al. 1997). A 10-s bolus of 2MeSATP (100 μM) increased the fluorescence from F_0 = 46 ± 4 to F_{max} = 133 ± 11, resulting in F_{max}/F_0 = 2.9 ± 0.2 (n = 15; Fig. 4). The time between the beginning of the bolus and F_{max} was 9.1 ± 0.6 s. These responses were not different from those induced by ATP, and cells responded repeatedly with subsequent F_{max} values continuously decreasing (n = 5).

α, β-Methylene ATP (α, β-meATP) has been identified as an agonist of $P2X_1$ receptors (MacKenzie et al. 1996) and various cloned P2X receptors (Fredholm et al. 1997). When pillar cells were subjected to a 10-s bolus of 100 μM α, β-meATP, there was no significant increase of fluorescence (F_{max}/F_0 = 1.06 ± 0.03; n = 6; Fig. 4).

The effect of purinergic antagonists

The presence of suramin (500 μM), an antagonist of both the P2X and P2Y receptor subtypes, inhibited the ATP-induced $[Ca^{2+}]_i$ increase (Fig. 5). After a 5-min preincubation of isolated cells with suramin, the resting $[Ca^{2+}]_i$ (47 ± 3 nM) was not different from the average resting $[Ca^{2+}]_i$ in HBSS. A 10-s bolus of 100 μM ATP did not increase the fluorescence (F_{max}/F_0 = 1.1 ± 0.01; n = 6). In contrast, 10 μM PPADS, a selective P2X antagonist, did not inhibit the response to ATP. After a 5-min preincubation, resting levels of $[Ca^{2+}]_i$ were unchanged (47 ± 5 nM) and the cells responded normally to a 10-s bolus of either ATP (F_{max}/F_0 = 2.3 ± 0.3; n = 8) or 2MeSATP (F_{max}/F_0 = 2.3 ± 0.3; n = 8). However, increasing the concentration of PPADS to 100 μM eliminated the response to ATP (F_{max}/F_0 = 1.1 ± 0.1; n = 3).

Cibacron Blue, a selective P2Y antagonist, attenuated the ATP-induced $[Ca^{2+}]_i$ increase. After a 5-min preincubation of the cells in 100 μM Cibacron Blue (resting $[Ca^{2+}]_i$ = 57 ± 6 nM), a 10-s bolus of ATP elicited a significantly lower F_{max}/F_0 ratio (1.5 ± 0.2; n = 7; $p < 0.05$). At 500 μM, the inhibitory effect was similar (F_{max}/F_0 = 1.5 ± 0.3; n = 4). In contrast, 20 μM Cibacron Blue was not effective (F_{max}/F_0 = 2.4 ± 0.6; n = 6).

The effects of NO donors

The effect of nitric oxide on the ATP-evoked $[Ca^{2+}]_i$ increase was investigated with NO donors. When the cells were preincubated with either 100 μM *S*NP or 0.1 μM DEA-NO for 20 min (Maragos et al. 1991; Gryglewski et al. 1989), resting $[Ca^{2+}]_i$ was 54 ± 5 nM (n = 9) and 56 ± 6 nM (n = 8), respectively, levels not different from the resting $[Ca^{2+}]_i$ in HBSS. The effect of a subsequent ATP application, however, was significantly attenuated. DEA-NO limited the ATP response to F_{max}/F_0 = 1.4 ± 0.1 while SNP was somewhat less effective, reducing the ATP response to F_{max}/F_0 = 2.0 ± 0.2 (Fig. 6).

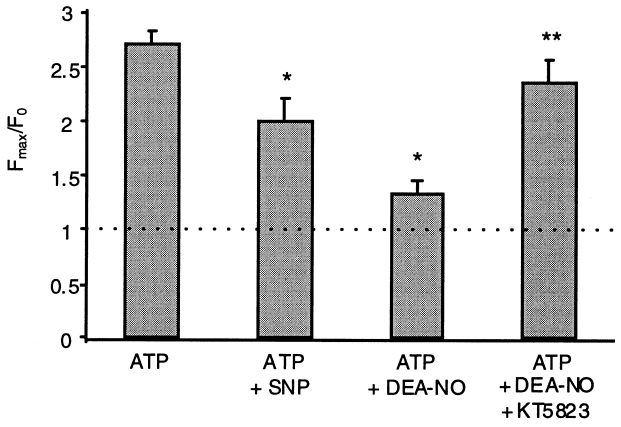

FIG. 6. Responses to ATP in the presence of NO donors and a protein kinase G inhibitor. ATP (100 μM) was applied to cells preincubated with NO donors and a protein kinase G inhibitor as described in the Methods section. The relative maximal fluorescence intensity (F_{max}/F_0) is shown as mean ± SEM. The dashed line indicates the resting fluorescence ($F/F_0 = 1$). Asterisks indicate that responses to ATP in the presence of 100 μM SNP ($n = 8$) or 0.1 μM DEA-NO ($n = 9$) are significantly different ($p < 0.05$) from ATP responses in their absence ($n = 27$). Double asterisks indicate that responses to ATP in the presence of 0.1 μM DEA-NO plus 1 μM KT5823 ($n = 6$) are significantly different from the responses to ATP plus DEA-NO ($p < 0.05$) but not different from ATP controls ($p > 0.05$)

To test the involvement of the cyclic GMP-dependent protein kinase (cGK) pathway in the action of NO donors, the cells were incubated with an inhibitor of cGK. KT5823 is a relatively selective, cell-permeable inhibitor of cGK with a K_i of 0.234 μM in contrast to its much higher K_i for protein kinase A (4 μM) and myosin light chain kinase (>10 μM) (Kase et al. 1987). When the cells were incubated with 0.1 μM DEA-NO in the additional presence of 1 μM KT5823, the maximum response to ATP was restored to 2.4 ± 0.2 ($n = 6$). The preincubation with DEA-NO in combination with KT5823 had no effect on the resting levels of $[Ca^{2+}]_i$.

DISCUSSION

In pillar cells of the guinea pig organ of Corti, $[Ca^{2+}]_i$ is increased by the extracellular application of ATP, and this response is attenuated by nitric oxide via the NO/cGMP/cGK pathway. This likens the regulation of calcium homeostasis in pillar cells to the mechanisms operating in Deiters' and Hensen's cells and suggests a concerted response of these supporting cells to humoral control.

Purinergic P2 mechanisms are suggested by the efficacy of ATP and the block by suramin, a general P2 receptor antagonist (Hoyle et al. 1990; Van den Abbeele et al. 1996; McMurray et al. 1998). The response to 2MeSATP and the absence of a response to α,β-meATP are consistent with an involvement of P2Y receptors since 2MeSATP is a P2Y agonist while α,β-meATP is a P2X agonist (Burnstock and Kennedy 1985). Also consistent with the participation of P2Y receptors are the block of the ATP response by Cibacron Blue and the failure of PPADS to attenuate the effect of ATP at a low concentration. Cibacron Blue is a selective P2Y antagonist at concentrations between 10 and 500 μM (Soediono and Burnstock 1994; Lim et al. 1997; McMurray et al. 1998; Filippi et al. 1999; Shalev et al. 1999). PPADS is a P2X antagonist whose specificity, however, is concentration-dependent. In rabbit vas deferens (Ziganshin et al. 1993), PPADS selectively inhibited the α,β-meATP-induced contraction at concentrations of 3–30 μM. At 10 μM, it was ineffective on pillar cells. Higher concentrations of PPADS (100 μM) may result in nonspecific antagonism to ATP (Sima et al. 1997).

Additional support for the involvement of a metabotropic mechanism in the increase in $[Ca^{2+}]_i$ is the response to ATP in Ca^{2+}-free medium. In this case, calcium should be released from intracellular stores, mediated by an activation of P2Y receptors coupled to G-proteins and the formation of inositol trisphosphate (IP_3). With time, EGTA will also deplete intracellular stores (Dulon et al. 1990). This is seen here in the significant decrease of $[Ca^{2+}]_i$ after a 20-min preincubation, explaining the absence of an ATP response after prolonged EGTA treatment. A mediation of increases in $[Ca^{2+}]_i$ by IP_3 receptors, in turn, is in agreement with the observations that ATP increases inositol phosphate production in the organ of Corti (Niedzielski and Schacht 1992; Ogawa and Schacht 1993, 1994) and that IP_3 receptors are present in cochlear supporting cells, including pillar cells (Tian and Schacht 1999).

While our results are consistent with a participation of P2Y receptors in ATP-induced increases of $[Ca^{2+}]_i$, other mechanisms may contribute. The diminished response to ATP in Ca^{2+}-free medium might result from a reduction of calcium influx via P2X receptors or voltage-gated channels. Chen et al. (1998) have shown an ATP-gated inward current in pillar cells which could depolarize the cell and activate voltage-gated calcium channels. In this context, the spatial pattern of $[Ca^{2+}]_i$ increase in pillar cells is reminiscent of global signals like those that can be elicited by voltage-gated channels in smooth muscle cells (Nelson et al. 1990) rather than localized signals that can be observed in outer hair cells in response to ATP (Mammano et al. 1999).

As far as P2X receptors are concerned, the pharmacological characteristics of P2X receptor subtypes are too complex to rule out their involvement at this point

(Fredholm et al. 1997; Kunapuli 1998; North and Surprenant 2000). For example, the $P2X_4$ receptor subunit could not be inhibited by PPADS, and α,β-meATP evoked a current that was only 13% of the response to ATP in oocytes expressing both $P2X_4$ and $P2X_6$ subunits (Lê et al. 1998). $P2X_2$ receptors are present in the apical portion of pillar cells (Housley et al. 1999) where they may contribute to local cation fluxes such as may be involved in the ATP-activated endocochlear shunt (Housley 1998; King et al. 1998). From the sum of our results (and in the absence of precise information on the distribution of ATP receptor subtypes and ion channels along pillar cells), we speculate on multiple pathways involved in $[Ca^{2+}]_i$ increases in pillar cells with a significant contribution by P2Y receptors.

The NO/cGMP/cGK pathway, as we have previously shown in Deiter's and Hensen's cells (Matsunobu and Schacht 2000), antagonizes the response to ATP in pillar cells. The fact that the protein kinase G inhibitor KT5823 reverses the suppressive action of NO on $[Ca^{2+}]_i$ suggests that the mechanism of action of NO is linked to protein kinase G, the final kinase in the pathway. The presence of this pathway in pillar cells is also consistent with the localization of the participating enzymes soluble guanylate cyclase and protein kinase G in these cells (Fessenden and Schacht 1997; Tian et al. 1999). The mechanism by which the calcium signal is attenuated remains speculative since protein kinase G may phosphorylate several relevant target proteins. An inhibition of Ca^{2+} release from internal Ca^{2+} stores would directly antagonize a P2Y-mediated calcium increase and is compatible with one of the known actions of protein kinase G, a phosphorylation of IP_3 receptors (Komalavilas and Lincoln 1994; Tertyshnikova et al. 1998). Protein kinase G also regulates the activity of several types of calcium channels (Meriney et al. 1994; Sumii et al. 1997; Yao et al. 2000), an action that might contribute to its overall effect. Finally, calcium export via a Ca^{2+} pump can be activated by protein kinase G (Vrolix et al. 1988).

The regulation of intracellular calcium levels by both purinergic receptors and NO implies the possibility of neurohumoral regulation in pillar cells and raises the question of the physiological significance of calcium mobilization in these structures. The microtubules produce shear resistance and stiffness in outer pillar cells (Tolomeo and Holley 1997). In inner pillar cells, the microtubules follow the curvature of the cell toward the outer pillar and outer hair cell (Tucker et al. 1993). Small changes in the elasticity of the microtubules would have a great impact on the micromechanics of the basilar membrane. However, microtubules in these cells are primarily long-lived and stable and may not undergo cycles of polymerization and depolymerization that could underlie a cytoskeletal rearrangement (Slepecky et al. 1995). On the other hand, the stability and architecture of microtubules are also controlled by crosslinking that is regulated by microtubule-associated proteins (MAPs) (Olmsted 1991; Dhamodharan and Wadsworth 1995; Gamblin et al. 1996). The activity of MAPs, in turn, can be regulated by calcium-dependent phosphorylation (Quinlan and Halpain 1996; Sato–Harada et al. 1996; Pettit and Fay 1998). We therefore speculate that purinergic stimulation and the subsequent rise in $[Ca^{2+}]_i$ change the stiffness of pillar cells and that this response can be modulated by NO. In combination with a similar effect on calcium mobilization in Deiters' cells, ATP and NO could be major modulators of the micromechanical properties of the basilar membrane.

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