P2 purinoceptor of the globular substance in the otoconial membrane of the guinea pig inner ear

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Suzuki, Hideaki, Katsuhsia Ikeda, Masayuki Furukawa, and Tomonori Takasaka. P2 purinoceptor of the globular substance in the otoconial membrane of the guinea pig inner ear. Am. J. Physiol. 273 (Cell Physiol. 42): C1533-C1540, 1997.—The biological characteristics of the globular substance, a precursor of otoconia, are unclear. In the present study, the ATP-induced internal free Ca$^{2+}$ concentration ([Ca$^{2+}$]) changes of the globular substance and the ATP distribution in the vestibular organ were investigated using a Ca$^{2+}$ indicator, fluo 3, and an adenine nucleotide-specific fluorochrome, quinacrine, by means of confocal laser scanning microscopy. [Ca$^{2+}$] showed a rapid and dose-dependent increase in response to ATP with a 50% effective concentration (EC$_{50}$) of 16.7 µM. This reaction was independent of external Ca$^{2+}$, indicating the presence of an internal Ca$^{2+}$ reservoir. Neither adenosine, α,β-methylene-ATP, 3′-O-(4-benzoylbenzoyl)-ATP, ADP, nor UTP evoked this reaction, whereas 2-methylthio-ATP induced an increase of [Ca$^{2+}$] with an EC$_{50}$ of 14.4 µM. Moreover, P2 antagonists, reactive blue 2 and suramin, and a phospholipase C inhibitor, U-73122, inhibited the ATP-induced [Ca$^{2+}$] increase. These findings indicate the presence of a P2Y purinoceptor on the globular substance. In addition, granular fluorescence was observed in the quinacrine-stained macular sensory epithelium, indicating the presence of ATP-containing granules in this tissue. These results suggest that a paracrine mechanism involving ATP may exist in the macula and that this mechanism regulates the biological behavior of the globular substance.

macula; vestibule; otoconia; adenosine 5′-triphosphate; quinacrine

THE MACULA IS A vestibular organ that is responsible for the perception of gravity and linear acceleration. It is composed of the sensory epithelium, which includes sensory hair cells, and the otoconial membrane. The otoconial membrane is situated above the sensory epithelium and consists of otoconia, the gelatinous layer, and subepithelial meshwork, a fine fibrous structure that connects the gelatinous layer with sensory cilia of the hair cells. Otoconia are biomineral bodies sitting on the gelatinous layer that add weight to the otoconial membrane, resulting in its deflection and, eventually, cilia deflection of hair cells according to the changes of direction in gravity.

Otoconia contain calcium carbonate as the mineral component and proteins with carbohydrates as the organic component. The mechanism of otoconial formation, a biomineralization process, is only partially understood, but it is thought to be different from the purely inorganic precipitation of crystal. The globular substance is a spherical structure floating in the gelatious layer of the otoconial membrane in the macula. This material is secreted from the macular sensory epithelium and is presumed to be a precursor of otoconia (12, 33). The morphological features of this substance have been studied by several authors (12, 33), and Harada (12) has detected its high Ca$^{2+}$ content with an X-ray microanalyzer. More recently, we observed the globular substance in a physiological buffer by means of confocal laser scanning microscopy and demonstrated that this substance is a membrane-enclosed structure and that this material manifests characteristics similar to those of the globular substance, i.e., it is produced by microapocrine secretion from osteogenic cells and is a membrane-enclosed structure (11).

Despite the significance of the globular substance in the process of otoconial formation, information about its biological reactions has so far been very limited. In the present study, we report the [Ca$^{2+}$] response of the globular substance induced by ATP and its analogs, providing new insight into the mechanism of otoconial formation. In addition, we also clarify the ATP distribution in the macula using an adenine nucleotide-specific fluorescent compound.

MATERIALS AND METHODS

Chemicals. Fluo 3-pentaacetoxymethyl ester (fluo 3-AM) was purchased from Molecular Probes (Eugene, OR). Adenosine, ADP, ATP, α,β-methyleneadenosine 5′-triphosphate (α,β-MeATP), 3′-O-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP), UTP, quinacrine, and ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) were obtained from Sigma Chemical (St. Louis, MO). 2-Methylthioadenosine 5′-triphosphate (2-MeS-ATP) and reactive blue 2 (RB2) were bought from Research Biochemicals International (Natick, MA). Suramin, U-73122, and U-73343 were purchased from Wako Pure Chemical (Osaka, Japan). PC-12 cells, derived from rat pheochromocytoma, were provided by Riken Cell Bank (Tsukuba, Japn). Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640, and fetal bovine serum (FBS) were obtained from GIBCO BRL Life Technologies (Palo Alto, CA).

Sample preparation. Adult albino guinea pigs, 6–10 wk old, were anesthetized by diethylether inhalation and decapitated. Temporal bones were collected, and the utricular maculae were dissected under a dissecting microscope in O$_2$-gassed artificial perilymph (APL) composed of (in mM) 150 NaCl, 3.5 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 2.3 tris(hydroxymethyl)aminomethane, 2.8 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, and 3 d-glucose (pH 7.4). The samples were then

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transferred onto a glass coverslip or into a thin-bottomed petri dish, and the otocional membrane was gently detached from the sensory epithelium by forceps. For the superfusion experiments to study [Ca\(^{2+}\)] response, the membrane on the glass coverslip was incubated with 3 μM fluo 3-AM as a free Ca\(^{2+}\) indicator in APL for 1 h at room temperature. The care and use of the animals were in accordance with the Guidelines of the Declaration of Helsinki.

Cell culture: PC-12 cells were cultured in DMEM supplemented with 10% FBS. For fibroblast culture, the dermis and subcutaneous tissue were excised from the abdominal skin of the guinea pig and cultured in RPMI 1640 containing 10% FBS. The samples were placed in thin-bottomed petri dishes and incubated in humidified 5% CO\(_2\)-95% air at 37°C until the cells grew nearly confluent.

Quinacrine staining. For the observation of the ATP distribution, the samples in the petri dish were incubated with 5 μM quinacrine in APL (for utricle) or in the culture medium (for cultured cells) for 30 min at room temperature as previously described (37) and then washed before confocal microscopic examination.

Superfusion experiment. Coverslips with fluo 3-loaded samples were placed in a superfusion chamber. Solutions were continuously saturated with O\(_2\) and pumped into the chamber at a flow rate of 0.8 ml/min by a peristaltic pump and removed by a siphon. Reagents were applied by changing the superfusion solutions.

Confocal laser scanning microscopy. The samples were directly observed under a laser scanning confocal imaging system (Bio-Rad MRC-600; Bio-Rad Microscience Division, Watford, UK) with an argon ion laser as the light source. The excitation wavelength was 488 nm. The objective was coupled to an inverted microscope (Olympus IMT-2; Olympus, Tokyo, Japan). The objective lens was a 60× oil immersion objective (SPlan Apo 60; Olympus) with a numerical aperture of 0.95. Emitted fluorescence, which passed through a long-pass filter (515 nm cutoff), was collected by a photomultiplier tube and displayed as a 768 × 512 pixel resolution image through a host computer (standard IBM PC-AT).

Dynamic changes in the intensity of fluo 3 fluorescence were observed every 2 min and displayed in a 256-step arbitrary fluorescence scale of 0 (no fluorescence) to 255 (most intense fluorescence). The obtained data were not converted to the absolute value of the internal Ca\(^{2+}\) concentration because of the error inherent in single-wavelength detection of fluorometric intensities and are therefore expressed as relative values. A value of relative fluorescence was calculated as follows:

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\text{Relative fluorescence} = \frac{(F - BG)}{(F_0 - BG)}
\]

where F is the intensity of the observed fluorescence, F\(_0\) is the intensity of the fluorescence at time zero, and BG is the background fluorescence.

Statistics: Data values are expressed as means ± SE. Statistical significance was analyzed using a two-tailed Student’s t-test, and a P value of <0.05 was considered significant.

RESULTS

ATP-induced [Ca\(^{2+}\)] response of the globular substance. Figure 1 represents pseudocolor images of typical ATP-induced [Ca\(^{2+}\)] increases in the globular substance. Fluo 3 fluorescence rapidly increased 2 min after the application of 100 μM ATP and then fell gradually (Fig. 1). Not all globular substances responded to ATP. Thirty-two of fifty globular substances (64%) showed a relative fluorescence value of 2.0 or more at the peak in response to 100 μM ATP, whereas the others did not. To quantitatively evaluate the [Ca\(^{2+}\)] responses, we calculated the arithmetic mean of relative fluorescence values of all globular substances that appeared in the confocal microscopic field. Mean relative fluorescence at the peak reached five- to sixfold the value of the resting level. The reaction was independent of external Ca\(^{2+}\), i.e., superfusion with Ca\(^{2+}\)-free APL supplemented with 1 mM EGTA (corresponding to 10−8 M Ca\(^{2+}\)) did not suppress this ATP-induced response (Fig. 2), indicating the release of Ca\(^{2+}\) from internal stores of the globular substance. There was no significant difference in the proportion of the responding population in the presence or absence of external Ca\(^{2+}\).

Neither adenosine, αβ-MeATP, BzATP, ADP, nor UTP evoked this reaction, whereas 2-MeS-ATP induced a significant increase in fluorescence (Fig. 3). The [Ca\(^{2+}\)] response was elicited by 10–300 μM ATP and 3–300 μM 2-MeS-ATP in a dose-dependent manner (Fig. 4). The concentrations that yielded the half-maximal response (EC\(_{50}\)) were 16.7 μM for ATP and 14.4 μM for 2-MeS-ATP.

P2 antagonists, RB2 and suramin, completely inhibited the ATP-induced [Ca\(^{2+}\)] increase (Fig. 5). Moreover, preincubation with 10 μM U-73122, a phospholipase C inhibitor (14), significantly inhibited the ATP-induced [Ca\(^{2+}\)] increase, whereas the same concentration of U-73434, an inactive analog of U-73122 (14), had no effect (Fig. 6).

These results indicate the presence of a P2 purinoreceptor on the globular substance. The pharmacological characteristics of the reactions strongly suggest that the receptor belongs to the P2Y family.

ATP distribution in the macula. Many fluorescent spots were observed in the sensory epithelium of the quinacrine-stained specimen (Fig. 7A). The fluorescence was most intense at a depth of 2–5 μm from the apical surface. The spots were 2–5 μm in diameter and 4–8 μm apart from one another. At a higher magnification, each fluorescent spot consisted of several smaller granules 0.4–0.9 μm in diameter (Fig. 7B). These findings indicate the presence of ATP-containing granules in the sensory epithelial cells. Meanwhile, the otoconial membrane showed little or no fluorescence of quinacrine.

Fluorescent granules were also seen in the cytoplasm of PC-12 cells (Fig. 7, D and E), which are known to have ATP-containing secretory vesicles (35), whereas fibroblasts from the abdominal skin of the guinea pig showed much less fluorescence (Fig. 7G). No fluorescence was detected in the specimens without quinacrine treatment that served as controls.

DISCUSSION

The present study is the first report demonstrating that extracellular ATP induces a [Ca\(^{2+}\)] increase in the globular substance. It is unusual that the dynamic
Fig. 1. ATP-induced internal free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) changes of the globular substance. Otoconial membrane of the utricular macula was dissected under a dissection microscope and incubated with 3 µM fluo 3-pentaacetoxymethylester (AM) in artificial perilymph (APL) for 1 h at room temperature. Sample was then placed in a superfusion chamber, and dynamic changes of the fluorescence were observed every 1 min under a confocal laser scanning imaging system (Bio-Rad MRC-600) as described in MATERIALS AND METHODS. APL was continuously saturated with O\(_2\) and pumped into the superfusion chamber at a flow rate of 0.8 ml/min; 100 µM ATP was applied by changing the superfusion solution at time 0. Each digit represents time (in min) from the application of ATP. Intensities of fluo 3 fluorescence in arbitrary scale are displayed in pseudocolor, as indicated in color calibration bar. Scale bar (top left) = 10 µm.
change of [Ca\textsuperscript{2+}] remained elevated throughout the exposure to ATP and even after the removal of ATP (Fig. 2). This would indicate that the globular substance cytoplasts lack a significant capacity for Ca\textsuperscript{2+} homeostasis and may suggest that this process is a vestigial response of the globular substance, which becomes nonliving otoconial bodies.

A number of authors have documented the biological actions of ATP in the inner ear. In vivo studies have shown that cochlear perilymphatic and endolymphatic perfusion with ATP alters cochlear electrical activity (15, 21) and that this effect is inhibited by purinergic antagonists (16, 21). In vitro experiments have shown that isolated inner ear component cells exhibit various physiological and biochemical reactions in response to ATP and its analogs. In terms of ATP responses, outer hair cells in the organ of Corti are one of the best-documented types of cells in the inner ear. Isolated outer hair cells contract in length (5, 29, 38), depolarize (2, 23), and show membrane currents (6, 18) and an intracellular Ca\textsuperscript{2+} increase (2, 25) in response to ATP. These reactions are also observed in the inner hair cells (8), supporting cells (2, 7), strial marginal cells (31, 34), and lateral wall epithelial cells (13) of the cochlea. At the morphological level, the ATP-binding sites of cochlear hair cells are the stereocilia, cuticular plate, and basolateral margins (20). Moreover, ATP and its analogs have been shown to reduce the K\textsuperscript{+} secretion of strial marginal cells (19) and induce inositol phosphate

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**Fig. 2.** ATP-induced [Ca\textsuperscript{2+}] changes of the globular substance. Otoconial membranes were treated as in Fig. 1. Samples were superfused with APL (○; n = 36) or with Ca\textsuperscript{2+}-free APL supplemented with 1 mM EGTA (□; n = 14); 100 µM ATP was applied by changing the superfusion solution as indicated in the graph. NS, not significant.

**Fig. 3.** Effects of purinergic agonists on [Ca\textsuperscript{2+}], response of the globular substance. Otoconial membranes were superfused with APL, and 100 µM of a purinergic agonist was applied by changing the superfusion solution. Each value represents mean ± SE of maximum relative fluorescence within 5 min after application of the agonist. αβ-MeATP, α,β-methylene-ATP; 2-MeS-ATP, 2-methylthio-ATP; BzATP, 3'-O-(4-benzoyl-benzoyl)-ATP. ** P < 0.01, *** P < 0.001.

**Fig. 4.** Dose-response relationships for ATP and 2-MeS-ATP. Otoconial membranes were superfused with APL, and various concentrations of ATP (○) or 2-MeS-ATP (□) were applied by changing the superfusion solution. Each value represents mean ± SE of maximum relative fluorescence within 5 min after application of the agonist. The 50% effective concentrations (EC\textsubscript{50}) are 16.7 and 14.5 µM for ATP and 2-MeS-ATP, respectively.

**Fig. 5.** Effects of P2 antagonists on ATP-induced [Ca\textsuperscript{2+}], response of the globular substance. Otoconial membranes were initially superfused with an antagonist dissolved in APL for 5 min, followed by exposure to both the antagonist and 100 µM ATP. Each value represents mean ± SE of maximum relative fluorescence within 5 min after application of ATP. RB2, reactive blue 2. ** P < 0.01.
release from the lateral wall and sensory epithelium of the cochlea (24, 27).

For vestibular organs, there are fewer studies on the effects of ATP. ATP-induced membrane currents, intracellular \(\mathrm{Ca}^{2+}\) increase, and cell motility have been reported in vestibular hair cells (28). ATP and its analogs have also been shown to elicit inositol phosphate release from the vestibular sensory epithelium (26), reduce the \(\mathrm{K}^+\) secretion of vestibular dark cells (34), and modulate the vestibular transepithelial potential (3).

These lines of evidence suggest that purine-mediated humoral mechanisms influence the function of the inner ear. The receptors that respond specifically to ATP but not to adenosine are referred to as P2 purinoceptors. P2 purinoceptors used to be pharmacologically classified into multiple subtypes, that is, \(\mathrm{P2X}_1\), \(\mathrm{P2Y}_1\), \(\mathrm{P2Z}_1\), \(\mathrm{P2U}_1\), \(\mathrm{P2T}_1\), and \(\mathrm{P2D}_1\). Studies with purinergic agonists revealed the presence of various types of P2 receptors in the cochlea and in the vestibule. \(\mathrm{P2X}_1\), \(\mathrm{P2Y}_1\), \(\mathrm{P2Z}_1\), and \(\mathrm{P2U}_1\) receptors are present in the cochlear lateral wall (13, 19, 27), \(\mathrm{P2X}_1\) in the organ of Corti (24, 26), \(\mathrm{P2Y}_1\) and \(\mathrm{P2U}_1\) in outer hair cells (25), \(\mathrm{P2Y}_1\) in vestibular dark cells (19), and \(\mathrm{P2Y}_1\) in the vestibular sensory epithelium (26) and semicircular canal (3). More recently, a new framework of P2 receptors has been established, based not only on the agonist potency order but also on the transduction mechanism and molecular structure. According to this nomenclature, P2 receptors are divided into \(\mathrm{P2X}\) (ligand-gated ion channels) and \(\mathrm{P2Y}\) (G protein-coupled receptors) families. Seven subtypes of the \(\mathrm{P2X}\) family (\(\mathrm{P2X}_{1-7}\)) and seven subtypes of the \(\mathrm{P2Y}\) family (\(\mathrm{P2Y}_{1-7}\)) have been identified to date (1, 4). The former \(\mathrm{P2Y}_{1}\) and \(\mathrm{P2U}_{1}\) receptors correspond to \(\mathrm{P2Y}_1\) and \(\mathrm{P2Y}_2\) in the new classification, respectively (1).

The present experiments with purinergic agonists and antagonists showed that a P2 receptor exists in the globular substance. The ATP-induced \([\mathrm{Ca}^{2+}]_i\) increase was independent of external \(\mathrm{Ca}^{2+}\) (Fig. 2), indicating that \(\mathrm{Ca}^{2+}\) was mobilized from an internal reservoir in the globular substance. The inhibition of the response by U-73122 (Fig. 6) indicates that the \(\mathrm{Ca}^{2+}\) mobilization is linked to a phospholipase C-dependent signaling mechanism, which is probably coupled to G protein. These results indicate that the purinoceptor of the globular substance belongs to the P2Y family and not to the P2X family. The rank order of agonist potency is similar to that of a cloned chick \(\mathrm{P2Y}_1\) receptor expressed in Xenopus oocytes (36) but different from that of a cloned turkey \(\mathrm{P2Y}_2\) receptor expressed in a human astrocytoma cell line (9) with respect to the inability of ADP to elicit the response in the present study. Therefore, the purinoceptor of the globular substance may be a variant of \(\mathrm{P2Y}_1\) or a new subtype. The subtype of the receptor could be identified by molecular cloning techniques. However, it is very difficult to cleanly isolate the globular substance from adjacent structures such as otocnia, the gelatinous layer, sensory epithelial cells, nonsensory epithelial lining cells including dark cells, and transitional cells. The sample in the present study is, therefore, too crude for biochemical, Northern blot, or polymerase chain reaction analyses.

The globular substance is a noncellular structure and does not possess a nucleus (33), suggesting that it is incapable of synthesizing proteins. Why, then, does this material have a purinoceptor? Electron microscopic studies have demonstrated that the globular substance is generated on the surface of the macular sensory epithelium by an apocrine-secretion-like mechanism (12). Moreover, it has been shown that a \(\mathrm{P2Y}_1\) (comparable to \(\mathrm{P2Y}_{1,2}\)) receptor is probably present in the vestibular sensory epithelium and that the ATP response of this tissue is accompanied by the release of inositol phosphate (26). When these observations are taken into account, the P2 receptor of the globular substance may have originated from that of macular sensory epithelial cells.

Although the role of intracellular ATP is well known, it has been thought that extracellular release of ATP rarely occurs because of its impermeability across the cell membrane. Nonetheless, it is also known that extracellular ATP influences many biological processes, such as platelet aggregation, vasodilation and constriction, neurotransmission, cardiac function, and smooth muscle contraction (10).

The source of ATP in the inner ear fluids is controversial. Because of a tight blood-labyrinth barrier in the normal inner ear, it seems unlikely that middle- to large-sized molecules in the systemic circulation directly enter into the inner ear fluids under physiological conditions. Extracellular ATP may exist in the inner ear via three sources. First, ATP may come from tissue injury or a pathological condition in the inner ear. ATP
Fig. 7. Confocal photomicrographs showing ATP distribution in the utricular macula and cultured cells. Samples were incubated with 5 µM quinacrine in APL (for utricle) or in the culture medium (for cultured cells) at room temperature for 30 min, washed, and then directly observed under a confocal laser scanning microscope as described in MATERIALS AND METHODS. A: confocal fluorescent image of the utricular sensory epithelium. Optical section image at a 3 µm depth from the apical surface shows many fluorescent spots. Scale bar = 25 µm. B: higher magnification of A. Each fluorescent spot is composed of several smaller granules, 0.4–0.9 µm in diameter. Scale bar = 5 µm. C: ordinary light microscopic image of PC-12 cells. Scale bar = 25 µm. D: simultaneous confocal fluorescent image of C. Fluorescent granules are seen in the cytoplasm of the cells. Scale bar = 25 µm. E: higher magnification of D. Scale bar = 5 µm. F: ordinary light microscopic image of cultured fibroblasts derived from guinea pig skin. Scale bar = 25 µm. G: simultaneous confocal fluorescent image of F. Fluorescence is much less than for the utricular sensory epithelium and PC-12 cells. Scale bar = 25 µm.
can be released from endothelial cells during the sudden breakage of blood vessels, from aggregating platelets by degranulation, and from red blood cells under conditions of ischemia and hypoxia (10). In fact, Munoz et al. (22) observed that ATP in the cochlear fluids is increased during hypoxia. Second, ATP could be coreleased with the putative neurotransmitters of the afferent (glutamate) or efferent (acetylcholine) systems that innervate sensory hair cells (7). There is accumulating evidence that ATP is released from sympathetic and parasympathetic nerve endings and acts as a cotransmitter of both acetylcholine and norepinephrine (17). However, direct evidence of this phenomenon in the inner ear is not available at present. In addition, because nerve endings are located on the basolateral surface facing the perilymphatic space of sensory hair cells, coreleased ATP is unlikely to reach the endolymphatic space where the globular substance lies. Third, ATP may be secreted from endolymphatic surface-lining cells. This type of ATP release would directly affect the globular substance, which floats in the endolymph. Using quinacrine, a fluorescent compound that preferentially binds to adenine nucleotides, particularly to ATP, White et al. (37) have demonstrated that stria marginal cells in the cochlear lateral wall have ATP-containing granules. With the use of the same method, we demonstrated the presence of an ATP-containing granular structure in the macular sensory epithelium, suggesting that an autocrine or paracrine mechanism involving ATP may exist in the macula to regulate the secretion and maturation of the globular substance. Interestingly, a similar purine-mediated mechanism has been shown to be involved in the regulation of ion channels of respiratory epithelial cells (30) and pituitary gland cells (32).

In conclusion, the ATP-induced \([Ca^{2+}]_i\) response of the globular substance and ATP distribution in the guinea pig macula were investigated by means of confocal laser scanning microscopy. ATP elicited a rapid and dose-dependent increase in \([Ca^{2+}]_i\) with an EC50 of 16.7 \(\mu\)M. This reaction was independent of external \(Ca^{2+}\), indicating that \(Ca^{2+}\) was mobilized from an internal reservoir in the globular substance. Experiments with purinergic agonists and antagonists and a phospholipase C inhibitor showed that this ATP-induced response was mediated by a purinoceptor that belongs to the P2Y family. Furthermore, granular fluorescence was observed in the quinacrine-stained macular sensory epithelium, indicating the presence of ATP-containing granules in this tissue. These results suggest that ATP may regulate the biological behavior of the globular substance via a paracrine mechanism. The biological significance of ATP and purinoceptors in the process of otoconial formation remains to be investigated in future studies.

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