Purinergic signaling in cochleovestibular hair cells and afferent neurons

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Abstract Purinergic signaling in the mammalian cochleovestibular hair cells and afferent neurons is reviewed. The scope includes P2 and P1 receptors in the inner hair cells (IHCs) of the cochlea, the type I spiral ganglion neurons (SGNs) that convey auditory signals from IHCs, the vestibular hair cells (VHCs) in the vestibular end organs (macula in the otolith organs and crista in the semicircular canals), and the vestibular ganglion neurons (VGNs) that transmit postural and rotatory information from VHCs. Various subtypes of P2X ionotropic receptors are expressed in IHCs as well as P2Y metabotropic receptors that mobilize intracellular calcium. Their functional roles still remain speculative, but adenosine 5'-triphosphate (ATP) could regulate the spontaneous activity of the hair cells during development and the receptor potentials of mature hair cells during sound stimulation. In SGNs, P2Y metabotropic receptors activate a nonspecific cation conductance that is permeable to large cations as NMDG⁺ and TEA⁺. Remarkably, this depolarizing nonspecific conductance in SGNs can also be activated by other metabotropic processes evoked by acetylcholine and tachykinin. The molecular nature and the role of this depolarizing channel

are unknown, but its electrophysiological properties suggest that it could lie within the transient receptor potential channel family and could regulate the firing properties of the afferent neurons. Studies on the vestibular partition (VHC and VGN) are sparse but have also shown the expression of P2X and P2Y receptors. There is still little evidence of functional P1 (adenosine) receptors in the afferent system of the inner ear.

Keywords ATP · Adenosine · Mammalian · Inner ear · Cochlea · Vestibular organ · Inner hair cell · Vestibular hair cell · Spiral ganglion · Vestibular ganglion

Introduction

Purinergic signaling is involved in various important physiological processes of the central nervous system including neurotransmission, neuromodulation, development, survival, and repair of neurons [1, 2]. Purinergic receptors are divided into P2 receptors that bind adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), and other extracellular nucleotides, and P1 receptors that are activated by adenosine. The family of P1 (adenosine) receptors is G-protein-coupled (metabotropic receptors) and signals by inhibiting or activating adenylate cyclase. The P2 (primarily ATP) receptor family is divided into P2X (ionotropic or ligand-gated) and P2Y (metabotropic) receptors. There are several subtypes for P2X (P2X₁–P2X₇, consecutively) and P2Y (P2Y₁–P2Y₁₄, not consecutively) that have different selectivity for agonists/antagonists. P2X receptors are generally believed to be involved in direct neurotransmission/modulation while P2Y receptors are mainly implicated in glia–neuron interactions. Purinergic signaling, principally P2, has also been demonstrated in

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special sense organs and their primary neurons related to vision, hearing, olfaction, and taste [3], but there still remains a large room for investigation, especially in the vestibular sensory system.

In this article, we review the studies on purinergic signaling in the afferent system of the eighth cranial nerve, i.e., the mammalian cochleovestibular hair cells and afferent neurons. The scope includes P2 and P1 receptors on the inner hair cells (IHCs) of the cochlea, the type I spiral ganglion neurons (SGNs) that convey auditory signals from IHCs, the vestibular hair cells (VHCs) in the vestibular end organs (macula in the otolith organs and crista in the semicircular canals), and the vestibular ganglion neurons (VGNs) that transmit postural and rotatory information from VHCs. The outer hair cells (OHCs) of the cochlea that amplify the movement of the basilar membrane with active motility are out of scope of the present review. Histological expression (mRNA and receptor protein) and physiological effects (in vivo and in vitro) will be discussed along with physiological and developmental implications.

Histological expression (mRNA and receptor protein) of P2 receptors in the cochlear partition (IHC and SGN type I) (Table 1)

IHC

The studies have neither been comprehensive nor consistent. Only P2X₂, P2X₃, and P2X₇ receptors have been investigated, and the results have discrepancy among species and between methods (in situ hybridization and immunohistochemistry). Part of such discrepancy may be due to the age of animals investigated, since transient expression in certain developmental stages has been shown in each subtype (P2X₂, P2X₃, and P2X₇). However, among them, consistent results show that the P2X ionotropic channels are localized at the endolymphatic surface [4], especially at the stereocilia [9], and Na⁺ influx through these channels was visualized using fluorescence [5]. More specifically, the P2X₂ receptor subtype was shown to be localized at the stereocilia of guinea pig hair cells [9], and therefore this may be the dominant P2X subtype.

SGN type I

All seven subtypes (P2X₁–P2X₇) have been shown to be expressed in adult SGNs. However, some subtypes (P2X₁, P2X₂, P2X₃) showed transiently stronger expression during the time period just before the onset of hearing (P12–P14 in rat), implying their developmental roles [6–8, 10, 11, 15].

P2 receptor expression can be modulated under environmental influence. Noise-induced upregulation of a certain

subtype (P2X₂) on the SGNs was reported [18]. Expression of ectonucleotidases (NTPDases) that hydrolyze ATP and ADP has also been reported in specific regions of the cochlea, including vasculature, neurons, hair cells, and supporting cells [19, 20]. Strong expression of NTPDase implies active purinergic signaling at these sites. In addition, differential expression between NTPDase1, which is a nucleoside diphosphatase and triphosphatase, and NTPDase2, which hydrolyzes nucleoside triphosphates preferentially, was observed. Stereocilia and the synaptic regions between IHC and SGN expressed both NTPDase1 and NTPDase2, whereas SGN body principally expressed NTPDase1. NTPDase3, a functional intermediate between NTPDase1 and 2, was also expressed in the cell bodies of the SGNs and the synaptic regions under IHCs [21].

Functional expression of P2 receptors in the cochlear partition

In vivo study

In vivo studies (Table 2) have exclusively been conducted using intracochlear perfusion with guinea pigs. In brief, cochleae of deeply anesthetized animals were opened in a minimally invasive manner and the scala tympani (perilymph) or scala media (endolymph) was perfused with small catheters and pumps. Physiological parameters such as cochlear microphonic (CM), endocochlear potential (EP), summing potential (SP), distortion product otoacoustic emissions (DPOAE), and compound action potentials (CAP) were recorded during perfusion, and the effects of perfused ligands or antagonists were evaluated. Most results indicated suppressive effects of ATP on the above physiological parameters in endocochlear perfusion [22, 23, 25, 27] but with an exception in which ATP analogs increased the endocochlear potential [26]. Moreover, perfusion with ATP antagonists (suramin, cibacron blue, and basilen blue: antagonists not specific for either P2X or P2Y) did not show consistent changes in the cochlear function [24], implying complex effects of P2 receptors in the cochlea. These controversial results can be attributed to simultaneous effects on multiple targets (hair cells, supporting cells, stria vascularis, etc.).

In vitro study

IHC

Functional P2X and P2Y receptors have first been demonstrated using spectrofluorometry recordings of intracellular calcium during ATP application (Table 3) [28]. Patch-clamp recordings revealed the expression of ionotropic (P2X) cation nonselective conductance mainly

Table 1 Histological expression (mRNA and receptor protein) of P2 receptors in the cochlear partition

Subtype	Species	Age	Target	Method	Findings	References
IHC						
P2	Guinea pig	Adult	Receptor	Fluorescence imaging	P2 receptors localized at the apical membrane	[4]
P2X	Guinea pig	N/A	Receptor	Fluorescence imaging	P2X ionotropic channels localized at the endolymphatic surface	[5]
P2X ₂	Rat	E12-Adult	mRNA	In situ hybridization	Positive for P10–P12	[6]
	Rat	Adult	Receptor	Immunohistochemistry	Negative	[7]
	Mouse	N/A	Receptor	Immunohistochemistry	Negative	[8]
P2X ₃	Guinea pig	Adult	Receptor	Immunohistochemistry	Positive at stereocilia	[9]
	Rat	E16-Adult	Receptor	Immunohistochemistry	Negative	[10]
	Mouse	E18-Adult	Receptor	Immunohistochemistry	Slightly positive for E18	[11]
P2X ₇	Rat	E14-Adult	Receptor	Immunohistochemistry	Positive for P0–P6	[12]
SGN (type I)						
P2	Guinea pig	Adult	Receptor	Autoradiography	Positive	[13]
P2X ₁ –P2X ₆	Rat	Adult	Receptor	Immunohistochemistry	All positive, strongest in P2X ₂	[14]
P2X ₁	Rat	E12-Adult	mRNA	In situ hybridization	Positive for E16–P6	[15]
P2X ₂	Mouse	N/A	Receptor	Immunohistochemistry	Positive	[8]
	Rat	P7-Adult	Receptor	Immunohistochemistry	All positive, strongest at synaptic region under IHC	[16]
	Guinea pig	Adult	Receptor	Immunohistochemistry	Positive at postsynaptic membrane under IHC	[9]
			mRNA	RT-PCR	P2X ₂₋₂ is the dominant splice variant	
	Rat	E16-Adult	Receptor	Immunohistochemistry	Positive, stronger at the synaptic region for E19–P6	[7]
	Rat	Adult	mRNA	In situ RT-PCR	Positive for a subpopulation of SGNs	[17]
			mRNA	RT-PCR	Identification of the third isoform, P2X ₂₋₃	
	Rat	E12-Adult	mRNA	In situ hybridization	Positive for E16-Adult, maximum at P8–P12	[6]
	Rat	E16-Adult	Receptor	Immunohistochemistry	Neuron body: positive for E16–P14	[10]
P2X ₃					Afferent fibers: positive for E18–P4	
	Mouse	E18-Adult	Receptor	Immunohistochemistry	Positive (body and fibers) for E18–P6	[11]
P2X ₇	Rat	E14-Adult	Receptor	Immunohistochemistry	Positive in neuron body for E18-Adult	[12]
					Positive at synaptic region under IHC for P0-Adult	

localized near the apical region (cuticular plate) of IHCs [29], since ATP-gated current had a larger amplitude and a shorter latency when the puff pipette was located at the cell apex rather than at the synaptic base (see Fig. 8 of [29]). Studies using fluorescent imaging of Na⁺ influx confirmed the apical location of ionotropic receptors, facing the endolymphatic compartment [4, 5]. To date, there have been few reports on differential expression of P2 receptors along the basilar membrane, i.e., basal, middle, and apical turns. For example, there was no difference in ATP conductance of IHCs among cochlear turns [30].

Furthermore, intracellular processes that regulate purinergic signaling have been reported in adult guinea pig

IHCs [35, 36]. ATP-induced intracellular Ca²⁺ responses are likely regulated by a negative feedback mediated by nitric oxide (NO), cyclic guanosine monophosphate (cGMP), and cGMP-dependent protein kinase.

SGN type I

As in IHCs, Ca²⁺ responses showed functional P2X and P2Y receptors [31], and electrophysiology demonstrated ionotropic (P2X) cation nonselective conductance [32, 33]. Moreover, a large conductance evoked by metabotropic (P2Y) receptors was reported [33]. A more detailed review of ATP-evoked conductances in SGNs will appear in the following section.

Table 2 In vivo studies on the cochlear partition

Species	Method	Findings	References
Guinea pig	Scala tympani (perilymph) perfusion with ATP	AP reduction	[22]
Guinea pig	Scala tympani (perilymph) perfusion with ATP	Reduction of CM, SP, DPOAE, and CAP	[23]
Guinea pig	Scala tympani (perilymph) perfusion with ATP antagonists	Complex effects, suppression of CAP	[24]
Guinea pig	Scala media (endolymph) perfusion with ATP	Suppression of EP and CM	[25]
Guinea pig	Scala tympani (perilymph) perfusion with ATP analogs	Enhancement of EP	[26]
Guinea pig	Scala media (endolymph) microinjection of ATP	Decrease of cochlear partition resistance	[27]

Regarding the regulation of ATP-induced intracellular Ca^{2+} mobilization related to NO, SGNs seem to display a different mechanism as compared to IHCs [37]. In SGNs, NO production due to Ca^{2+} influx acts in a positive feedback manner, and glucocorticoids have an enhancement effect on this process.

P2 receptors in the vestibular partition (VHC and VGN)

Compared to the cochlear partition, studies in the vestibular partition of mammals have been sparse. No in vivo electrophysiological recordings investigating the role of ATP in the mammalian vestibular organs have been reported. Histological expression of P2 receptor protein or mRNA has not been demonstrated in VHCs but there is one report of functional expression of P2 receptors [38]. In this study, functional P2 receptors were demonstrated in guinea pig VHCs by electrophysiology and Ca^{2+} responses but a detailed characterization was not completed, e.g., involvement of P2X receptors was evident but that of P2Y

receptors remained undetermined. In adult rat VGNs, immunohistochemistry demonstrated P2X₁–P2X₆ subtypes as in SGNs [14]. However, developmental changes of particular subtypes as observed with SGNs have not yet been demonstrated. As for in vitro physiological studies, Ca^{2+} responses revealed functional expression of P2X and P2Y receptors [39]. Moreover, we recently characterized electrophysiological properties of ionotropic (P2X) conductance in VGNs (Table 4) [40]. The characteristics of this conductance were similar to those observed in SGNs except that lowering extracellular pH, a procedure to evaluate modulation of the affinity of the ATP-binding site by extracellular protons [2], produced little effects in VGNs as compared to a response augmentation in SGNs [32].

P1 receptors in the inner ear

An immunohistochemical study showed expression of three subtypes of P1 adenosine receptors (A₁R, A_{2A}R, and A₃R) in both IHCs and SGNs of adult rats [41], but develop-

Table 3 In vitro studies on the cochlear partition

Species	Method	Findings	References
IHC			
Guinea pig	Ca^{2+} response	Demonstration of functional P2X and P2Y receptors	[28]
Guinea pig	Electrophysiology and Ca^{2+} response	P2X cation nonselective conductance localized near apex Characterization of P2Y-mediated Ca^{2+} response	[29]
Guinea pig	Electrophysiology	Similar ATP-evoked-conductance in all 4 turns of cochlea	[30]
SGN (type I)			
Guinea pig	Ca^{2+} response	Demonstration of functional P2X and P2Y receptors	[31]
Rat	Electrophysiology Patch clamp on cochlear slice (P3-P6)	P2X conductance (description in Table 4)	[32]
Rat	Electrophysiology Patch clamp on acutely isolated neurons	P2X and P2Y conductances (description in Table 4)	[33, 34]

Table 4 P2 conductances in SGN (type I) and VGN

P2X conductance			P2Y conductance		
SGN [32, 33]	Desensitizing cation nonselective conductance Impermeable to large cations as NMDG ⁺ ATP $\approx$ ADP > alpha, beta-me ATP > 2-MeS ATP Decay time constant $\tau \approx 2$ s Recovery after desensitization in 4 min Augmentation with lower pH	VGN [40]	Desensitizing cation nonselective conductance Impermeable to large cations as NMDG ⁺ ATP > alpha, beta-me ATP > ADP Decay time constant $\tau = 2-4$ s Recovery after desensitization in 3 min No effect with lower pH	SGN [33]	Large pore conductance that passes NMDG ⁺ and TEA ⁺ Activation of a common pathway with ACh and SP Sensitivity to pretreatment of ionomycin

mental changes of expression have not been reported. No protein or mRNA expression study for P1 receptors has been reported in the vestibular organs.

To date, there is little evidence of functional P1 receptors in the inner ear. In the cochlear partition, perfusion of adenosine in either perilymph or endolymph showed no changes in cochlear physiological parameters such as CAP [23, 25] and no in vitro study has been published. However, Vlajkovic et al. [42] (this issue; see also [43]) provides evidence that A1 receptor activation facilitates recovery from noise-induced hearing loss. In addition, prophylactic treatment with P1 receptor agonists confers otoprotection from noise damage [44, 45]. In the vestibular organs, there has been no in vivo study, and a direct application of adenosine on isolated VGNs in vitro failed to show any electrophysiological effects [40].

Physiological implication

Development

Important developmental roles played by purinergic (P2) receptors have recently been suggested [46, 47]. A specific multimeric combination of receptor subtypes (P2X₂₋₃ and P2X₃) was shown to contribute to synaptic reorganization to constitute accurate innervation pattern (SGN type I to IHC and SGN type II to OHC) just before the onset of hearing in rats, by inhibiting BDNF-mediated neuron development [47]. This corresponds to the transient expression of P2X₃ receptors reported previously [10, 11]. Another novel discovery is the contribution of purinergic signaling to the spontaneous firing activity of SGNs before the onset of hearing, which is important to neuronal survival and maintenance of tonotopic maps in the brain. It was shown that supporting cells adjacent to IHCs spontaneously release ATP that would depolarize the IHCs and trigger action potentials in the developing SGNs [46].

Signal transduction/modulation in IHC

Endolymph contains nanomolar levels of ATP [48], which is supposed to be derived from a vesicular storage in the lateral wall, i.e., stria vascularis [49], and/or to be released from supporting cells of the organ of Corti [50]. Localization of P2X receptors facing the endolymphatic surface suggests a regulatory role of endolymphatic ATP on the IHC receptor potentials. Indeed, ATP concentration increases in the endolymph during noise exposure [49]. P2X receptors at the apex of IHC may adaptively suppress sound transduction processes, as indicated by several in vivo studies [22, 23, 25].

Neuromodulation in SGN/VGN

Table 4 summarizes P2 conductances in SGN (type I) and VGN. In the two types of neurons of the inner ear, P2X ionotropic conductances share many properties including desensitization characteristics and ion permeability, except ligand preference (the order of response amplitudes to ATP and its analogs) and sensitivity to external pH. The small difference may be due to a distinct subtype configuration that forms different heteromultimeric receptor channels in SGNs and VGNs, although six subtypes (P2X₁–P2X₆) were identified in both SGNs and VGNs with similar patterns [14]. A preliminary in vitro study also identified a desensitizing (P2X) conductance in SGN type II innervating outer hair cells (OHCs), which had a similar amplitude and a similar desensitization time constant (2 s) with those of type I SGNs [51]. Moreover, it was recently shown that the function of SGN type II as a cochlear afferent can be modulated by ATP [52].

The metabotropic, P2Y-evoked conductance in SGNs has notable properties not reported in other primary sensory neurons of the cranial nerves. This slowly activating nonselective conductance is likely driven by a pore channel that can pass large cations such as NMDG⁺ and TEA⁺ [33].

Intriguingly, this large pore channel can also be activated by other metabotropic receptors, i.e., tachykinin receptors (NKR, ligand: substance P) and muscarinic acetylcholine (ACh) receptors (mAChR) as confirmed by mutual desensitization and nonadditive nature of responses. In detail, two different components in ATP-evoked currents were identified in acutely isolated SGNs from the rat cochlea: a rapid ionotropic (P2X) current and a slow metabotropic (P2Y) response. The two components could be isolated using a very short application of ATP. The first (P2X) current was mainly carried by Na^+ , but the second conductance (P2Y) had a large pore that passed very large cations (see Fig. 5 of [33]). This P2Y conductance shared many characteristics with ACh-evoked (muscarinic) and substance P-evoked conductances including the most effective block by ionomycin pretreatment (see Fig. 9 of [33] and Fig. 4 of [34]). The mechanism of this block remained uncertain, but it is possible that certain intracellular resources mobilized by intracellular calcium release are depleted by ionomycin. Mutual inhibition and nonadditivity of ligand-evoked conductances were demonstrated between each pair of the three ligands, i.e., ATP, ACh, and substance P. (See Fig. 10 of [33] and Fig. 5 of [34].) By these experiments, the P2Y receptor (P2YR)-mediated conductance was demonstrated to share the final effector channel with mAChR and NKR.

The molecular nature of this large-conductance nonspecific channel remains unknown, but this may be a member of the transient receptor potential (TRP) channel family, which has recently attracted researchers' attention. TRP channels, initially discovered in *Drosophila*, a fruit fly, form a loosely related superfamily of relatively nonselective cation channels with a diverse ionic selectivity [53, 54]. To date, TRP channels have been found ubiquitously expressed in various cell types also in vertebrates. TRP channels are comprised of six transmembrane segments with intracellular N- and C-terminals. Activation mechanisms are diverse: stimuli concerning vision, taste, olfaction, hearing, touch, thermosensation, and osmosensation, etc. The TRP superfamily, comprised of more than 30 cation channels, is divided into seven subfamilies: canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), polycystin (TRPP), mucolipin (TRPML), ankyrin (TRPA), and NOMPC (TRPN) families [53, 54]. Notably, TRPA1 has been suspected as a component of mechanotransduction channels at the hair bundles of sensory hair cells [55, 56]. In SGNs, TRP receptors have been shown to be expressed in the cell body by immunohistochemistry: TRPV1–6 [57–59], TRPC1–7, more intensely in TRPC1 and TRPC3 [60, 61], TRPM1, 2, 3, 6, 7, and 8 [62], TRPML1–3, TRPP2, 3, and 5 [63]. Modified expression of TRPV was reported following cochlear damage. Acoustic damage upregulated expression of TRPV1 in SGNs [64]. Kanamycin intoxica-

tion upregulated TRPV1 expression but downregulated TRPV4 expression [65]. Similarly, gentamicin challenge upregulated TRPV1 and 2 expression but downregulated TRPV3 and 4 expression in SGNs [66]. Developmental regulation of TRPC3 expression has recently been shown in the mouse cochlea including SGNs [67].

Among these subfamilies, the most promising candidate for the common effector channel activated by various metabotropic processes in SGNs is the TRPC channel. Indeed, there has been increasing evidence demonstrating TRPC channels as final effectors evoked by various metabotropic processes in other cell types. It has been suggested that mAChRs activate, via G-protein and phospholipase C (PLC), a nonselective cation conductance linked to TRPC5 channels in HEK cells [68]. Similarly, in neuronal PC12D cells, muscarinic receptors (M1mAChR) activated TRPC6 channels, mediated by protein kinase C [69]. In rat cardiomyocytes, P2Y₂ channels activated heteromeric TRPC3/7 channels mediated by G-protein and PLC beta [70], explaining the cause of arrhythmia after ischemic damage of cardiac cells. In HEK cells expressing both tachykinin receptors (NK2Rs) and TRPC3 channels, substance P evoked nonselective cation conductance [71]. Finally, both mAChR and P2YR were shown to activate common TRPC7 channels mediated by PLC and diacylglycerol, resulting in calcium influx in human keratinocytes [72]. Therefore, TRPC effector channels can be shared by multiple metabotropic processes evoked by different ligands, and further research targeting this type of TRP channel should be performed in SGNs.

The P2X ionotropic receptors are expected to play a direct neuromodulatory role, cooperating with glutamate receptors, since some reports showed strong expression of this receptor at the synaptic afferent boutons under IHCs [9, 16]. However, the question remains open whether ATP is coreleased with glutamate during exocytosis of the synaptic vesicles in IHCs.

In contrast, the role of the P2Y metabotropic receptors may be diverse, and we recall that these receptors have been shown to be expressed in the SGN perikaryons [33]. The P2Y-activated cation nonselective, depolarizing conductance, can reinforce neuronal excitability at the level of the perikaryon and facilitate the propagation of action potentials along the nerve fibers. Interestingly, it has recently been shown that P2Y receptors, cooperating with a simultaneously evoked P2X conductance, increased the neuronal firing rates in the mammalian cochlear nucleus [73]. Moreover, control of intracellular calcium can modify neuronal survival (trophic effect), and the neurons can recognize environmental pathologic events by sensing dispersed ATP via purinergic receptors (damage confrontation), thereby invoking (so far unknown) self-defense intracellular mechanisms. Finally, sharing a common

effector among different metabotropic receptors (P2YR, mAChR, NKR) implies an important role of this cellular process in SGNs, which remains to be elucidated.

Perspectives for future research

As described above, there is increasing evidence showing the importance of purinergic receptors (P2X) in cochlear development but their precise neuromodulatory roles in auditory physiology after cochlear maturation are still unclear. Studies on P2Y receptors in the inner ear are still sparse, and their physiological role also remains to be determined. In particular, the P2Y-associated nonspecific cation channels (TRP channel?), shared by other metabotropic receptors (mAChR and NKR), remain to be identified in SGNs. In the vestibular partition, since only a small number of works have been conducted in VHCs and VGNs, there is a large need of future investigation to identify the physiological role of these receptors in the vestibular system. Histological and functional investigation on the expression of P2 receptors in the vestibular partition (VHC/VGN) would certainly help to understand their role by comparison with the cochlear partition (IHC/SGN). As to P1 receptors, there is convincing evidence for the expression in IHCs and SGNs, with emerging physiological function. It would be of particular interest to see whether adenosine receptors, well known to modulate long-term potentiation (LTP) in central neurons, can also regulate neurotransmitter release in IHCs.

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