CHARACTERIZATION OF MUSCARINIC BINDING SITES IN THE ADULT AND DEVELOPING RAT COCHLEA

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Abstract—The maturation of the cholinergic innervation of the rat cochlea is associated with a transient increase in the muscarinic-receptor activated inositol phosphate synthesis. In order to investigate the mechanisms involved in this transient enhancement of the inositol phosphate response, the binding properties of the cochlear muscarinic receptors were studied during rat cochlear development. Incubating the membranes from 4-day-old, 12-day-old and adult cochleas with [³H]quinuclidinyl benzylate indicates that their respective, mean concentrations of cholinergic receptors are 454 ± 51 ($\pm$ SEM), 39 ± 2 and 42 ± 3 fmol/mg of protein. The dissociation constants at equilibrium are 207 ± 80 , 42 ± 7 and 28 ± 3 pM for the binding sites of the 4-day-old, 12-day-old and adult cochleas, respectively. Pharmacological characterization of the binding, using selective antagonists, shows that M3 cholinergic receptors are expressed in developing and adult cochleas. The data demonstrate that changes in muscarinic receptor affinity and number do not correlate with the previously observed peak of the inositol phosphate metabolism. The transient enhanced inositol phosphate response is therefore not due to changes in cholinergic receptors, but probably due to alterations involving the intrinsic activity of the phospholipase C and/or the efficacy of coupling of the transduction system.

Acetylcholine is the main neurotransmitter of the efferent auditory system (for review see Eybalin, 1993) that feeds back from the superior olivary complex to the cochlea. This neurotransmitter mediates slow metabotropic effects within target cells, via the activation of the muscarinic receptors (for reviews see Nathanson, 1987; Hulme *et al.*, 1990; Hosey, 1992). Their presence in the cochlea has been clearly demonstrated by both binding (James *et al.*, 1983; Whipple and Drescher, 1984; van Megen *et al.*, 1988) and functional studies (Guiramand *et al.*, 1990; Bartolami *et al.*, 1990, 1992a; Niedzielsky *et al.*, 1992). These latter investigations demonstrated that the

muscarinic receptors are linked to the inositol phosphate (IP) signalling pathway. M3 muscarinic receptors have been characterized in the 12-day-old rat cochlea (Guiramand *et al.*, 1990; Bartolami *et al.*, 1992a) and experiments using the polymerase chain reaction have identified mRNAs encoding for m1, m3 and m5 subtypes of muscarinic receptor in the mouse cochlea (Drescher *et al.*, 1992). The former two molecular forms of the cDNA-cloned receptors correspond to the M1 and M3 receptors, defined in functional studies by means of selective antagonists (Bonner, 1989; Hulme *et al.*, 1990).

During postnatal cochlear development, the activation of muscarinic receptors is characterised by a transient enhancement of the stimulated IP synthesis which peaks at day 12 (Bartolami *et al.*, 1990, 1992a). The transient increase of the IP response occurs concomitantly with both the formation of the efferent synapses on the outer hair cells (Lenoir *et al.*, 1980; Simmons *et al.*, 1990; Cole and Robertson, 1992) and the onset of physiological maturation of the cochlea (Uziel *et al.*, 1981; Puel and Uziel, 1987; Lenoir and Puel, 1987; Rybak *et al.*, 1992). Since a similar correlation between a muscarinic receptor activated IP synthesis and the efferent synaptogenesis has been

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Abbreviations AF-DX 116, 11-((2-((diethylamino)-methyl)-1-piperidinyl)acetyl)-5, 11-dihydro-6H-pyrido(2,3-b)-(1,4)-benzodiazepine-6 one; B_{max} , maximum concentration of binding site; 4-DAMP, 4-diphenylacetoxymethyl piperidine methiodide; IP, inositol phosphates; K_d , dissociation constant at equilibrium; K_i , inhibition constant at equilibrium; QNB, quinuclidinyl benzylate.

observed in the bird cochlea (Bartolami *et al.*, 1992b). These findings suggest that the IP turnover coupled to muscarinic receptors may be implicated in the functional maturation of the cochlea. In addition, the transient peak of the IP synthesis in response to activation of cholinergic receptors is concomitant with a period at which outer hair cells are hypersensitive to ototoxicity induced by aminoglycoside antibiotics (Pujol, 1986). This suggests a relationship between the aminoglycoside toxicity and the activation of the IP metabolism (Schacht, 1976, 1986; Bartolami *et al.*, 1993).

Altogether these observations indicate that the developmental pattern of the stimulation of muscarinic receptors is of importance for the physiology and pathophysiology of the cochlea. In the present study, we investigate whether the transient enhancement of the muscarinic receptor activated IP synthesis was due to developmental changes in binding properties of muscarinic receptors in the cochlea. The high affinity, but nonetheless non-specific antagonist quinuclidinyl benzylate (Yamamura and Snyder, 1974) was used to determine the concentration and affinity of muscarinic receptors in developing and adult rat cochleas. In addition, the binding sites were pharmacologically characterized using the selective antagonists pirenzepine, AF-DX 116 and 4-DAMP, respectively specific for M1, M2 and M3 muscarinic receptors (Hammer *et al.*, 1980; Ladinsky *et al.*, 1988; Barlow *et al.*, 1990). This study particularly focuses on three stages: 4-day-old, 12-day-old and adult which are representative of the three phases of the developmental profile of the muscarinic receptor activated IP synthesis. The first stage corresponds to the onset of the inositol phosphate response, the second one to the peak response, and third one to maturity of the response (Bartolami *et al.*, 1990, 1992a).

EXPERIMENTAL PROCEDURES

Materials

Tritiated quinuclidinyl benzylate (QNB, specific activity: 45.4 Ci/mmol) was purchased from New England Nuclear Boston, MA, atropine and pirenzepine from Sigma Chemical Co., St Louis MO and 4-diphenylacetoxy-*N*-methyl piperidine methiodide (4-DAMP) from Research Biochemical Inc. Natick, MA. Bovine serum albumin was obtained from Boehringer Mannheim, Indianapolis, IN and 11-((2-diethylamino)-methyl)-1-piperidinylacetyl)-5,11-dihydro-6H-pyrido(2,3-b)-(1,4)-benzodiazepine-6 one (AF-DX 116) was a gift from Boehringer Ingelheim (Reims, France). All other compounds were of analytical grade.

Dissection of the cochleas

Adult, 12 and 4-day-old Wistar rats were stunned and killed by decapitation. The cochleas were quickly dissected

out from the temporal bones, the stria vascularis were removed and the cochleas were collected in ice-cold phosphate buffered saline pH 7.4 (PBS: 50 mM Na₂HPO₄, 50 mM KH₂PO₄, 125 mM NaCl). The dissected cochleas were then composed of the organ of Corti, the spiral ganglion and the modiolus that contains the distal (peripheral) segment of the cochlear nerve.

Membrane preparation

The dissected cochleas were washed 4 times with PBS at 0–4 °C and then homogenized in PBS using an all glass homogenizer at the concentration of 12 cochleas/ml. The resulting homogenates were centrifuged at 300 g for 10 min, the supernatants containing the cochlear membranes were taken, and then stored at –70 °C before use.

Assays of [³H]QNB binding

The method was derived from the technique optimized by Yamamura and Snyder (1974). Membrane aliquots (320 µl) were mixed with 40 µl PBS and [³H]QNB at concentrations varying from 0.01 to 5 nM, and then incubated at 25 °C for 90 min. The binding reaction was stopped by filtration of the samples, under vacuum, onto Whatman GF/B filters and by washing the filters with 3 × 4 ml PBS at 0–4 °C. The amount of [³H]QNB retained/filter was measured by liquid scintillation counting with an efficacy of 50%. This procedure allows the determination of the quantity of total bound QNB. In order to measure the non-specific binding, 1 µM atropine was added to the incubation medium. In this condition, atropine is used at a 1000-fold higher concentration than the QNB concentration and therefore it competitively prevents the binding of QNB to muscarinic sites. In the presence of atropine, QNB binds solely to non-specific sites. The amount of specific bound QNB was obtained by subtracting the values of the non-specific binding from the total binding. Trials of displacement of the binding of [³H]QNB were conducted by adding the specific, muscarinic antagonists 4-DAMP (M3), pirenzepine (M1) and AF-DX 116 (M2) to the reaction medium. The results of the binding assays were normalized to the amount of protein present in the membrane preparations, which was measured according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Statistical analysis and data expression

The results were analysed using the LIGAND computer program (Munson and Rodbard, 1980) and were expressed either as ratios of the amount of bound QNB to the quantity of protein contained in the samples (fmol of QNB/mg of protein), or as percentages of the amount of specifically bound QNB. The data are the means ± SEM of three individual experiments, at least. Each experiment was performed in duplicate. The statistical significance of the data was determined using one way ANOVA.

RESULTS

Linearity of the [³H]QNB binding as a function of the concentration of cochlear membrane

Cochlear membranes obtained from adult, 12 and 4-day-old rat cochleas, were diluted in PBS at concentrations ranging from 2 to 12 cochleas/ml and used for binding assays with QNB at the concentration

of 0.08 nM. The specific QNB binding is linear for concentrations of cochlear membrane ranging from 111 to 186 $\mu\text{g/ml}$ (6–10 cochleas/ml) in the case of adult cochleas, from 57 to 143 $\mu\text{g/ml}$ (4–10 cochleas/ml) for the 12-day-old cochleas and from 52 to 317 $\mu\text{g/ml}$ (2–12 cochleas/ml) for the 4-day-old cochleas (data not shown). The mean amounts ($\pm\text{SEM}$) of protein/cochlea are $26.4 \pm 5.9 \mu\text{g}$, $14.3 \pm 1.8 \mu\text{g}$ and $18.6 \pm 2.2 \mu\text{g}$ for the 4-day-old, 12-day-old and adult cochleas, respectively.

Saturation of the [^3H]QNB binding

Membranes obtained from adult, 12-day-old and 4-day-old rat cochleas were diluted to concentrations of 149, 114 and 211 $\mu\text{g protein/ml}$ respectively (8 cochleas/ml) and incubated with increasing concentrations of [^3H]QNB. The saturation curves were established, for each stage of maturation and their respective Scatchard analyses were carried out. From these analyses, the binding constants were obtained (Table 1). The dissociation constants (K_d) are 207 ± 80 ($\pm\text{SEM}$), 42 ± 7 and 28 ± 3 pM for the muscarinic binding sites of the 4-day-old, 12-day-old and adult cochlear membranes, respectively. Each K_d value is significantly different from the other two ($P < 0.001$). The concentrations of binding sites (B_{max}) within the 4-day-old, 12-day-old and adult membrane preparations are, respectively, 454 ± 51 ($\pm\text{SEM}$), 39 ± 2 and 42 ± 3 fmol of QNB/mg of protein. The B_{max} values of the 12-day-old and adult cochlear membranes are not statistically different. The saturation of the specific binding is reached with concentrations of QNB starting at 0.1 nM in the cases of the adult and 12-day-old cochleas and, 0.5 nM for the 4-day-old cochlea. The Hill coefficients are in all cases less than but close to unity (Table 1).

Inhibition of the specific QNB binding by selective muscarinic antagonists

In this series of experiments, every membrane preparation was diluted to the concentration of 6 cochleas/ml. This concentration corresponds to about 111,

86 and 158 $\mu\text{g protein/ml}$ for the adult, 12-day-old and 4-day-old membrane preparations, respectively. The subtype-specific muscarinic antagonists pirenzepine (M1), AF-DX 116 (M2) and 4-DAMP (M3) were used to displace the specific QNB binding obtained by incubating the samples of adult and 12-day-old cochlear membranes with 0.08 nM of QNB and the 4-day-old membrane samples with 0.4 nM of QNB. These concentrations of QNB were selected from the saturation study.

As for the adult samples, the subtype-specific antagonists inhibit the specific binding of QNB, that is 38 ± 9 ($\pm\text{SEM}$) fmol of QNB/mg of protein, in a dose-dependent manner (Fig. 1) with a rank order of potency: 4-DAMP > AF-DX 116 > pirenzepine (Table 2). The inhibition constants (K_i) are significantly different, with $P < 0.01$ for 4-DAMP, pirenzepine and with $P < 0.05$ for AF-DX 116/pirenzepine and 4-DAMP/AF-DX 116.

In the 12-day-old preparation, the specific binding of QNB is 45 ± 10 fmol of QNB/mg of protein. It is inhibited in a dose-dependent manner (Fig. 2) with the following rank order of potency: 4-DAMP > AF-DX 116 = pirenzepine. The K_i values (Table 2) are

Fig. 1. Inhibition of the specific binding of QNB by muscarinic antagonists in adult cochlear membranes. The membranes, diluted to a concentration of $111 \pm 13 \mu\text{g/ml}$, were incubated with 0.08 nM of [^3H]QNB and exposed to the selective muscarinic antagonists 4-DAMP, AF-DX 116 and pirenzepine. The results are given as percentages of the specific binding of QNB, obtained in the absence of these muscarinic blockers, which is equal to 38 ± 9 ($\pm\text{SEM}$) fmol of QNB/mg of protein. The data points are means of 3 replications, at least. Each replication was performed in duplicate. The error bars are SEM and are not shown when they are smaller than the symbols.

Table 1 Constants of the binding of QNB at equilibrium, in the cochlear membranes

Age	n	K_d (pM)	B_{max} (fmol/mg)	Hill number
4-day-old	8	$207 \pm 80^{***}$	$454 \pm 51^{***}$	0.94 ± 0.02
12-day-old	6	$42 \pm 7^{***}$	39 ± 2	0.92 ± 0.05
Adult	6	28 ± 3	42 ± 3	0.97 ± 0.02

All the data are means $\pm\text{SEM}$ of experiments conducted in duplicate. The number of experiment is *n* and the number of tested QNB concentrations, experiment ranges from 5 to 11. *** different from its corresponding adult parameter with $P < 0.001$.

Table 2 Inhibitory parameters of the displacement of the specific binding of QNB in the cochlear membranes

Age	Antagonist	Free QNB (nM)	IC ₅₀ (nM)	K _i (nM)	Hill number
4-day-old	4-DAMP	0.40	126 ± 11	42.7 ± 3.7	1.08 ± 0.06
4-day-old	AF-DX 116	0.40	1820 ± 40	620.7 ± 13.6***	1.03 ± 0.08
4-day-old	Pirenzepine	0.40	6550 ± 250	2233.7 ± 85.2***	1.14 ± 0.04
12-day-old	4-DAMP	0.08	11 ± 2	3.7 ± 0.7	0.82 ± 0.02
12-day-old	AF-DX 116	0.08	500 ± 90	170.5 ± 30.7*	1.01 ± 0.02
12-day-old	Pirenzepine	0.08	588 ± 251	200.5 ± 85.6**	0.90 ± 0.05
Adult	4-DAMP	0.08	12 ± 2	3.1 ± 0.5	0.84 ± 0.08
Adult	AF-DX 116	0.08	189 ± 70	49.0 ± 18.1*	0.95 ± 0.17
Adult	Pirenzepine	0.08	752 ± 188	194.9 ± 48.8**	1.17 ± 0.09

The data are means ± SEM of experiments conducted in duplicate. At least 3 identical experiments were done in each case. K_i values were calculated by the equation of Cheng and Prusoff (1973): $K_i = (1 + C/A_0) \times IC_{50}$, with C being the concentration of free QNB, the K_i values are given in Table 2. For a given developmental stage, K_i values are different from the K_i value obtained with 4-DAMP, with $P < 0.001$ (***), $P < 0.01$ (**) and $P < 0.05$ (*)

significantly different for 4-DAMP/AF-DX 116 ($P < 0.01$) and for 4-DAMP/pirenzepine ($P < 0.05$).

Exposure of the 4-day-old membranes to antagonists also results in dose-dependent inhibitions of the specific binding of QNB, that is 479 ± 28 fmol of QNB/mg protein (Fig. 3). The relative order of potency is 4-DAMP > AF-DX 116 < pirenzepine. The K_i values of the three antagonists statistically differ ($P < 0.001$, in every case, Table 2). At all stages, the Hill numbers are close to unity, whatever the antagonist (Table 2).

DISCUSSION

Changes in muscarinic binding properties are not involved in the enhanced IP metabolism

The affinity of the cochlear muscarinic receptor increases during maturation, while the concentration of receptor decreases with age. When compared with the developmental pattern of the muscarinic receptor activated IP synthesis (Bartolami *et al.*, 1990, 1992a), the data demonstrate that binding properties of muscarinic sites and changes in the muscarinic stimu-

Fig. 2 Inhibition of the specific binding of QNB by muscarinic antagonists in membranes from 12-day-old cochleas. The membranes, diluted to a concentration of 86 ± 11 μ g/ml, were incubated with 0.08 nM of [3 H]QNB and exposed to the selective muscarinic antagonists 4-DAMP, AF-DX 116 and pirenzepine. The results are given as percentages of the specific binding of QNB, obtained in the absence of these muscarinic blockers, which is equal to 45 ± 10 ($\pm$ SEM) fmol of QNB/mg of protein. The data points are means of 3 replications, at least. Each replication was performed in duplicate. The error bars are SEM and are not shown when they are smaller than the symbols.

Fig. 3 Inhibition of the specific binding of QNB by muscarinic antagonists in membranes from 4-day-old cochleas. The membranes, diluted to a concentration of 158 ± 35 μ g/ml, were incubated with 0.4 nM of [3 H]QNB and exposed to the selective muscarinic antagonists 4-DAMP, AF-DX 116 and pirenzepine, used at various concentrations. The results are given as percentages of the specific binding of QNB, obtained in the absence of these muscarinic blockers, which is equal to 479 ± 28 ($\pm$ SEM) fmol of QNB/mg of protein. The data points are means of 3 replications, at least. Each replication was performed in duplicate. The error bars are SEM and are not shown when they are smaller than the symbols.

lation of the signalling pathway diverge during maturation of the auditory organ. For instance, the IP synthesis decreases from day 12 to adulthood whereas, during the same period, the receptor affinity increases and the concentration of muscarinic site does not change significantly (Fig. 4). Likewise, from day 4 to day 12 the IP formation increases while the concentration of muscarinic sites falls drastically (Fig. 4). Such a difference between muscarinic binding properties and muscarinic agonist-elicited IP turnover have also been shown during cortical ontogeny (Balduini *et al.*, 1987; Heacock *et al.*, 1987; Rooney and Nahorski, 1987). This conclusion rules out the possibility that the transient enhancement of the IP response peaking at day 12 (Bartolami *et al.*, 1990, 1992a), is due to an increase in either the concentration of muscarinic receptors or the affinity of the binding sites.

Pharmacology of the binding sites

Alternatively, a variation in the receptor phenotype could explain the transient, high IP response. The expression of M3 receptors, characterized at day 12 (Guiramand *et al.*, 1990; Bartolami *et al.*, 1992a), may decline and switch to the synthesis of muscarinic

receptors which are not coupled to phospholipase C, but rather linked to inhibition of adenylyl cyclase such as m2 or m4 receptors (Nathanson, 1987; Bonner, 1989; Hulme *et al.*, 1990; Hosey, 1992). However, the possibility of such a change in the muscarinic phenotype can be dismissed because the molecular analysis of mRNAs encoding cochlear muscarinic receptors did not detect m2 and m4 mRNAs in cochlear cells (Drescher *et al.*, 1992).

Drescher and co-workers (1992) also identified m1, m3 and m5 proteins mRNAs in adult mice cochleas, suggesting the presence of several muscarinic receptor subpopulations. In the present study, the very low affinity of pirenzepine for the binding sites argues against the functional expression of M1 receptors in the auditory organ. The absence of M1 receptors is further supported by an extended characterization which showed that both antagonist and agonist M1 ligands have a low affinity for IP synthesis-coupled muscarinic receptors in the cochlea (Bartolami *et al.*, 1992a). As for M5 receptors, the lack of a specific ligand prevents their pharmacological identification. On the other hand, in the present work Hill numbers are close to unity (Tables 1 and 2). In agreement with other surveys, this indicates the existence of solely one population of muscarinic sites (James *et al.*, 1983; Whipple and Drescher, 1984; van Megen *et al.*, 1988). This population is highly sensitive to 4-DAMP which is at least 15-fold more efficient than AF-DX 116 and pirenzepine in inhibiting the QNB binding (Table 2). Therefore, the cochlear muscarinic population is likely of the M3 subtype, as previously reported (Guiramand *et al.*, 1990; Bartolami *et al.*, 1992a). Together with mRNA analysis (Drescher *et al.*, 1992), our data suggest that the synthesis of M1 and M5 receptors may be regulated posttranscriptionally. In addition, M3 receptors have a pharmacology proper to the cochlea in that AF-DX 116 is a better antagonist than pirenzepine at the binding sites. Such a profile was also found *in vivo* (Kujawa *et al.*, 1993) and in patch-clamp studies using isolated outer hair cells (Kakchata *et al.*, 1993).

Concerning the muscarinic sites of the 4-day-old cochlea, their pharmacologic profile fits the M3 type but their K_d and K_i parameters are much higher than the ones generally obtained with M3 sites. This discrepancy may reflect a possible molecular maturation of muscarinic proteins during cochlear development, as has been shown in the retina (Cho and Klein, 1988).

Enhancement of phospholipase C activity

From the previous conclusions, it appears that the transitional peak activation of muscarinic receptors is

Fig. 4. Relationship between muscarinic binding properties and the stimulated IP formation during cochlear maturation. Concentrations and affinities of muscarinic receptors are obtained from the saturation studies. Receptor affinities were calculated from K_d values (affinity = $1/K_d$). Data obtained in each saturation experiment were expressed as percentage of the mean affinity and B_{max} values determined at adulthood. These percentage levels were combined for each developmental stage and their means ($\pm$ SEM, $n = 6$ at least) are shown. The adult mean B_{max} value is given in Table 1 and the adult mean affinity value is 38.81 ± 4.20 nM $^{-1}$. The IP responses are from Bartolami *et al.* (1992a, Fig. 1). The IP synthesis was stimulated by carbachol (1 mM), the basal level taken to be 100% of IP formation in adult cochleas is 0.043 ± 0.006 dpm 3 H-IPs/dpm [3 H]myo-inositol taken up/cochlea

accounted for by changes in activities of other proteins in the signalling pathway, lying downstream from muscarinic sites. These changes may be either an increase in the efficacy of coupling between receptors and phospholipases C, as reported in the developing cortex (Balduino *et al.*, 1987; Heacock *et al.*, 1987; Rooney and Nahorski, 1987) or an alteration in the intrinsic activity of phospholipase C.

Concerning coupling efficiency, besides regulation by interacting molecules, the improvement of the coupling efficiency could be due to the transitional expression of G proteins that are more potent in stimulating phospholipases C. Indeed, it has been reported that several G proteins able to activate phospholipases C with various efficiencies exist (Ashkenazi *et al.*, 1989; Lechleiter *et al.*, 1991). Alternatively, a variation in the turnover of G protein and/or phospholipase C could be involved. This would modify the stoichiometry of interactions between receptors, G proteins and phospholipases C, thus resulting in a greater number of phospholipases C activated by a single muscarinic receptor.

As for alterations of phospholipase C intrinsic activity, these modulations may be achieved by several mechanisms: (1) phosphorylation of phospholipase C by various kinases (Crooke and Bennet, 1989; Majerus *et al.*, 1990; Rhee, 1991; Guillon *et al.*, 1992), some of these kinases being present in the cochlea (Coling and Schacht, 1991); (2) regulation of the intracellular calcium level (Crooke and Bennet, 1989; Guillon *et al.*, 1992); (3) inhibition by phospholipids such as phosphatidylcholine (Majerus *et al.*, 1986); (4) regulation of phospholipase C translocation (Lee *et al.*, 1987) and (5) changes in the substrate supply due either to complex formations between substrate molecules and proteins such as alpha actinine, profilin and profilactine (Lassing and Lindberg, 1985; Goldshmidt-Clermont *et al.*, 1990; Fukami *et al.*, 1992) or to alteration of substrate synthesis (Guillon *et al.*, 1992). Actually, since an enhancement of the basal IP turnover parallels the transitional rise in the stimulated IP response (Bartolami *et al.*, 1990, 1992a), an alteration of the intrinsic activity of phospholipase C is favoured with regard to changes in the coupling efficiency.

In summary, the developmental divergence between the muscarinic binding properties and the pattern of stimulated IP metabolism suggests that changes in the properties of muscarinic receptors do not underlie the transient rise of the inositol phosphate response. Therefore, the transient increase, observed during the maturation of the rat cochlea, in both the basal and muscarinic receptor-mediated synthesis of

inositol phosphates appears to be likely due to an enhancement of the intrinsic activity of phospholipase C.

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