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Effects of ototoxins on quinuclidinyl benzylate binding in the rat cochlea

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Abstract

Ototoxins inhibit the muscarinic receptor-activated inositol phosphate synthesis in the rat cochlea. In order to study this inhibitory mechanism, we investigated the effects of the ototoxins ethacrynate, cisplatin, HgCl₂ and neomycin on [³H]quinuclidinyl benzylate binding to muscarinic receptors in adult and 12-day-old rat cochleas. The results are similar whatever the age: at concentrations that inhibit the inositol phosphate synthesis, ethacrynate is without effect. Neomycin only reduces [³H]quinuclidinyl benzylate binding at concentrations in the millimolar range. Cisplatin and HgCl₂ block the binding in a dose-dependent way. These results suggest that the block of the transduction system by cisplatin and HgCl₂ is due to direct interactions with muscarinic binding sites. Moreover, considering these data together with previous results, ethacrynate and neomycin may affect the phosphoinositide signalling pathway at targets including phosphoinositides and G proteins.

Key words: Muscarinic receptor; [³H]Quinuclidinyl benzylate; Ethacrynate; Neomycin; Cisplatin; Mercuric chloride; Ototoxicity

Ototoxins are molecules belonging to various classes (anti-cancer drugs, aminoglycoside antibiotics, mercurials, diuretics) that induce the loss of hearing and/or balance. Ototoxicity poses therefore a severe limitation on the clinical use of some of these drugs [9]. In the cochlea, several ototoxins have been shown to interact with the polyphosphoinositide turnover [3,19]. Muscarinic receptor-activated inositol phosphate synthesis is specifically inhibited by ototoxins in the cochlea while the basal turnover is not sensitive to these drugs [3]. These findings were obtained in 12-day-old rat cochleas, a stage when the cochleas are extremely sensitive to aminoglycoside-induced ototoxicity [17]; and when the synthesis of the inositol phosphates, coupled to muscarinic receptors, is transiently enhanced [2]. These data suggest that ototoxins reduce the activity of at least one of the main proteins in the polyphosphoinositide signalling pathway: i.e. muscarinic receptor, G protein and phospholipase C. Another alternative is the formation of a complex between

ototoxins and phosphoinositides which deprives phospholipase C of its phosphoinositide substrates [19]. Then, in order to shed more light on the mechanism(s) of the ototoxin-induced suppression of the carbachol-elicited formation of inositol phosphates, we assessed the effects of ototoxins on muscarinic receptors. This was achieved by investigating the effects of mercuric chloride, the diuretic ethacrynate, the anti-cancer drug cisplatin and the aminoglycoside neomycin on the binding of the antagonist quinuclidinyl benzylate (QNB) to muscarinic receptors [26], in membrane preparations of both adult and 12-day-old rat cochleas.

Membrane preparation. Adult and 12-day-old Wistar rats were anesthetized by inhalation of halothane before decapitation. The cochleas were dissected from temporal bones and the stria vascularis were removed. The dissected cochleas, thus composed of the organ of Corti, the spiral ganglion and the peripheral part of the cochlear nerve contained in the bony modiolus, were collected in phosphate buffered saline, pH 7.4 (PBS; 50 mM Na₂HPO₄, 50 mM KH₂PO₄, 125 mM NaCl) at 0–4°C. Cochleas were washed 4 times with PBS and then homogenized in PBS using an all glass homogenizer at the concentration of 12 cochleas/ml. Following a centrifugation at 300 × g for 10 min, the supernatant containing the

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cochlear membranes was taken, and then stored at -70°C before use.

Assays of [^3H]quinclidinyl benzylate binding. Previous studies demonstrated that membrane preparation concentrated from 6 to 10 cochleas/ml bind QNB linearly [4,24]. Thus, membranes were diluted in PBS at 6 cochleas/ml and aliquots ($320\ \mu\text{l}$) were mixed with $40\ \mu\text{l}$ PBS and [^3H]QNB (New England Nuclear, Boston, MA; specific activity: $45.4\ \text{Ci/mmol}$) at various concentrations and incubated at 25°C for 90 min. The binding reaction was stopped by filtration of the samples onto Whatman GF/B filters and by washing the filters with $3 \times 4\ \text{ml}$ PBS at $0-4^{\circ}\text{C}$. The amount of [^3H]QNB retained per filter was measured by liquid scintillation counting. This procedure allowed the determination of the total amount of bound QNB. In order to measure the non-specific binding, $1\ \mu\text{M}$ atropine (Sigma Chemical Co., St. Louis, MO) was added to the incubation medium. The amount of specifically bound QNB was obtained by subtracting non-specific binding from the total binding. Trials of displacement of bound QNB were conducted by adding the ototoxins to the medium. Here, it must be stressed that the ototoxins were used in concentration ranges at which they inhibit the phosphoinositide metabolism [3]. The results were normalized to the amount of protein present in the membrane preparation, which was measured according to Lowry and coworkers [14].

Data expression and statistical analysis. The results were analyzed using the LIGAND computer program [15]. They 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. Data are means \pm S.E.M. of 3 experiments, at least. Each individual experiment was composed of duplicated data points. The statistical significance of the data was determined using one-way ANOVA.

Properties of the QNB binding. The saturation of the QNB binding was obtained by incubating membranes with increasing concentrations of QNB. Saturation curves ($n = 6$) were subjected to Scatchard analyses for both stages of development (Fig. 1). The concentrations of binding sites (B_{max}) were 35 ± 3 (\pm S.E.M.) and 43 ± 5 fmol QNB/mg protein and the dissociation constants (K_d) were 42 ± 4 and 30 ± 1 pM for the 12-day-old and adult preparations, respectively. Both Hill coefficients were close to unity (0.96 ± 0.03 for 12-day-old membranes and 0.98 ± 0.06 for adult preparation).

Displacement of the QNB binding by ototoxins. In 12-day-old cochleas (Fig. 2, left), incubating the membranes with $0.08\ \text{nM}$ of QNB resulted in a specific QNB binding of 30 ± 7 fmol QNB/mg protein (\pm SEM) in controls and it corresponded to 0.44 ± 0.10 fmol QNB/cochlea. HgCl_2 caused a dose-dependent decrease in the QNB binding with an inhibition constant (K_i) of $4 \pm 14\ \mu\text{M}$. Cisplatin reduced the QNB binding in a dose-dependent

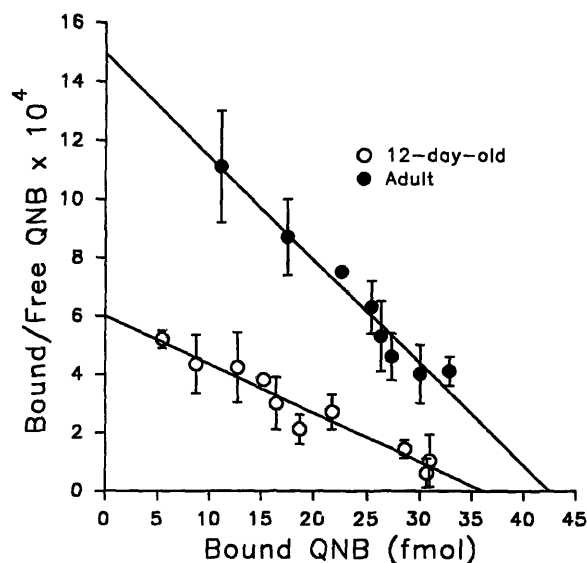


Fig. 1. Examples of Scatchard analysis the QNB binding in cochlear membranes from 12-day-old and adult rats. Membranes were incubated with increasing concentration of [^3H]QNB until saturation of the binding and saturation curves obtained from duplicated data-points were then subjected to Scatchard analysis. Error bars are S.E.M. of the duplicated data and are not shown when they are smaller than the symbols. These experiments were replicated 6 times for each developmental stage and the means B_{max} and K_d values obtained are given in the results.

way with a K_i value of $493 \pm 55\ \mu\text{M}$. Neomycin partially inhibited the QNB binding at concentrations above $1\ \text{mM}$; for instance it reduced the binding of $55 \pm 10\%$ at $10\ \text{mM}$ ($p < 0.01$). Ethacrynate, at all tested concentrations, had no effect on the specific binding.

In adult cochleas (Fig. 2, right), the specific QNB binding was 25 ± 6 fmol QNB/mg protein (\pm S.E.M.) in controls and it corresponds to 0.47 ± 0.11 fmol QNB/cochlea. The effects of ototoxins were similar to those observed in the immature stage: HgCl_2 and cisplatin suppressed in a dose-dependent manner the QNB binding with K_i values of 31 ± 8 and $220 \pm 16\ \mu\text{M}$, respectively. Neomycin at concentrations higher than $1\ \text{mM}$ partially decreased the QNB binding ($32 \pm 5\%$ of inhibition at $15\ \text{mM}$, $P < 0.01$). Ethacrynate was without effect on the QNB binding when applied at concentrations which blocks the inositol phosphate formation [3].

When comparing the data obtained at both stages of development, only cisplatin displaced QNB binding with a significant greater efficacy in the adult than in the immature preparation ($P < 0.01$). HgCl_2 seems to reduce QNB binding more efficiently in immature than adult preparation. However, the difference in the K_i values is not statistically significant.

The binding features of QNB to cochlear membranes are in line with the previous characterization of muscarinic binding sites during the rat cochlear development [4]. Indeed, K_d and B_{max} values and Hill numbers are in the

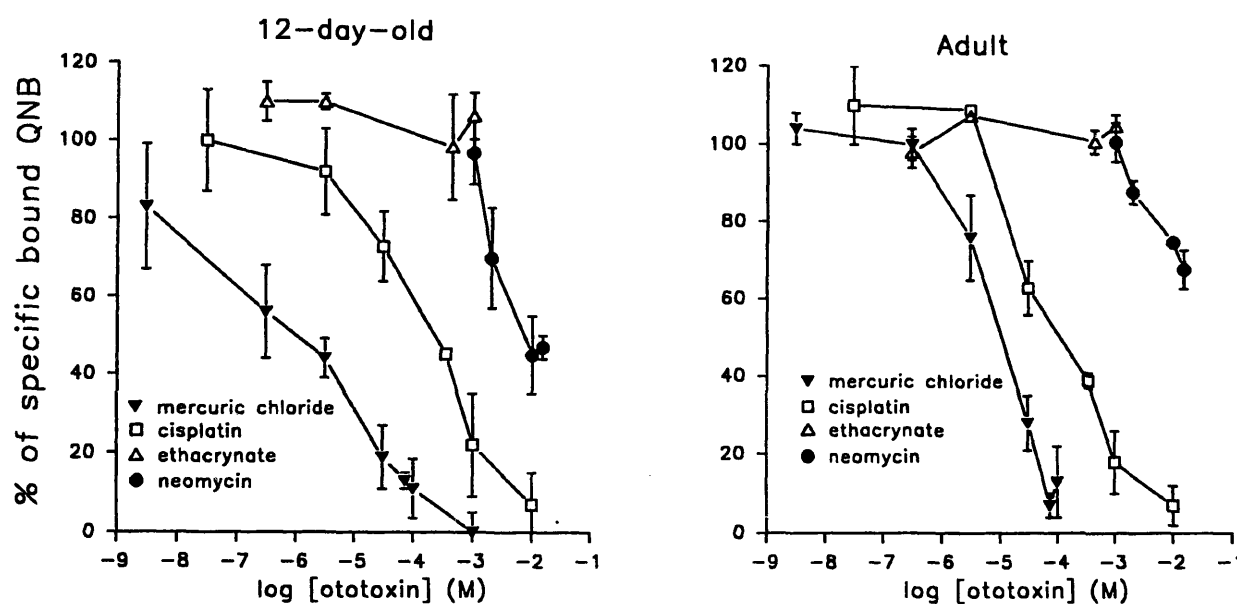


Fig. 2. Effects of ototoxins on specific QNB binding to cochlear membranes of 12-day-old (left panel) and adult rats (right panel). Tritiated QNB was used at 0.08 nM. The data are means \pm S.E.M. of, at least, 3 determinations conducted in duplicate and are expressed as percentages of the level of specific bound QNB, taken as 100%. The 100% levels correspond to 30 ± 7 and 25 ± 6 fmol of QNB/mg of protein for the immature and adult stages, respectively.

range of the corresponding binding parameters established in the developmental study (in 12-day-old preparation: $B_{\max} = 39 \pm 2$ fmol QNB/mg protein, $K_d = 42 \pm 7$ pM and Hill number = 0.92 ± 0.05 ; in adult preparation: $B_{\max} = 42 \pm 3$ fmol QNB/mg protein, $K_d = 28 \pm 3$ pM and Hill number = 0.97 ± 0.02 ; [4]). When comparing the effects of ototoxins on the QNB binding and on the inositol phosphate formation, reported earlier [3], it appears that cisplatin and HgCl_2 reduce the QNB binding with efficacies that seem comparable to those of their inhibitions of the muscarinic receptor-activated inositol phosphate synthesis [3]. Therefore, a direct action on muscarinic binding sites may explain the block by cisplatin and HgCl_2 of the inositol phosphate synthesis in the rat cochlea.

In the peripheral auditory system, muscarinic receptors are of the M3 subtype [4,18] and are particularly present at the cholinergic synapses between medial efferent axons and outer hair cells [6,10]. They are also presumably located at synapses between lateral efferent axons and afferent dendrites of auditory neurons [18]. These synapses are bathed by the perilymph that is likely to be the main route of ototoxins from the serum to target cells. Most pharmacokinetic surveys of ototoxins in the cochlea have been devoted to neomycin and other aminoglycosides [7,13,20,21,22,23]. In cochlear tissues, neomycin concentration was found not to exceed $100 \mu\text{M}$ [7]. Perilymphatic level of aminoglycoside amounts to $10\text{--}20 \mu\text{M}$ in experiments using drugs at concentrations higher than therapeutic dosages; while therapeutic treatment yields to perilymphatic concentrations of $4\text{--}7$

μM [20,21]. Perilymphatic levels measured in patients treated with aminoglycosides are close to $1 \mu\text{M}$ [13]. Therefore, it appears that the block of muscarinic binding sites by neomycin may not play a toxic role since it only occurs at 10 mM and above.

Ethacrynate and *N*-ethylmaleimide, a molecule that uncouples muscarinic signalling pathways [1,8,12,16,25], inhibit the muscarinic receptor-activated inositol phosphate synthesis in an additive manner in the rat cochlea [5], suggesting that they act on the same targets. In addition, *N*-ethylmaleimide affects neither the muscarinic receptor binding nor the basal metabolism of inositol phosphates [5] as does ethacrynate. Therefore, ethacrynate and *N*-ethylmaleimide block the signalling pathway in an identical fashion. Since *N*-ethylmaleimide has been shown to suppress muscarinic receptor-linked transduction systems by blocking G proteins in various models [1,8,12] and since ethacrynate seems to inhibit G proteins associated with adenylate cyclase in the cochlea [11], we propose that ethacrynate reduces the muscarinic receptor-activated inositol phosphate synthesis by blocking the coupling function of G proteins. The ethacrynate-induced uncoupling may also affect regions of the muscarinic receptor and the phospholipase C that are involved in interactions with the G protein. Moreover, ethacrynate may not act on the catalytic domain of phospholipase C since this drug does not affect the basal phosphoinositide metabolism [3].

In summary, the inhibition of the inositol phosphate turnover in the cochlea mediated by cisplatin and HgCl_2 seems to be due to a block of muscarinic binding sites.

For neomycin and ethacrynate, which have no significant effect on the binding site, the inhibition of the inositol phosphate release should be accounted for by different mechanisms. Actually, aminoglycosides could interact directly with phosphoinositides [19] while ethacrynate might inhibit the coupling mechanism of the signalling pathway.

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