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Sulphydryl-modifying Reagents Alter Ototoxin Block of Muscarinic Receptor-linked Phosphoinositide Turnover in the Cochlea

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Key words: rat, *N*-ethylmaleimide, cadmium, ototoxic drugs, inositol phosphates, muscarinic receptors

Abstract

In the 12-day-old rat cochlea, the synthesis of inositol phosphates (IPs) can be activated via M3 cholinceptors. This stimulation is blocked by ototoxins (mercury, ethacrynate, cisplatin, neomycin), drugs with side effects that lead to damage of hair cells and strial cells. As these toxic effects can be reversed *in vivo* by thiol molecules, we investigated whether modifications of thiol compounds could be involved in ototoxin-induced inhibition of the IP turnover in the cochlea. For this purpose, we assessed whether the sulphydryl-modifying reagents *N*-ethylmaleimide and cadmium modify the carbachol-stimulated formation of IPs in the 12-day-old rat cochlea. Both molecules inhibit the carbachol effect on a dose-dependent way without altering the basal metabolism of IPs. As cadmium may block some calcium channels, the effect of verapamil, another calcium channel antagonist, was tested. Verapamil (1–50 μ M) does not alter carbachol-evoked IP formation, suggesting that the inhibitory effect of cadmium is not due to a calcium influx block. Binding experiments with the muscarinic ligand quinuclidinyl benzylate (QNB) showed that the sulphydryl-modifying reagents do not displace QNB from binding sites. Combining ototoxins and reagents shows that *N*-ethylmaleimide acts synergistically with all ototoxins but ethacrynate while cadmium does so only with mercury. Both *N*-ethylmaleimide and cadmium have additive effects with ethacrynate. As a supplement, disulphide bond-modifying agents do not alter the carbachol-enhanced metabolism of IPs. These results suggest that molecules having thiol-modifying properties inhibit the carbachol-induced turnover of IPs without acting at the muscarinic sites. Since thiol modifiers and ethacrynate share similar features in both QNB binding and IP response it is hypothesized that they strike common targets, possibly G proteins.

Introduction

The ototoxins are drugs belonging to several biochemical classes: mercurials, anti-cancer drugs, 'loop' diuretics and aminoglycoside antibiotics. A feature that they have in common is that they provoke damage to cells in the cochlea. In this structure, their main targets are the strial cells of the lateral wall and the outer hair cells (OHCs) in the organ of Corti (for reviews see Hawkins, 1976; Rybak, 1986; Huang and Schacht, 1989). The mechanisms underlying ototoxicity are very complex and are currently the subject of considerable investigation. Recently, the blockage of the muscarinic receptor-linked inositol phosphate (IP) signalling pathway (Guiramand *et al.*, 1990; Bartolami *et al.*, 1990; Niedzielsky *et al.*, 1992) by various ototoxins (mercuric chloride, cisplatin, ethacrynate and neomycin) has been reported in the rat cochlea (Bartolami *et al.*, 1993). In addition, binding studies showed that all ototoxins except ethacrynate interact with muscarinic binding sites (S. Bartolami, M. Planche and R. Pujol, submitted for publication), and Schacht (1976, 1986) established that aminoglycosides interact with the phosphoinositide cycle by complexing

phosphatidylinositol 4,5-bisphosphate. It is, then, possible that ototoxins reduce the carbachol-activated IP response in several ways, including binding to precursors and occupancy of muscarinic sites.

Most ototoxins are also nephrotoxic, indicating that pathological processes in the cochlea and in the kidney could be the same. In this respect, ethacrynate, which is a sulphydryl-reactive diuretic (Koechel and Cafruny, 1975), has been proposed to cause nephrotoxicity via an alkylating step (Koechel *et al.*, 1984). The nephrotoxicity provoked by cisplatin can be prevented by sulphydryl molecules including the thiol metabolite WR1065 of the prodrug WR2721 (Treskes *et al.*, 1992a), diethyldithiocarbamate (Borch and Pleasants, 1979), 4-methylthiobenzoic acid (Boogaard *et al.*, 1991), *N*-benzyl-D-glucamine dithiocarbamate (Kiyozumi *et al.*, 1991) and glutathione (Zunino *et al.*, 1989). Moreover, similar observations are reported in the inner ear: the toxic metabolite of the aminoglycoside gentamicin (Huang and Schacht, 1990; Crann *et al.*, 1992), which promotes OHC death *in vitro*, is inactivated by glutathione (Garetz and Schacht, 1992).

Conversely, depletion of glutathione potentiates the ototoxicity induced by ethacrynate and the aminoglycoside kanamycin (Hoffman *et al.*, 1987, 1988). Lastly, mercuric chloride, which inhibits the carbachol-evoked formation of IPs in the rat cochlea (Bartolami *et al.*, 1993), is a well known sulphhydryl-modifying reagent (Vallee and Ulmer, 1972).

These observations indicate the existence of interactions between ototoxins and sulphhydryl group-bearing compounds in both ototoxicity and nephrotoxicity. A similar conclusion can be reached with cisplatin in other systems. For example, cisplatin-induced DNA platination is blocked by WR1065 and diethyldithiocarbamate (Treskes *et al.*, 1992b), there are strong interactions between cisplatin and the sulphhydryl residue cysteine (Lempers and Reedijk, 1990), and the resistance of cancer cell lines to cisplatin correlates with an increase in the cellular level of glutathione (Godwin *et al.*, 1992).

It appears, then, that ototoxins such as cisplatin, mercury and ethacrynate interact with molecules bearing thiol groups. The fact that ototoxins may inhibit carbachol-stimulated IP formation by acting on sulphhydryl group-bearing proteins is therefore a tempting hypothesis. The aim of this report was to assess, in the cochlea, the sensitivity of the carbachol-induced metabolism of IPs to molecules known to react with sulphhydryl groups. For this purpose, we studied the effect of *N*-ethylmaleimide and cadmium on the metabolism of IPs coupled to muscarinic receptors in the 12-day-old rat cochlea. This stage of maturation was chosen firstly because the carbachol-elicited turnover of IPs is maximal at day 12 (Bartolami *et al.*, 1990) and secondly because this age is within the critical period of enhanced susceptibility to ototoxicity (for review see Pujol, 1986). *N*-ethylmaleimide was selected both for its high potency as a sulphhydryl-modifying reagent and for its alkylating property, similar to that of ethacrynate, and cadmium was chosen because, besides being a sulphhydryl modifier (Vallee and Ulmer, 1972; Bruggeman *et al.*, 1992) and a calcium channel blocker (Tsien, 1983), it is also both a nephrotoxic compound (Nicholson *et al.*, 1983) and a heavy metal like cisplatin and mercury. Preliminary results have been presented (Bartolami *et al.*, 1992a).

Materials and methods

Reagents

Myo-[2-³H]inositol (specific activity 17.0 Ci/mmol) was purchased from Dositek (France) and tritiated quiniclidinyl benzylate (QNB, specific activity 45.4 Ci/mmol) from New England Nuclear. Atropine, carbachol, *N*-ethylmaleimide (NEM), neomycin, ethacrynic acid, cisplatin (*cis*-diamminedichloroplatinum II), DL-dithiothreitol, verapamil and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) were obtained from Sigma. All other compounds were of analytical grade.

Dissection of cochleas

Twelve-day-old Wistar rats were obtained from the Centre d'élevage de la Faculté de Pharmacie (Montpellier, France) and stunned before decapitation. The cochleas were dissected from temporal bones, the otic capsules and the stria vascularis were removed with the aid of a high-quality dissecting microscope. Under microscopic observation, the various regions of the cochlea including the organ of Corti and the spiral ganglion were easily recognizable, enabling the verification of morphological integrity of each cochlea. Damaged cochleas were discarded. The cochlear tissues were collected in Krebs–Ringer buffer (in mM: 125 NaCl, 3.5 KCl, 1.25 KH₂PO₄, 1.2 MgSO₄, 1.5 CaCl₂, 25 NaHCO₃ and 10 glucose). The dissected cochleas (consisting of the organ of Corti, the spiral ganglion and the modiolus) were

maintained in buffer equilibrated to pH 7.4 by bubbling with a mixture of O₂ 95% and CO₂ 5% (vol/vol) throughout the experiment.

Labelling of cochlear tissues

Incorporation of [³H]inositol into the cochleas was carried out at 37°C for 75 min in Krebs–Ringer buffer containing 1 mM cytidine and 10.8 μCi/ml myo-[2-³H]inositol. Afterwards, the cochleas were washed four times with Krebs–Ringer buffer and then individually distributed in test tubes containing 500 μl buffer. The tubes were transferred to a water bath thermostatically maintained at 37°C and continuously bubbled with the O₂/CO₂ mixture.

Stimulation of synthesis of IPs and purification of [³H]IPs

LiCl (10 mM) and the toxic agents neomycin, ethacrynate, mercuric chloride, cisplatin and/or sulphhydryl-modifying reagents NEM and cadmium chloride (where applicable) were added 15 min prior to stimulation by carbachol (1 mM). The muscarinic agonist was allowed to react for 20 min. The reaction was stopped by the application of 50 μl/tube of perchloric acid (72%) and by placing the tubes on ice. Cochlear homogenates, obtained by sonication, were centrifuged at 2000 *g* for 5 min. The supernatants containing the [³H]IPs were neutralized with 1.5 M KOH/0.075 M HEPES. After separation by anion-exchange chromatography, the [³H]IPs formed were measured in each cochlea as previously described (Guiramand *et al.*, 1990). The basal IP metabolism (basal control) was determined according to this procedure, in the absence of carbachol, ototoxins and sulphhydryl modifiers. The stimulated control level of IP formation was determined with carbachol (1 mM) in the absence of ototoxins and sulphhydryl modifiers. Since cadmium is also a calcium channel antagonist (Tsien, 1983), we tested the effect of verapamil, a calcium channel blocker on the carbachol-induced formation of IPs. Verapamil was applied together with lithium before the addition of carbachol. In addition, this study focusing on alterations of sulphate moieties was supplemented by assessment of the effects of DL-dithiothreitol and DTNB on the carbachol-induced IP responses. These molecules are disulphide bond modifiers.

Membrane preparation for binding experiments

Cochleas, dissected as indicated above, were washed four times with phosphate-buffered saline, pH 7.4 (PBS; 50 mM Na₂HPO₄, 50 mM KH₂PO₄, 125 mM NaCl) and then squashed into PBS with an all-glass homogenizer at the concentration of six cochleas/ml. The bony fragments were removed by centrifugation at 150 *g* for 5 min, and the supernatant containing the cochlear membranes was stored at –70°C before use.

[³H]QNB binding assays

Aliquots of the membrane preparation (320 μl) were mixed with 40 μl PBS and [³H]QNB at 0.08 nM and then incubated at 25°C for 90 min. Preliminary results have shown that at 0.08 nM the QNB binding is close to saturation in this preparation (Bartolami *et al.*, 1992b). The binding reaction was stopped by filtration of the samples on Whatman GF/B filters and washing the filters with 3 × 4 ml PBS at 4°C. The amount of [³H]QNB retained per filter was measured by liquid scintillation counting. This procedure allows the determination of the total amount of bound QNB. In order to measure the non-specific binding, 1 μM atropine was added to the incubation medium. The amount of specifically bound QNB was obtained by subtracting the value of the non-specific binding from the total binding. Trials of displacement of the bound QNB were conducted by adding the

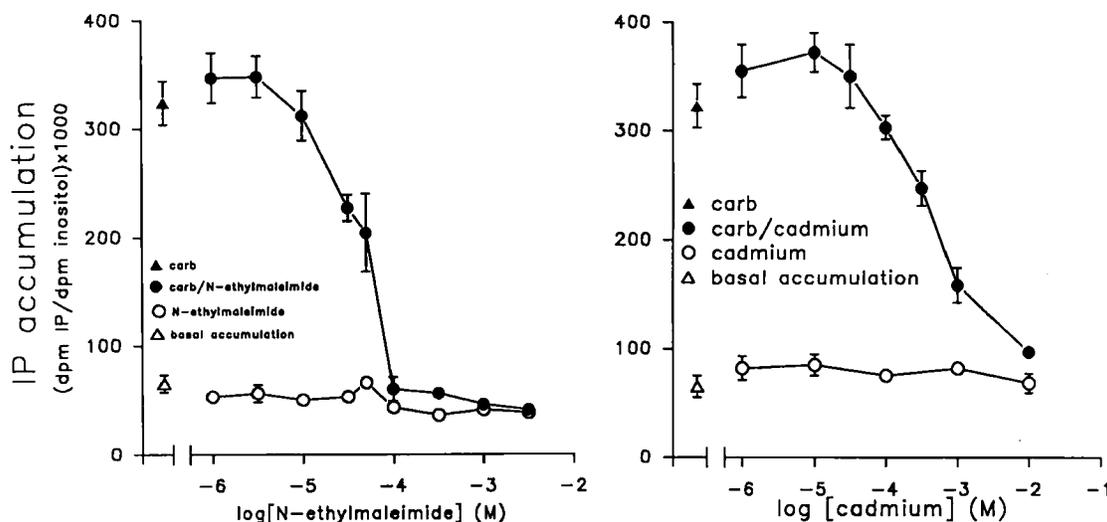


Fig. 1. Dose-dependent effects of *N*-ethylmaleimide and cadmium chloride on IP synthesis. Right, effects of NEM; left, effects of cadmium chloride. The amount of IPs formed in the presence of NEM or cadmium was measured in individual cochleas that were either stimulated by 1 mM carbachol or non-stimulated. The data are means \pm SEM of 6–12 individual replications. The basal IP synthesis is 60 ± 4 dpm IPs \times 1000/d.p.m. inositol and the carbachol (1 mM)-stimulated control level is 324 ± 20 d.p.m. IPs \times 1000/d.p.m. inositol. When error bar is not indicated, the width of the error bar is smaller than the thickness of the symbols. Carb, carbachol.

sulphydryl-modifying reagents cadmium chloride and NEM to the medium. The results of the binding assays were normalized to the amount of protein present in the membrane preparation, which was measured according to Lowry *et al.* (1951).

Data expression and statistical analysis

Data are expressed either as percentage of the level of specific QNB binding obtained in the absence of the tested molecules, or as $10^3 \times$ the ratio of the quantity of [3 H]IPs (in d.p.m.) accumulated to the amount of [3 H]inositol (in d.p.m.) taken up per cochlea (d.p.m. IPs \times 10^3 /d.p.m. inositol). The amount (d.p.m.) of [3 H]inositol taken up per cochlea was measured in the flow-through fraction of anion-exchange chromatography as described elsewhere (Guiramand *et al.*, 1990). When not indicated, the data points are means \pm SEM of at least six replications. Controls of the basal IP metabolism, carbachol-induced IP formation and specific bound QNB were included in each replication. The statistical significance of the data was determined by one-way ANOVA.

Results

Effects of NEM and cadmium chloride on the synthesis of IPs

Applications of either NEM or cadmium chloride do not change the basal metabolism of IPs at any concentration (Fig. 1). However, when the synthesis of IPs is stimulated by carbachol (1 mM), both sulphydryl-modifying reagents inhibited the formation of IPs in a dose-dependent manner. NEM is much more efficient than cadmium chloride in reducing the carbachol-induced accumulation of IPs (Fig. 1) since their respective IC_{50} values (concentrations of agents inhibiting 50% of the carbachol-induced IP accumulation) are 50 and 660 μ M. The maximum inhibitions are obtained with NEM at 0.1 mM and cadmium chloride at 3 mM.

Additive effects

In these tests, ototoxins and sulphydryl-modifying substances were used at concentrations smaller than the determined IC_{50} values (see above and Bartolami *et al.*, 1993), except for NEM which was used at its IC_{50} and neomycin for which the IC_{50} was not determined. For each test of additivity, the data were compared to their linear additivity values. These theoretical values were calculated by subtracting, from the stimulated control level (effect of carbachol alone), the sums of the individual levels of inhibition provoked by each individual drug, when applied on its own.

The addition of NEM at 50 μ M to cisplatin (0.3 mM), mercuric chloride (3 μ M) and neomycin (15 mM) results in a potentiation of the drug-elicited inhibitions of carbachol-induced synthesis of IPs (Fig. 2). With these two drug combinations, the response levels are low, achieving values significantly smaller than their respective, linear additive values. When NEM and ethacrynic acid (0.3 nM) are combined, they display an additive inhibitory effect (Fig. 2; the gap between the linear additivity value and the experimental data is smaller than the SEM of the data).

The combination of cadmium chloride (0.3 mM) with ethacrynic acid (0.3 nM) induces an additive inhibition of the carbachol-elicited accumulation of IPs (Fig. 2), whereas cadmium chloride does not potentiate the inhibitory effects of cisplatin (0.3 mM) and neomycin (15 mM). Indeed, the carbachol-induced synthesis of IPs reaches a level greater than the value of linear additivity for these combinations. When cadmium is associated with mercuric chloride (3 μ M) a potentiation is observed since the IP response level is less than the value of linear additivity for the latter pair (Fig. 2).

Effects of verapamil on cadmium chloride inhibition of the IP response

Co-exposing the cochleas to both cadmium chloride (1 mM) and verapamil (1, 10 and 50 μ M) causes a decrease in the level of inhibition in comparison to the magnitude of inhibition obtained when cadmium chloride is applied on its own (Fig. 3). Indeed, cadmium chloride

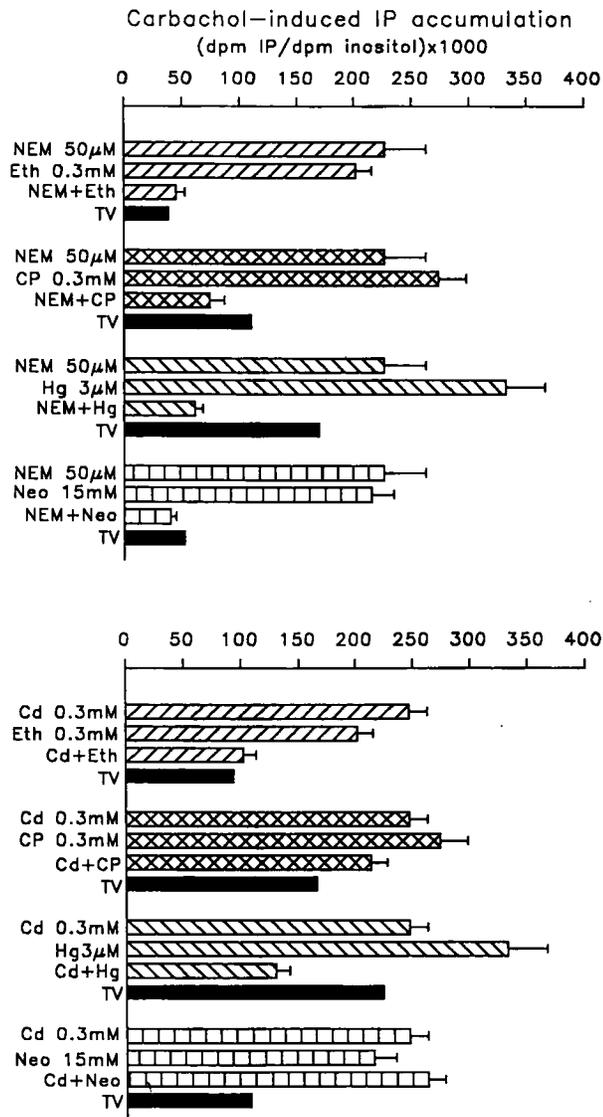


FIG. 2. Additivity tests between thiol modifier and ototoxins. Pairs of substances were applied to the cochleas prior to stimulation with 1 mM carbachol. Top, NEM and ototoxin combinations; bottom, cadmium and ototoxin combinations. The data are means \pm SEM of 6–18 individual replications. The basal IP synthesis is 70 ± 3 d.p.m. IPs \times 1000/d.p.m. inositol and the carbachol (1 mM)-stimulated control level is 390 ± 24 d.p.m. IPs \times 1000/d.p.m. inositol. All the data differ from the stimulated control level at $P < 0.01$. NEM, *N*-ethylmaleimide; Cd, cadmium chloride; Eth, ethacrynate; CP, cisplatin; Hg, mercuric chloride; Neo, neomycin; TV, theoretical value of linear additivity.

(1 mM) alone induces a $52 \pm 4\%$ mean inhibition of the carbachol-stimulated formation of IPs ($P < 0.001$), and the mean level of inhibition is reduced to $40 \pm 12\%$ ($P < 0.01$) by the addition of $1 \mu\text{M}$ verapamil. Combining cadmium with higher doses of verapamil increases the degree of protection of the muscarinic stimulation, with the mean cadmium block amounting to 12 ± 15 and $33 \pm 11\%$ in the presence of 10 and $50 \mu\text{M}$ verapamil respectively. At these verapamil concentrations, the levels of accumulation of IPs are not statistically different from the IP response evoked by 1 mM carbachol (Fig. 3). The application of verapamil on its own (1, 10 and $50 \mu\text{M}$) does not significantly alter the level of carbachol-induced synthesis of IPs (Fig. 3).

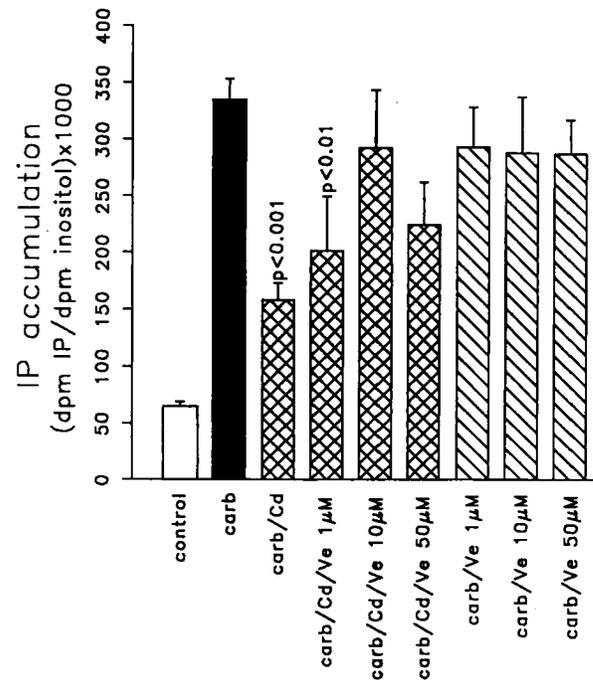


FIG. 3. Effect of verapamil on the cadmium chloride-induced blockage of stimulated IP metabolism. The cochleas were treated with verapamil and cadmium chloride (1 mM) 15 min before being stimulated by carbachol (1 mM). The data are means \pm SEM of six individual replications. The basal IP synthesis (white bar) is 65 ± 8 d.p.m. IPs \times 1000/d.p.m. inositol, and the carbachol (1 mM)-stimulated control level (black bar) is 335 ± 18 d.p.m. IPs \times 1000/d.p.m. inositol. Control, basal IP synthesis; carb, carbachol; Cd, cadmium chloride; Ve, verapamil. When not indicated, the effects of cadmium and/or verapamil are not statistically different from the carbachol (1 mM)-stimulated control level.

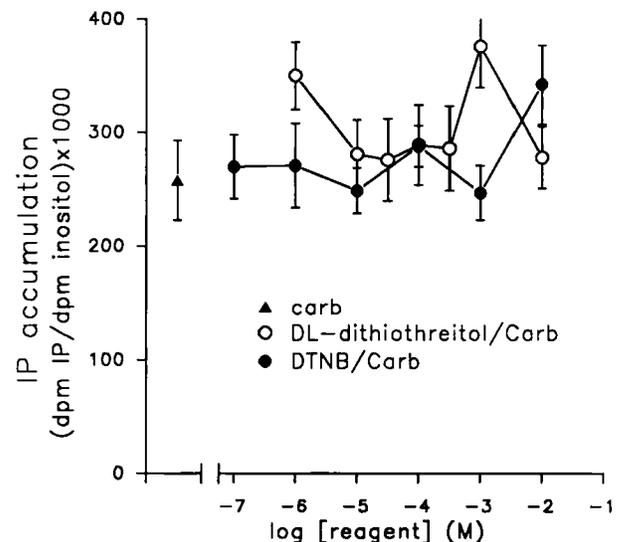


FIG. 4. Effects of DTNB and DL-dithiothreitol on stimulated IP synthesis. The disulphide bridge altering-reagents were applied 15 min before the enhancement of IP metabolism by 1 mM carbachol. The results are means \pm SEM of 6–9 individual replications. The basal IP synthesis is 62 ± 4 d.p.m. IPs \times 1000/d.p.m. inositol and the carbachol (1 mM)-stimulated control level is 268 ± 35 d.p.m. IPs \times 1000/d.p.m. inositol. When error bar is not indicated, the width of the error bar is smaller than the thickness of the symbols. Carb, carbachol.

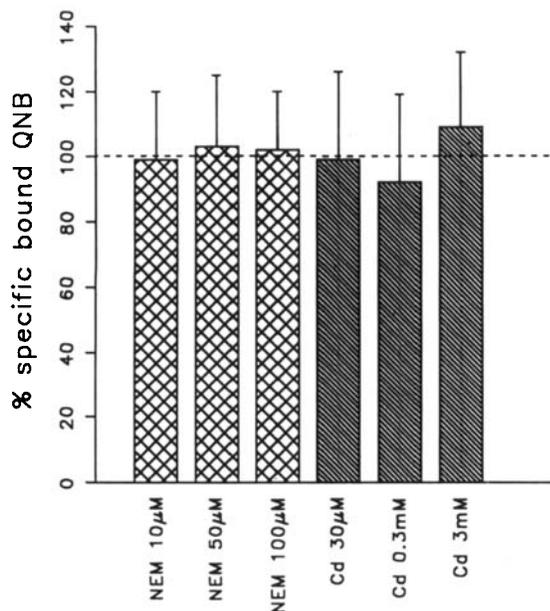


FIG. 5. Effects of the sulphhydryl-modifying-reagents on specific QNB binding in cochlear homogenate. QNB at 0.08 nM was added, together with the thiol modifiers, to the membrane preparation diluted at 6 cochleas/ml. The results of each determination were obtained from duplicated experimental points. The presented data are means \pm SEM of three individual determinations and are given as percentages of the level of specific QNB binding obtained in the absence of thiol modifiers. This level is taken as 100% and is equal to 133 ± 3 fmol QNB/mg of protein. Cd, cadmium.

Effects of disulphide bond altering-molecules on the carbachol-stimulated synthesis of IPs

Carbachol (1 mM)-activated IP metabolism is not significantly changed by either DL-dithiothreitol (from 0.1 μ M to 10 mM) or DTNB (from 1 μ M to 10 mM, Fig. 4).

Effects of NEM and cadmium chloride on QNB binding

Neither NEM (10 μ M, 50 μ M and 0.1 mM) nor cadmium chloride (30 μ M, 0.3 mM and 3 mM) have a significant effect on the specific binding of QNB (Fig. 5). In this set of experiments the mean specific QNB binding with the cochlear membranes is 133 ± 13 (\pm SEM) fmol QNB/mg protein or 0.90 ± 0.09 fmol QNB/cochlea. The effects of the disulphide bond-altering molecules DTNB and DL-dithiothreitol on QNB binding were not tested because of their failure to modify the carbachol-evoked IP response.

Discussion

In the 12-day-old cochlea, NEM and cadmium do not alter the basal metabolism of IPs but they block the turnover of IPs activated via muscarinic receptors. Their effects are not mediated by a direct action at the binding site of the cochlear muscarinic receptor, an observation that is in agreement with reports stating that NEM does not displace the QNB binding in rat brain (Carson, 1980; Korn *et al.*, 1983). Unlike the inhibitory effects of cadmium and NEM, pretreatments with the disulphide bond reagents DTNB and DL-dithiothreitol do not affect the carbachol-stimulated synthesis of IPs. The lack of effect of these compounds suggests that the muscarinic receptor-coupled IP turnover is either resistant to these molecules or that destruction of disulphide bonds (DL-dithiothreitol) and induction of disulphide bonds (DTNB)

do not affect this cochlear signalling pathway. This is supported by observations showing that DTNB has no effect on muscarinic binding in rat brain (Carson, 1980) and that DL-dithiothreitol does not alter carbachol-induced formation of IPs in 8-day-old rat brain synaptoneurosome (Vignes *et al.*, 1992). Simultaneous exposure of cochleas to ototoxins and *N*-ethylmaleimide or cadmium indicates that NEM acts synergistically with all ototoxins except ethacrynate to inhibit the carbachol stimulation. Conversely cadmium expresses synergism only with mercury but it does not potentiate the effect of cisplatin and neomycin. Synergism between thiol modifiers and neomycin, cisplatin and mercury indicate that the compounds may act on different targets in the signalling pathway, as shown by the fact that these three ototoxins block QNB binding (S. Bartolami, M. Planche and R. Pujol, submitted for publication) while the thiol modifiers do not affect muscarinic binding sites (Fig. 5). Interestingly, both cadmium and NEM induce additive inhibitions of the IP responses with ethacrynate, indicating that they may strike common targets.

Concerning cadmium, the possibility that it reduces the metabolism of IPs by blocking calcium influx (Tsien, 1983) should be considered since phospholipase C, which hydrolyses the membrane phosphoinositides into IPs, is sensitive to cytosolic calcium (Cooke and Bennett, 1989). However, the application of verapamil, an antagonist of voltage-sensitive calcium channels, does not significantly change the carbachol-induced synthesis of IPs in the rat cochlea. Therefore, assuming that all calcium channels are not closed, inhibition of phospholipase C via a decrease in calcium permeability may not be involved in the cadmium effect. Interestingly, cadmium and verapamil when co-applied decrease the level of inhibition of the carbachol stimulation, with respect to the one measured in the case of the cadmium exposure. Cadmium toxicity in GH₄C₁ cells is blocked by voltage-gated calcium channel antagonists, including verapamil (Hinkle *et al.*, 1987). These authors demonstrated that this toxicity developed in cells following the entry of cadmium through voltage-sensitive calcium channels. Therefore, calcium channel blockers protect the cells by decreasing the influx of cadmium through calcium channels. Consequently, this may explain why cadmium does not potentiate the inhibitory effects of either neomycin or cisplatin, since aminoglycosides and cisplatin block voltage-sensitive calcium channels (Dulon *et al.*, 1989; Saito *et al.*, 1991).

The block of the cochlear IP signalling pathway associated with cholinergic stimulation by NEM and cadmium may involve interactions with M3 muscarinic receptors, guanine nucleotide binding proteins (G proteins) and phospholipase C enzymes. Whatever the proteins involved, it is possible that cadmium and NEM may react preferentially with cysteine residues.

The M3 muscarinic receptors

Although muscarinic binding sites are not affected by NEM and cadmium, these molecules may interact with intracellular regions of the receptors. The sequence of the cloned rat m3 receptor, corresponding to the M3 pharmacological type, contains 13 cysteines (Bonner, 1989). Amongst them, three residues are located in the third intracellular loop, which is involved in the interaction with G proteins (Bonner, 1992). It is possible that the blockage of the thiol group of these cysteines may impede receptor-G protein coupling. Indeed, NEM has been shown to interfere with this coupling reaction in the rat brain (Korn *et al.*, 1983) and other systems (Harden *et al.*, 1982).

The G proteins

In the cochlea, G_t, G_s, G_i and G_o proteins are presumably present (Canlon *et al.*, 1991; Koch and Gloddek, 1991; Tachibana *et al.*,

1992), but Gp or Gq proteins, which activate phospholipase C in various systems (Ashkenazi *et al.*, 1989; Strathmann and Simon, 1990; Taylor *et al.*, 1991; Smrcka *et al.*, 1991), have not been detected yet. Anyway, whatever the type of G protein associated with the activation of the signalling pathway, one can hypothesize that cysteine residues of G proteins may be targets for cadmium and/or NEM. This idea is based on the observation that complexation by NEM of two cysteines of the alpha subunits of Go proteins results in inhibition of its GTPase activity (Winslow *et al.*, 1987). In addition, a conserved cysteine has been located in the 20 residue C-terminal segment of the alpha chain of Gi, Go and Gt proteins. The blockage of this cysteine may have drastic effects on the transduction system since the 20 amino acid segment is a contact site involved in G protein-receptor coupling (Sullivan *et al.*, 1987). Finally, the uncoupling effect of NEM, due to blockage of G proteins, has been evidenced in many systems (Harden *et al.*, 1982; Aronstam and Carrier, 1982; Korn *et al.*, 1983; Northup *et al.*, 1983; Winslow *et al.*, 1987; Freeman and Lau, 1991).

The phospholipase C enzymes

At least seven types of phospholipase C isozymes have been isolated, but none of them from the cochlea. The amino acid sequences were deduced from cDNA clones of four isozymes (Crooke and Bennet, 1989; Rhee *et al.*, 1989). The analysis of these sequences reveals that cysteines are present in the catalytic regions of all isozymes (Crooke and Bennet, 1989). This makes phospholipase C an additional putative target for NEM and/or cadmium. However, in cochleas exposed to both substances, no change in the basal IP turnover is obtained, therefore inhibition by NEM or cadmium due to a direct action on phospholipase C must be dismissed.

Implication in ototoxin effects

Concerning the mechanisms of ototoxin inhibition of the carbachol-induced IP response, previous observations suggest that neomycin may act by suppressing the supply of substrate to phospholipase C (Schacht, 1976, 1986), and both mercury and cisplatin may inhibit agonist-muscarinic receptor binding (Bartolami *et al.*, 1993). New information based on the present NEM effects sheds more light on the mechanism of action of ethacrynate on the stimulated IP response. Indeed, NEM, like ethacrynate, inhibits the carbachol effect but does not inhibit the QNB binding. Furthermore, NEM and ethacrynate block stimulated IP formation in an additive way, indicating that NEM and ethacrynate share the same target within the transduction system. Since NEM suppresses G protein activity and ethacrynate has been proposed to behave similarly in the cochlea (Koch and Gloddek, 1991), and since they interact similarly with the muscarinic receptor-coupled IP turnover, it is possible that ethacrynate diminishes carbachol-induced IP synthesis by uncoupling phospholipases C from cholinergic receptors via a direct action on G proteins.

Conclusions

The reported data show that cadmium and NEM inhibit *in vitro* the synthesis of IPs activated via the stimulation of M3 cholinergic receptors in the 12-day-old rat cochlea. The mechanisms of the inhibitory action of *N*-ethylmaleimide and cadmium seem to be different from those of the suppressing effects of neomycin, cisplatin and mercury. Conversely, ethacrynate and thiol modifiers, having identical inhibitory features, appear to react with the same targets, possibly the G proteins.

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Abbreviations

| | |
|------------------|---|
| DTNB | 5,5'-dithio-bis-2-nitrobenzoic acid |
| HEPES | <i>N</i> -(2-hydroxyethyl)piperazine- <i>N'</i> -(2-ethanesulphonic acid) |
| IC ₅₀ | 50% inhibitory concentration |
| IP | inositol phosphate |
| OHC | outer hair cell |
| PBS | phosphate-buffered saline |
| NEM | <i>N</i> -ethylmaleimide |
| QNB | quinclidinyl benzylate |

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