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An energy-dependent step in aminoglycoside ototoxicity: Prevention of gentamicin ototoxicity during reduced endolymphatic potential

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Guinea pigs received a bolus of gentamicin (10 mM for 5 min) by perilymphatic perfusion which normally led to an irreversible loss of the cochlear microphonic potential (CM). Various experimental conditions that reduced the endolymphatic potential (EP) were then superimposed on the gentamicin application. Reversible reductions in EP (and, concomitantly, in CM) were induced by asphyxia (3 min), intravenous furosemide (50 mg/kg), and perilymphatic perfusion of aminooxyacetic acid (10 mM). When the administration of gentamicin was initiated at the time of maximal EP reduction the usual irreversible gentamicin-induced decline of CM was prevented.

The results indicate that a metabolic process is essential in the expression of gentamicin toxicity. The data are consistent with the inhibition of an energy-dependent transport of the aminoglycoside. Alternatively, the data are also compatible with the hypothesis that entry of gentamicin into hair cells is prevented by a reduction in their transmembrane electrical potential.

aminoglycosides, ototoxicity, gentamicin, energy-dependent uptake, transmembrane potential

Introduction

Based on several lines of evidence, this laboratory has formulated a hypothesis to explain the acute and chronic ototoxic actions of aminoglycosides in the inner ear [16,20,21]. In the course of their action, the aminoglycosides appear to occupy at least two different cellular compartments. Perilymphatic perfusions of the guinea pig cochlea with gentamicin reduced the cochlear microphonic potential (CM) with an initial acute phase that was readily reversed by the addition of calcium; this was followed by a second phase insensitive to calcium [23]. Pharmacokinetic data demonstrated two different half-lives of gentamicin in cochlear tissues prior to and after the onset of chronic toxicity [25]. Consistent with these data would be a binding of gentamicin to the external cell surface as a first step (first compartment) and binding to intracellular components such as polyphos-

phoinositides in the second compartment [21,26]. It would have to be postulated that an uptake process transfers the drug from the outside to the inside of the cell. Some aspects of aminoglycoside uptake in the kidney [1,11] have been characterized but more detailed analyses have been carried out in bacteria where two distinct energy-dependent phases of uptake are documented [10]. There is no information on aminoglycoside uptake mechanisms in the cochlea.

The generation and maintenance of a transmembrane potential across the cytoplasmic membrane is a basic feature of all living cells. This potential and the distribution of ions associated with it play an essential role in membrane associated phenomena such as ionic conductance and transport processes. Recently, evidence has been presented that the magnitude of the transmembrane potential in bacterial cells is a determining factor in aminoglycoside uptake and bactericidal action [9]. Thus, more negative membrane potentials produced greater aminoglycoside uptake and enhanced bactericidal effects. A relationship of the transmembrane potential to aminoglycoside oto-

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toxicity is particularly attractive in view of the selective actions of aminoglycosides on outer hair cells which have been shown to have a more negative membrane potential than inner hair cells [8].

The present study was undertaken to test whether aminoglycoside actions are related to energy-dependent phenomena. The effects of gentamicin in perilymphatic perfusions on CM were studied under conditions of reduced endolymphatic potentials (EP). A reduction in EP by the experimental manipulations was assumed to be an indicator of reduced energy for the maintenance of transmembrane potentials and of transport processes of cochlear cells.

Materials and Methods

Experiments were performed on male guinea pigs (Hartley) weighing 250–400 g. Surgical and perilymphatic perfusion techniques were essentially as previously described [16,17,23]. An animal with a positive Preyer reflex was anesthetized with sodium pentobarbital (20 mg/kg body wt., i.p.), atropine sulfate (0.05 mg/kg body wt., s.c.) and Innovar (0.5 ml/kg body wt., i.m. containing 0.4 mg fentanyl and 20 mg droperidol/ml). Rectal temperature was maintained at $37 \pm 1^\circ\text{C}$ with a heating pad and artificial respiration provided through a tracheal cannula.

The EP was measured with a 3 M KCl micro-electrode inserted through the spiral ligament of the basal turn [14]. Perilymphatic perfusions were performed via glass capillaries implanted into the basal turn of scala tympani and scala vestibuli. The perfusion pipettes were placed prior to insertion of the EP recording electrode. The flow of perfusate was initiated after establishing a stable, positive EP. The CM was measured from a recording electrode in the scala tympani perfusion pipette and a reference electrode in the superficial masseter muscle. The CM was recorded in response to a sound stimulus of white noise, 20–4000 Hz, delivered to the external auditory meatus through an earphone coupled to an ear speculum. The sound intensity was adjusted to approximately 70 dB SPL to give an initial CM of 200–400 μV (RMS).

Perilymphatic spaces were perfused at a rate of

approximately 30 $\mu\text{l}/\text{min}$ with artificial perilymph containing 130 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 2 mM MgCl_2 , 0.1 mM NaH_2PO_4 , 10 mM NaHCO_3 , 5 mM glucose and 10 mM HEPES, final pH, 7.4. Gentamicin sulfate was dissolved in the artificial perilymph at a concentration of 10 mM and the pH adjusted with NaOH. After CM and EP had stabilized for 30 min the 'control perfusate' was switched to the 'experimental perfusate' containing drug. Gentamicin was perfused for 5 min whereafter perfusion with the control perfusate was resumed. Experiments were usually carried out for 90–120 min but perfusions without drugs could be extended for up to 3 h without major (< 20%) loss of CM. Animals with an initial CM of less than 200 μV or with an unstable CM and EP during the control period (loss of > 10%) were eliminated from the study. Amino-oxyacetic acid (10 mM) was dissolved in artificial perilymph and the pH adjusted with NaOH. Furosemide (50 mg/kg body weight) was injected over 90 s intravenously via a catheter in the left jugular vein. Anoxia was induced by shutting off the respirator. Furosemide and aminooxyacetic acid (AOAA) were purchased from Sigma. Gentamicin sulfate was a gift from the Schering Corporation (Bloomfield, NJ).

Results

Gentamicin at a concentration of 10 mM perfused as a bolus through the perilymphatic space for 5 min drastically reduced the microphonic potential but had no effect on the endolymphatic potential (Fig. 1 and Table I). The CM began to decline rapidly within 30 s after gentamicin entered the cochlea and continued to decline, although after the 5 min perfusion the perfusate no longer contained the drug. Consistently, a 60% reduction of CM was reached after 60 min. This irreversible action of gentamicin on CM and its lack of effect on EP have been described before [12,13].

Anoxia induced by turning the respirator off produced a rapid decline in EP and CM (Fig. 2). This effect rapidly and completely reversed if the respirator was turned back on after 3 min (Fig. 2 and Table I) consistent with reports by others [14]. When the perilymphatic space was perfused with

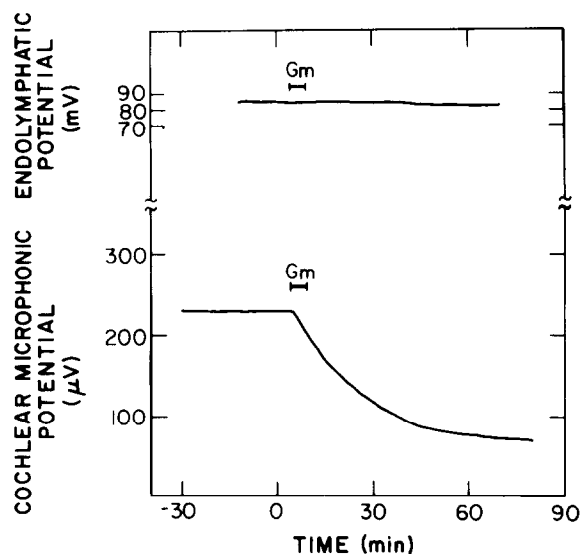


Fig. 1. Time course of gentamicin action on endolymphatic and cochlear microphonic potentials. Techniques for carrying out perilymphatic perfusions and recording EP and CM were as described in Materials and Methods. Following the control perfusion ('stabilizing period', -30 to 0 min), the perfusate

10 mM gentamicin for 5 min beginning at a time when EP and CM were suppressed by asphyxia the usual irreversible actions of gentamicin on CM were prevented (Fig. 2 and Table I). One hour after anoxia plus gentamicin, CM had recovered to $97 \pm 6\%$ of its pre-drug control value (Table I) whereas one hour after gentamicin alone, CM was $40 \pm 2\%$ of its pre-drug value (Table I).

Following intravenous injections of a bolus of furosemide (50 mg/kg), the endolymphatic potentials fell rapidly to near zero values (Fig. 3). The peak suppression of EP and CM occurred in less than 2 min. This was followed by a quick recovery of EP by about 20–30 mV, a plateau and then a gradual recovery to pre-injection values (Fig. 3). The CM followed a similar time course of recovery

was changed at the point indicated for 5 min to experimental perfusate containing 10 mM gentamicin (Gm). Curves are mean values from 4 animals (EP) and 5 animals (CM). Note the lack of effect of gentamicin on the EP.

TABLE I
EFFECT OF DRUG TREATMENTS ON COCHLEAR POTENTIALS

See Figs. 1–4 for experimental conditions.

A. Endolymphatic potential

Treatment	Control	Minimum (mV)	Recovered (mV)	Time ^a (min)	n
Gentamicin	80 ± 4	–	78 ± 3	60	4
Anoxia	82 ± 5	-11 ± 6	81 ± 4	18	4
Anoxia + Gm	80 ± 6	-3 ± 13	82 ± 5	18	6
Anoxia + Gm			80 ± 6	60	6
Furosemide + Gm	84 ± 4	12 ± 7	72 ± 5	90	2
AOAA + Gm	81 ± 11	18 ± 7	16 ± 3	60	4

B. Cochlear microphonics

Treatment	Minimum (% of control)	Recovered	Time ^a (min)	n
Gentamicin		40 ± 2	60	5
Anoxia	47 ± 10	100 ± 2	18	6
Anoxia + Gm	46 ± 11	97 ± 4	18	7
Anoxia + Gm		97 ± 6	60	7
Furosemide	44 ± 11	102 ± 8	90	4
Furosemide + Gm	44 ± 4	107 ± 8	90	3
AOAA	52 ± 10	72 ± 7	60	5
AOAA -- Gm	48 ± 10	73 ± 8	60	5
Gm -- AOAA	47 ± 4	48 ± 3	60	4

^a Time from beginning of treatment to measurement of recovered potential.

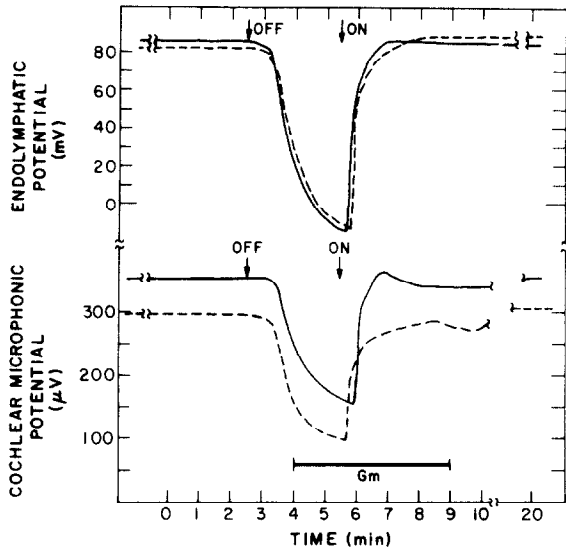


Fig. 2. Prevention of gentamicin-induced suppression of cochlear microphonic potentials during anoxic reduction of the endolymphatic potential. Experimental procedures were carried out as described in Materials and Methods. Following the control perfusion for 30 min the respirator was turned off at the time indicated by the arrow. 90 s later the control perfusate was changed to experimental perfusate containing 10 mM gentamicin (GM) for 5 min. Bar indicates time that gentamicin was in the perilymphatic space. After 3 min of asphyxia the respirator was turned back on. Thus, CM and EP were markedly reduced by anoxia at the time gentamicin entered the cochlea and remained suppressed for at least 2 of the 5 min of exposure to the drug. —, perfusions without gentamicin; - - - -, perfusions with gentamicin. Note that in contrast to results shown in Fig. 1 the CM recovers to pre-drug values.

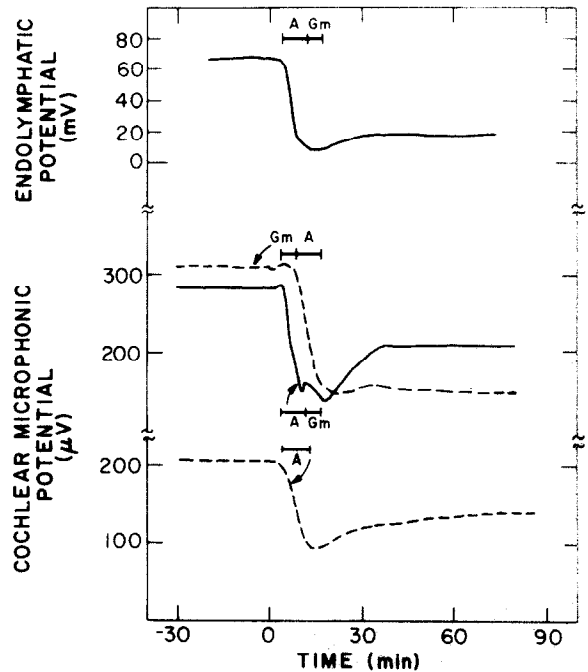
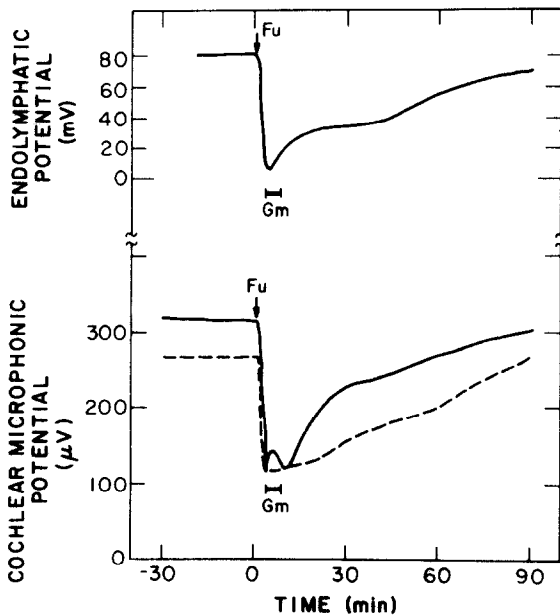


Fig. 4. Prevention of gentamicin-induced suppression of cochlear microphonic potentials during reduction of the endolymphatic potential by aminooxyacetic acid. Experimental procedures were as described in Materials and Methods. Following the control perfusion, drugs were introduced into the perfusate for the time and sequence indicated by the bars: A, aminooxyacetic acid (10 mM); Gm, gentamicin (10 mM). Note the contrast in the recovery of CM depending on the sequence of administration of the two drugs.

and returned to $102 \pm 8\%$ of its initial value by 90 min (Table I). These results, again, are compatible with previous reports by others [19]. The actions of 10 mM gentamicin were prevented when the 5 min perfusion of gentamicin was initiated at the time of peak suppression of EP by furosemide (Fig. 3 and Table I). With furosemide plus gentamicin,

Fig. 3. Prevention of gentamicin-induced suppression of cochlear microphonic potentials during reduction of the endolymphatic potential by furosemide. Experimental procedures were carried out as described in Materials and Methods. Following the control perfusion ('stabilizing period', -30 to 0 min), furosemide (Fu) was injected intravenously into the left jugular vein as a bolus over 90 s. At the point of maximum CM and EP suppression the control perfusate was changed to experimental perfusate containing 10 mM gentamicin (GM) for 5 min. Bar indicates time that gentamicin was in the perilymphatic space. —, perfusion without gentamicin; - - - -, with gentamicin. Again, note that in contrast to results shown in Fig. 1 the CM recovers to pre-gentamicin values.

CM had recovered to $107 \pm 8\%$ of its initial value by 90 min (Table I).

Perilymphatic perfusion of 10 mM AOAA rapidly suppressed EP and CM (Fig. 4). The effects of AOAA were reversible but required prolonged periods of perfusion with drug-free perfusate: by 60 min, CM had only recovered to $72 \pm 7\%$ of pre-AOAA values (Table I). These effects of AOAA on cochlear potentials are in keeping with reports of others [2,3]. The actions of 10 mM gentamicin on CM were prevented if the 5 min gentamicin perfusion began at the time of maximal EP and CM suppression (Fig. 4 and Table I) by AOAA. In contrast, if the administration of gentamicin preceded the perfusion of AOAA there was little, if any, recovery of CM after 60 min when compared to perfusions of gentamicin alone (Table I).

Discussion

The salient finding is that the expression of gentamicin toxicity requires an active metabolic process. This is the broadest interpretation of the fact that gentamicin toxicity is prevented when the drug is administered during a period of decreased endolymphatic potential. In this context the reduction of EP is taken as an indicator of interference with energy metabolism in cochlear structures. The question then arises what this active metabolic process is and how it affects the mechanism of aminoglycoside toxicity.

First we may consider the uptake of the drug. In the mechanism of the antibacterial activity of aminoglycosides it is clearly established that an active uptake process precedes the toxic effect of the drug [10] and uptake into the kidney also has an energy-dependent component [1]. For the inner ear such evidence still is lacking but results presented here are compatible with an active drug uptake system in the inner ear. When inhibited by such manipulations as asphyxia, the drug would not penetrate the cells and thus not be toxic.

The nature of the cochlear uptake system cannot be delineated from the present study. A target-specific inhibition of a transport system seems unlikely since such diverse agents as asphyxia, furosemide, and aminoxyacetic acid all produced a similar effect. Rather, since all agents

used inhibit metabolism as indicated by the decrease in EP, an interference with the energy supply to such a system may be the common action. Thus, any active carrier-mediated transport or ATP-dependent co-transport of aminoglycosides is compatible with the evidence. If, however, ATP levels remain largely unaffected in the hair cells during the acute phase of furosemide-induced reduction of EP as proposed for the stria vascularis [15] more restraints have to be applied to the model.

An alternative energy-dependent uptake of gentamicin could be driven by the transmembrane potential of the cells as demonstrated in bacteria [9]. The appeal of postulating a potential-related uptake in the inner ear is the apparent correlation between the susceptibility of inner and outer hair cells to aminoglycosides and their potential. Intracellular potentials of cochlear inner hair cells and supporting cells, however, show only small changes during transient asphyxia [4,18]. It would have to be assumed that under the conditions of the experiments the major action of the aminoglycosides is on the apical end of the hair cells: the reduction of the endolymphatic potential would lower the driving force for drug entry into the cells. Pharmacokinetic evidence for the route of access of aminoglycosides to endolymph is not entirely clear [24] and endolymph may not be the major compartment from which these drugs enter the hair cells after systemic injections [25].

While an inhibition of drug uptake seems the most direct explanation for the observed effects other interpretations are possible. For example, an inhibition of metabolism would also affect any metabolism of the drug itself. Drug metabolism may be a crucial determinant in toxicity as in the case of the nephrotoxic actions of the antineoplastic drug, *cis*-dichlorodiamine platin. The parent compound apparently has lower toxicity than its metabolites [7]. Considering the design of the experiments in this study it would have to be postulated that the initial step in aminoglycoside toxicity is a metabolic transformation and that the metabolite, but not the parent compound, will penetrate the cell to cause permanent damage. It is, however, generally assumed that aminoglycosides are not metabolized so that this interpretation appears less likely.

An apparent paradox in the current results is

the fact that the acute combination of two potentially ototoxic drugs results in reduced ototoxicity rather than a potentiation as observed when the same drugs are administered systemically. A potentiation has been reported for aminoglycosides in combination with both, furosemide [5] as well as aminooxyacetic acid [6]. Several reasons may account for this lack of interaction: gentamicin is given here by perilymphatic perfusions possibly eliminating the site of interaction; or, that in this acute combination neither drug has sufficient time to cause the effect that would lead to potentiation.

The postulate of an active uptake process for aminoglycosides in the cochlea fits well into the model we have previously proposed for their ototoxicity since phosphoinositides are considered to be present in the inner (cytoplasmic) surface of the membrane. The drug would first bind to negatively charged sites on the external plasma membrane such as acidic phospholipids. This action, antagonized by calcium, would account for the acute and reversible effects of the aminoglycosides. Following the uptake into the cells, the drugs bind to phosphoinositides, inhibit their function and disrupt membrane structure. The susceptibility of a tissue to aminoglycosides would then depend on both the presence of an uptake system and the presence of intracellular sites of action, most importantly, phosphoinositides.

The precise nature of the energy-dependent process involved in aminoglycoside toxicity remains to be elucidated. Both hypotheses raised, active carrier-mediated transport or transport by membrane potential, are amenable to further testing in vivo and in vitro. Our finding already points to the complexity of the aminoglycoside action in the inner ear and shows novel parallels between the cochlear and the renal and bacterial handling of the drug.

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