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Acetylcholine-induced Phosphorylation in Isolated Outer Hair Cells

MAGDOLNA SZÕNYI[1], PÉTER CSERMELY[2] and ISTVÁN SZIKLAI[3]

From the [1]Semmelweis University Medical School, ORL Clinic, [2]Institute of Medical Chemistry, Budapest, and [3]Debrecen University Medical School, ORL Clinic, Debrecen, Hungary

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Two groups of isolated, surviving outer hair cells (OHCs) of guinea pig cochleas ($n = 20$, for each group) were treated with 10 μM acetylcholine or acetylcholine plus strichnine (an α9 nAChR antagonist), respectively, under short-term tissue culture conditions. The protein content of the cell homogenates was separated by SDS-polyacrylamide gel electrophoresis, Western blotted and labelled with an antibody against phosphoserine residues. Signals were detected using the ECL system. Acetylcholine challenge of the OHCs resulted in a difference in the pattern of phosphorylated proteins from those of strichnine pretreated cells. A 220 kDa and a 120 kDa protein expressed a more intense phosphorylated state in the ACh group compared with the ACh plus strichnine group. The 220 kDa phosphoprotein is in the range of the cytoskeletal protein β-fodrin, whereas the 120 kDa fraction is similar to α-fodrin or an ankyrin isoform. Phosphorylation of proteins due to activation of the AChR by agonist can play a role in the signalling mechanism between receptor activation and increase in the electromotile capability of isolated OHCs. *Key words*: *acetylcholine*, *outer hair cells*, *cytoskeletal proteins*, *electromotility*, *ECL detection*.

INTRODUCTION

Acetylcholine (ACh), the efferent neurotransmitter on avian and mammalian inner ear receptor cells (1), acts on a special α9 nicotinic ACh receptor (nAChR) subtype, which shows mixed cholinergic and muscarinic pharmacological properties (2). The receptor activation allows a calcium influx (3, 4) and subsequent fast and slow cell responses. The slow response develops in approximately 20 s (5) and is probably mediated by a multiple-step signalling mechanism.

Outer hair cell (OHC) electromotility, a membrane voltage-driven, sound signal following cycle-by-cycle shortening and elongation of the OHCs in the audio frequency range, provides a force feedback to the basilar membrane and is assumed to be the cochlear amplifier itself (6), which is responsible for the sharp tuning and high sensitivity of the auditory system. Modulation of the efficacy (small-voltage gain) of this voltage-to-movement converter can change the auditory processing at threshold intensities. Conflicting observations are that efferent stimulation is known to inhibit cochlear processing, whereas ACh increases the gain and magnitude of electromotility in isolated OHCs (7, 8). This electromotile performance increase in ACh-challenged OHCs develops in approximately 20 s, as does the efferent stimulation-induced slow-response. It is demonstrated to be due to cell-stiffness, decrease of OHCs and is specific to ACh receptor activation (9).

The experiments described here aimed to explore some elements of the possible mechanism of the ACh-induced electromotile performance-increase in isolated mammalian OHCs. With respect to the ~ 20 s activation time for the slow ACh effect, an intermediate mechanism is suspected to be involved in the phenomenon. This was assumed to be a phosphorylation (10). Phosphorylation is a reversible and rapid covalent modification of molecules and its enzyme machinery is available in hair cells (11). Phosphorylation of presently unknown proteins in the cell membrane or in the subcortical lattice (cytoskeleton) may result in a change in the geometry or in the domain-assembly of the phosphorylated substrate proteins that decreases the cell-stiffness and concomitantly increases the magnitude of the OHC electromotility.

METHODS

Cell isolation and ACh stimulation

OHCs were isolated from young (3–6 week old) pigmented guinea pig ($n = 20$) cochleas. Animals were euthanized with chloroform anaesthesia. Organ of Corti segments were harvested, placed in a collagenase (Sigma, Type IV, 2 mg/ml) containing buffer solution (50 mM phosphate buffer with 10 mM Hepes, pH 7.4, 300 mosm) for 30 min. The organ of Corti segments were then transferred into fresh 100 μl buffer solution and were triturated. Dissociated OHCs were collected with a glass pipette and transferred to 20 μl buffer solution. ACh concentration was adjusted to 10 μM and the cells were incubated for 10 min at room temperature in the presence of 5 μl protein phosphatase inhibitor solution (0.4 mg/ml aprotinin, 4 mM sodium vanadate, 4 mM sodium molibdate, 40 mM β-glycerophosphate, 20 mM sodium pirophosphate, 8 mM EDTA, 8 mM PMSF) and then the samples were frozen. OHCs isolated from 10 animals were processed and frozen individually. Control group of OHCs from another 10 guinea pigs were treated in the same way, except, that 10

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μM strychnin (an inhibitor of the α9 nAChR) was added to the assay solution prior to ACh. Frozen specimens of OHCs were melted and combined separately according to ACh or ACh plus strichnine treatment. Further processing was the same for both groups.

Gel electrophoresis and Western blotting

Extracts of approximately 3,000 OHCs were prepared in an isolation buffer of 20 mM Hepes, pH 7.4, 0.4 mg/ml aprotinin, 4 mM sodium vanadate, 4 mM sodium molybdate, 40 mM sodium β-glycerophosphate, 20 mM sodium pyrophosphate and 8 mM EDTA by homogenization. Samples were treated by DNase I (Sigma) in the presence of 3 mM $MgCl_2$ for 60 min at 4°C to prevent gel-formation of sample DNA, as described previously (12). DNase I-treated samples were subjected to SDS-polyacrylamide gel electrophoresis using the discontinuous buffer system developed by Laemmli (13) in the presence of an additional 10 mM EDTA in Laemmli's sample buffer to ensure the complex formation of the magnesium ions. Proteins which were separated on 8% polyacrylamide gels were blotted to polyvinyl–pirrolidone membranes (Millipore, Bedford, MA, USA) as described (14) using the Miniblot apparatus from Bio-Rad. Phosphoproteins were detected using anti-phosphoserine primary antibodies (Sigma) (15) at a dilution of 1 : 2000. Anti-mouse secondary antibodies were conjugated to horseradish-peroxidase (DAKO A/S Glostrup, Denmark, dilution 1 : 5000) enhanced chemiluminescence (ECL) detection was performed with a kit obtained from Amersham (Amersham, UK).

RESULTS

Guinea pig OHCs were collected from the whole cochlea irrespective of cochlear turns. Two cell populations were pooled each from 10 animals. Approximately 150 cells were collected from one cochlea, consequently we had approximately 3,000 OHCs in both groups. Antibody against phosphoserine was used for detecting serine phosphorylation, which is known to be catalysed by protein kinases, such as PKA, PKC and CAMKII. Separation of the cell homogenates of the same number of OHCs to membrane and cytoplasm fractions did not result in an amount of proteins sufficient to obtain the sensitivity threshold of the ECL detection (unpublished observation).

Protein extraction of the OHCs incubated with ACh alone showed seven electrophoretic bands which are phosphorylated in serine residues. Treatment with ACh and strichnine simultaneously resulted in detection of only six serine-phophorylated proteins. Molecular mass (SDS electrophoretic pattern) of all 6 are identical with those proteins which are found in the extract of the ACh-treated cells (Fig. 1).

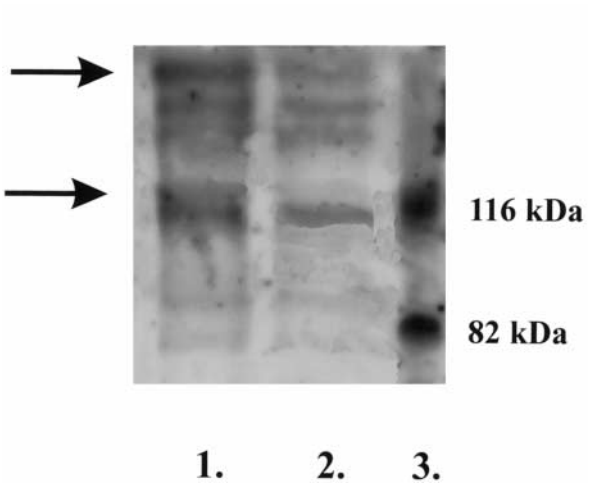

Fig. 1. Serine-phosphorylated proteins in the Western blotted protein extracts of OHCs separated by SDS-polyacrylamide gel electrophoresis. *In vitro* incubation of isolated OHCs with 10 μM ACh demonstrates two protein bands (220 and 120 kDa) with elevated serine-phosphorylation (1) compared with ACh and strichnine co-application pattern (2). Marker proteins are in the third lane (3).

Homogenates of those cells which were stimulated by ACh alone contain a 120 ± 5 kDa phosphorylated protein band which is not detected in the ACh plus strichnine treated OHCs (Fig. 1). Furthermore, the 220 ± 10 kDa protein fraction expressed a higher phosphorylated form in the ACh challenged group then in the ACh plus strichnine OHC preparation. These bands are not identical with those of DNase or

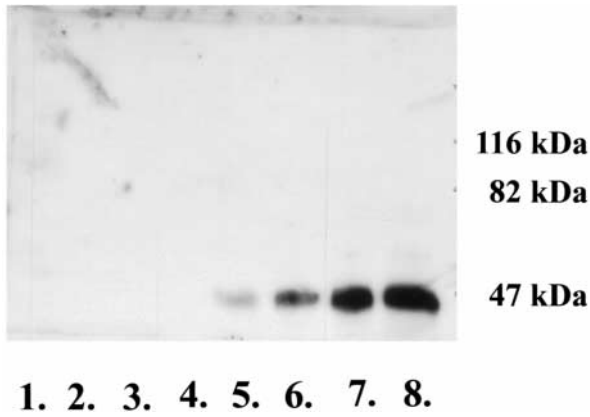

Fig. 2. Phosphoserine-dependent ECL signals of DNase and collagenase. Proteins were loaded at concentrations of 0.5, 1, 2, 4-times (lanes 1/5, 2/6, 3/7, 4/8, respectively) those used in the experiments. Note that phosphoserine of collagenase is under the detection limit and DNase is in the molecular mass range of 45 kDa.

collagenase (Fig. 2), which enzymes were added to the incubation solutions for preparation purposes.

DISCUSSION

Electromotility of the mammalian cochlear outer hair cells is assumed to be one of the functional bases in the peripheral auditory processing. Regulation of the electromotile gain at small voltages (at threshold sound intensities) is essential for high sensitivity of the system. The efficacy of the electromotile feedback is thought to be physiologically sufficient at the high frequencies. OHCs of this particular region in the cochlea, at the cochlear base, receive almost exclusively efferent innervation. The efferent neurotransmitter, ACh, however, proved to increase the electromotile activity of isolated OHCs (7, 8). This is exactly the opposite of what one would expect in the light of our present knowledge about the role of the efferent innervation, which is inhibitory, and the role of OHC electromotility, which is assumed to be enhancing the amplitudes of the basilar membrane vibrations, as the "cochlear amplifier" (6). Efferent influence upon the OHCs has been shown to act in 2 timescales. The fast (ms) effect is probably a hyperpolarization of the cell whereas the slow (efferent) ACh effect is due to a decrease in cell-stiffness (9). This slow ACh influence upon the electromotility is demonstrated to be evoked by phosphorylation as an intermediate mechanism between nAChR activation and the decrease in stiffness of the OHC (10).

Present findings, that ACh induces phosphorylation of two protein bands in living OHCs and that this phosphorylation can be inhibited by strychnine, the specific antagonist of the $\alpha 9$ nAChR argues for an nAChR activation-elicited phosphorylation mechanism. The candidate substrate proteins for this phosphorylation are presumably those which are linked to the cell cytoskeleton, cytoskeletal proteins or cell membrane proteins. The 220 ± 10 kDa band is in the molecular mass range of the cytoskeletal proteins: fodrine (240 kDa) and ankyrine (206 kDa). The latter binds either spectrin or fodrin as linkage protein to the plasma membrane and cytoskeleton (16, 17). Phosphorylation of spectrin is demonstrated to decrease the erythrocyte membrane mechanical stability, whereas dephosphorylation increases it (18). The 120 kDa protein can be α-fodrin (120 kDa) or protein 4.1, another linkage protein. Protein 4.1 is also known to be the substrate for phosphorylation and present in OHCs (19, 20). This protein has different isoforms and in the organ of Corti it was demonstrated to be present in two variants: a 140–150 kDa and a 105 kDa isoform (21).

Present findings in conjunction with previous results seem to support the possibility that a phosphorylation of presumably cytoskeletal proteins can take place in mammalian OHCs due to ACh stimulation and can result in a decrease in stiffness of the whole cell. This decrease in stiffness is responsible for the ACh-induced increase in the electromotile performance of the OHCs (9).

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Address for correspondence:
István Sziklai
ORL Clinic
Debrecen University Medical School
HU-4012 Debrecen
Nagyerdei krt. 98
Hungary
Tel/Fax: +36 52 414 763
E-mail: isziklai@jaguar.dote.hu