University Doctoral Thesis (Ph.D)

Electrophysiological properties of type I spiral ganglion neurones of the guinea pig

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INTRODUCTION

The auditory apparatus is responsible for the conversion of the acoustic stimuli, arriving in the form longitudinal pressure waves, to electrical impulses. These electrical impulses are, in turn, interpreted by certain parts of the central nervous system as acoustic sensation. One of the most important peripheral elements of the system is the cochlea, situated in the inner ear. The cochlea hosts the organ of Corti as well as the primary sensory neurones for audition. The sharp frequency coding, low activation threshold and broad perceivable frequency range are all characteristic features of the mammalian hearing apparatus. These properties are attributed to the uniquely evolved organ of Corti and to certain regulatory mechanisms. Activation of the receptor cells results in their depolarisation, which induces action potential firing in the afferent nerve fibres innervating the receptor cells. These afferent fibres are in fact the peripheral processes of the spiral ganglion neurones, whose cell bodies are situated in the modiolus. The central neurites of the same cells form the cochlear (or acoustic) nerve. The experimental data available suggest that the major steps of the perception of the auditory stimuli are the same in all mammalian species, although differences may exist between the audible spectrum of the individual species. Activation of the receptor cells and that of the spiral ganglion neurones are essential steps in the transformation of the auditory stimuli to acoustic sensation.

The specific sensory cells of auditory system are usually referred as hair cells. Two types of hair cells are distinguished; inner hair cells are regarded as the primary sensors of the apparatus, while the outer hair cells seem particularly important in regard of their specific tuning and amplifying functions. The two types of hair cells are provided by afferent innervation by two kinds of the spiral ganglion neurones. While the sensory innervation of the inner hair cells is ensured by the type I spiral ganglion cells, the afferent fibres reaching the outer hair cells belong to the type II spiral ganglion neurones.

The types of neurones of the spiral ganglion are not only different in terms of their projections, but they also differ from each other on the basis of their morphological and functional properties. The huge majority of the spiral ganglion cells can be classified as type I neurones (> 95 %). Type I neurones have somewhat elongated cell body, which is greater than 12 μm in diameter. Both the cell body and the processes possess thick myelin cover. Type II spiral ganglion cells are, in turn, relatively few in number, their round cell body is smaller than 12 μm in diameter, and they lack myelin cover.

Electrophysiological experiments, conducted on type I spiral ganglion cells demonstrated that these neurones produce rapidly adapting firing on stimulation, i.e. a single action potential at the beginning of a long lasting depolarising stimulus. Previous studies indicated that these cells possessed a variety of K^+ currents, and one of these components proved to be sensitive to the application of tetra-ethyl-ammonium (TEA), 4-aminopyridine (4-AP) and gadolinium ions.

It is a particularly important question from the point of view of the oto-rhino-laryngologists conducting everyday practice, whether the cochlear implant employed in the therapy of bilateral deafness produces hearing sensation with acceptable quality for the patients. As the cochlear implant is inserted directly into the cochlea and produces field stimulation of the spiral ganglion cells, it is imperative to describe and understand the most important electrical properties of the spiral ganglion cells themselves.

In the frame of the present work the following aims have been defined:

1. On the basis of the information available in the literature, we wished to work out a preparation and cell isolation technique, allowing the separation of healthy spiral ganglion neurones from the inner ear of the guinea pig.

2. We wanted to confirm the identity of the neurones separated by using immunocytochemistry. Features allowing the distinction between type I and type II spiral ganglion neurones were also sought.
3. We intended to record and identify the hyperpolarization-activated current(s) expressed by type I spiral ganglion cells. Description of the kinetic and pharmacological features of the current was also attempted.
4. Depolarization-activated K^+ currents were also investigated in the present work. Besides the description of these current components on type I spiral ganglion cells, attempts were made to separate the individual K^+ current components by using pharmacological tools.
5. Investigation of the action potential firing pattern of the type I spiral ganglion cells was also performed. Correlation was also sought between the action potential firing and the significance of the individual K^+ current components.

METHODS

Guinea pigs (both males and females) weighing 250-300 g were employed in the present study (with the permission of the local Ethical Committee). The experimental animals were anaesthetised by using pentobarbital (35 mg/kg i.p.) then decapitated. The subsequent steps of the preparation were performed in ice-cold, Na^+-free artificial cerebrospinal fluid (aCSF). The spiral ganglion was exposed by removing the temporal bone, followed by preparation of the bony cochlea and the modiolus. After breaking up the modiolus it became possible to reveal pieces of the spiral ganglion and to isolate the spiral ganglion cells. Neurone-specific enolase (NSE) and myelin-specific (S100 protein) immunostaining was carried out to identify the neurones and demonstrate the presence of myelin sheath, repectively.

During the electrophysiological experiments various channel blockers were employed. The drugs were applied by using a microperfusion system, allowing rapid exchange of the experimental solutions in the proximity of the cells.

The electrophysiological experiments were conducted by using the whole-cell configuration of the patch-clamp technique. The investigation of the steady-state activation parameters of the hyperpolarization-activated current was performed by using a double pulse protocol applied from a holding potential of -60 mV. First, a 500 ms long prepulse was delivered between –70 and –140 mV in 10 mV steps, then the amplitude of the tail-current was measured at –60 mV. The tail current amplitude was normalised to the maximum value obtained (measured after the –140 mV prepulse potential). These normalised values were then plotted as the function of prepulse potential.

RESULTS

1. Preparation of the spiral ganglion, cell isolation and identification of type I and type II spiral ganglion neurones

After the decapitation of the animals the temporal bone was removed, the bony cochlea revealed, then the spiral lamina was dislodged from the rest of the bony cochlea.

When the modiolus was removed it was broken up into several small pieces and the tissue chunks were transferred into an enzyme solution for 15-20 min. The cells were yielded by applying very gentle mechanical agitation. To make sure that the cells were firmly attached to the bottom of the experimental chamber, poly-D-lysine coating was employed. After the cell isolation both type I and type II cells were present in the experimental chamber. In order to make sure

that the electrophysiological measurements were conducted on type I cells only, those cells were selected, whose cell diameter was at least 10 µm, possessed remnants of the myelin sheath and had characteristic bipolar or unipolar appearance. Although these criteria could not explicitly rule out that occasionally and accidentally type II cells might have been patched, the relatively rare occurrence of type II cells made this very unlikely. In fact, out of the 51 isolated spiral ganglion neurones 7 cells were identified as "possible" or "likely" type II spiral ganglion neurones, and these nerve cells have been omitted from the data analysis.

To identify the cells yielded after the isolation procedure, immunocytochemical techniques were performed. Neurones were specifically labelled by using anti-neurone-specific enolase (anti-NSE) staining. Fig. 1B and C show the results of a neurone-specific-enolase immunoreaction. The cells which showed distinct immunopositivity had similar appearance to those identified as type I spiral ganglion neurones under phase-contrast optics. The presence of the myelin sheath (characteristic of type I spiral ganglion cells) was confirmed by using anti-S100 immunostaining.

2. Electrophysiological properties of type I spiral ganglion neurones.

The basic electrophysiological characteristics of the cells investigated in the present study were uniform, confirming that only type I cells were investigated. Including all the cells examined, the average whole cell capacitance was 9 ± 2 pF (n = 51); while the resting membrane potential was -62 ± 9 mV (n = 19). Considering the resting membrane potentials of the type I spiral ganglion neurones, in most of he further experiments a holding potential of -60 mV was applied.

2.1 Characterisation of the hyperpolarization-activated current of the type I spiral ganglion cells.

A slowly activating inward current could be observed when the type I spiral ganglion neurones were subjected to 2 s long hyperpolarizing stimuli from a holding potential of –60 mV to –140 mV in 10 mV steps. The activation of the current became obvious at –90 mV or more negative potentials. The current amplitude became higher, its activation became faster with the increasing negativity of the voltage steps. On the basis of the slow activation and some other features of this current it was recognised as a hyperpolarization-activated non-specific cationic current (I_h). Confirmation of this finding was sought by using CsCl and $BaCl_2$ in the experimental solution (1 mM in both cases). The hyperpolarization-activated current was effectively inhibited in the presence of Cs^+, while the blocking effect of Ba^{2+} was much less significant. The wash-out with normal aCSF achieved complete recovery of the current in either case.

A further (and in fact decisive) argument supporting the view that the hyperpolarization-activated component was indeed I_h, was its reversal potential. As I_h is a mixed cationic current its reversal potential is expected between -50 and -20 mV. In accordance with this expectation, the reversal potential of this current was –34 ± 7 mV.

2.2. Characterization and pharmacological separation of depolarization-activated ionic currents in type I spiral ganglion neurones

Type I spiral ganglion neurones produced outward currents in response to 200 ms long depolarizing pulses from a holding potential of –60 mV. Taking into account the ionic composition of the external and internal solutions these currents could be identified as K^+ currents. The outward current was shown to have a non-

inactivating (sustained) component whose activation was observable at depolarizations to –40 mV or beyond. When applying stronger depolarizations, besides this sustained component, an additional inactivating (transient) current appeared. The presence of the latter component was the most prominent around membrane potentials of +10 - +20 mV.

The classical way of separating the individual outward K^+ current components is the application of various channel blockers. In the present experiments TEA^+ decreased considerably the outward currents at concentrations of 1 and 10 mM, respectively. When analysing the kinetic features of the TEA^+-sensitive current it was found that the development of this component was mainly the consequence of activation of the delayed K^+ channels. However, comparison of the TEA^+-sensitive and TEA^+-resistant currents made it obvious that the TEA^+-insensitive current contained a significant non-inactivating component while the TEA^+-sensitive current had a remarkable inactivating component although the inactivation rate of the latter current was slower that that exhibited by the TEA^+-resistant current. This finding indicated that the type I spiral ganglion cells might possess transient outward K^+ currents as well.

In order to investigate the characteristics of the transient currents effects of various 4-aminopyridine (4-AP) concentrations on the outward K^+ currents were studied. It was established that in the presence of 100 μM 4-AP both the fast- and the slowly-inactivating current components disappeared leaving only a non-inactivating current to be observed. It was especially noticeable in these experiments that a significant fraction of the depolarization-activated K^+ current proved to be sensitive to relatively low concentrations of 4-AP (e.g. 100 μM).

To study the role of the 4-AP-sensitive currents in determining the firing pattern of type I spiral ganglion neurones, the responses of the cells to stimulation as well as the the 4-AP-induced modifications of these responses were recorded in current-clamp experiments. Under control conditions the neurones poduced only one action potential at the beginning of the stimulation and the character of their

response did not change even in the presence of 100 μM 4-AP (in other words, the investigated cells did not fire more than one action potential under the influence of the blocker). On the other hand, certain parameters of the action potentials were characteristically changed due the effects of 4-AP. Among the most important modifications were the increased amplitude, the slower repolarization and the finding that the plateu phase following the action potentials developed at more positive membrane potentials.

It is remarkable that 4-AP reduced the outward currents at concentrations as low as 30 μM. The presence of a highly 4-AP-sensitive current suggested that the type I spiral ganglion neurones might possess a dendrotoxin- (DTX-) sensitive current component as well. In some experiments, therefore, 200 nM DTX was added to the extracellular solution and it was found that this blocker inhibited approx. 30 % of the depolarization-activated outward currents. Moreover, the effects of DTX on the firing pattern of the type I spiral ganglion neurones were also studied. It was established that the application of DTX did not modify the fast-adapting firing character of these cells.

DISCUSSION

When starting the experimentation, one of the first tasks was to find the method most appropriate for isolating spiral ganglion neurones. Earlier papers reported on several studies carried out on various species but these reports focused mainly on presenting the experimental data and much less emphasis was laid on the details of obtaining the preparation. While working out the methods, we managed to establish an optimal procedure that enabled us to produce neurones suitable for recording ionic currents with considerable certainty. On comparing our technique to those reported earlier, several features of our approach are worth mentioning. In the case of the cell isolation procedures the biggest challenge and, in the same

time, the most frequently criticized effort is to prove that the membrane of the cells preserves its physiological structure and function. The most serious problem was to choose the appropriate enzymatic treatment. On the basis of preliminary experimental results, a combination of collagenase and pronase was found the most appropriate as these enzymes also helped the removal of the myelin sheet (the presence of the myelin makes impossible the formation of the patch-clamp configuration). Of course, when applying enzymatic treatment, it is always a real danger that overexposition somehow destroys the cell membrane. According to our experience, an incubation period of 15-20 minutes (at 37 °C) yielded the largest number of isolated neurones without damaging their integrity. When performing measurements in cell suspensions, a frequently seen complication is the instability of the cells, i.e. the fact that due to the constant perfusion they tend to change their position. To facilitate the adherence of the neurones, the glass surface was coated with poly-D-lysine.

The isolation procedure found most favourable under our experimental conditions is the result of several compromises. Nevertheless, it proved to be an appropriate method to produce isolated ganglion cells suitable for our purposes. The most important points, supporting this conclusion are the morphological characteristics, the physiological value of the resting membrane potential, the ability of firing action potentials, the development of different K^+ currents and the depolarization-induced increases of the cytoplasmic Ca^{2+} concentration (data not presented in the current theses).

In contrast to some other data available in the literature, we did not see heterogeneity in the electrophysiological properties of the guinea pig type I spiral ganglion neurones, as none of the cells investigated showed slowly adapting or non-adapting response.

One of the most important achievements of the present theses is the finding that there is a profound similarity between the K^+ currents of the type I spiral ganglion cells and of some other, more centrally situated neurones of the

auditory pathway (e.g. the bushy cells of the anterior part of the cochlear nucleus, principal cells of medial nucleus of the trapezoid body). All the indicated neurones possess a TEA^+-sensitive, non-inactivating current component and in their membranes the expression of a highly 4-AP sensitive, depolarization-activated K^+ channel could also be shown. It is also highly probable that these cells possess a DTX-sensitive current. These data indicate that a uniform ionic channel assembly might be responsible for the common membrane characteristics and, most importantly, for the generation of the rapidly adapting action potential pattern of the neurones mentioned above.

Beyond the listed similarities some differences between the discussed cells have to be mentioned, too. Although the presence of the DTX-sensitive current component in all the three neurone types is well established, its role in these neurones seems to be diverse. It is especially remarkable that in the bushy cells, in the neurones of the avian magnocellular nucleus and in the principal cells of medial nucleus of the trapezoid body, block of this current converts the fast-adapting response to a more slowly adapting one. A similar intervention (i.e. inhibition of the DTX-sensitive component) did not induce a similarly profound modification of the firing pattern of the type I spiral ganglion cells. One may conclude, therefore, that the DTX-sensitive current of the type I spiral ganglion neurones does not have such a definitive role in the generation of the rapidly adapting response as it was shown in the secondary and tertiary sensory neurones of the auditory pathway.

Parameters of the hyperpolarization-activated current (h-current) were identical with those reported earlier. One may conclude from this fact that the enzymatic treatment did not alter the membrane characteristics of the investigated cells. Several cellular functions may be attributed to the h-current, for example, this current may influence the resting membrane potential, it may prevent long-lasting inhibition of certain neurones and it may contribute to the spontaneous activity of some nerve cell types.

The DTX-sensitive current was shown to have a prominent role in generating the fast-adapting action potential pattern in several neurone types but a similar function could not be attributed to this current in the case of the type I spiral ganglion cells. On the other hand, the resting membrane potential became more positive in the presence of DTX and/or low 4-AP concentrations indicating that the ionic channels sensitive to these blockers might be partially activated at the resting membrane potential and, consequently, might contribute to the determination of the resting membrane potential of the type I spiral ganglion neurones.

The significance of the transient current remains to be clarified. This component did not play an important role in the determination of the firing characteristics. However, it is worth noting that the contribution of the transient currents to the whole K^+ current might be different in the soma and in the synaptic endings of the type I spiral ganglion neurones.

The fact very strict tonotopy can be demonstrated along the whole length of the auditory pathway (i.e. from the inner ear up to the auditory cortex) has high importance. From the practical point of view it makes necessary to construct and use cochlear implants that are able to stimulate those spiral ganglion neurones which correspond to the frequency of a given sound stimulus. Moreover, these tools have to produce firing patterns of the ganglion cells that show close resemblance to the activities developed in response to sound stimuli under physiological conditions. The present theses aimed at a better understanding of the membrane features and firing patterns of the spiral ganglion neurones, consequently – according to our intentions – the results may help to construct and produce more effective cochlear implants.

The thesis is based on publications as follows

1. Zs. Szabó, Cs. Harasztosi, I. Sziklai, G. Szűcs, Z. Rusznák, Ionic currents determining the membrane characteristics of type I spiral ganglion neurones of the guinea pig. Eur. J. Neurosci. 16, 1887-1895, 2002. **[IF:4,163]**
2. Zs. Szabó, Cs. Harasztosi, G. Szűcs, I. Sziklai, Z. Rusznák, A detailed procedure and dissection guide for the isolation of spiral ganglion cells of the guinea pig for electrophysiological experiments. Brain Res. Prot. 10, 139-147 2003. **[IF:1,109]**
3. Zs. Szabó, Cs. Harasztosi, I. Kovács, G. Szűcs, Z. Rusznák, I. Sziklai, Tengerimalacból izolált I. típusú ganglion spirale neuron depolarizációja által aktivál K^+ áramok jellemzése. Fül-, Orr-, Gégegyógyászat 49, 114-123, 2003.

Abstracts

1. Zs. Szabó, Cs. Harasztosi, G. Szűcs, Z. Rusznák, I. Sziklai, Ionic currents of type I spiral ganglion neurones of the guinea pig. Acta Oto-Rhyno-Laryng. Belg. 56, 286a, 2002.
2. Cs. Harasztosi, Zs. Szabó, Z. Rusznák, G. Szűcs, I. Sziklai, An improved procedure for the isolation of spiral ganglion cells of the guinea pig. Acta Oto-Rhyno-Laryng. Belg. 56, 287a, 2002.

Presentations, posters

1. Zs. Szabó, Cs. Harasztosi, G. Szűcs, I. Kovács, I. Sziklai, Z. Rusznák, Functional properties of ganglion spiral neurons isolated from the cochlea of the guinea pig MÉT 66. vándorgyűlése, Szeged, 06-08, 2001.
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5. Zs. Szabó, Cs. Harasztosi, G. Szűcs, Z. Rusznák, I. Sziklai, Ionic currents of type I spiral ganglion neurons of the guinea pig. 39th Inner Ear Biology Workshop, Liege, 08-10 2002.
6. Cs. Harasztosi, Zs. Szabó, Z. Rusznák, G. Szűcs, I. Sziklai, An improved procedure for the isolation of spiral ganglion cells of the guinea pig. 39th Inner Ear Biology Workshop, Liege, 08-10 09.
7. Zs. Szabó, Cs. Harasztosi, Z. Rusznák, I. Sziklai, Inhibition of potassium currents in spiral ganglion neurons of the guinea pig. Association for Research in Otolaryngology, 25, 601., Florida, USA, 2002.