Introduction

Sound energy is transmitted by the ossicles of the middle ear to the fluid compartments of the inner ear through the oval window, eventually changing the position of the basilar membrane relative to the tectorial membrane. Movement of the basilar membrane causes displacement of the stereocilia situated at the apical part of the hair cells (which are the sensory cells of the organ of Corti), resulting in the opening of the transduction channels. Activation of these channels allows K^+ entry and the generation of the transduction current. The actual effects of the depolarization of the hair cells depend on type of sensory cell in question.

There are two known types of the hair cells in the organ of Corti. Inner hair cells (IHC) are pear shaped cells with a centrally located nucleus. One row of inner hair cells runs along the cochlear duct. According to the prevailing theory, IHCs are the real sensory cells of the acoustic system. Outer hair cells (OHC), on the other hand, form another fundamental cell type of the organ of Corti. OHCs usually run in 3 rows, but in some cases a fourth and even a fifth row are found, and they are mainly innervated by efferent nerve terminals. OHCs are known to enhance and modulate the function of the inner hair cells.

The differences in the functions of the two cell types are the consequences of the different events that follow their depolarizations. In IHCs, depolarization of the cell is followed by neurotransmitter release at the hair cell base, which induces action potentials in the post-synaptic auditory fibre and the ascending auditory pathways. Since at low sound frequencies the frequency of the movement of the stereocilia matches the frequency of the sound stimulus, the hair cell—auditory nerve synapse is a highly active one. A synapse with rapid post-synaptic effect and equally rapid recovery is required to keep up with the frequency-related release of the neurotransmitter of the hair cells. Neurochemical and immunocytochemical evidence supports glutamate as the hair cell transmitter, acting on ionotropic glutamate receptors with subunit composition that is configured for rapid recovery. Glutamate may not be the only neurotransmitter utilised by the hair cells, and the presence of $P2X_2$ receptors in the auditory nerve suggests that ATP may also modulate auditory nerve activity, although its exact source remains unknown.

90-95% of the spiral ganglion cells (SGCs) are Type I SGCs that innervate the IHCs. They have oval and relatively large cell bodies, although there are significant interspecies differences in the latter parameter and its actual value is greatly influenced by the preparation technique. A special feature of Type I SGCs is that not only their processes but also their cell

bodies are myelinated, thus the soma is also part of the conduction pathway. A type I SGC has only one peripheral process, which is generally connected to a single (sometimes two) IHC. A single IHC at the same time can make synapses with 10-30 SGCs, the exact number depends on the species. Type I SGCs possess spontaneous activity, which is the result of both the leaking neurotransmitter release of the IHCs and the intrinsic membrane properties of these neurones. Besides the spontaneous and induced activity of the IHCs, the function of these synapses is regulated by various modulatory pathways and mechanisms (e.g. LOC) as well.

The functions of the OHCs are rather different from those of the IHCs. OHCs have a dual response to the transduction current generated by the opening of transduction channels in the stereocilia. The major response is a rapid change in the length and stiffness of the OHCs, which changes the shape of the organ of Corti, resulting in an enhanced transduction at the inner hair cells in that specific region of the cochlear spiral, increasing, therefore, the sensitivity and selectivity of the cochlea. Moreover, there is neurotransmitter release at the base of the OHCs, although this is relatively minor compared to that of the IHCs.

OHCs make synaptic contacts with Type II SGCs, which form only 5-10% of the SGC population. Their cell bodies are more spherical and smaller than those of the Type I neurones. A single Type II SGC innervates 3-10 OHCs, and they are unmyelinated (unlike their Type I counterparts). Although little is known about the OHC—Type II SGC synapse, there is evidence for the fundamental role of glutamate.

Spiral Ganglion

Since Type I and Type II SGCs have rather different functions, distinguishing them is very important. Several histological markers have been tested; e.g. anti-neuropeptide-Y and anti-parvalbumin antibodies for labelling Type I SGCs and anti-GFAP, anti-peripherin and anti-SAP (or anti-SAP108) antibodies for the specific labelling of Type II SGCs. The surprisingly high number of the employed antibodies also suggests that there is still no established marker with which the two neuronal types could be reliably distinguished. The problem is further complicated by the fact that neither Type I, nor Type II SGCs form homogeneous cell populations. Spiral ganglion neurones, like hair cells, are organized tonotopically, and several of their characteristics change gradually along the axis of the cochlea (e.g. cells situated closer to the basal region have generally larger cell bodies).

Besides the morphological differences there are also differences in the firing patterns of the SGCs. In general, SGCs fire a single action potential at the beginning of the stimulus when

subjected to suprathreshold stimulation. When high frequency stimulation is employed, the cells exhibit an adaptive behaviour: the action potential duration increases, the amplitude decreases, peak time decreases, and failures develop. Individual neurones, however, have varying latency and rate of adaptation, which depend on the type of the neurone and its localisation within the modiolus. Thus in the case of Type I SGCs, apical neurones (encoding lower frequency sounds) show longer latency and slower adaptation, whereas basal neurones (encoding higher frequency sounds) demonstrate short latency and rapidly adapting responses. Compared with Type I SGCs, Type II neurones showed slower accommodation, lower AP threshold and more prolonged responses to depolarizing current injections. Data concerning the changes in the firing pattern of Type II neurones along the axis of the cochlea are contradicting.

The results of some studies suggest that these morphological and electrophysiological differences along the axis of the cochlea are not pre-programmed into the cells; but they may result from extrinsic regulation – such as the local neurotrophin-3 (NT-3) concentration, which has a gradient along the axis, thus it can be the reason of the established firing differences.

Cochlear Nucleus

The next station in the processing of sound is the cochlear nucleus, which receives the information directly from the acoustic nerve. It is important to note that the role of the cochlear nucleus is not limited to simply passing on the signals, but the analysis of the auditory information begins here. During this process the various components of the auditory stimuli are relayed onto different pathways, which often run in parallel with each other, and feed information to the subsequent centres of the auditory system. It is known that some of these pathways can maintain the temporal coding of the acoustic signals; while in other ones temporal coding and high-fidelity transmission do not seem to be important.

After reaching the nucleus, spiral ganglion fibres branch into two separate bundles. The more rostral ones project to the anteroventral part of the cochlear nucleus (aVCN), whereas the more caudal descending fibres reach the dorsal cochlear nucleus (DCN) through the posteroventral part of the nucleus (pVCN). The most prominent neurones of the pVCN are the octopus cells. In the DCN four concentrically arranged layers can be distinguished. The most superficial area is the molecular layer (layer 1), containing mainly the axons of the granule cells, although some granule cell bodies are also situated here. Immediately below the

molecular layer, the fusiform (or the pyramidal) layer is found (layer 2), where the cell bodies of the pyramidal, granule, cartwheel and stellate cells are situated. In layer 3 the basal dendritic tree of the pyramidal cells is found, along with some vertical cells. The descending fibres of the acoustic nerve also terminate here and contact these structures. The deepest part of the nucleus (layer 4) consists of mainly the cell bodies of giant and large multipolar cells. There are two types of spiral ganglion fibres making contact with the cochlear nucleus: thick, myelinated fibres of type I SGCs, and the much thinner and unmyelinated fibres of type II SGCs. While the former ones contact some of the biggest cells of the cochlear nucleus complex (i.e. pyramidal and bushy neurones), the latter nerve fibres reach the granule cells, that distribute the received information via the extensive parallel fibre network. It is obvious that the most important source of information to the cochlear nucleus is the acoustic nerve, but it also receives acetyl-cholinergic modulatory projections from the superior olivary complex as well.

Overview of K$^+$-channels

K$^+$-channels have been first identified in the neuronal membrane due to their important roles in the action potentials. However, according to our present knowledge, they can be found in almost every cell type. Since the intracellular K$^+$-concentration is higher than in the extracellular space in almost every cell, opening of the K$^+$-channels stabilizes the resting membrane potential or it may hyper- or repolarize the membrane. Due to their ubiquitous occurrence and diversity, K$^+$-channels play important roles in many different physiological (as well as pathological) processes.

Voltage gated K$^+$-channels

Voltage gated K$^+$-channels (Kv channels) form one of the most numerous and most diverse group of the ion channels. In humans, the number of such subunits is over forty, but alternative splicing of the mRNA molecules further increases the number of the possible variations. The biophysical properties and functions of the Kv channels vary extensively, but in general, they are responsible for the repolarization and hyperpolarization of the membrane following an action potential and some of them may delay or even inhibit the genesis of an AP.

Functional Kv channels are made up of four subunits. Each subunit contains six transmembrane domains (S1-S6), the fourth of which is responsible for voltage sensing. The pore forming loop (P-loop) of these channels can be found between the S5 and S6 domains.

One of the reasons for the great diversity of the Kv-channels is the large number of the subunits, but it is further increased by the formation of homo- and heterotetramers *in vivo*.

Voltage gated K^+-channels can be divided into three major groups based on their sensitivity to depolarization and their inactivation properties: 1. dendrotoxin- (DTX-) sensitive, low voltage-activated (LVA) and slowly inactivating channels; 2. high voltage-activated, slowly (or not) inactivating channels producing the so called delayed rectifier current; 3. rapidly and voltage-dependently inactivating transient channels.

Low voltage-activated channels activate in response to small depolarizations from rest to ~ -60 mV (straddling the threshold for action potential generation). This current is generated by channels containing Kv1.1, Kv1.2 and/or Kv1.6 subunits.

Slowly inactivating delayed rectifier currents are produced by channels containing Kv1.3, Kv1.5, Kv2.1, Kv2.2, Kv3.1 or Kv3.2 α-subunits. These channels are activated by high voltage (HVA channels), meaning that they require substantial depolarization for activation. This current typically activates with significant delay, and shows no (or only very slow) inactivation. This current plays a crucial role in the repolarization phase following an AP, and thus in the genesis of brief action potentials, which is a fundamental feature in firing neurones capable of producing high frequency firing.

Transient outward K^+ currents (also termed A-currents) form the third functional class of outward K^+ currents. Both LVA (e.g. Kv4.2) and HVA (e.g. Kv3.4) channels can be found in this group. Their activation upon depolarization is followed by rapid (10-100 ms) inactivation, generating a transient conductance change.

These channels are often partially inactivated at the resting membrane potential, thus small changes of the membrane potential can strongly affect the degree of inactivation. A-current activation is, therefore, enhanced by hyperpolarization, so that it can modulate synaptic activity by altering the intrinsic excitability of the cell membrane. A-currents can transiently prolong the latencies of the first few spikes and/or they can change the interspike interval during an AP train. Inactivating currents might also play a role in synaptic integration by rapidly inactivating during an initial excitatory postsynaptic potential (EPSP), and reducing the threshold, thus a second coincident EPSP can trigger an action potential.

Hyperpolarization-activated cyclic nucleotide-gated cation channels

Hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channels belong to the Kv channel superfamily. These channels are activated by hyperpolarization (potentials negative to -50 - -60 mV) and the generated current is termed $I_{f(unny)}$ in the heart and I_h in neurones. In spontaneously active neurones (e.g. thalamocortical relay neurones) and in the pacemaking structures of the heart, the h-current plays a crucial role in generating the prepotential responsible for the rhythmic activity of these cells. This current is also important in several other cells that are not active spontaneously, because it helps in seting the resting membrane potential (e.g. photoreceptors of the eye), it can dampen the amplitude of depolarizing or hyperpolarizing responses, it may control the dendritic integration (e.g. hippocampal CA1 neurones) and it may also have a role in the regulation of synaptic transmission (e.g. cerebellar basket cells).

Similarly to Kv channels, functional HCN channels form tetramers. A single HCN subunit contains six transmembrane domains, the fourth of which is positively charged and is responsible for voltage sensing. In addition, every subunit contains a pore-forming P-loop, which contains the GYG amino acid triplet, which is thought to be the selectivity filter of K^+-permeable channels. Besides this GYG sequence, however, the amino acid sequence of the HCN channels and other K^+ channels considerably differ, and this may account for the relatively low K^+ selectivity of these channels.

cAMP (and cGMP too, though to a lesser extent) can greatly influence the activation of the h-current, by shifting the voltage dependence of its activation to more positive voltages. It is important to note that the regulation of I_h by cAMP does not require protein phosphorylation, but the response to cAMP is mediated by a cyclic nucleotide-binding domain (CNBD).

Four HCN subunits have been described so far (HCN1-4), having different kinetics, steady-state voltage dependence of activation and different sensitivity to the modulatory effects of cAMP. The diversity of the HCN channels is further increased by the possible formation of heterotetramers.

Aimsof the study

The various voltage gated K^+-currents play important roles in the neurones of the initial part of the auditory pathway.

1. In our experiments conducted on the cochlear nucleus we investigated:

1.a. The presence and distribution of certain Kv-subunits in the cochlear nucleus.

1.b. The Kv-channelsubunit pattern of the most important projection neurones of the cochlear nucleus.

1.c. The differences among the Kv-channelsubunit pattern of the most important projection neurones of the cochlear nucleus.

2. In our experiments conducted on guinea pig spiral ganglion our aims were:

2.a. The development of a technique that enables the confocal analysis of the SGCs and insures the secure differentiation between type I and II neurones.

2.b. We investigated the Kv- and HCN-subunit pattern of the SGCs.

2.c. We investigated the possible differences in the Kv- and HCN-subunit pattern between type I and type II cells.

2.d. We investigated the possible apico-basal gradients of the HCN-subunits.

Materials and methods

Experimental animals

Spiral ganglions were isolated from male, adult guinea pigs, weighing 400-700 g (n = 40). 20 guinea pigs were employed for the Western blot experiments; the cochleae of 69 animals were used for the wax-embedded and cochlear free floating preparation experiments.

Cochlear nuclei were prepared from young (11-17 days) and older (at least 25 days, but usually 1-3 months old) Wistar rats of both sexes. The data presented in this work were obtained from 36 animals (5 young and 31 older rats from both sexes); young animals were exclusively used for the investigation of the age dependence of the Kv1.2 subunit expression.

Preparation of the spiral ganglion from guinea pig

On the day of the experiments, animals were anesthetized with pentobarbital, decapitated, and the temporal bones were removed. They were then opened with a dental drill, making the cochlea visible. The separated modioli were fixed in 4% buffered formalin; the duration of the fixation period was determined according to the type of immunochemistry employed afterwards: it was 2 hrs when fluorescent immunohistochemistry was used, whereas the utilisation of the traditional wax-embedded tissue sections necessitated substantially longer, at least 24 hrs long fixation periods.

Preparation of the cochlear nucleus from rat

All dissection steps were carried out in ice-cold low-Na^+ artificial cerebrospinal fluid (aCSF). After the decapitation of the animal, the brain was quickly removed; the brain stem (containing the CN) was prepared and placed into 4% paraformaldehyde solution for 4 hours (4C). Following this, the fixed tissue was washed (3 × 10 minutes) in 0.1 M phosphate buffer (PB). Vibrating microtome was used to cut 50-60 μm thick slices.

Fluorescent immunochemistry

Samples were washed in PB buffer (10 min) and TBS (3 × 10 minutes) followed by blocking and permeabilisation (60 min), and were incubated with the primary antibodies (overnight, 4 °C). When the incubation with the primary antibodies was terminated, the slices were rinsed in TBS (3 × 10 min) and incubated with the appropriate fluorochrome-conjugated secondary antibody (3 hours at RT) followed by another washing step TBS (3 × 10 min). At the end of the procedure, the slices were mounted using a DAPI containing mounting medium.

Immunohistochemistry on wax-embedded preparations

Formalin-fixed modioli were decalcified using Biodec R (Bio-Optica, Milan, Italy) for 8 hrs at RT, embedded in paraffin-wax, and followed by the preparation of traditional histological sections. Although the decalcination process alone usually acted as a suitable antigen retrieval (AR) method, in some cases the intensity of the reaction was enhanced by more intense AR methods.

When the primary antibody was raised in rabbit, the endogenous peroxidase activity was blocked with 3% aqueous H_2O_2 (10 min, RT). Slides were washed in PBS and blocked. Tissue sections were then incubated with the primary antibody (4 °C, overnight). On the second day of the experiments, slices were rinsed in PBS (3 × 5 min), incubated with the EnVision system, and visualised by employing a VIP SK4600 kit or DAB. Slight counterstaining was performed with methyl green.

If the primary antibody was produced in goat (anti-HCN1 and anti-HCN4), both horseradish peroxidase- and alkaline-phosphatase-based reactions were employed. When the alkaline-phosphatase-based technique was utilised, the AR was followed by three washes in Tris-buffer. Tissue sections were blocked and incubated with the primary antibody (4 °C, overnight). On the second day of the experiments, sections were rinsed with TB (3 × 5 min), followed by incubation with the biotinylated secondary antibody. The sections were then rinsed in TB and incubated with alkaline phosphatase - conjugated streptavidin. At the end of the procedure, slides were rinsed with TB and the immunoreaction was visualized using fuchsine. On certain occasions slight counterstaining was performed with methyl green. Peroxidase-based immunoreactions were carried out as described above.

Western blot

Protein concentrations of the samples were determined by the BCA method. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked and incubated with the appropriate primary and secondary antibodies. The immunoreactive bands were visualized by enhanced chemiluminescence and detected using BioMax light film or LAS 3000 Intelligent Dark Box. Immunoblots were quantified by densitometry.

Retrogade labelling

In the course of the present experiments the aim was to label all projection neurones of the DCN that targeted the inferior colliculus. For this purpose tetramethylrhodamine-dextran crystals were applied into sagittal incisions made on the ventral surface of the brain stem at the level of the trapezoid body. To avoid photobleaching of the retrograde tracer, all steps of the labelling procedure were performed in a dark room.

The application of the rhodamine was immediately followed by the incubation of the brain stem in normal aCSF (8-12 hrs), which was continuously oxygenated. At the end of the procedure, the preparation was fixed in 4% paraformaldehyde solution (4 °C for 12 hrs) and 50-60 μm thick sagittal slices were cut, which subsequently underwent the immunolabelling procedure as described above.

Results

Distribution of A-type current generating subunits in the CN (Kv4.2, Kv4.3, Kv3.4)

Both the DCN and VCN showed Kv4.2-specific positivity. The Kv4.2-labelling was present on the cell bodies and processes of the bushy cells. Octopus cells were positive, too. Moreover, in double labelling experiments some of the Kv4.2 positive dots showed co-localisation with the synaptophysin positive synaptic terminals raising the possibility of presynaptically localised Kv4.2 channels. The acoustic nerve fibres, however, proved to be Kv4.2 negative.

The DCN demonstrated stronger immunolabelling than the VCN, especially in some deeper areas and in the granule cell region. In the latter area besides the granule neurons small, spherical, synaptophysin positive structures could be observed that demonstrated Kv4.2 positivity as well, and were identified as glomerular synapses. Only few of the giant cells (1/5) expressed this subunit, whereas the majority of the pyramidal neurones proved to be Kv4.2 positive.

Presence of the Kv4.3 subunit could be demonstrated in both the DCN and VCN. This subunit — like the Kv4.2 subunit — was present on the cell bodies and processes of the bushy cells, as well as on the octopus cells.

The granule cells of the DCN also expressed the Kv3.4 protein, yet the ependyma layer covering the surface of the nucleus proved to be negative. All investigated giant cells (8/8) were positive, and the single pyramidal cell that could be convincingly identified was Kv4.3 positive as well.

The investigation of the expression pattern of the Kv3.4 subunit showed that it is more strongly expressed in the CN than in the nearby regions of the brain stem. The octopus and bushy neurones of the VCN proved to be positive, whereas the acoustic nerve fibres were negative.

In the granule region of the DCN not only the neurones but also the aforementioned glomerular synapses possessed Kv3.4 immunopositivity. Giant cells (n = 3) did not show significant labelling, whereas a positively identified pyramidal neurone was positive.

Distribution of low voltage-activated (LVA), dendrotoxin-sensitive channels in the CN (Kv1.1, Kv1.2, Kv1.6)

Several areas of the nucleus showed strong Kv1.1 positivity, like the deep and fusiform layers of the DCN, as well as the octopus cell region of the VCN, and the bushy neurones were also labelled.

Only a portion of the pyramidal (6/9) and giant cells (18/29) proved to be Kv1.2 positive, whereas the granule neurones did not show significant immunolabelling.

The investigation of the distribution of the Kv1.2 subunits appeared to be particularly interesting, as a controversy was present in the literature concerning the ontogenetic alterations of the expression pattern of this subunit in the CN. Thus in the experiments regarding the distribution of the Kv1.2 subunits, specific emphasis was laid upon contrasting the labelling patterns observed in young and older animals. Our results showed that the

intensity of the Kv1.2-specific immunopositivity was more prominent in the bushy and octopus neurones of the older animals, and while in younger animals the immunolabelling appeared to be primarily intracellular, in old animals it was particularly intense on the cell surface.

Granule cells of the DCN proved to be Kv1.2 negative. At the same time most pyramidal (6/7) and several giant cells (14/34) expressed this channel subunit. The age-dependent expression of the Kv1.2 subunit could also be noticed in the DCN: it was more prominent in older animals.

The Kv1.6-specific immunolabelling in the VCN was stronger than in the DCN. Both the cell bodies and processes of the bushy and octopus neurones were positive. Granule cells did not show significant Kv1.6 labelling, however most pyramidal and giant cells (6/8 and 8/12, respectively) expressed the subunit.

Distribution of the delayed rectifier current generating Kv3.1 subunit in the CN

Although both the DCN and the VCN showed definite Kv3.1 immunopositivity, the intensity of the labelling was appreciably stronger in the VCN than in the DCN. The bushy and octopus cells of the VCN showed prominent immunolabelling.

The majority of giant (27/33) and pyramidal cells (12/15) were positive, too. The immunopositivity of the granule cells was low compared with the other investigated projection cells of either the DCN or the VCN.

Discussion

One aim of the present work was to provide a comprehensive description of the Kv subunit expression of positively identified neurones of the CN. Although the presence of certain Kv channel proteins has been detected before and their distribution patterns described, this is the first study employing experimental animals of the same species and age to describe the precise, cell-specific distribution of 7 different Kv subunits. Besides the general importance of detecting the presence or absence of certain subunits, and thus allowing the prediction of the membrane properties of the individual types of cells, it was demonstrated that some Kv subunits are preferentially expressed by the various types of CN neurones.

Kv subunit expression pattern in the cochlear nucleus

The presence of all three low voltage-activated DTX-sensitive channel subunits could be demonstrated on the bushy neurones of the VCN, and they may play an important role in the rapidly adapting nature of these neurones. This observation is in good agreement with the hypothesis that several subunits are responsible for generating this current in the bushy cells. Both LVA (Kv4.2 and Kv4.3) and HVA (Kv3.4) channel subunits take part in producing the transient current of bushy cells. The delayed rectifier current is present in various portions of the auditory pathway, including the bushy neurones, which demonstrated strong Kv3.1b immunopositivity.

Octopus cells form the other major projection cell type of the VCN. Similarly to the bushy neurones octopus cells also express all three LVA DTX-sensitive subunits. Moreover, the expressions of the Kv1.1 and Kv1.2 subunits were so prominent that they may even be used as markers assisting cell identification in future studies. All three investigated subunits responsible for the transient currents were expressed by the octopus neurones and they were characterised by strong Kv3.1 positivity as well. Our results confirm that these cells possess all three major voltage-gated K^+-current types.

The granule cells of the DCN expressed none of the LVA DTX-sensitive subunits and their Kv3.1-specific immunolabelling was also negative. However, all three investigated transient current producing subunits were expressed by these neurones, with the Kv4.2-specific immunopositivity being the most prominent in the whole nucleus. Moreover, our work demonstrated the Kv4.2 and Kv3.4 positivity of the glomerular synapses of the CN, resembling the same findings in the cerebellum and further supporting the view about the common origin and close morphological similarity between the CN and the cerebellum.

Last but not least, on the basis of the heterogeneity concerning their Kv expression patterns, it is suggested that neither the pyramidal nor the giant neurones form homogenous cell populations, thus it cannot be ruled out that functional/morphological subgroups may exist within these cell classes. It is worth mentioning that most giant cells were strongly Kv3.1 positive, but the aforementioned heterogeneity could be observed in the case of this subunit, too.

In the case of the Kv1.2 channel subunit, besides studying the distribution of the protein within the nucleus, we also investigated developmental changes occurring in the Kv1.2 expression pattern. The expression of the Kv1.2 subunit was noted in the CN isolated from both young and old animals, however the intensity of the Kv1.2-specific immunopositivity

increased with the age of the animals. Moreover, a characteristic translocation of the Kv1.2 subunits could also be observed, especially in the cases of the octopus and bushy cells: while in young animals the Kv1.2 immunopositivity appeared to be present intracellularly, in older animals very strong immunopositivity could be noted in the cell surface membrane.

Results

Morphological characteristics of the guinea pig spiral ganglion neurones in cochlear free-floating preparations

We have developed a new preparation that allowed the reconstruction of the three-dimensional structure of the SGC nests. Confocal Z-stack images were used to determine the morphological characteristics of the SGCs, which allowed the investigation of the SGCs from any desired angle. The measured morphological parameters were: largest diameter, cross-sectional area, circumference and elongation factor. SGCs could be divided into two populations: large and small cells. The morphological parameters of large cells (n = 100) were: average diameter: $19.9 \pm 2.4 \, \mu m$, cross-sectional area: $220{,}6 \pm 35{,}6 \, \mu m^2$, circumference: $56{,}5 \pm 5{,}6 \, \mu m$, elongation factor: $1{,}42 \pm 0{,}23$; whereas in small cells (n = 11) they were $14{,}2 \pm 1{,}0 \, \mu m$, $142{,}3 \pm 28{,}4 \, \mu m^2$, $43{,}8 \pm 4{,}8 \, \mu m$ and $1{,}15 \pm 0{,}28$ respectively. In the immunohistochemical experiments both large and small cells proved to be NSE positive, however only large cells showed S-100 labelling, whereas small cells were negative, suggesting that only the former neurones were myelinated. Based on the morphological and immunohistochemical data, large cells were recognised as type I, whereas small cells were regarded as type II cells of the spiral ganglion.

Morphological characteristics of the guinea pig spiral ganglion neurones in wax-embedded preparations

When the basic morphometric data were determined in wax-embedded tissue sections and compared to those obtained from the free-floating preparation, it was obvious that the more prolonged formalin fixation and the application of the histological protocols substantially reduced all relevant parameters of the SGCs, changed their shape and they also decreased the differences between the smaller and larger cells. Besides the alterations affecting cell

morphology, in the wax-embedded preparations precise cell identification was further complicated by the fact that unless the cell nuclei were clearly stained, it was not possible to determine whether the sectioning plane was running at or near the central plane of the soma. Consequently, without this information it was impossible to precisely determine the greatest diameter (or the other parameters) of the cells.

It could be concluded, therefore, that the cochlear free-floating preparation more effectively conserved the geometry and size of the SGCs than the traditional wax-embedded preparation, thus the new technique allowed more reliable distinction between the larger (type I) and smaller (type II) cell populations.

Low-voltage activated, DTX-sensitive K+ current producing subunits (Kv1.1, Kv1.2, Kv1.6)

Our experiments conducted on both cochlear free floating and wax-embedded preparations showed that SGCs expressed all three DTX-sensitive Kv-subunits on both their cell bodies and processes. The Kv1.1 and Kv1.6 subunits were present in the majority (>95%) of the neurones and no remarkable difference could be observed between type I and type II cells. In the case of the Kv1.2 subunit however, almost all type I neurones proved to positive, whereas only about half of the small cells expressed this subunit. Moreover, significant differences in the level of the Kv1.2 expression of the individual neurones could be noted.

A-type current producing subunits (Kv1.4, Kv4.2, Kv4.3, Kv3.4)

The investigation of the presence and distribution of the Kv1.4 subunits was especially inviting in the present study, due to two reasons: on the one hand, Kv1.4 is a subunit which may associate with the DTX-sensitive Kv subunits, and it can, therefore, effectively modify the kinetical properties of the current produced by these Kv subunits. On the other hand, previous studies reported on the absence of Kv1.4 immunopositivity in the rat cochlear nucleus, so it was an interesting question whether the Kv1.4 subunit is generally absent in the first section of the auditory pathway, including the spiral ganglion. In our experiments we demonstrated, that spiral ganglion neurones were Kv1.4 positive, their labelling intensities were variable, and it mostly affected the cell bodies. Neural processes were weakly positive.

In the cases of the Kv4.2 and Kv4.3 subunits we found, that most SGCs expressed them and that these channel subunits were mainly located on the cell bodies of the neurones, although some processes were also labelled.

The presence of the HVA K^+-current producing Kv4.3 subunit could also be demonstrated in most SGCs, however the labelling intensities of the individual neurones showed great variability and it was present mainly on the soma only.

No significant difference could be observed between type I and type II cells in the expression pattern of any of the investigated Kv-subunits (Kv1.4, Kv4.2, Kv4.3, Kv3.4).

Delayed rectifier current producing subunits (Kv3.1b, Kv3.2)

Out of the several Kv subunits known to contribute to the assembly of delayed rectifier K^+ channels, the presence of Kv3.1b and Kv3.2 subunits was tested in the present work. The majority of the SGCs proved to be Kv3.1 positive. The immunopositivity was present on the cell bodies and processes as well, and the cell surface always showed distinctly stronger positivity than the cytoplasm. No difference between type I and type II cells could be noted in the case of this subunit either.

Unlike the strong and wide-spread distribution of the Kv3.1 subunit, the other delayed-rectifier current producing channel subunit tested in the present work (Kv3.2) was not expressed by the SGCs. This observation was made in both the cochlear free-floating and wax-embedded preparations; suggesting that this subunit plays only negligible (if any) roles in the genesis of the delayed rectifier K^+ current of the SGCs.

Validation of HCN-specific antibodies

In the present work the presence and distribution of all four known HCN channel subunits were tested on guinea pig SGCs. Since we had very limited experience with the antibodies employed in the present work, validation of the immunohistochemistry results was sought by employing Western blotting on whole spiral ganglion samples. Each HCN-specific antibody produced a single immunoreactive band at or near the molecular weights expected, and no labelling could be observed when the primary antibodies were preincubated with their respective blocking peptides.

HCN subunit expression pattern of the spiral ganglion cells

The expression of all four HCN-subunits could be demonstrated on both cochlear free floating and wax-embedded preparations. All four HCN-subunits were present on the great majority of the SGCs, and both the cell bodies and the processes were labelled. Interestingly, while no difference could be observed between type I and type II cells, the labelling intensity of the individual (even neighbouring) neurones showed great variability.

Apico-basal gradients of the HCN1 and HCN3 expressions

The results of the immunolabellings performed on whole modioli did not show strong difference between the intensity of the immunoreactions presented by SGCs situated near the apex or base of the spiral ganglion. However, since no densitometry or other image analysis was employed in the present work, the lack of prominent difference did not necessarily mean that there might not be differences in the amount of HCN subunits at the protein level, especially in the light of the fact that the labelling intensity showed considerable cell to cell variability.

To reveal the presence of possible apico-basal gradients of the HCN subunit expression, Western blot experiments were conducted using tissue samples obtained from the apical, intermediate and basal thirds of the modiolus, and all results were densitometrically evaluated.

Out of the four HCN subunits tested, the results describing the expressions of the HCN1 and HCN3 subunits showed clear dependence on the position of the cells along the axis of the modiolus: the amounts of the HCN1 and HCN3 proteins were 3.5 ± 0.9 and 2.9 ± 0.5 times greater in the apical than in the basal thirds of the modiolus, respectively.

Discussion

In this work a new technique, termed cochlear free-floating preparation has been introduced, that has several advantages over previously employed techniques. On the one hand since the fixation time is shorter and there is no need for decalcification or antigen retrieval, the morphology of the SGCs are better preserved, which also makes it easier to differentiate between type I and type II cells. On the other hand while in wax-embedded samples only one section, thus only a small portion of the SGCs can be tested and only small segments of the

processes are visible, in the cochlear free floating preparations the confocal Z-stack images show the three dimensional structure of the SGC nests and the processes can be followed over long distances.

Transient current producing K$^+$ channel subunits in the guinea pig spiral ganglion

The presence or absence of the transient K$^+$ current expressed by the SGCs is perhaps one of the most controversial issues of the field. In some studies no such inactivating currents could be detected either on acutely isolated or on cultured SGCs suggesting that the relevant channels might not be present at all, or they were expressed by the neurites only, which had been lost during the preparation. In other studies however, a transient current component could be observed, moreover it has also been described that the expression of a transient current showed significant time dependence. The availability and function of the transient K$^+$ current expressed by rat SGCs were also clearly demonstrated in a cochlear slice preparation and it could also be demonstrated in the same work on isolated cells, indicating that the earlier suggestion about the preferential localisation of the appropriate channels on the neurites of the SGCs might not be correct. Signs of activation of a transient K$^+$ current were also demonstrated on confirmed type II cells in a cochlear slice preparation.

Kinetical analysis targeting the transient current expressed by the SGCs suggested that it was composed of multiple current components, raising the possibility that several Kv subunits might contribute to the assembly of channels producing transient K$^+$ currents, but only the presence of Kv4.2 subunits has been demonstrated so far, using murine SGCs maintained in tissue culture. Kv4.2 expression was also found in the present study. Moreover, the presence of Kv4.3 subunits was also demonstrated in our study, suggesting that Kv4.2/Kv4.3 heterotetrameric channels may be present in the cell surface of the SGCs. Besides Kv4.2 and Kv4.3, the presence of Kv1.4 subunits should also be mentioned, which may associate with other members of the Kv1 family (most notably Kv1.1, Kv1.2 and Kv1.6), contributing to the genesis of a DTX-sensitive current.

While channels containing Kv4.2, Kv4.3 or Kv1.4 subunits produce LVA transient currents, Kv3.4 channels produce transient current activating at more positive membrane potentials. On the basis of the kinetics characterising the rate of recovery and inactivation of the transient current expressed by the SGCs, it has been suggested that Kv3.4 channels may also be expressed by them; however this study is the first to give experimental evidence confirming this hypothesis. Although the exact role of the transient K$^+$ current components produced by

the spiral ganglion cells is still a matter of debate, they may ensure frequency encoding, may be the targets of various modulatory mechanisms that may affect the functional properties of the SGCs and they may be important for the temporal integration of the cochlear amplifier activity of the type II cells, where the presence of a transient K^+ current was confirmed, too.

DTX-sensitive K^+ currents expressed by the SGCs

The presence of a DTX-sensitive K^+ current component seems to be a prominent feature of the relay neurones of the auditory pathway. Functionally, this current may be important for the ability of the cells to produce high frequency firing, and it was shown that inhibition of this component dramatically altered the firing pattern of the rapidly accommodating SGCs, evoking the genesis of a series of action potentials in response to sustained depolarization. LVA K^+ channels may prevent excessive depolarization and hyperexcitability, which may be particularly important for the high frequency and faithful information processing ability of the SGCs. In the present work, it was shown that the Kv1.1, Kv1.2 and Kv1.6 subunits were expressed by the guinea pig SGCs. It has also been suggested that the molecular assembly of the K^+ channels responsible for the genesis of this current may involve Kv1.4 subunits as well, and the somatic expression of this subunit found in the present work is clearly in line with this suggestion.

Delayed rectifier K^+ currents expressed by the SGCs

The ability of the SGCs to produce high frequency firing is only partially explained by the presence of the DTX-sensitive current. As this activity pattern necessitates very rapid and powerful repolarization, delayed-rectifier type K^+ currents must also have prominent roles in ensuring the characteristic firing properties of the SGCs. Several K^+ channel subunits can contribute to the assembly of this current; in the present work we investigated the presence of the Kv3.1b and Kv3.2 subunits. The present work provides the first immunochemical evidence for the expression of Kv3.1b subunits in the guinea pig spiral ganglion. Our results also showed that the Kv3.2 subunit is not present in these neurones.

Presence and subunit composition of HCN channels of the spiral ganglion cells

The presence of I_h has been reported in several species. Although the function of this current is not clear, it may be responsible for a certain portion of the intrinsic activity produced by type I spiral ganglion cells, it may depolarize the membrane during the hyperpolarization phase following the action potential, and it may also reduce the membrane time constant, substantially increasing, therefore, the rate of membrane potential changes produced by these cells. I_h may be involved in setting the resting membrane potential of the SGCs, and the interplay between certain low voltage-activated, DTX-sensitive K^+ currents and I_h may have prominent roles in determining the actual membrane potential and hence the responsiveness of the cells. In the present work the presence of all four known HCN-subunits was demonstrated on guinea pig SGCs.

Our immunohistochemical results showed that although almost every SGC expressed HCN-subunits, the intensity of the expression showed significant cell to cell variability even in neighbouring neurones. According to a hypothesis the heterogeneity of the I_h could be the result of the differential phosphorylation of the HCN subunits. The present results also indicate that the differential expression of the individual HCN subunits may also contribute to this phenomenon.

Beyond investigating the channel subunit expression of the individual neurones we demonstrated that the HCN1 and HCN3 subunits were differentially expressed along the axis of the cochlea: their level of expression was higher in the apical areas of the modiolus than in the basal areas.

Differences between type I and type II neurones

There are contradictory results in the literature concerning the functional differences of type I and type II cells. The results demonstrated in the present study cannot answer this question either, but the lack of marked difference in the expression patterns of the Kv and HCN subunits suggests that perhaps huge and striking differences may not be expected between the membrane properties and firing behaviours of type I and type II cells. However, the difference between the Kv1.2 expression of type I and type II cells described here is noteworthy. Although it would be tempting to suggest that the slower accommodating response and lower activation threshold of some of the type II cells is the consequence of their reduced (or lacking) Kv1.2 expression, it must be noted that several other mechanisms – not investigated in the present work – may also crucially affect the membrane parameters of these neurones. Future studies, aiming at the investigation of the significance of Kv1.2 subunits will be

required, therefore, to substantiate the hypothesis that their absence or presence may have functionally important impact on the firing properties of the type II cells.

Summary

In the present work we studied the presence and distribution of several voltage gated K^+-channels in two sections of the initial part of the auditory pathway: the cochlear nucleus and the spiral ganglion. We investigated the expression pattern of seven voltage gated K^+-subunits in the cochlear nucleus and its major projection neurones. Our results may help to predict the membrane properties of the individual types of cells. Besides investigating the channel subunit pattern we demonstrated that some Kv subunits are preferentially expressed by the various types of CN neurones, some of which could be even used as markers assisting cell identification in future studies.

Our results showed that two major cell types of the cochlear nucleus, the pyramidal and giant neurones do not form homogenous cell populations concerning their Kv expression patterns, thus it cannot be ruled out that functional/morphological subgroups may exist within these cell classes.

We also demonstrated the Kv4.2 and Kv3.4 positivity of the glomerular synapses of the CN. Since glomerular synapses were found to be Kv4.2 positive in the cerebellum as well, these findings further support the view about the common origin and close morphological similarity between the CN and the cerebellum.

The other section of the auditory pathway investigated in the present study was the spiral ganglion. We developed a new preparation for the investigation of the SGCs, that better preserved the morphology of the neurones and allowed the three dimensional analysis of the cells.

In our experiments we investigated the presence and distribution of nine Kv- and all four known HCN-subunits on guinea pig SGCs. We demonstrated, that spiral ganglion cells expressed LVA DTX-sensitive, delayed rectifier and A-type current producing voltage gated K^+-channel subunits. We also demonstrated, that all four known HCN-subunits were present on the SGCs and that the expression levels of these proteins showed significant cell to cell variability. Moreover we demonstrated that the HCN1 and HCN3 subunits were differentially expressed along the axis of the cochlea: their levels of expression were higher in the apical areas of the modiolus than in the basal areas.

Publications

Publications providing the basis of the thesis:

1. *Rusznák Z,* **Bakondi G***, Pocsai K, Pór A, Kosztka L, Pál B, Nagy D, Szucs G.*
   Voltage-gated Potassium Channel (Kv) Subunits Expressed in the Rat Cochlear
   Nucleus.
   J Histochem Cytochem. **56**(5):443-65 **IF: 2,449**

2. **Bakondi G***, Pór Á., Kovács I, Szűcs G, Rusznák Z.*
   Voltage-gated K$^+$ channel (Kv) subunit expression of the guinea pig spiral ganglion
   cells studied in a newly developed cochlear free-floating preparation
   Brain Res. **1210**: 148-162. (elektronikus formában hozzáférhető) **IF: 2,341**

3. **Bakondi G***, Pór Á., Kovács I, Szűcs G, Rusznák Z.*
   HCN subunit expression pattern of guinea pig spiral ganglion cells
   Bírálat alatt

Other publications not used in the thesis:

1. *Pocsai K, Pal B, Pap P,* **Bakondi G***, Kosztka L, Rusznak Z, Szucs G.* Rhodamine
   backfilling and confocal microscopy as a tool for the unambiguous identification of
   neuronal cell types: a study of the neurones of the rat cochlear nucleus.
   *Brain Res Bull.***71**(5):529-38. **IF: 1,684**

2. *Pocsai K, Kosztka L, Bakondi G, Gonczi M, Fodor J, Dienes B, Szentesi P, Kovacs I,
   Feniger-Barish R, Kopf E, Zharhary D, Szucs G, Csernoch L, Rusznak Z.*
   Melanoma cells exhibit strong intracellular TASK-3-specific immunopositivity in both
   tissue sections and cell culture.
   *Cell Mol Life Sci.***63**(19-20):2364-76. **IF: 4,655**

3. *Rusznák Z, Bakondi G, Kosztka L, Pocsai K, Dienes B, Fodor J, Telek A, Gönczi M,
   Szucs G, Csernoch L.*
   Mitochondrial expression of the two-pore domain TASK-3 channels in malignantly
   transformed and non-malignant human cells.
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Combined impact factor of the publications: 13,38