PhD THESES

Application of immunocytochemical, immunohistochemical and morphological methods for the investigation of the distribution and potential significance of various K^+ channel subunits

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1. INTRODUCTION

1.1. The structure and classification of K^+ channels

The activity of the cell surface K^+ channels can significantly affect the electric properties of the cells expressing them, including the actual value of the resting membrane potential, the excitability of excitable structures, the neurotransmitter release in synapses and the efficiency of synaptic transmission. According to some recent experimental results, however, besides their "classical" electrogenic functions, K^+ channels are capable of influencing such general cell functions, as differentiation, proliferation and apoptosis. Moreover, the activity of K^+ channels may also increase the tolerance of various cells to hypoxia. K^+ channels may also have important roles in certain pathological processes, demonstrated by the fact that overexpression of some K^+ channels has been reported in malignant tumours of the prostate, colon, lung, breast and skin. Convincing data support the hypothesis that K^+-channels are not merely present in these tumours, but they may have causative roles in their pathogenesis.

The molecular structure of K^+ channels is rather diverse; on the basis of the number and structure of the subunits they are composed of, they can be divided into three major superfamilies. The most numerous K^+ channel family contains the voltage-gated (or depolarization-activated) K^+ channels (Kv channels). Kv channels are composed of four subunits, each containing six transmembrane domains. Voltage-gated K^+ channels have particularly important functions in the genesis of both the repolarization and after-hyperpolarizaton following action potentials (AP), and in delaying or even preventing the firing of action potentials, thus they are essential in determining the shape, duration and frequency of APs.

The two remaining superfamilies of K^+ channels are the inward rectifier K^+ channels and the two-pore-domain (TPD) K^+ channels. Channels belonging to the latter group differ considerably from any other known potassium channels in their molecular structure, behaviour and function. Generally, the subunits that form the functional TPD K^+ channel molecules contain four transmembrane domains and two pore forming loops arranged in tandem, and the functional molecule is eventually formed by the dimerisation of the individual subunits.

One of the subgroups within the TPD channel superfamily is formed by the TASK channels (TWIK- [twin-pore domain in weak inward rectifier K^+ channels]

related, acid-sensitive K^+ channels). TASK channels are extremely sensitive to alterations of the extracellular pH: the acidification of the extracellular milieu evokes their closure. Molecular biological techniques and functional investigations revealed that the pH-sensitivity of the TASK channels is ensured (at least partially) by a histidine residue situated at the 98th position (rather close to the outer mouth of the K^+ permeable pore).

The pharmacology of the TASK channels is markedly different from that of the other K^+ channels, and it deserves special attention that certain members of the TASK family are activated by some volatile anaesthetics, resulting in the hyperpolarization, and thus reduced excitability of the neurones expressing them. This observation provides an excellent explanation of the well-known sleep-inducing and central nervous system inhibiting effects of halothane.

TASK channels have numerous physiological functions; amongst others they might have roles in rendering hypoxia sensitivity to the peripheral chemoreceptor cells, in the secretory function of the zona glomerulosa cells of the rat adrenal gland, and in the regulation of neurotransmission in the central nervous system. TASK-1 and TASK-3 channels also participate in the induction of apoptosis in some cells.

More and more experimental data support the theory that besides their physiological functions, some K^+ channels also play roles in certain pathological processes. Convincing data suggest that the activity of cell surface K^+ channels is essential in cell proliferation, and it also seems more and more obvious that it can be important in both the genesis and growth of certain human tumours. With respect of their roles in the tumourgenesis, TASK-3 channels seem to be especially important. Their effect is so significant that the gene encoding the TASK-3 channel protein (Kcnk9) is already officially considered as a protooncogene. However, the mechanism by which TASK-3 channels exert their tumourigenic functions is still unknown. According to one conception, the activation of cell surface K^+ channels hyperpolarizes the cell membrane, resulting in an increased electrochemical gradient of Ca^{2+}, leading to an increased Ca^{2+} influx through the background Ca^{2+} channels and thus having substantial effect on cell proliferation and differentiation. Another possible explanation is that the efflux of K^+ through the cell membrane decreases the intracellular K^+ concentration, which directly alters the activity of some enzymes (i.e. caspases). It is also possible that the presence of TASK-3 channels might increase the resistance of the cells against hypoxia. In experiments conducted on cultured neuroblastoma cells, the

overexpression of TASK-3 significantly increased the resistance of cells against hypoxia and serum deprivation, which can be an apparent advantage for tumour cells situated in the central, poorly vascularised region of solid tumours. However, the mechanism by which TASK-3 channels promote the survival of cells under hypoxic conditions is still unclear.

1.2. The significance, structure and main cell types of the cochlear nucleus

During the perception of sound, the primary firing pattern of the auditory system is decoded. An important and early step in this process is the distribution of AP firing patterns arriving along the fibres of the acoustic nerve amongst neuronal networks. The network properties of these circuits allow them to decode the incoming activity on the basis of the various parameters of the sound (volume, pitch, tone, sound source localisation). According to the available data, the structure of the cochlear nucleus (CN) enables its neuronal networks to perform this task. Just like in the case of any other central nervous system structure with such a complex cellular composition, several questions are to be answered regarding the cochlear nucleus: How many neuronal types can be distinguished in the nucleus? What functional properties characterise the individual neurones of the cochlear nucleus? Although the investigations carried out in the last decades were still unable to answer all these questions, a conception about the structure of the cochlear nucleus is taking shape, which seems to be relevant in the cases of several mammalian species.

It is generally accepted that the cell types of the cochlear nucleus possess different morphological and functional characteristics, synaptic connections, and membrane properties. The anteroventral part of the nucleus contains mainly bushy and stellate cells, whereas the posterior part is dominated by the octopus neurones. The dorsal part of the nucleus is composed of four distinct, concentric layers; the most superficial one is the molecular layer, containing mainly the axons of granule cells and sparsely the cell bodies of the granule cells, too. This is followed by the layer of fusiform or pyramidal cell layer, containing pyramidal, granule, cartwheel and stellate cells. In the third layer the basal dendritic trees of the pyramidal cells and some of the so-called vertical neurones are found, along with some descending fibres of the acoustic

nerve making contact with every cell that is present here. In the deepest layer of the CN the cell bodies of the giant and multipolar cells are situated.

1.3. Aims of the present work

The main questions and problems arising during the planning and execution of the present study were the followings:

1. Is it possible to describe the behaviour of a certain cell type, and is it possible to predict its firing pattern on the basis of the types and composition of Kv subunits expressed in its cell surface membrane?

1.a. Which Kv subunits are present in the bushy neurones?

1.b. Is it possible to establish an acceptable algorithm allowing the identification of enzymatically isolated bushy cells?

2. In the course of the experiments in free-floating slices aiming at the determination of the distribution of Kv subunits in the CN, it became necessary to find a technique suitable for the determination of the morphology of neurones in brain slices. For the sake of this, the following questions had to be answered:

2.a. Is it possible to selectively label the projection neurones of the cochlear nucleus?

2.b. Is it possible to confirm that Purkinje-like cells (PLCs) project into the cerebellum?

2.c. Is it possible to work out a general system that allows easier identification of the various types of the cochlear neurones?

2.d. Is it possible that neurones are misidentified without occurring to the investigator that the classification was wrong?

3. During the above experiments the demand for the validation of a novel TASK-3 specific antibody arose, which we combined with the examination of TASK-3 expression of melanoma malignum cells present in tissue sections and in cell cultures. At the beginning of the work answers for the following questions were sought:

3.a. Is the new antibody specific and applicable? Do the results obtained by its application correspond to those yielded when other TASK-3 specific antibodies are applied?

3.b. What are the optimal reaction conditions and what kind of antigen retrieval is required when paraffin-embedded tissue samples are employed?

3.c. Do the results yielded by immunocytochemical and immunohistochemical methods correspond to each other?

3.d. Are there any differences in the TASK-3 expression of benign melanocytes and malignant melanoma cells?

2. MATERIALS AND METHODS

The investigation of bushy cells was performed by using either enzymatically isolated neurones or free-floating slices. Immunochemical and whole-cell voltage-clamp experiments were applied to detect the Kv1.1-, Kv1.2-, Kv1.3-, Kv1.6-, Kv3.1b-, Kv3.2-, Kv3.4-, Kv4.2- and Kv4.3 subunit expressions. Two subunit-specific blockers were employed in the electrophysiology experiments: phrixotoxin-2 (PaTx2) is reported to be specific for K^+ channels containing Kv4.2 and/or Kv4.3 subunits and BDS-I (blood depressing substance) is known to be specific for K^+ channels consisting of Kv3.4 subunits.

In the experiments aiming at the morphological analysis of the projection neurones of the cochlear nucleus, the cell bodies of the investigated cells were labelled with tetramethylrhodamine-dextran applied to incisions corresponding to their axonal projections. This method took advantage of the fact that the dye was transported by both anterograde and retrograde axonal transports. At the end of the procedure, 50-60 μm thick slices were cut from the brain stem containing the rhodamine filled neurones, and the slices were subjected to a confocal analysis.

In the experiments aiming at the detection of the presence of TASK-3 channels and the validation of a newly developed hTASK-3 antibody, both paraffin-embedded melanoma sections and cultured melanoma cell lines were utilised. The specificity of the antibody was confirmed by Western blot technique, as well as C2C12 and HEK293 cells, transiently and stably transfected with a hTASK-3 channel encoding plasmid, respectively.

3. RESULTS

3.1. K^+ channel subunits expressed by the bushy neurones of the cochlear nucleus

Bushy cells of the cochlear nucleus have rather complex dendritic arborisation, and they are situated exclusively in the aVCN. Bushy neurones produce a rapidly adapting response (type II response) on sustained depolarization, enabling them to maintain the temporal pattern of the incoming stimuli arriving along the acoustic nerve (primary-like firing). This feature of the bushy cells seems to be particularly important in the sound source localisation. The characteristic firing pattern of bushy neurones is partly the consequence of their low membrane time constant, resulting in their ability to generate extremely rapid responses after the excitatory stimuli. In the present study we wanted to know whether the unique membrane properties of the bushy cells are explained by either the presence of certain specific K^+ channel subunits or by a unique spatial distribution of these channels.

Some of the experiments were conducted on enzymatically isolated bushy neurones. As the rat cochlear nucleus contains numerous cell types, we tried to work out a method that allows the unambiguous identification of the bushy neurones. According to our cell identification protocol, we only identified cells as bushy cells if they had a round or slightly elongated cell body of approximately 20 μm diameter, and if they had at least one, but maximum two processes. If there was only one process emerging from the cell body, it had to have a rich arborisation, as only bushy neurones possess such dendritic tree. Another prerequisite for positive identification was that cells with two processes had to possess a branching and a non-branching one.

During the investigation we performed preadsorption control experiments on a regular basis, which were always carried out simultaneously with the positive reaction. The digital images of the bushy cells identified as the results of the positive and control experiments were prepared with identical exposure time and camera gain. The application of this technique not only enabled us to confirm the positivity of the immunoreaction, but we could also perform a semiquantitative evaluation of the reaction intensity. During the evaluation of the intensity of the immunoreaction, the intensity of the preabsorbtion control reaction was considered as a reference to which the intensity of the positive reaction was compared.

The results of the immunocytochemical experiments conducted on enzymatically isolated bushy neurones showed that they express several subunits responsible for the genesis of transient K^+ currents. The Kv3.4 subunit was present both on the soma and on the processes, demonstrating a characteristic, patchy pattern. The application of the Kv4.3-specific antibody gave similar results, but the labelling mainly concentrated in and around the nucleus. We demonstrated the presence of the Kv4.2 subunit on the soma and processes as well, and found that the bushy neurones of the cochlear nucleus do not express the Kv1.4 subunit.

Bushy neurones are known to produce a dendrotoxin-sensitive current component induced by depolarisation. As the subunits sensitive to DTX are Kv1.1, Kv1.2 and Kv1.6, their presence was also tested. The presence of all three subunits was demonstrated with Kv1.1 and Kv1.6 being present primarily on the soma, whereas Kv1.2 was expressed both on the cell body and processes in a considerable amount. It also could be observed that among the three antibodies tested, the expression of Kv1.6 was the weakest.

Earlier electrophysiological experiments have pointed out that bushy neurones have a powerful repolarizing potential, which suggested that Kv subunits responsible for delayed K^+ currents strongly affect their membrane properties. In this study we examined the presence of three such subunits on bushy cells. Our results showed that the Kv3.1 subunits are the main components of the delayed K^+ channels. The Kv1.3 specific immunoreaction could also be detected, but it was somewhat weaker. The application of the Kv3.2 specific antibody yielded the least significant reaction. The experiments were extended to check for the presence and expression of TASK-1. Regardless of the age of the experimental animals tested, the TASK-1 channel was always present in the bushy cells, and the intensity of the TASK-1-specific immunoreaction continuously increased with the age of the experimental animal.

In order to test the reliability of the immunoreactions conducted on freshly isolated neurones, some of the experiments were performed on free-floating CN slices as well. As the results of the two methods were in complete accordance, both the cell identification protocol and the interpretation of the immunoreaction results proved to be correct and reliable.

Besides the morphological examinations, functional experiments were also carried out on bushy cells in isolated brain slices. In these electrophysiological experiments a holding potential of -80 mV was used, and 100 ms long depolarising

voltages were delivered to -10, +10 and +30 mV. We found that PaTX2, a known specific blocker of channels containing Kv4.2 and/or Kv4.3 subunits unambiguously decreased the transient K^+ currents. The selective inhibition of the inactivating current component was especially evident when depolarisations to -10 and +10 mV were used. When a stronger depolarisation (to +30 mV) was applied, the steady-state current component was also partially inhibited. In a different set of experiments BDS-I, a specific blocker of Kv3.4 subunits was applied. This blocker also proved to be highly effective in reducing the peak of the transient current. The combined application of PaTX2 and BDS-I in the extracellular solution strongly reduced the transient current, although it did not abolish it entirely, which suggests that besides Kv4.2/Kv4.3 and Kv3.4, other K^+ channel subunits also participate in the formation of the transient currents of the bushy neurones.

Our data show that the presence of Kv3.1 subunits, and their contribution to the general membrane properties are highly important in the function of bushy neurones and in shaping their characteristic firing pattern. This subunit is responsible for generating a delayed K^+ current, which in turn, is essential for the fast repolarization after the action potentials, and thus it enables cells to fire action potentials with high frequency. Kv3.1 subunits are mainly, though not solely, responsible for the delayed currents of bushy neurones. As bushy cells showed definite, although weaker immunopositivites for the Kv1.3 and Kv3.2 immunopositivities than for the Kv3.1, it could be concluded that these subunits also take part in the formation of delayed K^+ currents making it possible to achieve quick repolarization.

According to our data, bushy cells also express several Kv subunits that are responsible for transient, rapidly inactivating K^+ currents (formerly known as A-current). Immunocytochemical, immunohistochemical data and functional experiments carried out with the application of specific blockers to Kv3.4 and Kv4.2/4.3 subunits pointed out that these subunits all participate in the formation of the transient currents of the bushy cells; whereas the presence and contribution of Kv1.4 subunits is negligible.

Another important result of the current theses is the demonstration of TASK-1 immunopositivity of bushy cells. Although the presence of this channel in the auditory system has already been shown on mRNA level, we were the first to report on the TASK-1 protein expression by the bushy cells. The neuronal function of background K^+ channels (including the TASK-1 channels) is known to be the regulation of the resting K^+ permeability, thus these channels have a crucial impact on the input

resistance and thus the excitability of the cells. It can be assumed, therefore, that TASK-1 channels have an important role in maintaining the low input resistance, and thus the fast membrane time constant and quick response properties of the bushy cells.

Although the application of enzimatically isolated neurones had several advantages, it also had some severe drawbacks. We concluded that the only way out of this difficulty we were facing was the regular application of the free-floating slice technique, as the preservation of both the cell morphology and localisation provided significant help for the reliable cell identification. Amongst the several possibilities, we considered the application of a fluorescent dye, rhodamine, which seemed to be particularly advantageous as it is transported in both antero- and retrograde directions along the axons when introduced into a laesion made at the appropriate position of the brain stem, thus it is capable of filling up the cell bodies belonging to the severed axons, allowing the selective labelling of certain cell types of the cochlear nucleus.

3.2 Morphological analysis of the projection neurones of the cochlear nucleus after retrograde labelling with rhodamine

The morphological and functional studies aiming at the projection neurones of the cochlear nucleus have been in the focus of various studies for a long time. One of the projection neurones, termed octopus cell is situated in the pVCN. Its name reflects its characteristic appearance: these neurones have several thick dendrites which tend to protrude from one particular side of the body, resembling the shape of an octopus. The significance of this arrangement of their processes becomes evident when one takes into account that these dendrites cross the descending branches of acoustic nerve at the point where the cochlear fibres show the most significant degree of convergence, allowing the octopus cells to make contact with the highest possible number of these axons. The octopus cells have extraordinarily low input resistance, thus they have brief and very short latency postsynaptic potentials. Octopus cells, therefore, fire action potentials only if the stimuli come at the same time in a more or less synchronised fashion. On the basis of this observation octopus cells are regarded as “coincidence detectors”.

The main cells of the second layer are the pyramidal cells. They possess triangular somata, an axon as well as a basal and an apical dendritic tree. The apical dendrites intrude the most superficial, molecular layer of the nucleus, where they make several contacts with the parallel fibre network of the granule cells. The cell body and

the distal dendritic tree are located in the third layer of the DCN and they receive inputs from the descending acoustic fibres. Considering that cochlear, somatosensory and descending information from the higher centres all reach the granule cells, this double afferentation of the pyramidal cell suggests the possibility of a significant degree of integration. The same conclusion can be reached when one considers that pyramidal cells can generate rather different firing patterns. Moreover, depending on the intensity and type of the stimuli as well as the actual value of the resting membrane potential, the very same cell can also change its firing pattern.

The giant cells have the largest cell bodies in the entire cochlear nucleus complex, and they are found in both the VCN and DCN. The dendritic arborisation of the giant cells covers a huge area in the CN, often extending to 500-600 μm. This anatomical arrangement suggests that giant cells can receive and integrate information from a huge number of acoustic fibres and interneurones. When stimulated by depolarizations with suprathreshold intensity, giant cells produce large, overshooting APs that are followed by two afterhyperpolarisations.

The PLCs are found in the DCN, and they are likely to be projection neurones. As their name suggests, they are relatives of the cereballar Purkinje cells, and some authors even believe that PLCs are ectopic Purkinje cells. The soma of the PLCs faces the outer surface of the DCN, whereas the large and rich dendritic arborisation runs towards the centre of the nucleus. There is only limited amount of information available about the synaptic inputs of the PLCs, but there are data suggesting that their axons may reach the cerebellar nuclei.

Rhodamine backfilling and subsequent confocal analysis was employed to seek morphological features which may facilitate correct cell identification of the principal projection neurones of the cochlear nucleus. The backfilling of cells with rhodamine provided information about the projection pathways of the neurones tested, and allowed their isolated labelling making it possible to describe the location of the cell body within the cochlear nucleus. Moreover, the confocal analysis of the individual cells not only gave the possibility to reconstruct the three-dimensional appearances of neurones, but also enabled the investigation of the selected neurones from unusual planes and perspectives, too. One of the most important aims of the present experiments was to establish the chances of misidentification of the neurones when they are not viewed in the optimal plane.

It was established that PLCs have spherical or slightly elongated somata (diameter: 32 ± 4 μm [n=8]) and extremely rich dendritic arborisation. Moreover, it was also shown that PLCs do project to the cerebellum.

Bushy cells could usually be easily identified as they appeared in a columnar fashion amongst the intranuclear fibres of the acoustic nerve in the VCN. Using the slices prepared from the cochlear nucleus, the individual bushy cells could be reconstructed, possessing globular or slightly elongated cell bodies (diameter: 22 ± 3 μm, ranging between 18 and 25 μm; n = 22), and having two processes emerging from roughly the opposite poles of the soma.

Octopus neurones were identified as cells having rather variable appearances, although they usually had slightly elongated somata, with an average diameter of 26 ± 4 μm (ranging between 18 and 32 μm; n = 20). Most of the octopus cells investigated in the present study had three processes, but some of them possessed 2, 4 or 5 appendages.

Possibly the most easily recognisable cell type of the cochlear nucleus complex is the giant cell, having huge cell bodies and at least three processes. The average diameter of the giant cells in the present work was 37 ± 9 μm (n = 31), ranging between 27-64 μm; thus giant cells having greater than 60 μm diameter soma are not uncommon in the CN. In some cases the cell bodies of the giant cells had thin, polygonal cell body. Although the giant cells were usually easily recognisable, but their position and orientation in the cochlear slices occasionally resulted in misleading morphological features.

The pyramidal cells of the CN are rather similar to the giant cells, and the differentiation of these cells often presents severe problems. Considering the fact that pyramidal cells possess triangular cell body from which three processes emerge, giant and pyramidal cells may have very similar appearances, especially if the giant neurone has three processes (as approximately half of the giant cells had in this study). If this is the case, their location in the CN (pyramidal cells are situated in the second layer of the DCN, whereas the giant cells are scattered in both the VCN and the deep part of the DCN), as well as the diameter of the cell body (pyramidal cells are usually 20 μm in diameter, giant cells are much bigger) may help in their identification. The diameter of the cell body is a rather obvious clue helping the unambiguous classification of the two cell types. In the present work, pyramidal cells were found to be 23 ± 3 μm in diameter (n = 16), whereas giant cells possessing three processes (thus being the ones that are the

most difficult to distinguish from the pyramidal cells) were generally larger, 34 ± 7 μm (n = 15). Even more importantly, the diameters of the pyramidal and 3-processed giant neurones ranged between 18-27 μm and 27-53 μm, respectively; indicating that triangular cells with less than 25 μm somatic diameter most likely belong to the pyramidal cell population, whereas larger cells can be safely classified as giant neurones.

The confocal analysis of the individual cells not only gave the possibility to reconstruct their three-dimensional appearances, providing, therefore, a better understanding of the arrangement and relative position of their somata and processes within the nucleus, but it also allowed the investigation of the images of the cells from planes which are usually not preferred when performing morphological studies. This manipulation emphasised the plausible hypothesis that in many cases the plane selected for slicing may so substantially alter the morphological characteristics of the cells that it may eventually lead to false cell identification.

Considering the cell identification problems described in this study, it seems apparent that confocal microscopy is extremely useful in the reliable classification of the different cell types of the cochlear nucleus. Our results point out that this approach in not only suitable for the morphological identification of cochlear nucleus neurones, but they raise the possibility that revision of some earlier data might be necessary because of the likely false identification of certain cell types.

3.3 TASK-3 expression of melanoma malignum cells

The gene encoding the human TASK-3 channel *(kcnk9)* is situated on the long arm of the 8th chromosome (8q24.3); the biosynthesis of the specific mRNA is encoded by two exons. Although it is positioned in the proximity of the of a well-known protooncogene called *c-myc*, the separate amplification of the *kcnk9* was shown in several tumour tissue samples, which points out the role of TASK-3 channels in malignant transformation. TASK-3 channels are practically insensitive to the traditional K^+ channel inhibitors, but they can be effectively blocked by ruthenium red and some metal ions, such as zinc and copper. Considering that the zinc and copper concentrations of the extracellular fluid can increase significantly in some diseases and injuries of the central nervous system and in some metabolic disorders, the effects of these ions on TASK channels can be of great clinical importance.

In the frame of the current study three TASK-3 specific antibodies were examined (one of them being a recently developed monoclonal antibody) under immunocytochemical and immunohistochemical conditions. These antibodies may be applicable in a large-scale, retrospective study aiming at revealing the possible correlations between the biological, histopathological, and clinical parameters of malignant tissue samples and the distribution and intensity of their TASK-3 expression patterns.

In the first part of the study, experiments were conducted on formalin-fixed, paraffin-embedded melanoma malignum tissue samples. As the consequence of the formalin fixation, special attention was paid to the selection of the optimal antigen retrieval technique, especially in the light of our earlier observation that the TASK-3 specific antibodies tend to give false negative or weak immunoreactions on wax-embedded sections. Intense TASK-3 immunoreaction could be observed in the melanoma malignum cells of tissue sections, whereas no immunopositivity could be detected in the connective tissue. Metastatic melanoma malignum cells also proved to be TASK-3 positive. The application of all three TASK-3 antibodies resulted in strong immunopositivity with similar distribution pattern. It is important to note that although the immunopositivity occasionally seemed to be associated with the cell surface membrane, in most cases the strongest labelling was present intracellularly, often giving a definitely granular pattern. A prominent perinuclear immunopositivity was also frequently seen, and in the cases of tumourous giant cells or dividing forms strong immunopositivity was often situated between the individual nuclei. In some cases the reaction was the strongest at one pole of the nucleus. The observable processes of the melanoma cells also showed strong immunopositivity.

As the presence and function of TASK-3 channels are thought to be associated with the genesis of certain malignant tumours, we investigated whether TASK-3 immunopositivity was a unique feature of melanoma cells, distinguishing them from healthy melanocytes, or whether melanocytes also possessed noticeable TASK-3 immunopositivity. Similarly to the melanoma cells, most melanocytes also showed strong TASK-3 positivity, which often gave a distinct granular pattern. The distribution of immunoreaction was basically the same as in the melanoma sections (intense perinuclear labelling), and similarly to the melanoma sections, the nuclear polymorphism (the presence of labelled and unlabelled nuclei) was also noted in tissue sections obtained from benign naevi.

In the next phase of the investigations we wanted to test the applicability of the antibodies, and to demonstrate the TASK-3 expression of melanoma cells under immunocytochemical conditions as well. At this stage of the experiments, three established melanoma malignum cell lines were employed: one was derived from a primary cutaneous melanoma, and two from metastatic melanomas. All three TASK-3 specific antibodies tested gave strong and consistent immunopositivities on both the primary and the metastatic cell lines. Regardless of the antibody employed, the cytoplasmic immunopositivity usually concentrated on one pole of the nucleus or (in the cases of polynuclear cells) between the nuclei giving a reticular pattern; but in some cases the cell surface membrane was also labelled.

To provide further evidence of the validity of the immunoreactions, Western-blot experiments were conducted using protein samples prepared from melanoma cell cultures. In complete accordance with the results of the immunocytochemical and immunohistochemical experiments, protein samples prepared from the nuclear fraction gave a much stronger specific band than the total protein preparation, appearing at a position corresponding to the molecular weight of the human TASK-3 channel monomer. This observation confirmed that the nuclear TASK-3 immunopositivity was indeed the consequence of the unusual localisation of the protein.

Several positive control experiments were also employed in the present work. As the first step of these control reactions, immunohistochemical labelling of the rat adrenal gland was performed, because functional studies have already demonstrated the presence of TASK-3 channels in the cell membrane of the zona glomerulosa cells. In these experiments, an intense immunolabelling was seen in the zona glomerulosa, and a less powerful, but clearly present positivity was also noted in the adrenal medulla. However, neither the connective tissue encapsulating the adrenal gland, nor the other two layers of the adrenal cortex demonstrated considerable positivity. It is important to note that the nuclei of the zona glomerulosa cells showed no TASK-3 positivity at all, whereas the cell surface membrane exhibited particularly intense immunoreaction, suggesting as the TASK-3 specific antibodies employed in the present work were capable of recognising TASK-3 channels in the cell membrane. Consequently, the relatively weak surface membrane positivity of melanoma cells was not the consequence of the inadequate binding of the antibodies, but it indicated that the channel protein was present here in relatively low quantities.

In the next step, cells normally not expressing TASK-3 channels were transfected with a vector encoding the TASK-3 channel protein (both transient and stable transfection experiments were carried out), and then subjected to TASK-3 specific immunoreaction. We demonstrated that the successfully transfected cells gave strong and specific immunoreactions in both transfection systems.

Besides the positive control experiments described above, one more method was used to confirm the validity of the nuclear TASK-3 positivity of melanoma malignum cells, and to show that this unusual distribution of the TASK-3 channel protein was not an artefact. In these experiments melanoma cells were transfected with a newly constructed plasmid encoding a TASK-3-GFP (Green Fluorescent Protein) fusion protein. The successfully transfected cells could be easily recognized by their intense green fluorescence. Moreover, the distribution of the green fluorescence was similar to that observed in the immunocytochemistry experiments: it was present in the cytoplasm and in the nuclei (but it spared the nucleoli) and it concentrated in the terminal regions of the processes. This observation clearly showed that the TASK-3 channel protein can enter the nucleus of the melanoma malignum cells.

In conclusion, on the basis of our experimental data it could be concluded that all three TASK-3 specific antibodies were specific and applicable, and that the unusual immunolabelling pattern seen in melanoma malignum cells actually reflected the true distribution of the TASK-3 channels. In the light of these experimental data, it seems to be an important question why TASK-3 channels are not present in the expected quantity in the cell surface membrane of the melanoma cells. A possible explanation of this phenomenon might be that the intracellular trafficing of the channel protein requires an interaction with the 14-3-3 adapter protein. Though the mechanism by which the adapter protein can ensure the proper translocation of the TASK-3 channels into the cell surface membrane is still unclear, it is known that the lack of the proper interaction between the two proteins results in a decreased rate of translocation of TASK-3 into the cell surface membrane. It requires further examinations, however, to determine whether the inappropriate interaction between the TASK-3 channel protein and the 14-3-3 adapter protein is the consequence of the absence of 14-3-3 protein from the melanoma cells, it is due to a mutation in the binding site of the TASK-3 channel protein or it is the result of any other reason yet to be considered.

Publications providing the basis of the present theses

Full papers

1. Pál B., Pór Á., Pocsai K., Szűcs G., Rusznák Z. (2005) Voltage-gated and background K^+ channel subunits expressed by the bushy cells of the rat ventral cochlear nucleus. Hearing Res. 199: 57-70. (***IF: 1,578***)

2. Pocsai K., Kosztka L., Bakondi G., Gönczi M., Fodor J., Dienes B., Szentesi P., Kovács I., Feniger-Barish R., Kopf E., Zharhary D., Szűcs G., Csernoch L., Rusznák Z. (2006) Melanoma cells exhibit strong intracellular TASK-3-specific immunopositivity in both tissue sections and cell culture. Cell. Mol. Life Sci. 63: 2364-2376 (***IF: 4,582***)

3. Pocsai K., Pál B., Pap P., Bakondi G., Kosztka L., Rusznák Z., Szűcs G. (2007) 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. Bulletin 71: 529-538 (***IF: 2,481***)

Abstracts

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