PhD THESES

INVESTIGATION OF THE DISTRIBUTION AND SIGNIFICANCE OF TWO POTENTIALLY ONCOGENIC PROTEINS (TASK-3 AND PKC) IN HEALTHY AND PATHOLOGIC TISSUES

Dr. Ilona Kovács

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Tutor: Dr. Zoltán Nemes

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1. INTRODUCTION

1.1. K^+ channels and their significance in the cellular function – what the microelectrodes do not talk about...

1.1.1. Classification and molecular composition of the "classical" $K^{\pm}$ channels

K^+ channels do not only play decisive roles in the physiological functions of the excitable and non-excitable cells, but their significance in the genesis of various pathological functions has also been pointed out. The rather diverse functions of the K^+ channels are explained by the enormous diversity of the molecules belonging to this group of channel proteins. It has been established that depending on the number of subunits forming the functional K^+ channels as well as on the basis of the structure of the individual subunits the various types of K^+ channels (which usually possess markedly different functions) fall into major groups or superfamilies.

The voltage gated K^+ channel superfamily (Kv channels) was discovered first, and at present this group contains the largest number of K^+ channels. Although the biophysical properties, electrical characteristics and functions of the channels belonging to this group are extremely diverse, their molecular structure is roughly the same and they can be activated by membrane depolarization. The Kv channels consist of 4 individual subunits, each of them possessing 6 transmembrane domains and a single loop, which is known to be responsible for the formation of the K^+ permeable pore. As the consequence of the unique molecular composition of these channels, the Kv channels are often referred as 6TM-P channels in the literature.

Since 1949 we know that there are membranes where the K^+ permeability may increase in response to either depolarization or hyperpolarization. Specific studies pointed out that the hyperpolarization-activated channels fall into two major categories, as one can distinguish between the "classical" inward rectifier K^+ channels and the hyperpolarizatiuon-activated non-specific cationic channels.

Similarly to the members of the Kv superfamily, the inward rectifier K^+ channels (Kir) are also assembled from 4 individual subunits. These subunits possess 2 transmembrane domains and a single loop situated between them, thus these channels are frequently referred as 2TM-P channels. Kir channels have a variety of functions,

their activity may contribute to the development of the resting membrane potential (although they cannot maintain it on their own), and following the activity of the skeletal muscle they may also ensure the entry of K^+ into the sarcoplasm from the transverse tubules.

1.1.2. The twin-pore $K^{\pm}$ channels – a dogma dies

The first report about a new class of K^+ channels emerged in 1995. These K^+ channels (usually termed twin-pore-domain K^+ channels) strongly differed from any type of K^+ channel which had been known before. This molecule not only differed from the more conventional types of K^+ channels in terms of its molecular structure and organisation, but in terms of its functions and behaviour as well.

One of the most important differences that the twin-pore channel subunits possess two, rather than one pore-forming loops, arranged in a tandem fashion. Moreover, unlike the previously known K^+ channels where the production of the functional molecule requires tetramerisation, these channels form dimers. Specific experiments proved that this class of K^+ channels has several subgroups showing some degree of sequence homology. Numerous subgroups have been described within the tandem-pore domain K^+ channel family, (i.e. TWIK, TREK, TRAAK, TRESK, THIK, TALK), amongst which the so called TASK channels seem to be particularly important.

The specific experiments conducted in the past few years have demonstrated that the presence and activity of the TASK channels are essential in the genesis of the resting membrane potential of the mammalian cells, and they also contribute to the determination of the input resistance and excitability of these cells. Moreover, TASK channels are rather sensitive to the alterations of the extracellular pH, as a downward shift of the pH results in the closure of these channels. The pharmacology of the TASK channels also proved to be rather surprising, as they are resistant to the conventional K^+ channel blockers (Cs^+, Ba^{2+}, 4-aminopyridine, tetraethyl-ammonium), whereas they are blocked in the presence of some local anaesthetics (such as the lidocaine, bupivicaine). It appears to be a quite important discovery that certain volatile anaesthetics (i.e. halothane) activate some TASK channels (hitherto TASK-1 and TASK-2 channels have been shown to be activated by halothane). Considering this, it seems to be a logical theory that this newly discovered K^+ channel family may represent the long but so far unsuccessfully sought targets of the volatile anaesthetics.

Although a remarkable advance was achieved recently concerning the biophysical properties and molecular biology of the TASK channels, their exact tissue distribution and detailed functions are not very well known. One of the possible (and quite important) functions of the TASK channels is related to the functions of the peripheral chemoreceptors, as it has been demonstrated that the type I receptor cells express TASK channels. Moreover, it has also been shown that the presence of oxygen can influence the activity of the TASK channels and thus the function of the cell. However, the mechanism of the oxygen action on the TASK channels is not known yet. TASK channels play crucial roles in the regulation of the aldosterone secretion as well.

Besides the functions listed above, TASK channels participate in the regulation and/or completion of such cellular events, whose presence has not been connected to ionic channels or to the functions of the ionic channels before. It turned out that the expression and function of the TASK-1 and TASK-3 channels play important roles in the induction of apoptosis, and (somewhat controversially) the genomic amplification of the TASK-3 channels has been demonstrated in some malignant tumours affecting various human tissues (mainly in the colorectal region and breasts).

It seems to be a rather important discovery that the cell proliferation inducing effect of the functional TASK-3 channels could be demonstrated in mouse fibroblast culture. Artificial overexpression of the TASK-3 channel proved to be clearly tumorigenic, which could be (at least partially) explained by the reduced rate of apoptosis in the affected cells. Some authors raised the possibility that the expression of the TASK-3 channels could somehow increase the hypoxia tolerance of the cells, thus it could enhance the growth of the malignant tumours. It is very important to point out that the above effects occurred only in the presence of functional channels. When the cells were transfected with a mutant version of the TASK-3 channel, which was not permeable for K^+, the tumour inducing effects could not be observed. Another piece of evidence emphasising the significance of the functionality of the TASK-3 channels in the tumour induction is the experimental finding demonstrating the lack of oncogenic potential in the presence of certain drugs capable of more or less selectively inhibiting the TASK-3 channels (i.e. ruthenium red).

Unfortunately, despite the exciting (and definitely important) experimental findings concerning the oncogenic potential and possible other roles of the TASK-3 channels, our knowledge about the exact tissue localisation and distribution of the TASK-3 channels is rather limited. There are not too many data available regarding the

TASK-3 channel expression pattern of the animal tissues, and our knowledge concerning the TASK-3 expression of the human tissues is particularly superficial. As the anti-TASK-3 primary antibodies became commercially available, there is a fair chance that more experimental data would be collected in the not too distant future. However, at present there are no studies available which attempted the thorough investigation of the TASK-3 channel distribution in healthy tissue samples and in various types of malignant tumours, which tried to relate some sort of functional significance to the presence of the TASK-3 channels in the human tissues, which wanted to describe the correlation between the histological and clinical appearance of the malignant tumours and their TASK-3 expression pattern, and (last but not least) which attempted to test and characterise the commercially available antibodies and tried to complete the rather tedious (thus not particularly exciting) but very exhausting work to find the optimal conditions for the immunoreactions, including the quest for the most effective antigen retrieval technique.

1.1.3. Significance of certain $K^{\pm}$ channels in the genesis of malignant tumours

The tumorogenic role of the TASK-3 channels, explained in the previous chapter, is very significant, but not unique. There are several lines of evidence suggesting that various (sometimes voltage-gated) K^+ channels may have distinguished roles in the regulation of cell proliferation and apoptosis as well as in the development of some malignant tumours. It is known that the proliferation of human lymphocytes may be inhibited with the application of certain K^+ channel blockers, and - even more interestingly - the growth of certain malignant tumours could be reduced when K^+ channels were blocked (such data exist for malignant melanoma, small-cell lung cancer, certain types of breast and prostate cancers).

It is still not clear, however, what the actual connection is between the presence and activity of the K^+ channels and the regulation of cell apoptosis or induction of malignant tumours. Some authors believe that K^+ channels affect cell division only indirectly – on one hand via the alteration of the intracellular Ca^{2+} concentration caused by the activity of K^+ channels, and on the other hand via the cell volume changes induced by their activity. The experimental finding, however, that the activation of certain K^+ channels induced cell apoptosis, and this process could be prevented in the

presence of K^+ channel blockers but could not be altered when drugs affecting the activity of the Ca^{2+} and Cl^- channels were applied, argues against this hypothesis.

2.0. The protein kinase C enzymes and their functions

1.2.1. General characterisation of the protein kinase C isoenzymes

While the discovery of the tumorigenic potential, cell apoptosis and differentiation regulating functions of the TASK-3 channels is fairly recent, the significance of the protein kinase C (PKC) isoenzyme family in these processes has long been established. The PKC family is an important representative of the serine/threonine kinases. The PKC isoenzymes (at least 11 different enzymes are known up to now) fall into four major groups. The activity of the various members of the "classical" group (cPKCα, β_I, β_{II} és γ) requires the presence of Ca^{2+} as well as either diacyl-glycerol (DAG), or its exogenous analogue phorbol-ester. The non-Ca^{2+} dependent or "novel" PKC isoenzymes (nPKCδ, ε, η and θ) may be fully activated with phorbol-esters or DAG even in the absence of Ca^{2+}. The third group contains the "atypical" PKC isoenzymes (aPKC; ζ and λ/ι), whose activation requires neither Ca^{2+} nor phorbol-esters. The activation and structural features of the PKCμ are so different from the rest of the PKC isoenzymes that it forms a different subgroup of its own.

The molecular events following the activation of the PKC isoenzymes begin with the binding of the activator molecules to the regulatory domain, resulting in the translocation of the activated enzyme to an intracellular structure (cell membrane, Golgi complex, nuclear envelope, cytoskeletal components). At the end of the process that catalytic domain phosphorylates the enzyme-specific substrate (i.e. cytoskeletal proteins, ionic channels, receptors, transcription factors, kinases, phosphatases). To our present knowledge, permanent activation of the PKC isoenzymes does not develop, because the intracellular proteolytic enzymes cleave the activated PKC isoenzyme, thus inactivate it.

Although there is not a single cell type which would not possess at least one type of PKC isoenzyme, not all types of PKC isoenzymes are present in a particular cell type; thus the PKC isoenzymes show species-, tissue- and cell-specific distribution

pattern. The presence of PKCα, δ, ε and ζ is ubiquitous, whereas PKCγ is present almost exclusively in the brain. The PKCη is most common in the T-cells, the PKCθ seems to be the most important PKC isoenzyme of the skeletal muscle.

The PKC isoenzymes are capable of influencing a broad spectrum of physiological functions. Amongst these one may emphasise their significance in the regulation of cell proliferation, cell differentiation, apoptosis, their role in the mediator production, establishment of the electrophysiological characteristics of the cell membrane of the excitable cells, in the maintenance of the integrity and function of the central nervous system, their contribution to the defence mechanisms of the body – and the list is far not complete.

It has been known for a long time that the PKC isoenzymes show not only structural, activational and distributional heterogeneity, but their regulation and biological functions may also significantly differ from each other. Moreover, the individual types of PKC isoenzymes may not only contribute differently to a particular cell function, but they often have opposite roles. Considering this, it is not really surprising that the alterations of the expression patterns of the various PKC isoenzymes have been related to the genesis of malignant tumours affecting the keratinocytes, breasts, uterus and prostate.

A valuable piece of evidence pointing to the possible tumorigenic role of the PKC isoenzymes is the experimental finding that (after prior initiation) tumours could be induced when the tumour-promoter phorbol-esters had been injected into the skin. The complexity of the process is clearly indicated by the fact that in the absence of prior initiation PMA inhibited cell proliferation *in vitro*, reduced the expression of the early, whereas increased that of the late differentiation markers, thus it directed the cells towards terminal differentiation.

As it can be seen, the alterations of the PKC isoenzyme pattern may have significant roles in the genesis of the malignant tumours, but neither the nature of this alteration pattern, nor its connection to the tumour grade has been clarified yet.

1.1.2. Multiple roles of the PKC isoenzymes in the regulation of the proliferation and differentiation

The investigations conducted in the past few years pointed out that several types of PKC isoenzymes may have roles in the various cellular processes; and this

observation applies for their significance in the regulation of the cell proliferation and differentiation as well. It has been proved that certain cells may be forced towards differentiation by increasing the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$), and this process absolutely requires the activation of PKC. To our knowledge PKCα plays a crucial role in the regulation of this phenomenon. Specific experiments have pointed out that the overexpression of the PKCδ and η isoenzymes inhibited cell proliferation, and at the same time it stimulated cell differentiation. As the consequence, their overexpression could reduce the growth of certain artificially induced malignant tumours. It should be noted that the PKCε isoenzyme had an opposite effect.

The cPKCα seems to have a central role in the calcium- and confluency-induced terminal differentiation, as it stimulated cell apoptosis and differentiation both *in vivo* and *in vitro*, and - in the same time – it inhibited cell proliferation. The cPKCβ seems to have an opposite role (at least in the keratinocytes), as the proliferation of the cPKCβ overexpressing cells increased, whereas the apoptotic processes were inhibited thus it exhibited definite tumorogenic effects.

The nPKCε proved to be a crucially important positive regulator of the cell proliferation. The overexpression of the nPKCε resulted in pathological proliferation (hyperproliferative transformation), whereas the down-regulation of this enzyme (or the dominant overexpression of the negative mutant) inhibited cell proliferation and induced differentiation. The nPKCδ functions as the positive regulator of the cell differentiation and apoptosis, and (in parallel with these effects) it inhibits cell proliferation and tumour growth. It is a particularly interesting observation that in Ha-ras overexpression induced malignant transformation (thus in the case of the increased proliferation capacity and reduced differentiation tendency) nPKCδ disappeared from the cells.

3.0. Aims of the present work

It was a fundamental aim of the present work to investigate the presence, distribution and significance of the aforementioned potentially oncogenic proteins in healthy and tumorous tissue samples. The following problems have been specifically studied:

1. In the first step of the experiments testing of a newly developed, polyclonal anti-TASK-3 antibody was intended. Particular attention was given to the confirmation of the specificity of the antibody, and the most effective antigen retrieval method applicable on formalin-fixed, paraffin-embedded tissue sections was also specifically sought.
2. After working out the optimal conditions for the antibody, the investigation of the distribution of the TASK-3 channels was performed in the human gastrointestinal tract. The gastrointestinal tract was selected as the first subject of the present study, because we wished to describe the TASK-3 expression pattern of the malignant tumours occurring here (sadly, the number and frequency of such tumours is so high that it made the gastrointestinal tract a straightforward selection...).
3. We wished to investigate the TASK-3 expression pattern of the human pancreas. This project appeared to be especially interesting in the light of the fact that the highest concentrations of the TASK-5-specific mRNA was observed in this tissue, and the TASK-5 channels are very closely related to the TASK-3 type. As TASK-3 channels close on extracellular acidification, their presence might have indicated that they are involved in the regulation of the pancreatic hormone secretion.
4. In the presence of positive experimental data, we wanted to perform the investigation of the TASK-3 expression in the hormone producing cells of the islets of Langerhans, after completing A- and B-cell specific double labellings.
5. We wished to investigate whether malignant tumours showed any sort of difference in terms of their TASK-3 specific labelling patterns compared to the physiological tissue samples. We hoped that correlation might be found after investigating the TASK-3 expression of a high number of breast cancer cases between the TASK-3 labelling of the tumours and their histological features.
6. We wanted to check for possible differences between the TASK-3 expression patterns (intensity and/or distribution of the labelling) of the tissue samples obtained via different methods (core-biopsy, surgical intervention).
7. By investigating transitional cell bladder carcinomas, we wanted to investigate the PKC isoenzyme pattern of the bladder epithelium showing tumorous malformation. Possible correlations between the grade of the tumours and their PKC pattern were also studied.

8. We wished to see whether the PKC isoenzyme pattern could anyhow characterise the grade of the tumours, in the hope that the PKC pattern may serve as a prognostic marker.

2. MATERIALS AND METHODS

Most of the tissue samples employed in the present work were obtained during surgical interventions (with the exception of the cerebellar sample and core-biopsies) for intra- or postoperative histopathological diagnosis. After removing the tissue samples, they were either frozen or fixed in 4 % buffered formaldehyde (24 h), then they were paraffin-embedded. Some of the tissue samples obtained from the breast were removed via core-biopsy. These samples were immediately transferred to the fixative (4 % formaldehyde), unlike the tissue samples excised during surgical intervention, as these tissue blocks were kept in physiological saline for as long as 20-30 minutes prior to fixation.

The immunohistochemistry was performed on 4 μm thick histological sections, in the cases of both the frozen and paraffin-embedded samples. The ideal antigen retrieval (AR) method was sought by using human cerebellar samples as positive controls, and this AR was employed in the cases of the other tissue samples if frozen sections were not available. If frozen samples could be obtained, then the results of the immunolabelling achieved on these sections were always compared to those performed on tissue sections undergoing routine histological preparation (formalin fixation, paraffin embedding) and antigen retrieval. This protocol allowed the confirmation of the adequacy of the AR technique.

In order to confirm the specificity of the primary antibodies, always two different primary antibodies were employed in the present work, targeting different epitopes of the channel protein, situated far from each other, and the immunolabellings were compared. A further confirmation of the specificity was achieved by the regularly performed preadsorption control experiments, which never resulted in appreciable immunolabelling. Besides the immunohistochemistry, western blotting was also preformed.

Either single (visualised with DAB) or double immunolabellings (visualised with New Fuchsin and DAB) were performed. The investigations were conducted on tissue samples isolated from the gastrointestinal tract (submandibular gland, stomach, colon, pericolonic lymphatic tissue, pancreas) and on tissue specimens of the mamillary gland if both the core-biopsy and the surgically removed sample were available.

The PKC isoenzyme pattern expressed by cancers of the urinary bladder was investigated by using Western blotting. This part of the present work was conducted on

transitional cell bladder tumours isolated from 23 patients (17 male and 6 females). The tissue samples were removed by means of transurethral resection, partial bladder resection or cystectomy, control specimens were obtained during prostatectomy. Half of the excised tissue samples underwent routine histopathological preparation in order to determine the grade and stage (according to the TNM system) of the tumours. The other half of the samples was frozen in liquid nitrogen, then serial sections were prepared from the tumour by using a cryomicrotome until the lamina propria of the bladder was reached. This manoeuvre was essential to make sure that only the epithelial structures were investigated in the present study. Densitometry was employed to quantify the results of the Western blot experiments, which allowed us to determine the PKC content of the various samples thus the results obtained in the individual groups could be directly compared.

3. RESULTS

3.1 Immunhistochemical investigation of the TASK-3 channel distribution – finding the optimal conditions for the immunolabelling

When the experiments concerning the TASK-3 channels were commenced, the optimalisation of the immunoreaction was performed first, by using human cerebellar sections as positive controls. Besides determining the optimal dilution of the primary antibody, we demonstrated that an appropriate AR technique is essential if the immunolabelling was performed on formalin-fixed, paraffin-embedded tissue sections. The lack or inappropriate selection of the AR technique resulted in false-negative reactions. We also showed that the primary antibodies employed in the rest of the present work gave optimal immunolabelling if the tissue sections were exposed to heat in a slightly acidic or alkalic environment.

3.2. Immunohistochemical investigation of the TASK-3 channel distribution in the human gastrointestinal tract

When the TASK-3 immunopositivity pattern of the human gastrointestinal tissue samples were investigated, particularly strong immunolabelling of the colonic mucosa (and primarily the epithelial cells) could be observed. Strong TASK-3 positivity could also be seen in the neural elements belonging to the myenteric plexus, whereas the surrounding smooth muscle layer produced only a weak immunoreactivity which was present mainly in the nuclear-perinuclear regions of the smooth muscle cells. The results of the Western blot experiments conducted on the same tissue were in perfect agreement with these observations, resulting in an intense, thick band when homogenised tissue yielded from the colonic mucosa was employed. When the tissue lysate produced from the smooth muscle layer was used for the Western blotting, the immunoreactive band was much weaker and thinner.

Intense immunolabelling could also be observed in the gastric mucosa, with no prominent regional differences. Similarly to the situation observed in the large intestine, the smooth muscle layer of the stomach produced weak TASK-3 labelling only, which was primarily present in the proximity of the cell nuclei. The presence of the TASK-3 proteins could also be confirmed in malignant tumours arising from the

stomach and large intestine. The TASK-3 labelling was the strongest in the cytoplasm, as well as in or around the nuclei of the cancer cells. These observations were confirmed with Western blot experiments too.

Distinct and strong immunolabelling could be observed in the submandibular gland (it was the strongest in the ductal epithelium), in lymphatic tissues situated along the gastrointestinal tract and in the pancreas. In the latter structure a very interesting, clinically noteworthy distribution pattern could be revealed. Although the exocrine part of the pancreas (including the ductal epithelium) was definitely TASK-3 positive, the intensity of the immunolabelling of the islets of Langerhans was even stronger. Double immunolabelling experiments proved that the TASK-3 channels are present in both the A- and B-cells of the pancreas.

3.3. Immunohistochemical investigation of the distribution pattern of the TASK-3 channels in breast cancers

Besides investigating the TASK-3 positivity of the healthy tissue samples, the TASK-3 expression was also studied in various breast cancer specimens as well. The present study was conducted on those tissue samples where both the core-biopsy and surgical preparation were available. This part of the study was conducted in the hope that correlation could be found between the histological features, receptor pattern and prognostic factors of the breast cancers and their TASK-3-specific labelling.

Our results indicate that the TASK-3 cannels are expressed by the tissue sections prepared from breast cancers. The healthy ductal and glandular epithelium showed obvious TASK-3 positivity, and the cancer cells were also strongly labelled. Although the no correlation could be found between the grade of the tumours and their TASK-3 expression pattern, a very interesting difference could be noted between the distribution of the TASK-3 channels when the core biopsies and the surgical preparations were compared. When the core-biopsies were investigated, the TASK-3 labelling was the most prominent in the cytoplasm of the tumour cells, whereas most of the nuclei were definitely negative. However, when the tissue sample was studied which was obtained from the same area of the patient but it was excised during surgical intervention, the cytoplasmic immunolabelling was still present but the nuclei also showed rather strong and definite immunopositivity.

This difference between the TASK-3 immunopositivity pattern of the core-biopsies and surgical preparations could also be observed in the cases of benign lesions. The glandular portion of the breast proved to be strongly TASK-3 positive (regardless of the method of the excision of the tissue samples), and the immunopositivity could also be observed inside the secretory granules. The nuclear labelling, however, was always more pronounced if the tissue block was obtained during surgical intervention.

3.4. Alterations of the PKC isoenzyme pattern in malignant bladder tumours

The Western blot analysis of the tissue samples isolated from bladder cancers revealed that the bladder epithelium expresses five different types of PKC isoforms (cPKC α and β, nPKCδ and ε, as well as aPKCζ). The other members of the PKC family were not detected in the tissue lysate. Although no difference could be seen in the PKC isoenzyme patterns of the healthy epithelium and tumour samples, the quantity of the PKC isoenzymes showed marked, statistically significant, grade-dependent alterations.

We demonstrated in the present work that in G1 grade cancers only the quantity of the PKCζ increased in a statistically significant manner, the amount of the other PKC isoenzymes did not change relative to the control samples. In the cases of G2 and G3 grade tumours, however, the quantity of every PKC isoenzyme showed some sort of statistically significant alteration. In G2 and G3 cancers the amount of both the cPKCβ and nPKCδ reduced significantly compared to either the control samples or to the G1 tumours ($p < 0.05$). No statistically significant difference could be seen, however, between the G2 and G3 grade cancers.

The investigation of the remaining three isotypes revealed that their quantity definitely increased as the tumour progressed. Unlike the cPKCa and aPKCζ isoenzymes whose amount gradually increased as the tumour developed, the quantity of the nPKCε was significantly higher in G2 and G3 grade cancers than in the control samples or in the G1 tumour, but no significant difference could be observed between the G2 and G3 grade tumours.

These experimental findings suggest that the various types of PKC isoenzymes may have opposite roles in the genesis of the malignant tumours. It seems to be

particularly interesting that this kind of antagonism is present even in the cases of PKC isoenzymes which belong to the same class. It is worth noting that the quantities of the "conventional" or Ca^{2+} dependent PKCα and PKCβ, as well as the "novel" PKCδ and PKCε showed opposite changes as the tumour progressed. This finding suggests that the alterations observed here did not merely occur as the consequence of the changes of the activation mechanisms of the major classes but they were caused by modifications which could selectively affect the individual isoenzymes.

4. DISCUSSION

4.1. Immunohistochemical investigation of the distribution of the TASK-3 channels – conditions of the immunoreactions and specificity of the labelling

In the present work we performed the evaluation of the commercially available human anti-TASK-3 specific antibodies, and we worked out the optimal conditions for the immunolabelling. Our results on frozen and paraffin-embedded tissue samples pointed out that the TASK-3 channels may have significant roles even in such tissues, where the amount of TASK-3 specific mRNA had been detected in relatively small quantities. We also demonstrated that the TASK-3 channels are present in the human gastrointestinal tract, where the TASK-3 expression of the epithelial cells and neural elements seems to be particularly powerful, but the channels are present in other structures as well. Last, but not least, we showed that the TASK-3 channels are expressed in tissue samples excised from the breast, and the distribution of the TASK-3 immunolabelling pattern depends on method of the removal of the sample (whether core-biopsy or surgical intervention was the method of the sampling).

As the TASK-3 specific primary antibodies are now commercially available, this and similar projects, trying to work out the optimal conditions for the immunoreactions, and aiming at the confirmation of the specificity of the primary antibodies have great practical significance. The investigation and mapping of the oncogenic effect of the TASK-3 channels in human tissue samples require retrospective studies on formalin-fixed and paraffin-embedded samples. There is a great disadvantage of the formalin-fixation, however, as the fixative reacts with the various side-chains of the protein molecules producing methylene-bridges. However, these cross-bridges (which, in fact, are responsible for the fixative effect) not only stabilise and immobilise the protein molecules, but they may alter their structures to such an extent that their ability to react with the specific antibodies may be severely compromised (or even completely demolished). These changes may result in weak or false-negative immunolabelling. These problems are known in the everyday histopathological work, and they are dealt with by using various antigen retrieval techniques, when the tissue samples are exposed to altered pH, increased temperature or a weak proteolytic treatment is employed (or the

combination of these mechanisms) to expose the hidden epitopes of the fixed protein molecules.

Specific emphasis was laid upon the confirmation of the specificity of the reactions (thus the specificity of the primary antibodies). As knock-out animals were not available, which could have been used for the unambiguous demonstration of the specificity of the immunolabelling, two different primary antibodies were employed in the present work, targeting two different epitopes of the TASK-3 channels which situated far form each other. Our observation that the same immunolabelling pattern developed with both primary antibodies, and only intensity differences occurred, clearly pointed out that they detected the same target protein, as the probability that the two primary antibodies non-specifically recognised the same structures is truly negligible.

The results of the preadsorption control experiments and the Western blotting provided further valuable evidence arguing for the specificity of the primary antibodies.

4.2. The possible physiological functions of the TASK-3 channels in the gastrointestinal tract

The interpretation of the results of the present work cannot be complete without discussing the possible functional consequences of the TASK-3 channel distribution reported here. Our results suggest that TASK-3 channels may have important roles in the K^+ secretion of the colonic epithelium. Similarly to the large intestine, a substantial portion of the K^+ transport may be performed via the TASK-3 channels in the gastric mucosa and in the exocrine portion of the pancreas. This seems to be an especially tempting suggestion in the light of the fact that in the Cl^--secretory epithelium (such as the pancreas) the movement of K^+ provides the driving force for the Cl^- movement. The roles of certain Ca^{2+}-activated K^+ channels have been suggested in this process, but the findings of the present work suggest that TASK-3 channels may also be involved in the Cl^- secretion of the ductal epithelium in the pancreas.

One of the most interesting findings of the present work concerns the strong TASK-3 expression of the insular cells of the pancreas, which indicates that these channels may be intimately involved in the regulation of the insulin and/or glucagon secretion of the pancreas. An indirect piece of evidence supporting this hypothesis is the experimental finding found several years ago, whose explanation is still not clear. It has been found that alterations of the extracellular pH could influence the hormone

production of the B cells. As TASK channels are known to be closed by extracellular acidification, they may contribute to the depolarization of the B cells, thus they may have importance in the increased insulin secretion accompanying acidosis.

The above observations may have great clinical importance. On the basis of the oncogenic potential of the TASK-3 channels, it has been suggested that TASK-3 blockers (such as the ruthenium red) might be employed in the therapy of malignant tumours. However, the experimental findings described in this work suggest that those substances which inhibit the function and/or expression of the TASK-3 channels may have several, hitherto not considered side-effects, and they may severely compromise the physiological functions of the gastrointestinal tract and the regulation of the hormone secretion of the pancreas.

4.3. The significance of the TASK-3 channels in malignant tumours – does it matter whether core-biopsy or surgical specimen?

We found in the present experiments (in accordance with the results of other authors) that the TASK-3 channels are present in malignant tumours as well. It was demonstrated here that the TASK-3 expression of the tissue samples obtained in the forms of core biopsies is predominantly cytoplasmic, and most of the nuclei did not show immunolabelling. The intracellular localisation of the immunoreaction suggests that these channels may be present in the endoplasmic reticulum in large quantities. This phenomenon is rather similar to those described earlier in transfected COS cells and in cultured astrocytes about the distribution of TASK-1, TASK-2 and TASK-3 channels. We found it particularly noteworthy that the TASK-3 specific labelling often showed marked perinuclear distribution, suggesting that these channels may be present in the perinuclear cysternae, and perhaps they are part of them. It is still not clear, however, what the function of these channels is in the intracellular membranes. One might assume that they form a rapidly and easily accessible TASK-3 channel pool, which may be immediately transported to the desired destination when the need arises. It cannot be excluded either that these channels have definite functions even within the endoplasmic reticulum as (amongst others) they may ensure the compensatory K^+ movement accompanying Ca^{2+} efflux from the sarcoplasmic reticulum.

Unlike the core-biopsies, when the tissue samples were removed during a surgical intervention, the TASK-3 specific immunolabelling was not only confined to

the cytoplasm of the cells, but strong labelling could also be observed in the nuclear region of the cells. It is not clear, however, whether the labelling is really within the nucleus, it is caused by the intense labelling of the nuclear membrane or perhaps it reflects the strong immunoreaction of the perinuclear cysternae of the endoplasmic reticulum. As both the type of the tissue samples and the technical details of the immunolabelling are the same in the cases of the core-biopsies and surgically removed tissue samples, the most obvious methodical problems/differences/errors can be ruled out which might explain the alterations. It is important to note, however, that a significant difference can be found between the handling of the tissue samples, depending on the method of tissue excision. In the case of the core-biopsies the patients do not undergo general anaesthesia and the removed tissue samples are immediately transferred into the fixative. On the other hand, when the tissue sample is removed during a surgical intervention, the patients receive some form of general anaesthesia, and the tissue samples are kept in physiological saline for a certain period of time (sometimes as long as 20-30 min), and they are transferred into the fixative only after this period. This difference arises the possibility that the TASK-3 channels are translocated to the proximity of (or into) the nucleus as the consequence of the tissue hypoxia or acidosis (or the release of various tissue factors produced as the consequence of these effects). This observation seems to be particularly interesting in the light of the fact that it has been suggested that the presence and activity of the TASK-3 channels might increase the hypoxia tolerating ability of the cells.

4.4. Correlation between malignant urinary bladder tumours and their PKC isoenzyme pattern

One of the most interesting findings of the present work is the confirmation of the grade-dependent quantitative changes of the PKC isoenzymes of the transitional cell tumours of the bladder epithelium. The observations presented here suggest that (similarly to other tissues) the PKC isoenzymes may have central roles in the malignant transformations of the bladder epithelium. Our experimental findings indicate that the individual types of PKC isoenzymes may have opposite roles in the tumour genesis. It seems to be particularly interesting that this kind of antagonism is present even in the cases of PKC isoenzymes belonging to the same class, indicating that the alterations observed here did not occur as a mere consequence of the changes of the activation

mechanisms of the major classes but they were caused by modifications which could selectively affect the individual isoenzymes.

On the bases of the experiments presented here it seems to be a real possibility that after studying even larger number of cases, the tendencies outlined in this work may be refined. Moreover, as more experimental data will be obtained about the PKC isoenzyme pattern of the tumours, valuable data will be gained about the progression (or perhaps the prognosis) of the tumours. Moreover, it seems to be an important task to verify whether the changes in the PKC isoenzyme pattern are the cause or the consequence of the malignant transformation of the cells.

4.5. Concluding remarks...

In the present theses the tissue distribution of two potentially oncogenic proteins has been investigated along with their possible roles in the genesis of malignant tumours. Both proteins are ubiquitous with a broad variety of physiological and pathological functions. Both proteins are present in healthy tissues, thus their oncogenic potential cannot be the mere consequence of their presence but it may be caused by the possible alterations of their distribution and function. The results of the present theses pointed out that the amount of certain types of PKC isoenzymes increased, whereas the quantity of other types reduced in cells showing malignant transformation. These observations suggest that certain highly specific PKC isoenzyme agonists and/or antagonists may influence the growth of the malignant tumours, and may help in achieving tumour regression. Our results about the TASK channels also indicate, however, that (although the oncogenic potential of the TASK-3 channels is widely accepted nowadays) these molecules are present in the healthy tissues as well, thus their specific blockers (which are often regarded as potential anti-tumour drugs) may severely upset the physiological functions of the entire body. Considering these, our results support the seemingly pessimistic (but, unfortunately, quite realistic) view that much more work is required to make the tiniest advance towards the development of drugs which may effectively prevent the development (or may induce the regression) of malignant tumours without severely compromising the physiological functions of the body.

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