Purinergic signal transduction in cellular differentiation and malignant transformation: the special role of the $P2X_7$ receptor

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Debrecen, 2006

Introduction

The role of Ca^{2+} in differentiation, proliferation, apoptosis and malignant transformation

Ca^{2+} ions of the cytoplasm not only regulate short-term cell functions (for example contractility, exocytosis) but also processes determining the long-term fate of cells, such as differentiation (determination of pluri- or multipotent cells to gain some special function and the process of obtaining the required phenotype), proliferation (multiplication of cells), apoptosis (programmed cell death) and malignant transformation (the process of healthy cells becoming malignant). The multiple role of calcium is mediated by Ca^{2+}-binding proteins that alter cell functions. These Ca^{2+}-binding molecules exert their effects either without phosphorilation (for example the Ca^{2+} sensor synaptotagmin in the exocytosis of vesicules) or are able to activate (calmodulin, activator of the calmodulin dependent protein kinase and calcineurin) or are themselves kinases (protein kinase C) that alter their degree of activity depending on the saturation of their Ca^{2+}-binding sites. As a result of the activation of the primary target molecules a phosphorilation/dephosphorilation cascade leads to the modification of practically any of the signal transduction pathways of the cell and, in the end, regulating its vital functions. Therefore the alteration of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is able to promote proliferation or inhibit differentiation and apoptosis, which – solely or in combination – can result in malignant transformation.

Skeletal muscle differentiation

The precursors of the skeletal muscle cells of the trunk and the limbs are the undifferentiated mesenchymal cells of the mesoderma. The skeletal muscle cells of the limb buds migrate into the limbs after delamination and then start intense proliferation. At the same time, the process of determination starts,

resulting in myoblasts that have started differentiation towards skeletal muscle but are not terminally differentiated yet. The fusion of myoblasts gives rise to the terminally differentiated myotubes in two waves, the first one resulting in the so-called primary myotubes. During the second wave, along with the innervation of skeletal muscle, secondary myotubes are formed around the primary myotubes. Further myoblasts fuse with the myotubes, which finally leads to the formation of differentiated myofibres. The sequential events of delamination-migration-proliferation-determination-differentiation-muscle fibre formation are under tight genetic control. These processes are regulated by the activation or inhibition of transcription factors, receptors or secreted molecules, or the increase or decrease of their transcription.

De novo formation of skeletal muscle takes place during muscle growth and regeneration postnatally, too. The main source of skeletal muscle in adults is a pool of cells situated under the basal lamina of fibres. Based on their characteristics during *in vitro* culturing, and their surface and intracellular markers, two types cells can be found: muscle-derived stem cells (MDSCs) and satellite cells. MDSCs belong to the pluripotent stem cells usually found in the bone marrow and other tissues, and they participate in muscle regeneration either directly or through forming satellite cells. Satellite cells are quiescent myoblasts under the basal lamina of muscle fibres and they can differentiate into skeletal muscle much more readily than MDSCs. After the lesion of muscle they get activated and become myoblasts, then myofibres to make up for the lost fibres.

Melanomagenesis

As a result of the benign local proliferation of the melanocytes found in the stratum basale of the epidermis, the naevus tissue is formed containing modified melanocytes called naevocytes. Either naevocytes or melanocytes can suffer mutations that lead to the activation of oncogenes or the inactivation of tumor

suppressor genes, resulting finally in malignant transformation, giving rise to one of the most malignant tumor types, the malignant melanoma. According to their pigmentation melanomas can be melanotic and amelanotic, out of which the latter is generally considered more malignant, as the absence of the production of melanin indicates dedifferentiation. Since it is a frequent tumor that shows early dissemination and is highly therapy resistent, malignant melanoma has been the subject of intense research.

Purinergic signal transduction pathways

It was in 1929 that Szent-Györgyi and Drury first reported the effects of extracellular adenosin, and since then it has become clear that ATP, UTP and their derivatives, released into the extracellular space either as cotransmitters via exocytosis or during cell laesions, mediate several fast short-term as well as slow trophic effects. As examples of the former, one can think of their regulating function in thrombocyte aggregation or exocytosis, whereas their long-term effects include the regulation of proliferation, differentiation and apoptosis.

There are two groups of the cell surface, purine- and pyrimydine-activated (e.g. ATP, ADP, UTP etc.) P2 purinergic receptors: ionotropic P2X and metabotropic P2Y receptors. *P2X receptors* are formed by the homo- or heteromultimerization of the $P2X_{1-7}$ subunits, therefore there are 7 subtypes of homomeric P2X receptors. The ionic channel is made up of 3 to 6 subunits. Upon binding ATP on the extracellular binding site, a cation channel opens which increases the $[Ca^{2+}]_i$ partly from the extracellular space and partly from the intracellular stores released due to the activation calcium-induced calcium release (CICR) mechanism. Moreover, the opening of P2X receptors depolarizes the cells, which is the source of the entrance and release of further calcium. P2Y receptors are 7-transmembrane-domain, G-protein coupled metabotropic receptors. So far 8 types of mammalian P2Y receptors have been cloned, of

which the earliest-known 4 subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$) are G_q-protein coupled, lead to the release of IP_3 and thus increases the $[Ca^{2+}]_i$, whereas the recently discovered subtypes activate the adenyl cyclase via G_s-proteins ($P2Y_{11}$) or inhibit it via G_i-proteins ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) and alter the intracellular cAMP concentration.

The special characteristics and role of the $P2X_7$ receptor ($P2X_7R$)

The $P2X_7R$ is different from the other P2X receptors from several points of view and it has received special attention in the past decade following its identification as a proapoptotic receptor, which has been proven in both healthy and immortalized cell types in several *in vitro* studies. After the activation of the $P2X_7R$ a complex cellular response follows that can be divided into three major events, each of which is directly related to the induction of apoptosis. First, the $P2X_7R$ is a Ca^{2+}-permeable ionic channel that opens a large pore upon repeated or prolonged activation and becomes permeable to molecules up to 900 Da in weight. Second, after the activation of the $P2X_7R$, independently of the increase of the intracellular calcium concentration, several apoptotic enzymes become activated, such as caspase-1, caspase-3, caspase-9, IL-1β and through the latter numerous other enzymes. Third, the binding of ATP to the $P2X_7R$ leads to membrane blebbing, which is the morphological alteration of cells followed by cell death. This can be the result of the rise in $[Ca^{2+}]_i$ but it has been shown that blebbing can take place independently of changes in $[Ca^{2+}]_i$, too. The reorganization of the cytoskeleton is mediated by the long intracellular C-terminal chain that is in direct contact with several cytoskeletal and other intracellular structures. Upon binding of the agonist, these relationships are altered resulting in the characteristic morphological changes.

Aims of the experiments

The work presented here deals with the P2-receptor-mediated effects of ATP on the long-term processes of proliferation, differentiation and apoptosis. The problem was approached from two directions: we studied the *physiological* proliferation and differentiation of healthy cells and the *pathological* processes of malignant transformation as the manifestation of abnormalities in proliferation, differentiation and apoptosis.

With our experiments we aimed to answer the following groups of questions:

1. What alterations in the extracellular ATP- and KCl-depolarization-evoked cell responses take place during the differentiation of *immortalized myoblasts* into myotubes / muscle fibres? Is there any interaction between the two signalling pathways, and if yes, how? Do the differentiation-dependent alterations of the two signalling pathways differ when differentiation is induced by diverse stimuli? If yes, do both the depolarization- and the extracellular ATP-evoked responses go through different forms of development? Which purinergic receptors are responsible for these alterations? Does the $P2X_7R$ have any function in these cells?

2. What are the changes that take place in the purinergic signalling of *primary satellite cells* during the differentiation into myotubes / muscle fibres? What characterises the responses of these cells to extracellular ATP and is this response affected by the sustained depolarization of the cell? Does the $P2X_7R$ have an important role in the purinergic signalling of primary skeletal muscle cells?

3. Are *melanocytes or melanoma cells* responsive to extracellularly applied ATP? Do they differ in their responsiveness? Which purinergic receptors underlie the differences, is the $P2X_7R$ among them? What function does the

recently identified ryanodine receptor have in the Ca^{2+} homeostasis of these cells, does it interact with the purinergic signal transduction?

4. How does the pharmacological modification of the purinergic receptors of malignant melanoma affect the in vitro proliferation, differentiation and apoptosis? Can the course of the disease be modified in *animal experiments* by the application of the agonists / antagonists of the purinergic receptors?

Materials and methods

1. Cells and cell culture

1.1. Culturing the C2C12 mouse skeletal muscle cell line: Cells were cultured on DMEM medium containing 15% FCS, antibiotics and antimycotics. Differentiation was induced by DMEM containing 5% FCS and 5% HS. Clones of C2C12 cells stably overexpressing the PKCα and PKCδ isoforms were cultured in DMEM containing 15% FCS. This medium also contained 500 μg/ml G418 (Geneticin) to select the transfected cells.

1.2. Preparation of primary mouse satellite cell culture: The limbs of 2- to 8-day-old newborn mice provided the tissue that was digested in phosphate buffer containing 1 mg/ml tripsin and 0.75 mg/ml collagenase II. After centrifugation the sedimented cells were resuspended in Ham F-12 medium containing 10% FCS, antibiotics and antimycotics and spread on sterile cover slips. On the third day of culturing, the medium was switched to DMEM containing 2% FCS, 2% HS, antibiotics and antimycotics to promote differentiation.

1.3. Culturing primary human melanocytes: Healthy human melanocytes were isolated by dispase-tripsin digestion from skin samples of patients that had undergone plastic surgery. Cells were cultured in a medium of the following ingredients: 100 ml AIM-V Lymphocyte Medium, 100 ml Keratinocyte-SFM, 400 μl BPE, 1μl hrEGF, 58.44 mg L-glutamate, 5 ml FCS, 50 NE/ml penicillin,

50 μg/ml streptomicin and1.25 μg/ml Fungizone. Cells used for experiments had been passaged no more than six times.

1.4. Culturing human melanoma cell lines: We used three cell lines: the HT168-M1 and the HT199 cell lines form tumors in SCID mice and are metastatic in diverse metastasis models, whereas the WM35 line does not give metastases, although even these cells are tumorigenic in SCID mice. Cell lines were cultured in RPMI-1640 medium containing 10% FCS, 2 mM L-glutamate, antibiotics and antimycotics. Every experiment was carried out on 3- to 4-day-old cultures.

2. Following the changes in $[Ca^{2+}]_i$

2.1. Single cell fluorescent calcium measurements: Fura-2 loaded into cells was alternately excited by 340- and 380-nm light by the DeltaScan™ apparatus of Photon Technology International (PTI). Emitted light was detected at 510 nm by a photomultiplier. The ratio of the fluorescence measured after excitation at the two wavelengths ($R=F_{340}/F_{380}$) provided the intracellular calcium concentration. The parameters of the transient changes in the $[Ca^{2+}]_i$ (amplitude, latency, maximal rate of rise, time constant of decay, time to peak, full width at half maximum) were determined by a computer program written in our laboratory for this purpose (PTIana).

2.2. Calculation of the calcium flux (FL): Calcium flux was defined as the flux of calcium ions into the myoplasm from the extracellular space and the intracellular stores. FL was determined according to the formula $FL=d(Ca_{tot}+Ca_{transzp})/dt$,where Ca_{tot} denotes all the calcium in the myoplasm, and $Ca_{transzp}$ stands for the amount of calcium transported by the calcium removal mechanisms (i.e. the pumps), which is proportionate to the relative saturation of the pumps ([Ca-pumpa]/[pumpa]), where the proportionality coeffitient is the maximal rate of removal (PV_{max}). PV_{max} was determined separately for each cell examined, using an exponential fit on the descending segment of a KCl-evoked calcium transient, at least three seconds after the peak.

3. Immunodetection methods

3.1. Immunocytochemistry and immunohystochemistry: Cells were fixed in acetone for 5 min, permeabilized in 0.1% Triton-X-100 for 10 min, then blocked in 1% BSA for 30 min. Both primary and secondary antibodies were used for 1 h at room temperature, nuclei were stained with DAPI or propidium iodide. Whwn making double staining, we applied the two primary antibodies at the same time, similarly to the two secondary antibodies. Negative control were made by omitting the primary antibodies. Cells were then examined with a Zeiss LSM 510 META laser scanning confocal microscope or a Nikon Eclipse E600 fluorescent microscope. During immunohystochemical examinations, the technique was different at the following points: *fixation*: methanol, 10 min; *permeabilization*: did not take place; *washing*: PBS, 3x5 min; *blocking*: 1% BSA and 10 times diluted goat serum, at room temperature, 2 h; *primary antibody*: 60 min, 37°C; *washing*: PBS, 6x10 min; *secondary antibody*: biotin-conjugated anti-mouse IgG, 1:100, 40 min, at room temperature; *washing*: PBS, 3x5 min; *marking*: strepdavidin-FITC, 1:100, 40 min, at room temperature.

3.2. Western immunoblot: protein content of harvested and sonicated samples was determined by modified BCA protein assay. After SDS-PAGE, samples were transferred to nitrocellulose membranes. Blocking with 5% powdered milk was followed by the use of anti-purinergic receptor and anti-desmin primary and then HRPO-conjugated secondary antibodies. Immunoreactive bands were detected using the ECL Western blotting detection kit on light-sensitive films. Optical densities were measured with the Imare-Pro Plus 4.5 analysing software and normalized to the control. The cytochrome c signal of membranes was routinely checked.

4. The siRNA technique

3-day-old cells in primary culture were transfected with FITC-conjugated anti-$P2X_4$, -$P2X_5$, -$P2X_7$ and -$P2Y_1$ receptor siRNA using the cationic transfecting reagent jetSI-ENDO according to the manufacturers instructions. Experiments were carried out after 48 hours. JetSI-ENDO-treated cells, in case of which the siRNAs were omitted, were used as control. The degree of the remainder of the translation of target genes was checked by Western blot.

5. Measurement of ionic currents and membrane potential

Extracellular ATP-induced transmembrane ionic currents were detected using the patch-clamp technique in the whole-cell configuration. Patch micropipettes were made of borosilicate glass capillaries and an Axopatch 200A amplifier was used. Electric stimulation, sampling and analysis was carried out using a pCLAMP 6.0.4. software. Solutions were changed by a perfusion system of constant speed.

6. Examination of cellular proliferation and cell death

6.1. MTT cell proliferation assay: 0.5 mg/ml MTT was added to 6-day-old cells cultured in 96-well-plates at a density of 5000 cells/well. Following a 2-hour incubation at 37 °C, the concentration of formazan crystals was determined colorimetrically in a BioRad Model 550 Microplate Reader at 570 nm, according to the instructions of the manufacturer.

6.2. Flow cytometric apoptosis assay: apoptosis was induced in cultured melanoma cells using 1 μM methoxy estradiol. To test the effect of ATP, some of the cultures were treated with methoxy estradiol plus 180 μM ATP. After 48 h, cells were suspended, fixed in 70% ethanol, and then incubated for 2 hours with propidium iodide and RNAse. CyFlow folw cytometer and a Flow Max software were used to determine the apoptotic cell fraction sub-G_o/G_1.

6.3. Determination of the necrotic cell fraction – trypan blue exclusion test: Equal amounts of the cell suspension and 0.4% trypan blue solution were mixed, incubated for 3 min, then counting blue and non-blue cells in Burker's chamber showed the necrotic cell fraction.

7. Tumor growth and metastasis formation in an in vivo animal model

HT168-M1 melanoma cells were suspended, centrifuged, washed, resuspended in Medium 199, then $5x10^4$ living tumor cells in 50 µl were injected into three groups of SCID mice intrasplenically. Each group contained 6 to 8 mice. Starting from the 7th day after the inoculation, the first group received 100, the second 500 µg/kg/day $ZnSO_4$ in PBS per os for 12 days, whereas the third – control – group received the same amount of PBS only. Mice were sacrificed by Na pentobarbital overdose on day 28, then the number of liver metastases and the size of the spleen as an indicator of the size of the primary tumour was determined.

8. Statistical analysis

To compare groups of experimental data Student's t-test was used, significant difference was declared at $p<0.05$.

Results

1. Alterations in purinergic signalling during the differentiation of the C2C12 mouse myoblast cell line

1.1. Dose and age dependence of the effect of ATP and KCl-depolarization: In the C2C12 cell line, the dose-response relationship of ATP was determined by fitting a Hill function. The half effective dosis (EC_{50}) was found to be 143 µM and the Hill coeffitient (n) 1.05. When the age dependence of the maximally effective 180 µM ATP was examined, the Ca^{2+} transients displayed

monotonous increase as differentiation proceded: the amplitude of 2-day-old myoblasts was 111±25 nM (n=13), whereas in the case of 9-day-old, differentiated myotubes the amplitudes were found to be 231±35 nM (n=24). In the case of the most developed, 9-day-old myotubes biphasic transients appeared, that is, a fast rising and declining component of large amplitude was followed by a slower and smaller component.

After determining the dose-response relationship of the effect of KCl-depolarization, the maximally effective 120 mM KCl was further tested. When examining the age dependence, we found that the youngest cells did not respond to depolarization at all, but between day 2 and day 5 their responsiveness appeared, and by day 5 it reached the nearly maximal amplitude of 200 nM, and in the course of further development it hardly increased any further.

1.2. The effect of ATP and KCl in the absebce of extracellular Ca^{2+}: As a next step, we determined the proportion of the calcium transient that is caused by the entrance of extracellular calcium into the cytoplasm. Since repeated applications of ATP lead to significant desensitization (the second transient being 85, whereas the third 70% of the first transient), while repeated depolarizing pulses did not cause desensitization, the amplitudes of second transients normalized to the first control transient were compared in the presence and in the absence of extracellular calcium. In the case of ATP, the removal of extracellular calcium decreased the relative amplitude from 86±6% to 76±4%, while the same manipulation resulted in a drop from 113±10% to 87±2% in the case of KCl-depolarization.

1.3. Alterations in the ATP- and KCl-induced responses during PKCα- and PKCδ-overexpression induced differentiation: Besides the proper modification of the serum content of the culture medium (SID, serum induced differentiation), differentiation can also be induced by the overexpression of certain PKC isoforms (PKCID, PKC induced differentiation). The next step in our experiments was to compare the alterations experienced during SID and

PKCID. MTT proliferation tests and the determination of the expression level of the differentiation marker desmin showed that PKCα induces differentiation and inhibits proliferation, while PKCδ behaves inversely, that is, inhibits differentiation and promotes proliferation.

2-day-old myoblasts overexpressing one of the other of the above-mentioned PKC isoforms were stimulated with 180 μM ATP. We found that the $[Ca^{2+}]_i$ of control and PKCδ-overexpressing cells hardly changed, but the PKCα-overexpressing cells produced large calcium transients even at this early stage of differentiation. Similar experiments on the same cell types at day 9 of culturing showed that in control cells differentiation had taken place and calcium transients appeared, but the responsiveness of PKC-overexpressing cells did not change as compared to day 2. By the end of PKCID, transients failed to become biphasic, which was typical in cells that have completed the SID programme.

Examining the effects of KCl-depolarization we found that the voltage-dependent responses of PKCδ-overexpressing clones failed to improve further after reaching some minimal responsiveness, whereas in PKCα-overexpressing, early-differentiating clones, voltage-dependent responses were missing at day 2, when purinergic signals were already present at their maximum, but were present in 9-day-old cells. These transients were similar both in kinetic parameters and in amplitude to depolarization-evoked responses of cells that had undergone SID.

1.4. Expression pattern changes during PKCID and SID: The receptor expression pattern of 2-day-old, undifferentiated cells was compared with cells that had undergone SID and PKCID. On control myoblasts, $P2X_4$, $P2X_7$ and $P2Y_2$ receptors were found, the $P2Y_4$ receptor was missing from these cells. As differentiation proceded, the expression of the $P2X_4$ receptor decreased significantly, below 10% of the original value, independent of the stimulus that had induced differentiation. The expression of the $P2X_7$ receptor remained practically unchanged in PKCα-overexpressing cells, but increased four-fold

during SID. The level of the $P2Y_2R$ increased little in the first 2 days of PKCID but decreased to 25% by the end of SID. The most obvious, not only quantitative but also qualitative change could be observed in the case of the $P2Y_4$ receptor, as this receptor was completely missing from undifferentiated 2-day-old myoblasts but its strong expression could be detected at the end of SID.

2. Alterations of purinergic signalling during the differentiation of primary cultured mouse skeletal muscle cells

2.1. Age dependence of the effect of ATP: According to the results of our patch-clamp experiments, 40-s-long applications of ATP failed to cause desensitization in the ATP-evoked current (I_{ATP}) either in cells with 5 to 10 nuclei, or in cells with more than 10 nuclei. At the same time, the amplitude of the inward I_{ATP} was significantly different: the average of the current density measured on cells with 5 to 10 nuclei proved to be greater than 1.5 pA pF^{-1}, while this value on cells with more than 10 nuclei was smaller than 0.25 pA pF^{-1}. The resting membrane potential of more differentiated cells was more negative (-80 mV, as compared to -60 mV in cells with 5-10 nuclei), and ATP caused much smaller depolarization.

According to fluorescent calcium measurements and FL calculations, the $[Ca^{2+}]_i$ of the undifferentiated cells (with less than 5 nuclei) became stabilized on a higher level, then it returned to the former resting level. In the background of these changes we found a fast rising and then fast declining first (FL_{peak}), and a sustained second ($FL_{plateau}$) component. In cells with 5 to 10 nuclei we found similar changes, but FL_{peak} was steeper and narrower. In cells with more than 10 nuclei, neither $[Ca^{2+}]_i$, nor FL displayed the early rising phase, upon the addition of ATP only the delayed phase was present.

2.2. The effect of ATP in the absence of extracellular Ca^{2+}, and on depolarized cells: Following a control ATP stimulus, cells were permanently depolarized with KCl, then ATP was readministered into the extracellular

solution. On cells with less than 5 nuclei, depolarization itself failed to evoke a calcium transient, but the ATP application afterwards resulted in a decreased calcium response. This, however, was not statistically different from the decrease caused by desensitization. Examining the undelying changes in FL we found that the fast component was practically abolished by the depolarization, while the delayed slow component was not significantly different from what was seen in desensitized cells. Similar results were seen on more differentiated cells with more than 5 nuclei, but the drop in the amplitude of the calcium transient was significant in this case.

When we removed extracellular calcium instead of depolarizing the cell, we detected similar phenomena: the amplitude of the calcium transient was decreased and it did not cause significant change in the sustained phase of the FL. FL_{peak} was much less affected in either case, in the case of cells with 5 to 10 nuclei we did not see any significant change as compared to experiments carried out in extracellular solutions containing calcium.

2.3. Purinergic receptor subtypes shaping the biphasic calcium transient: Immunocytochemical staining indicated that $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_4$ receptors are present on our cells. The $P2X_2$ receptor, whose presence had been shown in developing rat skeletal muscle, was missing from the mouse myoblasts we used.

10 µM suramin incompletely, whereas 300 µM suramin completely blocked the ATP-evoked ionic currents, proving that the effect of ATP causing the changes in current and membrane potential is mediated by P2X receptors. Suramin also inhibited the increases in $[Ca^{2+}]_i$ and FL in a dose-dependent manner, but it did not abolish the calcium transient completely. As the only P2Y receptor that is resistent to suramin is the $P2Y_4$, this observation proved the presence of this rceptor subtype on our cells.

After the examination of suramin that is able to block nearly all the P2 receptors, we decided to test the specific agonists of certain receptor subtypes:

BzATP for $P2X_7$, 2-MeS-ADP for $P2Y_1$ and UTP for the $P2Y_{2/4}$ receptors. BzATP was found to be similar to ATP, as far as the effect on the two phases of FL is concerned. In the experiments with 2-MeS-ADP and UTP calcium transients and the FL_{peak} became significantly smaller, while the drop in the amplitude of the $FL_{plateau}$ was not statistically significant, revealing that the metabotropic P2Y receptors should be responsible for the second phase of the transient.

Cells were then transfected with siRNA against the $P2X_4$, $P2X_5$, $P2X_7$ and $P2Y_1$ receptors. The suppression of the $P2X_4$ receptor was unsuccessful, while the $P2X_5$ receptor was only slightly suppressed. As opposed to this, the level of the $P2Y_1$ receptor was decreased by nearly 40% and that of the $P2X_7$ receptor was decreased by 56%. The most significantly suppressed $P2X_7$ receptor, that we thought to be one of the most important subtypes in these cells based on the previous experiments, proved to be very important. The amplitude of the calcium transients and FL_{peak} dropped to one fourth of the formerly seen level, and even the amplitude of the sustained phase vas halved.

2.4. Further examination of the role of the $P2X_7R$ in ATP-evoked ionic currents: The next set of experiments aimed to determine the contribution of the $P2X_7R$ to ATP-evoked and P2X mediated ionic currents. Cells were treated with ATP of lower than saturating concentrations and then the same concentration of the specific $P2X_7R$ agonist BzATP was used, which resulted in a current larger than the control. When cells were pretreated with the $P2X_7R$ specific antagonist oxidized ATP or were formerly transfected with anti $P2X_7R$ siRNA, the same amount of ATP induced significabtly smaller ionic currents and the second BzATP stimulus evoked a response that was even smaller than the decreased I_{ATP}. The currents evoked by BzATP in control cellas and in the cases when the $P2X_7R$ was selectively inactivated were significantly different, showing a 7- to 10-fold difference.

3. Special characteristics of the purinergic signalling of malignant melanoma cells

3.1. Appearance of purinergic signalling in the course of melanomagenesis: All attempts carried out by us proved that melanocytes do not respond to extracellular ATP by changing their intracellular calcium concentration. The same concentration of ATP, on the other hand, induced calcium transients that could be repeatedly evoked without desensitization. Most of the cells even became more and more sensitive to the stimuli and produced ever greater transients. The second transient was, on average, double the first one.

3.2. Identification of pharmacologically normal $P2X_7$ receptors in melanoma cells: The examination of three melanoma cell lines revealed that the only purinergic receptor convincingly positive in all cell lines was the $P2X_7R$. Comparing the expression of the receptor in melanocytes and melanoma cells we found that in the case of melanoma cells the staining was much more intense and the summation picture showed extranuclear positivity in the latter. Confocal microscopy demonstrated that the $P2X_7R$ was also localized in the plasma membrane. (The cause and significance of the nuclear positivity seen in melanocytes and melanoma cells is as yet unknown.) Finally, overexpression was also proved by Western blot. Testing the specific agonist (BzATP) and antagonists (Brilliant Blue G, Zn^{2+}) of the receptor and recording the phenomenon of sensitization, we managed to identify the receptor also functionally as normal $P2X_7R$, at least as far as the effect on $[Ca^{2+}]_i$ is concerned.

3.3. The effect of the $P2X_7R$ of melanoma cells on apoptosis, necrosis and metastasis formation: To test the effect of the receptor on apoptosis, apoptosis was induced in melanoma cell cultures by 2-methoxy estradiol (2ME) and the rate of apoptotic cells was determined after 48 h. Then the effect of ATP on cell death was checked. Our results clearly demonstrated that ATP was antiapoptotic in these cells, as opposed to what was formerly known about this receptor: 2ME

induced apoptosis in more than 50% of the cells and this apoptotic rate was halved by ATP. At the same time, the antiapoptotic effect of ATP was eliminated by the $P2X_7R$ antagonist BBG, while the potent $P2X_7R$ antagonist $ZnSO_4$, if applied alone, promoted apoptosis and necrosis in a dose dependent manner. To see if $ZnSO_4$ was able to exert anti-tumour effect in an *in vivo* animal model, we injected melanoma cells into the spleen of SCID mice and measured the weight of the primary, as well as the number of the metastatic tumours. $ZnSO_4$ applied *per os* did not affect the size of the primary tumour but decreased the number of liver metastases dose dependently.

3.4. Ryanodine receptor overexpression, dysfunction and RyR-$P2X_7R$ interaction in melanoma: As recently the overexpression of the type 2 ryanodine receptor has been demonstrated, and, furthermore, the interaction of the RyR2 and purinergic signalling has been proposed in the literature, we investigated whether the RyR and the $P2X_7R$ would interact in our melanoma cell lines. We found the RyR in the endoplasmic reticulum and, in part of the cells, in the cytoplasm membrane. Ryanodine and caffeine proved to be ineffective in modifying $[Ca^{2+}]_i$ in any way. Nevertheless, 10 μM ryanodine significantly inhibited the ATP-evoked calcium transients. As ryanodine is not known to directly interact with any of the purinergic receptors, these results definitely suggest that the RyR2 and the $P2X_7R$ are in some kind of interaction.

Discussion

1. Alterations in the purinergic signalization of C2C12 cells during differentiation and proliferation

Our experiments on the C2C12 mouse skeletal muscle cell line have shown, that during differentiation, the responsiveness of muscle cells to extracellular ATP increases gradually. Moreover, the most mature cells show a qualitative change in ATP-evoked calcium response: the calcium transient becomes

biphasic. In the case of PKCα–induced differentiation, the purinergic response resembles the late, slow component of the biphasic response seen with SID, however, the first, fast component is missing. A strong correlation with the parameters of depolarization evoked transients indicate that the administration of ATP to mature cells could also raise $[Ca^{2+}]_i$ levels via activation of voltage-dependent processes during the first phase of the transient. This mechanism was further supported by our detailed analysis of changes in the calcium levels of primary cultures, slightly different in kinetics, but similar in morphology, using voltage and membrane potential measurements. The absence of the fast voltage dependent first component during the early stages of development is most probably due to the number of $P2X_7$ receptors, which, up to day 9, is too small to create an inward flow sufficient to depolarize the cell to the threshold of the action potential. Immunocytochemical and Western blot analyses supported this hypothesis: mature cells after SID expressed almost four times more $P2X_7$ receptors.

Studying the changes during PKCID, we found, that while the depolarization-evoked response showed none, the ATP-evoked response showed two major changes as compared to SID. First, a purinergic response similar in amplitude and kinetics to the second component of the biphasic response described at the end stage of SID appeared as early as culture day 2. Second, the fast phase did not appear on day 9. Apparently, PKCα overexpression enabled a fast development in purinergic signal transduction well before the appearance of the voltage-dependent response. However, as the apparatus capable of sensing and reacting to voltage changes developed, no links formed betweeen the purinergic and voltage directed signalization.

Theoretically, excessive intracellular PKCα can decrease the purinergic signal in two ways: by inhibiting either the expression, or the function of purinergic receptors. Unlike the development during SID, purinergic receptor distribution in PKCα-overexpressing cells showed hardly any alterations in line

with the observation that ATP-evoked responses were already maximal. The lack of an increase in $P2X_7$ receptor expression could in fact underlie the absence of the action potential. Another possibility theoretically is that PKCα modifies the function of P2 receptors by phosphorilation, since every P2X receptor has a PKC phosphorilation site at its intraellular domain.

Overproduction of PKCδ in myoblasts increased cell proliferation and inhibited cell differentiation. Even mature cultures showed no response to ATP, which proves that the acceleration of proliferation was independent of purinergic signallization.

2. Purinergic signalization of mouse primary cultured skeletal muscle cells during differentiation and proliferation

In the case of the largest myotubes and muscle fibres, the fast first phase of the calcium response disappears. This phenomenon lead us to the conclusion that ATP is only transiently neccessary in muscle differentiation and regeneration, its role as a signal transducing molecule rapidly decreases at the end of the process. Presumably, these changes occur parallel to the decrease in extracellular ATP concentrations that originated from ruptured cells in case of muscle injury.

The role of voltage dependent channels in the development of the early, fast phase of the ATP-evoked calcium transients is supported by the finding, that depolarized cells (that is, cells with inactivated ion channels) displayed suppressed FL_{peak} , whereas $FL_{plateau}$ showed no changes at all. Our voltage measurements proved that ATP causes a significant inward flow of cations through the plasma membrane of these cells. This induces a change in the membrane potential (depolarization to -40 mV) large enough to activate voltage-dependent channels, resulting in an action potential. In the case of the most mature myotubes and skeletal muscle fibres, along with the changes in voltage-dependent and purinergic processes. The resting membrane potential becomes

more and more negative. Together with the reduction of the ionic currents at the late steges of development, this results in the inability of ATP to evoke an action potential and thus in the disappearance of theearly phase of the calcium transient.

Voltage-dependent mechanisms, due to their relatively fast inactivation, cannot play a role in the late component of the ATP-evoked calcium transients, and the the $FL_{plateau}$. The role of the P2X receptors, and most importantly, the non-desensitizing $P2X_7R$ creating the $FL_{plateau}$ cannot be excluded. However, since the $FL_{plateau}$ showed no significant decrease in the absence of extracellular Ca^{2+} ions (which is not true in the case of FL_{peak}), whereas in the presence of 300 μM suramin, after administration of ATP, the slow phase of Ca^{2+} transient could still be detected even when I_{ATP} was zero, we can conclude, that the latter component is mainly caused by the activation of P2Y receptors.

Using immunocytochemistry, we detected $P2X_4$, $P2X_5$, and $P2X_7$ ionotropic, and $P2Y_1$ and $P2Y_4$ metabotropic receptor subtypes in our cultures. It is of great significance, that former works do not mention $P2X_4$ and $P2X_7$ receptors as receptor subtypes typical of skeletal muscle. Using current measurements, detection of $[Ca^{2+}]_i$, analysis of the early and late phases of the Ca^{2+} flux, in the presence and absence of the specific agonist (BzATP) and antagonist (o-ATP) of $P2X_7$, with and without its suppression with siRNA, we have proven, that the $P2X_7$ receptor undoubtedly plays the most important role in ATP-induced depolarization during the differentiation of primary cultured myoblasts into myotubes. The end of the development or regeneration process is marked by the disappearance of that Ca^{2+} transient component, which the $P2X_7$ receptor accounts for.

3.Purinergic signalization in malignant melanoma

Our studies showed that during melanomagenesis, melanocytes previously irresponsive to ATP become sensitive to purinergic stimuli and express the $P2X_7$ receptor in large quantities. Regarding the calcium response to ATP, these

receptors were found to be normal in every aspect. Yet, instead of their normal proapoptotic effect, the receptors observed on melanoma cells inhibited apoptosis and necrosis in our experiments. Thus, we concluded, that something must influence the apoptotic function of these receptors. Searching for a possible modulator, our attention was drawn to RyR, which also had been found overexpressed in melanoma cells. We demonstrated, that in melanoma cells, RyR is found in the endoplasmic reticulum, and probably in the plasma membrane as well. The latter is a rather unusual localization for this receptor, but similar localization has been described in cardiac muscle cells, smooth muscle cells and osteoclasts. Neither in melanocytes, nor in melanoma cells was the administration of the known agonists of the RyR followed by a change in the intracellular calcium concentration. Dysfunctional, overexpressed receptors are not a rarity in tumour cells, so we most probably deal with an abnormally functioning channel.

After the independent analysis of the functioning, overexpressed $P2X_7R$, and the dysfunctioning RyR2, we administered molecules effecting these two receptors simultaneously, and found, that in the presence of ryanodine, the amplitude of ATP-induced calcium response decreased. Since the direct action of ryanodine on the $P2X_7$ receptor is highly unlikely, a possible scenario is that ryanodine, binding to the RyR decreases the ATP-induced calcium entry through the $P2X_7$ receptor. The question is: how?

The response is difficult, since both proteins play central roles in forming two large heterogenic receptor structure, which are in complex interactions with their molecular neighbourhoods. Because of the complex signal transduction in the case of one, and the uncertainty of the subcellular localization of the other, it is difficult even to imagine how the RyR and the $P2X_7R$ interact with each other. The two basic possibilities are direct mechanical connection and indirect interaction. On the one hand, we can assume that the two receptor conglomerates are in close vicinity if we accept the hypothesis that the

overexpressed RyR is also found in the plasma membrane. On the other hand, however, we cannot exclude that the RyR in the ER gets in touch with the $P2X_7R$ of the cytoplasma membrane, either via the cytoskeleton, or directly (this kind of direct interaction with a surface membrane protein is characteristic of the RyR). In any of the above listed possibilities, we can imagine that when ryanodine binds to its receptor, the conformation of RyR and thus the $P2X_7R$ is altered, which finally leads to the decrease in the inward flow of calcium.

If we accept the interaction of these two receptors in melanoma, the following sequence of events can be envisioned: a potentially damaging noxa (e.g. UV irradiation) reaches the cell, which – among other effects – activates oncogenes or inactivates tumour suppressor genes. This leads to the activation of the apoptosis program of the cell, and as its part, the $P2X_7R$ is expressed and the cell dies. In those cases, however, when another mutation causes the RyR to be overexpressed by chance, signal transduction through the $P2X_7R$ is modified and the RyR-$P2X_7R$ functional complex exerts antiapoptotic effect, meaning the survival of the damaged cell and promoting the tumour. The antiapoptotic effect can come about in at least two ways. First, it is possible that the overexpressed (and possibly mutant or abnormally localized) RyR inhibits the $P2X_7R$ mediated calcium response even in its resting form, without binding any agonist or antagonist, thus the agonists of the $P2X_7R$ cause the $[Ca^{2+}]_i$ rise to a level that is lower than that leading to apoptosis, therefore alternative, antiapoptotic signaling pathways are activated. Second, the RyR-$P2X_7R$ interaction could inhibit other $P2X_7R$ functions (e.g. cytoskeletal reorganization or production of interleukins) that are necessary for causing cell death.

Finally, it is important to pinpoint the molecules that could activate the antiapoptotic functions of the RyR and the $P2X_7R$ *in vivo*, as these are necessary for cell death to take place in the body, too. In the case of the $P2X_7R$ the most obvious candidate is ATP, which can get into the extracellular space in two ways: either as cotransmitter from nerve terminals, which is not likely in

melanoma cells and melanocytes as they are not innervated (but is a real possibility in the course of skeletal muscle differentiation when muscle becomes innervated and neurotransmission begins), or from decomposing melanocytes and melanoma cells. This is certainly possible in melanoma, since it is a fast growing tumour, neovascurarization is always lagging behind and therefore ischaemia and necrosis is always present. As far as the RyR is concerned, we can suppose that it can affect the $P2X_7R$ constantly without binding an agonist or antagonist (RyR-$P2X_7R$ functional complex). If, however, it can only interact with the purinergic signalling pathway after binding some agent, the possible candidates are Ca^{2+} ions and ATP as its physiological *in vivo* regulators, even though we do not know how this regulation might take place.

Summary

The theses of this Ph.D. dissertation summarise the results of our research into the alterations observed during the differentiation of skeletal muscle cells and the malignant transformation of human melanocytes.

We have shown that when C2C12 mouse myoblasts and primary cultured mouse skeletal muscle cells are induced to differentiate by the appropriate alteration of the serum content of their culturing medium, the responsiveness of cells to extracellular ATP increases and the Ca^{2+} transients as well as the underlying Ca^{2+} fluxes become biphasic. The early peak component is initiated by the opening of ionotropic P2X receptors, especially the $P2X_7R$, followed by inward ionic currents, depolarization of the cell, initiation of an action potential and thus the activation of voltage-gated processes. This phase is sensitive to the removal of extracellular Ca^{2+} ions, to preceding and sustained membrane depolarization and to the inhibition of P2X receptors. Its kinetic parameters resemble those of the depolarization-induced Ca^{2+} transients, and it is rapidly terminated as voltage-gated channels inactivate. On C2C12 cells this early peak component appears only at the most differentiated stage, but if differentiation is induced by the overexpression of PKCα, the early phase fails to appear even in these cells (whereas the delayed phase can be detected with maximal amplitude early in differentiation). In the latter group of cells, the increase in the expression of $P2X_7R$ is also lacking and the responses of cells resemble the transients of the ones that have undergone transfection with anti-$P2X_7R$ siRNA displaying a suppressed early phase, which underlines the importance of the $P2X_7$ subtype during the peak component. In the case of primary cultured skeletal muscle cells, the biphasic shape of the Ca^{2+} transient appears already at an early stage, but, as a sign of the involution of purinergic signalling, the large peak disappears in the most developed myofibres and only the sustained phase can be detected. The latter component of the transient is produced by the release

of Ca^{2+} from the SR following the activation of metabotropic P2 receptors ($P2Y_2R$ and $P2Y_4R$ on C2C12, while $P2Y_1R$ and $P2Y_4R$ on primary myotubes).

As compared to control healthy melanocytes, ATP sensitivity appeared in each of our melanoma cell lines as a gain of new function. Our experiments have shown that all the three melanoma cell lines express the $P2X_7R$, which, localised in the cytoplasma membrane and having the typical pharmacological characteristics of $P2X_7$ receptors, is responsible for the ATP-evoked Ca^{2+} transients. Unlike in other tissues, however, the activation of the receptor exerts antiapoptotic effect, while its inhibition promotes apoptosis and necrosis *in vitro* and inhibits metastasis formation *in vivo*. The altered function of $P2X_7R$ can be related to the overexpression of the ryanodine receptor in melanoma cells. We have demonstrated that RyR is localised in the ER as well as in the cytoplasma membrane. Its well-known agonists are unable to activate the receptor, but ryanodine significantly decreased the amplitude of the $P2X_7R$-mediated Ca^{2+} transients. It cannot be excluded that the pathologically overexpressed RyR modifies the function of the $P2X_7R$ in a way that the latter becomes a protective factor for the cell, thus leading to malignant transformation.

The results of the work discussed in this dissertation prove that diverse cell types make use of the purinergic signalling pathway as the regulator of processes critical in the determination of the fate of the cell, such as differentiation to a specialised function or avoiding apoptosis. We have also demonstrated that the $P2X_7$ receptor subtype is a key player in directing cells between proliferation and differentiation, as well as apoptosis and survival.

Publications

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