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Proliferation of retinal glial (Müller) cells: Role of P2 receptors and potassium channels*

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Abstract

Müller glial cells of the vertebrate retina express purinergic P2 receptors; activation of these receptors causes an increase of the intracellular free calcium concentration and a subsequent activation of calcium-dependent potassium channels in the plasma membranes. The expression of these receptors varies among the animal species. Müller cells of all species investigated so far express metabotropic P2Y receptors while human Müller cells additionally express ionotropic $P2X_7$ receptors. The expression level of functional purinergic receptors in Müller cells of the rabbit retina is dependent on the developmental stage. At the early postnatal stage, the vast majority of immature radial glial cells show calcium responses upon exposure to extracellular ATP whereas in the adult retina, only a minority of Müller cells shows such responses. However, in experimental proliferative retinal diseases, the ATP responsiveness increases significantly in correlation to alterations of the potassium conductances of the membrane. A similar disease-dependent up-regulation was found in human Müller cells, in respect to the expression of functional $P2X_7$ receptors. Purinergic receptors are suggested to be implicated in the regulation of both retinal precursor cell proliferation in the developing retina and reactive Müller cell proliferation in the diseased adult retina.

I. Purinergic receptors on Müller cells

Since the first description on Müller cells of the tiger salamander retina by Keirstead and Miller [1], it becomes more and more obvious that adenosine 5'-triphosphate (ATP) is one of the major extracellular signaling molecules affecting Müller cell physiology. In the mature mammalian retina, ATP is released via extracellular calcium-dependent exocytosis, putatively from neuronal presynapses [2]. Another possible major source of extracellular ATP in the retina are glial cells. After different kinds of stimuli which induce calcium waves within the retinal glial cell network (particularly, after mechanical stress), ATP is released into the extracellular space [3]. The subsequent ATP-evoked autocrine and paracrine stimulation of purinergic receptors on glial cells is thought to be one main mechanism for mediating extraneuronal long-range calcium signaling in the retina [4]. These intercellular glial calcium waves alter the spontaneous and light-evoked activity of retinal ganglion cells [5].

Generally, extracellular ATP may activate two different families of purinergic receptors: G protein-coupled P2Y receptors and P2X receptors which represent ligand-gated ion channels [6] Müller cells may express receptor subtypes of both the metabotropic and ionotropic receptor families; however, the expression patterns of functional P2

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receptors on Müller cells are apparently species-dependent. Human Müller cells express both P2Y and $P2X_7$ receptors [7-9]. The activation of P2Y receptors causes transient release of calcium ions from inositol 1,4,5-triphosphate (IP_3)-gated intracellular stores [8] while activation of $P2X_7$ receptors opens calcium-permeable receptor channels in the Müller cell membrane [9]. Both events result in an increase of the intracellular free calcium concentration. The calcium increase has two consequences on the plasma membrane conductance of human Müller cells: first, they activate calcium-dependent potassium channels of big conductance (BK channels) which is reflected by a current increase at positive membrane potentials in whole-cell patch-clamp records (Fig. 1A). Second, in a subpopulation of human Müller cells, non-specific cation channels open in response to the intracellular calcium increase after P2Y receptor activation. In whole-cell records, this is reflected by current increases at negative membrane potentials (Fig. 1B). Likely, these current increases are caused by opening of calcium-activated, calcium-permeable cation channels which were firstly described to be present in cultured human Müller cells [10].

In contrast to human Müller cells, Müller cells from The rabbit retina apparently express only P2Y receptors [11]. Activation of these receptors by extracellular nucleotides transiently increases BK currents but does not cause any opening of cation channels (Fig. 1C). A second difference between human and rabbit Müller cells is the percentage of cells that express functional P2 receptors. Almost all Müller cells from human retinae which were investigated so far (electrophysiologically and by calcium imaging) expressed functional P2 receptors, whereas only a minority of investigated Müller cells from the adult healthy rabbit retina (approximately 10%) showed current or calcium responses to extracellular ATP (Fig. 2E) [11,12]. Similarly, in Müller cells from the rat retina, only 5 to 24% of the cells investigated (depending on the age of the animals) showed calcium responses to extracellular ATP [13], likely via activation of $P2Y_1$ receptors [14]. On the other hand, cation currents which would reflect P2X receptor activation, were never observed in Müller cells of the rat [15]. This expression pattern of functional P2 receptors is very similar to that observed in murine Müller cells where only a small subpopulation of cells showed calcium responses to extra-

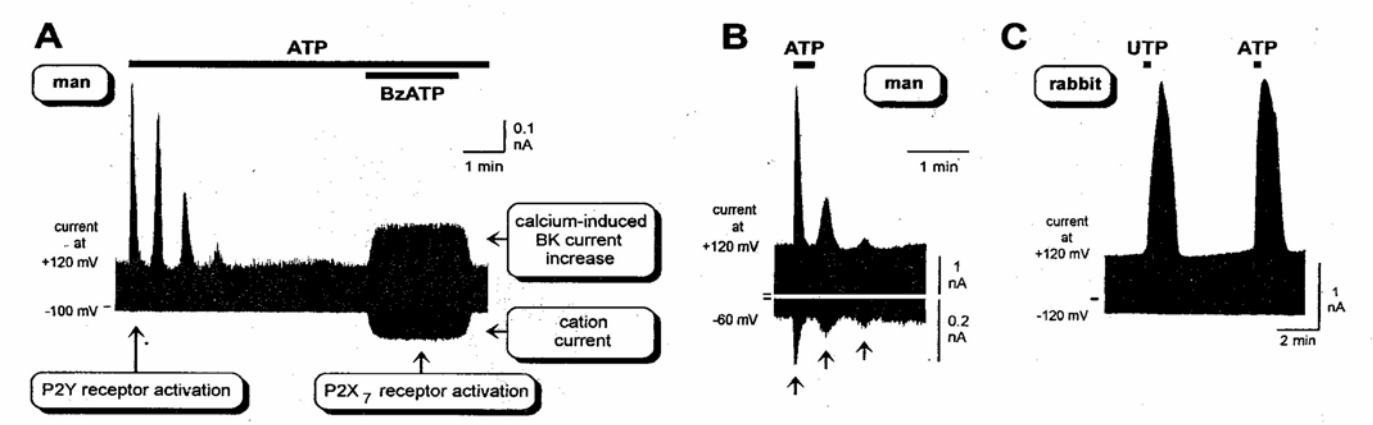

Fig. 1: *Müller cells express purinergic P2 receptors, and activation of these receptors alters plasma membrane conductances. Whole-cell patch-clamp records in acutely isolated Müller cells from human (A,B) and rabbit retinae (C), respectively. The cells were isolated from PVR retinae. At positive potentials, BK current increases are visible which are caused by P2 receptor-mediated elevation of the intracellular free calcium concentration. Increases of the currents at negative potentials reflect opening of non-specific cation channels.* **A.** *Human Müller cells express both P2Y and $P2X_7$ receptors (7). Activation of P2Y receptors by extracellular ATP (100 µM) induces repetitive transient increases of BK currents (at +120 mV) which reflects repetitive release of calcium from intracellular stores. Stimulation of $P2X_7$ receptors by 2'-/3'-O-(4-benzoylbenzoyl)-ATP (BzATP, 50 µM) results in opening of receptor channels which mediate non-selective cation currents. Through $P2X_7$ receptor channels, extracellular calcium can enter the Müller cell interior and activates subsequently BK channels which is reflected by the current increase at +120 mV.* **B.** *In another human Müller cell, activation of P2Y receptors by extracellular ATP (100 µM) has two consequences on the plasma membrane conductance: the receptor-mediated repetitive release of calcium from intracellular stores evokes transient increases of BK currents (at +120 mV) and transient increases of cation currents (at -60 mV; arrows). These cation currents do not reflect activation of P2X receptor subtypes since buffering of intracellular calcium blocks the activation of these currents, and these currents were also observed after application of glutamate and UTP, for example (8).* **C.** *In a rabbit Müller cell, extracellular nucleotides (at 100 µM) evoke transient increases of BK currents at positive potentials and no current alterations at negative potentials. The small bars at the left side of the traces indicate zero-current potentials.*

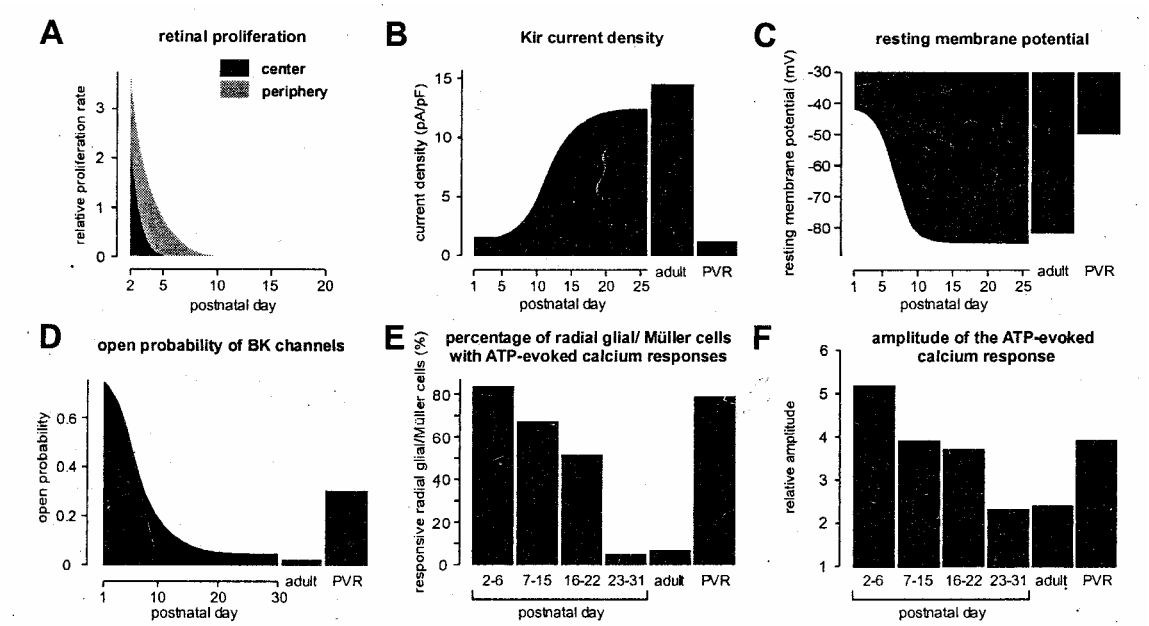

Fig. 2. *The potassium currents and the responsiveness to extracellular ATP of rabbit Müller cells alter in relation to retinal proliferation.* ***A.*** *In the first postnatal week, decreasing precursor cell proliferation can be observed in dependence on the site within the retina (44).* ***B.*** *In the second and third postnatal weeks, immature radial glial cells differentiate into mature Müller cells, as indicated by the increasing amplitude of whole-cell Kir currents (27).* ***C.*** *The Müller cell differentiation is accompanied by a hyperpolarization of the plasma membrane at resting conditions (27).* ***D.*** *In correlation to the decreasing precursor cell proliferation and to the Müller cell differentiation, the activity of BK channels decreases postnatally (27).* ***E.*** *The percentage of radial glia/Müller cell endfeet which respond to application of extracellular ATP with an increase of the intracellular free calcium concentration decreases significantly postnatally (12).* ***F.*** *The amplitude of the ATP-evoked calcium responses in radial glia/Müller cell endfeet decreases in the course of the postnatal retinal development (12). In Müller cells from PVR retinae, the physiological parameters alter in opposite direction, i.e., onto values which can be observed in neonatal rabbit retinae (11,26,40,56).*

cellular ATP (O. Uckermann, unpublished observation). The failure to detect P2X receptor-mediated currents in rat Müller cells is puzzling since the presence of mRNA for P2X receptor subtypes has been described [16]. Perhaps, the data reflect different functional states rather than different expression levels of the receptors. Nevertheless, the expression of functional P2 receptors is a general phenomenon of rat (at least, subpopulations of) Müller glial cells while receptor expression may differ among the species qualitatively as well as quantitatively

One feature of $P2X_7$ receptor channels in other cell systems is their ability to form relatively large non-selective pores which are permeable to molecules of up to 900 Da [17,18]. For example, lucifer yellow, a small molecule which permeates $P2X_7$ receptor-induced pores, entered 2'-/3'-O-(4-benzoylbenzoyl)-ATP (BzATP)-treated astrocytes [19]. However, $P2X_7$ receptors of human Müller cells lack the ability to form such large pores [9]. The functional significance of this peculiarity is unclear.

II. Potassium channels

The main membrane conductance of Müller cells in the healthy adult retina is mediated by inwardly rectifying potassium (Kir) channels [20-23]. These Kir channels were suggested to mediate spatial buffering potassium currents thought to be crucially involved in the extracellular potassium homeostasis mediated by Müller cells [24,25]. The high open probability of these channels, above a wide potential range [21,23,26,27], allows these channels to play a crucial role in the maintenance of the membrane potential at hyperpolarized values, i.e., close to the equilibrium potetential of potassium ions (at approximately -80 mV at resting conditions). Blocking the Kir channels in Müller cells by barium ions results in a significant membrane depolarization (to approximately -40 mV) [20,28,29]. The very negative membrane potential of Müller cells is essential for the activity of electrogenic neurotransmitter uptake carriers, e.g., for glutamate [30,31] and for γ-aminobutyric acid [32,33]. Therefore, the expression level of Kir currents was suggested to reflect the differentiation degree of Müller cells [34].

However, Müller cells may display also depolarization-activated potassium currents such as A-type and delayed rectifying potassium currents [20,22,27,35] and calcium-activated potassium currents mediated by BK channels [36]. BK channels are activated mainly by membrane depolarization and by an increase of the intracellular calcium concentration [36,37], but also by several other fac-

tors such as the second messenger, arachidonic acid (38), and by increases of the intracellular pH [37], as well as by phosphorylation by the protein kinase A [37,39] while phosphorylation by the protein kinase C inhibits the BK channel activity in Müller cells [39].

The expression level of BK channels in Müller cells from the healthy retina was observed to be species-dependent. All electrophysiologically investigated Müller cells from healthy human retinae expressed BK channel-mediated currents (8), and approximately 90% of freshly isolated Müller cells from the healthy rabbit retina [40] and only approximately 50% of porcine Müller cells were observed to express BK currents [41]. On the other hand, in sheep, rat, guinea pig, and horse retinae, only a minority of Müller cells expressed these currents (Pannicke, Bringmann, unpublished results).

III. Potassium channels and Müller cell proliferation

During the ontogenetic development of murine and rabbit retinae, precursor cells proliferate until approximately the end of the first postnatal week (Fig. 2A) [42-46]. Subsequently (predominantly, during the second and third postnatal week), immature radial glial cells differentiate into mature Müller cells. Müller cell differentiation occurs in correlation with the development of light-induced ganglion cell activity (between postnatal days 7 and 20 in the rabbit retina [47]. Electrophysiologically, the main alteration of plasma membrane conductance occurring during Müller cell differentiation is an enhancement of Kir channel-mediated currents (Fig. 2B) [15,27,48]. The increasing expression of functional Kir channels in the Müller cell membrane is accompanied by a hyperpolarization of the plasma membrane [15], in the mean from -40 mV in the first postnatal week to approximately -80 mV in adult rabbit cells (Fig. 2C) [27]. In correlation with the decreasing retinal precursor proliferation and with the ongoing Müller cell differentiation, the BK channel activity (measured at resting conditions) falls to very low values during this time (Fig. 2D) [27]. These data indicate that the Kir channel expression is especially high in Müller cells of the healthy mature retina, and suggest that the BK channel activity may support the proliferation of retinal precursor cells.

Support for the latter assumption comes from data obtained in cases of Müller cell gliosis and from proliferation studies on cultured Müller cells. Müller cell gliosis is regularly accompanied by a re-entry of Müller cells into the proliferation cycle [49]. For example, after experimental retinal detachment, Müller cells begin to proliferate within one day; maximal proliferation is observed three to four days after detachment [50,51]. N-Methyl-D-aspartate-induced excitotoxicity in postnatal chicken retinae causes a fast transient Müller cell proliferation which is accompanied by a dedifferentiation of Müller cells into retinal progenitor-like cells [52]. Proliferative vitreoretinopathy (PVR) is characterized by the uncontrolled long-lasting intraocular proliferation of different cell types, among others, of Müller cells [53-55]. Proliferative Müller cell gliosis is accompanied by alterations in potassium channel expression and activity [34]. Müller cells from detached rabbit retinae display a reduction of their Kir currents by 40%, and a slight depolarization of the plasma membrane, already two days after detachment [40]. The membrane conductance changes are much stronger in Müller cells from PVR retinae; here, the Kir currents are almost completely down-regulated (Fig. 2B) and the plasma membrane is strongly depolarized, in the mean to -40 to -60 mV (Fig. 2C) [11,26,40,56]. On the other hand, a ten-fold increase of the BK channel activity at the resting membrane potential was observed in human Müller cells from PVR retinae if compared to control retinae [26]. All these changes of potassium currents in Müller cells from PVR retinae indicate that a dedifferentiation of these cells occurs, and suggest a role of these alterations in the induction or maintenance of Müller cell proliferation [34].

In cultured Müller cells, BK channels are crucially involved in the maintenance of proliferation [36]. Exposure of Müller cells to a mitogenic conditioned medium increased the activity of their BK channels [36]. Iberiotoxin, a specific inhibitor of BK channels [57], blocks the proliferation of cultured Müller cells induced by various agonists [7,58] such as the epidermal growth factor (EGF) [59].

IV. Purinergic receptors and Müller cell proliferation

The postnatal differentiation of rabbit Müller cells is not only accompanied by changes in the potassium currents but also by a strong decrease of their calcium responsiveness, particularly to extracellular ATP [12]. Both the number of Müller cells that respond to ATP with an increase of their intracellular free calcium concentration (Fig. 2E) and the amplitude of their calcium responses (Fig. 2F) decrease postnatally. In the first postnatal week, more than 80% of the radial glia endfeet showed ATP-evoked calcium responses while in the adult retina, only 7% of the Müller cell endfeet were responsive [12]. However, Müller cells derived from eyes in which a PVR was induced experimentally show a strong up-regulation of their responsiveness to extracellular ATP; approximately 80% of the Müller cell endfeet were responsive in such retinae (Fig. 2E) [11]. The ATP effects were mediated via activation of UTP-sensitive P2Y receptors [11,12]. These data may implicate purinergic receptors in the support of both precursor cell proliferation during retinal development and Müller cell proliferation in the diseased retina.

Observations in other animal species and other disease models may support this assumption. In the early embryonic chick retina, a stimulation of P2Y receptors evoked intracellular calcium responses [60]. The amplitude of these purinergic calcium responses decreased in parallel to the decline of the mitotic activity of retinal precursor cells (i.e., before synaptogenesis). Moreover, a decline of the ATP concentration in the chick amniotic fluid was observed during retinal development [61]. ATP is released from organ cultures of embryonic chick retinae, and extracellularly applied ATP increases the DNA synthesis rate in these organ cultures via stimulation of purinergic recep-

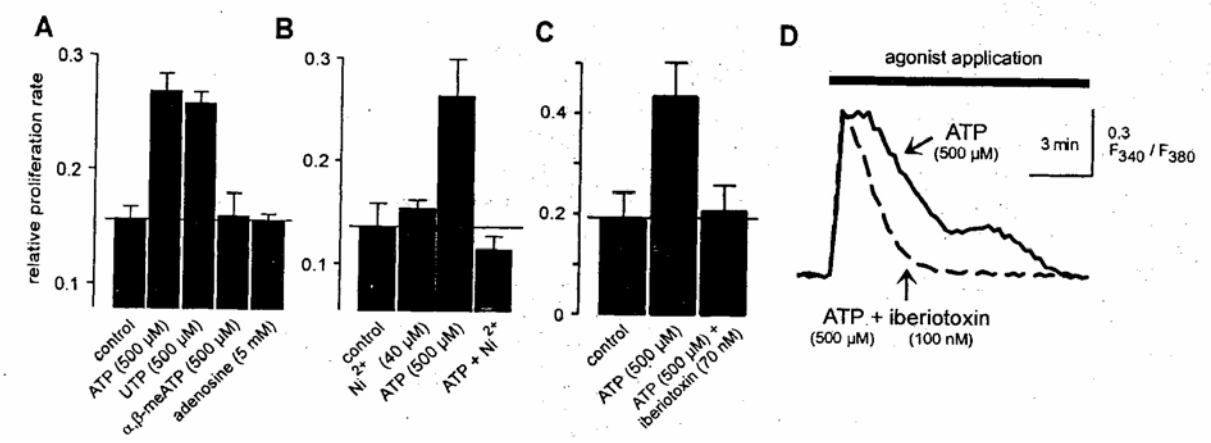

Fig. 3: *The BK channel activity supports the mitogenic effects of extracellular ATP in primarily cultured Müller cells of the guinea pig (58).* ***A.*** *Treatment with extracellular ATP or UTP, but not with α,ß-methylene adenosine 5'- triphosphate (α,ß -meATP), a P2X receptor agonist, or with adenosine, a P1 receptor agonist, increased the proliferation rate, indicating the involvement of UTP-sensitive P2Y receptors in the mitogenic effect of ATP.* ***B.*** *Nickel ions, which block calcium-permeable ion channels, inhibit the mitogenic effect of ATP, indicating that an influx of calcium ions from the extracellular space is involved in this effect.* ***C.*** *The BK channel blocker iberiotoxin inhibits the mitogenic effect of ATP.* ***D.*** *The ATP-evoked intracellular calcium transient is shortened by co-application of iberiotoxin indicating a supporting role of the BK channel activity on the calcium influx from the extracellular space into the Müller cells.*

tors, at the developmental stage when Müller and bipolar precursor cells proliferate [61,62]. These data are in line with the assumption that P2Y receptors, activated by autocrine or paracrine release of ATP, are involved in the regulation of proliferation of retinal precursor cells. During PVR in the human eye, but not in the non-proliferative human retinal degeneration due to choroidal melanoma, Müller cells enhance their responsiveness to activation of ionotropic $P2X_7$ receptors [7,8] which was significantly correlated with other signs of gliosis, e.g. with the down-regulation of Kir currents [7]. This indicates that a higher ATP responsiveness may reflect a stronger gliosis. However, an enhanced Müller cell responsiveness to extracellular ATP seems to be a more general feature of Müller cell gliosis. In a dispase-model of experimental retinopathy in rabbits which is accompanied by alterations of the microvasculature, by photoreceptor cell death, and by intraretinal proliferation, but not by development of a PVR, Müller cells showed an up-regulation of their calcium and BK current responsiveness to ATP via activation of UTP-sensitive P2Y receptors [63]. There was a relation between the decrease of the Kir currents and the increase of the ATP responsiveness, suggesting that both features of gliosis may occur in parallel. An up-regulation of ATP-evoked calcium responses in Müller cells was also observed after Borna disease virus infection in rats [13].

Extracellular ATP increases the proliferation rate of cultured Müller cells [7,58,64]. In guinea-pig Müller cells, ATP stimulates the proliferation via activation of UTP-sensitive P2Y receptors (Fig. 3A). Here, extracellular ATP evokes two intracellular signaling pathways: a calcium-dependent pathway [58] and a receptor tyrosine kinase-dependent pathway [64].

The ATP-evoked proliferation of cultured Müller cells is dependent on an influx of extracellular calcium ions into the Müller cells (Fig. 3B). The increase of the intracellular calcium concentration is necessary to activate different calcium-dependent enzymes such as protein kinase C and calpains [58]. BK channels are crucially involved in the regulation of the ATP-induced Müller cell proliferation since the BK channel inhibitors iberiotoxin (Fig. 3C) or charybdotoxin block the ATP effect [7,58]. Based on calcium imaging experiments, it was suggested that the BK channel activity supports the calcium influx from the extracellular space through open calcium release-activated calcium channels, as indicated by the fact that iberiotoxin leads to significantly shortened intracellular calcium transients (Fig. 3D) [58]. A similar depressing effect of iberiotoxin was observed in regard to EGF-induced calcium transients [59]. As previously shown in other cell-systems, BK channel activity leads to a hyperpolarization of the plasma membrane which increases the electrochemical driving force for calcium influx into the cells [65]. One function of BK channels in Müller cells may be to enhance the calcium entry after receptor stimulation, via hyperpolarization of the membrane. An enhanced calcium entry would subsequently increase the proliferation rate. A correlation has been described between the duration of ATP-evoked calcium transients and the rate of Müller cell proliferation [58]. However, other functions of the ATP-evoked stimulation of the BK channel activity are conceivable as well, e.g., a role in volume [66] and cell shape regulation which accompany proliferation and migration, and which are partially mediated by potassium efflux through channels.

In cultured Müller cells, stimulation of P2Y receptors leads to a transactivation of at least two different receptor tyrosine kinases: of the platelet-derived growth factor-α (PDGF-α) receptor and of the EGF receptor tyrosine kinases [64]. These transactivations are mediated by subsequent release of PDGF and of heparin-binding EGF from the Müller cells. The EGF receptors receive mitogenic signals from both tyrosine kinase (PDGFα) receptors and G protein-coupled (P2Y) receptors, and a tyrosine phosphorylation of the EGF receptor seems to be the final step for several different extracellular mitogenic signals at the level of Müller cell plasma membrane.

It is probable that purinergic receptors and receptors for growth factors may collaborate in early retinal development and in proliferative Müller cell gliosis. Late retinal precursor cells show enhanced proliferative responses to EGF receptor activation [67], and the PDGFα receptor has been implicated in the generation of PVR [68] while inhibition of the PDGFα receptor has been shown to attenuate experimental PVR [69]. Various physiological relationships between purinergic and growth factor receptors have been described in other cell systems. Receptor tyrosine kinases were found to inhibit the protein kinase C-mediated desensitization of P2Y receptors [70]; interleukin-1ß and tumor necrosis factor-α may enhance the expression of P2Y receptors [71,72]; and, vice versa, extracellular nucleotides may enhance the effects of growth hormones [73], e.g., via increasing the release of trophic factors and growth factors [74]. Moreover, an involvement of extracellular ATP in retinal differentiation may be assumed since this signaling molecule also has been shown to exert trophic effects on both astrocytes [73,74] and neurons [75]. During gliosis, enhanced ATP responsiveness may be a step in a trophic response of Müller cells (as an attempt of wound healing) initiated by serum invasion or by (signals released due to) degeneration of retinal neurons. However, very little is presently known about the involvement of purinergic receptors in retinal precursor proliferation, retinal differentiation, and retinal diseases, and future studies are urgently required to reveal the importance of purinergic receptors and their signaling mechanisms in proliferation and differentiation events of the retina.

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