

Early Cellular Responses of Purine Nucleoside-mediated Protection of Hypoxia-induced Injuries of Neuronal PC12 Cells

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Abstract: Hypoxia in brain may lead to cell death by apoptosis and necrosis. In parallel adenosine, a powerful endogenous neuroprotectant is formed. We wanted to investigate the effect of adenosine and its purine nucleoside relatives, inosine and guanosine on early cellular responses to hypoxia. O₂-sensitive neuronal PC12-cells were subjected to chemical hypoxia induced with rotenone, an inhibitor of mitochondrial complex I. Loss of viability after hypoxic insult was impressively rescued by adenosine, guanosine and inosine. PC12-cells mainly express the A2A adenosine receptor. Its inhibition with a specific antagonist (CSC) induced cell death of PC12-cells, which could be salvaged by adenosine but not with guanosine or inosine. We have previously demonstrated the important role of mitogen activated protein kinases 1/2 (p42/44 MAPK) in purine-mediated rescue. In this study we were interested in the involvement of protein kinases whose activities mediate these processes, including protein kinase A (PKA), phosphoinositide 3-kinase (PI3-K) and protein kinase C-related kinases (PRK 1/2). Pharmacological inhibition of PKA and PI3-K increased hypoxia-induced toxicity and likewise also affected the rescue by purine nucleosides. Nerve growth factor (NGF) and purine nucleosides induced an activation of PRK 1/2, which to our knowledge indicates for the first time that these kinases are potentially involved in purine nucleoside-mediated rescue of hypoxic neuronal cells. Results suggest that A2A receptor expressing cells are mainly dependent on the purine nucleoside adenosine for their rescue after hypoxic insult. In addition to PKA, PI3-K is an important effector molecule in A2A-mediated signaling and for the rescue of PC12-cells after hypoxic insult.

Key words: Hypoxia, neuronal cell death, adenosine, neuroprotection, phosphoinositide 3-kinase, protein kinase C-related kinases, mitogen activated protein kinases

INTRODUCTION

Adenosine, the final metabolite in the stepwise dephosphorylation of ATP, is produced and released in the central nervous system in response to ischemia and hypoxia molecules^[1, 2] and stimulation of adenosine receptors was hypothesized to result in an effective treatment of stroke^[3-4], for a review^[5]. Likewise, inosine and guanosine were shown to induce neurite outgrowth and preserve glial cell viability^[6-8]. Despite vigorous studies, many aspects of the mechanisms involved in purine-based protection are still unclear. In this study we wanted to investigate the effect of purine nucleosides on early cellular responses to hypoxia, induced by rotenone, a mitochondrial complex I inhibitor^[9-12].

For a test model O₂-sensitive clonal rat pheochromocytoma (PC12)-cells, which are widely used as a model system for sympathetic ganglion-like neurons^[13,14] and exposures to hypoxia^[15] were used.

PC12- cells express abundant A2A adenosine receptors^[16-18], which have been shown to affect these cellular responses to hypoxia^[3,19]. PC12-cells differentiate into sympathetic neurons upon treatment with NGF^[20], which requires activation of p42/44 MAPK and induction of gene expression^[21]. Under certain circumstances purine nucleosides were shown to cooperate with NGF-mediated pathways^[22-24]. Recent data in our hands revealed that p42/p44 MAPK was strongly activated by purine nucleosides especially adenosine. Hence we have put a special emphasis on the study of the impact of purine nucleosides on the early signaling elements leading to the activation of p42/44 MAPK following hypoxic insult.

MATERIALS AND METHODS

Determination of apoptosis: Cells were cultured in FM (RPMI 1640, 10 % horse-serum, 5 % fetal-calf-serum, 1 % Pen/Strep and 1 % L-glutamine). Stimulations were done in LM (RPMI 1640, 1.25 %

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horse-serum, 0.625 % fetal-calf-serum, 1 % Pen/Strep and 1 % L-glutamine). Within 30 min^[10] after addition of rotenone (10 µM), cells were stimulated with 500 µM adenosine, guanosine and inosine (Sigma). For inhibitor studies, nucleoside transport inhibitor S-(4-Nitrobenzyl)-6-thioinosine (NBFI, 10 µM, Sigma), adenosine A2A receptor antagonist 8-(3-Chlorostyryl) caffeine (CSC, 10 µM Sigma), A1 receptor antagonist 8-Phenyltheophylline (8-PT, 100 nM, Sigma), the cAMP-dependent protein kinase inhibitor H89 (5, 10 and 20 µM, Sigma) and the PI3-Kinase inhibitor LY294002 (25 µM, Sigma) were used. For CSC experiments, cells were starved in LM one day before stimulation. All pharmacological inhibitors were added 2 h, CSC 30 min prior to tests.

Fluorescence analysis: Cells were stained after 24 h. For microscopic analysis cells were fixed with 4 % PFA before staining and for FACS scan after staining with 2 % PFA. Primary antibodies: A2A, A2B, A1 (Chemicon); secondary antibody: Alexa Fluor 488 F(ab') fragment of goat-anti-rabbit (Eubio).

Calculation of neurite outgrowth: Cells were stimulated with NGF-β (5 ng mL⁻¹), purines (500 µM), rotenone (10 µM) and LY294002 (25 µM). On day 3 neurite-bearing-cells (neurites longer than one time cell diameter) were counted and averaged from 3 microscopic fields.

Immunoblots: Detection was done with following antibodies: phospho-PRK 1/2 and phospho-p42/44 MAPK (New-England-Biolabs), total-PRK 1 (BD Transduction Laboratories) and total-p42/44 MAPK (New-England-Biolabs). For quantification, blots were scanned with Molecular Dynamics Personal Densitometer SI scanner and ratios (active divided through total) were calculated.

Statistical analysis: All values in figures were expressed as the mean ± SEM. One-tailed Mann-Whitney-test was used. *p*-values of < 0,05 were considered statistically significant.

RESULTS AND DISCUSSION

Chemical hypoxia induced apoptosis of neuronal PC12-cells (Fig. 1a). In accordance with previous studies, purine nucleosides, especially adenosine, impressively counteracted this process (Fig. 1b). Recent results in the literature indicated that hypoxia-induced membrane responses of PC12-cells are likely to be mediated via activation of the A2A adenosine receptors^[16, 19]. In line with these findings cells express high levels of A2A but only marginal amounts of A2B and A1 receptors (Fig. 2a-b). Pharmacological inhibition of the A2A receptor by CSC enhanced cell death of hypoxic PC12-cells. While addition of

adenosine potently rescued cells, guanosine and inosine were ineffective (Fig. 2c). We therefore suppose that adenosine is the most important neuroprotectant for neuronal PC12-cells. However, there may be circumstances under which inosine can activate at least some of the receptors^[25] and provides a larger activation than adenosine^[5,11,26]. In line with these findings inosine was the only purine nucleoside used in this study, which was affected by the A1 receptor antagonist 8-PT (Table 1). Guanosine, does not bind to the adenosine receptor, but recent studies showed the existence of specific G-coupled receptors for guanosine^[27]. Others^[28] however, observed, that the mitogenic activity of guanosine was partly inhibited by antagonists of A1 and A2B adenosine receptors. Alternatively, inhibition of nucleoside transport by NBFI improved viability of cells (data not shown) and significantly increased adenosine-mediated rescue of hypoxic cells (Table 1), presumably due to the fact that inhibition of adenosine uptake via nucleoside transport increased A2A receptor-mediated signaling^[29]. Our own results therefore confirm the hypothesis that adenosine mainly acts via adenosine receptor-mediated signaling^[30], whereas many aspects of the mechanisms involved in inosine – and guanosine- based protection still remain unclear.

NGF triggers intracellular signaling cascades including MAPK and PI3-K, reviewed in:^[31] and promotes the differentiation of neuronal cells e.g. PC12-cells, a well-studied model of growth factor actions^[13,20,32-34]. Like NGF, the ability of PKA to differentiate PC12-cells is associated with a sustained activation of MAPK. As seen earlier, neurite outgrowth is down regulated under hypoxia and purine nucleosides support NGF -mediated rescue. Co localization of NGF receptors and A2A receptors suggests a potential cross-interaction between their signaling pathways^[22]. Along with previous findings^[23,35-39], earlier results by our lab, have demonstrated that purine nucleosides rapidly activate p42/44 MAPK and in combination with NGF led to a further additive enhancement. Under hypoxic conditions we could also observe the cooperative effects of NGF and purine nucleosides on p42/44 MAPK activation (Fig. 3a). PC12-cells were further treated with LY294002 and for control purpose with PD098059, a specific p42/44 MAPK inhibitor^[40]. Administration of LY294002 led to the inhibition of adenosine-mediated p42/44 MAPK activation, whereas inosine- and guanosine-mediated activation of p42/44 MAPK was less effected (inosine) or even enhanced (guanosine) (Fig. 3b). Our results are in line with earlier data^[39], which showed that PI3-K is required for activation of Rap1, which may represent an integral part of a direct path to p42/44 MAPK phosphorylation. As expected, MEK-1 inhibitor PD098059 reduced the purine nucleoside-mediated activation of p42/44 MAPK (Fig. 3c).

In this study we were interested to further illuminate the role of PKA- and PI3-K-mediated signaling pathways for NGF- and purine nucleoside-mediated effects.

Table 1: Effect of pharmacological inhibitors on purine-mediated rescue

Treatment	A+R	G+R	I+R
-----	63,55 ± 5,52 % (n=5)	37,55 ± 3,59 % (n=5)	42,48 ± 3,19 % (n=5)
8-PT 100 nM	70,58 ± 4,93 % (n=5)	40,20 ± 5,43 % (n=5)	32,40 ± 7,22 % (n=5)
NBTI 10 µM	79,95 ± 1,84 % (n=5) ⁽¹⁾	48,53 ± 8,64 % (n=5)	39,50 ± 10,86 % (n=5)

Cells were incubated with NBTI, 8-PT, rotenone (R, 10 µM) and together with adenosine (A+R), guanosine (G+R) and inosine (I+R) in a concentration of 500 µM. FACS scan followed. Statistical difference (1) p=0,0079 NBTI+A+R vs. A+R

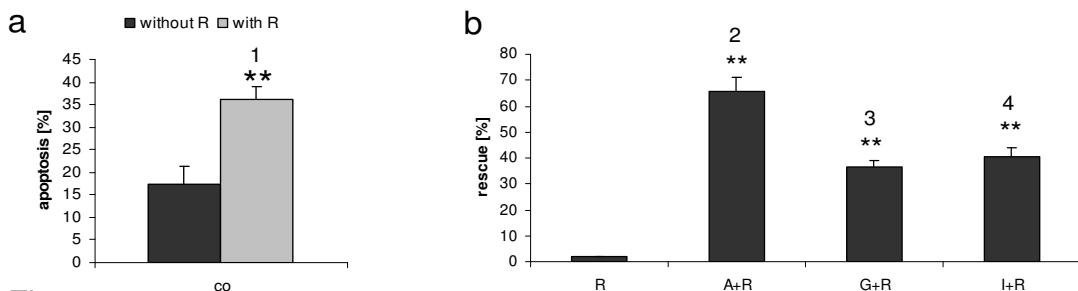


Fig. 1: a-b Purine nucleoside-mediated rescue of rotenone-induced apoptosis of PC12-cells: Cell death was induced by rotenone (R, 10 µM) for 24 h (a). Further incubation was done with 500 µM adenosine (A+R), guanosine (G+R) and inosine (I+R) for 24 h (b). Quantification was achieved by PI staining and FACS scan. Significant difference: (1) p= 0.0040 rotenone vs. control, (2) and (3) p=0.0040 and (4) p=0.0079 purines+rotenone vs. rotenone; n=5

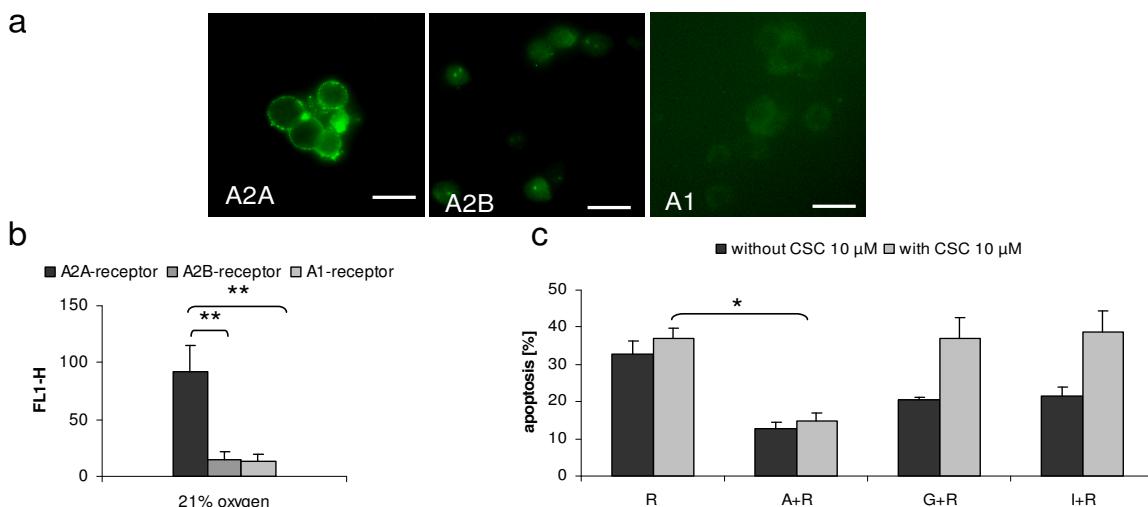


Fig. 2: a-c Receptor staining: Cells were incubated in FM under normoxic conditions (21 % O₂) for 24 h and stained for A2A, A2B or A1. Cells were analyzed on a Zeiss Axioplan-2-Fluorescence-Microscope; scale bar, 10µm (a). For quantification, FACS analysis was used. Fluorescence intensity (FL1-H) of unspecific binding was subtracted from values obtained for primary antibody (b). Cells were stimulated with CSC (10 µM), rotenone (R, 10 µM) and purines 500 µM: adenosine (A+R), guanosine (G+R) and inosine (I+R) (c). After 24 h, PI staining and FACS scan followed. Significant difference: (b) double-asterix (**) p=0.0079; (c) asterix (*) p=0.0143; (b) n=3, (c) n=5

The importance of PKA for PC12-cell viability was studied by use of the pharmacological inhibitor H89 (5, 10 and 20 µM). With increasing concentration H89 induced a rising toxicity and inhibition of the purine-mediated rescue. At a concentration of 10 µM, toxicity could still be reduced to some extent by adenosine and

to a minor extent also by inosine (Fig. 4a). Data corroborate our earlier results obtained after the inhibition of the A2A receptor and suggest that A2A and cAMP-mediated signaling is essential for the survival of neuronal PC12-cells. Likewise inhibition of PI3-K with the pharmacological inhibitor LY294002

increased rotenone-mediated toxicity and significantly inhibited adenosine -mediated rescue of hypoxic cells (Fig. 4b). In accordance with these data, LY294002 decreased adenosine- and guanosine- yet not inosine-mediated rescue of neurites (Fig. 4c). This observation is in line with studies mentioned before, suggesting that

the PI3-K/Rac pathway is one of the early signals that mediate NGF-induced neuronal differentiation in PC12-cells^[41], for a review^[22].

In PC12-cells, a not yet elucidated inhibitory signalling link between NGF receptors and RhoA was suggested, reviewed in^[42].

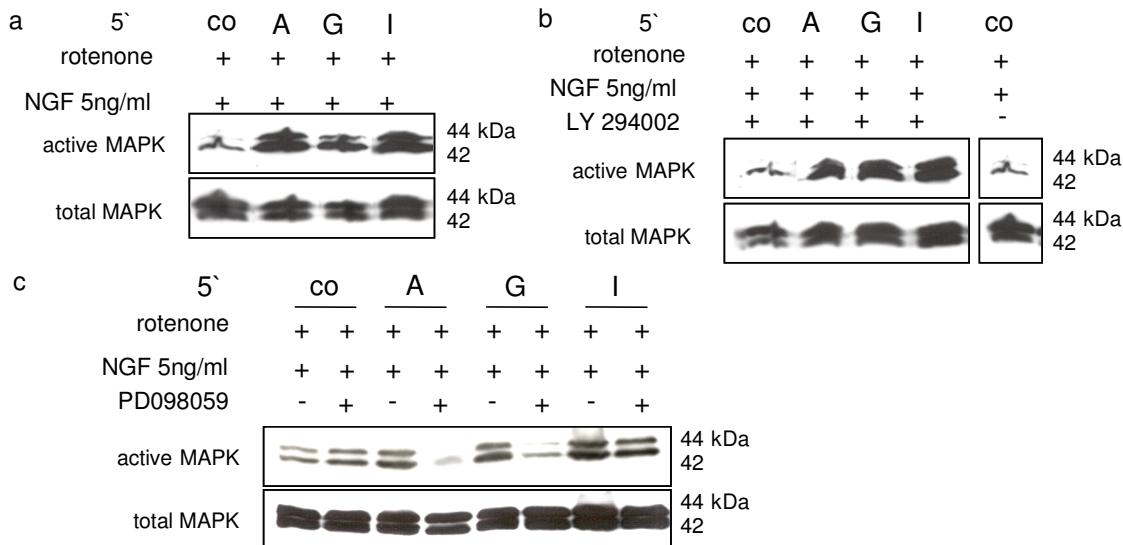


Fig. 3: a-c Effect of LY294002 and PD098059 on p42/44 MAPK activity in hypoxic PC12-cells: Cells were stimulated in serum-free medium for 5 min with NGF-β 5 ng mL⁻¹ and rotenone 1 μM (co), additional with 500 μM adenosine (A), guanosine (G) and inosine (I) (a), +/- LY294002 25 μM (b) and +/- PD098059 (c)

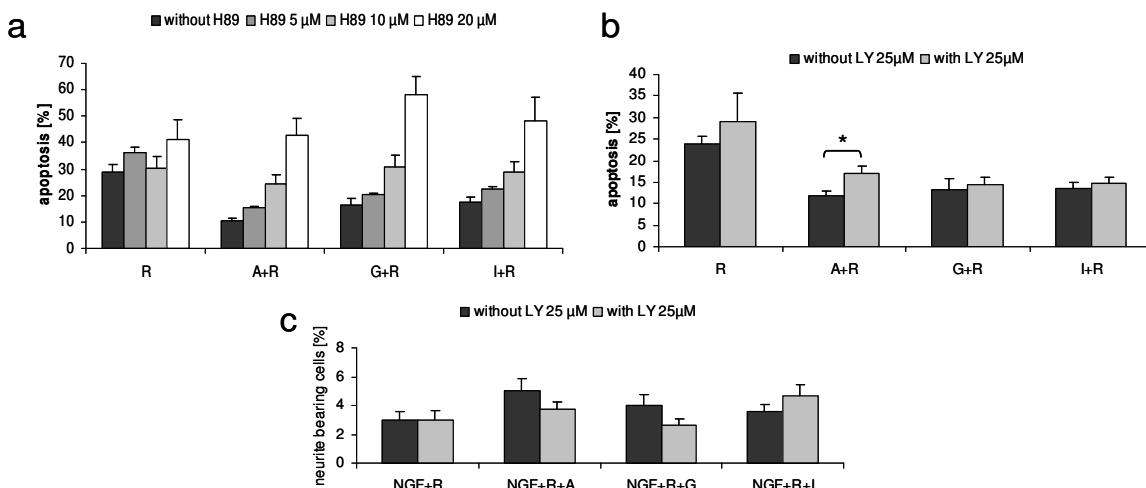


Fig. 4: a-c Effect of H89 and LY294002: For inhibitor studies (a - b), cells were treated in LM with the inhibitors ((a) H89 5, 10 and 20 μM; (b) LY294002 25 μM) for 2 h. Stimulation with rotenone (R, 10 μM) and purine nucleosides 500 μM: adenosine (A+R), guanosine (G+R) and inosine (I+R) followed. PI staining and FACS scan was done. Statistical difference: asterix (*) p=0.0476; n=6. For the neurite outgrowth assay, stimuli were added together to adherent cells. On day 3 neurite positive cells were counted and evaluated (c)

Their data indicate that NGF -mediated activation of the TrkA receptor stimulates PI3-K, which in turn increases Rac1 activity to induce transient RhoA inactivation during the initial phase of neurite outgrowth. Among direct Rho effectors are the 'protein

kinases N' (PKNs), termed also 'protein kinase C-related kinases' (PRK 1/2), reviewed in^[43]. Authors further showed that activation of PRK 1/2 involves 3-phosphoinositide-dependent protein kinase-1 (PDK1) and that the interaction of PRK 1/2 with PDK1 was

shown to be dependent upon Rho. Consistent with the finding that PKN and PRK 2 are effectors for Rho is the observation that PKN and PRK 2 can enhance or mediate changes in the actin cytoskeleton and gene transcription^[44]. We were therefore interested in the role of PRK 1/2 (p120 /140). At first we studied, whether PRK 1/2 activity may be influenced by NGF. Indeed, as revealed in immunoblots, addition of NGF led to the concentration dependant activation of PRK 1/2 (Fig. 5a).

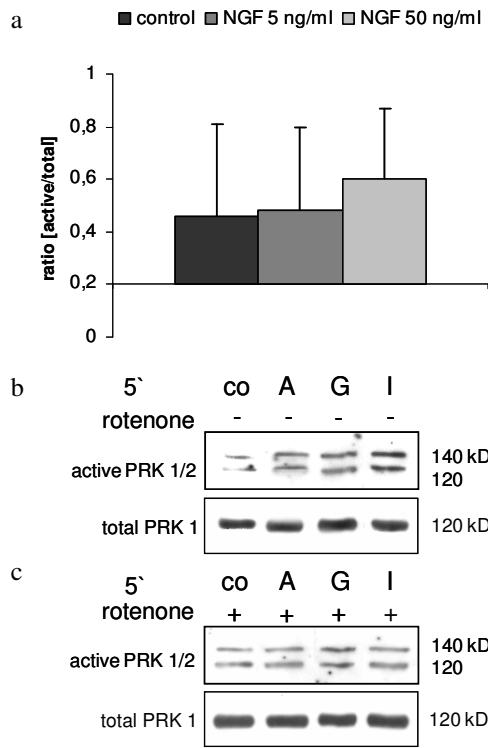


Fig. 5a-c: PRK 1/2 activation in PC12-cells: Normoxic cells were stimulated with/without NGF- β 5 and 50 ng mL $^{-1}$ for 5 min (a, n=6). In addition cells were incubated with serum-free medium (co), with 500 μ M purine nucleosides: adenosine (A), guanosine (G), inosine (I) and +/- rotenone 1 μ M for 5 min (b – c, n=5). Immunoblots were analyzed with specific antibodies to PRK 1/2

In view of the fact discussed above that NGF apparently induces transient RhoA inactivation this result appears paradoxical. Similarly purines led to the activation of PRK 1/2, whereby inosine was the most potent activating component (Fig. 5b). Under hypoxia a higher basic and guanosine-mediated activity of PRK 1/2 was observed, whereas adenosine- and inosine-mediated kinase phosphorylation was reduced (Fig. 5c). NGF activated PRK 1/2 in hypoxic PC12-cells and the activation was further increased by a combination of NGF with purine nucleosides (Fig. 6a). Inhibition of

PI3-K with LY294002 led to the reduction of NGF+adenosine- and NGF+inosine- but not NGF+guanosine-mediated PRK 1/2 activation (Fig. 6b). These data are in line with our findings that LY294002 inhibited adenosine and inosine but not guanosine – mediated MAPK activation. As discussed in previous publications it was suggested that inosine and guanosine exert its receptor-independent effects probably via PKN that has been implicated in mediating NGF-induced differentiation^[45,46] and induction of gene

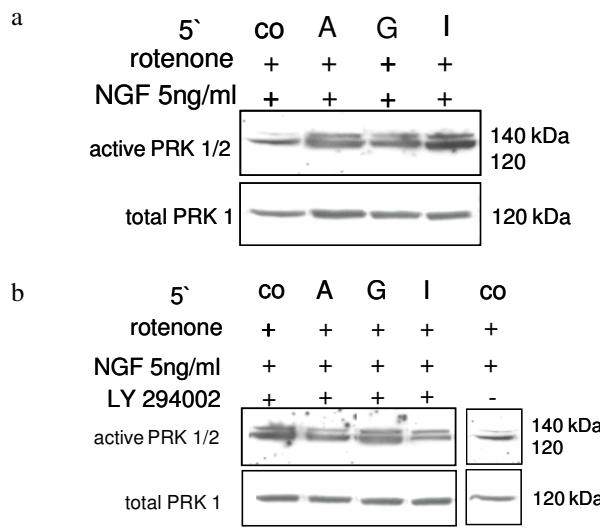


Fig. 6: a-b Effect of PI3-K inhibition on PRK 1/2 activation in hypoxic PC12-cells: Cells were treated with rotenone 1 μ M. Additional stimulation was achieved by using NGF- β 5 ng mL $^{-1}$, 500 μ M adenosine (A), guanosine (G) and inosine (I) (a). Furthermore LY294002 25 μ M (b) was used

expression^[47-48]. PKN termed by this group^[46,49] is a protein kinase of an apparent molecular mass of 45-47 kDa, which is rapidly activated by NGF and other agents and was selectively inhibited by purine analogs. We could however not detect an inhibiting effect of the inhibitor 6-TG on purine-mediated PRK 1/2 activation. Therefore it remains questionable, whether the earlier detected PKN (p 45-47) and the PKN alias PRK (p120/140) molecules are exactly the same kinases, apart from the fact that both of them are activated by NGF. This interesting question will be clarified in future studies.

In summary results in this study demonstrated that in PC12-cells, adenosine and guanosine strongly promote viability and in combination with NGF also neurite outgrowth after hypoxic insult. In line with previous findings results revealed that PI3-K and PKA are essential survival kinases for neuronal PC12-cells. For adenosine-mediated rescue of viability and neurite outgrowth MAPK activation is essential. Guanosine- and inosine-mediated rescue however, is apparently more independent of MAPK activation. PRK 1/2 are

activated by NGF and purine nucleosides and might be involved in the regulation of the cytoskeleton^[43]. Results, showing the positive impact of inosine, adenosine and guanosine on viability and neurite outgrowth of PC12-cells, support current studies on the possibility of novel neuronal cell regeneration therapies and confirm the important role of purine nucleosides in neuronal pathologies involving hypoxic insult.

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