Schwann Cells Metabolize Extracellular 2',3'-cAMP to 2'-AMP

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ABSTRACT

The 3',5'-cAMP–adenosine pathway (3',5'-cAMP→5'-AMP→adenosine) and the 2',3'-cAMP–adenosine pathway (2',3'-cAMP→2'-AMP/3'-AMP→adenosine) are active in the brain. Oligodendrocytes participate in the brain 2',3'-cAMP–adenosine pathway via their robust expression of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; converts 2',3'-cAMP to 2'-AMP). Because Schwann cells also express CNPase, it is conceivable that the 2',3'-cAMP–adenosine pathway exists in the peripheral nervous system. To test this and to compare the 2',3'-cAMP–adenosine pathway to the 3',5'-cAMP–adenosine pathway in Schwann cells, we examined the metabolism of 2',3'-cAMP, 2'-AMP, 3'-AMP, 3',5'-cAMP, and 5'-AMP in primary rat Schwann cells in culture. Addition of 2',3'-cAMP (3, 10, and 30 μ M) to Schwann cells increased levels of 2'-AMP in the medium from 0.006 ± 0.002 to 21 ± 2 , 70 ± 3 , and 187 ± 10 nM/ μ g protein, respectively; in contrast, Schwann cells had little ability to

convert 2',3'-cAMP to 3'-AMP or 3',5'-cAMP to either 3'-AMP or 5'-AMP. Although Schwann cells slightly converted 2',3'-cAMP and 2'-AMP to adenosine, they did so at very modest rates (e.g., 5- and 3-fold, respectively, more slowly compared with our previously reported studies in oligodendrocytes). Using transected myelinated rat sciatic nerves in culture medium, we observed a time-related increase in endogenous intracellular 2',3'-cAMP and extracellular 2'-AMP. These findings indicate that Schwann cells do not have a robust 3',5'-cAMP–adenosine pathway but do have a 2',3'-cAMP–adenosine pathway; however, because the pathway mostly involves 2'-AMP formation rather than 3'-AMP, and because the conversion of 2'-AMP to adenosine is slow, metabolism of 2',3'-cAMP mostly results in the accumulation of 2'-AMP. Accumulation of 2'-AMP in peripheral nerves postinjury could have pathophysiological consequences.

Introduction

Extracellular adenosine, by activating cell-surface, G protein–coupled adenosine receptors, exerts wide-ranging effects on many organ systems (Fredholm, 2010; Fredholm et al., 2011). For example, the receptor-mediated effects of extracellular adenosine influence numerous physiologic variables, including heart rhythm (Shryock and Belardinelli, 1997), neurotransmission [both in the central nervous system (CNS) (Boison, 2007; Sperlagh and Vizi, 2011) and peripheral nervous system (PNS) (Shepherd and Vanhoutte, 1985; Richardt et al., 1996)], vascular tone (Headrick et al., 2013), cell survival postinjury (Forman et al., 2006), and immune system responses (Sitkovsky et al., 2004; Eltzschig, 2009; Ohta and Sitkovsky, 2009; Linden, 2011). These effects of adenosine suggest opportunities to develop drugs that affect organ systems by influencing extracellular adenosine levels or adenosine receptor activation. Thus, it is important to

understand the mechanisms controlling extracellular adenosine levels within specific cellular environments.

An important determinant of extracellular adenosine levels is the rate of adenosine production in the extracellular compartment. In this regard, the classic pathway mediating extracellular adenosine formation is the conversion of extracellular ATP to adenosine (via the sequential actions of the ecto-enzymes CD39 and CD73) (Eltzschig and Carmeliet, 2011; Eltzschig et al., 2012; Eltzschig, 2013). However, accumulating evidence suggests that, in addition to extracellular ATP, extracellular cAMPs also can be converted to adenosine. Specifically, studies show that both extracellular 3',5'-cAMP (Jackson and Raghvendra, 2004) and extracellular 2',3'-cAMP (Jackson, 2011) [positional isomer of 3',5'-cAMP recently discovered to exist in intact organs and in vivo (Jackson et al., 2009; Ren et al., 2009; Verrier et al., 2012)] can serve as a source of extracellular adenosine production. Hormonal activation of adenylyl cyclases results in intracellular production of 3',5'-cAMP, which is actively transported to the extracellular compartment and metabolized to 5'-AMP and then to adenosine (i.e., the extracellular 3',5'-cAMP–adenosine pathway) (Jackson and Raghvendra, 2004). Cellular injury/energy depletion triggers the breakdown of RNA by RNases, producing intracellular 2',3'-cAMP that

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ABBREVIATIONS: AMPCP, α,β -methylene-adenosine-5'-diphosphate; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; DPSPX, 1,3-dipropyl-8-(p-sulphophenyl)xanthine; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline; PNS, peripheral nervous system; TNAP, tissue nonspecific alkaline phosphatase.

is transported to the extracellular compartment where it is converted to 2'-AMP and 3'-AMP, which in turn are metabolized to adenosine (i.e., the extracellular 2',3'-cAMP-adenosine pathway) (Jackson, 2011).

Our recent studies demonstrate that, of the two extracellular cAMP-adenosine pathways, the extracellular 2',3'-cAMP-adenosine pathway is more active in the brain than the extracellular 3',5'-cAMP-adenosine pathway. In this regard, the brain converts extracellular 2',3'-cAMP to 2'-AMP and 3'-AMP and metabolizes extracellular 2'-AMP and 3'-AMP to adenosine (Verrier et al., 2012). Recent studies suggest that, in the brain, the conversion of extracellular 2',3'-cAMP to 2'-AMP is mediated mostly by 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (Verrier et al., 2012, 2013). This conclusion is based on the observations that, in CNPase knockout mice, the conversion of exogenous 2',3'-cAMP to 2'-AMP is impaired, and traumatic brain injury increases extracellular 2'-AMP and adenosine much less in CNPase knockout mice compared with wild-type mice (Verrier et al., 2012). Likely, the abundance of oligodendrocytes in the brain is the reason that CNPase is critical to the CNS extracellular 2',3'-cAMP-adenosine pathway. This concept is based on the facts that oligodendrocytes express large amounts of CNPase (this protein is the third most abundant protein in the myelin sheath), and oligodendrocytes isolated from CNPase knockout mice generate less 2'-AMP and adenosine from 2',3'-cAMP (Verrier et al., 2013).

Although oligodendrocytes are the myelinating glia cells in the CNS, in the PNS, Schwann cells are the myelinating glia and provide both trophic and structural support to axons of the peripheral nerves (Jessen and Mirsky, 2005). During development, the premyelinating Schwann cells form a one-to-one relationship with axons and undergo elaborate morphologic changes. The process of myelination requires vast increases in the amount of both lipid membrane and myelin-specific proteins necessary to support the mature myelin. These proteins include myelin protein zero, myelin basic protein, peripheral myelin protein 22, and, importantly, CNPase (Patzig et al., 2011).

Since myelinated nerves in the PNS are surrounded by Schwann cells that express CNPase, it seems likely that myelinated nerves in the PNS have an active 2',3'-cAMP-adenosine pathway. This may be quite important because adenosine attenuates pain (Sawynok and Liu, 2003; Hayashida et al., 2005) and suppresses neuroinflammation (Tsutsui et al., 2004). Potentially, then, damage to myelinated nerves in the PNS could activate a 2',3'-cAMP-adenosine pathway that inhibits pain signals and reduces neuroinflammation. Therefore, in the present experiments, we sought to determine if the PNS, similar to the CNS, has a functional 2',3'-cAMP-adenosine pathway, and to determine whether the 2',3'-cAMP-adenosine pathway is more robust in the PNS as compared with the 3',5'-cAMP-adenosine pathway, as is the case in the CNS. To accomplish these goals, we conducted experiments in both primary rat Schwann cells and ex vivo rat sciatic nerves. In these experiments, S100 (Dong et al., 1999) and CNPase (Sprinkle, 1989) immune reactivity were used as Schwann cell markers.

Materials and Methods

Cell Culture. Primary rat Schwann cells were isolated as previously described (Verrier et al., 2009). In brief, postnatal day 2

rat pups were anesthetized, and the sciatic nerves were removed and placed in ice-cold Hanks' balanced salt solution (HBSS). The nerves were then dissociated with trypsin and collagenase for 15 minutes at 37°C with occasional shaking. The protease treatment was repeated two more times, and then Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA) with 10% fetal calf serum (FCS; Fisher Scientific, Waltham, MA) was added to quench the trypsin. The protease-treated nerve preparation was then passed through an 18-gauge needle several times, and then a 23-gauge needle until the solution was homogenous. The cells were passed through a 40- μ m cell filter device, pelleted via centrifugation, and then grown in 10% FCS/DMEM containing 10 μ M cytosine β -D-arabinofuranoside hydrochloride (Sigma-Aldrich, St. Louis, MO) for 4 days to remove fibroblast contamination. Cells were then treated with trypsin, split 1:4, and further cultured in Schwann cell media (10% FCS in DMEM, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 μ M forskolin, 10 μ g/ml glial growth factor) on poly-D-lysine-coated plates. To induce Schwann cell differentiation, we used a previously characterized "defined" medium [DMEM-F12, 100 U/ml penicillin, 100 μ g/ml streptomycin, 100 μ g/ml bovine serum albumin, N2 supplement (Life Technologies), 38 ng/ml dexamethasone, 50 ng/ml thyroxine, and 50 ng/ml tri-iodothyronine] for 72 hours (Cheng and Mudge, 1996).

Immunofluorescence. Primary Schwann cells grown on poly-D-lysine-coated eight-well glass culture slides were used for immunofluorescence experiments to confirm the purity of our Schwann cell cultures. Approximately 25,000 primary Schwann cells were seeded into each well and allowed to proliferate for 2 days. The cells were washed twice with phosphate-buffered saline (PBS) and then incubated in 4% paraformaldehyde for 15 minutes, washed again three times, and then incubated in 100% methanol at -30°C for 5 minutes. Cells were washed again and blocked for 1 hour with 5% goat serum in PBS. Primary antibodies were incubated overnight at 4°C. We used the Schwann cell markers S100 (antibody MAB079-1 used at 1:1000 dilution; Millipore, Billerica, MA) and CNPase (antibody ab6319 used at 1:500 dilution; Abcam, Cambridge, England) to demonstrate purity of the cultures. Fluorochrome-conjugated secondary antibodies (used at 1:500 dilution; Life Technologies) were used to visualize the specific staining, and 4',6-diamidino-2-phenylindole was used for nucleus detection.

Western Blot. To detect the levels of proteins expressed in Schwann cells cultured in normal growth media compared with the defined media, Western blot analysis was performed as previously described (Verrier et al., 2009). In brief, three separate 10-cm plates of cells per media (growth and defined) were harvested in radiologic immunoprecipitation assay buffer containing phosphatase and protease inhibitors. Proteins were then subjected to polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking in 5% milk/Tris-buffered saline with 0.05% Tween-20, the blots were incubated with rabbit anti-tissue non-specific alkaline phosphatase (TNAP; antibody NBP1-95392 used at 1:5000 dilution; Novus Biologicals, Littleton, CO), mouse anti-CNPase (antibody ab6319 used at 1:500 dilution; Abcam), or rabbit anti- α -tubulin (antibody 21445 used at 1:1000 dilution; Cell Signaling Technology, Danvers, MA) overnight. The blots were then washed three times in Tris-buffered saline, and the appropriate horseradish peroxidase conjugated secondary antibody (used at 1:10,000 dilution; Life Technologies) was applied in 5% milk/Tris-buffered saline with 0.05% Tween-20 for 2 hours, and then the blots were incubated with chemiluminescent substrate and exposed to film.

Ex Vivo Sciatic Nerve Study. To detect endogenous cAMPs, AMPs, and adenosine production from transected nerves, both sciatic nerves were isolated from four 6-week-old rats and used ex vivo (Barrientos et al., 2011). The nerves were kept in cold HBSS, cleaned of any residual muscle or connective tissues, and then cut into 3-mm sections with a sterile razor. The nerve sections were then transferred to 24-well plates (two nerve pieces per well) containing DMEM, and incubated at 37°C for the times indicated. At the indicated time points, the medium was harvested and incubated at 100°C for

2 minutes (to denature any enzymes present in the medium) and stored at -80°C for later purine analysis. The nerve sections were immediately frozen, crushed under liquid nitrogen, and then processed for purine analysis by extracting purines with ice-cold 1-propanol.

Metabolism Studies. To examine purine metabolism, experiments were performed as previously described (Verrier et al., 2011). In brief, 100,000 primary rat Schwann cells per well of a 24-well plate were grown in defined medium for 48 hours and then washed twice with HEPES-buffered HBSS and treated with 0.5 ml of PBS with HEPES (25 mM) and NaHCO_3 (13 mM) in the presence and absence of substrates (2',3'-cAMP, 3'-AMP, 2'-AMP, 3',5'-cAMP, or 5'-AMP; Sigma-Aldrich). Where indicated, enzyme inhibitors were included in the treatment [3-isobutyl-1-methylxanthine (IBMX), broad spectrum phosphodiesterase inhibitor (Beavo and Reifsnnyder, 1990); 1,3-dipropyl-8-(p-sulfophenyl)xanthine (DPSPX), ecto-phosphodiesterase inhibitor (Tofovic et al., 1991); α,β -methyleneadenosine-5'-diphosphate (AMPCP), ecto-5'-nucleotidase (CD73) inhibitor (Zimmermann, 1992)]. After a 60-minute incubation at 37°C , the medium was collected and immediately incubated in 100°C water bath for 2 minutes to denature enzymes. Samples were then stored at -80°C until assayed by mass spectrometry. Total protein content of a least six wells per 24-well plate was measured using the Thermo Scientific Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

Effects of DPSPX on CNPase Activity. To investigate whether DPSPX is a CNPase inhibitor, we used purified recombinant human CNPase (item number TP307038; OriGene, Rockville, MD) and measured the conversion of 2',3'-cAMP to 2'-AMP in the absence and presence of DPSPX. Twenty-five nanograms of CNPase enzyme and $30\text{ }\mu\text{M}$ 2',3'-cAMP were used per reaction. The concentrations of DPSPX were 1 and 10 mM, based on previous studies (Verrier et al., 2012). CNPase, 2',3'-cAMP, and DPSPX (where indicated) were combined in 1.5-ml centrifuge tubes and incubated at 37°C for 30 minutes. Then, the tubes were placed in a 100°C water bath for 2 minutes to denature the enzyme and stop the reaction. The samples were immediately placed in an -80°C freezer until they were processed for measurement of 2'-AMP by mass spectrometry.

Analytical Methods. Purines were measured using a modification of our previously published method (Jackson et al., 2009). Samples were spiked with a heavy-isotope internal standard ($^{13}\text{C}_{10}$ -labeled adenosine; Medical Isotopes, Pelham, NH), and purines were resolved by reversed-phase liquid chromatography (UPLC BEH C18 column, $1.7\text{-}\mu\text{m}$ beads, $2.1 \times 150\text{ mm}$; Waters, Milford, MA) and quantified using a triple quadrupole mass spectrometer (TSQ Quantum Ultra; Thermo Fisher Scientific, San Jose, CA) operating in the selected reaction monitoring mode with a heated electrospray ionization source. The mobile phase was delivered with a Waters Acquity ultra-performance liquid chromatographic system and consisted of linear gradient changes involving two buffers: buffer A, 1% acetic acid in water; buffer B, methanol. The mobile phase flow rate was $300\text{ }\mu\text{L}/\text{min}$. The gradient (A/B) was as follows: from 0 to 2 minutes, 99.6%/0.4%; from 2 to 3 minutes, to 98.0%/2.0%; from 3 to 4 minutes, to 85.0%/15.0%; from 4 to 6.5 minutes, to 99.6%/0.4%. Instrument settings were as follows: sample tray temperature, 10°C ; column temperature, 50°C ; ion spray voltage, 4.0 kV; ion transfer tube temperature, 350°C ; source vaporization temperature, 320°C ; Q2 collision-induced dissociation gas, argon at 1.5 mTorr; sheath gas, nitrogen at 60 psi; auxiliary gas, nitrogen at 35 psi; Q1/Q3 width: 0.7/0.7 units full-width half-maximum; scan width, 0.6 units; scan time, 0.01 seconds. The m/z for the parent ions and daughter ions, the collision energy, and retention time of measured purines are shown in Table 1. The limit of detection for purines in this assay system is estimated to be 0.2 nM.

Statistical Analysis. Data were analyzed by 1-factor analysis of variance or Student's *t* test as appropriate. The criterion of significance

TABLE 1

Parameters for selected reaction monitoring

Purine	Parent Ion	Collision Energy	Daughter Ion	Approximate Retention Time
	<i>m/z</i>	<i>volts</i>	<i>m/z</i>	<i>min</i>
$^{13}\text{C}_{10}$ -Adenosine	278	19	141	3.35
2',3'-cAMP	330	28	136	2.34
3',5'-cAMP	330	28	136	3.68
5'-AMP	348	19	136	1.72
3'-AMP	348	19	136	2.16
2'-AMP	348	19	136	3.10
Adenosine	268	19	136	3.29

was $P < 0.05$. All values in text and figures are means $\pm$ S.E.M. Data were normalized to nanomolar per microgram of protein.

Results

Schwann Cell Culture Purity and CNPase Expression. To ensure the purity of our primary Schwann cell cultures, cells isolated from rat pups (postnatal day 2) were grown on poly-D-lysine-coated glass chamber slides and probed for known Schwann cell markers. All the cells in the culture were positive for the Schwann cell markers S100 (Dong et al., 1999) (Fig. 1A) and CNPase (Sprinkle, 1989) (Fig. 1B). In vivo myelin genes, including CNPase, are upregulated when Schwann cells differentiate. Accordingly, we used a previously characterized "defined" medium to promote differentiation in vitro (Cheng and Mudge, 1996). In the present study, we validated the method by using western blot to examine the expression levels of CNPase in Schwann cells cultured only in growth medium versus cells differentiated for 72 hours in defined medium. The levels of CNPase were approximately twice as high in cells differentiated in defined medium compared with those cultured only in growth medium (Fig. 1, C and D), whereas the expression of TNAP was unchanged. For the metabolism studies, we used the defined medium to promote Schwann cell differentiation.

2',3'-cAMP and 3',5'-cAMP Conversion to 2'-AMP, 3'-AMP, and 5'-AMP by Schwann Cells. Figure 2 illustrates the ability of Schwann cells to metabolize extracellular 2',3'-cAMP and 3',5'-cAMP to their respective AMPs. When Schwann cells were incubated with increasing concentrations of extracellular 2',3'-cAMP, there was a robust and concentration-dependent increase in the amount of 2'-AMP formed (Fig. 2A). The maximal amount of 2'-AMP detected was $187.2\text{ nM}/\mu\text{g}$ protein obtained with $30\text{ }\mu\text{M}$ of 2',3'-cAMP. The Schwann cells formed 3'-AMP from 2',3'-cAMP at a rate almost 100-fold less than that of 2'-AMP (Fig. 2B). As expected, there was no formation of either 2'-AMP or 3'-AMP from incubation with any concentration of extracellular 3',5'-cAMP. 3',5'-cAMP was able to form only trace amounts of 5'-AMP, 10-fold less than 3'-AMP and 1000-fold less than 2'-AMP (both from 2',3'-cAMP) (Fig. 2C). There was no appreciable amount of 5'-AMP from extracellular 2',3'-cAMP.

In an effort to elucidate the identities of the phosphodiesterases expressed by Schwann cells that perform the various cAMP to AMP reactions, we used well characterized phosphodiesterase inhibitors (IBMX and DPSPX) in the presence of either 2',3'-cAMP or 3',5'-cAMP. The conversion of 2',3'-cAMP to 2'-AMP was not inhibited by 1 mM of IBMX (a cell-permeable, pan-phosphodiesterase inhibitor) but was significantly

Fig. 1. Schwann cell culture purity and CNPase expression. Cultures were probed with the Schwann cell markers S100 red (A) and CNPase green (B) to confirm the purity of the primary rat Schwann cell cultures (4',6-diamidino-2-phenylindole, blue; scale bar = 50 μ m). (C) Western blots for TNAP, CNPase, and α -tubulin on Schwann cell lysates from cultures grown in either growth or defined medium. Defined medium did not alter expression of either TNAP or α -tubulin, but increased expression of CNPase. (D) Quantification of CNPase expression between the cultures grown in growth medium versus defined medium (normalized to α -tubulin as a loading control) revealed a *significant increase in CNPase cultures grown in defined medium. Ab, antibody; CNP1, CNPase isoform type 1; CNP2, CNPase isoform type 2.

inhibited by 41% by 1 mM of the non-cell-permeable phosphodiesterase inhibitor DPSPX (Fig. 3A). The conversion of 2',3'-cAMP to 3'-AMP was not inhibited by 1 mM of either IBMX or DPSPX (Fig. 3B). In contrast, the formation of 5'-AMP from 3',5'-cAMP was nearly abolished by 1 mM of IBMX or DPSPX (Fig. 3B).

Conversion of 2',3'-cAMP, 3',5'-cAMP, 2'-AMP, 3'-AMP, and 5'-AMP to Adenosine by Schwann Cells. Next we sought to determine the ability of Schwann cells to convert the individual cAMPs and AMPs to adenosine. Although Schwann cells were able to metabolize some 2'-AMP, 3'-AMP, and 5'-AMP (Fig. 4A) and 2',3'-cAMP (Fig. 4B) to adenosine, these reactions were very slow and inefficient. For example, based on our previously published studies in oligodendrocytes (Verrier et al., 2013), oligodendrocytes were approximately 3-, 2.5-, 29-, and 5-fold more efficient than Schwann cells with regard to metabolizing 2'-AMP, 3'-AMP, 5'-AMP, and 2',3'-cAMP, respectively, to adenosine. Moreover, Schwann cells had no ability to convert 3',5'-cAMP to adenosine. Although clearly Schwann cells have limited capacity to convert cAMPs and AMPs to adenosine, we examined the effects of the known CD73 inhibitor AMPCP (0.1 mM) and TNAP inhibitor levamisole (1 mM) on the conversion of the AMPs to adenosine. AMPCP had no effect on the conversion of either 2'-AMP (Fig. 5A) or 3'-AMP (Fig. 5B) to adenosine. In contrast, AMPCP inhibited the formation of adenosine from extracellular 5'-AMP (Fig. 5C). Levamisole did not affect the metabolism of the AMPs to adenosine (Fig. 5).

Formation and Release of Endogenous cAMPs and AMPs from Sciatic Nerves. Utilizing an ex vivo nerve transection model, we next sought to determine if the sciatic nerve releases endogenously produced 2',3'-cAMP. For these experiments, adult rat sciatic nerves were isolated and cut into small sections and incubated for either 0 (basal), 1, or 3 hours. As shown in Fig. 6A, when the nerve sections were incubated for 1 and 3 hours in serum-free medium, the levels of 2',3'-cAMP inside the nerve increased when compared with

baseline samples. In addition, the levels of 2'-AMP increased in the medium significantly at 1 and 3 hours (Fig. 6B); however, 3'-AMP was not detected in the medium at any time point. Similar to 2',3'-cAMP, levels of 3',5'-cAMP also increased within the injured nerve at 1 and 3 hours (Fig. 6C). However, in contrast to 2'-AMP, levels of 5'-AMP in the medium actually decreased at 1 and 3 hours (Fig. 6D). Adenosine levels in the nerve did not change over time (Fig. 7A), whereas adenosine levels in the medium declined at 3 hours (Fig. 7B).

Effects of DPSPX on CNPase Activity. Because in Schwann cells, DPSPX (1 mM) decreased the metabolism of exogenous 2',3'-cAMP to 2'-AMP by 41%, we entertained the hypothesis that DPSPX is a CNPase inhibitor. Indeed, as shown in Fig. 8, 1 mM of DPSPX inhibited the production of 2'-AMP from 2',3'-cAMP by recombinant human CNPase by 35%, suggesting that most of the inhibition of 2'-AMP production by DPSPX in Schwann cells was mediated by direct inhibition of CNPase. At 10 mM, DPSPX reduced CNPase activity by 75%.

Discussion

A major objective of the present investigation was to test the hypothesis that Schwann cells are capable of metabolizing 2',3'-cAMP to 2'-AMP. The motivation for this hypothesis was the fact that CNPase is highly expressed in Schwann cells (Sprinkle, 1989), a finding confirmed in our Schwann cell cultures. The present study clearly demonstrates that indeed Schwann cells can metabolize extracellular/exogenous (i.e., added to the culture medium) 2',3'-cAMP to 2'-AMP. Importantly, Schwann cells have little ability to convert extracellular 2',3'-cAMP to 3'-AMP or to metabolize extracellular 3',5'-cAMP to either 5'-AMP or 3'-AMP. In addition, Schwann cells per se have very limited capacity to convert any of the AMPs to adenosine. These findings suggest that if 2',3'-cAMP is synthesized and released into the extracellular compartment, any Schwann cells in the vicinity would rapidly metabolize the 2',3'-cAMP to 2'-AMP but would likely not

Fig. 2. Schwann cell 2',3'-cAMP and 3',5'-cAMP metabolism. Line graphs show the concentration-dependent effects in Schwann cells of 2',3'-cAMP and 3',5'-cAMP on the extracellular levels of 2'-AMP (A), 3'-AMP (B), and 5'-AMP (C). For visual comparisons of 2'-AMP versus 3'-AMP and 5'-AMP, the main graphs show results using the same y-axis scale. However, since levels of 3'-AMP and 5'-AMP were very low, also shown are the levels of 3'-AMP and 5'-AMP using an expanded (magnified) y-axis scale. Values represent the mean $\pm$ S.E.M., $n = 6$ for each group. ^a $P < 0.05$ versus basal. ANOVA, analysis of variance.

metabolize 2'-AMP further to adenosine. Our results also suggest that if 3',5'-cAMP is made and released into the extracellular compartment, any Schwann cells in the vicinity would not metabolize 3',5'-cAMP to 5'-AMP and would likely not metabolize 5'-AMP further to adenosine.

Endogenous 2',3'-cAMP is formed intracellularly from the breakdown of RNA (Thompson et al., 1994), which occurs rapidly following cell injury, and is transported to the extracellular compartment. The fact that Schwann cells can metabolize extracellular 2',3'-cAMP to 2'-AMP, but only slightly to 3'-AMP, suggests that 2',3'-cAMP produced within peripheral axons would, upon transport to the extracellular compartment, be metabolized mostly to 2'-AMP. Our

observation that nerve injury (transection of nerves followed by incubation) is associated with a time-dependent increase in intracellular 2',3'-cAMP and extracellular 2'-AMP, but not extracellular 3'-AMP, is consistent with this prediction. The observation that Schwann cells have little ability to metabolize extracellular 3',5'-cAMP to 5'-AMP suggests that 3',5'-cAMP produced within peripheral axons would not, even upon transport to the extracellular compartment, be metabolized to 5'-AMP. Our finding that nerve injury is associated with a time-dependent increase in intracellular 3',5'-cAMP, but not extracellular 5'-AMP, is also consistent with the results in Schwann cells. Finally, the fact that Schwann cells have very limited ability to convert any of the AMPs to adenosine is

Fig. 3. Effects of phosphodiesterase inhibitors on metabolism of 2',3'-cAMP and 3',5'-cAMP in Schwann cells. Bar graphs show the effects of IBMX (1 mM) and DPSPX (1 mM) on Schwann cell metabolism of 2',3'-cAMP to 2'-AMP (A), 2',3'-cAMP to 3'-AMP (B), and 3',5'-cAMP to 5'-AMP (C). Values represent the mean $\pm$ S.E.M., $n = 6$ for each group. ^a $P < 0.05$ versus PBS; ^b $P < 0.05$ versus no inhibitor; ANOVA, analysis of variance; No Inhib, no inhibitor present in the medium.

entirely consistent with the observation that nerve injury does not increase extracellular adenosine (which in fact decreases over time). However, adenosine production by peripheral nerves may occur via capsaicin-sensitive sensory afferents (Liu et al., 2002).

Taken together, the results of the present study suggest that peripheral myelinated nerves, when subjected to injury, generate 2',3'-cAMP, which is transported to the extracellular compartment and converted by Schwann cells to 2'-AMP. This mechanism may importantly affect the response of peripheral axons to injury. Recent studies show that 2',3'-cAMP activates mitochondrial permeability transition pores, a process that induces apoptosis and necrosis (Azarashvili et al., 2009). Therefore, when axons are injured, the metabolism of extracellular 2',3'-cAMP to 2'-AMP by Schwann cells would

Fig. 4. Schwann cell AMP and cAMP metabolism. Line graphs show the concentration-dependent effects in Schwann cells of AMPs (A) and cAMPs (B) on the extracellular levels of adenosine. Values represent the mean $\pm$ S.E.M., $n = 6$ for each group. ^a $P < 0.05$ versus basal; ANOVA, analysis of variance.

increase the concentration gradient between intracellular neuronal 2',3'-cAMP and extracellular 2',3'-cAMP. This in turn would facilitate the removal and inactivation of 2',3'-cAMP from the local environment. This role of Schwann cells may be critical to the preservation of nerve function and recovery postinjury. Indeed, CNPase knockout mice develop motor dysfunction with aging (Lappe-Siefke et al., 2003). Although this certainly involves CNS mechanisms, the lack of CNPase in Schwann cells could conceivably also contribute to motor nerve dysfunction. Future studies are required to test this hypothesis.

Of potential pharmacological interest is the observation that Schwann cells appear to have little ability to metabolize any of the AMPs to adenosine. This finding is corroborated by our observations that in injured nerves, extracellular levels of adenosine fall, rather than rise, rapidly with time. Adenosine is an important anti-inflammatory nucleoside that suppresses multiple aspects of both the innate and adaptive arms of the immune system, resulting in the reduced production and release of inflammatory cytokines (Hasko and Cronstein, 2004). In fact, there is a growing body of evidence supporting the concept that regulatory T cells suppress effector T cells mainly via generating adenosine (Mandapathil et al., 2010; Ohta and Sitkovsky, 2014). The lack of adenosine production by injured peripheral nerves would allow more aggressive immune responses in the vicinity of the injured nerves, leading to loss of function and slower recovery. Additionally, in the PNS, extracellular adenosine is very much implicated in pain signaling post nerve injury, with the A₁ receptor being the primary adenosine receptor involved in inhibiting pain transmission (Johansson et al., 2001). Our findings suggest then that pharmacological approaches to increase adenosine levels in the vicinity of injured peripheral nerves may be useful for

Fig. 5. Effects of CD73 inhibitor and alkaline phosphatase inhibitor on Schwann cell metabolism of AMPs to adenosine. Bar graphs show the effect of AMPCP (CD73 inhibitor; 0.1 mM) and levamisole (TNAP inhibitor; 1 mM) on the conversion of 2'-AMP (A), 3'-AMP (B), and 5'-AMP (C) to adenosine by the primary Schwann cell cultures. Values represent the mean $\pm$ S.E.M., $n = 6$ for each group. $^aP < 0.05$ versus PBS; $^bP < 0.05$ versus no inhibitor. ANOVA, analysis of variance; No Inhib, no inhibitor present in the medium.

Fig. 6. Production of endogenous 2',3'-cAMP, 2'-AMP, 3',5'-cAMP, and 5'-AMP by sciatic nerves. Ex vivo transected sciatic nerve pieces were incubated for 0, 1, or 3 hours. At the indicated times, the medium was collected and the nerve sections were extracted. Both medium and nerve extracts were analyzed by liquid chromatography–tandem mass spectrometry for 2',3'-cAMP (A), 2'-AMP (B), 3',5'-cAMP (C), and 5'-AMP (D). Values represent the mean $\pm$ S.E.M., $n = 7$ for each group. $^aP < 0.05$ versus PBS; ANOVA, analysis of variance.

preventing subsequent immune-response injury of damaged nerves and to modulate peripheral pain signaling. These hypotheses require further investigation.

It is notable that, in Schwann cells, the major metabolite of 2',3'-cAMP is 2'-AMP. It is quite possible that 2'-AMP has pharmacological effects on axons that are completely independent of its metabolism to adenosine. It is tempting to speculate that 2'-AMP (or 3'-AMP) may be an agonist for adenosine receptors, as has been shown for 5'-AMP (Rittiner et al., 2012), and that this accumulation of 2'-AMP can activate the A_1 receptor postinjury and thus suppresses nociceptive signaling. However, because little is known regarding the pharmacology of 2'-AMP, at present, we cannot speculate as to whether 2'-AMP levels should be augmented or reduced to protect myelinated nerves from injury. Importantly, inhibition of either CD73 or alkaline phosphatase in Schwann cells does not alter the metabolism of 2'-AMP to adenosine, suggesting that these ecto-enzymes, at least in Schwann cells, normally play little role in the metabolism of 2'-AMP. Therefore, pharmacological manipulation of these ecto-enzymes may not alter 2'-AMP levels in Schwann cells.

Inhibition of phosphodiesterase with IBMX inhibits the modest production of 5'-AMP from 3',5'-cAMP, but does not alter the production of 2'-AMP or 3'-AMP from 2',3'-cAMP. Importantly, DPSPX (1 mM) abolishes the production of 5'-AMP from 3',5'-cAMP, reduces the conversion of 2',3'-cAMP to 2'-AMP, but does not affect the metabolism of 2',3'-cAMP to 3'-AMP. These findings suggest that the phosphodiesterases that metabolize 3',5'-cAMP to 5'-AMP, 2',3'-cAMP to 3'-AMP, and 2',3'-cAMP to 2'-AMP are distinct. It is likely that DPSPX potentially blocks the ecto-enzyme that converts 3',5'-cAMP to 5'-AMP and less potentially inhibits the ecto-enzyme that converts 2',3'-cAMP to 2'-AMP. In this regard, our

Fig. 7. Production of endogenous adenosine by sciatic nerves. Ex vivo transected sciatic nerve pieces were incubated for 0, 1, or 3 hours. At the indicated times, the medium was collected and the nerve sections were extracted. Both medium and nerve extracts were analyzed by liquid chromatography–tandem mass spectrometry for adenosine. Adenosine did not change in nerve extracts (A) but decreased in the medium (B). Values represent the mean $\pm$ S.E.M., $n = 7$ for each group. ^a $P < 0.05$ versus PBS; ANOVA, analysis of variance.

experiments with recombinant CNPase demonstrate that DPSPX is a direct inhibitor of CNPase. Since 1 mM of DPSPX similarly reduces the conversion of 2',3'-cAMP to 2'-AMP by recombinant CNPase and Schwann cells (35% versus 41%), it is likely that, in Schwann cells, CNPase is the enzyme responsible for the metabolism of 2',3'-cAMP to 2'-AMP. Ideally, this concept should be confirmed in Schwann cells isolated from CNPase knockout mice. Although we currently have a colony of CNPase knockout mice, culturing sufficient numbers of Schwann cells from mice to perform these kinds of metabolism studies is challenging.

Currently, there is no selective or potent inhibitor of CNPase. Our novel finding that high concentrations of DPSPX block CNPase provides a starting point for designing more potent and selective inhibitors of this enzyme. Low concentrations DPSPX block adenosine receptors (Daly et al., 1985); therefore, ideally it would be important to synthesize DPSPX analogs that have increased affinity for CNPase yet decreased affinity for adenosine receptors. Such analogs would be invaluable in determining the role of CNPase in physiology and pathology. Moreover, a potent and selective CNPase inhibitor might have therapeutic benefits. For example, if future studies indicate a detrimental role of 2'-AMP in peripheral nerve injury, a drug similar to DPSPX (but more potent) may be an option to reduce 2'-AMP production in response to peripheral nerve injury. Importantly, our previous studies in rat preglomerular vascular smooth muscle cells (Jackson et al., 2010) and mouse microglia and astrocytes (Verrier et al., 2011) show that DPSPX partially blocks the conversion of 2',3'-cAMP to 2'-AMP (but not 3'-AMP) in those cell types. In light of our new finding that DPSPX partially

Fig. 8. Inhibition of CNPase activity by DPSPX. Bar graph shows the effect of 1 and 10 mM DPSPX on recombinant human CNPase metabolism of 2',3'-cAMP to 2'-AMP. Values represent the mean $\pm$ S.E.M., $n = 6$ for each group. ^a $P < 0.05$ versus no inhibitor; No enzyme, no CNPase present; No Inhibitor, no DPSPX present in the medium.

blocks CNPase activity, these results likely mean that CNPase is involved in forming 2'-AMP in a variety of cell types.

In conclusion, the present study reveals that Schwann cells metabolize 2',3'-cAMP mostly to 2'-AMP, with very little subsequent conversion of 2'-AMP to adenosine. Thus, Schwann cells do not have a complete extracellular 2',3'-cAMP–adenosine pathway (2',3'-cAMP $\rightarrow$ 2'-AMP $\rightarrow$ adenosine), but do have a partial pathway (2',3'-cAMP $\rightarrow$ 2'-AMP). In addition, Schwann cells have neither a complete nor partial 3',5'-cAMP–adenosine pathway (3',5'-cAMP $\rightarrow$ 5'-AMP $\rightarrow$ adenosine). Finally, peripheral nerves in response to injury produce 2'-AMP (likely from Schwann cells acting on 2',3'-cAMP) but cannot maintain high levels of extracellular adenosine (likely because Schwann cells have little ability to metabolize AMPs to adenosine). Future work should focus on whether the conversion of 2',3'-cAMP to 2'-AMP is protective, detrimental, or both, and whether pharmacological approaches to increase adenosine or decrease 2'-AMP levels in the vicinity of injured peripheral nerves would protect from dysfunction and accelerate recovery.

Authorship Contributions

Participated in research design: Verrier, Kochanek, Jackson.

Conducted experiments: Verrier.

Performed data analysis: Verrier, Jackson.

Wrote or contributed to the writing of the manuscript: Verrier, Kochanek, Jackson.

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