Measurement of purine release with microelectrode biosensors

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Abstract Purinergic signalling departs from traditional paradigms of neurotransmission in the variety of release mechanisms and routes of production of extracellular ATP and adenosine. Direct real-time measurements of these purinergic agents have been of great value in understanding the functional roles of this signalling system in a number of diverse contexts. Here, we review the methods for measuring purine release, introduce the concept of microelectrode biosensors for ATP and adenosine and explain how these have been used to provide new mechanistic insight in respiratory chemoreception, synaptic physiology, eye development and purine salvage. We finish by considering the association of purine release with pathological conditions and examine the possibilities that biosensors for purines may one day be a standard part of the clinical diagnostic tool chest.

Keywords Biosensor · Real-time measurement · Epilepsy · Stroke · Chemosensory mechanisms · Development

The need for real-time purine measurement

The traditional paradigm of synaptic transmission involves exocytotic release of transmitter from a defined presynaptic

structure into the narrow gap of the synaptic cleft where it diffuses to activate receptors on the closely apposed postsynaptic membrane. Release is time-locked to the presynaptic action potential and results in the predictable occurrence of postsynaptic events on defined time scales. While capturing the essence of synaptic transmission, this paradigm is an oversimplification. For example, transmitters such as GABA [1] and glutamate [2] can spill out of the synaptic cleft to have more diffuse and widespread actions than this classical model suggests. Under pathological conditions, transmitters and neurotransmitters, such as glutamate [3], can be released by the reversal of Na⁺-dependent concentrative transporters.

The purines, ATP and adenosine, depart from this paradigm even more comprehensively. ATP can indeed be released at synapses [4, 5]. However, in the CNS, it seems that it acts mainly as a cotransmitter [6, 7], and there are very few synapses where it acts as the principal transmitter. ATP is also released by glial cells [8, 9] for the most part via exocytosis [10, 11] but see [12]. However, ATP can also be released via a variety of channel-mediated mechanisms (for example, via gap junction hemichannels [13–15] and volume-activated channels [16, 17]). Receptors for ATP are widespread throughout the CNS; yet, the cellular sources of ATP release to activate these receptors are not immediately obvious. In addition, although ATP can be broken down to ADP and further to adenosine, these breakdown products are themselves active at particular receptor subtypes.

The routes for adenosine release and the cellular sources seem to be even more mysterious than those for ATP. For example, there is abundant evidence that adenosine can arise from the breakdown of previously released ATP [18]. The accumulation of adenosine under these conditions will depend on the characteristics and distribution of the ectonucleotidases (a diverse set of proteins encoded by at

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least three gene families) present in the extracellular space. These enzymes are not necessarily arranged in a discrete anatomical structure; thus, the appearance of adenosine in the extracellular space may be widespread and diffuse.

There is also evidence that adenosine can be released in an activity-dependent manner [19, 20], although whether this represents direct adenosine release or production via the prior release of ATP and extremely rapid conversion to adenosine remains, at least for the time being, unclear [21]. Under pathological conditions, it seems that adenosine is indeed released directly [22, 23], although how this occurs and the molecules and transporters involved still have not been settled.

Given that much of purinergic signalling occurs outside of traditional synaptic structures and that there may not be any convenient electrophysiological correlates of purine release that can be measured, a method for directly measuring real-time release of ATP and adenosine has great potential value in the analysis of this extracellular signalling system. We recognized this need many years ago and invented and applied the first biosensors to the study of purinergic signalling [24, 25].

Desirable measurement characteristics

Before describing the new biological insights that have arisen from direct measurement of purine release, it is worthwhile to consider the characteristics of a useful measurement system. Any measurements should be capable of resolving changes in analyte concentration over meaningful time scales. Although conventional synaptic transmission occurs at millisecond time scales, the more diffuse actions of purines are considerably slower—seconds to minutes. The measurements should be spatially resolved—depending on the exact nature of the signalling involved, appropriate scales range from the size of the synaptic cleft to the gross extracellular space. The measuring system should be sensitive over a physiologically relevant concentration range and capable of providing a quantitative measurement. Once again, the meaningful concentration range is very broad, ranging from the nanomolar to the low micromolar. Finally, a good measurement system should be minimally invasive. Increasingly many investigators desire measurement of chemical signalling in freely moving awake animals as they perform behavioural tasks. From this brief listing of desirable measurement characteristics, it should be readily apparent that no single measurement system can fulfil the entire range of requirements. For example, a measurement system capable of monitoring purine release at the synaptic cleft may be unsuited to measurement in the general extracellular space (and vice versa); a highly sensitive measurement system capable of resolving nanomolar concentrations may lose linearity at higher concentration levels.

Measurement methods

Microdialysis

One of the most widely used methods for purine measurement is microdialysis whereby a small microdialysis probe is implanted into a tissue. Slow perfusion of dialysate collects low-molecular-weight compounds in the vicinity of the probe. Analysis of the dialysate is usually by HPLC or a related method. This method has the advantage that it can be used to detect several substances at once and is potentially quantitative [26, 27]. However, the temporal resolution is limited usually to the minute time scales. Also, because the probes are implanted, a significant tissue reaction can occur (reactive gliosis), and this can create a local microenvironment where the concentration of transmitters and other signalling substances can be significantly perturbed [28, 29].

Luciferase

The bioluminescent enzyme luciferase has been the oldest method used to measure the release of ATP [30]. Indeed, this method is still used today [8, 14]. It has the advantage of being highly specific. However, there are significant disadvantages: the amount of light produced is low necessitating the use of highly sensitive cameras and comprehensive measures to eliminate stray sources of light if real-time spatially resolved measurements are to be attempted; it can be hard to obtain quantitative data; and it is not easy to use *in vivo*, but this has been achieved [31]. This method can only measure ATP.

Sniffing

Another method that has been used is to exploit ligand-gated ATP channels (usually P2X₂ receptors) as tiny biosensors of ATP release. This method has been used in two ways—in isolated membrane patches (“patch sniffing”) [32] or in whole cells (“sniffer cells”) [33]. As this method exploits the very receptors that are involved in ATP signalling, it will detect ATP concentrations in an appropriate range. It is capable of providing good temporally resolved data. However, quantitation is hard to achieve with this method, and it is practically limited to use *in vitro*. Once again, while this is well suited to ATP, it has not been achieved for adenosine.

Recombinant receptors

Genetic expression of modified recombinant receptors targeted to particular cells and subcellular structures is an exciting new method that is highly suited to the analysis of ATP release at the synapse. This has been currently achieved by fusing a green fluorescent protein-based Ca²⁺

sensor onto a P2X receptor [34]. As these receptors are highly permeable to Ca^{2+} , the nearby sensor gives an excellent signal related to receptor activation (and hence ATP release). This method has resolved synaptic release of ATP and is potentially capable of exquisite spatial resolution. However, it has the disadvantage that the expression of exogenous P2X receptors within neurons will alter their integrative and signalling properties, lessening its utility of use in vivo or in system-level investigations of purinergic signalling. In principle, other receptors, especially those that are G-protein coupled, could be adapted to provide FRET signals proportional to ligand binding and could be adapted as genetically encoded biosensors. This could be particularly useful for measurements of adenosine, but to our knowledge, this has not yet been achieved.

Microelectrode biosensors

Our particular approach has been to develop microelectrode biosensors for the purines [35, 36]. While there are many ways biosensors can operate (indeed luciferase, patch sniffing, and recombinant receptors are all biosensing approaches for the detection of purines), we have developed electrochemical biosensors based around oxidases to provide a real-time signal for both ATP and adenosine and downstream purines. The basic principle is that an enzymatic cascade recognizes the purine of interest and produces a product (H_2O_2) proportional to the analyte concentration. This product can then be detected electrochemically at the microelectrode surface.

As neither “ATP oxidase” nor “adenosine oxidase” exists, we have developed two enzymatic cascades to achieve detection. For ATP, this relies upon the enzymes glycerol

kinase and glycerol-3-phosphate oxidase. If glycerol is provided at a level that saturates glycerol kinase (>0.5 mM), the reaction becomes solely dependent on ambient ATP concentrations [36]. The product of the first step, glycerol-3-phosphate, is the substrate for the second, peroxide producing, enzyme (Fig. 1a) that gives the final readout proportional to ATP concentration.

An alternative ATP detection cascade has been reported: hexokinase and glucose oxidase [37]. Here, the hexokinase converts glucose to glucose-6-phosphate, which is not a substrate for glucose oxidase (Fig. 1b). Thus, the signal is manifested as a loss of the glucose oxidation current. This can work well in vitro, but this biosensor necessarily has to operate at glucose concentrations around the K_m for glucose oxidase and is therefore also glucose sensitive. Thus, in vivo, where local glucose concentrations may well change with time, this detection cascade cannot be used with confidence that it will report only ATP concentrations as opposed to a combination of changes in ATP and glucose concentrations. For adenosine, we use a cascade of three enzymes: adenosine deaminase, nucleoside phosphorylase and xanthine oxidase (Fig. 1c) to produce an electrochemical signal proportional to adenosine concentration [35].

A key point to remember is that multienzyme biosensors are sensitive to the substrates of all enzymes present in the cascade. Any measurement protocol must therefore take this into account. One way to do this is to compare the signal derived from biosensors having the complete measurement cascade, with reference sensors that lack the first enzyme. Other controls are also possible and have been previously described in detail [38].

A further requirement for the practical application of biosensing to physiological measurements is that the biosensors should be selective for the compound(s) of

Fig. 1 Enzyme cascades for electrochemical detection of ATP and adenosine. Each cascade results in a change in production of H_2O_2 that is proportional to the analyte concentration. Note that in (b) ATP results in a *loss* of H_2O_2 as the presence of ATP would drive the hexokinase reaction at the expense of gluconolactone and H_2O_2

interest. While this can be conferred by the enzymes involved, electrochemical biosensors have a complication all of their own. In general, unless special technical measures are taken, these biosensors operate at rather positive potentials necessary for the oxidation of H_2O_2 . Unfortunately, at these potentials, many other compounds, some of which are common in physiological systems, will also oxidize and generate a current unrelated to the specific analyte—so-called electrochemical interference.

There are several ways round this [38]. One is to use rather complex electrode designs which involve electrochemical mediators so that the operating potential can be reduced to the point where very few compounds are capable of being oxidized (or reduced) to give a nonspecific current. This solution may be very useful for diagnostic applications (see later) but in general greatly slows the response time of the biosensor, a significant disadvantage for applications to physiological signalling. A common alternative is the use of a permselective barrier layer that excludes interferences, but still permits the detection of the enzymatically produced H_2O_2 . Because all the layers involved can be very thin, this latter method has the advantage of not slowing biosensor response time (Fig. 2).

No matter what strategy is used to enhance the selectivity of electrochemical biosensors, no method gives an absolute guarantee of perfect selectivity. For example, mediators may react directly with an interference to give a spurious signal and permselective layers may not effectively exclude very low-molecular-weight interferences (e.g., nitric oxide). Therefore, any use of biosensors should be accompanied by well-performed controls to check the veracity of the observed signal. These issues have been considered in detail previously [38]. A very useful and

powerful approach to employing biosensors as part of an experimental programme is to make predictions from the biosensor recordings that can subsequently be tested by an independent experiment, for example, by use of selective antagonists to block the predicted actions of purines.

New biological insight from purine measurements

The real reason for introducing any new analytical technology to the study of biological systems is the new insight into mechanism that such technologies empower. We therefore briefly review a series of studies where a direct measurement of purine release has led to significant advance and provide a table giving abbreviated details of studies conducted to date (Table 1).

CO_2 chemosensory transduction

The control of PCO_2 in arterial blood is a vital physiological function that regulates the pH of all bodily fluids. Both peripheral and central chemosensors measure PCO_2 and cause adaptive changes in breathing to closely regulate its level. The predominant theory of chemoreception (“reaction theory”) put forward by Winterstein and Loeschcke proposes that PCO_2 is measured only via consequent changes in pH [39, 40]. However, other molecules could potentially signal changes in PCO_2 – CO_2 itself and HCO_3^- , and these alternatives have rather been neglected. In the brain, it is clear that the ventral surface of the medulla oblongata (VMS) contains at least some of the central chemoreceptors; however, other nuclei such as the locus coeruleus have also been proposed [41, 42]. At the VMS, Loeschcke and Mitchell proposed rostral, intermediate and caudal chemosensitive areas [43, 44]. The rostral area appears to correspond to the retrotrapezoid nucleus. Guyenet and colleagues have adduced considerable evidence for the existence of chemosensitive neurons in this nucleus that appear important in the regulation of breathing, at least in the neonate [45, 46]. Neurons of the medullary raphe have also been shown to be chemosensitive [47, 48] and associated with blood vessels in a manner highly suggestive of a chemosensory function [48].

Nevertheless, there remains considerable uncertainty over the mechanisms of CO_2 chemoreception and the competing claims of the various proposed nuclei and neurons. Application of ATP biosensing to the analysis of chemoreception has been very productive. This showed that during hypercapnia, ATP is released specifically from the classical chemosensitive areas at the ventral surface of the medulla (Fig. 3a) and that the release occurred before the adaptive changes in breathing [49]. This gave rise to the prediction that ATP may be released from the chemo-

Fig. 2 Schematic of a microelectrode biosensor. The electrode 7–50 μm in diameter protrudes from a glass body. The sensing tip is covered with multiple layers designed to enhance the selectivity and biocompatibility of the biosensor. The enzymatic layer contains the cascades of Fig. 1 and thereby impart sensitivity to the desired analyte by producing changes in H_2O_2 in proportion to the concentration of the analyte. The H_2O_2 can permeate the permselectivity layer to be detected at the microelectrode surface (usually polarized at 500–600 mV with respect to an Ag/AgCl reference electrode). Interferences are unable to permeate the inner and outer permselectivity layers

Table 1 Examples of the use of microelectrode biosensors for the measurement of ATP or adenosine in biological tissues

Brain area or tissue	Functional context	Analyte	References
Spinal cord	Locomotion	Adenosine, ATP	[24, 35, 36]
Medulla oblongata	Hypoxia–respiration	Adenosine	[107]
Medulla oblongata (preBotzinger complex)	Control of respiration	ATP	[108]
Medulla oblongata (nucleus tractus solitarius)	Afferent control of breathing	ATP	[109]
Medulla oblongata (ventrolateral)	Hypoxia	ATP	[110]
Medulla oblongata (ventral surface)	H ⁺ /CO ₂ chemosensory control of breathing	ATP	[15, 49, 52, 53]
Medulla oblongata (nucleus tractus solitarius)	Hypothalamic defence reaction	Adenosine	[111]
Cerebellum	Activity-dependent release	Adenosine, ATP	[19, 20, 112, 113]
Hypothalamus	Induction of fever	ATP, adenosine	[114]
Hypothalamus	Glucosensing by tanycytes	ATP	[115]
Hippocampus	CO ₂ sensitivity and seizures	Adenosine	[78]
Hippocampus	Seizure generation and control	Adenosine, ATP	[72, 80]
Cortex	Spreading depression	ATP	[116]
Hippocampus	Hypoxia, ischaemia	ATP, adenosine	[22, 25, 67, 68, 70, 117, 118]
Thalamus	Deep brain stimulation	Adenosine	[119]
Striatum	Deep brain stimulation	Adenosine	[120]
Cultured cells	Ischaemic preconditioning	Adenosine	[121]
Neuronal progenitor cells	Proliferation and differentiation	ATP	[122]
Retina	Neural development	ATP	[123]
Amphibian embryo	Early development	ATP	[64]
Carotid body	Chemosensitivity	ATP	[124] ^a
Ileum –myenteric plexus	Cannabinoid action	Adenosine	[125]
Intestinal tissue	ATP release in ileum	ATP	[126] ^a
Skeletal muscle arterioles	Origins of ATP release from arterioles	ATP	[127]

^a ATP measurement by the cascade in Fig. 1b; all other examples utilize the cascade of Fig. 1a

Fig. 3 ATP release during CO₂ chemosensory transduction. **a** Schematic of the ventral surface of the medulla oblongata showing sites of in vivo ATP release (black circles) and no ATP release (open circles) relative to known landmarks and major blood vessels (7n 7th nucleus, XII XIIth nerve, py pyramids) in an anaesthetized, ventilated rat. Sample records of ATP release (Net ATP) for different locations shown on the right in combination with end-tidal CO₂ and integrated phrenic nerve activity (PNG). Reproduced with permission from [49].

b In vitro recordings of ATP release, measured from the caudal location near to the XIIth nerve, evoked by changes in PCO₂ at constant extracellular pH. Note that a reduction of PCO₂ from an initial level of 35 mmHg causes a drop in ATP release at the surface of the medulla and that there is a resting ATP tone, visible when the sensors are removed from the surface of the medulla at the end of the recording (top trace, dotted lines). Reproduced with permission from [15]

sensory cells and act as the initial trigger for neuronal activation. Application of ATP antagonists to the sites of ATP release gives support to this hypothesis by considerably reducing the extent of the adaptive changes in breathing in response to hypercapnia [49]. The question of how this ATP release may relate to known populations of chemosensitive neurons was initially vexatious, with the evidence suggesting no effect of ATP antagonists (in the neonate) on the chemosensory responses of retrotrapezoid neurons [50]. However, this negative evidence has more recently been overturned—when challenged with a CO₂ stimulus in the neonate (as opposed to a pH stimulus), a significant component of the retrotrapezoid neuron responses is indeed ATP receptor-dependent [51]. At adult stages, the response of these neurons is almost entirely dependent on the prior release of ATP [52].

However, ATP biosensing has had two even more profound consequences for understanding of CO₂ chemosensory mechanisms. Through pursuit of CO₂-dependent ATP release in isolated slices of the VMS (Fig. 3b), a new chemosensory transducer has been identified that appears directly sensitive to CO₂ [15, 53]. This is connexin26 (Cx26) which is correctly located to sense changes in PCO₂ (in the leptomeninges, the astrocytes of the marginal glial layer at the ventral surface and in cells adjacent to penetrating blood vessels). CO₂-dependent ATP release in these slices depends upon Cx26, and the expression of Cx26 in a cell line is sufficient by itself to recapitulate CO₂-dependent ATP release. Recordings from isolated patches show that Cx26 will respond to alterations of PCO₂ at a constant pH. The model emerging from these studies is that Cx26 is both the conduit for ATP release and the transducing molecule capable of directly detecting PCO₂. For the first time, this gives a mechanistic alternative or addition to the reaction theory that has dominated the field for so long.

In parallel with this work, Gourine and colleagues have examined the sensitivity of astrocytes of the VMS to pH [52]. They have demonstrated that changes in pH can evoke Ca²⁺ waves in these astrocytes that depend on the prior release of ATP (probably in this case via exocytosis). By expressing channelrhodopsin2 in astrocytes, they have shown that activation of astrocytes by these light-sensitive channels will enhance breathing through an ATP receptor-dependent mechanism.

The initial and continued application of ATP biosensing to CO₂ chemosensory transduction has thus had a transformative effect: identification of new cellular components of the reflex machinery; reevaluation of the roles of some of the known components, and identification of a new transducing molecule causally related to respiratory chemoreception that is directly sensitive to CO₂.

Direct activity-dependent adenosine release

The prevailing orthodoxy is that except under pathological conditions, adenosine is not directly released from neurons, but arises instead from the catabolism of previously released ATP. Indeed, there is an abundance of extracellular enzymes capable of mediating this conversion of ATP all the way to adenosine [18, 54–57]. Although there are several examples of activity-dependent adenosine release [21], these have largely been viewed as arising from exocytotic release of ATP and subsequent conversion to adenosine. One of the problems of assessing the mechanisms underlying adenosine release is that ionotropic adenosine receptors do not exist; therefore, adenosinergic miniature PSCs, indicative of quantal release which would conclusively settle the issue, cannot be observed.

Adenosine biosensors have sufficient sensitivity and temporal resolution to measure spike-evoked adenosine release in the cerebellum [19]. The use of biosensors provides an equivalent measure to the postsynaptic potentials observed at the synapses mediated by more conventional transmitters. The biosensor recordings reveal that adenosine release has the characteristics of exocytosis—tetrodotoxin and Ca²⁺ dependence [19]. Furthermore, adenosine release depends on spike width and can exhibit short-term plasticity more typically connected with conventional synaptic potentials. For example, at very short intervals (50 ms), adenosine release undergoes paired pulse facilitation, while at longer intervals (30–120 s), it undergoes paired pulse depression [20] (Fig. 4). These data are even more suggestive of an exocytotic release mechanism, which can undergo depletion and rather slow restocking [20].

It remains possible that this activity-dependent adenosine release conforms to the current orthodoxy of prior synaptic ATP release and conversion to adenosine. However, under the same conditions where adenosine release is observed, no corresponding “upstream” ATP release has been resolved with ATP biosensors, even when the adenosine release has been greatly enhanced by use of 4AP to broaden the presynaptic spikes. The data at present would suggest that if this does indeed occur, the conversion must be localised extremely close to the sites of adenosine release and must be very efficient. The current lack of potent and selective inhibitors of the enzymes that break ATP down has not allowed rigorous testing of the two competing mechanisms to date. Nevertheless, the use of biosensor recordings has revealed a richness to activity-dependent adenosine release that was unknown before the application of these methods.

Eye development

The gene network, involving *Pax6*, *Six3*, *Rx1* and *Lhx2* (sometimes referred to as the eye-field transcription

Fig. 4 Spike-evoked adenosine release displays short-term plasticity. **a** Adenosine release evoked by a single spike in the parallel fibres of a cerebellar slice. **ai** raw trace, *inset* shows the decay of the signal can be fitted by a single exponential process, *green line* is a minimal theoretical model of the data (rapid initial rise of adenosine followed by exponential decay, details in [20]); and **aai** deconvolution of the signal (effectively to remove the slowest component of the decay due to diffusion), *green line* is deconvolution of the minimal theoretical model. **b** Demonstration of short-term plasticity of adenosine release during paired stimuli at different interpulse intervals. **bi** raw records at intervals indicated below, *inset* showing that the decay can again be fitted by a single exponential process, **bii** the deconvoluted records. At intervals of 30 s or longer, adenosine release undergoes paired pulse depression. At very short intervals (50 ms), adenosine release exhibits paired pulse facilitation. Reproduced with permission from [20]

factors), that determines the development of the eye has been well characterised in a number of organisms [58–60]. *Pax6* and its orthologues have been referred to as master regulators of eye development and are central to the gene networks involved in a number of different organisms, including *Drosophila*. This has given rise to the notion that

despite their very different outward appearance, the vertebrate and invertebrate eyes represent divergent evolution from a common precursor [61, 62].

The mechanisms by which the gene network for eye development becomes activated remain an area of active investigation [63]. However, a role for ATP signalling is now evident [64]. Masse et al. reported that overexpression of *NTPDase2* in the anterior part of the embryo could induce ectopic eye formation—an effect that was increased by simultaneous overexpression of the $P2Y_1$ receptor. Manipulation of the expression of *NTPDase2* altered the expression of the gene network for eye development. In particular, simultaneous knockdown of the expression of *NTPDase2* and the $P2Y_1$ receptor could almost completely abolish *Pax6* and *Rx1* expression. Even more convincingly, this combined knockdown could, in some cases, completely prevent the development of the eye. These observations demonstrate that endogenous purinergic signalling by ADP, generated by *NTPDase2* and acting on the $P2Y_1$ receptor, is responsible for the activation of the gene network that determines eye formation.

In the same study, the authors used ATP biosensors to measure ATP release at the site of future eye formation around the time of activation of the eye-field transcription factors (Fig. 5). What was surprising is that they observed a short period (a few minutes) of ATP release in the eye field. While the use of ATP biosensors was secondary to the fundamental discovery of the importance of purinergic signalling in eye development, the direct measurement of ATP release in the very early embryo has nevertheless introduced the concept that rather discrete and brief signalling events can have very long-term consequences. In this context, a signal lasting only a few minutes activated a gene network that stays active for many hours. Interestingly, the eye-field transcription factor network is not a

Fig. 5 Transient release of ATP in the eye field of the developing embryo. *ATP* and *Null* (no enzyme control) biosensors were placed in the anterior part of the developing neural plate in the eye fields, and a second *ATP* biosensor at a more posterior location (*inset*). Recordings were from embryonic stage 11 (before expression of the eye-field transcription factor genes) to 12.5. Midway through, a transient period of ATP release was observed in the eye field (*main traces* and *inset*).

This ATP may represent the trigger (after conversion to ADP) for expression of the eye-field transcription factor genes. Note that ATP release does not occur at this time in the posterior location, although at later times, there is a clearly substantial ATP release in this location that may be involved in the control of the development of the future nervous system. Reproduced with permission from [64]

simple sequential chain of gene activation, but involves both positive feedback from downstream genes to activate upstream genes and positive self-regulation of gene expression. *Pax6* for example activates *Six3*, *Lhx2* and *tll*, but these genes in turn upregulate expression of *Pax6*, which is also able to reinforce its own expression. It is, therefore, highly plausible that a self-reinforcing gene network such as this would only require a relatively brief signal to initiate stable expression over many hours [65].

Purine salvage

The fact that adenosine can be detected in peripheral blood during cerebral ischemia in humans [66] potentially ushers in an era of clinical diagnosis based upon purines (see below). However, from the point of view of cellular bioenergetics, this means that the ability of the brain to resynthesise ATP after metabolic stress is compromised, which may form the basis of a depletable pool of adenosine that we have described previously [67, 68]. This is due to the reliance of the brain on the purine salvage pathway, an energy-efficient means by which to form ATP from its metabolites. This occurs via two main pathways, the action of hypoxanthine-guanosine-phosphoribosyltransferase (HGPRT), which converts hypoxanthine to IMP, and adenine phosphoribosyltransferase (APRT), which converts adenine to AMP. Both these pathways rely upon 5-phosphoribosyl-1-pyrophosphate (PRPP) for the ribose group of adenine nucleotides. Importantly, exogenous D-ribose can be administered to increase the PRPP pool [69].

A graphic example of the loss of ATP metabolites and persistently depressed ATP levels can be found in the widely used brain slice preparation. Since their inception, brain slices have been criticised for their ATP levels, which are typically 50% lower than values observed in vivo. We have recently addressed this issue [70] to investigate whether the reduction in ATP reflects loss of salvageable metabolites during brain slice preparation, analogous to injury to the intact brain in vivo, or whether this reflects a fundamental metabolic handicap that brain slices suffer from.

To address this, we provided brain slices with the purine salvage substrates D-ribose and adenine (RibAde). We found that brain slice ATP levels were restored to close to reported in vivo values indicating that: (a) the reduction of tissue ATP levels reflected the loss of salvageable metabolites during preparation and (b) that brain slices retain the capacity to synthesise ATP from precursors. Moreover, we were able to show, using adenosine biosensors, that the greater ATP pool translated into greater activity-dependent adenosine release (Fig. 6), which raised the threshold for the induction of long-term potentiation. This release seemed to be independent of prior release of ATP as it

Fig. 6 Demonstration of the efficacy of purine salvage pathways in restoration of adenosine release from brain slices. **Top:** Metabolism of ATP and purine salvage pathway for adenine and D-ribose utilization: APRT adenine phosphoribosyltransferase, HGPRT hypoxanthine phosphoribosyltransferase. Solid lines indicate direct routes, and dashed lines indicate indirect routes. The reactions of the purine salvage pathway are catalyzed by HGPRT and APRT. **b Lower:** Preincubation of hippocampal slices for 2 h with D-ribose (1 mM) and adenine (50 μ M; RibAde) results in greater adenosine (Ado) release in response to three periods of theta burst stimulation (arrows). Concentration of adenosine in nanomolar indicates that the signal recorded reflects adenosine and metabolites such as inosine. Reproduced with permission from [70]

was insensitive to the ecto-ATPase inhibitor POM1 [71], but sensitive to a combination of equilibrative nucleoside transporters (ENTs) inhibitors [70]. In these experiments, sensors were invaluable as they allowed the kinetics of enhanced adenosine release to be established, which would not have been possible through the use of receptor antagonists. Furthermore, they permitted the effects of inhibition of ecto-ATPases and ENTs on adenosine release to be established directly since the commonly used surrogate for adenosine release, the depression of excitatory synaptic transmission, is inhibited nonspecifically by POM1 [71], ARL 67156 [72] and by ENT inhibition [67, 73]. Given that both D-ribose and adenine have been used safely in humans, these studies offer the possibility that RibAde treatment may be of value in a range of neurological conditions characterised by reductions in tissue ATP, as D-ribose has been for the energetically compromised heart, in vivo and in humans [74].

From basic science to clinical applications

There is a fundamental relationship between adenosine and downstream purines and cellular metabolism. Under conditions of metabolic stress, intracellular adenosine can rise, and this will efflux the cells via equilibrative transporters. This has given rise to the general notion that adenosine (and downstream purines) may be useful biomarkers of pathologies in the CNS. However, lack of a rapid and convenient measurement system in whole blood has hindered larger-scale studies aimed at establishing the diagnostic value of

purines more clearly. In this section, we briefly review three clinical areas in which purinergic signalling is involved and where purine-based diagnostics may ultimately have a valuable role.

Epilepsy and purines

The seminal observation that adenosine levels rise in the hippocampi of humans during epileptic seizures [75] has been instrumental in our understanding of the role of adenosine during seizure activity [76, 77]. It is now abundantly clear that adenosine exerts a profound anticonvulsant influence, primarily via the inhibitory adenosine A₁ receptor since adenosine A₁ receptor antagonists greatly enhance seizure activity. Indeed, this is exploited clinically through the use of xanthines, such as caffeine, aminophylline and theophylline, and the use of hyperventilation, which reduces extracellular adenosine [78], to enhance seizures associated with electroconvulsive therapy [79].

However, there are few direct demonstrations of adenosine release during seizure activity [77], which may reflect its brief and/or localised nature, which may be below the spatial or temporal resolution of commonly used techniques, such as microdialysis. Application of microelectrode biosensors has revealed low micromolar adenosine release during brief (10–15 s) electrically electrographic seizures in the CA1 region of hippocampal slices [80], which is exaggerated when seizure activity is enhanced when A₁ receptors are blocked [80]. Addressing the issue of whether this adenosine arises from prior metabolism of ATP, Lopatar et al. found that neither electrically-evoked nor KCl-induced electrographic seizures were associated with appreciable ATP release, either as measured with ATP biosensors or via the use of pharmacological tools [72]. This does not exclude the possibility that some seizure phenotypes may be associated with ATP release [81].

Foetal hypoxia and hypoxanthine

A sizeable literature supports the notion that hypoxanthine is a marker of foetal hypoxia and asphyxia [82–90]. Continual measurement of the unborn child's heart rate during labour is currently the main tool in judging its well-being in utero. Foetal distress is well known to be associated with abnormal patterns in the cardiotachogram (CTG) during labour. In prospective assessment of CTG traces, post-delivery measurement of hypoxanthine in umbilical cord blood correlated strongly with the degree of distress indicated in the CTG traces. Hypoxanthine also correlates well with the APGAR score (a simple five-criterion measure of neonatal viability). In babies that have suffered neurological damage as a result of hypoxia, hypoxanthine levels rise to such an extent that it is secreted

in greatly elevated quantities in urine for many hours (>40 h) following birth [84]. Indeed, hypoxanthine has been proposed as a marker of periventricular leukomalacia [91], the commonest form of neural defect resulting from hypoxia at birth (and which depends upon A₁ receptors [92]).

The potential value of hypoxanthine as a biomarker for foetal hypoxia remains unclear, probably for technical reasons. The measurements of hypoxanthine were not performed at the point of care, necessitating treatment and storage of blood for later analysis (usually by spinning down the red blood cells and freezing the plasma). This raises the prospect that the labile purines may undergo further breakdown or sequestration during processing and storage prior to their determination. There is some disagreement over what constitutes the normal levels of hypoxanthine, and there can be great variability in hypoxanthine levels between individuals. Poor sample treatment, with variable degrees of purine degradation during treatment and storage, is likely to have contributed to this.

Purines as a biomarker of stroke and related conditions

Adenosine has been proposed as a neuroprotective metabolite in the brain, produced during metabolic stress and able to simultaneously shut down potentially damaging glutamatergic transmission that can result in extensive cell death [93, 94]. There are many studies both in vitro and in vivo that demonstrate the release of adenosine during ischemia and its in vitro analogue (oxygen–glucose deprivation) [22, 25, 67, 68, 77, 95–99]. Interestingly, adenosine can be released very early in the onset of an ischemic insult at points where, at least in vitro, complete recovery of neuronal and synaptic function is possible. This implies that rather than being a “death signal,” adenosine is an indicator of distress. This very early production of adenosine before the onset of cell death makes it an attractive potential biomarker for conditions such as transient ischemic attack and stroke.

Plasma adenosine levels increase in patients suffering from chronic heart failure (ischemic and non-ischemic), and the degree of elevation correlated with the severity of the condition. Elevated adenosine levels in blood also accompany transient ischaemic attacks and stroke and can remain elevated for a few days following the initial insult [100]. An interesting approach to the evaluation of adenosine, hypoxanthine and lactate as markers of cerebral ischemia was provided in a study looking at these analytes in venous outflow from the brain (jugular vein) during carotid endarterectomy [66]—a procedure that can transiently occlude blood supply to the brain. This procedure effectively provides a controlled and timed insult to the brain. Thus, blood analyte measurements can be made before,

during and after the insult. This analysis showed that adenosine was a much more sensitive indicator of the degree of brain hypoxia compared to lactate.

Challenges of purine blood measurement

ATP, adenosine and downstream purines have a very short half-life in blood [101]. This is because there are many paths of purine degradation and uptake in whole blood. What makes the analysis of blood purines even harder is that red blood cells contain high levels of ATP (1–2 mM, [102]). This can be released in response to hypoxia [103] and cell deformation or damage [104] during sample collection. This means that sample handling of blood for the analysis of purines is of the utmost importance if reliable measurements are to be obtained. The luxury of storage and leisurely measurement is not an option for the purines.

In this circumstance, a point of care measurement device has obvious utility. While not eliminating the need for care in obtaining blood samples (to avoid damaging the red blood cells), an ability to measure purine levels rapidly in whole blood would be extremely useful and bypass issues of sample stability. Biosensors have obvious utility in this context—for example, blood glucose has been reliably measured with biosensors for decades. Although the expected concentrations of purines in blood under control conditions are very low, during pathological conditions, they do rise to micromolar levels. The challenge of using biosensors with whole blood is that potential interferences such as ascorbate and urate are present at two to three orders of magnitude higher concentration [105]. Any successful measurement system must therefore be highly selective and able to discriminate submicromolar to low micromolar concentrations of ATP or adenosine from the much bigger interference signal. We have achieved this with mediated biosensors based upon the hexametallo cyanate mediator Ruthenium Purple [106] opening the door to systematic evaluation of blood purines in different subsets of patients on a scale that will be much greater than has hitherto occurred.

Concluding remarks

It is more than 10 years since the publication of the first biosensor measurements of purine release during physiological activity in the nervous system [24]. Since then, the technology has developed considerably further, especially in terms of miniaturization and selectivity, and has been applied to the study of many different physiological and pathological phenomena. The real-time measurement of both ATP and adenosine release has proven to be an

extremely useful tool in understanding the functions mediated by these signalling agents. We are at the cusp of applying purine measurement biosensor technologies to routine measurement of the purines in blood which has the potential to exploit these important molecules as biomarkers for a range of important pathologies that include stroke and related conditions.

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