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Nonsynaptic and nonvesicular ATP release from neurons and relevance to neuron–glia signaling

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Abstract

Studies on the release of ATP from neurons began with the earliest investigations of quantal neurotransmitter release in the 1950s, but in contrast to ATP release from other cells, studies of ATP release from neurons have been narrowly constrained to one mechanism, vesicular release. This is a consequence of the prominence of synaptic transmission in neuronal communication, but nonvesicular mechanisms for ATP release from neurons are likely to have a broader range of functions than synaptic release. Investigations of activity-dependent communication between axons and myelinating glia have stimulated a search for mechanisms that could release ATP from axons and other nonsynaptic regions in response to action potential firing. This has identified volume-activated anion channels as an important mechanism in activity-dependent ATP release from axons, and renewed interest in micromechanical changes in axons that accompany action potential firing.

Keywords

ATP release; Neuron–glia interactions; Axon volume; Intrinsic optical signals; Activity-dependent development; Synaptic-vesicle release; Neurotransmitter release; Axon swelling; Learning; Volume-activated anion channel; White matter

1. Introduction

1.1. A brief history of ATP release

In 1927 Cyrus Fiske and Yerlagadda Subbarow, working at Harvard Medical School, applied a new colorimetric assay they had developed for determination of inorganic phosphate to an extract of freshly dissected cat muscle. They observed a curious delay in color production. “The time required to reach a constant reading was about thirty minutes, whereas ordinarily the full color (relative to the standard) has developed within four minutes or less [1].” Either something in the extract was inhibiting the reaction or something in muscle was generating inorganic phosphate. They concluded from further experiments that an organic molecule in muscle must be generating phosphate. Furthermore, they reported that electrical stimulation of muscle or depriving the tissue of oxygen depleted the “labile phosphorus”, from the tissue. “The outcome appears likely to throw light on a field of biochemistry never before suspected of being in any way related to phosphoric acid.” Two years later, in 1929, they identified the compound as adenosine 5'-triphosphate (ATP), which generates inorganic phosphorus by hydrolysis of its three phosphate atoms [2].

That same year, Albert Szent-Györgyi and Alan Drury found that substances they isolated from muscle and injected intravenously into dogs and other animals disturbed the cardiac rhythm, dilated the coronary artery, and inhibited smooth muscle contraction in the intestine [3]. They concluded that the molecule in muscle extract responsible for the effects was “adenylic acid.” In a footnote they reported, “Through the kindness of C. H. Fiske we have been able to test the action of the pyrophosphoric or diphosphoric ester of adenylic acid. Its action is similar to that of adenylic acid and adenosine.” (p. 218).

Thus in the same year, the two fundamental actions of this versatile biological molecule were discovered: as the energy source for metabolism and biochemical reactions, and as an extracellular signaling molecule. In 1930, Subbarow, was awarded the PhD degree. He later discovered the antibiotic tetracycline, the anticancer drug methotrexate, and isolated the vitamin folic acid, among many other important pharmacological discoveries [4]. Szent-Györgyi would receive the Nobel Prize in 1937 for discovering vitamin C.

Research on the extracellular signaling function of ATP lagged considerably behind research on ATP as a cellular energy source, because the biology of cell signaling could not be elucidated by biochemistry alone, but also because release of ATP from a cell seemed incongruous with its vital role as the cellular energy source. In his review paper published in 1982, titled “Does ATP Cross the Cell Plasma Membrane?” [5], Irshad Chaudry concluded that experimental evidence clearly established this fact, but the mechanisms were unclear. He argued that the critical role of ATP in cellular respiration and energy required that ATP must be able to cross membranes of intracellular organelles, so in principle, regulated movement of ATP across the plasma membrane seemed highly plausible.

1.2. Release of ATP from non-neuronal cells

The discovery of ATP from muscle extracts and its action in stimulating heart rate and dilating arteries suggested a possible signaling role for the molecule in increasing blood supply to muscle tissue during exertion. Boyd and Forrester studied several substances that were released by muscle into a bathing solution, which when applied to frog heart would increase the heart rate. In 1968 they identified ATP as one of these molecules liberated from muscle without tissue damage [6]. Later studies showed that ATP was released into the bloodstream of humans after exercise [7–9]. In the 1970s and 1980s fluid shear force [10], and hypoxia [11,12] and various ligands [13] were shown to stimulate release of ATP from muscle or endothelial cells, but whether this was a specific secretory mechanism or a non-specific increase in membrane permeability was unclear.

Today many mechanisms for ATP release from cells have been identified and they are activated by a wide range of stimuli [for reviews 14–18]. These ATP release mechanisms include damage to the cell membrane, mechanical stress, hypoxia, inflammation, several agonists, and electrical excitation of neural tissue. In non-neuronal cells, several cellular mechanisms for ATP release have been identified and studied extensively, including exocytosis, gap junction hemichannels, ion channels, the cystic fibrosis transmembrane conductance regulator, P2X7 receptors, and nucleoside transporters. This is in marked contrast to ATP release from neurons where essentially only one mechanism of ATP release has been investigated.

1.3. Release of ATP from neurons

The earliest studies showed that electrical stimulation of axons liberated ATP [19,20]. These studies were contemporaneous with the first suggestion that neurotransmitter release was quantal, first proposed by Del Castillo and Katz in 1957 [21], and the discovery of the synaptic vesicle in 1954 [22,23]. Studies on synaptosomes showed that ATP was present in

high concentrations inside synaptic vesicles [24–26], and chromatography [27,28], radioactive tracer studies [29–31], and firefly chemiluminescence assay [32,33], showed that ATP was released by neurons in the CNS and PNS. Electrophysiological studies in the 1980s and 1990s, too numerous to list comprehensively here, firmly established ATP as a neurotransmitter throughout the nervous system [34–37 and Lalo et al., this issue]. As late as 1996, however, Hamann and Attwell disputed the evidence that ATP was released from synaptic vesicles [38]. This was based on their studies failing to detect ATP release from brain slice unless the stimulus voltage was raised to extreme levels that damaged cell membranes. Although they used methods similar to those used previously (detecting firefly chemiluminescence with a photomultiplier tube), the sensitivity of earlier studies showing release of ATP from hippocampal slice [32] was far greater than that of Hamann and Attwell (6.2×10^{-11} M vs. 1×10^{-6} M ATP). Recently the ATP transporter that concentrates ATP into vesicles has been identified [39], removing any doubt that ATP can be released by vesicular mechanisms. There was some challenge in the 1970s and 1980s to the vesicular mechanism of neurotransmitter release in general [40–42], but exocytosis was and remains almost universally accepted as the mechanism whereby neurotransmitters and neuromodulators are released from neurons.

1.4. ATP release from axons and extra-synaptic regions

As the defining feature of the neuron doctrine, the synapse has long been the focus of research on neurotransmission and neuronal plasticity. Synaptic release of neurotransmitter has also guided thinking about many other aspects of cell signaling in the nervous system, such as the effects of neural activity on development of neurons and glia. However, many developmental events regulated by neural activity, for example, axon outgrowth, cell proliferation, migration, and differentiation, operate before synaptogenesis. (See for example [43].) When calcium imaging revealed responses in glia (Schwann cells and astrocytes) that were initiated by electrical stimulation of neurons, spillover of transmitter released into the synaptic cleft was understood to be the basis for activity-dependent neuron–glia communication [44–46]. Likewise, regulation of synaptic transmission by glia was considered the focus of glial influence on information processing in neural circuits [47–49]. ATP quickly emerged early as one of the primary intercellular signaling molecules activating calcium responses in glia, and in communication between all types of glial cells [50,51]. However, calcium imaging showed that glia situated along axon segments could also respond to neurotransmitters and ATP released by axons during electrical stimulation [52–54], and that impulse activity, by releasing ATP from axons, could affect proliferation, differentiation, and myelination of Schwann cells [52,55] and oligodendrocytes [56,57]. Although synapses are not present in nerve segments, release of ATP and other neurotransmitters from axons was assumed to be by exocytosis of vesicles with the axolemma [54], or, in the case of glutamate, by reversal of neurotransmitter reuptake mechanisms [53]. In support of the vesicular release of ATP from axons, removal of extracellular calcium was found to inhibit the glial calcium responses to axon stimulation [54].

A more specific test has shown that ATP can be released through a nonvesicular mechanism from neurons firing action potentials. After treatment with botulinum toxin A, which prevents vesicle fusion with the plasma membrane by cleaving SNAP-25, ATP is still released from neurons by action potential firing induced by electrical stimulation (Fig. 1).

2. Nonvesicular release of ATP from neurons

2.1. ATP release induced by mechanical stimulation

Mechanical stimulation of cells is widely appreciated as a potent way to induce ATP release from many types of non-neuronal cells [58], and such stimulation is often used for experimental studies in place of “natural” stimulation [50,59]. However, even from the earliest study of ATP release from neurons in response to electrical stimulation [20], it was evident that the release apparatus was extremely sensitive to mechanical stimulation. “These results show that mechanical as well as electrical stimulation results in the liberation of ATP” [20, p. 498]. This, however, was thought to result from mechanical stimulation inducing axon firing.

2.2. ATP release through volume-activated anion channels in non-neuronal cells

Some types of chloride channels in the cell membrane of astrocytes and other non-neuronal cells are activated by membrane stretch (volume-activated anion channels, VAAC). Some of these channels are permeable to ATP^{4-} which can be released together with water to restore normal cell volume after cell swelling under hypo-osmotic conditions [60]. Cell swelling from hypoxia can also release ATP through VAAC. Small organic anions, including glutamate, GABA, and taurine, are also released through these channels upon activation by cell swelling. The molecular identity of these channels is not yet clear, but they can be characterized by their pharmacological and electrophysiological properties, see Table 1. Much less is known about VAACs in excitable cells, where chloride channels have an additional function in maintaining resting membrane potential and excitability. Recent studies (described below) show that action potentials can release ATP from neurons through these channels.

3. ATP release from axons through channels activated by action-potential induced axon swelling

3.1. Optical and volume changes in axons during excitation

From the earliest studies of electrical excitation in nerve fibers it was evident that many changes in physical properties of axons closely track the voltage fluctuation during action potential firing. This includes rapid heating and cooling [61–63], mechanical displacement of the axon membrane [64–76], and changes in light transmission through axons [77–84]. The rapid changes in light transmission and membrane displacement are caused by transmembrane flux of ions (notably Na^+ and Ca^{2+}) and water accompanying the action potential. This is believed to alter the submembrane cytoplasm and osmotic pressure, causing submicroscopic axon swelling, which reduces light scattering through axons [78,84,85]. Flexoelectric deformation of curved cell membrane can also contribute to membrane displacement during excitation [86]. In response to trains of action potentials, axonal swelling increases from the accumulating transmembrane flux of ions and re-distribution of water molecules across the cell membrane [87]. Such microscopic volume changes in axons firing action potentials can cause mechanical changes in tissue, including physical contraction of the frog spinal cord [69], dorsal root ganglia [70], olfactory bulbs [63], amphibian skin [88], invertebrate axons [64,73,89,90], and mammalian neurohypophysis [76,83] after trains of action potentials, and in the squid retina in response to light [62].

Axon displacement in response to single action potentials has been reported in other experiments using more sensitive and rapid measurement methods, such as atomic force microscopy [74–76], laser interferometry [65,75], optical coherence tomography [90], and fiberoptic displacement probes and micromechanical displacement sensors [88]. These

physical phenomena accompanying axon depolarization were not known to have biological significance, but new research shows that ATP is released through VAACs by action potential-induced axon swelling [91]. The amount of ATP released from neurons in response to action potential firing is a linear function of the increase in light transmission through axons caused by action-potential-induced axon swelling (Fig. 2). This is consistent with a causal relation between activity-dependent axon swelling and ATP release. This is confirmed by experiments showing that release of ATP from neurons is inhibited by electrical stimulation in the presence of drugs blocking VAACs (Fig. 3).

The pharmacological profile of chloride channel blockers that inhibit action potential-induced ATP release from axons is consistent with activation of the maxi-anion channel, although the results strongly suggest that a number of different chloride channels may be activated by axon swelling during excitation to release ATP [91,92]. The maxi-anion channel is a prominent pathway for ATP release from astrocytes and other cells [60,93,94] in response to cell swelling under hypotonic conditions or ischemia. It has a large single-channel conductance of 300–400 pS and a 1.3-nm-radius pore that allows efflux of small intracellular organic anions, including glutamate (0.35-nm radius), and ATP (0.6 nm radius) [93].

3.2. Biological significance of ATP release through volume-activated anion channels in axons

The significance of ATP release through VAACs activated by axonal firing potentially has a broader scope than processes regulated by ATP release from synapses. Many types of cells in the nervous system have membrane receptors for ATP [17], including astrocytes, Schwann cells, oligodendrocytes, endothelial cells, microglia, neuronal cell bodies and dendrites, and progenitor cells (for example NG2 glia [95], which can differentiate into astrocytes, oligodendrocytes, and neurons). Most of these cells are not located within the proximity of synapses where they could be activated by spillover of ATP spreading from the synaptic cleft.

Vesicular release of ATP is well established from astrocytes and other non-neuronal cells, showing that a synaptic specialization is not necessary for cells to release ATP from vesicles [51,96–98]. Release of ATP from individual vesicles or clusters of vesicles along axons or other extra-synaptic regions of neurons would be expected, and this has been demonstrated from the cell body of sensory neurons [99,100]. However, this new research shows that nonvesicular ATP release from neurons also occurs. These two mechanisms of ATP release may be differentially activated by different patterns or different types of stimulation, and they may participate to different extents in various activity-dependent biological processes.

ATP release from neurons might participate in such diverse functions as activity-dependent effects on development, cell differentiation, vasculature [101] and immune responses or signaling participating in chronic pain [102,103] in response to neural impulse activity, and in pathological conditions such as spreading cortical depression [104], and neuroinflammation [105]. Nonsynaptic release of ATP would allow activity-dependent communication between axons, glia, vascular, and other cells that are not coupled to neurons by synapses [106]. Nonsynaptic mechanisms of activity-dependent communication could enable glial development and function outside synaptic regions to be regulated by axonal firing arising spontaneously in developing neural networks or through environmental experience.

Previous research has shown that action potentials in unmyelinated DRG axons cause release of ATP which signals to myelinating glia (Schwann cells and oligodendrocytes) to regulate their development and myelination [52,55–57]. Traditionally myelin was primarily

of interest to those concerned with demyelinating disease. However, these new findings showing that myelination can be controlled by electrical impulse activity, together with human brain imaging showing changes in fiber tracts after learning, reveals a new mechanism of learning beyond regulation of neurotransmitter release at synapses [107]. Myelination of an axon increases impulse conduction velocity roughly 50 times; thus the increased transmission speed will have profound effects on information processing in that neural circuit [108,109]. The human brain continues to form myelin throughout childhood and into early adult life, suggesting a role of myelin in learning and strengthening neural circuits through experience. In summary, studies of ATP release from axons and communication with myelin-forming glia are expanding research on the mechanisms of learning beyond the synapse, to include the transmission of information through the entire network involved in carrying out a complex cognitive function [107–109].

4. Conclusions

Many nonvesicular mechanisms of ATP release from non-neuronal cells are known. Among these, ATP release through VAACs in response to hypotonic stress is well established. New research shows that these same channels can be activated in neurons by electrical impulse activity, which causes microscopic axon swelling. This nonsynaptic, and nonvesicular ATP release is a new mechanism for activity-dependent release of neurotransmitter from axons with special relevance to ATP and neuron–glia signaling outside synapses.

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Fig. 1.

Nonvesicular release of ATP from neurons. Action potentials (10 Hz, red bar) cause release of ATP from mouse dorsal root ganglion neurons after cleavage of SNAP-25, a protein that is required for fusion of synaptic vesicles with the cell membrane. Immunoblot (inset) confirms enzymatic degradation of SNAP-25 after incubation in botulinum toxin A (BoNT). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a loading control. Reprinted from [91] with permission

Fig. 2. ATP release is highly correlated with the increase in light transmission through axons accompanying action potential firing. The increased light transmission is caused by microscopic axon swelling ($p < 0.0001$; $r^2 = 0.983$; $n = 12$). Reprinted from [91] with permission

Fig. 3. Pharmacological blockers of volume-regulated anion channels, including NPPG (green) and glibenclamide (yellow), inhibit action potential-induced release of ATP from neurons (black). Reprinted from [91] with permission. NPPB (5-nitro-2-(3-phenylpropylamino)-benzoate).

Table 1

Guide to chloride channels with emphasis on those implicated in release of ATP and small amino acids during cell volume regulation.

Maxi-anion channel

- Activated by cell swelling
- Large-conductance (400 pS), 1.3 nm pore radius
- ATP and glutamate release from non-excitable cells
- Involved in cell volume regulation
- Gene identity unknown, VDAC (voltage-dependent anion channel) gene is disputed as maxi-anion channel

VRAC

(volume-regulated anion channel)

Also known as: volume regulated channel (VRC), volume sensitive osmolyte-anion channel (VSOAC), volume expansion-sensing channel (VSOR)

- Activated by cell swelling and displays voltage-dependent ATP conductance
- Intermediate conductance (90 pS at positive potentials)
- 0.63 nm pore radius
- Participates in regulating cell volume
- Involved in efflux of organic osmolytes including glutamate from non-excitable cells
- These channels may not be a single entity
- The gene identity is unclear

CFTR

(cystic fibrosis transmembrane conductance regulator)

- cAMP regulated Cl^- channel
- Implicated in ATP release
- Regulates several conductances including CaCC, VRAC, ORCC, and TRPV4
- 0.6–1 nm pore radius

CIC family

- 9 genes with alternative splicing
- Forms homo and heterodimeric channels
- Located on intracellular organelles and the plasma membrane
- CIC2 is activated by cell swelling
- The relation of CIC3 to VRAC is disputed
- Heterodimers of CIC 3/5 is a candidate for VAAC

CACC

(calcium-activated chloride channel)

- The molecular identity is unclear, bestrophins (4 genes) and anoctamins (10 genes) have been implicated Modulated by CamKII

Other chloride channels

Ligand-gated chloride channels (GABA, glycine receptors), acid-activated chloride channels (SLC26A7 gene candidate), cGMP-dependent CaCC, intracellular chloride channels (CICs), ORCC (outwardly rectifying chloride channel), transporter-associated (CICs), calcium-activated chloride channel (CICA family).

Modified from [91,110,111].