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ATP as a Cotransmitter in Sympathetic Nerves and Its Inactivation by Releasable Enzymes

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ABSTRACT
ATP and norepinephrine (NE) are cotransmitters released from many postganglionic sympathetic nerves. In this article, we review the evidence for ATP and NE cotransmission in the rodent vas deferens with special attention to the mechanisms involved in removing the cotransmitters from the neuroeffector junction. Although the clearance of NE is well understood (e.g., the primary mechanism being reuptake into the nerves), the clearance of ATP is just beginning to be explained. The general belief has been that ATP is metabolized by cell-fixed ectonucleotidases. It now seems, however, that when ATP is released from nerves as a transmitter there is a concomitant release of nucleotidases that rapidly degrade ATP sequentially to ADP, AMP, and adenosine, thereby terminating the action of ATP. In the guinea pig vas deferens, there appear to be at least two enzymes, one that converts ATP to ADP and ADP to AMP (an ATPDase) and a second enzyme that converts AMP to adenosine (an AMPase). An important feature of this process is that the transmitter-metabolizing nucleotidases are released into the synaptic space as opposed to being fixed to cell membranes. A preliminary characterization of these enzymes suggests that the releasable ATPDase exhibits some similarities to known ectonucleoside triphosphate/diphosphohydrolases, whereas the releasable AMPase exhibits some similarities to ecto-5′-nucleotidases.

Cotransmission in Vas Deferens
The vas deferens is supplied with a dense sympathetic innervation, and stimulation of the nerves results in a biphasic mechanical response that consists of an initial rapid twitch, followed by a maintained contraction (see Westfall and Westfall, 2001). The evidence is now clear that the first phase of the response is mediated mainly by ATP acting on postjunctional P2X receptors, whereas the second phase is mediated mainly by NE acting on α1-adrenoceptors (Fig. 1). Activation of P2X receptors by ATP results in the production of excitatory junction potentials that summate to produce action potentials, which then propagate from one smooth muscle cell to another (Sneddon et al., 1982; Sneddon and Westfall, 1984). The calcium influx that results from depolarization leads to the generation of a major component of the first phase of the contraction. The activation of the α1-adrenoceptors by NE results mainly in the second maintained phase of the contraction, by a mechanism that involves the release of intracellular calcium by a phosphoinositide path-

ABBREVIATIONS: NE, norepinephrine; NPY, neuropeptide Y; ENPPases, ectonucleotide pyrophosphatase/phosphodiesterases; ENTPDases, ectonucleoside triphosphate/diphosphohydrolases; EFS, electrical field stimulation; ADO, adenosine; ARL 67156, 6-N-N-diethyl-β,γ-dibromomethylene-0-ATP.
The cotransmitters are apparently released from the same types of nerves because pretreatment with 6-hydroxydopamine, an agent that specifically destroys adrenergic nerves, abolishes both phases of the neurogenically induced biphasic contraction (Fedan et al., 1981).

In addition to ATP and NE, neuropeptide Y (NPY) has been found in some sympathetic nerves, and the release of NPY, along with ATP and NE, has been demonstrated in response to nerve stimulation of the guinea pig vas deferens (Kasakov et al., 1988). Although it is clear that ATP and NE are cotransmitters in the vas deferens, being responsible for the phasic and tonic contractions of the smooth muscle, NPY seems to play a modulatory role by influencing the release and the postjunctional actions of ATP and NE (Stjarne et al., 1986; Bitran et al., 1991).

**Prejunctional Modulation of Cotransmitter Release**

Probably all nerves that release NE have prejunctional $\alpha_2$-adrenoceptors that, when stimulated, reduce the release of NE. Stimulation of these “autoreceptors” in the vas deferens of the mouse, rat, and guinea pig reduce not only the release of NE but also the release of ATP (Brown et al., 1983; Sneddon and Westfall, 1984; Driessen et al., 1993). Endogenously released NE, however, has a greater influence on its own release than that of ATP. Interestingly, certain $\alpha_2$-adrenoceptor agonists (e.g., xylazine) produce a greater reduction of ATP release than of NE release (Westfall et al., 1996a). These results suggest that the release of ATP and NE can be differentially regulated, and therefore, the cotransmitters may be differentially released (vide infra).

ATP also seems to function as a neuromodulator, causing a reduction of transmitter release in the peripheral and central nervous systems (Silinsky et al., 1998; Stone et al., 2000). The ability of antagonists of P1 receptors (receptors for adenosine) to reduce the inhibition of transmitter release by ATP has supported the popular view that the effect of ATP is caused by its metabolism to adenosine, which then activates P1 receptors (Kirkpatrick and Burnstock, 1992). Adenosine has long been known to reduce transmitter release in vas deferens (Hedqvist and Fredholm, 1976) and other tissues (see Paton, 1981). Although it is likely that a portion of the effect of ATP is due to the formation of adenosine, there is intriguing evidence indicating that nucleotides can act per se to inhibit transmitter release without first being degraded to adenosine (Shinozuka et al., 1988; von Kugelgen et al., 1989; Forsyth et al., 1991; Todorov et al., 1994b). The specificity of the receptor type upon which ATP acts directly has not been fully elucidated. Suggestions range from a P2Y type (von Kugelgen et al., 1989; von Kugelgen and Starke, 1991) to a unique hybrid receptor with some features of both P1 and P2 receptors. Shinozuka et al. (1988) have referred to this receptor as a P3 receptor. This concept of prejunctional autoreceptors is shown schematically in Fig. 1.
Release of the Cotransmitters

Even though there is now a consensus that neurotransmission commonly involves the release of several neurotransmitter substances (see Furness et al., 1989; Hokfelt et al., 1992; Lundberg, 1996), questions remain about whether storage and release of cotransmitters occur in or from common sites. In the case of the sympathetic cotransmitters ATP and NE, the initial concept was based on an analogy with adrenal chromaffin cells that release ATP and catecholamines in the same ratio in which they are stored in the chromaffin secretory granules. Consequently, it has been assumed that the sympathetic nerves also store ATP and NE in the same synaptic vesicles, and therefore, upon release, the two cotransmitters are presented at their specific receptors simultaneously and in constant proportions (Stjärne, 1989, 1994; Brock et al., 1997; Brock and Cunnane, 1999).

When the time courses of release of endogenous ATP and NE from the sympathetic nerves of vas deferens and the time courses of release of ATP and catecholamines from adrenal chromaffin cells were compared, different patterns were observed (Todorov et al., 1994a, 1996). Adrenal chromaffin cells release ATP and catecholamines continuously and in a constant molar ratio. The sympathetic nerves, on the other hand, release ATP transiently only at the beginning of a train of nerve stimulation. The release of NE occurs later in the train and, once started, is maintained throughout the course of nerve stimulation (Todorov et al., 1996; Mihaylova-Todorova et al., 2001). These findings with sympathetic nerves were, to our knowledge, the first direct evidence that ATP and NE are not always released simultaneously. It seems that the cotransmitter composition of the “cocktail” of neurotransmitters released from the sympathetic nerves changes during the course of stimulation. Early in the train, the cocktail contains mainly ATP, ATP, and NE in the middle and almost exclusively NE near the end of the train of stimuli. In addition, there is a good correlation between this dissociated temporal pattern of release of cotransmitters and the temporal pattern of the mechanical response of the tissue. The purinergic twitch contraction of the vas deferens seems to reflect the early and transient release of ATP, whereas the adrenergic tonic contraction reflects the delayed and sustained release of NE. To explain the temporal disparity of the release of the cotransmitters, we have suggested that the sympathetic nerves may store ATP and NE in separate vesicles and release them via independent mechanisms (Todorov et al., 1994a, 1996). Recently, the concept of separate storage and differential release of the cotransmitters ATP and NE has been receiving growing support (Brock et al., 2000; Stjärne, 2001).

Inactivation of Transmitters

For neurotransmission to be effective, the neurotransmitters, once released, need to be inactivated or removed from the neuroeffector junction. For amine transmitters, such as NE, dopamine, and serotonin, the mechanism by which this occurs is well understood. The transmitters are taken back up into the nerve by specific high-affinity transporters that clear the amines from the synapse (see review by Amara and Kuhar, 1993). The amine transporters are important drug targets in that a number of clinically useful antidepressants and antianxiety drugs have as their mechanism of action inhibition of neuronal reuptake.

A different process terminates the action of the neurotransmitter acetylcholine. The enzyme acetylcholinesterase, which is associated with pre- and postjunctional membranes, forms a complex with acetylcholine and reacts to release choline, which is then taken up by the nerve. Drugs that inhibit the activity of acetylcholinesterase have important therapeutic uses in myasthenia gravis, glaucoma, paralytic ileus, atony of the urinary bladder, and enhancing cognition in victims of Alzheimer’s disease (Taylor, 2001).

The mechanism of clearance of ATP from the synapse is not as well understood as the mechanisms for removal of the autonomic neurotransmitters NE and acetylcholine. The general belief has been that ATP, once it is released into the neuroeffector junction, is metabolized by extracellularly directed, membrane-bound nucleotidases to ADP, AMP, and adenosine (Gordon, 1986; Zimmermann, 1992; Plesner, 1995). The rate of metabolism of ATP by these ecto-ATPases is relatively slow compared with synaptic events (Pearson et al., 1985; Plesner, 1995). Work by Todorov et al. (1996) suggested that a process other than metabolism of ATP by membrane-bound ecto-ATPases may be involved in terminating the action of ATP. For example, superfusion of the sympathetically innervated vas deferens preparation with ATP revealed that, over a 1 minute superfusion period, there was essentially no metabolism of the nucleotide, although metabolism of ATP would have been expected if ecto-ATPases were the primary inactivation mechanism. If the sympathetic nerves were stimulated during the superfusion period, however, there was virtually complete degradation of ATP (Todorov et al., 1997). This phenomenon is illustrated in Fig. 2.

Releasable Nucleotidases

The nerve stimulation-related metabolism of ATP is associated with the release of nucleotidases that breakdown ATP, as well as ADP and AMP, to adenosine. The enzyme activity that overflows the tissue preparation during nerve stimulation remains stable in the superfusate and has allowed a preliminary characterization of the kinetics and sensitivity to antagonists of these nucleotidases (Westfall et al., 2000a,b; Mihaylova-Todorova et al., 2002).

Inhibition of the propagation of action potentials with tetrodotoxin, suppression of postganglionic sympathetic neurotransmission with guanethidine, or inhibition of excytosis by omission of extracellular Ca$^{2+}$ all prevented the release of nucleotidase activity, strongly suggesting that the sympathetic nerves are the source of the enzymes (Todorov et al., 1997). Interestingly, the time course of release and modulation of release by presynjuctional receptors of the nucleotidases indicates that the enzyme activity is coreleased with ATP and not with NE (Mihaylova-Todorova et al., 2001). These results suggest that the proteins carrying the enzyme activity originate from ATP-storage vesicles as opposed to catecholamine vesicles.

At this point, it is not known whether these nucleotidases represent a heretofore unidentified type of enzyme or whether these are known enzymes and the ability to be released and to metabolize transmitter ATP are newly recognized features. In an attempt to clarify this issue, a number of known nucleotidases have been considered as candidates. For example, there
are nucleotidases that might be expected to be involved in some way in the process of neurotransmission, such as the vacuolar H+-transporting ATPase, the Na+-K+-ATPase, the multidrug resistance channel, and the cytosolic N-ethylmaleimide-sensitive fusion protein. All of these, however, have been rejected as candidates based on the fact that specific antagonists, namely bafilomycin, ouabain, orthovanadate, and N-ethylmaleimide failed to affect the activity of the releasable nucleotidases (Todorov et al., 1997; Fig. 3).

There are a variety of other enzymes that have the potential to dephosphorylate extracellular nucleotides, including phosphatases, nucleotide pyrophosphatases, phosphodiesterases, and ecto-nucleotide triphosphate diphosphohydrolases. Most of these enzymes are fixed to cell membranes with the catalytic site facing the extracellular space where they can metabolize extracellular nucleotides and are, therefore, referred to as ecto-enzymes. There is also evidence that some ectophosphatases could be released from cell membranes upon activation of endogenous phospholipases and cleavage of a glycosylphosphatidyl-inositol linkage anchoring the protein to the cell membrane (Hooper, 1997).

In an attempt to more completely understand the nature of the releasable nucleotidases, Mihaylova-Todorova et al. (2002) examined the effects of several pharmacological agents known to inhibit various ecto-enzymes. Known inhibitors of phosphatases, such as levamisole and phosphatase inhibitor cocktail II (Sigma-Aldrich), St. Louis, MO), however, do not affect the activity of the releasable nucleotidases (Fig. 3). Also, 3-isobutyl-1-methylxanthine, a nonspecific phosphodiesterase antagonist, failed to inhibit the ATPase and AMPase activities of the releasable nucleotidases. Additionally, para-nitrophenyl thymidine monophosphate, a preferred substrate of ecto-nucleotide pyrophosphatase/phosphodiesterases (ENPPases) had no influence on the ATP metabolism by releasable nucleotidases indicating that ENPPases do not contribute to this phenomenon (Fig. 3).

The releasable nucleotidases seem to have some similarities with members of the mammalian ecto-ATPase CD39 gene family. It has recently been suggested that this family of enzymes be referred to as ENTPDases (Zimmermann et al., 2000). An example of a similarity is that, 6-N,N-diethyl-β,N-dibromomethylene-d-ATP (ARL 67156) inhibits the activity of ecto-ATPases expressed by blood (Crack et al., 1995) and...
smooth muscle cells (Westfall et al., 1996b), as well as the ATPase activity of releasable nucleotidases from guinea pig (Fig. 3; Todorov et al., 1997; Westfall et al., 2000b; Mihaylova-Todorova et al., 2002) and rabbit vas deferens (Westfall et al., 2000a). Furthermore, suramin inhibits ecto-ATPase activity in neuronal and non-neuronal tissues (Bultmann et al., 1996; Marti et al., 1996) and also inhibits the activity of the releasable nucleotidases (Fig. 3; Todorov et al., 1997; Mihaylova-Todorova et al., 2002). Therefore, the releasable nucleotidases share similarities with the ENTPDases. There are some differences as well, however. Those members of ENTPDase family that exhibit the greatest degree of ATPase activity (in comparison with GTPase or UTPase activity) are membrane-bound proteins, whereas those members that are potentially soluble (and therefore potentially releasable) hydrolyze ATP poorly (see Mihaylova-Todorova et al., 2002 for additional discussion and original references). Moreover, none of the members of the ENTPDase family hydrolyze AMP to adenosine, whereas the releasable nucleotidases of the guinea pig vas deferens exhibit prominent AMPase (or 5'-nucleotidase) activity. Interestingly, known inhibitors of ecto-5'-nucleotidases, such as α,β-methylene ADP and concanavalin A inhibit the metabolism of AMP to adenosine by releasable nucleotidases (Fig. 3). This suggests that releasable nucleotidases, in addition to exhibiting similarities to ENTPDases, also exhibit similarities to 5'-nucleotidases. Furthermore, these two activities of the releasable nucleotidases, i.e., an ATPase and 5'-nucleotidase activity, can be differentially inhibited. Suramin inhibits only the ATPDase and not the 5'-nucleotidase activity, whereas α,β-methylene ADP and concanavalin A inhibit the metabolism of AMP but not ATP by the releasable nucleotidases. ARL 67156 inhibits both ATPDase and AMPase activity of the releasable nucleotidases (Mihaylova-Todorova et al., 2002; Fig. 3).

Based on the evidence obtained to date, it seems that at least two enzymes, an ATPDase and an AMPase, that work cooperatively to breakdown extracellular ATP to adenosine are released from the sympathetic nerves of the guinea pig vas deferens (curiously, there is preliminary evidence that the nucleotidases released from the sympathetic nerves of the rabbit vas deferens lack AMPase activity (Westfall et al., 2000b)). The ATPDase exhibits pharmacological similarities to known ENTPDases, whereas the AMPase resembles, from a pharmacological prospective, ecto-5'-nucleotidase. The concept of releasable nucleotidases, along with cell-fixed ecto-enzymes, is shown schematically in Fig. 1.

**Implications for Drug Development**

Extracellular adenine nucleotides and nucleosides are now known to be involved in a plethora of physiological functions and pathological conditions including neurotransmission and neuromodulation, platelet aggregation and hemostasis, pulmonary function, nociception, and auditory and ocular function to name a few (Abbracchio and Burnstock, 1998; Burnstock and Williams, 2000). As the diverse actions of purines have become increasingly recognized, there has been a growing interest in how they produce their effects. This has led to an active investigation of the cell surface receptors upon which the purines act as well as of potentially useful agonists and antagonists of adenosine receptors and P2 receptors. In addition to receptor agonists and antagonists, another pharmacological approach, which has received less attention, would be to develop agents that would affect the extracellular concentration of the endogenous purines by influencing their metabolism.

A good example of this latter approach relates to platelet aggregation. As pointed out by Zimmermann (1999), there is growing evidence for a specific ecto-ATPase of the CD39 gene family associated with endothelial cells that limits the extent of platelet aggregation by converting ADP, which induces platelet aggregation, to adenosine, which has antiaggregation activity (Kaczmarek et al., 1996; Marcus et al., 1997). The hypothesis that CD39/NTPase 1 is a key thromboregulatory factor has been supported by in vivo experiments with NTPDase 1-null (CD39 −/−) mice (Enjoji et al., 1999; Imai et al., 1999). Furthermore, a recombinant form of this ecto-ATPase, which is soluble, has been shown to block ADP-induced platelet aggregation in vitro and has potential as a therapeutic agent for patients with thromboembolic disorders (Gayle et al., 1998).

There may be situations where it is useful to enhance purinergic neurotransmission, just as it is for adrenergic, dopaminergic, serotonergic, and cholinergic neurotransmission. In this regard, Westfall et al. (1996b, 1997) have shown that ARL 67156 enhances neurotransmission in vas deferens and urinary bladder presumably, in part, by preventing the rapid breakdown of ATP by the neurally released nucleotidases. There is also evidence that sympathetic nerves in blood vessels release nucleotidases (e.g., rat caudal and rabbit saphenous artery; L. D. Todorov and S. T. Mihaylova-Todorova, unpublished results). Thus, neurotransmission in blood vessels may be influenced by pharmacological manipulation of the nucleotidases. As more is learned about the releasable nucleotidases and as specific inhibitors are developed, one might expect the emergence of a class of drugs that will be to purinergic neurotransmission what amine reuptake inhibitors are to adrenergic and serotonergic neurotransmission and what cholinesterase inhibitors are to cholinergic neurotransmission. In addition to potential therapeutic applications for enzyme inhibitors, there may be a place for recombinant forms of the neuronal-releasable nucleotidases, in analogy with the recombinant CD39 ecto-ATPase being investigated as an antiplatelet aggregatory agent.

As a closing thought, it may be fortuitous that there are multiple types of ecto- and releasable nucleotidases. This provides some hope that system specific enzymes and inhibitors can be developed.

**References**


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