EctoNucleotidase in Cardiac Sympathetic Nerve Endings Modulates ATP-Mediated Feedback of Norepinephrine Release

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ABSTRACT

ATP, coreleased with norepinephrine, affects adrenergic transmission by acting on purinoceptors at sympathetic nerve endings. Ectonucleotidases terminate the actions of ATP. Previously, we had preliminary evidence for ectonucleotidase activity in cardiac sympathetic nerve terminals. Therefore, we investigated whether this ectonucleotidase might influence norepinephrine release in the heart. Sympathetic nerve endings isolated from guinea pig heart (cardiac synaptosomes) were rich in \( \text{Ca}^{2+} \)-dependent ectonucleotidase activity, as measured by metabolism of exogenously added radiolabeled ATP or ADP. By its inhibitor profile, ectonucleotidase resembled ectonucleotide triphosphate diphosphohydrolase 1 (E-NTPDase1). Exogenous ATP elicited concentration-dependent norepinephrine release from cardiac synaptosomes (EC\(_{50}\) 0.96 \( \mu \text{M} \)). This release was antagonized by the P2X receptor antagonist pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (10 \( \mu \text{M} \)) and potentiated by the P2Y receptor antagonist 2',3'-dideoxy-\( \text{N}^\alpha\)-methyladenosine-3',5'-diphosphate (MRS 2179) (30 \( n\text{M} \)). Norepinephrine release promoted by ATP was also potentiated by the nucleotidase inhibitor 6-N,N-diethyl-\( \beta\)-\( \gamma\)-dibromomethylene-\( \delta\)-adenosine-5'-triphosphate (ARL67156) (30 \( \mu\text{M} \)) and blocked by a recombinant, soluble form of human E-NTPDase1 (solCD39). In contrast, ARL67156 had no effect on norepinephrine release induced by the nonhydrolyzable analog, \( \alpha, \beta\)-methyleneadenosine-5'-triphosphate (\( \alpha, \beta\)-MeATP). Depolarization of cardiac synaptosomes with K\(^+\) elicited release of endogenous norepinephrine. This was attenuated by PPADS and solCD39 and potentiated by MRS 2179 and ARL67156. Importantly, our results demonstrate that facilitation of ATP-induced norepinephrine release from cardiac sympathetic nerves is a composite of two autocrine components: positive, mediated by P2X receptors, and negative, mediated by P2Y receptors. Modulation of norepinephrine release by coreleased ATP is terminated by endogenous as well as exogenous ectonucleotidase. We propose that ectonucleotidase control of norepinephrine release should provide cardiac protection in hyperadrenergic states such as myocardial ischemia.

In adrenergic nerve cells, norepinephrine (NE) is copackaged in vesicles with ATP. Both NE and ATP are released in parallel during sympathetic neurotransmission (von Kugelgen et al., 1994; Sneddon et al., 1999). Unlike NE, those actions are terminated predominantly by re-uptake into nerve endings by a specific transporter (Amara and Kuhar, 1993), ATP, once released, is metabolized extracellularly by ectonucleotidases via sequential conversion to ADP and AMP, then to adenosine by 5'-nucleotidase (Zimmermann and Braun, 1999) and eventually to inosine and hypoxanthine. Ectonucleotidases are therefore a key element in purinergic transmission because they modulate the ultimate biologic effects of released nucleotides.

Preliminary data from our laboratory indicate that sympathetic nerve endings from guinea pig heart contain \( \text{Ca}^{2+} \)-dependent ectonucleotidase activity (Sesti et al., 2001). This is in agreement with recent cytochemical evidence in rat heart (Zinchuk et al., 1999).

In addition to its postsynaptic effects, ATP affects adrenergic transmission by acting on purinoceptors at sympathetic nerve endings (Burnstock, 1999). In primary cultures of dissociated rat superior cervical ganglion neurons, ATP-gated ionotropic P2X purinoceptors (P2XR) are known to enhance NE exocytosis, whereas metabotropic G-protein-coupled P2Y

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ABBREVIATIONS: NE, norepinephrine; ARL67156, 6-N,N-diethyl-\( \beta\)-\( \gamma\)-dibromomethylene-\( \delta\)-adenosine-5'-triphosphate; DEPC, diethylpyrocarbonate; E-NTPDase1, ectonucleotide triphosphate diphosphohydrolase 1; HBS, HEPES-buffered saline; \( \alpha, \beta\)-MeATP, \( \alpha, \beta\)-methyleneadenosine-5'-triphosphate; 2'-MeATP, 2',3'-dideoxy-\( \text{N}^\alpha\)-methyladenosine-3',5'-diphosphate; MRS 2179, 2',3'-dideoxy-\( \text{N}^\alpha\)-methyladenosine-3',5'-diphosphate; PPADS, pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid; TLC, thin layer chromatography.
purinoceptors (P2YR) may attenuate it. This suggests that endogenous ATP acts by an autocrine feedback mechanism on cardiac sympathetic terminals from which it is released.

In this investigation, we demonstrate that ATP modulates NE release from cardiac sympathetic nerve endings and that this action of ATP is controlled by an ectonucleotidase activity that we identified in cardiac synaptosomes.

Materials and Methods

Preparation of Cardiac Synaptosomes. Male Hartley guinea pigs (250–300 g) were sacrificed by cervical dislocation under light anesthesia with CO₂ vapor in accordance with institutional guidelines. The rib cage was dissected away and the heart was rapidly excised, freed from fat and connective tissue, and transferred to a Langendorff apparatus. Spontaneously beating hearts were perfused through the aorta for 15 min at constant pressure (40 cm of H₂O) with Ringer’s solution at 37°C saturated with 100% O₂ (pH 7.5) (Seyedi et al., 1997). This procedure ensured that no blood remained in the coronary circulation. At the end of the perfusion, the hearts were minced in ice-cold 0.32 M sucrose containing 1 mM EGTA, pH 7.4. Minced tissue was digested with 40 mg of collagenase per 10 ml of HEPES-buffered saline solution (HBS) per gram of wet heart weight for 45 min at 37°C. HBS contained 1 mM pargyline (monoamine oxidase inhibitor) to prevent enzymatic destruction of synaptosomal NE (Seyedi et al., 1997). After low-speed centrifugation (10 min, 120g, 4°C), the resulting pellet was suspended in 10 volumes of 0.32 M sucrose and homogenized with a Teflon/glass homogenizer. The homogenate was centrifuged (10 min, 650g, 4°C), and the pellet was rehomogenized and recentrifuged. The pellet containing cellular debris was discarded, and supernatants from the last two centrifugations were combined and aliquotted into 12 tubes for centrifugation (20 min, 20,000g, 4°C).

NE Release from Cardiac Synaptosomes. Each pellet, which contained cardiac synaptosomes, was resuspended in HBS, pH 7.4, to a final volume of 1 ml in the presence or absence of drugs. HBS contained 1 mM pargyline and tropolone (catechol-methyltransferase inhibitor) and 1 µM each of atropine (muscarinic antagonist), desipramine (NE transporter inhibitor), and yohimbine (α₂-adrenoceptor antagonist). Each suspension was incubated in a water bath at 37°C for 5 min either in the absence or presence of solCD39, a recombinant soluble form of human E-NTPDase1/CD39 (Gayle et al., 1998), or the E-NTPDase inhibitor, 6-N,N-diethyl-N-1998), or the E-NTPDase inhibitor, 6-

To establish the characteristics of the ectonucleotidase activity, we used inhibitors selective for phosphatases and ectonucleotidases [1 or 10 mM sodium azide, 1 mM DEPC, 0.1 mM sodium orthovanadate, 50 µg/ml concanavalin A, 1 mM tetrasirole, and 100 µM ARL67156]]. Inhibitors were added in 10-µl aliquots to wells (96-well plates) containing 10 µl of synaptosomal suspension, prior to addition of 30 µl of mastermix that contained 50-µM radiolabeled assay substrate, 3 mM CaCl₂, and 3 mM MgCl₂ (final concentrations). Orthovanadate and DEPC were freshly prepared just prior to addition to synaptosomes. All stock solutions were prepared in bis-Tris-propane buffer, pH 8.5. Plates were incubated for 3 or 5 min at 37°C. Samples were processed for quantitation of nucleotidase activity, as described above. Results were expressed as percentage of control values (100% – no inhibitor). Data presented are averages ± S.E.M. of nine observations obtained in three separate experiments.

Drugs. [8-14C]ATP and [8-14C]ADP were purchased from PerkinElmer Life Sciences (Boston, MA). Silica gel 60 F254-precoated TLC plates were obtained from EM Scientific (Gibbstown, NJ). All other (bio)chemicals used were purchased from Sigma-Aldrich (St. Louis, MO). SolCD39 was a generous gift from Drs. C. R. Maliszewski and R. B. Gayle III (Immunex Corp., Seattle, WA).

Results

Release of Synaptosomal NE by Exogenous ATP and Analogos. Incubation of cardiac synaptosomes with ATP (0.01–30 µM for 5 sec) caused a concentration-dependent increase in the release of endogenous NE that reached a maximum of 16% above basal level (EC₅₀ 0.96 µM; Fig. 1). The ATP analog 2-MeSATP (0.1–300 nM) also caused a concentration-dependent increase in NE release that reached a maximum of 14% (EC₅₀ 8.58 nM; Fig. 1). Another ATP analog, α, β-MeATP (0.03–3 µM), caused a concentration-dependent increase in NE release that reached a maximum of 22% (EC₅₀ 0.13 µM; Fig. 1).

In the presence of the P2YXR antagonist PPADS (10 µM), the NE-releasing effect of ATP was potentiated when MRS 2179, which MRS 2179 acts as a P2XR antagonist (Brown et al., 2000) (Fig. 3). In contrast, the NE-releasing effect of ATP was attenuated by MRS 2179 at the 30 µM concentration, at which MRS 2179 acts as a P2XR antagonist (Brown et al., 2000) (Fig. 3).
ARL67156 and by the E-NTPDase solCD39. The E-NTPDase inhibitor ARL67156 (30 μM) potentiated the NE-releasing effects of ATP and 2-MeSATP. As shown in Fig. 4, A and B, ATP and 2-MeSATP elicited a greater release of synaptosomal NE in the presence than in the absence of ARL67156, indicated by the upward shifts in the concentration-response curves. In contrast, ARL67156 did not modify the NE-releasing effect of α, β-MeATP (Fig. 4C).

The recombinant, soluble form of human E-NTPDase1/CD39 (solCD39, 3 nM), markedly attenuated the ATP-induced release of synaptosomal NE. As shown in Fig. 4A, ATP elicited a much smaller release of NE in the presence of solCD39 than in its absence.

EctoNucleotidase Activity in Cardiac Sympathetic Nerve Endings: Ca2+ Dependence and Inhibition Profile. Using our standard nucleotidase assay system (see Materials and Methods), we found cardiac synaptosomes to be rich in Ca2+-dependent ectonucleotidase activity. As shown in Fig. 5, suspensions of cardiac synaptosomes metabolized ADP at a rate of 15 nmol/min/mg of protein when Ca2+ was present in the incubation mixture. In contrast, when extracellular Ca2+ was chelated by EDTA, ectonucleotidase activity was suppressed by more than 80% (Fig. 5).

Shown in Fig. 6 are the effects of various inhibitors on cardiac synaptosomal nucleotidase activities. Neither ATPase nor ADPase activity was inhibited by the Na+/K+ ATP-ase inhibitor ouabain, the alkaline phosphatase inhibitor tetramisole,
the adenosine uptake inhibitor dipyridamole, the inhibitor of P-type ATPases orthovanadate, or the lectin concanavalin A. Synaptosomal ADPase (but not solCD39 ADPase) activity was inhibited by the adenylate kinase inhibitor P1,P5-di-(adenosine-5'-H11032)-pentaphosphate. Activity was inhibited by sodium azide, the histidine and tyrosine modifier DEPC, and the selective ectoATPDase inhibitor ARL67156. For comparison, we determined in parallel the inhibitor profile of solCD39, the recombinant soluble form of human E-NTPDase1. Our data show a similar pattern of inhibition, with the exception of pronounced stimulation of solCD39 nucleotidase activity by concanavalin A, and, to a lesser extent, by ouabain and dipyridamole. In synaptosomes, the ATPase/ADPase activity ratio was 1.74, whereas for solCD39 this ratio was 0.79. These data suggest a general similarity in enzyme characteristics between synaptosomal nucleotidase activity and solCD39, implying that the synaptosomal enzyme is of the E-NTPDase1 type.

Endogenous ATP Modulates NE Release. Depolarization of cardiac synaptosomes with K⁺ (5 min, 3–100 mM) elicited release of endogenous NE, which increased dose-dependently to ~30% above basal level (Fig. 7). MRS 2179 (30 nM, a concentration at which MRS 2179 is considered a selective P2Y₁R antagonist (Boyer et al., 1998)] augmented K⁺-induced NE exocytosis, as indicated by the leftward shift of the K⁺ concentration-response curve (Fig. 7A). In contrast, in the presence of 10 μM PPADS, a P2XR antagonist, K⁺-induced NE exocytosis was markedly attenuated at all K⁺ concentrations used (Fig. 7A). The E-NTPDase inhibitor ARL67156 (30 μM) also augmented NE exocytosis, as indicated by a leftward shift of the K⁺ concentration-response curve (Fig. 7B). Conversely, in the presence of 3 nM solCD39 (our recombinant, soluble form of human E-NTPDase1/CD39), NE exocytosis was reduced, as indicated by a marked downward shift of the K⁺ concentration-response curve (Fig. 7B).

Discussion

Our studies reveal a novel pathway that regulates NE release from cardiac sympathetic nerve terminals. ATP, co-released with NE, activates presynaptic P2XR and promotes NE exocytosis. An endogenous ectonucleotidase that we identified in cardiac sympathetic nerve endings metabolically deletes released ATP and thereby effectively decreases NE release.

Our data demonstrate that exogenous ATP evokes the release of NE from sympathetic nerve endings within a few seconds of its administration. This suggests that a transmitter-gated ionotropic receptor (P2XR) is involved (Ralevic and Burnstock, 1998). Indeed, we found that α, β-MeATP, which does not act at P2Y (Ralevic and Burnstock, 1998; Nörenberg and Illes, 2000), was more potent than ATP in releasing NE (see Fig. 1). In addition, the NE-releasing effect of ATP, 2-MeSATP, and α, β-MeATP was inhibited by PPADS (see Fig. 2) at a concentration (10 μM) at which this compound
functions as a selective P2XR antagonist (Kim et al., 2001). Although our data indicate that the NE-releasing effect of ATP and 2-MeSATP is due to P2XR activation, a possible P2YR component cannot be ruled out. If present, such a component would be expected to partially reduce the NE-releasing effect of ATP and 2-MeSATP, but not that of /H9251, /H9252-MeATP, which, as mentioned above, does not activate P2YR.

Conversely, we found that ATP can also diminish NE release by acting on presynaptic inhibitory P2YR. In fact, at a concentration of 30 nM, at which MRS 2179 acts as a selective P2Y1R antagonist (Brown et al., 2000), the NE-releasing effect of ATP was potentiated (see Fig. 3). In contrast, at a 1000-fold greater concentration, at which MRS 2179 antagonizes the effects of ATP at P2XR (Brown et al., 2000), the NE-releasing effect of ATP was inhibited (see Fig. 3).

Our data clearly indicate that endogenous ATP, released with NE upon depolarization of cardiac sympathetic nerve endings, modulates NE release by activating presynaptic facilitatory and inhibitory purinergic receptors. Indeed, the P2XR antagonist PPADS markedly inhibited NE exocytosis, whereas the P2Y1R antagonist MRS 2179 potentiated it (see Fig. 7A).

Our data demonstrate that a membrane nucleotidase plays a significant role in terminating the effects of exogenous ATP on sympathetic nerve endings. Indeed, the ectonucleotidase inhibitor ARL67156 (Crack et al., 1995) potentiated the P2XR-mediated effects of nucleotides that are metabolized by ectonucleotidase (i.e., ATP and 2-MeSATP) (Plesner, 1995), but not those of /H9251, /H9252-MeATP, which is not an ectonucleotidase substrate (Welford et al., 1987) (see Fig. 4). Importantly, solCD39, a recombinant soluble form of human E-NTPDase1 (Gayle et al., 1998), markedly attenuated the P2XR-mediated effects of ATP (see Fig. 4A).

A novel aspect of our study is the discovery that cardiac sympathetic nerve terminals express a nucleotidase activity that bears general similarity to that of solCD39 (see Figs. 5 and 6). This implies that the synaptosomal nucleotidase is of the E-NTPDase1 type. This enzyme is of importance in the regulation of NE release from cardiac sympathetic nerve terminals, as revealed by our data. Indeed, K+-induced depolarization of the synaptosomal membrane elicited much more NE release in the presence of the nucleotidase inhibitor ARL67156 than under control conditions. Conversely, in the presence of solCD39, NE exocytosis was markedly attenuated (see Fig. 7B). This indicates that endogenous ATP, re-
leased by depolarization of sympathetic nerve endings, exerts predominantly a facilitatory autocrine effect on NE release. This modulatory action is resultant of two components: positive, P2XR-mediated, and negative, P2YR-mediated. The modulatory action of ATP is terminated by E-NTPDase, both pre- and postsynaptically (see Fig. 8). Thus, by metabolizing released ATP, E-NTPDase ultimately controls the release of NE.

We hypothesize that E-NTPDase at the sympathetic nerve endings plays a protective role in hyperadrenergic states such as myocardial ischemia, which is characterized by an exaggerated release of NE. By decreasing the concentration of ATP at the nerve endings, E-NTPDase will not only reduce activation of facilitatory P2XR but also favor activation of low-threshold inhibitory P2YR, thus curtailing NE release. Indeed, we have preliminary evidence that depolarization of cardiac synaptosomes with K⁺ enhances their E-NTPDase activity (C. Sesti, R. Levi, M. J. Broekman, and A. J. Marcus, unpublished results). Furthermore, inhibition of E-NTPDase with ARL67156 enhances NE release in a myocardial ischemia/reperfusion model, whereas administration of solCD39 reduces it (Levi et al., 2001).

Metabolism of adenine nucleotides yielded AMP as the main end product in our system (data not shown). Apparently, 5'-nucleotidase is not particularly active in cardiac synaptosomes, which is similar to observations on PC12 cells (Vollmayer et al., 2001). Nevertheless, it seems likely that 5'-nucleotidase in adjacent endothelial (Marcus et al., 1991) or smooth muscle cells would generate adenosine in situ, which would then act on A₁ purinoceptors on cardiac sympathetic nerve endings culminating in decreased NE release (Imamura et al., 1994, 1996; Seyedi et al., 1997).

Enhanced adrenergic activity and NE release are known causes of clinical cardiac dysfunction, arrhythmias, and sud-

Fig. 7. Release of endogenous NE from guinea pig heart synaptosomes by depolarization with K⁺. A, potentiation by the P2Y₁R antagonist MRS 2179 and blockade by the P2X₇R antagonist PPADS. Synaptosomes were incubated for 5 min with increasing concentrations of K⁺ in the absence (control) or presence of 30 nM MRS 2179 or 10 μM PPADS (n = 4 and 4). Points represent mean increases in NE release above basal level (±S.E.M.). Basal NE level: 1.12 ± 0.06 pmol/mg of protein (n = 8). B, potentiation by the E-NTPDase inhibitor ARL67156 and inhibition by the recombinant, soluble form of human E-NTPDase1/CD39 (solCD39). Synaptosomes were incubated for 5 min with increasing concentrations of K⁺, in the absence (control) or presence of 30 μM ARL67156 or 3 nM solCD39 (n = 16 and 8, respectively). Points represent mean increases in NE release above basal level (±S.E.M.). Basal NE level: 1.17 ± 0.11 pmol/mg of protein (n = 24). When not visible, error bars are included in the symbol.

Fig. 8. Schematic representation of the role of E-NTPDase in modulating NE release from cardiac sympathetic nerve terminals.
den cardiac death in myocardial ischemia (Braunwald and Sobel, 1988; Dart and Du, 1993; Kübler and Strasser, 1994; Benedict et al., 1996). Thus, our results identify a novel protective role for the E-NTPDase at cardiac sympathetic nerve terminals and suggest that negative modulation of ATP-mediated NE release by solCD39 may offer a novel therapeutic approach to myocardial ischemia and its consequences.

References


