Ectonucleoside Triphosphate Diphosphohydrolase1/CD39, Localized in Neurons of Human and Porcine Heart, Modulates ATP-Induced Norepinephrine Exocytosis

Takuji Machida, Paul M. Heerdt, Alicia C. Reid, Ulrich Schäfer, Randi B. Silver, M. Johan Broekman, Aaron J. Marcus, and Roberto Levi

Departments of Pharmacology (T.M., U.S., R.L.), Anesthesiology (P.M.H.), and Physiology and Biophysics (A.C.R., R.B.S.), and Division of Hematology and Medical Oncology, Departments of Medicine (M.J.B., A.J.M.) and Pathology (A.J.M.), Weill Medical College of Cornell University, New York, New York; and Division of Hematology and Medical Oncology, Department of Medicine (M.J.B., A.J.M.), VA New York Harbor Health Care System, New York, New York

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ABSTRACT

Using a guinea pig heart synaptosomal preparation, we previously observed that norepinephrine (NE) exocytosis was attenuated by a blockade of P2X purinoceptors, potentiated by inhibition of ectonucleoside triphosphate diphosphohydrolase-1 (E-NTPDase1)/CD39, and reduced by soluble CD39, a recombinant form of human E-NTPDase1/CD39. This suggests that norepinephrine and ATP are coreleased upon depolarization of cardiac sympathetic nerve endings and that ATP enhances norepinephrine exocytosis by an action modulated by E-NTPDase1/CD39 activity. Whether E-NTPDase1/CD39 is localized to cardiac neurons and modulates norepinephrine exocytosis in intact heart tissue remained untested. We report that E-NTPDase1/CD39 is selectively localized in human and porcine cardiac neurons and that depolarization of porcine heart tissue elicits ω-conotoxin-inhibitable release of both norepinephrine and ATP. Inhibition of E-NTPDase1/CD39 with ARL67156 markedly potentiated ATP release, demonstrating that E-NTPDase1/CD39 is a major determinant of ATP availability at sympathetic nerve terminals. Notably, inhibition of E-NTPDase1/CD39 enhanced both ATP and NE exocytosis, whereas administration of soluble CD39 reduced both ATP and NE exocytosis. The strong correlation between ATP and norepinephrine release was abolished in the presence of the purinergic P2X receptor (P2XR) antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS). We conclude that released ATP governs norepinephrine exocytosis by activating presynaptic P2XR and that this action is controlled by neuronal E-NTPDase1/CD39. Clinically, excessive norepinephrine release is a major cause of arrhythmic and coronary vascular dysfunction during myocardial ischemia. By curtailing NE release, in addition to its effects as an antithrombotic agent, soluble CD39 may constitute a novel therapeutic approach to ischemic complications in the myocardium.

It is now well established that norepinephrine (NE) and ATP function as cotransmitters at peripheral adrenergic neuromediator junctions (Burnstock, 1999; Sneddon et al., 1999). Whether NE and ATP are released from the same site or from different varicosities (Driessen et al., 1993; Westfall et al., 1996; Bobalova and Mutafova-Yambolieva, 2001; Stjarne, 2001; Brock and Tan, 2004), ATP is likely to modulate NE release in either case (von Kugelgen et al., 1999; Sperlágh et al., 2000; Sesti et al., 2002). Indeed, once released, neurotransmitter ATP promotes NE release by activating presynaptic P2X purinoceptors (P2XRs) (Boehm, 1999; Sperlágh et al., 2000; Sesti et al., 2002; Queiroz et al., 2003).

Two different and independent mechanisms terminate the effects of the two transmitters: NE is removed from the synaptic cleft primarily by reuptake into the nerve terminals by a specific transporter (Amara and Kuhar, 1993), whereas ATP is metabolized by nucleotidases (Zimmermann and Braun, 1999; Westfall et al., 2002). Using sympathetic nerve endings isolated from guinea pig heart (i.e., cardiac synaptosomes), we previously demonstrated that activation of ATP-gated ionotropic presynaptic P2XRs promoted NE exocytosis (Sesti et al., 2002). This effect was increased by inhibition of endogenous ectonucleotidase (E-NTPDase1) and diminished

ABBREVIATIONS: NE, norepinephrine; P2XR, purinergic P2X receptor(s); E-NTPDase1, ectonucleoside triphosphate diphosphohydrolase1; solCD39, recombinant, soluble form of human E-NTPDase1/CD39; KHS, Krebs-Henseleit solution; ARL67156, 6-N,N-diethyl-β-γ-dibromomethylene-β-adenosine-5′-triphosphate; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid; ω-CTX, ω-conotoxin; PBS, phosphate-buffered saline; Ab, antibody; ANOVA, analysis of variance.
by the addition of soluble CD39 (solCD39), a recombinant form of human E-NTPDase1 (Sesti et al., 2002; Marcus et al., 2003). Furthermore, depolarization of synaptosomes with K+ evoked NE exocytosis, which was also potentiated by inhibition of E-NTPDase1 and attenuated by administration of solCD39.

These findings suggest that ATP released by depolarization of sympathetic terminals enhances NE exocytosis and that E-NTPDase1 plays an important role in adrenergic neurotransmission in the heart. This notion was based on results obtained in synaptosomes isolated from guinea pig heart, but it required verification and amplification in an intact tissue preparation. Accordingly, we have now studied the localization of CD39 in human and porcine heart. In addition, we studied the role of E-NTPDase1 in exocytosis of NE and ATP in porcine heart, the physiology of which more closely resembles the human heart (Appel et al., 2001). Our findings have now directly demonstrated the presence of E-NTPDase1/CD39 in cardiac neurons and indicate that, by terminating the action of transmitter ATP, E-NTPDase1/CD39 negatively modulates NE exocytosis in the heart.

Materials and Methods

Sources of Human and Porcine Cardiac Tissue

**Human.** Specimens of right atrium (i.e., surgical waste tissue) were obtained from patients undergoing cardiopulmonary bypass (two males, ages 60 and 63 years), following a protocol approved by our Institutional Review Board. At the time of surgery, a sample of atrial appendage measuring ~1 cm3 was removed from the atriotomy site and rapidly transported to the laboratory in ice-cold oxygenated Krebs-Henseleit solution (KHS) composed of the following:

- 118.2 mM NaCl
- 4.83 mM KCl
- 2.5 mM CaCl2
- 2.37 mM MgSO4
- 1.0 mM KH2PO4
- 25 mM NaHCO3
- 11.1 mM glucose

**Porcine.** Under a protocol approved by the Institutional Animal Care and Use Committee, tissue harvested from eight female Sinclair pigs weighing approximately 30 kg were used for the study. After sedation with intramuscular tiletamine/zolazepam (2.2 mg/kg), animals were deeply anesthetized with isoflurane (1.3% end tidal) and 100% oxygen. The chest was opened via median sternotomy, the pericardium was opened widely, and the heart was sur-
In human sections that were probed with only a mouse monoclonal human anti-CD39 antibody (1:100), the corresponding 2nd Ab was Alexa Fluor 488 goat anti-mouse IgG (1:400). For colocalization studies, both a rabbit anti-synapsin I antibody, a specific label of neurons (De Camilli et al., 1983), and a mouse monoclonal human anti-CD39 antibody were applied to human heart sections at a dilution of 1:100. For these sections, the 2nd Abs used were Alexa Fluor 594 donkey anti-rabbit IgG (red) (1:1200) and Alexa Fluor 488 donkey anti-mouse IgG (green) (1:600).

Porcine heart sections that were colabeled with the mouse monoclonal human anti-CD39 antibody (1:100) and goat polyclonal anti-synapsin Ia/b antibody (1:400) were subsequently stained with the following 2nd Abs: Alexa Fluor 488 donkey anti-mouse IgG (green) (1:600) and Alexa Fluor 594 donkey anti-goat IgG (red) (1:1200).

Tissue sections were examined with an inverted epifluorescent microscope (Nikon Diaphot; Morrell Instrument Co., Melville, NY) interfaced to a frame-transfer type cooled charge-coupled device (Roper Scientific, Duluth, GA) and processed with MetaFluor/Meta-Morph software (Universal Imaging Corporation, Downingtown, PA). Digital images were imported into Adobe Photoshop (7.0) for minimal processing.

**Drugs**

SolCD39 was a generous gift from Drs. C. R. Maliszewski and R. B. Gayle III (Amgen/Immunex, Seattle, WA). ARL67156 was purchased from A. G. Scientific, Inc. (San Diego, CA). Donkey serum was purchased from Abcam (Cambridge, MA). Mouse monoclonal anti-human CD39 antibody BU61 was purchased from Accurate Chemical & Scientific (Westbury, NY). Goat polyclonal anti-synapsin Ia/b antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other antibodies were purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Statistics**

Values are expressed as mean ± S.E.M. Analysis by one-way ANOVA was followed by post hoc testing (Dunnett’s test). A value of \( P < 0.05 \) was considered statistically significant.

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**Results**

Since E-NTPDase1/CD39 metabolizes neurotransmitter ATP, if E-NTPDase1/CD39 were localized to cardiac neurons, it could curtail the potentiation of NE exocytosis by neurotransmitter ATP. Therefore, we initially investigated the localization of E-NTPDase1/CD39 in the human heart. For this purpose, we used mouse monoclonal antibody BU61 directed against human CD39. We immunostained frozen sections of surgical specimens of human right atrium with BU61. As shown in Fig. 1A, positive immunostaining was limited to distinct nerve-like structures. This suggested a selective distribution of E-NTPDase1/CD39 in cardiac nerves. To confirm this, we colabeled sections of human atria with an anti-synapsin antibody (synapsin being a protein specific to neurons; De Camilli et al., 1983) and the anti-CD39 antibody. We found that CD39 and synapsin were colocalized in cardiac nerves, as shown in Fig. 1, B–D. Given the close similarity of human and porcine cardiac tissues (Appel et al., 2001), and because of limited availability of human heart specimens, we next extended these studies to porcine atrial tissue. As in the human heart, CD39 and synapsin colocalized in porcine cardiac nerves (Fig. 1, E–G).

We next studied the role of E-NTPDase1/CD39 in the exocytosis of NE and ATP. As shown in Fig. 2, K⁺-induced depolarization of porcine atrial (A) and ventricular (B) tissue elicited release of NE and ATP. In response to 50 and 100 mM K⁺, NE release increased ~2- and ~8- to 9-fold above control values, respectively, in both atria and ventricles. With 50 and 100 mM K⁺, ATP release increased ~2- and ~5-fold in the atria and ~3 and ~9-fold in the ventricles, respectively. In the presence of the N-type Ca²⁺ channel inhibitor ω-conotoxin (ω-CTX) (10 and 100 nM), the increase in the release of both NE and ATP in response to 50 mM K⁺ was abolished, indicating that this release was exocytotic. The P2XR antagonist PPADS (100 μM) and the ectonucle-
otidase inhibitor ARL67156 (60 μM), alone or in combination, did not change basal NE release in atrial tissue (Fig. 3A). However, when porcine atrial tissue was depolarized with 50 mM K+, PPADS attenuated NE exocytosis as a function of its concentration (by 30 and 73%, at 30 and 100 μM, respectively; Fig. 3B). PPADS (100 μM) did not modify basal or K+-stimulated (50 mM) ATP release (Fig. 3, C and D), whereas 60 μM ARL67156, either alone or in combination with 100 μM PPADS, doubled ATP release, both under control and K+-stimulated conditions (Fig. 3B). PPADS did not modify the increase in ATP release caused by ARL67156 (Fig. 3, C and D).

As shown in Fig. 5A, incubation with solCD39 markedly decreased both basal (90%, at 10 μM) and K+-stimulated (50 mM) ATP release (50 and 80%, at 1 and 10 μM, respectively) in porcine atrial tissue. In the same preparations, 10 μM solCD39 did not modify basal NE release, but it reduced the K+-induced NE exocytosis by 22% at 0.1 μM and by 45% at both 1 and 10 μM (Fig. 5B).

Shown in Fig. 6 is the relationship between the release of ATP and NE in porcine heart tissue in response to K+-depolarization, either under control conditions or in the presence of PPADS, ARL67156, and solCD39. Thus, we have demonstrated a highly significant correlation between the K+-evoked release of ATP and that of NE, independently of whether ATP release was enhanced by 60 μM ARL67156 or was diminished by incubation with 10 μM solCD39. In con-
trast, in the presence of 100 μM PPADS, NE release remained constant independently of the changes in ATP released. This lack of correlation in the presence of PPADS suggested that blockade of P2XR prevented ATP-induced modulation of NE release.

Discussion

ATP functions as a neurotransmitter in peripheral sympathetic nerves (von Kugelgen et al., 1994; Sneddon et al., 1999) and central sympathetic system (Poelchen et al., 2001) and also modulates release of other transmitters, such as NE (Burnstock, 1999). Using a synaptosomal preparation, i.e., a high-speed pellet of a collagenase-treated guinea pig heart homogenate containing “pinched-off” sympathetic nerve terminals, we proposed that, once released from these nerve endings, ATP amplifies NE release via a positive feedback mechanism initiated by P2XR activation (Sesti et al., 2002). We hypothesized that this ATP-dependent amplification of NE release could be influenced by E-NTPDase1/CD39, an enzyme that inactivates ATP, thereby interrupting the ATP-initiated positive feedback loop (Sesti et al., 2002). Whether E-NTPDase1/CD39 is localized to cardiac neurons and modulates NE exocytosis in intact cardiac tissue remained untested. The present investigation was designed to answer these questions.

We have now directly demonstrated the presence of E-NTPDase1/CD39 in cardiac nerves by immunohistochemistry. To confirm that E-NTPDase1/CD39 is localized in cardiac nerves, we used colocalization methodology using an anti-synapsin antibody to identify neuronal structures (De Camilli et al., 1983) and a mouse monoclonal antibody directed against human CD39. With this technique, we determined that in human and in porcine cardiac tissue, specific CD39 staining is selectively limited to neurons (Fig. 1). Notably, the staining could include both membrane-fixed (Gordon, 1986; Zimmermann and Braun, 1999) and releasable nucleotidases (Todorov et al., 1997; Westfall et al., 2002). Colocalization of CD39 with synaptophysin, demonstrating the presence of CD39 in neurons, had been previously reported in rat brain (Wang and Guidotti, 1998).

The presence of NTPDase1 and 2 was previously reported in murine cardiac tissues (Sevigny et al., 2002). NTPDase1 was localized in the endothelium, whereas NTPDase2 was found in pericytes (Sevigny et al., 2002). Also, mRNA for CD39L2 (NTPDase6) was found to be expressed in myocytes and capillary endothelial cells in human heart (Yeung et al., 2000). In the present study, using an anti-NTPDase1 antibody, we found immunostaining only in neurons in human and porcine cardiac tissue. The lack of staining in the microvasculature may be due to species differences. Although we did not search for other nucleotidases (e.g., NTPDase2), there are no reports in the literature of NTPDase2 in human and porcine heart.

Because of availability and similarity to human heart (Appel et al., 2001), we chose to use porcine cardiac tissue to
Fig. 4. Effects of the P2X receptor antagonist PPADS and the E-NTPDase1/CD39 inhibitor ARL67156 on NE and ATP exocytosis in porcine ventricle. A, neither 60 μM ARL67156 (ARL) nor 100 μM PPADS affected basal NE release. B, PPADS attenuated NE exocytosis evoked by depolarization with 50 mM K⁺, whereas 60 μM ARL67156 enhanced it. PPADS attenuated the ARL67156-induced potentiation of NE exocytosis. C, ARL67156 (60 μM) enhanced basal ATP release, whereas 100 μM PPADS had no effect on either basal or ARL67156-potentiated ATP release. D, ARL67156 (60 μM) enhanced the K⁺-induced ATP exocytosis, whereas PPADS did not modify it, whether in the presence or absence of ARL67156. Columns represent mean values (±S.E.M.; n = 4–19 for A and B; n = 4–20 for C and D). **, P < 0.01 versus corresponding basal or K⁺ control NE and ATP release by unpaired t test. †, ††, and †††, P < 0.05, 0.01, and 0.001 versus corresponding K⁺-evoked NE exocytosis by one-way ANOVA followed by Dunnett’s test.

Fig. 5. Effects of solCD39 (CD39) on ATP and NE exocytosis in porcine atrium and ventricle. A and B, administration of solCD39 (1 and 10 μM) decreased basal ATP release as well as K⁺-evoked (50 mM) ATP exocytosis from atrium (A) and ventricle (B). C, administration of solCD39 did not modify basal NE release, but it decreased K⁺-induced ATP exocytosis in both atrium (C) and ventricle (D). Columns represent mean values (±S.E.M.; n = 4–18, n = 4–20, n = 4–20, and n = 4–19 for A–D, respectively). **, P < 0.01 versus corresponding basal ATP release; † and ††, P < 0.05 and 0.01, versus corresponding K⁺-evoked NE and ATP exocytosis by one-way ANOVA followed by Dunnett’s test.
Ca²⁺ and ventricular, initiated release of both NE and ATP, and evoked depolarization of porcine cardiac tissue, both atrial presence of 50 mM K⁺ were diminished due to metabolism by exogenous

bition of E-NTPDase1/CD39 with ARL67156, NE exocytosis correlated positively with the quantities of ATP released. (Queiroz et al., 2003).

overflow in rat vas deferens, whereas PPADS inhibits it availability at sympathetic nerve endings. Consistent with ARl67156 markedly potentiated the release of ATP, demonstrating the possibility that ATP might modulate its own release exocytosis in either atrial or ventricular tissue, thus excluding the possibility that ATP might modulate its own release via P2XR. In contrast, inhibition of E-NTPDase1/CD39 with ARL67156 markedly potentiated the release of ATP, demonstrating that E-NTPDase1/CD39 is required for metabolic disposition of released ATP and thereby determines ATP availability at sympathetic nerve endings. Consistent with our results, ARL 67156 potentiates electrically evoked NE overflow in rat vas deferens, whereas PPADS inhibits it (Queiroz et al., 2003).

Notably, we found that the magnitude of NE exocytosis correlated positively with the quantities of ATP released. Indeed, when exocytosed ATP levels were increased by inhibition of E-NTPDase1/CD39 with ARL67156, NE exocytosis also increased; conversely, when the quantities of released ATP were diminished due to metabolism by exogenous solCD39, NE exocytosis was markedly reduced. The correlation between ATP and NE release thus remained constant during various experimental conditions, irrespective of whether ATP release was increased or decreased (Fig. 6). This correlation suggested that released ATP regulates NE exocytosis by activating presynaptic P2XR, as noted in previous studies (Boehm, 1999; Sperlah et al., 2000; Sesti et al., 2002, 2003; Queiroz et al., 2003). Indeed, we found that in the presence of the P2XR antagonist PPADS, the curve relating NE release to that of ATP was shifted downwards, demonstrating that for the same quantities of ATP much less NE was released (Fig. 6). This strengthens the hypothesis that coreleased ATP modulates NE exocytosis via P2XR activation.

The important regulatory role played by E-NTPDase1/CD39 in P2XR-mediated modulation of NE exocytosis is clearly established by our results. Inhibition of this ectonucleotidase with ARL67156 potentiated the release of both ATP and NE, whereas administration of solCD39 depressed it. Excessive NE release is a major cause of arrhythmic and coronary vascular dysfunction in myocardial ischemia (Braunwald and Sobel, 1988; Benedict et al., 1996; Levi and Smith, 2000). By curtailing NE release, in addition to its antithrombotic effects (Marcus et al., 2003), solCD39 may offer a novel therapeutic approach to ischemic clinical conditions. Indeed, our previous data indicated that solCD39 reduces ischemia-induced efflux of NE not only in synaptosomal preparations but also in the intact perfused heart (Sesti et al., 2003). This signifies that the circulation of the heart is in intimate contact with its neuronal system, raising the possibility that systematically administered high molecular weight agents affecting neuronal activity, such as solCD39, may induce immediate and beneficial effects under conditions of ischemia. As a therapeutic agent, solCD39 is likely to be more advantageous than a P2XR antagonist. Unlike P2XR antagonists, solCD39 will decrease ATP concentrations at sympathetic nerve endings, thus not only reducing activation of facilitatory P2X receptors but also favoring activation of low-threshold inhibitory P2Y receptors (Boehm and Kubista, 2002; Sesti et al., 2002).

Another possible application of solCD39 is in congestive heart failure. This disorder is usually accompanied by enhanced adrenergic discharge in the arteriolar bed. This results in an increase in peripheral resistance and afterload, producing further deterioration of cardiac function (Benedict et al., 1996; Esler and Kaye, 1998). By limiting excessive NE release, solCD39 should be a valuable adjunct in the management of cardiac failure.

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References


Address correspondence to: Dr. Roberto Levi, Department of Pharmacology, 1300 York Ave., Room LC419, Weill Medical College of Cornell University, New York, NY 10021. E-mail rlevi@med.cornell.edu