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Ecto-nucleotidase in Sympathetic Nerve Endings Modulates ATP and Norepinephrine Exocytosis in Myocardial Ischemia

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Abbreviations used:
ARL67156, 6-N,N-diethyl-β-γ-dibromomethylene-D-adenosine-5'-triphosphate; DMI, desipramine; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; HBS, HEPES-buffered saline; NE, norepinephrine; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid; P2XR, purinergic P2X receptor; solCD39, recombinant soluble form of human E-NTPDase1/CD39; TLC, thin layer chromatography.

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Abstract

We recently reported that ATP, co-released with norepinephrine (NE) from cardiac sympathetic nerves, increases NE exocytosis via a positive feedback mechanism. A neuronal ecto-nucleotidase (E-NTPDase) metabolizes the released ATP, decreasing NE exocytosis. Excessive NE release in myocardial ischemia exacerbates cardiac dysfunction. Thus, we studied whether the ATP-mediated autocrine amplification of NE release is operative in ischemia and, if so, whether it can be modulated by E-NTPDase and its recombinant equivalent, solCD39. Isolated, guinea-pig hearts underwent 10- or 20-min ischemic episodes, wherein NE was released by exocytosis and reversal of the NE transporter, respectively. Further, to restrict the role of E-NTPDase to transmitter ATP, sympathetic nerve endings were isolated (cardiac synaptosomes) and subjected to increasing periods of ischemia. Availability of released ATP at the nerve terminals was either increased via E-NTPDase inhibition or diminished by enhancing ATP hydrolysis with solCD39. P2X receptor blockade with PPADS was used to attenuate the effects of released ATP. We found that, in short-term ischemia (but, as anticipated, not in protracted-ischemia, where NE release is carrier-mediated), ATP exocytosis was linearly correlated with that of NE. This indicates that by limiting the availability of ATP at sympathetic terminals, E-NTPDase effectively attenuates NE

exocytosis in myocardial ischemia. Our findings suggest a key role for neuronal E-NTPDase in the control of adrenergic function in the ischemic heart. Because excessive NE release is an established cause of dysfunction in ischemic heart disease, solCD39 may offer a novel therapeutic approach to myocardial ischemia and its consequences.

The release of norepinephrine (NE) from sympathetic nerves in the heart is regulated by several pre-synaptic receptors (Boehm and Kubista, 2002). Of these, some are inhibitory (e.g., α_2-adrenergic, adenosine A_1 and histamine H_3 receptors)(Imamura et al., 1996) and others, facilitatory (e.g., angiotensin AT_1 and purinergic P2X receptors; P2XR)(Seyedi et al., 1997; Sesti et al., 2002; Seyedi et al., 2002). Each of these receptors can be activated in an autocrine or paracrine mode by the respective endogenous ligand. Local concentrations of the ligand are reduced by re-uptake into nerve endings or by metabolic hydrolysis, as in the case of NE (Amara and Kuhar, 1993) and ATP (Zimmermann and Braun, 1999), respectively.

We recently reported that ATP, co-released with NE from cardiac sympathetic nerve terminals, increases NE release via a positive feedback mechanism (Sesti et al., 2002). Furthermore, an endogenous ecto-nucleotidase (E-NTPDase), which we identified in cardiac sympathetic nerve endings, metabolically deletes released ATP, thereby effectively decreasing NE release (Sesti et al., 2002). Inasmuch as excessive NE release in hyperadrenergic states, such as acute myocardial ischemia, can further exacerbate cardiac dysfunction (Levi and Smith, 2000), we studied whether the ATP-mediated autocrine positive feedback amplification of NE

release operates in ischemic conditions and, if so, to what extent it can be modulated by E-NTPDase.

To this end, we chose the isolated, spontaneously beating heart, subjected to either 10- or 20-min periods of ischemia, as the experimental model. In these two time frames, NE is released by exocytosis and reversal of the NE transporter, respectively (Hatta et al., 1999). Further, to eliminate possible effects of ATP produced by myocytes and endothelial cells, and to restrict the role of E-NTPDase to transmitter ATP released from sympathetic terminals, we isolated sympathetic nerve endings from the heart (cardiac synaptosomes) and subjected them to progressively increasing periods of ischemia. We report here that in short-term ischemia, characterized by exocytosis of NE and ATP, E-NTPDase limits the availability of released ATP at sympathetic nerve terminals. We conclude that E-NTPDase modulates the P2XR-mediated, positive feedback mechanism that exacerbates ischemic NE release.

Materials and Methods

Preparation of Cardiac Synaptosomes

Male Hartley guinea pigs (250-300 g) were sacrificed by cervical dislocation under light anesthesia with CO_2 vapor in accordance with Institutional guidelines. The rib cage was dissected away, and the heart 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 H_2O) with Ringer's solution at 37°C saturated with 100% O_2 (pH 7.5) (Seyedi et al., 1997). This procedure ensured that blood did not remain in the coronary circulation. At the end of perfusion, the hearts were minced in ice-cold 0.32 M sucrose containing 1 mM EGTA, pH 7.4. The minced tissue was digested with 40 mg collagenase per 10 ml of HEPES-buffered saline solution (HBS) per gram of wet heart weight for 45 min at 37°C. HBS contained 50 mM HEPES, pH 7.4, 144 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose and 1 mM pargyline (monoamine oxidase inhibitor) to prevent enzymatic degradation of synaptosomal NE (Seyedi et al., 1997). After low-speed centrifugation (10 min, 120 *g*, 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, 650 *g*, 4°C) and the pellet re-homogenized and re-

centrifuged. The resulting pellet, containing cellular debris, was discarded and supernatants from the two centrifugations were combined and aliquotted into 12 tubes for centrifugation (20 min, 20,000 ***g,*** 4°C).

Each pellet, containing cardiac synaptosomes, was resuspended either in HBS pH 7.4 (normoxic conditions) or in glucose-free HBS, containing the reducing agent sodium dithionite (ischemic conditions) to a final volume of 0.5 ml in the presence or absence of drugs. Each suspension was incubated in a water bath at 37°C either in the absence or presence of the NE transporter inhibitor desipramine (DMI); a recombinant soluble form of human E-NTPDase1/CD39 (solCD39) (Gayle, III et al., 1998); the E-NTPDase inhibitor, 6-N,N-diethyl-β-γ-dibromomethylene-D-adenosine-5'-triphosphate (ARL67156) and the P2XR antagonist, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS). These agents were added at the concentrations indicated, 5 min prior to ischemia or normoxia. In each experiment, one sample was untreated (control, basal NE release) and incubated for the same length of time under both conditions.

Induction of Ischemia in Cardiac Synaptosomes

Ischemia was induced by incubating synaptosomes for 10, 20, 30 or 70 min in glucose-free HBS bubbled with 95% N_2 and 5% CO_2, containing sodium dithionite (3 mM, P_{O_2} ~0 mmHg, pH ~7.3; ischemic release)(Seyedi et al., 2002). Matched synaptosomes were incubated for

an equivalent period of time with oxygenated (95% O_2 and 5% CO_2) HBS (normoxic release).

Following incubation, each sample was centrifuged (20 min, 20,000 *g*, 4°C) and the supernatant assayed either for NE content by high-performance liquid chromatography with electrochemical detection (Seyedi et al., 1997) or ATP content by luciferin-luciferase luminometry (see below). The pellet was assayed for protein content by a modified Lowry procedure (Seyedi et al., 1997). Data were expressed as pmol/mg protein for NE and fmol/mg protein for ATP (mean ± S.E.M.; n = number of observations).

Ischemia/Reperfusion in Isolated Hearts

Guinea-pig hearts were isolated as described above and perfused for 30-min (stabilization period) prior to ischemia. Normothermic 10-min or 20-min global ischemia was then induced by complete cessation of coronary perfusion, followed by a 20-min reperfusion period (Hatta et al., 1999). The coronary effluent was collected into tubes, every 5 min for a total of 15 min, prior to ischemia and every 2 min following ischemia. When DMI, solCD39, ARL67156 or PPADS was used, the heart was perfused with the compound for the duration of the experiment. Hearts were weighed at the end of the experiment. Samples of coronary effluent were assayed for NE and ATP, by HPLC with electrochemical detection and

luciferin-luciferase luminometry, respectively. Data were expressed as pmol of NE or ATP released/gram of wet weight (mean ± S.E.M., n = number of hearts).

Quantification of ATP release by luciferin-luciferase assay

ATP levels were measured with a firefly luciferin-luciferase assay-based commercial kit (ATP Bioluminescence Assay Kit HS II, Roche Diagnostics Corp., Indianapolis, IN). Samples (50 µL) of each supernatant (synaptosomal preparations) or coronary effluent (isolated hearts) were pipetted into appropriate test tubes, placed in a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA) and processed by autoinjection of 50 µL of luciferin/luciferase reagent. ATP concentrations were calculated from a calibration curve constructed the same day using ATP standards included in the kit. The optimal detection range was between 10^{-10} and 10^{-16} moles of ATP. The amount of ATP was expressed as pmol/g heart in ischemia/reperfusion experiments and as fmol/mg protein in the experiments with synaptosomes.

Radio-TLC Assay for E-NTPDase activity

Exogenous ATP was added to samples of synaptosomes or solCD39 and its metabolism was measured. Samples were incubated in 96-well plates with 50 µM [^{14}C]ATP in 50 µL assay buffer [15 mM Tris, 134 mM NaCl, 5 mM glucose, pH 7.4, containing 3 mM $CaCl_2$] for 5 min at 37°C.

With the 96-well plate on ice, PPADS (30 μM) was added to samples. The assay buffer "master mix", containing radioactive substrate, was then added. The 96-well plate was then transferred to a 37°C water bath. To stop the reaction, the plate was again placed on wet ice and 10 μL “stop solution” (160 mM disodium EDTA, pH 7.0, 17 mM ADP, and 0.15 M NaCl) was immediately added to each well to block further nucleotide metabolism. Following centrifugation to remove particulate material, 40 μL of supernatant was removed and stored at -20°C prior to separation of nucleotides, nucleosides, and bases by TLC using the solvent system isobutanol/1-pentanol/ethylene glycol monoethyl ether/NH_4OH/H_2O (90:60:180:90:120, v/v). Radioactivity was quantitated by radio-TLC scanning (Instantimager; Packard BioScience; Meriden, CT) (Marcus et al., 1997) (Drosopoulos et al., 2000; Sesti et al., 2002). Values were calculated as averages of triplicate measurements following subtraction of buffer blanks (consistently less than 1% of total radioactivity). Data were expressed as percentage of ATP metabolized.

Chemicals and Drugs

SolCD39 was a generous gift from Drs. C.R. Maliszewski and R.B. Gayle, III (Immunex Corp, Seattle, WA). All other chemicals were purchased from Sigma-Aldrich, St. Louis, MO.

Statistical Analysis

Statistical analysis was performed using GraphPad Software (Prism 3.00 and InStat 3.01). Statistical comparisons between groups were made by one-way ANOVA followed by post-test analysis with the methods of Bonferroni (isolated hearts) and Tukey (synaptosomes).

Results

Overflow of NE and ATP at reperfusion following 10-minute ischemia in guinea-pig hearts.

NE overflow into the coronary effluent of isolated guinea-pig hearts during 20-min reperfusion, following a 10-min period of global ischemia, increased from an undetectable pre-ischemic level to 4.52 ± 0.37 pmol/g (± S.E.M.; n = 6) (Fig. 1A). In the presence of the NE transporter inhibitor DMI (300 nM), NE overflow after ischemia was further increased by ~50%, indicating that the increase in NE overflow resulted from exocytotic NE release. When hearts were perfused with the P2XR antagonist PPADS (30 μM), NE overflow after ischemia was reduced by ~43%. NE overflow was also reduced (~45%) in the presence of the recombinant soluble form of human E-NTPDase1/CD39, solCD39 (10 nM). Perfusion with the E-NTPDase inhibitor ARL67156 (30 μM) elicited a ~43% increase in NE overflow, whereas ARL67156 and PPADS in combination decreased NE overflow by ~35% (Fig. 1A).

ATP overflow into the coronary effluent of the same guinea-pig hearts increased three fold from a pre-ischemic level of 10.93 ± 1.70 to a post-ischemic level of 29.67 ± 1.29 pmol/g (± S.E.M.; n = 6) (Fig. 1B). In the presence of PPADS and ARL67156, either alone or in combination, ATP overflow after ischemia increased ~6-fold from the respective pre-

ischemic levels. In hearts perfused with solCD39, ATP overflow was undetectable both before and after ischemia.

Overflow of NE and ATP at reperfusion following 20-minute ischemia in guinea-pig hearts.

NE overflow into the coronary effluent of isolated guinea-pig hearts during 20-minute reperfusion, following a 20-minute period of global ischemia, increased from an undetectable pre-ischemic level to 608.50 ± 39.62 pmol/g (± S.E.M.; n = 6) (Fig. 2A). In the presence of the NE transporter inhibitor DMI (300 nM), NE overflow after ischemia was decreased by ~84%. This indicated that the increase in NE overflow resulted from carrier-mediated NE release, due to reversal of the NE transporter. In contrast, when hearts were perfused either with the P2XR antagonist PPADS (30 μM), or with the recombinant soluble form of human E-NTPDase1/CD39, solCD39 (10 nM), or with the E-NTPDase inhibitor ARL67156 (30 μM), NE overflow after ischemia was the same as in the absence of these agents (Fig. 2A).

ATP overflow into the coronary effluent of the same guinea-pig hearts increased 2-fold from a pre-ischemic level of 11.70 ± 0.20 to a post-ischemic level of 24.25 ± 0.21 pmol/g (± S.E.M.; n = 6) (Fig. 2B). In the presence of PPADS or ARL67156, ATP overflow after ischemia increased

~3-fold from the respective pre-ischemic levels. In hearts perfused with solCD39, ATP overflow was undetectable both before and after ischemia.

Shown in figure 3 is the lack of correlation between the overflow of ATP and that of NE in 30 and 24 guinea-pig hearts subjected to 10- (panel A) and 20-min ischemia (panel B), respectively, both in the absence and in the presence of PPADS, ARL67156 and solCD39.

NE and ATP release from ischemic cardiac sympathetic nerve terminals.

Sympathetic nerve terminals (cardiac synaptosomes) were isolated from guinea-pig hearts and incubated for 10, 20, 30 and 70 minutes, either under normoxic or ischemic conditions (see Methods). Ischemia caused a marked increase in NE and ATP release, in the picomolar and femtomolar range, respectively. NE release progressively increased with the time of exposure to ischemia, while the increase in ATP release progressively declined (Fig. 4). In the presence of the NE transporter inhibitor DMI (300 nM), NE release was enhanced by ~35% after 10-min ischemia but was reduced by ~65% after 30-min ischemia (Figs. 5A and 7A). This indicated that NE release was exocytotic during the first 10 minutes of ischemia and carrier-mediated, due to reversal of the NE transporter, in the subsequent 20 minutes. In contrast, DMI had no effect on ATP release (Figs. 5B and 7B).

Exocytotic release of NE and ATP from cardiac synaptosomes during 10-min ischemia.

Incubation of cardiac synaptosomes for 10 minutes under ischemic conditions elicited a ~64% increase in the release of NE above basal normoxic conditions. This increase was reduced by ~40% by the P2XR antagonist PPADS (10 μM) or by the recombinant soluble form of human E-NTPDase1/CD39, solCD39 (3 nM). In contrast, the E-NTPDase inhibitor ARL67156 (30 μM) potentiated by ~50% the increase in NE release caused by 10-min ischemia. When ARL67156 and PPADS were used in combination, the ischemia-induced increase in NE release was attenuated by ~40% (Fig. 5A).

In the same synaptosomes, a 10-min period of ischemia elicited a 4-fold increase in ATP release above the normoxic state. This increase was reduced by ~50% in the presence of PPADS, and was completely suppressed by solCD39. In contrast, the increase in ATP release induced by 10-min ischemia was further potentiated by 2.5-fold in the presence of ARL67156. When ARL67156 and PPADS were used in combination, the ischemia-induced increase in ATP release was attenuated by ~40% (Fig. 5B). In normoxic conditions, DMI, PPADS, ARL67156 and solCD39 had no effect on NE and ATP release.

Shown in Figure 6 is the correlation between the release of ATP and that of NE from synaptosomes isolated from 16 guinea-pig hearts and subjected to 10-min ischemia, either in the absence or presence of various drugs. NE release was directly correlated with ATP release (r^2=0.843). Notably, agents that decreased (PPADS, solCD39, ARL67156+PPADS) or increased (ARL67156) ATP release also decreased or increased NE release.

Release of NE and ATP from cardiac synaptosomes during 30-min ischemia.

Incubation of cardiac synaptosomes for 30 minutes under ischemic conditions elicited a ~2-fold increase in NE release above baseline normoxic conditions. This increase was inhibited by ~15% in the presence of either PPADS (10 μM), solCD39 (3 nM), or PPADS and ARL67156 in combination. In contrast, ARL67156 (30 μM) potentiated the ischemia-induced increase in NE release by ~17% (Fig. 7A).

In these synaptosomes, 30-min of ischemia elicited a 2.5-fold increase in ATP release above the normoxic state. This increase was reduced by ~30% in the presence of PPADS and was completely suppressed by solCD39. In contrast, ARL67156 elicited a ~6-fold increase in ischemia-induced ATP release. When ARL67156 and PPADS were used in combination, the increase in ATP release was attenuated by ~45%

(Fig. 7B). In normoxic conditions, DMI, PPADS, ARL67156 and solCD39 had no effect on NE and ATP release. There was no correlation between ATP and NE release (data not shown).

Effects of PPADS on E-NTPDase activity.

Although PPADS is classified as a selective P2XR antagonist (Kim et al., 2001), we examined the possibility that it could attenuate the hydrolysis of ATP (Heine et al., 1999; Zimmermann, 2000). If so, this would explain the increase in ATP overflow in the presence of PPADS reported in Figs. 1B and 2B. Therefore, we investigated the effect of PPADS on the metabolism of ATP by either synaptosomal E-NTPDase or solCD39. We found that in the presence of PPADS (30 μM), the metabolism of ATP by endogenous synaptosomal E-NTPDase was inhibited by 25.65 ± 0.65 % (± S.E.M.; n = 6). When metabolism of ATP was induced by solCD39, PPADS inhibited it by 63.65 ± 2.85 % (± S.E.M.; n = 6).

Discussion

We previously reported the presence of ecto-nucleotidase (E-NTPDase) activity in cardiac sympathetic nerve terminals (Sesti et al., 2002). We determined how this ecto-nucleotidase, by hydrolyzing ATP released from the nerves, reduced the availability of ATP at presynaptic ionotropic P2XR that facilitate NE release. Thus, we demonstrated that a function of this ecto-nucleotidase was to attenuate NE exocytosis from cardiac sympathetic nerve endings (Sesti et al., 2002). These studies have now been extended to ecto-nucleotidase in ischemia-reperfusion models in the whole heart and in sympathetic nerve endings. In these models, NE release can be either exocytotic, or carrier-mediated, due to reversal of the NE transporter (Hatta et al., 1999).

In short-term ischemia models, in both the isolated heart and cardiac synaptosomes, NE exocytosis was augmented by inhibition of E-NTPDase with ARL67156. Moreover, NE exocytosis was markedly reduced either by enhancing the hydrolysis of released ATP with solCD39, or by blocking P2XR with PPADS. Interestingly, when ARL67156 was combined with PPADS, the blockade of P2XR prevented ARL67156-induced potentiation of NE release. Collectively, these findings support the concept that ATP, released from sympathetic nerve endings in the heart, activates pre-junctional P2XR in an autocrine mode. This positive feedback mechanism amplifies NE exocytosis not only in physiological, but also in

pathophysiological conditions, such as myocardial ischemia. Indeed, we found that in cardiac synaptosomes subjected to 10-min ischemia, the release of NE, in the picomolar range, correlated linearly with that of ATP in the low femtomolar ATP concentration range. This correlation did not apply to isolated hearts during 10-min ischemia. In all likelihood, this occurred because the large overflow of ATP during reperfusion (high picomolar range) reflected highly increased ATP production by sources other than sympathetic nerve terminals, such as myocytes and endothelial cells (Bodin and Burnstock, 2001). Indeed, only transmitter ATP would be expected to exert positive feedback modulatory action on the nerve terminals.

In our long-lasting ischemia models, both in the isolated heart and in cardiac synaptosomes, released ATP did not modulate NE release. In these models, NE release is mainly carrier-mediated and Ca^{2+}-independent; i.e., NE is “carried” out of sympathetic nerve endings via the NE transporter operating in an outward direction (Hatta et al., 1999; Seyedi et al., 2002). This occurs because, with prolonged ischemia, the intra-axonal concentrations of Na^{+} and free NE greatly increase, thus inducing a reversal of the NE transporter (Levi and Smith, 2000) and promoting a massive increase in NE release (i.e., 100-fold greater than exocytotic NE release in short-term ischemia). Inasmuch as vesicular NE is not involved in this type of non-exocytotic, Ca^{2+}-independent NE release, one would

expect ATP to be unable to affect it by an action on pre-synaptic ionotropic P2XR. In fact, neither blockade of P2XR with PPADS, nor hydrolysis of ATP with solCD39, nor an increase in ATP availability by inhibition of E-NTPDase with ARL67156, modified carrier-mediated NE release in protracted ischemia in the isolated heart model. In contrast, as anticipated, inhibition of the carrier system with DMI induced a very effective blockade of this type of NE release.

The idea that transmitter ATP is likely to modulate exocytotic, but not carrier-mediated NE release is further supported by our findings with ischemic synaptosomes. We found that, in this model, NE release progressively increased with the time of exposure to ischemia, but the release of ATP progressively decreased. This occurred because with continued ischemia there was a progressive shift from exocytotic to carrier-mediated NE release. Unlike NE, there is no neuronal transporter for ATP; therefore, the release of transmitter ATP can only be exocytotic (von Kugelgen et al., 1994; Bodin and Burnstock, 2001). In fact, the release of ATP elicited by ischemia did not increase like that of NE, but actually diminished with time, reflecting the progressive loss of exocytosis. Indeed, we found that the promotion of NE release by co-released ATP and its modulation by neuronal ecto-nucleotidase, which were very evident in cardiac synaptosomes after 10 min of ischemia, were greatly diminished after 20 additional minutes of ischemia.

In the presence of PPADS, the overflow of ATP from isolated hearts, following both 10- and 20-min ischemia was increased. This finding was not initially anticipated from the known sole antagonism of P2XR by PPADS (Kim et al., 2001). Indeed, we found that PPADS, at the concentration used in these ischemia models (30 μM), also inhibited ATP hydrolysis by either solCD39 or synaptosomal nucleotidase. This effect was evident only in ischemic hearts, where ATP overflow reflects a generalized production of ATP by various cellular sources in addition to sympathetic nerves. In fact, in ischemic synaptosomes, where the release of ATP is exclusively from nerve endings, PPADS did not enhance it. In fact, PPADS markedly decreased ATP release from synaptosomes made ischemic for 10 min, and moderately decreased it in those kept ischemic for 20 min. We interpret this finding as an indication that, once released, transmitter ATP activates pre-junctional P2XR, promoting the exocytosis of both NE and ATP.

It is conceivable that the cardiac synaptosomal fraction may also contain other terminals, such as parasympathetic and non-myelinated sensory terminals (Seyedi et al., 1997; Seyedi et al., 1999), which could release ATP (Bodin and Burnstock, 2001) during ischemia. This ATP might further amplify NE exocytosis from sympathetic nerve endings in a paracrine mode.

In conclusion, our findings support a key role for neuronal E-NTPDase in the control of adrenergic function in the ischemic heart. We demonstrate that by limiting the availability of released ATP at sympathetic nerve terminals, E-NTPDase modulates the P2XR-mediated, positive feedback mechanism that enhances NE and ATP release. Enhanced adrenergic activity and excessive NE release are known causes of clinical cardiac dysfunction in myocardial ischemia (Braunwald and Sobel, 1988; Dart and Du, 1993; Kübler and Strasser, 1994; Benedict et al., 1996). Therefore, our results identify a protective role for the E-NTPDase at cardiac sympathetic nerve terminals and suggest that by mitigating ATP-mediated NE release, solCD39 may offer a novel therapeutic approach to myocardial ischemia and its consequences. Moreover, solCD39 strongly inhibits human platelet aggregation induced by ADP, collagen, arachidonate, or TRAP (thrombin receptor agonist peptide) (Marcus et al., 2003). In a murine model of stroke, driven by excessive platelet recruitment, solCD39 reduced the sequelae of stroke, without an increase in intracerebral hemorrhage (Pinsky et al., 2002). Thus, in the heart, solCD39 has the potential not only of attenuating NE release and its dysfunctional consequences, but also of impeding the transition from myocardial ischemia to infarction.

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Footnotes

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Figure Legends

Figure 1. Overflow of NE (panel A) and ATP (panel B) into the coronary effluent of isolated guinea-pig hearts subjected to 10-min global ischemia, followed by 20-min reperfusion. Hearts were perfused in the absence of drugs (control) or with DMI (300 nM), PPADS (30 μM), solCD39 (10 nM) or ARL67156 (30 μM). ARL67156 was perfused either alone or in combination with PPADS. In panel A, each bar (mean ± S.E.M.; n = 6) represents the cumulative overflow of NE into the coronary effluent during reperfusion following ischemia. Before ischemia, NE overflow was below detection level. In panel B, for each bar (mean ± S.E.M.; n = 6), the gray area represents the overflow of ATP before ischemia, whereas the open area represents the increase in ATP overflow during reperfusion. ATP overflow was below detectable range in the presence of solCD39. **P<0.01 and ***P<0.001, significantly different from control (NE) and from own pre-ischemic level (ATP), by ANOVA with Bonferroni's test for post-hoc analysis. Drugs were added to the perfusion medium 20 min before ischemia. When not visible, error bars are included in the bar.

Figure 2. Overflow of NE (panel A) and ATP (panel B) into the coronary effluent of isolated guinea-pig hearts subjected to 20-min global ischemia, followed by 20-min reperfusion. Hearts were perfused in the absence of

drugs (control) or with DMI (300 nM), PPADS (30 μM), solCD39 (10 nM) or ARL67156 (30 μM). In panel A, each bar (mean ± S.E.M.; n = 6) represents the cumulative overflow of NE into the coronary effluent during reperfusion following ischemia. Before ischemia, NE overflow was below detection level. In panel B, for each bar (mean ± S.E.M.; n = 6), the gray area represents the overflow of ATP before ischemia, whereas the open area represents the increase in ATP overflow during reperfusion. ATP overflow was below detectable range in the presence of solCD39. ***P<0.001, significantly different from control (NE) and from own pre-ischemic level (ATP), by ANOVA with Bonferroni's test for post-hoc analysis. Drugs were added to the perfusion medium 20 min before ischemia. When not visible, error bars are included in the bar.

Figure 3. Lack of correlation between cumulative NE and ATP overflow during reperfusion following 10-min (Panel A) and 20-min ischemia (Panel B) in isolated guinea-pig hearts. NE overflow, both in the presence and the absence of drugs, was plotted against ATP overflow measured under the same conditions. Correlation coefficients (r^2) were 0.109 (panel A, 30 hearts) and 0.008 (panel B, 24 hearts). Each point is the mean of 4 observations. In all likelihood, the lack of correlation reflected a greatly increased ATP production in the whole heart by cellular sources other than sympathetic nerve terminals (see Discussion).

Figure 4. Time course of the release of NE (panel A) and ATP (panel B) from cardiac synaptosomes. Synaptosomes were incubated for 10, 20, 30 and 70 minutes either in normoxic or ischemic conditions. Bars (mean ± S.E.M.) represent the release of NE or ATP during each incubation period. For each bar (n = 16), the gray area represents release in normoxia and the open area represents the increase in release due to ischemia. When not visible, error bars are included in the bar.

Figure 5. Release of NE (panel A) and ATP (panel B) from cardiac synaptosomes during 10-min ischemia. Synaptosomes were incubated for 10 min either under normoxic or ischemic conditions, in the absence (control) or presence of DMI (300 nM), PPADS (10 μM), solCD39 (3 nM) or ARL67156 (30 μM), alone or in combination with PPADS. Each bar (mean ± S.E.M.; n = 16; control, n = 64) represents the release of NE or ATP during 10-min incubation under ischemic conditions. The dotted lines represent NE and ATP release during normoxia, 0.857 ± 0.012 pmol/mg of protein and 0.326 ± 0.032 fmol/mg of protein, respectively. Ischemic ATP release was undetectable in the presence of solCD39. ***P<0.001 *vs.* control, by one-way ANOVA with Tukey post-test. When not visible, error bars are included in the bar.

Figure 6. Correlation between NE and ATP release from cardiac synaptosomes induced by 10-min ischemia. NE release either in the

absence (control) or in the presence of drugs was plotted against ATP release measured in the same conditions. Points represent 16 observations in the presence of each drug, and 64 in the absence of drugs (control). The line was calculated by linear regression analysis. Correlation coefficient was r^2= 0.843 (P<0.001).

Figure 7. Release of NE (panel A) and ATP (panel B) from cardiac synaptosomes during 30-min ischemia. Synaptosomes were incubated for 30 min either in normoxic or ischemic conditions in the absence (control) or presence of DMI (300 nM), PPADS (10 μM), solCD39 (3 nM) or ARL67156 (30 μM) alone or in combination with PPADS. Each bar (mean ± S.E.M.; n = 16; control, n = 64) represents the release of NE or ATP during 30-min incubation in ischemic conditions. The dotted lines represent NE and ATP release during normoxic conditions, 1.150 ± 0.002 pmol/mg of protein and 0.295 ± 0.004 fmol/mg of protein, respectively. Ischemic ATP release was undetectable in the presence of solCD39. **P<0.01 and ***P<0.001 *vs.* control, by one-way ANOVA with Tukey post-test. When not visible, error bars are included in the bar.

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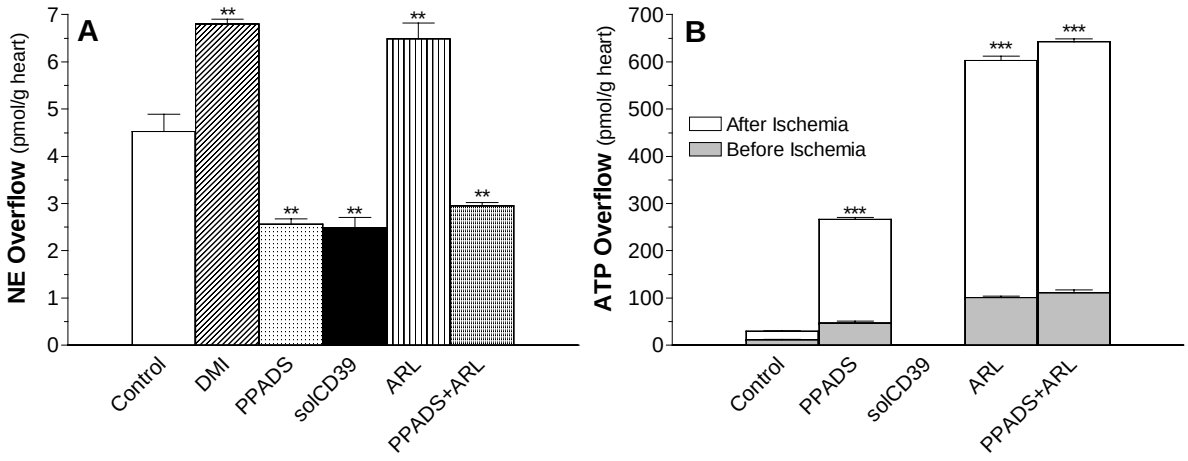

FIGURE 1

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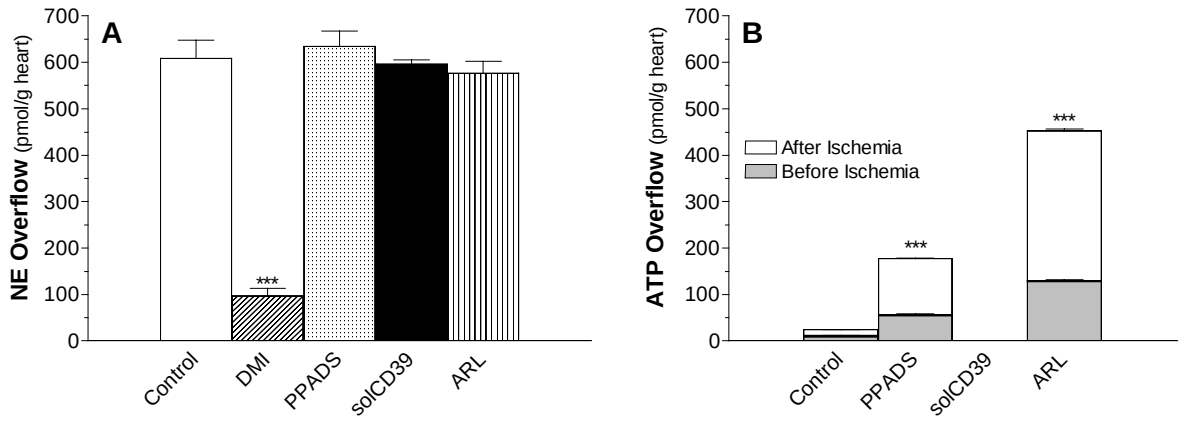

FIGURE 2

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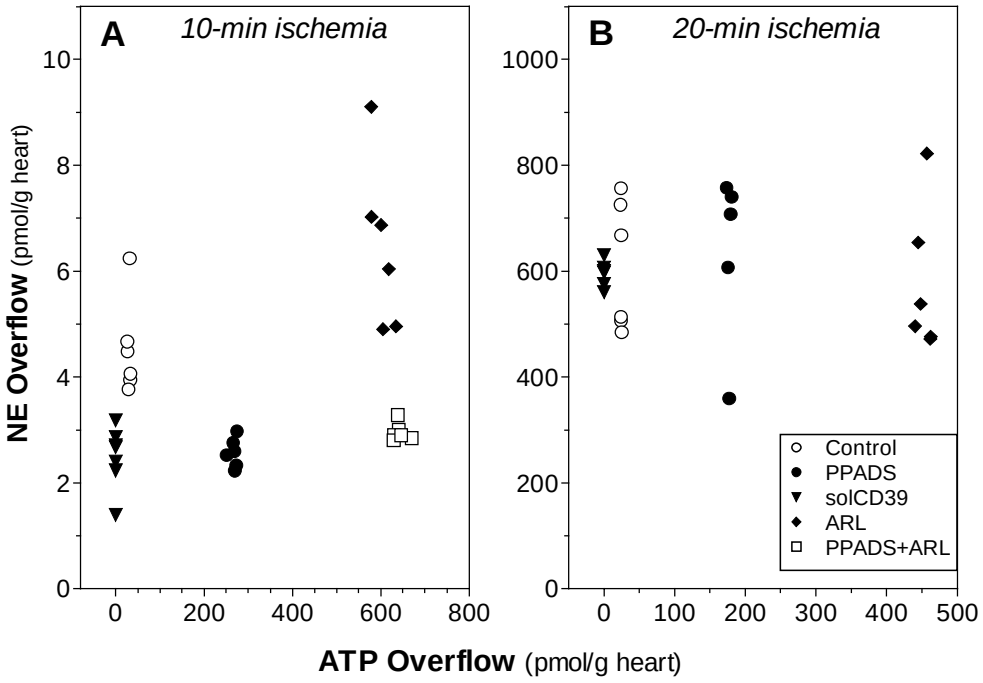

FIGURE 3

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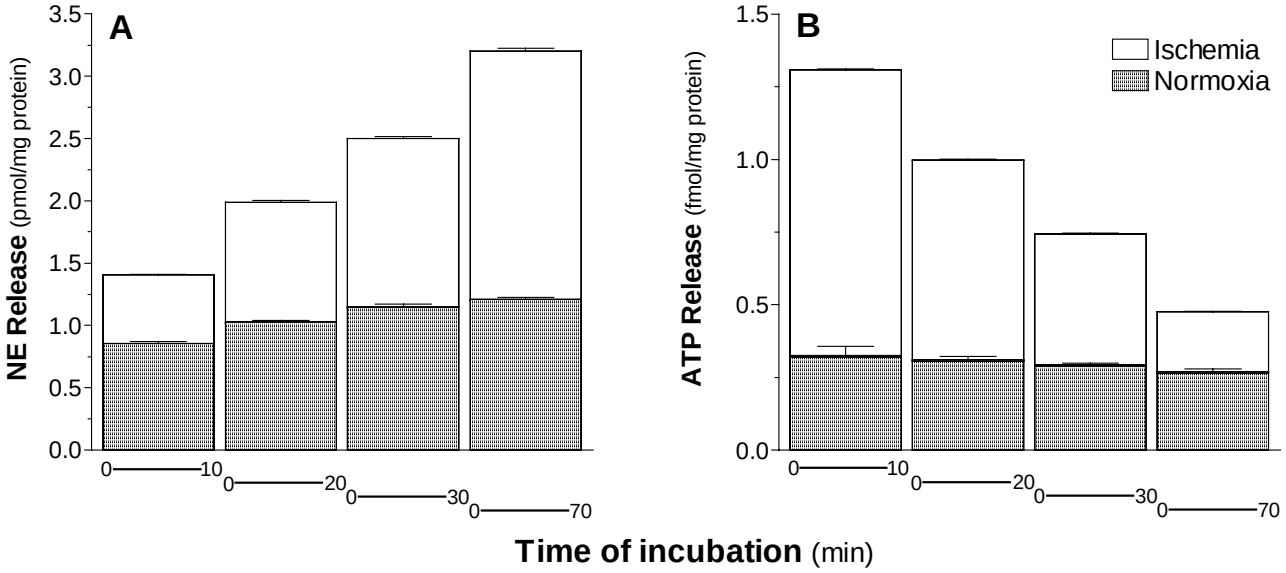

FIGURE 4

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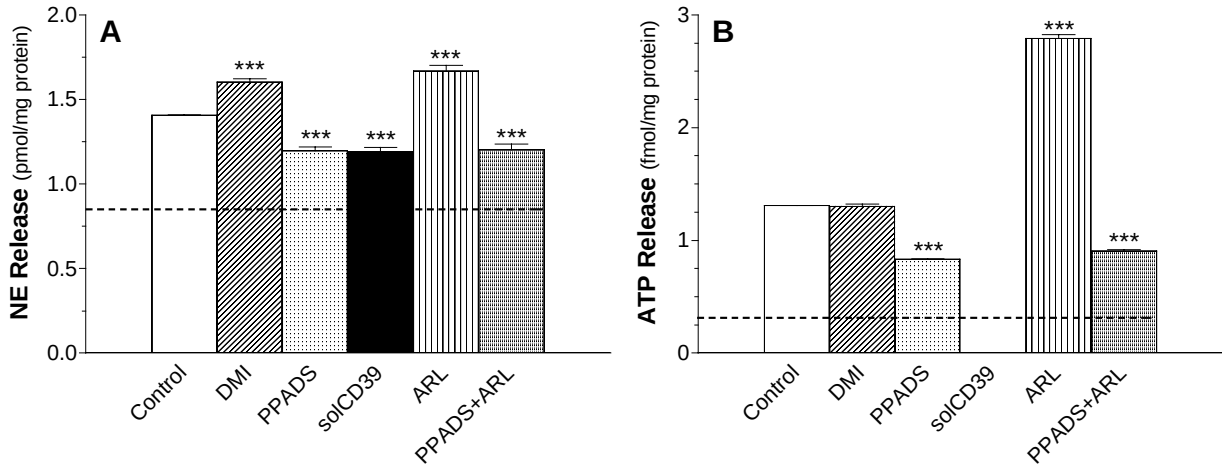

FIGURE 5

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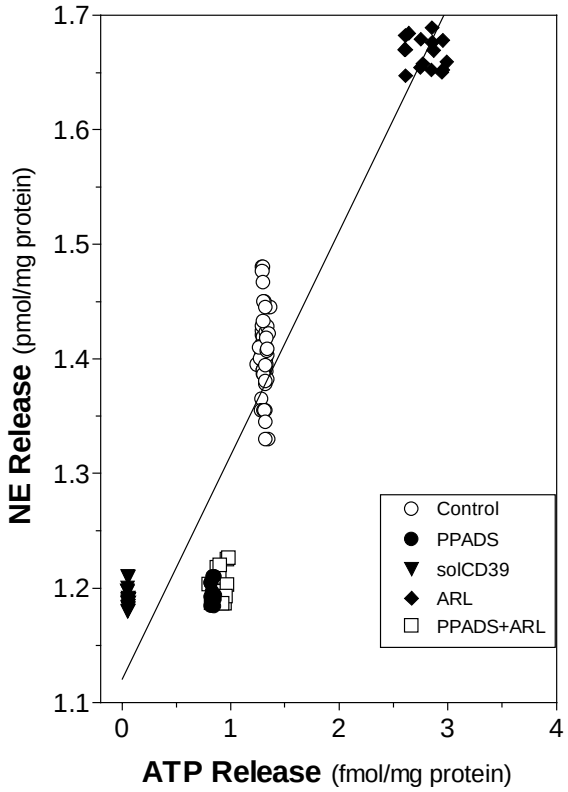

FIGURE 6

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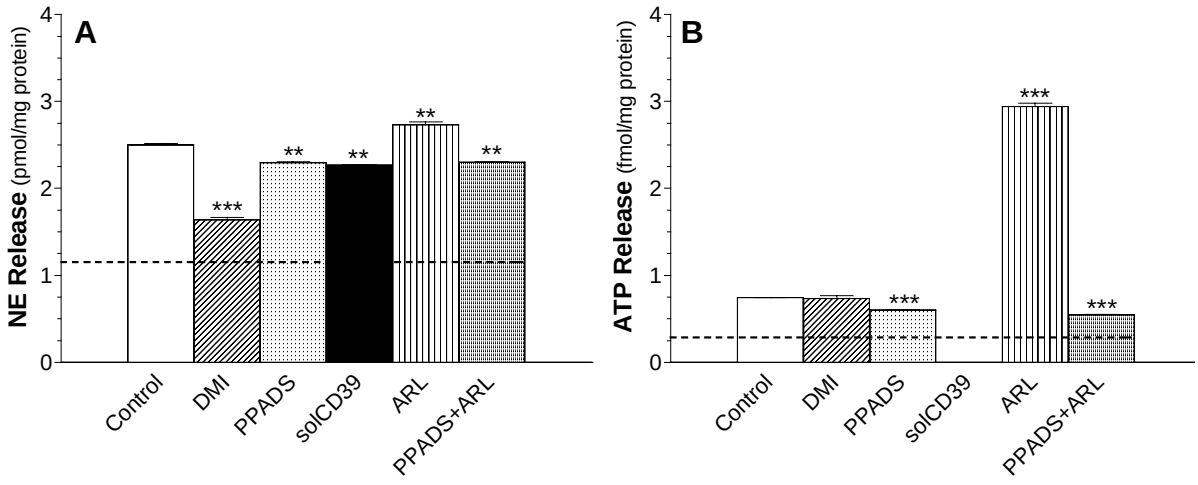

FIGURE 7