Endogenous adenosine contributes to renal sympathetic neurotransmission via postjunctural A1 receptor-mediated coincident signaling

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Jackson EK, Cheng D, Tofovic SP, Mi Z. Endogenous adenosine contributes to renal sympathetic neurotransmission via postjunctural A1 receptor-mediated coincident signaling. Am J Physiol Renal Physiol 302: F466–F476, 2012. First published November 23, 2011; doi:10.1152/ajprenal.00495.2011.—Adenosine A1 receptor antagonists have diuretic/natriuretic activity and may be useful for treating sodium-retaining diseases, many of which are associated with increased renal sympathetic tone. Therefore, it is important to determine whether A1 receptor antagonists alter renal sympathetic neurotransmission.

In isolated, perfused rat kidneys, renal vasoconstriction induced by renal sympathetic nerve stimulation was attenuated by 1) 1,3-dipropyl-8-p-sulfophenylxanthine (xanthine analog that is not a selective adenosine receptor antagonist, but is cell membrane impermeable and thus does not block intracellular phosphohydrolases), 2) xanthine amine congener (xanthine analog that is a selective A1 receptor antagonist), 3) 1,3-dipropyl-8-cyclopentylxanthine (xanthine analog that is a highly selective A1 receptor antagonist), and 4) FK453 (nonxanthine analog that is a highly selective A1 receptor antagonist). In contrast, FR113452 (enantiomer of FK453 that does not block A1 receptors), MRS-1754 (selective A2B receptor antagonist), and VUF-5574 (selective A3 receptor antagonist) did not alter responses to renal sympathetic nerve stimulation, and ZM-241385 (selective A2A receptor antagonist) enhanced responses. Antagonism of A1 receptors did not alter renal spillover of norepinephrine.

Because A1 receptor activation stimulates sodium reabsorption, it is not surprising that blockade of A1 receptors decreases sodium reabsorption and increases sodium excretion (i.e., A1 antagonists are diuretics). Indeed, selective blockade of renal A1 receptors rapidly (within minutes) and markedly (3- to 10-fold) increases urinary sodium excretion in animals and humans with little or no effect on potassium excretion (30, 31, 63). For this reason, A1 receptor antagonists represent a new class of diuretics that may prove useful for the treatment of a variety of cardiovascular disorders. Indeed, A1 receptor antagonists are in development as diuretics for the management of chronic and acute heart failure (27) and may be useful for other renal indications such as treatment of liver cirrhosis (28), hepatorenal syndrome (27), and prevention of radiocontrast media-induced nephropathy (27).

A concern, however, with regard to using A1 antagonists as diuretics or in renal diseases in general is that blockade of A1 receptors theoretically could augment renal sympathetic neurotransmission, an effect that could reduce renal blood flow, glomerular filtration, and electrolyte excretion. In many organ systems and tissues, prejunctional A1 receptors attenuate norepinephrine (NE) release from sympathetic nerve varicosities (16). Importantly, sympathetic nerve stimulation not only releases NE, but also ATP (a cotransmitter with NE and a precursor of adenosine) as well as enzymes involved in the metabolism of ATP to adenosine (64). Thus, renal sympathetic nerve stimulation (RSNS) would likely increase both NE and adenosine in neuroeffector junctions of the renal microcirculation, and A1 receptor antagonists, by blocking prejunctional A1 receptors, could possibly prevent prejunctional inhibition of NE release by endogenous adenosine formed in the neuroeffector junction and therefore might augment renal sympathetic neurotransmission.

Counter to the aforementioned deduction, one can construct a line of reasoning leading to the diametrically opposite conclusion that A1 receptor antagonists might attenuate, rather than augment, renal sympathetic neurotransmission. In this regard, early studies suggest that adenosine augments renal vasoconstriction induced by NE. Hedqvist and Fredholm (24) reported in 1976 that in the isolated,perfused rabbit kidney adenosine caused a concentration-dependent increase in vasoconstrictor responses to RSNS and exogenous NE, yet decreased the release of NE evoked by nerve stimulation. In 1978, these investigators extended their findings by demonstrating that the effects of adenosine in the rabbit kidney were blocked by theophylline (25) (a low-potency, nonspecific adenosine receptor antagonist) and that theophylline per se markedly inhibited (by ~60%) vasoconstrictor responses to RSNS, yet caused only a modest increase (by ~25%) in RSNS-induced NE release. These results suggested a dual role for endogenous adenosine in sympathetic neurotransmission in

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the rabbit kidney, i.e., modest prejunctional inhibition of NE release but profound augmentation of postjunctional responses to released (and exogenous) NE. Yoneda et al. (65) using an in vivo dog kidney preparation, similarly concluded that endogenous adenosine inhibits RSNS-induced NE release and facilitates NE-induced renal vasoconstriction. However, neither administration of exogenous adenosine nor blockade of adenosine receptors with theophylline affected RSNS-induced renal vasoconstriction, suggesting offsetting prejunctional vs. postjunctional effects of exogenous and endogenous adenosine. Ekas and co-workers in 1981 (12) and 1983 (13) reported that in isolated, perfused rat kidneys exogenous adenosine inhibited RSNS-induced vasoconstriction and NE release, but had little effect on responses to exogenous NE.

Surprisingly, in the last 20 years, the role of endogenous adenosine in renal sympathetic neurotransmission has received little attention, and therefore our knowledge in this area has remained stagnant. However, since these early pioneering studies, there have been three profound changes: 1) it is now widely appreciated that adenosine can act on multiple receptor subtypes that have disparate effects, 2) a broad array of highly potent and selective adenosine receptor antagonists and agonists for the adenosine receptor subtypes is now available, and 3) A1 receptor antagonists are now being developed as a novel class of diuretics and renal drugs. Therefore, it seems appropriate to revisit the role of adenosine in renal sympathetic neurotransmission using modern pharmacological tools to more precisely define in particular the role of A1 adenosine receptors in renal sympathetic neurotransmission.

METHODS

Animals. Kidneys for isolation and perfusion were obtained from male Sprague-Dawley rats weighing ~400 g. Animals were purchased from Charles River (Wilmington, MA). The Institutional Animal Care and Use Committee approved all procedures. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Drugs. 1,3-Dipropyl-8-p-sulfophenylxanthine [DPSX; xanthine analog that is a nonselective adenosine receptor antagonist that does not penetrate cell membranes (57)], xanthine amine congener [XAC; xanthine analog that is a selective A1 receptor antagonist (37)], 1,3-dipropyl-8-cyclopentylxanthine [DPCCXP; xanthine analog that is a highly selective A1 receptor antagonist (37)], ZM-241385 [nonxanthine analog that is a highly selective A2A receptor antagonist (37)], MRS-1754 [nonxanthine analog that is a highly selective A2B receptor antagonist (37)], VUF-5574 [nonxanthine analog that is a highly selective A3 receptor antagonist (37)], XV-5735 [nonxanthine antagonist that does not penetrate cell membranes (57)] and therefore does not inhibit intracellular phosphodiesterases] on responses to RSNS. Perfused kidneys were subjected to two periods of RSNS (9 Hz for 5 min) separated by 35 min. In one group, DPSX (300 μmol/l) was added to the perfusate 15 min before the second stimulation period (DPSX group) and in another group DPSX was not added (control group).

Protocol 1. Many xanthine derivatives inhibit intracellular phosphodiesterases (1) and thereby increase intracellular 3',5'-cAMP levels and reduce vascular responses to most vasoconstrictors. Therefore, as an initial test of the role of adenosine in renal sympathetic neurotransmission, we examined the effects of DPSX [an adenosine receptor antagonist that does not penetrate cell membranes (57) and therefore does not inhibit intracellular phosphodiesterases] on responses to RSNS. Perfused kidneys were subjected to two periods of RSNS (9 Hz for 5 min) separated by 35 min. In one group, DPSX (300 μmol/l) was added to the perfusate 15 min before the second stimulation period (DPSX group) and in another group DPSX was not added (control group).

Protocol 2. To confirm and extend the results from protocol 1, the concentration-dependent effects of DPSX (30, 100, and 300 μmol/l) on renal sympathetic neurotransmission were assessed by eliciting perfusion pressure responses to RSNS during four stimulation periods (9 Hz for 5 min) separated by 15 min. In the control group, no treatments were added to the perfusate, whereas in the treatment group DPSX was added to the perfusate between the first and second, second and third, and third and fourth periods to provide the indicated concentrations in the perfusate.

Protocol 3. Although DPSX is a useful pharmacological tool to assess the role of cell surface adenosine receptors, DPSX is not selective for the A1 receptor, but instead it antagonizes all four adenosine receptor subtypes. To ascertain whether the effects of DPSX were mediated by blockade of A1 receptors, we next examined the effects of XAC [xanthine derivative that is selective for the A1 receptor (37), DPCPX [xanthine derivative that is highly selective for the A1 receptor (37)], FK453 [nonxanthine derivative that is highly selective for the A1 receptor (39, 40), and FR113452 [the enantiomer of FK453 that does not block the A1 receptor (39, 40)]. The effects of these agents on RSNS responses were assessed at concentrations of 10, 100, and 1,000 mmol/l using the same experimental design as described for protocol 2.

Protocol 4. To determine whether the adenosine receptor antagonists augmented NE release, renal venous perfusate was collected before and during RSNS (9 Hz for 5 min) in control kidneys or kidneys pretreated for 15 min with 300 μmol/l of DPSX or 1,000 mmol/l of XAC. A similar experiment was performed comparing the effects of 1,000 mmol/l of FR113452 vs. FK453 on NE release.

Protocol 5. To determine whether other adenosine receptor subtypes contribute to renal sympathetic neurotransmission, we compared the effects of DPCPX [highly selective A1 receptor antagonist (37)], ZM-241385 [selective A2A receptor antagonist (37)], MRS-1754 [selective A2B receptor antagonist (37)], and VUF-5574 [selective A3 receptor antagonist (60)] on responses to RSNS. To detect subtle changes in RSNS responses, we employed a paired experimental design in which both kidneys from the same rat were removed and perfused separately but simultaneously. After a rest period (30 min), a test response (5 Hz RSNS) was elicited. For each pair of kidneys, 15 min before initiating experimental RSNS, one kidney received vehicle only and the other kidney received one of the four antagonists (1,000...
Protocol 6. Adenosine can be formed in the neuroeffector junction due to release of the cotransmitter ATP, which can be rapidly metabolized by nucleotidases to adenosine (64). If this is the main source of adenosine that participates in renal sympathetic neurotransmission, then blockade of A1 receptors should attenuate vasoconstrictor responses to RSNS (which would release ATP) but might not attenuate vasoconstrictor responses to exogenous NE (which would not release cotransmitters and therefore adenosine levels might not be elevated). To test this prediction, perfused kidneys were subjected to RSNS at 3, 5, and 7 Hz (5-min stimulation periods) and to exogenous NE (5-min infusions) at 100, 175, and 275 nmol/l in the absence and then presence of DPCPX (100 nmol/l). Responses were elicited at 10-min intervals.

Protocol 7. Because we did not observe any effects of the A1 antagonists on NE release, we hypothesized that the A1 receptor participates in renal sympathetic neurotransmission via coincident signaling with NE (i.e., we postulated that A1 receptor activation enhances NE-induced renal vasoconstriction). To test this hypothesis, renovascular responses to exogenous NE were elicited with 5-min intrarenal artery infusions of increasing concentrations of NE to find a concentration of NE that would increase perfusion pressure ~20 mmHg. The average concentration of NE required to achieve basal responses in this range was 199 ± 11 nmol/l. Next, the concentration of NE that increased renal perfusion pressure to the desired target range was administered to the kidneys six times (5-min infusions with 10-min rest periods between responses). The first three responses were basal responses in the absence of any treatments to establish the reproducibility of the NE-induced renal vasoconstriction. Next, in two groups of kidneys, CCPA [a highly selective A1 receptor agonist (37)] was infused into the renal artery to achieve increasing concentrations of CCPA of 1, 3, and 10 nmol/l, and responses to NE were elicited 10 min into the infusion of each concentration of CCPA. One of these two groups was cotreated with DPCPX (1,000 nmol/l; CCPA + DPCPX group), whereas the other only received CCPA (CCPA group). In a third group, neither CCPA nor DPCPX was administered, yet responses to NE were elicited every 10 min (time control group).

Protocol 8. Our previous work indicates that in the renal microcirculation, the mechanism by which Gi pathway activators augment vasoconstrictor responses to Gq pathway activators is via coincident signaling at the level of PLC leading to activation of the PKC → c-src → PI3K pathway (33). To test whether this mechanism of coincident signaling mediates the ability of A1 receptors [which are coupled to Gi (49)] to enhance renovascular responses to NE [which are mediated by α1-adrenoceptors which are coupled to Gq (9)], kidneys were pretreated with either U73122 [1 μmol/l; phospholipase C inhibitor (3)], U73343 [1 μmol/l; inactive analog of U73122 (50)], GF109203X [3 μmol/l; protein kinase C inhibitor (38)], PPI [1 μmol/l; c-src inhibitor (22)], or wortmannin [0.2 μmol/l; PI3K inhibitor (61)]. Because 3-phosphoinositide-dependent protein kinase-1 (PKD1) is activated by PI3K (48), we also examined the effects of OSU-03012 [0.5 μmol/l; inhibitor of PKD1 (66)]. After 15 min of pretreatment, renovascular responses to exogenous NE were elicited with 5-min intrarenal artery infusions of increasing concentrations of NE to find a concentration of NE that would increase perfusion pressure ~20 mmHg. Next, the concentration of NE that increased renal perfusion pressure to the desired target range was administered to the kidneys (5-min infusion). This response was a basal response in the presence of pretreatment with the inhibitors but in the absence of CCPA. Next, CCPA was infused into the renal artery to achieve a concentration of CCPA of 3 nmol/l and responses to NE were again elicited 10 min into the infusion of CCPA.

Protocol 9. Our previous work demonstrates that in both the isolated, perfused rat kidney (46) and mouse kidney (52), RSNS increases the release of adenosine into the renal venous perfusate. However, we have not yet examined the effects of exogenous NE on adenosine release from isolated, perfused kidneys. Therefore, in protocol 9, NE was infused into the renal artery for 5 min at 175 nmol/l, and renal venous perfusate was collected during the last min of the treatment. After a rest period of 10 min, this protocol was repeated with a higher concentration of NE (275 nmol/l).

Statistics. Data were analyzed by one- or two-factor ANOVA, with post hoc comparisons using a Fisher’s least significant difference (LSD) test or by Student’s unpaired or paired t-test as appropriate. The criterion of significance was P < 0.05. All values in text and figures are means ± SE.

RESULTS

Protocol 1. DPSPX is an adenosine receptor antagonist that does not penetrate cell membranes and therefore does not inhibit intracellular phosphodiesterases. In the DPSPX group, the perfusion pressure response to RSNS was significantly reduced in the presence of DPSPX (Fig. 1A) and this effect was not due to degradation in the responsiveness of the preparation as evidenced by the fact that responses in the control group were maintained. DPSPX did not affect baseline perfusion pressure.

Protocol 2. Responses to RSNS (expressed as a percentage of the initial response) were stable in the control group, whereas a concentration-dependent reduction in responses to RSNS was observed in the DPSPX group (Fig. 1B). DPSPX did not affect baseline perfusion pressures.

Protocol 3. XAC (xanthine derivative selective for the A1 receptor), DPCPX (xanthine derivative highly selective for the A1 receptor), and FK453 (nonxanthine derivative highly selective for the A1 receptor) inhibited responses to RSNS (Fig. 2, A, B, and C, respectively) and the inhibition was similar to that observed for DPSPX. In contrast to FK453, FR113452 (an enantiomer of FK453 that does not block A1 receptors) did not significantly alter vasoconstrictor responses to RSNS (Fig. 2D). Neither XAC, DPCPX, FK453, nor FR113452 affected baseline perfusion pressures.

Protocol 4. RSNS caused a robust increase in the concentration of NE in the renal venous perfusate of control kidneys, and this response was not altered by high concentrations of either DPSPX or XAC (Fig. 3A). As shown in Fig. 3B, the increase in NE concentration in the renal venous perfusate induced by RSNS was similar and not significantly different in FR113452-treated vs. FK453-treated kidneys.

Protocol 5. DPCPX (Fig. 4A) significantly attenuated responses to RSNS; in contrast, ZM-241385 (selective A2A receptor antagonist) increased responses to RSNS at high frequencies of stimulation (Fig. 4B). Neither MRS-1754 (selective A2B receptor antagonist) nor VUF-5574 (selective A3 receptor antagonist) significantly affected vasoconstrictor responses induced by RSNS (Fig. 4, C and D, respectively). Neither DPCPX, ZM-241385, MRS-1754, nor VUF-5574 affected baseline perfusion pressures.

Protocol 6. As shown in Fig. 5, DPCPX significantly inhibited responses to RSNS (Fig. 5A) but not to exogenous NE (Fig. 5B). DPCPX did not affect baseline perfusion pressure.
Protocol 7. As shown in Fig. 6, basal responses to exogenous NE were stable during the first three basal periods in all three groups. In the CCPA (highly selective A1 receptor agonist) group, CCPA concentration dependently enhanced the vasoconstrictor response to NE such that at 10 nmol/l, the NE response was augmented fivefold. CCPA per se did not alter baseline perfusion pressure. In the presence of DPCPX, CCPA did not affect the responses to NE, and the responses to NE were stable in the time control group.

Protocol 8. CCPA (3 nmol/l) in the absence of inhibitors enhanced the vasoconstrictor response to NE (Fig. 7A). CCPA per se did not alter baseline perfusion pressure. The ability of CCPA to enhance the vasoconstrictor response to NE was attenuated in the presence of U73122 (phospholipase C inhibitor), GF109203X (protein kinase C inhibitor), PP1 (c-src inhibitor), wortmannin (PI3K inhibitor), or OSU-03012 (inhibitor of PDK1; Fig. 7, B, C, D, E, and F, respectively). In contrast to these inhibitors, neither U73343 (inactive analog of U73122) nor the antioxidant apocynin (10 μmol/l) attenuated the ability of CCPA to enhance NE-induced vasoconstriction [perfusion pressure response to NE: in absence of inhibitors, 24 ± 2 and 89 ± 11 mmHg before and during CCPA, respectively (n = 6); in U73343-treated kidneys; 21 ± 3 and 96 ± 8 mmHg before and during CCPA, respectively (n = 3); in apocynin-treated kidneys; 27 ± 1 and 116 ± 19 mmHg before and during CCPA, respectively (n = 6)].
Protocol 9. NE at 175 and 275 nmol/l increased perfusion pressure from 50/1100 to 90/1100 and to 90/1100 mmHg, respectively (n = 6). However, NE did not significantly (P > 0.2400) increase adenosine levels (basal levels of adenosine were 22/1100 ng/ml; in the presence of 175 ng/l of NE, adenosine levels were 27/1100 ng/ml; in the presence of 275 nmol/l of NE, adenosine levels were 30/1100 ng/ml).

DISCUSSION

The present study supports the conclusion that endogenous adenosine, via agonism of A1 receptors, contributes to renal sympathetic neurotransmission. The evidence for this conclusion is that nonselective blockade of cell surface adenosine receptors with DPSPX and selective antagonism of A1 receptors with three different antagonists (XAC, DPCPX, and FK453) attenuates vasoconstrictor responses to RSNS. DPSPX, XAC, and DPCPX are xanthine derivatives, and therefore it is conceivable that they share off-target effects due to the xanthine component of their chemical structure that accounts for their ability to attenuate responses to RSNS. However, the fact that FK453, a nonxanthine drug, also inhibits RSNS responses makes this possibility remote. Moreover, the possibility of off-target effects of FK453 is remote because FK453, a nonxanthine drug, also inhibits RSNS responses during the third and fourth periods. The responses to RSNS during the second, third, and fourth periods are expressed as a percentage of the basal response during the first period in the absence of any treatments. Basal perfusion pressures (before treatments) were 58 ± 4, 52 ± 3, 53 ± 1, 55 ± 3, and 55 ± 2 mmHg for the CON group, XAC group, DPCPX group, and FR113452 group, respectively. Basal perfusion pressures were not affected by either XAC, DPCPX, FK453, or FR113452. RSNS responses during the first period (before treatments) were 71 ± 5, 69 ± 16, 101 ± 9, 86 ± 7, and 87 ± 9 mmHg for the CON group, XAC group, DPCPX group, FK453 group, and FR113452 group, respectively. *P < 0.05 compared with CON group in corresponding RSNS period. The P values in the figure are from 2-factor ANOVA. Values represent means ± SE for the indicated number of experiments (n).

Fig. 2. Bar graphs show effects of increasing concentrations of xanthine amine congener (XAC; A), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; B), FK453 (C), or FR113452 (D) on perfusion pressure responses to RSNS. The experiment entailed 4 periods of RSNS, and in the XAC, DPCPX, FK453, or FR113452 groups, but not the CON group, XAC, DPCPX, FK453, or FR113452 was added to the perfusate between the first and second periods, second and third periods, and third and fourth periods. The responses to RSNS during the second, third, and fourth periods are expressed as a percentage of the basal response during the first period in the absence of any treatments. Basal perfusion pressures (before treatments) were 58 ± 4, 52 ± 3, 53 ± 1, 55 ± 3, and 55 ± 2 mmHg for the CON group, XAC group, DPCPX group, FK453 group, and FR113452 group, respectively. Basal perfusion pressures were not affected by either XAC, DPCPX, FK453, or FR113452. RSNS responses during the first period (before treatments) were 71 ± 5, 69 ± 16, 101 ± 9, 86 ± 7, and 87 ± 9 mmHg for the CON group, XAC group, DPCPX group, FK453 group, and FR113452 group, respectively. *P < 0.05 compared with CON group in corresponding RSNS period. The P values in the figure are from 2-factor ANOVA. Values represent means ± SE for the indicated number of experiments (n).
neurotransmission in the in situ blood-perfused rat mesentery, antagonism of adenosine receptors with DPSPX does not alter noradrenergic neurotransmission (32, 41). Therefore, with regard to the peripheral sympathetic nervous system, the presynaptic effect of A1 receptor activation is likely more of pharmacological interest, rather than physiological importance.

Most likely the mechanism by which endogenous adenosine participates in renal sympathetic neurotransmission involves coincident signaling at the postjunctional membrane. Coincident signaling is the convergence of signaling pathways such that one pathway augments the effects of the other pathway because of synergistic actions on a protein coincident detector (54). In this regard, A1 receptor activation is known to augment angiotensin II-induced renal vasoconstriction (42, 47). Since both NE and angiotensin II signal via Gq-coupled receptors [α1-adrenoceptors (9) and angiotensin II AT1 receptors (44), respectively], the A1 receptor would also likely enhance NE-induced renal vasoconstriction. Also, A1 receptors are Gi-coupled receptors (49), and studies by our lab demonstrate coincident signaling between Gq-coupled receptors (for example, AT1 receptors and vasopressinV1 receptors) and Gi-coupled receptors (for example, Y1 receptors and α2-adrenoceptors) in the renal microcirculation resulting in potentiation of Gq-induced renal vasoconstriction by the Gi signal transduction pathway (10, 11, 17). Thus, taken together, this line of reasoning would predict coincident signaling in the renal microcirculation between released adenosine, via A1 receptor-induced activation of Gi, and released NE, via α1-adrenoceptor-induced activation of Gq. Therefore, blockade of A1 receptors would be expected to inhibit RSNS-induced renal vasoconstriction by inhibiting coincident signaling by postjunctional receptors. In support of this conclusion, our results show that CCPA, a highly potent and highly selective A1 receptor agonist, causes a profound increase in the vasoconstrictor response to exogenous NE. Also, our previous work indicates that in the renal microcirculation, the mechanism by which Gi pathway activators augment vasoconstrictor responses to Gq pathway activators is via coincident signaling at the level of PLC leading to activation of the PKC → c-src → PI3K pathway (33). The present study demonstrates that inhibition of each of the steps in this pathway (PLC, PKC, c-src, and PI3K) blocks the CCPA-induced augmentation of renovascular responses to NE. Moreover, blockade of PDK1, the major downstream transducer for PI3K (48), also abolishes CCPA-induced enhancement of renovascular responses to NE. These findings are highly supportive of the following mechanism for the coincident signaling pathway between A1 receptors and NE: PLC-activated activators is via coincident signaling at the level of PLC-activated activators (most likely by “direct” vasoconstriction independent of NE. This conclusion is based on the observations that CCPA, a potent and selective A1 receptor agonist, does not per se affect baseline perfusion pressure in the isolated, perfused rat kidney at 1, 3, or 10 nmol/l; yet CCPA remarkably enhances the vasoconstrictor response to NE at these same concentrations. In support of this concept, our previous work demonstrates that in the isolated, perfused rat kidney blockade of α1-adrenoceptors with either prazosin or phentolamine eliminates vasoconstrictor responses to RSNS at 3 to 9 Hz (for 3 min) (46). If adenosine in the neuroeffector junction were causing direct vasoconstriction, then RSNS at these frequencies should induce a response, albeit reduced, in the presence of α1-adrenoceptor blockade.

Interestingly, in isolated, perfused mouse arterioles, A1 receptor activation causes profound vasoconstriction (23), suggesting “direct” vasoconstriction. Importantly, even in this model system, A1-induced renal vasoconstriction is mediated by β2-subunit-induced activation of PLC (23). As reviewed by Selbie and Hill (54), β subunits released by Gi-coupled receptors can synergize with αq subunits released by Gq-coupled receptors to cause a more robust activation of PLC (most likely PLC-β isoforms) and its downstream transducer molecules including PKC. Although in reconstituted systems (55) or overexpressing systems (67) β subunit directly stimulates

Fig. 3. A: bar graph shows effects of RSNS on the renal venous perfusate concentration of norepinephrine in nontreated kidneys (CON) and in kidneys treated with either DPSPX (300 µmol/l) or XAC (1,000 nmol/l). The P value is from 1-factor ANOVA comparing CON vs. DPSPX vs. XAC groups. Basal levels of norepinephrine before RSNS in the CON, DPSPX-treated, and XAC-treated groups were not significantly different (55 ± 23, 93 ± 24, and 164 ± 68 pg/ml, respectively). B: bar graph shows effects of RSNS on the renal venous perfusate concentration of norepinephrine in kidneys treated with either FR113452 (1,000 nmol/l) or FK453 (1,000 nmol/l). Basal levels of norepinephrine before RSNS in the FR113452-treated and FK453-treated groups were not significantly different (96 ± 19 and 52 ± 20 pg/ml, respectively). The P value is from Student’s unpaired t-test comparing FR113452 vs. FK453 groups. For both panels, values represent means ± SE for the indicated number of experiments (n).
PLC-β, in tissues and cells under physiological conditions, Gi-coupled receptors generally exert minimal “direct” PLC activation per se, but rather significantly enhance the effects of Gq-coupled receptors on PLC activation via a mechanism that is inhibited by pertussis toxin and subunit scavengers (54). The mechanism of this interaction may in part be due to subunits inhibiting the ability of PLC-β to activate the GTPase activity of αq (5).

We hypothesize that A1 receptors require coincident signaling to cause potent vasoconstriction and that the partners in the coincident signaling process can be many different endogenous agonists that also activate PLC depending on the physiological context. So the point is not that the interaction is specific, but that it is important in the renal sympathetic neuroeffector junction. With regard to renal sympathetic neurotransmission, NE would be the primary coincident signaling partner simply because NE is released into and achieves high concentrations in the same biophase in which adenosine is formed. Also, although we did not measure or control angiotensin II in the present study, the fact that 1-adrenoceptor blockade abolishes RSNS responses eliminates the possibility that adenosine is enhancing neurotransmission in the isolated, perfused rat kid-

Fig. 4. Line graphs show effects of DPCPX (A), ZM-241385 (B), MRS-1754 (C), or VUF-5574 (D) on perfusion pressure responses to RSNS. These experiments employed a paired experimental design in which both kidneys from the same rat were removed and perfused separately but simultaneously. Of each pair, 15 min before initiating RSNS, one kidney received vehicle only (DMSO) and the other kidney received one of the four antagonists (1,000 nmol/l). A frequency-response relationship was elicited by stimulating at 2, 3, 4, 5, 6, 7, 8, 9, and 10 Hz for 2 min at 15-min intervals. Responses were normalized to a 5-Hz test response that was elicited before drug treatments. A: basal perfusion pressures (before treatments) were 51 ± 2 and 53 ± 2 mmHg for the CON group and DPCPX group, respectively. Basal perfusion pressures were not affected by DPCPX. Test RSNS responses (before treatments) were 75 ± 6 and 85 ± 10 mmHg for the CON group and DPCPX group, respectively. B: basal perfusion pressures (before treatments) were 50 ± 2 and 46 ± 4 mmHg for the CON group and ZM-241385 group, respectively. Basal perfusion pressures were not affected by ZM-241385. Test RSNS responses (before treatments) were 82 ± 12 and 83 ± 15 mmHg for the CON group and ZM-241385 group, respectively. C: basal perfusion pressures (before treatments) were 45 ± 2 and 48 ± 3 mmHg for the CON group and MRS-1754 group, respectively. Basal perfusion pressures were not affected by MRS-1754. Test RSNS responses (before treatments) were 97 ± 4 and 111 ± 6 mmHg for the CON group and MRS-1754 group, respectively. D: basal perfusion pressures (before treatments) were 47 ± 2 and 44 ± 2 mmHg for the CON group and VUF-5574 group, respectively. Basal perfusion pressures were not affected by VUF-5574. Test RSNS responses (before treatments) were 105 ± 20 and 102 ± 20 mmHg for the CON group and VUF-5574 group, respectively. *P < 0.05 compared with CON group at corresponding frequency of RSNS. The P values in the figure are from repeated-measures 2-factor ANOVA. Values represent means ± SE for the indicated number of experiments (n).
ney by coincident signaling with angiotensin II. This is expected since in the isolated, perfused rat kidney renin substrate is not provided and any released renin would not recirculate to the preglomerular microcirculation. However, in vivo it is entirely possible that coincident signaling occurs between A1 receptors and AT1 receptors due to sympathetically driven renin release and accumulation of renin with subsequent formation of angiotensin II.

Adenosine in the neuroeffector junction could arise from adenosine production triggered by NE acting on α-adrenoceptors or β-adrenoceptors on postjunctional cell surfaces. Indeed, in the isolated, perfused rat kidney blockade of adrenergic receptors reduces RSNS-induced renal venous adenosine and inosine secretion (46). Alternatively, as Westfall and co-workers (64) elegantly demonstrated, adenosine can be formed in the neuroeffector junction due to release of the cotransmitter ATP, which can be rapidly metabolized by releasable nucleotidases (which undergo exocytosis along with ATP) to adenosine (the Westfall mechanism). If the main source of the adenosine that participates in renal sympathetic neurotransmission is due to NE-induced activation of postjunctional adrenergic receptors, then blockade of A1 receptors might be expected to attenuate vasoconstrictor responses to both exogenous NE and RSNS. On the other hand, if the main source of adenosine is via exocytosis of ATP and nucleotidases followed by metabolism of ATP to adenosine, then blockade of A1 receptors would attenuate vasoconstrictor responses to RSNS but would not attenuate vasoconstrictor responses to exogenous NE (which would not release cotransmitters and therefore not engage the Westfall mechanism). Our results are consistent with the Westfall mechanism because DPCPX attenuates vasoconstrictor responses to RSNS, but not to exogenous NE. However, it is also conceivable that the differential effects of A1 receptor antagonism on responses to exogenous NE vs. RSNS could be due to relatively selective expression of vasoconstrictor A1 receptors in the postjunctional surface of the neuroeffector junction vs. relatively selective expression of vasodilatory A2A (4) and A2B (15) receptors (which would counteract the effects of A1 receptors) in the noninnervated vascular muscle cell membranes in the kidney microcirculation. Because exogenously administered NE would mostly engage noninnervated vascular smooth muscle cell surfaces, whereas NE released from sympathetic nerve terminals would stimulate mostly innervated vascular smooth muscle cell surfaces, this too could explain the fact that DPCPX attenuates responses to RSNS, but not to exogenous NE. Finally, it is conceivable that NE stimulates postjunctional release of adenosine in the renal neuroeffector

Fig. 5. Line graphs show effects of DPCPX on frequency-dependent perfusion pressure responses to RSNS (A) and concentration-related perfusion pressure responses to exogenous norepinephrine (NE; B). Basal perfusion pressure was 63 ± 3 mmHg and was unaffected by DPCPX. *P < 0.05 compared with CON group at corresponding RSNS frequency. The P values in panels are from 2-factor ANOVA. Values represent means ± SE for the indicated number of experiments (n).

Fig. 6. Bar graph shows perfusion pressure responses to exogenous norepinephrine before (basal) and during treatment with increasing concentrations of 2-chloro-N6-cyclopentyladenosine (CCPA) with or without cotreatment with DPCPX. Some kidneys were not treated with either CCPA or DPCPX (time control). Basal perfusion pressures (before treatments) were 57 ± 4, 61 ± 2, and 60 ± 3 mmHg in the CCPA group, CCPA + DPCPX group, and time control group, respectively, and were not affected by the treatments. *P < 0.05 compared with the third baseline response (basal #3) of CCPA group. **P < 0.05 compared with responses in CCPA group during corresponding period. The P values in graph are from 2-factor ANOVA. Values represent means ± SE for the indicated number of experiments (n).
junction via α-adrenoceptors or β-adrenoceptors, but does so to a much lesser extent outside of the neuroeffector junction. These three possible explanations for why A1 antagonism reduces responses to RSNS, but not responses to exogenous NE, are not mutually exclusive.

The present study also shows that blockade of A2B and A3 receptors has little if any effect on renal sympathetic neurotransmission. In contrast, antagonism of A2A receptors augments responses to RSNS. This is not unexpected because renal A2A receptors are vasodilatory (4) and blockade of A2A receptors would be expected to enhance responses to RSNS.

In addition to adenosine/A1 receptor interactions, it is conceivable that ATP/P2 receptor interactions also participate in renal sympathetic neurotransmission; the present study does not address this possibility. In this regard, ATP directly activates P2 receptors, and agonism of some P2 receptor subtypes elicits intense renal vasoconstriction (20, 21). For example, by contracting renal vascular smooth muscle cells in afferent arterioles, ATP participates in renal autoregulation via activation of P2X1 receptors (29). Presently, however, the literature regarding the role of ATP/P2 receptor interactions in renal sympathetic neurotransmission is sparse and contradictory, with some investigators concluding that ATP, via P2 receptors, is a direct cotransmitter in the renal sympathetic nervous system (62), while others conclude that ATP/P2 receptor interactions do not participate in renal sympathetic neurotransmission (14, 53).

The advantage of the isolated, Tyrode’s-perfused kidney system is the absence of variables that could be affected by treatments and cloud data interpretation. Nonetheless, there are caveats with this model system. First, basal perfusion pressures are lower than arterial pressure because the kidneys are perfused with Tyrode’s solution, which has much lower viscosity compared with blood (2). Despite this, the isolated, perfused rat kidney is characterized by high vascular sensitivity to vasoconstrictors (17, 36, 51) and the vascular response of the preparation is similar to that observed in vivo (34). Second, Tyrode’s solution is a poor oxygen carrier. However, the preparation is stable with regard to reproducible responses and the basal secretion of purines is low (46), suggesting that impaired oxygen delivery is not influencing vascular responses or adenosine production. Third, reactive oxygen species may be formed in the perfusate or by the kidney; however, in the present study addition of the antioxidant apocynin did not alter the ability of CCPA to enhance vasoconstrictor response to NE, suggesting that the results were not an artifact of oxidative stress.

In two phase II clinical trials, the A1 receptor antagonist rolodylline (KW-3902) demonstrated promise as a diuretic in acute decompensated heart failure with renal impairment or diuretic resistance (6, 19). Similarly, in a third phase II trial in patients with chronic congestive heart failure, rolodylline exerted beneficial effects (8). Surprisingly, in a large phase III clinical trial in patients with acute heart failure and renal impairment, rolodylline did not significantly affect the primary endpoint, although the percentage of patients experiencing treatment success was significantly increased (43), and a subsequent analysis of this study (45) showed that rolodylline administration was associated with an increase in the proportion of patients showing early relief from dyspnea and with a
numerically lower mortality at 14 and 30 days, largely because of reduced heart failure mortality. These studies speak to the need of being able to identify patients most likely to benefit from these drugs if and when they are clinically available. The present study suggests that biomarkers of increased renal sympathetic tone may usefully serve this purpose. Finally, the present results suggest that A1 antagonists may be beneficial in renal diseases and hypertension associated with increased renal sympathetic tone.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: E.K.J. and Z.M. conception and design of research; E.K.J., D.C., S.P.T., and Z.M. analyzed data; E.K.J. and S.P.T. interpreted results of experiments; E.K.J. prepared figures; E.K.J. drafted manuscript; E.K.J., D.C., S.P.T., and Z.M. edited and revised manuscript; E.K.J., D.C., S.P.T., and Z.M. approved final version of manuscript; D.C., S.P.T., and Z.M. performed experiments.

**REFERENCES**


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ENDOGENOUS ADENOSINE IN RENAL SYMPATHETIC NEUROTRANSMISSION


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