Phosphatase inhibitor cantharidin blocks adenosine $A_1$ receptor anti-adrenergic effect in rat cardiac myocytes

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Narayan, Prakash, Robert M. Mentzer, J r., and Robert D. Lasley. Phosphatase inhibitor cantharidin blocks adenosine $A_1$ receptor anti-adrenergic effect in rat cardiac myocytes. Am. J. Physiol. Heart Circ. Physiol. 278: H1–H7, 2000.—Experiments were performed to examine whether the protein phosphatase inhibitor cantharidin blocks the anti-adrenergic effect of adenosine $A_1$ receptor stimulation. In electrically stimulated adult rat ventricular myocytes loaded with the intracellular calcium concentration ($[Ca^{2+}]_i$) indicator fluo-3, isoproterenol (10 nM) increased systolic $[Ca^{2+}]_i$ by 46%, increased twitch amplitude by 56%, and increased total cellular cAMP content by 140%. The adenosine $A_1$ receptor agonist 2-chloro-N$^6$-cyclopentyladenosine (CCPA) reduced isoproterenol-stimulated $[Ca^{2+}]_i$, contractility, and cAMP by 87 and 80%, respectively, but reduced CAMP content by only 18%. Cantharidin had no effects on myocyte $[Ca^{2+}]_i$, contractility, or cAMP in the absence or presence of isoproterenol but blocked the effects of CCPA on $[Ca^{2+}]_i$, and contractility by ~44%. Cantharidin had no effect on CAMP attenuation of isoproterenol-induced increases in cAMP. Pretreatment with CCPA also reduced the increase in contractile parameters produced by the direct cAMP-dependent protein kinase A (PKA) activator 8-bromoAMP. These results suggest that activation of protein phosphatases mediate, in part, the anti-adrenergic effect of adenosine $A_1$ receptor activation in ventricular myocardium.

There are reports however indicating that the anti-adrenergic effect of adenosine $A_1$ receptor activation cannot entirely be accounted for by this pathway (9, 10, 22, 24). Using several adenosine $A_1$ receptor analogs, Gupta et al. (9) and Neumann and colleagues (24) reported a dissociation between the reductions in isoproterenol-stimulated phosphorylation of phospholamban and troponin I and contractility versus the reduction in cAMP content in isolated guinea pig ventricular myocytes. These authors hypothesized that adenosine $A_1$ receptor activation exerted its anti-adrenergic effect via the stimulation of protein phosphatases. Additional support for this hypothesis is provided by studies with the muscarinic-cholinergic agonist acetylcholine, which exerts an anti-adrenergic effect similar to adenosine $A_1$ receptor (18). It has been reported that acetylcholine reduces the phosphorylation of phospholamban in isolated guinea pig ventricular myocytes stimulated with either the phosphodiesterase inhibitor isobutylmethylxanthine or the cAMP analog 8-bromocAMP (8-BrcAMP) (11) and increases protein phosphatase activity by decreasing protein phosphatase inhibitor-1 activity in isoproterenol-stimulated, isolated perfused guinea pig hearts (10). The selective phosphatase inhibitors okadaic acid and cantharidin block the acetylcholine attenuation of isoproterenol-stimulated sarcoplasmal calcium channel activity in guinea pig ventricular myocytes (13). Although there are no reports of protein phosphatase inhibitor effects on adenosine $A_1$ receptor signal transduction in cardiac myocytes, it has been reported that protein phosphatase inhibitors block adenosine receptor-mediated effects in polymorphonuclear leukocytes and bovine adrenal chromaffin cells (19, 26).

The purpose of the present study was to determine whether the protein phosphatase inhibitor cantharidin blocks the anti-adrenergic effects of adenosine $A_1$ receptor activation on $[Ca^{2+}]_i$, contractility, and cAMP content in isoproterenol-stimulated rat ventricular myocytes.

METHODS

All animals in this study received humane care according to the guidelines set forth in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, National Institutes of Health Publication (No. 85-23, 1996) and according to the guidelines of the Department of Laboratory Animal Resources, University of Kentucky.

Isolation of myocytes. Ventricular myocytes were enzymatically dissociated from male Sprague-Dawley rats (350–400 g) by a previously described method (21) with minor modifica-

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ADENOSINE has no direct inotropic effects in mammalian ventricular myocardium but reduces β-adrenergic receptor-stimulated increases in L-type calcium channel activity, action potential duration, intracellular calcium concentration ($[Ca^{2+}]_i$) transient, and twitch amplitude (3, 4, 8). It is well recognized that this anti-adrenergic effect of adenosine is mediated via adenosine $A_1$ receptor coupling to a pertussis toxin-sensitive guanine nucleotide binding (G) protein, presumably $G_1$ or $G_0$ (4, 22), but there are conflicting reports of the mechanism(s) downstream to the activation of this inhibitory G protein. From the observations that adenosine $A_1$ receptor activation reduces catecholamine-stimulated adenylyl cyclase activity and cellular cAMP levels, it was hypothesized that increased cAMP was the primary mediator of the anti-adrenergic effect of adenosine $A_1$ receptor activation (4, 5, 12).
To assess the effects of the various treatments on the kinetics of excised and retrogradely perfused with an HEPES buffer. Rats were heparinized (500 U ip) and anesthetized with H2 ADENOSINE A1 RECEPTOR AND PROTEIN PHOSPHATASES

derivative of the Ca2+ loaded at room temperature with the acetoxymethylester K+ A-23187 calcium ionophore) in a calcium-free Krebs solution 75-W xenon arc lamp through an epifluorescence attachment [Ca2+]i. Fluorescence intensity was analyzed by custom-built software (Coyote Bay). The free cytosolic calcium concentration occurred in all the calcium transients. 

For simultaneous measurement of contractility, the myocyte was continuously illuminated with low intensity light from the illumination optics of the microscope and its image collected by the CCD camera. Changes in cell length were quantified via edge-motion detection with a video dimension analyzer (Coyote Bay). Contractile amplitude, expressed as a percentage of baseline twitch amplitude, velocity of shortening (+0.1L/min), and velocity of relengthening (−0.1L/min) were computed as described by Spinale et al. (30).

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Protocols. Myocytes were field stimulated throughout the experimental protocols at 0.5 Hz via platinum electrodes using a Grass SD9 stimulator (Grass Instruments, Quincy, MA). Each myocyte served as its own control. An equilibration period of 10 min was allowed for each myocyte following which baseline measurements were obtained. This ensured the presence of steady-state [Ca2+]i, and contractions. The following groups were studied (n = no. of myocytes): group 1: isoproterenol (n = 6); group 2: CCPA + isoproterenol (n = 6); group 3: cantharidin + isoproterenol (n = 4); group 4: cantharidin + CCPA + isoproterenol (n = 6); group 5: propranolol + isoproterenol (n = 4); group 6: cantharidin + propranolol + isoproterenol (n = 4); group 7: 8-BrcAMP (n = 4); and group 8: CCPA + B-BrCAMP (n = 4).

In the first group, myocytes were exposed to two treatments with isoproterenol (10 nM, 2 min) separated by a 10-min washout period. In the second group, recovery from the first exposure to isoproterenol was followed by treatment with the adenosine A1 receptor agonist 2-chloro-N6-cyclopentyladenosine (CCPA, 200 nM, 5 min). Myocytes were reexposed to isoproterenol in the presence of CCPA. Additional cells (n = 4) in this group were treated with the combination of CCPA and the selective adenosine A1 receptor antagonist 8-cyclopropyl-1,3-dipropylxanthine (DPCPX, 200 nM, 5 min). They were then exposed to isoproterenol in the presence of CCPA + DPCPX.

In group 3, the effects of the serine-threonine phosphatase inhibitor cantharidin (15) were examined on isoproterenol-stimulated [Ca2+]i, and contractility. After the first exposure to isoproterenol, myocytes were treated for 5 min with cantharidin (500 nM) before the second isoproterenol treatment. In group 4, after recovery from the first exposure to isoproterenol, myocytes were treated with CCPA + cantharidin before the second isoproterenol exposure. Groups 5 and 6 utilized similar protocols to test whether cantharidin altered the effects of the β-blocker propranolol (300 nM, 5 min).

Groups 7 and 8 tested whether CCPA altered the effects of the cAMP analog 8-BrcAMP. In group 7, myocytes were exposed to two treatments with 8-BrcAMP (1 nM, 5 min) separated by a 20-min washout period. In group 8, recovery from the first exposure to 8-BrcAMP was followed by treatment with CCPA. Myocytes were then reexposed to 8-BrcAMP in the presence of CCPA.

CAMP assay protocol. Parallel experiments were performed to determine CCPA and propranolol effects on isoproterenol-induced increases in cellular cAMP levels. After isolation, myocytes, suspended in room temperature (25°C) HEPES buffer (2–3 mg protein/ml), were incubated for a 30-min period with adenosine deaminase (2 U/ml, Sigma) and the phosphodiesterase inhibitor rolipram (50 µM) to block the effects of endogenous adenosine and cAMP phosphodiesterases, respectively (29). The cell suspension was then aliquoted into 1-ml Eppendorf tubes and divided into the following groups: control (n = 8); isoproterenol (n = 8); CCPA + isoproterenol (n = 6); cantharidin + isoproterenol (n = 4); cantharidin + CCPA + isoproterenol (n = 6); and propranolol + isoproterenol (n = 4).


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[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})(R_{max} - R)}{}
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where R is the measured fluorescence intensity (corrected for background fluorescence), Rmin is the fluorescence at zero calcium, Rmax is the fluorescence under saturating calcium concentrations (2.5 mM), and Kd is the dissociation constant. Rmin was derived by exposing myocytes (treated with 10 µM A-23187 calcium ionophore) in a calcium-free Krebs solution containing 10 mM EGTA, and Rmax was derived by exposing the myocytes to Krebs solution containing 2.5 mM CaCl2 (no EGTA). A Kd of 400 nM was used (25). The rate of decline of the calcium transient at any time point is proportional to the instantaneous [Ca2+]i and is reflective of the cellular mechanisms responsible for returning [Ca2+]i, to diastolic levels (8). To assess the effects of the various treatments on the kinetics of [Ca2+]i, the rate of decline at 200 nM [Ca2+]i, (−0.1Ca2+/dt) was calculated. The value of 200 nM was chosen because this concentration occurred in all the calcium transients.
Treatment times and concentrations were identical to the [Ca^{2+}], and contractility protocols. A subset of myocytes was used to study the effects of CCPA (n = 2), cantharidin (n = 2), and propranolol (n = 2) on cAMP levels. After treatments, the cell suspension was centrifuged at 1,000 g for 1 min and the supernatant discarded. Myocytes were then lysed with 0.1 N HCl, and the suspension was centrifuged at 3,000 g for 1 min. The resulting supernatant was used to measure cellular cAMP (immunoassay kit, R&D Systems, Minneapolis, MN), and the pellet was used to measure total cell protein using a modification of the Lowry assay (Bio-Rad, La Jolla, CA). Total cAMP was expressed as picomoles per milligram protein.

Materials. Isoproterenol, 8-BrcAMP, cantharidin, and propranolol were obtained from Sigma Chemical. CCPA and DPCPX were obtained from RBI (Natick, MA). Isoproterenol was used from fresh stock aliquots of 100 µM (dissolved in double distilled H2O), which were discarded at the end of the experiment. 8-BrcAMP, CCPA, and propranolol were dissolved in double distilled H2O; cantharidin and DPCPX were dissolved in dimethylsulfoxide. The vehicle concentrations did not exceed 0.05% in the buffer preparation.

Data analysis. Data are expressed as means ± SE (n, number of myocytes). Because none of the pretreatments (CCPA, DPCPX, cantharidin, and propranolol) influenced myocyte [Ca^{2+}], or contractility, comparison of data in these groups was done by single-factor analysis of variance (ANOVA) followed by Newman-Keuls post hoc analysis. Analysis of cAMP levels among the groups was done by ANOVA followed by Newman-Keuls post hoc analysis. A P value < 0.05 was considered statistically significant.

RESULTS

None of the treatments influenced diastolic [Ca^{2+}], (which averaged ~120 nM), and hence only systolic [Ca^{2+}], values, which averaged ~450 nM under baseline conditions, are reported. Myocytes were shortened by ~10% of their resting cell length, +6L/1t averaged 90.2 ± 5 µm/s, and −6L/1t averaged 75 ± 3 µm/s under these conditions. A 2-min infusion of isoproterenol increased systolic [Ca^{2+}], by 46 ± 2% (Fig. 1A) and −δCa_{200/6} from 0.969 ± 0.03 to 1.672 ± 0.06 µM/s (P < 0.05 vs. baseline). Isoproterenol increased twitch amplitude by 56 ± 4% (Fig. 1B), +6L/1t by 140 ± 8.8%, and −6L/1t by 95 ± 4%. The effects of isoproterenol were completely reversible because both the magnitude and kinetics of [Ca^{2+}], and contractility recovered to baseline on washout of the drug. A subsequent exposure to isoproterenol produced effects identical to the first (data not shown).

In the second group of myocytes, a 5-min treatment with CCPA had no effect on systolic [Ca^{2+}] (433 ± 17 nM), −δCa_{200/6}, twitch amplitude, +6L/1t, and −6L/1t. As seen in Fig. 1, A and B, in the presence of CCPA, isoproterenol produced only an ~10% increase in both systolic [Ca^{2+}], and contractility. The anti-adrenergic effect of adenosine A1 receptor activation was also manifest in the kinetics of [Ca^{2+}], decline and contractility. In the presence of CCPA, isoproterenol increased −δCa_{200/6} from 1.041 ± 0.08 to 1.20 ± 0.12 µM/s, +6L/1t by 61 ± 8%, and −6L/1t by 44 ± 3% (P < 0.05 vs. isoproterenol values). The anti-adrenergic effect of CCPA was negated by the adenosine A1 receptor antagonist DPCPX (Fig. 1, A and B).

It was then examined whether the phosphatase inhibitor cantharidin inhibited the anti-adrenergic effect of CCPA. Treatment of myocytes with cantharidin (500 nM, 5 min) did not have any significant effects on systolic [Ca^{2+}], twitch amplitude or the kinetic parameters. Exposure to isoproterenol in the presence of cantharidin produced effects not significantly different from exposure to isoproterenol alone (Fig. 2, A and B). This dose of cantharidin also did not affect the response to a higher dose of isoproterenol (100 nM) because systolic [Ca^{2+}], increased to 820 ± 17 nM and twitch amplitude increased by 74 ± 2% (n = 2) in the absence or presence of cantharidin. Importantly, in myocytes pretreated with CCPA + cantharidin, exposure to isoproterenol (10 nM) increased both systolic [Ca^{2+}], and the extent of shortening by ~40% (P < 0.05 vs. CCPA + isoproterenol, Fig. 2, A and B). Isoproterenol increased −δCa_{200/6} from 0.891 ± 0.06 to 1.449 ± 0.1 µM/s, +6L/1t by 114 ± 10%, and −6L/1t by 92 ± 6% in myocytes pretreated with cantharidin + CCPA (P <
These results clearly demonstrate a blockade of the anti-adrenergic effect of CCPA with cantharidin. The role of cellular cAMP in the anti-adrenergic effect of CCPA was examined in parallel experiments. Under baseline conditions, total cellular cAMP averaged 4.84 ± 0.58 pmol/mg protein. Exposure to isoproterenol increased cAMP 2.4-fold to 11.6 ± 0.4 pmol/mg protein. Treatment with CCPA alone had no effect on baseline cAMP (4.04 ± 0.39 pmol/mg protein), but in cells treated with CCPA + isoproterenol, cAMP increased to 9.5 ± 0.83 pmol/mg protein. Whereas this was only an 18% decrease from the isoproterenol group, it was significantly different from both baseline and isoproterenol groups (Fig. 3). In the cantharidin and cantharidin + isoproterenol groups, cellular cAMP contents did not differ from baseline and isoproterenol groups, respectively. In myocytes pretreated with CCPA + cantharidin and exposed to isoproterenol, cAMP content averaged 9.8 ± 0.5 pmol/mg protein, a value similar to the CCPA + isoproterenol group (Fig. 3).

A series of experiments was performed to determine whether the protein phosphatase inhibitor cantharidin altered the effects of the β-blocker propranolol. A 5-min treatment with propranolol (300 nM) had no effect on systolic [Ca^{2+}]_{i} and contractility but completely inhibited the effects of isoproterenol (Fig. 4, A and B). Propranolol alone had no effect on baseline cAMP (3.9 ± 0.14 pmol/mg protein) but blocked the isoproterenol effects of CCPA on Iso-stimulated total cellular cAMP content. See METHODS for details; n ≥ 4 myocytes in each group. Results are means ± SE. *P < 0.05 vs. control. #P < 0.05 vs. Iso.
Adenosine A1 receptor activation may completely block the isoproterenol-induced increase in particulate cAMP, which may not be observed when measuring total cellular cAMP. However, it has been reported that the muscarinic receptor agonist carbachol, which exerts a similar anti-adrenergic effect as CCPA, did not reduce total or particulate cAMP levels in isoproterenol-stimulated isolated perfused rat hearts (32). Given the similarities between the muscarinic M2 and adenosine A1 anti-adrenergic effects, it is likely that A1 receptor antagonism of the contractile effects of β-adrenergic receptor stimulation cannot entirely be accounted for by a reduction in cAMP content.

The two effects distal to cAMP, which A1 receptor activation could modulate, are cAMP-dependent protein kinase A (PKA) and a protein phosphatase. Because adenosine does reduce cAMP levels, it is expected that this would be associated with reduced PKA activity. This is indeed the case, although, as is the case with cAMP levels, there is a significant dissociation between A1 receptor modulation of PKA activity and contractile effects. In perfused rat hearts, application of adenosine decreased the isoproterenol-stimulated increase in PKA activity by only 30%, whereas contractility was reduced by 75% (5). In guinea pig myocardium, adenosine A1 receptor activation reduced isoproterenol-stimulated force development with little or no change in PKA activity (10). Studies on the anti-adrenergic effect of muscarinic M2 receptor stimulation indicate a similar disparity between catecholamine-induced increases in both the phosphorylation of regulatory proteins and contractility and alterations in PKA activity (10, 11).

In mammalian myocardium PKA and Ca2+-calmodulin-dependent protein kinase phosphorylate serine-threonine residues on regulatory proteins such as phospholamban, a process that plays a physiological role in both the inotropic and kinetic parameters of contractility (20). At least two distinct enzymes, protein phosphatases 1 (PP1) and 2A (PP2A), by virtue of dephosphorylation of these regulatory proteins, reverse the effects of protein kinases (2, 6, 23, 28). Gupta et al. (10) reported that both acetylcholine and the adenosine A1 agonist PIA attenuated isoproterenol-induced increases in the activity of protein phosphatase inhibitor-1, an inhibitor of PP1 (28), in guinea pig ventricular myocardium. The authors suggested that adenosine and acetylcholine increase PP1 activity by inhibiting catecholamine-stimulated protein phosphatase inhibitor-1 activity (10). These data resulted in the hypothesis that the anti-adrenergic effects of muscarinic M2 and adenosine A1 receptor activation are due, in part, to the stimulation of a serine-threonine phosphatase. A direct stimulatory effect of adenosine on protein phosphatases has been reported in bovine adrenal chromaffin cells and polymorphonuclear leukocytes (19, 26).

The results of the present study provide additional support for this hypothesis, because the protein phosphatase inhibitor cantharidin blocked the anti-adrenergic effect of CCPA. Cantharidin alone had no effect on the baseline magnitudes and kinetics of the calcium transient or contractility nor did it influence the actions
of isoproterenol (at two different concentrations) on these parameters. Cantharidin alone did not alter cellular cAMP content nor did it affect cAMP levels in myocytes treated with isoproterenol or CCPA + isoproterenol. Cantharidin also did not modify the anti-adrenergic action of the β-adrenergic receptor blocker propranolol. However, cantharidin blocked the anti-adrenergic effects of CCPA to the same extent as the A1 antagonist.

Cantharidin, an extract from blister beetles, is an inhibitor of serine-threonine protein phosphatases. In vitro studies indicate that cantharidin has a higher affinity for PP2A [inhibitory constant (K_i) = 0.13 μM] than for PP1 (K_i = 1.7 μM). Only at higher concentrations (>10 μM) does cantharidin appear to inhibit protein phosphatases 2B and 2C (15). Cantharidin has been used in numerous studies to examine the role of protein kinases and phosphatases in myocardial excitation-contraction coupling (7, 15–17, 23). At concentrations >3 μM, cantharidin exerts a positive inotropic effect in cardiac muscle by increasing transsarcolemmal calcium flux (23). In the present study however cantharidin exerted no direct effects on contractility or [Ca^{2+}]. Although it is possible that cantharidin could have altered PKA activity or increased the sensitivity of PKA to cAMP, cantharidin did not potentiate any of the effects of a submaximal dose (10 nM) of isoproterenol.

The results obtained in the 8-BrcAMP protocols provide additional evidence supporting our hypothesis that adenosine exerts significant anti-adrenergic effects distal to adenylyl cyclase. Incubation of ventricular myocytes with 8-BrcAMP, which acts by stimulating PKA to cAMP, cantharidin did not potentiate any of the PKA-dependent effects of CCPA to the same extent as the A1 antagonist.

Increases of cyclic AMP by 10.220.33.4 on March 31, 2017 http://ajpheart.physiology.org/ Downloaded from

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