Activation of P2Y1 and P2Y2 nucleotide receptors by adenosine 5′-triphosphate analogues augmented nerve-mediated relaxation of human corpus cavernosum

Serap Gur, PhD; Wayne J.G. Hellstrom, MD, FACS

Abstract

Introduction: Adenosine 5′-triphosphate (ATP) is a ubiquitous cellular energy source. We evaluated the effect of ATP and its analogues on nonadrenergic and noncholinergic relaxation in precontracted human corpus cavernosal smooth muscle (HCCSM).

Methods: We obtained specimens of human corpus cavernosum (HCC) from patients undergoing penile prosthesis surgery (patient age 46–70 yr, *n* = 17) with prior approval from the local institutional review board. Isolated HCC strips were placed in organ baths containing Krebs solution and functional experiments were conducted. Immunohistochemical localization studies were performed to establish the presence of purinergic P2X1, P2Y1 and P2Y2 receptors in HCC.

Results: The amplitude of relaxation induced by electrical-field stimulation (EFS) on HCC was significantly increased after exposure to ATP (P2X and P2Y agonists), 2-MeSATP (P2Y1 agonist), and uridine 5′ triphosphate (P2Y2 agonist), but not α,β-methylene ATP (P2X1 agonist). The P2X1 antagonist pyridoxal-5′-phosphate-6-azophenyl-2′, 4′-disulfonate, and the nonspecific P2Y antagonist, reactive blue 2, did not inhibit the potentiated response of EFS on HCC. Although immunoreactivity for both P2Y1 and P2Y2 receptors was localized abundantly in HCC, there was only low-level immunostaining for the P2X1 receptor.

Conclusion: These data demonstrate that nerve-mediated relaxation of HCCSM strips precontracted with phenylephrine in organ bath preparations is amplified by stimulating purinergic P2Y1 and P2Y2 receptors. Although nucleotides are important regulators of HCCSM tone, these observations suggest an independent purinergic relaxing mechanism in the HCC, separate from the better known nitrergic system.

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Résumé

Introduction : L'adénosine 5′-triphosphate (ATP) est une source d'énergie cellulaire générale. L'effet de l'ATP et de ses analogues sur le relâchement non adrénergique et non cholinergique du muscle lisse précontracté du corps caverneux a été évalué.

Méthode : Des échantillons de corps caverneux humains (CCH) ont été obtenus à partir de patients porteurs d'une prothèse pénienne (âgés de 46 à 70 ans, *n* = 17) avec l'approbation préalable du comité d'éthique de l'établissement. Des bandes isolées de CCH ont été placées dans des bains organiques contenant une solution de Krebs et des expériences fonctionnelles ont ensuite été réalisées. On a eu recours à des tests de localisation immunohistochimique pour déceler la présence des récepteurs purinergiques P2X1, P2Y1 et P2Y2 dans les échantillons de CCH.

Résultats : L'ampleur du relâchement produit par stimulation électrique des échantillons de CCH a été significativement accrue après exposition à l'ATP (agoniste des récepteurs P2X et P2Y), à la 2-MeSATP (agoniste du récepteur P2Y1) et à l'UTP (agoniste du récepteur P2Y2), mais pas à la α,β-MeATP (agoniste du récepteur P2X1). L'antagoniste du récepteur P2X1, le pyridoxal-5'-phosphate-6-azophényl-2′, 4′-disulfonate, et l'agoniste non spécifique du récepteur P2Y, le bleu chimiquement réactif, n'ont pas inhibé la réponse potentialisée par stimulation électrique des bandes de CCH. Même si une immunoréactivité des récepteurs P2Y1 et P2Y2 a été grandement notée dans les bandes de CCH, on n'a obtenu qu'une faible immunocoloration pour le récepteur P2X1.

Conclusion : Ces données montrent que le relâchement par voie nerveuse des bandes de CCH précontractées par phényléphrine dans des bains organiques est amplifié par la stimulation des récepteurs purinergiques P2Y1 et P2Y2. Bien que les nucléotides constituent des facteurs importants de régulation du tonus des CCH, ces observations portent à croire à l'existence d'un mécanisme indépendant de relâchement purinergique distinct du système nitrergique mieux connu.

Introduction

Adenosine 5′-triphosphate (ATP) is present in every cell as a major energy source.[1] Since Burnstock proposed the classification of P2 purinoceptors, extracellular ATP has been recognized as a vital extracellular signaling molecule that elicits diverse biological responses from many organ and tissue systems.[2–4] Adenosine 5′-triphosphate acts via P2 receptors, which are classified as G protein–coupled P2Y receptors, and the ligand-gated cation channel P2X receptors.[5] Researchers have identified 7 subtypes of P2X receptor and 5 subtypes of mammalian P2Y receptor.[2]

Adenosine 5′-triphosphate is a nitric oxide (NO)–activating agonist that induces penile tumescence.[6] Early studies in isolated corpus cavernosum smooth muscle

(CCSM) preparations demonstrated that ATP and other purines decreased both basal and phenylephrine-stimulated tension.[7,8] Intracavernosal injection of ATP in dogs increased intracavernous pressures leading to penile erection.[9] Lee and coworkers[10] suggested that P2X receptors acted during the detumesence process. Obara and colleagues[11] demonstrated the expression of P2Y1 receptors in endothelial cells that line the lacunar spaces and blood vessels of the penis. Other studies have also noted that ATP was a potent and NO-independent relaxant of human and rabbit CCSM.[12] Relaxation of human corpus cavernosum smooth muscle (HCCSM) occurred by stimulation of P2Y purinoceptors via NO release.[6] Thus the exact role of ATP and/or its specific agonists in the mechanism of erection remains undocumented. It is interesting to recognize that ATP, the signaling molecule of the purinergic system, can nonspecifically augment NO production in human corpus cavernosum (HCC).

The aim of this study was to establish whether ATP and its analogues could affect the nonadrenergic and noncholinergic (NANC) nerve-mediated relaxation responses of HCC. Additionally, immunohistochemical labelling and light microscopy was employed to demonstrate the immunoreactivity of P2X1, P2Y1 and P2Y2 receptors in the human corporal endothelium and smooth muscle.

Methods

Source of HCC

Cavernosal tissues were obtained from patients with erectile dysfunction (ED) (patient age 46–70 yr, $n = 17$) undergoing penile prosthesis surgery. All studies were conducted under institutional review board guidelines. Human corpus cavernosum tissue biopsies were placed in an ice-cold Krebs solution and transported immediately to the laboratory for in vitro experiments. In our study the specimens of HCC were obtained from 16 male patients during penile prosthesis inflammation. Four samples were obtained from patients with hypertension and hypercholesterolemia, 5 samples were obtained from patients after radical prostatectomy, 4 samples were obtained from patients with Peyronie disease and 4 samples were obtained from patients with diabetes mellitus.

Organ chamber studies

Strips of HCC tissue (1 × 1 × 8 mm) were immersed in 20-mL organ chambers containing Krebs solution (containing, in mM, sodium chloride: 118.1; potassium chloride: 4.7; potassium dihydrogen phosphate: 1.0; magnesium sulfate: 1.0; sodium bicarbonate: 25.0; calcium chloride: 2.5; and glucose: 11.1), maintained at 37°C, and aerated with 95% oxygen, 5% carbon dioxide, pH 7.4. The muscle strip ends were tied with silk to a wire connected to a force transducer on one end and fixed with silk ties to a metallic support on the other end, and vertically mounted under 1 g resting tension. The preparations were allowed to equilibrate for a minimum of 90 minutes and the bath medium was replaced every 15 minutes. Changes in isometric tension were recorded on a chart polygraph. Relaxation responses were tabulated after adding increasing concentrations of study compounds to strips precontracted with 10 μM phenylephrine (Phe).

For electrical-field stimulation (EFS), the strips were stimulated for 10 seconds with 2 parallel platinum electrodes at 20 Hz frequency as square-wave pulses of 50 V (0.8 ms) delivered by a current amplifier and a stimulator. Before EFS components, in an attempt to determine the relaxation response elicited by the NANC nerve component, the tissues were treated with adrenergic nerve blocker guanethidine (10 μmol/L) and muscarinic receptor blocker atropine (1 μmol/L) for 30 minutes to eliminate the adrenergic and cholinergic components.

In the organ bath experiments, HCCSM strips were precontracted with 10 μM Phe. After reaching the plateau level of Phe-induced contractile response, ATP (10^{-4}M), uridine 5′ triphosphate (UTP, 10^{-5} M), 2-methylthioATP (2-MeSATP, 10^{-5} M), and α,β-methyleneATP (10^{-5} M) were added to the organ bath and EFS (20 Hz) was applied to the HCCSM strip. Responses to ATP and its analogues were recorded before and after administration of P2X antagonist PPADS, reactive blue 2 (RB2), nonspecific NOS inhibitor Nomega-nitro-L-arginine methyl ester (L-NAME, 100 mM), and soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4] oxadiazolo [4,3-a]quinoxaline-1-one (ODQ, 10 μM).

Immunohistochemistry

Upon receipt from the operating room, HCC tissues were sectioned in 12-μm thick slices using a cryostat and placed on gelatin-coated slides. Sections were treated with 50% methanol containing 0.4% hydrogen peroxide for 10 minutes. Nonspecific protein binding sites were blocked with 10% normal horse serum (NHS) diluted 1:50 in phosphate-buffered saline containing 0.1% (weight/volume) bovine serum albumin. Slides were incubated with rabbit primary polyclonal antibody at a dilution of 1:200 (in 10% NHS) at room temperature for 1 hour. Samples were then washed and incubated for an additional 30 minutes with biotinylated secondary antibody (Dako), followed by a further 30-minute incubation with an avidin-biotin-conjugated horseradish peroxidase (Dako), and then a substrate (DAB, Vectastain, Vector Laboratories) for 5 minutes. Harris hematoxylin was

used as a counterstain, and negative control slides were stained with only secondary antibody. The images were visualized under light microscopy (Leica DM4000B and DFC 280 colour digital camera system, Leica Microsystems).

Drugs

Phenylephrine, ATP, 2-MeSATP, UTP, α,β-meATP, pyridoxyl 5-phosphate 6-azophenyl-2,4-disulfonic acid (PPADS), RB2, L-NAME, and ODQ were purchased from Sigma Chemical Company. Drugs were dissolved in distilled water at the time of each experiment. P2X1, P2Y1, and P2Y2 rabbit polyclonal antibodies were purchased from Santa-Cruz Biotechnology, Inc.

Statistical analysis

All data are expressed as means and standard errors. Maximal relaxation of EFS to agonists were expressed as percentage inhibition on Phe-stimulated contractions (10 μm). Statistical analysis of data was performed using one-way analysis of variance (ANOVA) with repeated-measures followed by Bonferroni posttest performed using the Prism 4 statistical analysis packages for Windows (GraphPad Software). A p value < 0.05 was considered to be significant.

Results

Electrical-field stimulation, acetylcholine and ATP response on HCC strips

Figure 1 illustrates the nerve-mediated, endothelium-dependent acetylcholine-induced and NANC-ATP-induced relaxation responses of HCC tissues. A 10^{-3} M concentration of ATP produced greater relaxation of HCC (88%) compared with acetylcholine (ACh) and EFS-induced relaxation.

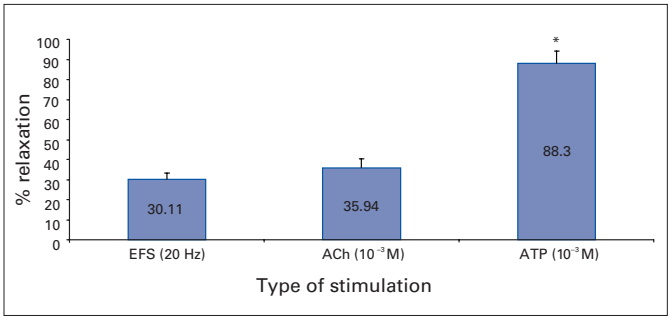

Fig. 1. Nonadrenergic and noncholinergic nerve-mediated or electrical field stimulation (EFS, 20 Hz), acetylcholine (ACh)–induced (10^{-3} M) and adenosine 5′-triphosphate (ATP, 10^{-3} M) induced relaxations in human corpus cavernosum. Results are expressed as percent relaxation of the phenylephrine (10 μ) contraction, and are shown as means and standard errors for 8–10 experiments. * $p < 0.001$, which is significantly different from EFS-induced relaxation, paired Student t test.

Effect of ATP and UTP on EFS-induced relaxation responses in HCC strips

Electrical-field stimulation–induced relaxation in HCCSM was 2-fold over the control value, in the presence of the nonspecific P2 receptor agonist ATP (2.27, Fig. 2A), P2Y1 agonist 2-MeSATP (2.45, Fig. 2B) and P2Y2 agonist UTP (2.47, Fig. 2C). P2X agonist α,β-meATP did not affect the EFS response at 20 Hz (Fig. 3).

Effects of antagonism with NOS inhibitor L-NAME, selective sGC inhibitor ODQ, PX1 receptor inhibitor PPADS and P2Y nonspecific inhibitor RB2 in HCC strips

Not all drugs affected the HCC response to EFS (at 20 fre-

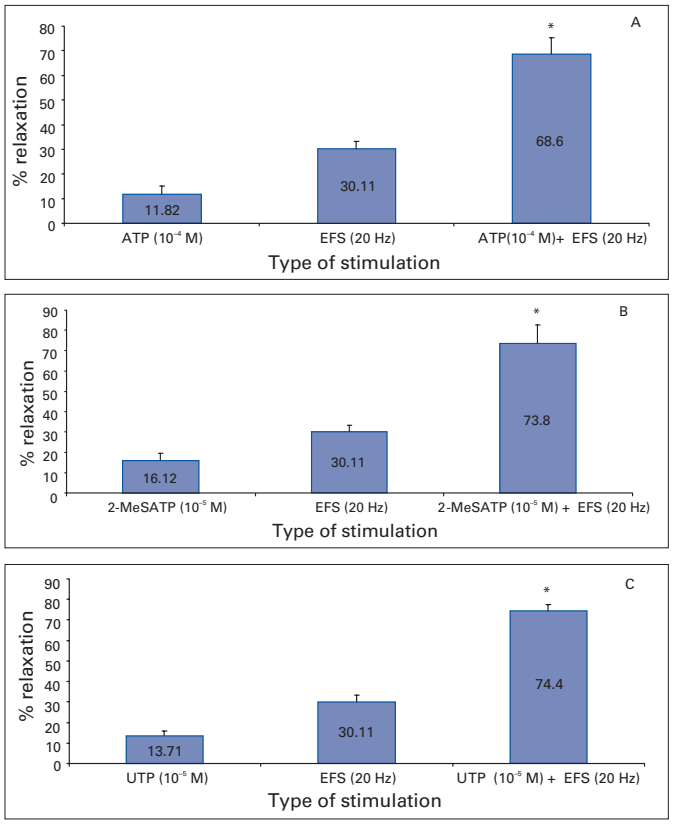

Fig. 2. Effect of adenosine 5′-triphosphate (ATP, 10^{-4} M) **(A)**, 2-methylthioATP (2-MeSATP, 10^{-5} M) **(B)** and uridine 5′ triphosphate (UTP, 10^{-5} M) **(C)** on the nonadrenergic and noncholinergic (NANC) nerve-mediated relaxations to electrical field stimulation (EFS, 20 Hz) in human corpus cavernosum (HCC) strips. Results are expressed as percent relaxation of phenylephrine (10 μ) contraction, and are shown as means and standard errors for 8–10 experiments. * $p < 0.001$, which is significantly different when the drug is not present, paired Student t test.

quency) in the presence of ATP or an agonist (data not shown).

Immunohistochemistry

Human penile tissues showed minimal staining for P2X1 receptors on HCCSM (Fig. 4A). Both P2Y1 receptor (Fig. 4B) and P2Y2 receptor (Fig. 4C) immunoreactivity were present on HCCSM.

Discussion

There is no pharmacological evidence for the role of purinergic mechanisms in nerve-mediated relaxation of HCCSM. This communication reports that exogenous ATP (a non-selective P2 receptor agonist) and its analogues can potentiate nerve-mediated relaxation responses of HCCSM with involvement of P2Y1 and P2Y2 receptors, but not P2X1 receptors.

Adenosine 5′-triphosphate markedly augments nerve-mediated relaxation in organ bath preparations of HCCSM strips contracted with Phe. It should be noted, however, that 2 types of purinergic receptors, P2X and P2Y, may be involved in the augmented effects of ATP on HCCSM. This data showed that 2-MeSATP (P2Y1 agonist, adenine nucleotide) and UTP (P2Y2, uridine nucleotide) produced equal enhancements of EFS-relaxation upon their exogenous application, similar to ATP; thereby documenting the expression of P2Y1 and P2Y2 receptors in the HCCSM. It is understood that P2Y receptors are coupled via G-protein to phospholipase C resulting in inositol 1,4,5-trisphosphate generation and Ca^{2+} release from intracellular stores or stimulation/inhibition of adenylate cyclase.[13] P2Y receptors also mediate proliferative signaling pathways downstream. For instance, both UTP and ATP induce proliferation of cultured human keratinocytes and rat aortic smooth muscle cells.[14,15] Cultured rat mesangial cell proliferation has been shown to be mediated in part by P2Y2 receptors.[16] Thus the role of the P2Y2 receptor may mediate trophic effects in HCC. In a study using HCCSM strips procured from men with ED undergoing penile prosthesis implantation, an active P2Y1 agonist (adenosine-5′-O-[2-thiodiphosphate]) caused smooth-muscle relaxation.[6] Obara and colleagues[11] showed that the P2Y1 receptor is expressed in endothelial cells that line the lacunar space and blood vessels, yet is not expressed in CCSM cells and urethras of normal rats. P2Y1 receptors are observed throughout the CCSM in our studies. These differences may be due to the difference in species or pathophysiological status of the human penis. By contrast, P2X1 purinergic agonist, α,β-meATP, did not cause any augmentation of EFS-induced nerve-mediated relaxation, suggesting an absence of a P2X receptor effects. P2X1 antagonist, PPADS, did not inhibit the potentiated response of EFS on HCC and there was low-level immunostaining for the P2X1 receptor in the tissue used.

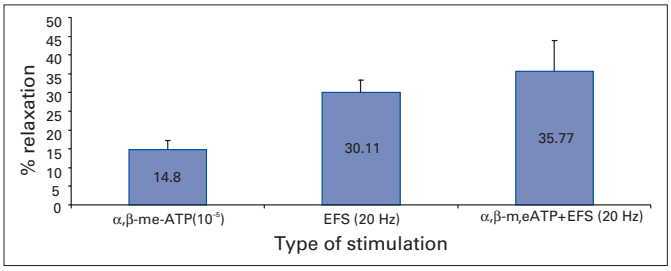

Fig. 3. Effect of α,β-methylene adenosine 5′-triphosphate (meATP, 10^{-4} M) on the nonadrenergic and noncholinergic nerve-mediated relaxations to electrical field stimulation (EFS, 20 Hz) in human corpus cavernosum strips. Results are expressed as percent relaxation of phenylephrine (10 μ) contraction, and are shown as means and standard errors for 8–10 experiments. * $p < 0.001$, significantly different from absence of drug, paired Student *t* test.

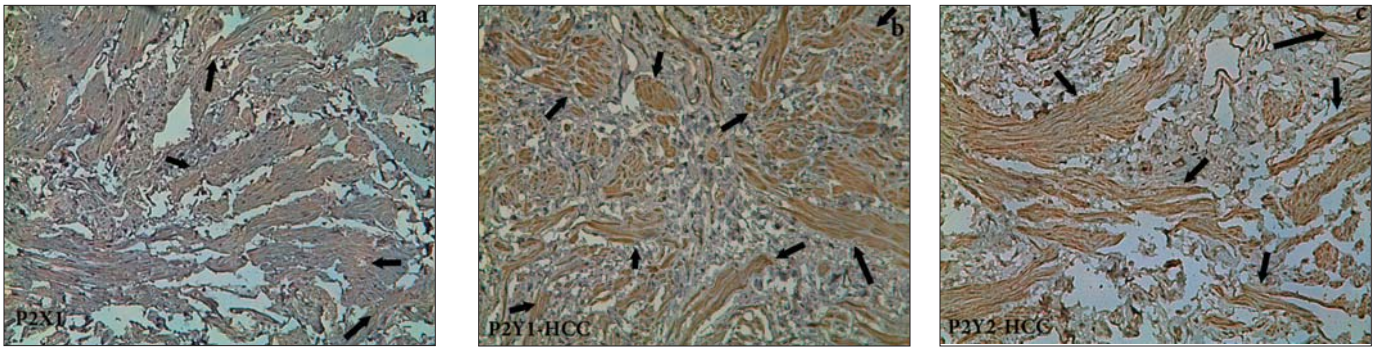

Fig. 4. Representative immunohistochemical staining of transverse section of human corpus cavernosum smooth muscle (original magnification × 10). Immunostained for P2X1 **(A)**, immunostained P2Y1 **(B)** and immunostained P2Y2 **(C)**. We noted the abundant staining for P2Y1 and P2Y2 (black arrows) receptors of smooth muscle membrane and less staining for P2X1 receptors in cavernosal smooth muscle as observed by 4 independent observers.

Low-level P2X receptors seen in immunohistochemistry pictures may play a role in producing vasoconstriction, even with high levels of sympathetic activity that occur in hypertension diabetes and benign prostatic hyperplasia. In our study we suggest that P2X receptors are unable to directly mediate corporal tone. The impact of a low-level and lack of P2X-mediated constriction in penile smooth muscle is unknown. Hence, enhancement of EFS-induced relaxation may be related to hyperpolarization mediated via metabotropic receptors rather than ionotropic receptors. As of yet, there is no published data to support this hypothesis; however, immunohistochemical studies will likely support our in vitro observations. Immunohistochemical analysis with an antibody against the P2Y1 receptor subtype revealed that functional P2Y1 and P2Y2 purinoceptors were intensely expressed in CCSM.

The physiological relevance of NO enhancing EFS-evoked relaxation was explored. The ATP augmented response to EFS was unaffected by L-NAME or by ODQ, which argues against the theory of a serial cascade involving NO production by ATP. Filippi and coauthors[12] showed that ATP acted as a potent NO-independent relaxing agent for human and rabbit corpus cavernosum. On the other hand, others found that, in rabbit CCSM tissue, ATP played an important role in smooth-muscle relaxation, but its actions were independent of the endothelium.[7] Our previous data supported an independent NO-related mechanism.[17] Nevertheless, ATP enhanced the effect of the NO pathway, but appears not to be involved in activation. This suggests that purinergic receptors are coupled to a novel signaling pathway. Perhaps purinergic nerves operating through P2Y1 and P2Y2 purinoceptors may be responsible, in part, for the nonnitrergic NANC relaxation in HCCSM in vitro.

We observed that P2X purinoceptor antagonist, PPADS, and the P2Y antagonist, RB2, did not inhibit nonnitrergic NANC relaxation caused by 20 Hz electrical stimulation. Similarly, Filippi and colleagues[12] showed that the P2X and P2Y antagonists PPADS and RB2 did not modify ATP's direct relaxation response on HCC. Besides insensitivity to a putative antagonist such as RB2, the purinergic receptor is influenced by pharmacological tools interfering with the classical transduction pathway of P2Y receptors. Thus the identification of the receptor subtypes involved in the ATP relaxation effect by EFS in HCC remains to be established.

The relaxations induced by ATP are not blocked by L-NAME, suggesting that ATP cannot stimulate the release of NO and that NO is not the final neurotransmitter mediating muscle relaxation. Conceivably there may not be any involvement concerning interaction between neuronal release of ATP and NO by EFS. This suggests that NO is not one of the major inhibitory neurotransmitters in HCC. Thus ATP has also been demonstrated to be an inhibitory neurotransmitter in HCC. It seems that NO and ATP act independently, exerting their effects directly on the penile smooth muscle.

In conclusion, these initial findings extend our knowledge on purinergic mediators affecting penile erection/detumescence and could possibly identify a new area for the investigation of ED drugs. Further studies to assess the mechanistic role of ATP and ATP receptors in penile erection are needed.

From the Department of Urology, Tulane Health Sciences Center, New Orleans, La.

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Competing interests: None declared.

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Correspondence: Dr. Wayne Hellstrom, Professor of Urology, Chief, Section of Andrology, Tulane University Health Sciences Center, Department of Urology, 1430 Tulane Ave., SL-42, New Orleans LA 70112; fax 504 988-5059; whellst@tulane.edu