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DETRUSOR OVERACTIVITY INDUCED BY INTRAVESICAL APPLICATION OF ADENOSINE 5'-TRIPHOSPHATE (ATP) UNDER DIFFERENT DELIVERY CONDITIONS IN RATS

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Runninghead: ATP-mediated mechanisms for bladder overactivity

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Abstract

Objectives: We investigated the effects of intravesical application of adenosine 5'-triphosphate (ATP) on bladder activity to elucidate the role of urothelial barrier function and ecto-adenosine triphosphatase (ecto-ATPase) activity in the ATP-mediated mechanism inducing detrusor overactivity.

Methods: Continuous cystometry via an intravesical catheter inserted from the bladder dome was performed in conscious female rats.

Results: ATP solutions adjusted to pH 6.0 did not elicit significant detrusor overactivity at a concentration of 60 mM while in bladders pretreated with protamine sulfate (10 mg/ml) to increase urothelial permeability, ATP solution (pH 6.0) induced detrusor overactivity by decreasing intercontraction intervals (ICI). These irritant effects of ATP after protamine treatment were antagonized by P2X receptor antagonists, such as pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonic acid (70 μmol/kg) and 2',3'-O-(2,4,6, trinitrophenyl) adenosine 5'-triphosphate (30 μmol/kg), and these were also suppressed in rats pretreated with systemic capsaicin (125 mg/kg s.c.). α,β-methylene ATP (5 mM, pH 6.0) or ATP (60 mM, pH6) following intravesical infusion of 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (5 mM, pH 6.0), an ecto-ATPase inhibitor, induced detrusor overactivity without protamine pretreatment, but the reductions in ICI was smaller compared with ATP after protamine treatment.

Conclusions: Low permeability of bladder epithelium and ecto-ATPase activity can prevent

ATP activation of subepithelial P2X receptors to induce bladder overactivity. Thus, enhanced penetration of endogenous ATP due to urothelial damage may contribute to urinary frequency and bladder pain in hypersensitive bladder disorders such as interstitial cystitis.

Introduction

Recent studies have demonstrated that ATP is also released from urothelial cells by mechanical stretch of the bladder, and that bladder epithelium obtained from patients or cats with interstitial cystitis (IC) can release higher amounts of ATP compared with controls[1-4]. Mice lacking the $P2X_3$ receptor subunit, which is normally localized in suburothelial afferent nerves, reportedly had bladder hyporeflexia[5]. Thus, P2X purinergic receptor-mediated transduction mechanisms in bladder afferent pathways seem to play an important role in the control of bladder function under normal and pathological conditions such as IC.

It was reported that ATP infused intravesically at concentrations ranging 0.1-10mM can induce detrusor overactivity through an activation of bladder afferent pathways[6,7]. However, ATP produces a acidic solution (pH 3-4), and it is therefore possible that detrusor overactivity following acidic ATP solution can be induced not only by ATP-induced afferent sensitization, but also by activation of acid-sensitive receptors such as TRPV1 capsaicin receptors[8] and/or by damaging the bladder epithelium that may enhance ATP penetration into the subepithelial layer.

Thus, we sought to investigate in further detail the ATP-mediated mechanisms inducing detrusor overactivity using pH-adjusted ATP solution, especially focusing on epithelial barrier function and ecto-adenosine triphosphatase (ecto-ATPase) activity.

Materials and Methods

Animals

Female Sprague-Dawley rats (weighing 170-240 g) were used. The protocol for this study complies with the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Laboratory Animal Resources, and was approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Cystometry

Under halothane anesthesia, a PE-10 polyethylene catheter (Clay Adams, Parsippany, NJ) was inserted into the right jugular vein, and the free end of catheter was tunneled subcutaneously to the back of the neck. Then, a PE-50 catheter was inserted through the bladder dome and tightened by surgical suture. Thereafter, rats were placed in restraining cages and allowed to recover from anesthesia for 2 hours. The intravesical catheter was connected to a pressure transducer and a syringe pump for infusing solutions. Phosphate buffered saline (PBS) was infused (0.04 ml/min) continuously into the bladder until the interval of micturition cycles became stable. Then three micturition cycles were analyzed as baseline cystometric parameters. Intercontraction intervals (ICI), maximal voiding pressure (MVP), pressure threshold for voiding (PT) and baseline pressure (BP) were measured.

Drug administration

In the first group of rats, ATP (Sigma Aldrich Co., St. Louis, MO) dissolved in PBS (60 mM, n = 7), adjusted to pH 6.0 using sodium hydroxide, was infused intravesically following a

one hour intravesical instillation of PBS solution, which was also adjusted to pH 6.0, after a control period with normal PBS to examine whether pH-adjusted ATP solution can induce bladder overactivity.

In the second group of rats, protamine sulfate (PS; Sigma) dissolved in PBS (10 mg/ml) was instilled into the bladder for one hour to increase the permeability of bladder epithelium[9], and then PBS (pH 6.0) was infused one hour. Thereafter, ATP (20, 40 and 60 mM, pH 6.0, n = 7, respectively) was instilled intravesically. P2X receptor antagonists, pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; 70 μmol/kg, n = 5) (Tocris Cookson Inc., Ballwin, MO) or 2',3'-O-(2,4,6, trinitrophenyl) adenosine 5'-triphosphate, trisodium salt (TNP-ATP; 30 μmol/kg, n = 6) (Sigma) were injected intravenously five minutes before starting ATP (60 mM) infusion.

The experiments using PS and ATP (60mM, pH 6.0) were also performed in rats pretreated with systemic capsaicin (n = 6). Capsaicin (Sigma) was administered to rats in a solution (20 mg/ml) given subcutaneously in divided doses on 2 consecutive days: 25 and 50 mg/kg on the first day and 50 mg/kg on the second day. Four days after the first injection, cystometry was performed. To evaluate the effectiveness of capsaicin pretreatment, an eye wipe test was performed.

In the next series of experiments, the role of ecto-ATPase on the surface of bladder epithelium was evaluated using α,β-methyleneadenosine 5'-triphosphate (α,β-methylene ATP, 5 mM, Sigma), more stable analog of ATP, adjusted to pH 6.0, which was infused intravesically

after PBS (pH 6.0) with or without pre-infusion of PS (n = 6 and 4, respectively). In another group of rats, 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, Sigma) dissolved in PBS (5 mM, pH 6.0) was instilled into the bladder for one hour to inhibit ecto-ATPase activity, followed by ATP (60 mM, pH 6.0) with DIDS (5 mM) solution (n = 6).

In these experiments, micturition cycles from 10 to 30 minutes after starting intravesical infusion of ATP or α,β-methylene ATP were analyzed because detrusor overactivity was consistently observed during this period without apparent desensitization; whereas micturition cycles from 10 to 60 minutes after starting intravesical administration were measured for protamine sulfate, PBS (pH 6.0) and DIDS infusion.

Statistics

Results are given as mean ± SEM. Student's two-tailed *t*-test was used for comparisons of cystometric parameters before and after drug administration in the same animal. One-way ANOVA followed by Dunnett's multiple comparison test was used for comparisons of different dosages of ATP after PS infusion, and a parametric test (unpaired *t*-test) was used to test for differences in cystometric parameters between different protocol groups. For all statistical tests, $P < 0.05$ was considered significant.

Results

Effect of intravesical ATP adjusted to pH 6.0

PBS (pH 6.0) alone infused into the bladder did not alter any cystometric parameters. Similarly, application of ATP solutions (60 mM) adjusted to pH 6.0 did not elicit significant detrusor overactivity (Table 1).

Effect of ATP (pH 6.0) after PS infusion

When PS was infused intravesically, bladder overactivity was observed as evidenced by decreased ICI (Figure 1A). Thus, following PS infusion, PBS (pH 6.0) was infused intravesically, which partially normalize the cystometric parameters. Intravesical instillation of ATP (20, 40 and 60 mM) after PS treatment induced detrusor overactivity evidenced by a dose-dependent reduction in ICI 5-10 minutes after starting infusion (Figure 1A, B). At 60 mM of ATP, ICI was decreased to 52.3 ± 8.1%, PT was decreased and BP was increased significantly (Figure 1B, Table 1). In addition, the ATP-induced reduction in ICI after PS treatment was significantly greater compared with the ICI changes after ATP infusion without PS infusion (Figure 1B, Table 1).

Effect of P2X receptor antagonist

PPADS, given intravenously before ATP infusion, antagonized the ATP-induced ICI reduction (Figure 1B, Table 1). ICI, MVP, PT and BP were not changed significantly, and the changes of ICI and PT were significantly smaller compared to those without the antagonist. However, 30 minutes after PPADS administration, ICI started gradually decreasing to reach the

similar level of those obtained without the antagonist. Another purinergic receptor antagonist, TNP-ATP, also suppressed detrusor overactivity induced by intravesical ATP (Figure 1B, Table 1). Intravesical ATP application after TNP-ATP decreased ICI to 75.5 ± 5.2%, however, the changes were significantly smaller compared to those without the antagonist, and the changes of PT was also significantly smaller compared to those without the antagonist (Table 1).

Effect of ATP in capsaicin pretreated rats

In capsaicin-pretreated rats, intravesical infusion of ATP solution following PS tended to decrease ICI (85.5 ± 6.1% of control) and PT (Figure 1B, Table 1), but without statistical significance. However, these changes were significantly smaller when compared to the ICI and PT reductions in normal rats. No responses were observed in the eye wiping test with capsaicin in these animals.

Effect of intravesical α,β-methylene ATP (pH 6.0) with or without PS treatment

α,β-methylene ATP induced a significant decrease in ICI without PS pre-infusion (ICI; 86.9 ± 3.9%, Table 2), and after intravesical infusion of PS, α,β-methylene ATP induced greater bladder overactivity by reducing ICI to 70.5 ± 4.8% (Table 2), which was significantly different from the ICI reduction by α,β-methylene ATP without PS pre-infusion (Figure 2, Table 2).

Effect of ATP after DIDS infusion

When DIDS, an ecto-ATPase inhibitor, was infused intravesically, MVP increased significantly, but ICI did not change. ATP solution with DIDS instilled intravesically after DIDS induced detrusor overactivity with a significant decrease in ICI to 84.0 ± 3.2% (Table 2).

This change of ICI was significantly greater than the change after ATP infusion without PS, but was significantly smaller than the ATP-induced ICI change seen after PS infusion (Figure 2).

Comment

The bladder permeability barrier is located in the apical membrane of superficial layer of epithelial cells, so-called umbrella cells[10]. Intravesical application of PS has been used to induce bladder epithelial injury[9], which permeabilize the apical membranes of theurothelium[9]. Acetate, propionate, butyrate or succinate salts at pH 4.4 alters transepithelial permeability of the rabbit urothelium, but not at pH 5.0[11], and in our study, ATP (pH 6.0) alone did not induce significant detrusor overactivity. It therefore seems likely that ATP in pH 6.0 solution cannot penetrate the intact bladder epithelial layer, and that increased permeability of bladder epithelium is needed for ATP to activate suburothelial afferent nerves. However, in previous studies, ATP infused intravesically at concentrations ranging 0.1-10mM induced detrusor overactivity[6,7]. Although the pH of ATP solutions was not described in these studies, ATP produces a very acidic solution ranging pH 3-4. Therefore, it is possible that acidic ATP solution can induce detrusor overactivity not only by ATP-induced afferent sensitization, but also by activation of acid-sensitive receptors and/or increased ATP penetration into the subepithelial layer due to the damage of the bladder epithelium or the increased amount of uncharged ATP in the acidic solution.

On the other hand, α,β-methylene ATP induced detrusor overactivity without PS treatment in our and other studies[6]. This may be explained by the difference of stability of ATP and α,β-methylene ATP. It has been reported that, in the extracellular space of the urothelium and smooth muscles, including detrusor, ATP is broken down by ecto-ATPase[12], but, in the

guinea-pig urinary bladder, the rate of degradation of α,β-methylene ATP by ecto-ATPase is less than 25% compared with ATP[12]. In our study, when DIDS, an inhibitor of ecto-ATPase[13], was infused intravesically, the following application of ATP (pH 6.0) induced detrusor overactivity without pre-infusion of PS. Rong *et al*. also showed that intravesical application of α,β-methylene ATP increased multifiber discharges in the mouse bladder, suggesting that α,β-methylene ATP can penetrate the urothelium[14]. Taken together, it is assumed that ATP (pH 6.0) with co-application of DIDS or α,β-methylene ATP can penetrate bladder epithelium to induce detrusor overactivity without changing urothelial permeability and that, in addition to urothelial barrier function, ecto-ATPase activity in the urothelium also contributes to the suppression of ATP-induced detrusor overactivity. In addition, since DIDS is also known to be an inhibitor of Cl^- transport[15], it is possible that DIDS-induced Cl^- transport inhibition might increase sensitization of C-fiber bladder afferents by ATP although further studies are needed to clarify this point.

In the rat, bladder sensory neurons express homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors[16], and $P2X_3$ staining has been detected in suburothelial afferent nerves of the wall of bladder and ureter[17]. Deletion of the $P2X_3$ gene results in urinary bladder hyporeflexia evidenced by increased voiding volume[18] and decreased bladder afferent sensitivity in response to increasing intravesical pressure[5]. Furthermore, deletion of the $P2X_2$ gene, and loss of heteromeric $P2X_{2/3}$ receptors, also results in a marked urinary bladder hyporeflexia due to increased thresholds for bladder contractions during bladder filling[19]. King *et al*. reported that,

the $P2X_3$ receptor blockade raised pressure and volume thresholds for the micturition reflex, whereas the $P2X_1$ receptor blockade diminished motor activity associated with voiding[20]. In our study, intravesical infusion of ATP after PS induced detrusor overactivity characterized by reductions in ICI and PT, but not MVP, which were antagonized by PPADS and TNP-ATP. PPADS is a selective P2X receptor antagonist[21], and TNP-ATP has been demonstrated to be a potent nanomolar antagonist for the recombinant rat $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptors[22]. Overall, these data are likely to indicate that intravesical application of ATP after PS treatment activated $P2X_3$ and $P2X_{2/3}$ receptors located on subepithelial nerve fibers which induce the reduction in ICI and PT although there is also the possibility that intravesical ATP may have its effects through stimulating the urothelial cells and increasing urothelial release of various mediators to indirectly activate subepithelial sensory nerves.

Afferent pathways innervating the rat urinary bladder consist of myelinated Aδ-fibers and unmyelinated C-fibers[23], and the majority of C-fiber bladder afferent neurons are capsaicin sensitive[24]. Yiangou *et al.* have also demonstrated the presence of TRPV1-IR and $P2X_3$-IR nerve fibers in rat bladder suburothelium[25]. In the present study, the reduction of ICI after intravesical ATP infusion in capsaicin-pretreated rats was significantly smaller compared with normal rats, indicating that the majority of $P2X_3$ and/or $P2X_{2/3}$ receptors activated by intravesical ATP are located on capsaicin-sensitive C-fiber afferents.

Recent studies demonstrated that bladder epithelium obtained from patients with IC can release higher amounts of ATP during stretch[3]. In addition, increased urothelial permeability

due to urothelial damage has been documented to be an important mechanism for inducing painful symptoms in IC[26]. It has also been reported that detrusor smooth muscles from patients with unstable or obstructed bladders had lower ATPase activity than stable bladders[27]. Thus, the damage of urothelial barrier function and/or decreased levels of ecto-ATPase activity could contribute to C-fiber desensitization by endogenous ATP released from bladder epithelium in patients with IC or bladder overactivity.

Conclusions

Intravesically applied ATP in pH 6.0 solution does not penetrate the intact bladder epithelial layer in the rat because of permeability barrier and ecto-ATPase of the urothelium. Therefore, increased permeability of bladder epithelium is needed for exogenously applied ATP to activate subepithelial P2X receptors in order to sensitize C-fiber afferents and induce detrusor overactivity.

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Legends

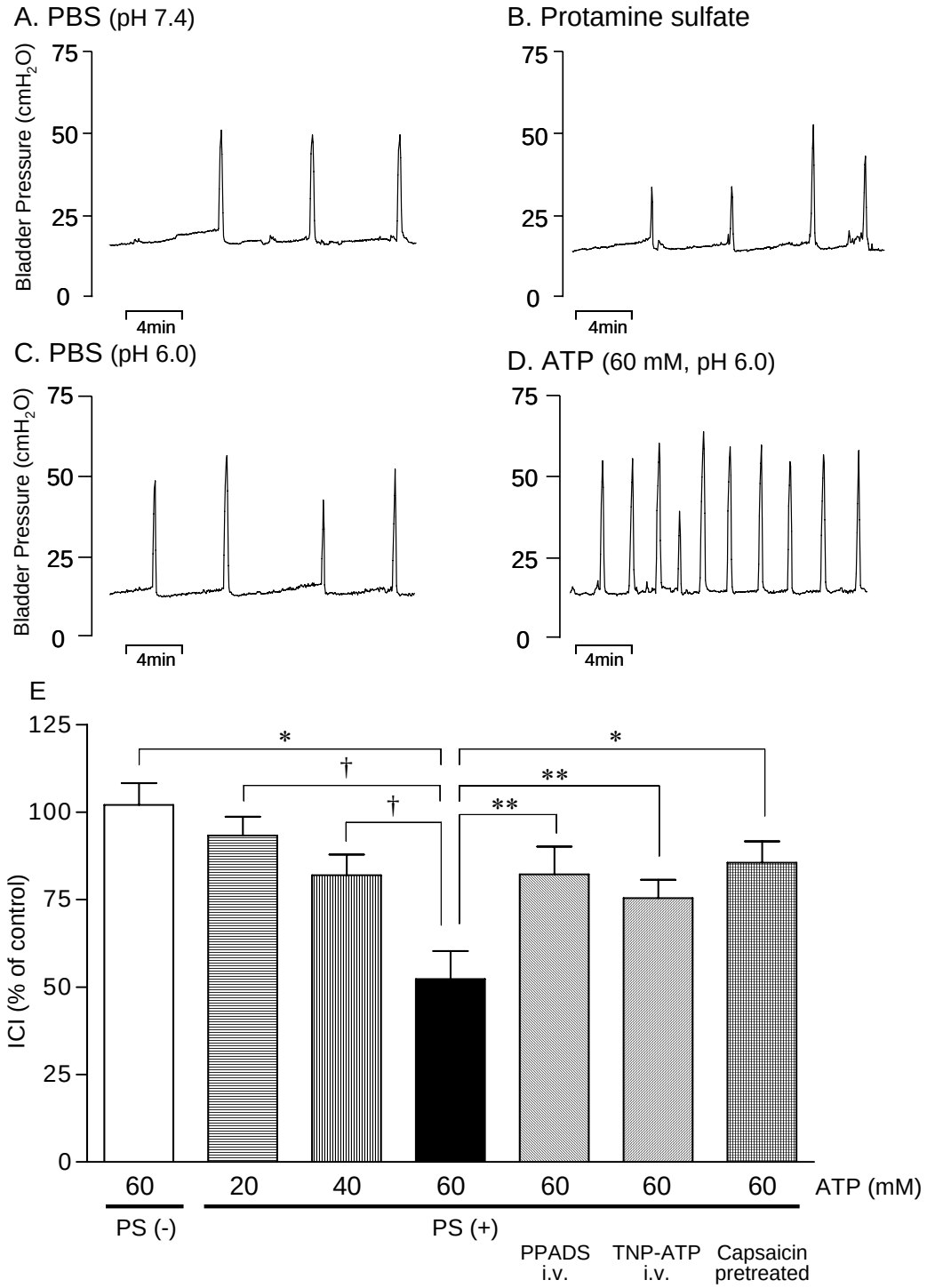
Figure 1: Effects of sequential intravesical application of PBS (pH 7.4), protamine sulfate (10 mg/ml), PBS (pH 6.0) and ATP (60 mM, pH 6.0) on bladder activity in conscious normal rats (A). Effects of intravesical instillation of ATP (60 mM without protamine sulfate pre-infusion, and 20, 40, 60 mM with protamine sulfate pre-infusion, and 60 mM with protamine sulfate after PPADS or TNP-ATP intravenous injection) on ICI expressed as % of the value during PBS (pH 6.0) infusion after protamine treatment in conscious normal and capsaicin pretreated rats (B). n = 7 (without protamine sulfate), n = 7 (20, 40, 60 mM), n = 5 (60 mM after PPADS i.v.), n = 6 (60mM after TNP-ATP i.v.), n = 6 (capsaicin pretreated rats). PS; protamine sulfate pre-infusion.

* $P < 0.01$, ** $P < 0.05$ (unpaired *t*-test), †$P < 0.01$ (Dunnett's multiple comparison test)

Figure 2: Comparison of the effects of intravesical instillation of ATP (60 mM, pH 6.0) with or without protamine sulfate (PS, 10 mg/ml) or DIDS (5 mM) (left), and the effects of α,β-methylene ATP (5 mM, pH 6.0) with or without protamine sulfate (right) on ICI under different conditions in conscious normal rats. Data are expressed as % of the value during PBS (pH 6.0) infusion. PS; protamine sulfate pre-infusion. Presence and absence of the pre-treatment with PS or DIDS are indicated by plus (+) and minus (-), respectively.

* $P < 0.01$, ** $P < 0.05$ (unpaired *t*-test)

* $P < 0.01$, ** $P < 0.05$ (unpaired *t*-test), †$P < 0.01$ (Dunnett's multiple comparison test)


A. PBS (pH 7.4)
B. Protamine sulfate
C. PBS (pH 6.0)
D. ATP (60 mM, pH 6.0)
Bladder Pressure (cmH2O)
75
50
25
0
4min
E
ICI (% of control)
125
100
75
50
25
0
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†
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60
20
40
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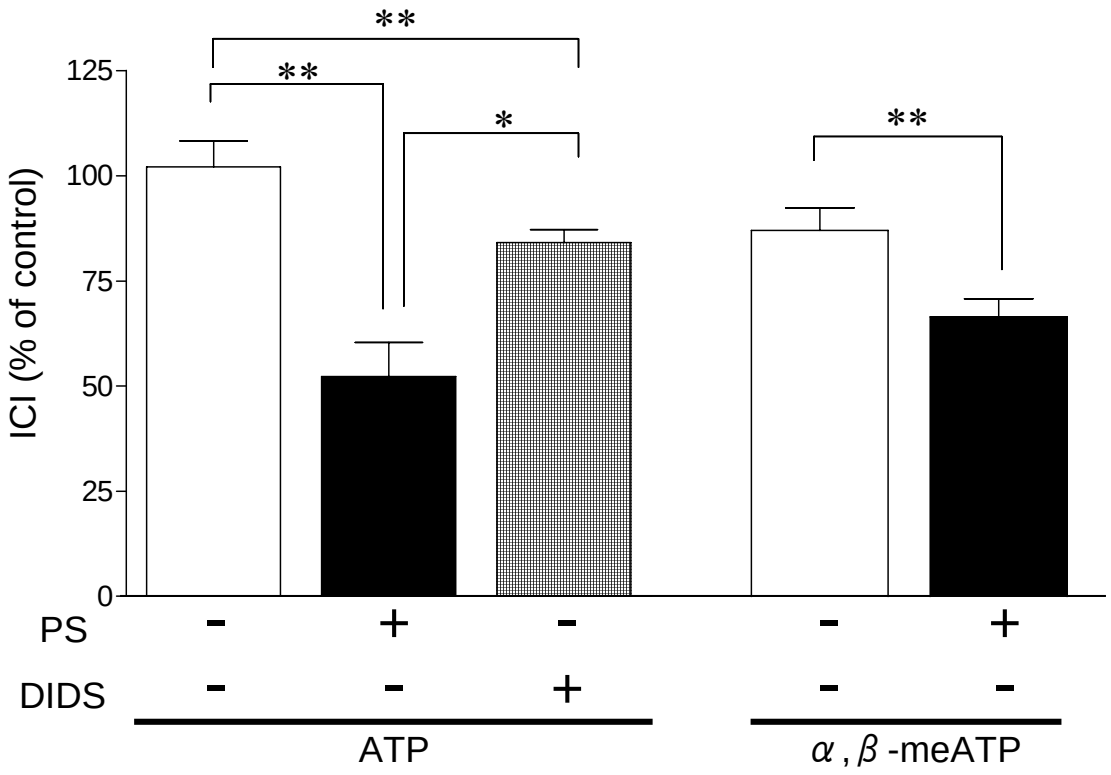
ATP (mM)
PS (-)
PS (+)
PPADS i.v.
TNP-ATP i.v.
Capsaicin pretreated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*
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125
100
75
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ICI (% of control)
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-
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ATP
α , β -meATP