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Original Article

Ectonucleotidases and purinergic receptors in mouse prostate gland

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Abstract: Objectives: Extracellular ATP/ADP and its metabolite adenosine play crucial roles in cellular signaling by interacting with P2 and P1/adenosine receptors. These signaling molecules are regulated by ectonucleotidases, which convert ATP/ADP into adenosine. While recent studies suggest impaired ATP hydrolysis in the aging prostate, the expression and function of ectonucleotidases and purinergic receptors in the prostate gland remain unclear. This study aims to characterize the expression patterns of purinergic enzymes and receptors in the mouse prostate and investigate their functional implications. Methods: Mouse prostate glands were isolated and analyzed using immunofluorescent staining and microscopy imaging with specific antibodies to detect purinergic enzymes and receptors. Functional studies were conducted to assess prostate smooth muscle contraction in response to purinergic agonists, particularly α,β-meATP and ATPγS. Results: Our analysis revealed distinct expression patterns of purinergic enzymes and receptors in the prostate: Ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1) and P2X1 receptors were predominantly localized in prostate smooth muscle cells, ENTPD2 and ecto-5'-nucleotidase (NT5E) in prostate interstitial cells, and alkaline phosphatase (ALPL) in prostate epithelial cells. Notably, ENTPD1 was identified as a key ectonucleotidase expressed in mouse prostate smooth muscle cells. Functionally, P2X1-mediated smooth muscle contraction was triggered by α,β-meATP. However, ATPγS induced contraction even after P2X1 desensitization, suggesting the involvement of additional P2Y receptors. Further analysis confirmed the presence of P2Y1, P2Y2, and P2Y11 receptors in mouse prostate smooth muscle, likely mediating the ATPγS-induced contraction. Conclusions: This study provides a comprehensive characterization of purinergic signaling components in the mouse prostate. The identification of ENTPD1 in smooth muscle cells and the functional role of multiple P2Y receptors in smooth muscle contraction highlight potential regulatory mechanisms of prostate function. These findings lay the groundwork for future research on purinergic signaling in prostate physiology and its potential implications in age-related dysfunction, both in rodents and humans.

Keywords: Purinergic signaling, smooth muscle contractility, P2Y receptor, alkaline phosphatase, mouse prostate

Introduction

The prostate gland, a male organ composed of tubular structures, plays a crucial role in fertility. Luminal epithelial cells secrete fluids that mix with sperm to create semen, while stromal smooth muscle cells contract forcefully to expel semen into the urethra during ejaculation [1]. Dysregulation of prostate function can lead to benign prostatic hyperplasia (BPH), prostatitis, and even cancer, which are significant morbidities in the aging population [2]. For instance, BPH affects 50% of men over 50 and >80% of men over 70. Despite these high prevalence rates, the molecular physiology of the prostate and the pathogenesis of its disorders remain incompletely understood, highlighting the need for deeper investigation into the underlying mechanisms to develop better therapeutic strategies [3].

Purinergic signaling plays numerous important roles, including in inflammation and immune responses, muscle contractility, gluconeogenesis and metabolism, cell proliferation and differentiation, tumor metastasis, and cancer therapy [4]. This signaling comprises a dynamic and complex interactive network. Cell-released signaling molecules like ATP and ADP bind to P2 purinergic receptors of the P2X (1-7) and P2Y (1, 2, 4, 6, 11-14) families, initiating downstream signaling. ATP and ADP are further

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converted into adenosine by enzymes. Ectonucleoside triphosphate diphosphohydrolases (ENTPD1, 2, 3, 8), a group of cell surface ectonucleotidases sequentially convert extracellular ATP to ADP and then to AMP, while ecto-5'-nucleotidase (NT5E) and alkaline phosphatase (ALPL) convert AMP to adenosine. Adenosine binds to P1 or adenosine receptors (A1, A2a, A2b, A3) [5].

Physiologically, purinergic contractility accounts for part of nerve-mediated prostate contraction, although the detailed mechanism of how this signaling network impacts prostate gland function is not fully understood [6-10]. However, abnormal ATP levels and purinergic receptor function have been reported in both animal models and human patients with prostate dysfunctions. For example, in rodent models of prostatitis, elevated levels of P2X2, P2X3, and P2X7 might mediate prostatic pain sensation [11, 12]. Increased expression of P2X1 in aged mice could cause an increase in prostate muscle tone [9, 13]. In human BPH patients, increased ATP release and impaired ATP hydrolysis have been observed, along with a significant reduction in P2X2 and P2X3 proteins [13]. These studies suggest that purinergic signaling in the prostate gland plays an important role in prostate physiology and pathophysiology.

Over the decades, our group investigates molecular pathways leading to BPH, focusing on the regulation of steroid 5-alpha reductase in androgen and prostate growth [14-18]. In this study, we broaden our research by determining various purine-converting enzymes and purinergic receptors in the mouse prostate gland. We were the first to identify that ENTPD1, the primary enzyme responsible for converting ATP and ADP to AMP, is predominantly expressed in mouse prostate smooth muscle cells. Additionally, we discovered a novel purinergic contractility in the smooth muscle of the mouse prostate, potentially mediated by P2Y receptor(s). These findings provide a foundation for further mechanistic insights into how purinergic signaling regulates prostate function and dysfunction.

Materials and method

Materials

Unless otherwise specified, all chemicals were obtained from Sigma (St. Louis, MO) and were of reagent grade or higher. α,β-meATP (Cat. #: 3209) and NF 546 (Cat. #: 3892) were purchased from R&D system (Minneapolis, MN, USA). ATPγS (Cat. #: NU-406) and ADPβS (Cat. #: NU-433) were obtained from Jena Bioscience (Thuringia, Germany).

Animals

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) aged 12-16 weeks old were used in this study. All animal studies were performed in adherence with U.S. National Institutes of Health guidelines for animal care and use and with the approval of the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee (Protocol #: 2022-049). Mice were housed in standard polycarbonate cages and maintained on a 12:12-h light-dark cycle at 25°C with free access to food and water.

Isolation of mouse prostate glands

A midline abdomen incision was made to expose and remove the entire prostate along with the seminal vesicle, bladder, urethra, and vas deferens. The tissue was placed in a petri dish with PBS solution, and the prostate was carefully dissected out under an Olympus dissecting microscope. The isolated prostate tissue was then used for either myography study or immunofluorescence staining.

Immunofluorescence staining and microscopy imaging

The isolated whole prostate gland was fixed in 4% (wt/vol) paraformaldehyde for 2 hours at room temperature. The fixed tissue was then treated with 30% (w/v) sucrose solution for another 2 hours, cryoprotected, and frozen in OCT compound at -80°C. The tissue was sectioned (5 μm) and incubated with primary antibodies (1:100) overnight at 4°C. Depending on the number and species of the primary antibodies, the sections were then incubated with Alexa Fluor 488- and/or Alexa Fluor 555-conjugated secondary antibodies (diluted 1:100), and nuclei were stained with DAPI. Imaging was performed on an Olympus BX60 fluorescence microscope with a 40×/0.75 objective. Images (512 and 512 pixels) were saved as TIFF files and imported into Adobe Illustrator 28.1. In this study, each prostate tissue was sectioned to obtain slides with approximately four sections of tissue per slide. Each antibody staining was

Table 1. List of antibodies and dyes used for immunofluorescent staining

Antibody or dye	Catalog #	Host animal	Company
ENTPD1	AF4398	Sheep	R&D System
P2X1	APR-001	Rabbit	Alomone Lab
ENTPD2	AF5797	Sheep	R&D System
CD34	AB8158	Rat	ABCAM
ENTPD3	AF4464	Sheep	R&D System
NT5E	AF4488	Sheep	R&D System
ALPL	AF2910	Goat	R&D System
P2Y1	H120	Rabbit	Santa Cruz
P2Y2	AB10270	Rabbit	ABCAM
P2Y11	A24482	Rabbit	ABclonal
Alexa 488 Donkey anti-Sheep	A-11015	Donkey	ThermoFisher
Alexa 488 Donkey anti-Rabbit	A-21206	Donkey	ThermoFisher
Alexa 555 Donkey anti-Rat	A-78945	Donkey	ThermoFisher
Alexa 488 Donkey anti-Goat	A-11055	Donkey	ThermoFisher
Rhodamine Phalloidin	PHDR1		Cytoskeleton
Antifade mounting medium with DAPI	H-2000-10		Vector Laboratories

repeated at least twice to ensure consistency of the staining results.

Antibodies

Detailed information on antibodies and dyes used for immunofluorescent staining is listed in **Table 1**.

Myography

Each male mouse has two long anterior prostate lobes. A longitudinal midline cut of the prostate yields two tissue preparations for myograph studies, with one suture tied to the tip of the anterior prostate lobe and the other to the dorsal lobe end. Prostate strips were then mounted in an SI-MB4 tissue bath system (World Precision Instruments, Sarasota, FL, USA). Force sensors were connected to a TBM 4M transbridge (World Precision Instruments), and the signal was amplified by a PowerLab (AD Instruments, Colorado Springs, CO, USA) and monitored through Chart software (AD Instruments). Prostate strips were gently stretched to optimize contraction force, then pre-equilibrated for at least 1 h. All experiments were conducted at 37°C in physiological saline solution (in mM: 120 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 15.5 $NaHCO_3$, 1.2 NaH_2PO_4, 11.5 glucose, and 2.5 mM $CaCl_2$) with continuous bubbling of 95% O_2/5% CO_2. Contraction force was sampled at 2000/s using Chart software. Prostate strips were treated with agonists or antagonists, and/or subjected to electrical field stimulation (EFS).

Electrical field stimulation

Prostate strip EFS was carried out by a Grass S48 field stimulator (Grass Technologies, RI, USA) using standard protocols previously described: voltage 50 V, duration 0.05 ms, trains of stimuli 3 s, and frequencies 20, and 50 Hz [19, 20].

Statistical analyses

All data are expressed as means ± SD or presented as box-and-whisker plots, with whiskers extending from the minimum to maximum values. Statistical analysis was performed using Student's t-test to compare different stimulations against the non-stimulated baseline. A *p*-value of <0.05 was considered statistically significant.

Results

ENTPD1 and P2X1 receptors are predominantly expressed in prostate smooth muscle cells

ENTPD1 is a major enzyme that converts ATP and ADP to AMP. Immunostaining and imaging demonstrated the expression of ENTPD1 in the prostate gland (**Figure 1A**). By counterstaining the actin filament with rhodamine-phalloidin

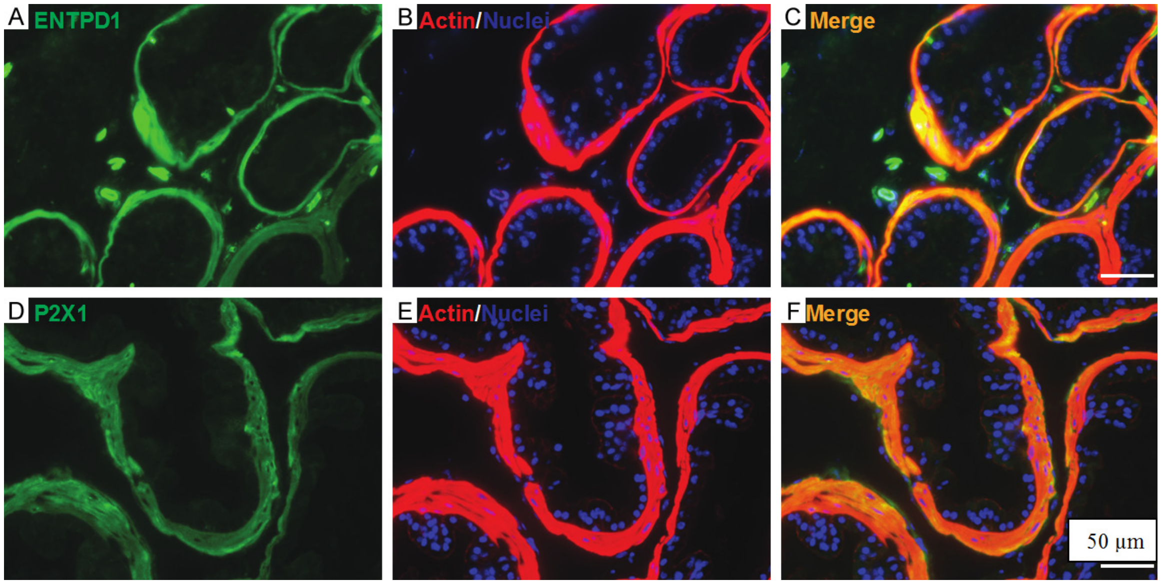

Figure 1. Expression of Ectonucleoside triphosphate diphosphohydrolases 1 (ENTPD1) and P2X1 receptors in the smooth muscle of mouse prostate. Mouse prostate tissue was labeled with specific anti-ENTPD1 and anti-P2X1 (green in D) antibodies. (A-C) ENTPD1 Expression: (A) Green fluorescence indicates ENTPD1 localization. (B) Red fluorescence marks Actin (smooth muscle), while blue fluorescence (DAPI) labels nuclei. (C) Merge: ENTPD1 appears primarily in smooth muscle regions, overlapping with actin signals. (D-F) P2X1 Expression: (D) Green fluorescence represents P2X1 receptor localization. (E) Red fluorescence for Actin, and blue for nuclei (DAPI). (F) Merge: P2X1 is predominantly expressed in the smooth muscle layer, colocalizing with actin. Scale bar, 50 μm. These representative images were obtained from a minimum of three mice.

and nuclei with DAPI, we identify its cellular localization clearly. As shown in **Figure 1B**, rhodamine-phalloidin binds to actin-rich smooth muscle cells, indicated by a strong bright red signal. In contrast, epithelial cells, aligned inside the muscle bundles, exhibit relatively large, round nuclei with faint red actin signaling outlining their membranes. ENTPD1 expression is predominantly found in the smooth muscle bundles of the prostate gland. It is also strongly expressed in a subset of cells in the interstitial space, likely the endothelial cells of the vasculature, as previously reported in other systems [21, 22]. The P2X1 receptor, an ATP-activated cation channel, is solely expressed in smooth muscle cells in the prostate gland, consistent with previous reports that the P2X1 receptor mediates prostate contractility [7-9]. The key innovation in our report is the identification of ENTPD1 as predominantly expressed in mouse prostate smooth muscle cells.

ENTPD2 but not ENTPD3 is expressed in prostate interstitial cells

ENTPD2 is another major purine-converting enzyme located on the extracellular surface. As shown in **Figure 2A-C**, ENTPD2 expression in the prostate gland is not colocalized with smooth muscle cells or epithelial cells. Instead, it is found in cells with long, thin filaments adjacent to the smooth muscles. To identity these cells, we labeled them with the interstitial cell marker CD34. **Figure 2D-F** demonstrate that ENTPD2 is nicely colocalized with CD34 signaling, indicating that these ENTPD2-positive cells are interstitial cells in the prostate gland.

ENTPD3, another enzyme with a similar function, is expressed in the basolateral membrane of bladder epithelial cells (**Figure 3D-F**). However, ENTPD3 expression was not detected in any cell type within the prostate gland (**Figure 3A-C**).

NT5E and ALPL are differentially expressed in the prostate gland

NT5E is an enzyme that specifically converts AMP to adenosine, while ALPL is a non-specific enzyme converting ATP/ADP to AMP and adenosine. Interestingly, NT5E is only present in cells morphologically similar to ENTPD2-positive cells, but not in smooth muscle or epithe-

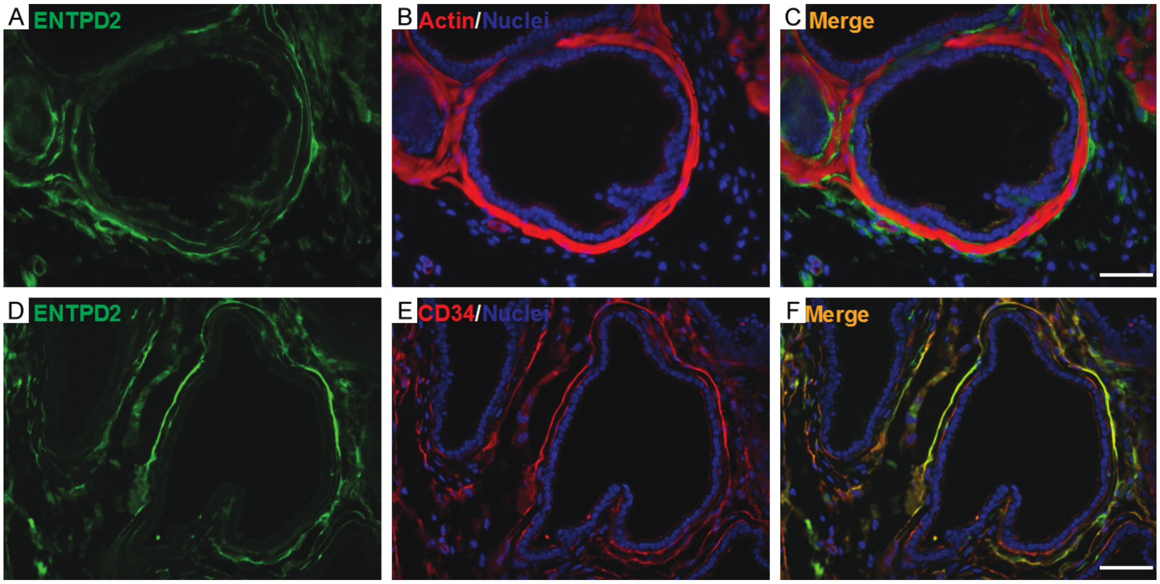

Figure 2. Expression of ENTPD2 in mouse prostate interstitial cells. Mouse prostate tissue was labeled with specific anti-ENTPD2 (green in A and D) antibody. (A-C) ENTPD2 and Actin Expression: (A) Green fluorescence shows ENTPD2 expression. (B) Red fluorescence marks Actin, highlighting smooth muscle structures, while blue (DAPI) labels nuclei. (C) Merge: ENTPD2 appears primarily in interstitial regions surrounding the smooth muscle layer, with minimal colocalization with actin. (D-F) ENTPD2 and CD34 Expression: (D) Green fluorescence represents ENTPD2 localization. (E) Red fluorescence marks CD34, a marker for interstitial cells, while blue (DAPI) stains nuclei. (F) Merge: ENTPD2 partially colocalizes with CD34-positive interstitial cells. Scale bar: 50 μm.

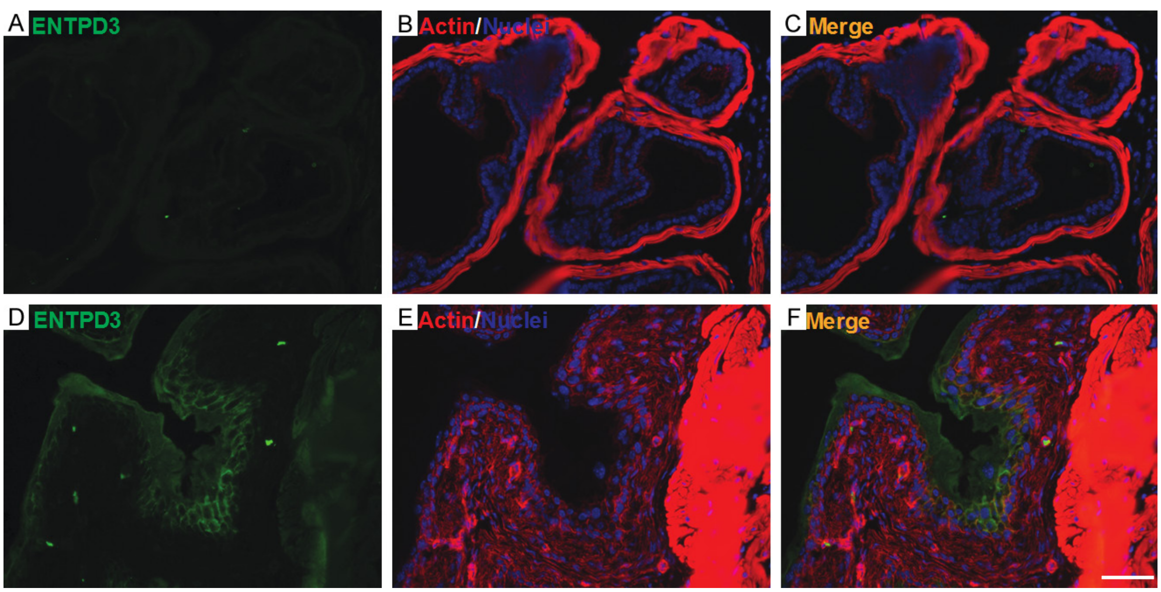

Figure 3. Absence of ENTPD3 in mouse prostate gland. Mouse prostate (A-C) and bladder (D-F) tissues were labeled with specific anti-ENTPD3 antibody (green in A and D). (A) ENTPD3 was not observed in prostate epithelial cells. (B) Red fluorescence marks Actin, primarily labeling smooth muscle structures, while blue fluorescence (DAPI) highlights nuclei. (C) Merged image of ENTPD3 and Actin. (D) ENTPD3 expression in the bladder tissue, localized on the basolateral membrane of bladder urothelial cells (serving as a positive control). (E) Red fluorescence marks Actin. (F) Merged image of ENTPD3 and Actin. Scale bar: 50 μm.

lial cells (**Figure 4A-C**), indicating its expression in prostate interstitial cells. In contrast, ALPL is strongly expressed in the prostate epithelial cell membrane, particularly on the apical mem-

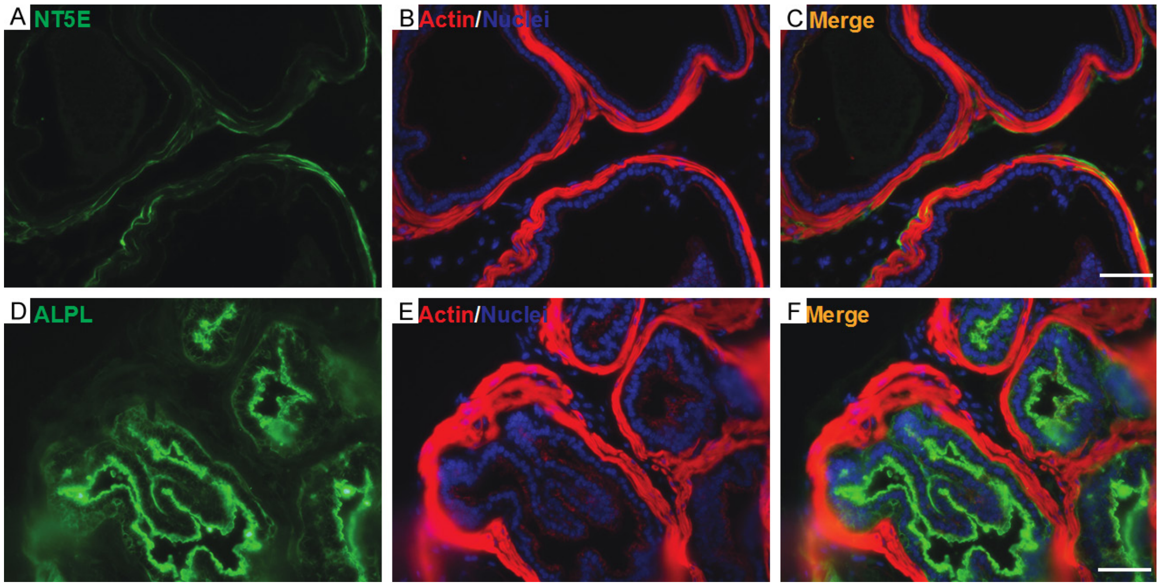

Figure 4. Expression of NT5E (ecto-5'-nucleotidase) and ALPL (alkaline phosphatase) in the prostate gland. A-C. NT5E Expression in Mouse Prostate. A. Green fluorescence represents NT5E expression. B. Red fluorescence labels Actin, primarily marking smooth muscle cells, while blue fluorescence (DAPI) highlights nuclei. C. Merge: NT5E signaling did not colocalize with prostate smooth muscle but mimicked the ENTPD2 expression pattern, indicating localization in interstitial cells. D-F. ALPL Expression in Mouse Prostate. D. Green fluorescence shows ALPL expression. E. Red fluorescence labels Actin. F. Merge: ALPL is highly expressed in the prostate epithelial cell membrane, particularly the apical membrane facing the lumen. Scale bar: 50 μm.

brane surface (**Figure 4D-F**), suggesting an important role for ALPL in converting luminal ATP/ADP molecules into adenosine in the prostate gland.

The mouse prostate gland exhibits purinergic contractility

We performed myography studies to determine whether the mouse prostate can respond to purinergic stimulation. As shown in **Figure 5A**, **5E**, the mouse prostate generates a significant contraction force in response to 100 mM KCl-induced depolarization, with peak force reaching approximately 10 mN. The mouse prostate also produces significant contraction force in response to electrical field stimulation (**Figure 5B**, **5E**). The addition of 10 μM α,β-meATP induced a noticeable contraction that decayed quickly (**Figure 5C**, **5E**). This rapid decay is due to the activation and desensitization of P2X1 receptors in smooth muscle cells, a well-known characteristic of the P2X1 receptor [23]. However, after 10-15 minutes of α,β-meATP-induced desensitization, the subsequent addition of 25 μM ATPγS generated another noticeable contraction force (**Figure 5D**, **5E**), suggesting that different receptor(s) mediate this purinergic contractility.

In addition to activating P2X receptors, ATP also activates P2Y1, P2Y2, and P2Y11 receptors [24]. We tested NF546, a selective agonist for the P2Y11 receptor, but 15 μM NF546 produced no measurable response. We also tested the involvement of ADP-activated P2Y receptors by stimulating the prostate tissue with ADPβS, which yielded no observable contraction force (**Figure 5E**).

P2Y receptors are potential candidate targets for novel purinergic contractile force

The observation of a novel ATPγS-induced contraction force suggests the potential presence of P2Y1, P2Y2, and P2Y11 receptors in the prostate gland (**Figure 5**). Consequently, we performed further immunofluorescent localization studies. Interestingly, all three ATP-activated P2Y receptors were detected in the prostate gland, specifically in the prostate smooth muscle cells (**Figure 6A-I**). This supports their potential role in mediating prostate smooth muscle contractility.

Discussion

ATP is a well-characterized neurotransmitter but is also released from tissues due to

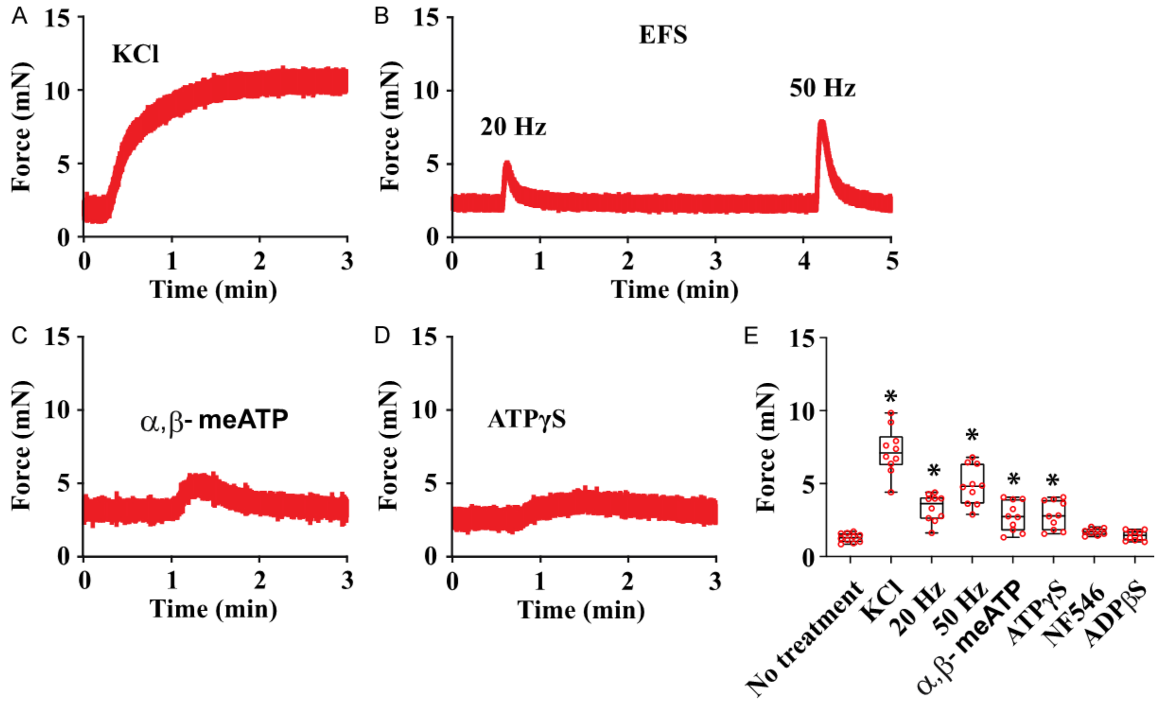

Figure 5. Purinergic contraction forces in mouse prostate tissue. (A-D) Representative traces of prostate smooth muscle contraction from male mice (n=8-10 tissue preparations) in response to various stimuli: KCl depolarization (A), electrical field stimulation (EFS) (B), P2X receptor agonist α,β-meATP (C), and P2 receptor agonist ATPγS (D). (E) Summary data of purinergic contraction forces presented as boxes-and-whiskers. The centerline represents the median, the box encompasses the interquartile range (IQR, 25th to 75th percentile), and the whiskers extend from the minimum to the maximum values. Data were analyzed using the Student's *t*-test. *P<0.05.

mechanical stress, injury, or noxious stimuli. Excessive extracellular ATP can cause abnormal pain sensation, elevated muscle tone, and even tissue damage. Therefore, extracellular purine kinetics must be tightly regulated to maintain proper cellular functions [4]. For example, neuronally released ATP needs to be quickly recycled or degraded to avoid continuous activation of corresponding P2 receptors. This is achieved by cell surface purine-converting enzymes. Decreased purine hydrolysis activity has been reported in the prostates of aged mouse and guinea pig [9, 10].

In the intestinal mucosa, the expression of ENTPD1 and ENTPD2 is cell-selective, playing a crucial role in regulating gut motility, epithelial barrier function, and neuromuscular transmission [25]. Similarly, our study revealed distinct expression patterns of purine-converting enzymes across the three major types of prostate cells (**Figures 1**, **2**, and **4**). Previous studies have shown that ENTPD1 mediates smooth muscle contraction in the mouse bladder [26]. Consistent with this, we observed abundant ENTPD1 expression in mouse prostate smooth muscle tissue. However, in contrast to the bladder, these cells lack the enzyme necessary to convert AMP to adenosine, suggesting tissue-specific differences in purinergic signaling. Additionally, prostate interstitial cells express both ENTPD2 and NT5e, which facilitate the conversion of ATP/ADP to AMP and adenosine. This finding aligns with studies in vascular smooth muscle cells, where ENTPD2 and NT5e have been identified as key regulators of extracellular nucleotide metabolism, highlighting a conserved role of these ectonucleotidases in regulating nucleotide levels across various tissues [27]. Prostate epithelial cells exhibit strong ALPL expression, with notably high apical localization, suggesting a role in regulating lumen ATP concentration within the prostate gland. By mediating extracellular nucleotide hydrolysis, ALPL contributes to various physiological functions [28]. Furthermore, in prostate cancer, epithelial-derived ALPL plays a crucial role in controlling cell death and epithelial plasticity [29], highlighting its potential significance in disease progression. The functionality of

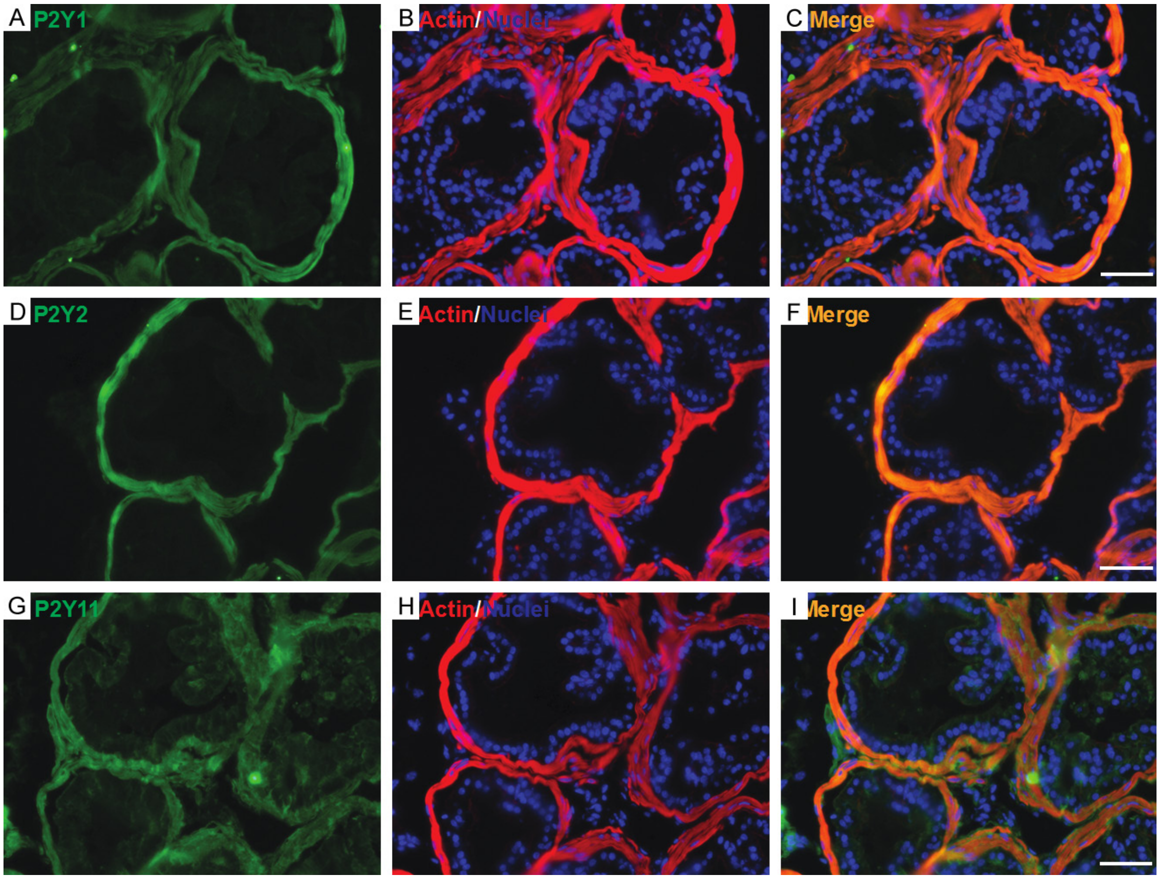

Figure 6. Expression of ATP-sensitive P2Y receptors in mouse prostate smooth muscle. (A, D, G): Mouse prostate tissues labeled with specific antibodies against P2Y1 (green in A), P2Y2 (green in D), and P2Y11 (green in G) receptors. (B, E, H): Prostate smooth muscle labeled with a red marker. (C, F, I): Merged images showing colocalization of P2Y receptors (green) with prostate smooth muscle (red), resulting in yellow signals. Nuclei are stained blue with DAPI. Scale bar: 50 μm.

these differential expression patterns of purine-converting enzymes remains unclear but suggests an important role in prostate function.

There are very few reports on mouse prostate smooth muscle contractile function [9, 30]. Unlike the gastrointestinal or urinary bladder smooth muscle, which aligns longitudinally or circularly to allows optimized stretching of isolated muscle strips, prostate smooth muscle displays an irregular alignment around the glands. This anisotropy makes it impossible to stretch prostate smooth muscle on a tissue holder. Indeed, when prostate tissue was stretched, the tension increased quickly and did not decay significantly over time (data not shown). In contrast, to the tension in gastrointestinal or urinary bladder smooth muscle tissue increases slowly and decays over time when stretched.

It is well known that smooth muscle stretch or length is a critical factor in generating maximal contraction force [31], while muscle with un-optimized length generates less force. Based on this theory, the contractile force of prostate muscle might be significantly smaller than that of gastrointestinal or urinary bladder smooth muscle tissue. This seems to be true, as the force generated by mouse prostate tissue in our study was significantly less than the reported forces generated by smooth muscle tissue in other systems [20, 32]. Nonetheless, we detected significant mouse prostate contraction in our experimental setting, with maximal force reaching approximately 10 mN in response to 100 mM KCl stimulation (**Figure 5A**).

The significance of purinergic contraction force in prostate smooth muscle remains debated in both rodents and humans. However, it is generally accepted that the P2X1 receptor is present in prostate smooth muscle and mediates ATP-induced contraction [6-10]. We have confirmed its presence in mouse prostate smooth muscle and demonstrate a noticeable contractile function in response to agonist 10 μM α,β-meATP (**Figures 1D-F**, **5C**). Interestingly, after P2X1 desensitization, a small but clear contraction force occurs in response to ATPγS, but not ADPβS (**Figure 5D**, **5E**). This led us to identify multiple P2Y receptors in mouse prostate smooth muscle (**Figure 6**).

P2Y1 and P2Y2 receptors are coupled with Gq protein, which can activate phospholipase C and inositol triphosphate pathways, leading to smooth muscle contraction in the esophagus [33, 34]. Whether P2Y1 and P2Y2 receptors in mouse prostate smooth muscle have a similar function requires further investigation.

Smooth muscle plays an important role in prostate function by expelling seminal fluid. It is also an important drug target in BPH treatment by blocking the adrenergic alpha1 receptor [35], which relaxes prostate smooth muscle tone. The increase in extracellular ATP and the decline in purine-converting enzyme activity in the aging prostate suggest a potential novel therapeutic approach for BPH [13]. Further studies are needed to determine whether these purine-converting enzymes are also present in the human prostate and whether these novel P2Y receptors exist and are functional in both mouse and human prostates.

In summary, we have identified multiple purine-converting enzymes in mouse prostate glands with differential localizations. Additionally, we have identified P2Y receptors in mouse smooth muscle that potentially mediate purinergic contractility. The presence of these molecules suggests that purinergic signaling may play a role in various prostate functions and dysfunctions, including inflammation, proliferation, metabolism, and muscle contraction.

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Disclosure of conflict of interest

None.

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