P2X1 receptors localized in lipid rafts mediate ATP motor responses in the human vas deferens longitudinal muscles

Running Title: P2X1Rs in human vas deferens localize in rafts

Summary sentence: ATP elicits contractions of human vas deferens longitudinal muscles mediated by P2X1 receptors distributed in raft domains.

Key words: P2X1 receptors, P2X receptor desensitization, human vas deferens, sympathetic co-transmission, ATP-induced contractility, P2X1 receptors in lipid rafts.

Verónica Donoso,3,4 Andrés Norambuena,3 Camilo Navarrete,3 Inés Poblete,3 Alfredo Velasco,5 and Juan Pablo Huidobro-Toro2,3,4

3Laboratorio de Nucleótidos, Departamento de Fisiología, Facultad de Ciencias Biológicas, P. Universidad Católica de Chile y, Santiago, Chile
4Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile
5Departamento de Urología, Clínica Santa María, Santiago, Providencia, Chile

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2Correspondence: J. Pablo Huidobro-Toro, Laboratorio de Nucleótidos, Departamento de Fisiología, Facultad de Ciencias Biológicas, P. Universidad Católica de Chile, Casilla 114-D, Santiago, Chile. E-mail: jphuidobro@bio.puc.cl

ABSTRACT
To assess the role of the P2X1 receptors (P2X1R) in the longitudinal and circular layers of the human vas deferens, ex-vivo isolated strips or rings from tissue biopsies were prepared to record isometric contractions. To ascertain its membrane distribution, tissue extracts were analyzed by immunoblots following sucrose gradient ultracentrifugation. ATP, alpha,beta-methylene ATP or electrical field stimulation elicited robust contractions of the longitudinal layer, but not of the circular layer which evidenced inconsistent responses. Alpha,beta-methylene ATP generated stronger and more robust contractions than ATP. In parallel, prostatic segments of the rat vas deferens were examined. The motor responses in both species were not sustained but decayed within the first min, showing desensitization to additional applications. Cross desensitization was established between alpha,beta-methylene ATP or ATP-evoked contractions and electrical field stimulation-induced contractions. Full recovery of the desensitized motor responses required more than 30 min and showed a similar pattern in human and rat tissues. Immuno blot analysis of the human vas deferens extracts revealed a P2X1R oligomer of approximately 200 kDa under non reducing conditions, whereas dithiothreitol-treated extracts showed a single band of approximately 70 kDa. The P2X1R was identified in ultracentrifugation fractions containing 15-29 % sucrose; the receptor localized in the same fractions as flotillin-1, indicating that it regionalized into smooth muscle lipid rafts. In conclusion, ATP plays a key role in the human vas deferens contractile responses of the longitudinal smooth muscle layer, an effect mediated through P2X1Rs.

INTRODUCTION
Sympathetic nerve endings comprise a dense network around the smooth muscle layers of the vas deferens. The stimulation of these peripheral nerve terminals evokes the release of adenosine
5′-triphosphate (ATP), noradrenaline (NA), neuropeptide Y, along with β-nicotinamide adenine dinucleotide [1,2]. These sympathetic co-transmitters are released by exocytosis from the sympathetic nerve varicosities [2,3]. The electrically evoked contractions elicited by nerve ending stimulation are biphasic; the excitatory junctional potential, due mainly to ATP in rodent models, accounts for the phasic component whereas the tonic phase is due essentially to NA activity [4,5]. Several α1-adrenoceptor subtypes contribute to the tonic muscular component [6]. Whereas the rodent vas deferens has been successfully used as a model to study different aspects of sympathetic co-transmission, little is known about the human tissue sympathetic co-transmission. The functional role of ATP as a co-transmitter in human vas deferens transmission remains unclear. Reports, based on studies performed mainly on the circular layer of the tissue discarded the role of ATP since the contractions in response to nerve stimulation were antagonized by α1-adrenoceptor antagonists, suggesting the lack of a purinergic component in the human vas deferens co-transmission [7-10]. Moreover, Banks et al [11] showed evidence favoring the involvement of P2X1 receptors (P2X1R) and α1-adrenoceptors in the motor contractions of the human vas deferens, although no dissection of the tissue muscle layers was achieved. More recently Amobi et al [12] addressed for the first time the functional role of ATP in both the circular and longitudinal layers of the human vas deferens motor activity. They demonstrated the prominent role of the purinergic motor component in the longitudinal layer of the human vas deferens. However, at present, no data is available about the cell membrane distribution of the P2X1R in this tissue; whilst studies using cell lines have shown that the P2X1R is not homogeneously distributed along the cell membrane surface [13], but regionalized into lipid rafts. These microdomains establish a discrete and dynamic assembly of cholesterol, glicosphingolipids and other membrane lipids and structural proteins such as caveolin and flotilin that might be relevant for its functional activity. Understanding the localization of the P2X1R in the plasma membrane is significant since rafts are commonly associated with signaling platforms, consonant with the role of ATP as a sympathetic co-transmitter acting in conjunction with α1-adrenoceptors which couple to G trimeric proteins, enriched in raft domains.

α,β-methylene ATP (α,β-mATP) is a preferred and potent P2X1R ligand, which is relatively resistant to ATPases activity. A single or repeated exposure(s) to ATP or α,β-mATP, causes within minute, or less, loss of sensitivity to further purinergic applications; this condition is referred to as desensitization [14], which is particularly prominent for the P2X1 or P2X3Rs mediated responses in almost every cell type examined. Considering the highest affinity of α,β-mATP for the P2X1R, and that it causes a fast and more profound desensitization than ATP, the latter agonist has been classically used as a pharmacological tool to describe P2X1R-mediated responses [14, 15], not under estimating that α,β-mATP may interact, albeit with reduced affinity, with purinoceptors other than the P2X1R subtype.

To test the hypothesis that ATP through P2X1R activation is an endogenous transmitter of the human vas deferens motor responses, we used P2X1R desensitization as a pharmacological tool to identify the role of the P2X1R in motor responses in both the longitudinal and circular layers of the human vas deferens. In addition, we also hypothesized that the P2X1R is not homogeneously distributed in the smooth muscle cell membranes of this tissue, but micro regionalized into lipid rafts, a plasma membrane subdomain containing key proteins involved in intracellular cascades relevant to the maintenance of the tissue motor tone. To these aims we recorded the tension evoked by either exogenous ATP applications or that elicited by electrical
field stimulation (EFS) of the tissue nerve endings and evaluated the role of purinergic mechanisms in the human tissue motor responses. Moreover, by means of selective antibodies and electrophoresis separation, we identified the P2X1R in the human tissue, consistent with its first cloning from the rat tissue [16]. Using sucrose gradient ultracentrifugation, we ascertained the putative micro regionalization of the P2X1R in the plasma cell membrane of smooth muscles from the human vas deferens. In sum, the present results highlight the role of ATP and its P2X1Rs in the human vas deferens motor activity particularly in the tissue longitudinal smooth muscle layer.

**MATERIAL AND METHODS**

*Collection of human and rat tissues*

Ethical approval and written informed consent from all patients for the use of tissues for laboratory research was obtained prior to hospitalization. Approval from the Ethical Committee of Clínica Santa María for investigations using human biopsies was obtained prior to initiating the project. Experiments were performed in agreement with the norms and rules of the Helsinki Declaration. Nineteen out of the 23 human vas deferens biopsies were obtained from healthy fertile men undergoing elective vasectomies (males aged 28-45 years), 4 tissues were from prostatectomy patients. The vasectomy protocol included extraction of an excised short segment; coagulation and ligation of both ends [17, 18]. Four sample specimens were from patients programmed for surgery due to prostatic pathology (the age of these patients ranged from 55-68 and averaged 60 years).

Biopsies were transported to the laboratory in sterile saline generally within one hour or less after surgery. In the lab, tissues were immediately immersed Tyrode buffer gassed with carbogen mixture (95% O₂/5% CO₂), specimens were dissected free of connective tissue/blood vessels and strips (longitudinal muscle preparations, aprox 25 mm long) or rings (circular layer muscles, 3-5 mm width) were mounted in the bath chambers for muscular bioassays. Two segments from a same biopsy (initially a longitudinal segment and a ring, but later we used only tissue strips) were mounted in a doubled jacketed 7 ml organ bath, maintained with Tyrode buffer at 37°C. The tissues were incubated in buffer gassed with carbogen mixture to maintain Tyrode at pH 7.4 and provide rapid mixing of the drugs once applied to the tissue bath. The motor responses were recorded by means of force displacement transducers, connected to a Grass model 79D Polygraph Instrument; the contractile responses were displayed in recording paper. The tissues were equilibrated at 1.5 g of basal tension for an hour prior to initiating the experimental protocols; during this period, buffer was changed every 15 min and tension was checked to assure the basal smooth muscle tension was maintained throughout. The bath was a conventional organ bath chamber as reported previously by our group [2, 4]; drugs could easily be added from the top and the buffer was rapidly eliminated by removal of the 7 ml bath content through a bottom device, a maneuver denoted as W (meaning drug washout) in the recording panels.

In view of the limited access to human biopsies, supplementary protocols were conducted in isolated rat vas deferens preparations. Animal studies were conducted to add pharmacological meaningful data to the studies with human tissue and to compare the experimental results between two species. To this purpose, Sprague Dawley male rats (300 ± 20 g) raised at the Faculty Animal Reproduction Laboratories were used. Rats were anesthetized with a mixture of...
ketamine:xylazine (75:2.5 mg/kg ip); once the rats were deeply anesthetized, a midline incision exposed the abdominal cavity; vas deferens were dissected, only the segment near the prostate was used for the recording of isometric contractions, since ATP produces larger contractions in this segment of the ductus. Buffer and details of the recording conditions were as described previously [2]. Rats were sacrificed by a KCl overdose followed by pneumothorax. The protocols were previously approved by the Faculty Ethical and animal Care Committee for Experimental Research, as part of CARE program project PFB 12/2007 and Fondecyt project 1110672.

Protocol descriptions

Recording of motor activity elicited by transmitters applications or electrical field stimulation (EFS). Prior to testing the tissue responses to purinergic agents, all tissues were challenged with a standard of 70 mM KCl, a stimulus which generated a rapid motor response of more than 1 g tension in the longitudinal strips and between 0.6-0.8 g in the ring preparations; human tissues that did not develop this tension were eliminated. The experiments always concluded with a 70 mM KCl challenge to assure the biopsies were viable during the recording period.

Concentration-response curves; studies with receptor agonists and antagonists. Once human or rat tissues responded to KCl applications, contractions elicited by exogenous ATP (30-30000 µM), α,β-mATP (1-16 µM), or 1 µM noradrenaline were routinely examined. To assess whether structural ATP analogues also elicited motor responses, evidencing receptor selectivity, rat strip preparations were challenged with ATP structural analogs such as ADP, AMP or adenosine to elicit the contractions of the longitudinal smooth muscle layer. Moreover, to examine the ATP receptor subtype selectivity, we examined the muscle tension elicited by 40 µM ATP-induced challenges before and after rat strip preparations were pre incubated for 30 min with NF 449 in concentrations varying from 1 to 100 nM, an alleged selective and potent P2X1R antagonist [19]. Initially, separate preparations examined the effect of only two concentrations of the antagonist; for example 1 nM or 30 nM. In all cases the motor effect elicited by a challenge of 40 µM ATP was recorded before and after incubation with the antagonist (n=6 each). In a second separate set of preparations, tissues were preincubated with either 10 or 100 nM NF 449 before and after tissue challenge with 40 µM ATP (n=6 each). In parallel muscle trips preparations, similar protocols were conducted, except we examined the antagonist activity of 1 to 100 µM suramin a non-selective purinoceptors antagonist (n=6 each). As with NF 449, all preparations were challenged before and 30 min after antagonist pre incubation with 40 µM ATP. In this particular case, each tissue served to examine the effect of a single concentration of suramin. Finally we compared the potency of NF 449 and suramin to block the motor effects of 40 µM ATP challenges. Additionally, ATP-concentration-response experiments (n=4) were performed in the same tissue in the absence first and 30 min after tissue pre-incubation with 10 nM NF 499. To this aim, ATP concentration-response curves (n=4) were performed in a same tissue before and after tissue incubation with 10 nM NF 449; results are expressed as the g of tension developed by parallel tissues.

Electrical field stimulation (EFS) protocol. To examine the motor responses elicited by EFS, the tissue nerve endings were stimulated through platinum wires placed parallel to the tissue strips; the electrodes were connected to a Grass S4 stimulator. The tissues were stimulated with EFS of 1.6 or 15 Hz (1ms duration and supramaximal 70V for 30 sec). To ascertain the whether
the electrically evoked contractions and relaxations are of neuronal origin and required voltage-
dependent sodium channels, biopsies were pre-treated initially with 100 nM TTX 5 min prior to
the EFS. In view of the scarce blockade of the electrically evoked motor responses, experiments
were repeated in the same biopsies applying 390 nM TTX for 5 min prior to the EFS protocol.
Immediately thereafter, the tissues were rinsed to eliminate the toxin and examine whether the
TTX-induced blockade was reversible.

**Desensitization protocols; recovery of the ATP motor activity after sustained α,β-mATP
exposure and cross desensitization between ATP motor responses and the contractions
induced by electrical field stimulation**

**Desensitization protocol.** To desensitize the P2X1R and examine the motor responses elicited by
EFS, desensitization protocols were performed by sequential applications of either 400 µM ATP
or 16 µM α,β-mATP generally spaced 5 or 6 min apart without changing the bath
solution. Once the motor responses to further agonist applications were abrogated, the receptor-
mediated motor response was considered desensitized [14]. To assess desensitization specificity,
tissues were challenged with 70 mM KCl immediately 6 minutes after the last α,β-mATP
application without tissue washout.

**Recovery from desensitization.** After 16 µM α,β-mATP application, a 400 µM ATP challenge
was applied; 1 min thereafter, the buffer was rinsed off. Tissues were added with 400 µM ATP 5,
20 or 40 min to assess the time required to recover the initial 400 µM ATP-evoked tension
elicited prior to the 16 µM α,β-mATP. In these protocols, care was taken to rapidly rinse off the
testing ATP applications to avoid further receptor desensitization elicited each testing ATP
addition. Parallel experiments were repeated with rat vas deferens strip preparations.

**Cross desensitization assays.** To assess cross desensitization between the motor responses
induced by α,β-mATP and those elicited by EFS or the 70 mM KCl-induced motor activity, the
motor response elicited by EFS or KCl were recorded prior to and following α,β-mATP-induced
desensitization. Parallel experiments were repeated in rat vas deferens strip preparations to
compare qualitatively the results after desensitization.

**P2X1R immunoblotting**

Human vas deferens biopsies were homogenized in the presence of different protease inhibitors;
protein extracts were prepared and processed for P2X1R immunoreactivity through western
blotting. A parallel protocol examined P2X1R desensitized tissues by prior exposures to 16 µM
α,β-mATP. At least 3 biopsies from separate patients were examined under reducing conditions,
added with 1 mM dithiothreitol (DTT) before extract heating; the electrophoresis buffer also
contained 1 mM DTT). The P2X1R antibody was used at a 1:10 000 dilutions. Immunoblots
characteristically evidenced a single band of the expected molecular size; occasionally
additional, nonspecific bands were observed.

**Analysis of P2X1R microdomain localization using sucrose gradients**

To assess the possible lipid raft insertion of the P2X1R, three separate biopsies of the human vas
derefers were used as control tissues, and three additional tissues were exposed to repeated
applications of 16 µM α,β-mATP (referred to as “desensitized” tissues). The homogenate was
adjusted to 45% sucrose by the addition of 0.5 ml of 70% sucrose and placed in the bottom of the ultracentrifuge tube. A 5 to 35% discontinuous sucrose gradient was formed above and centrifuged at 180,000 g for 16 hours. Eleven fractions of 360 µl each were collected from the top of the centrifuge tube and precipitated by adding 36 µl of 100% trichloroacetic solution. Proteins were separated by SDS-PAGE transferred to polyvinylidene difluoride membranes, and incubated with the P2X1R antibody (1/10 000 dilution) or the flotillin-1 antibody [16]. Quantitative analysis of immunoblots was performed with Image J software (http://rsbweb.nih.gov/ij/), following digitalized blots scanned in a VISTA-T630 UMax scanner driven by Adobe Photoshop CS (Adobe system, Mountain View, CA); this procedure was previously used by [20].

**Drugs and antibody providers**
ATP, α,β-methylene ATP, noradrenaline, suramin, tetrodotoxin, peptidase inhibitors such as leupeptin, pepstatin, PMSF and antipain were purchased from Sigma-Aldrich (St. Louis, MO, USA); NF 449 was obtained from Tocris Bioscience, (Ellisville, MO, USA). Analytical grade reagents for buffer preparations were obtained from Merck (Darmstadt, Germany). All molecular biology reagents and buffers were obtained from Gibco (BRL Life Tech., CA, USA). Actin, P2X1R, and flotillin1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Data analysis**
All quantitative data are expressed as the mean ± standard error of the mean (S.E.M.). The differences were evaluated for statistical significance (P < 0.05). Significance was determined by Student’s t-test or ANOVA with Dunnett’s multiple comparison tests.

**RESULTS**

**Purinergic motor activity elicited by exogenous ATP or noradrenaline applications or EFS in both muscular layers of the human vas deferens**
Exogenous ATP or α,β-mATP applications evoked rapid phasic motor responses in strips of human vas deferens biopsies; however, in rings prepared from a same biopsy, the purinergic motor responses were significantly smaller and rather inconsistent. In both preparations, the contractions elicited by purinergic agents were brief and decayed within 20-30 sec (see representative traces in Figure 1); statistical data analysis of this set of data is provided in Figure 2. In some preparations, we noted that ATP elicited the fast contraction component followed by relaxation below basal tension. The magnitude of the 16 µM α,β-mATP-induced contractions was larger than that elicited with 400 or 4000 ATP µM (see tracings in Figure 1 and statistical analysis of this data in Figure 2). In view of the physiological impact of sympathetic co-transmission in this tissue, we also examined the motor response elicited by 1 µM noradrenaline; strips showed a 5.4-fold larger contraction than the rings (P<0.05, Figure 2), resulting in a common pattern with the purinergic agonists. In contrast, the KCl-induced contractions were not significantly different between these two preparations; the tension elicited by the strips was only 1.5-fold larger than that elicited by the rings (1.34 ± 0.28 vs 0.85 ± 0.14 g of tension, Figure 2), indicating that the rings were viable and developed similar tension as the strips. EFS of the human tissue nerve endings evoked fast motor responses in both strips and rings. Consonant with the motor effects elicited by ATP, EFS-induced contractions were not sustained during the 30-sec stimulation period; in 4 out of 6 strips and 2 out of 3 rings we observed that the
contractions were followed by relaxations below basal tissue tension (see representative tracings in Figure 1B and D). Consistently, the 15 Hz nerve-mediated contractions of the longitudinal layer were 7.5-fold larger than those developed in the circular layer (3.7 ± 0.3 vs 0.5 ± 0.13 g, n = 5, P<0.001, Figure 2). In support of the neuronal origin of the EFS-evoked motor responses, tetrodotoxin (TTX) reduced the motor effects; while 100 nM TTX only weakly antagonized the EFS-induced contractions in the human biopsies, it abolished those of the rat preparations. Considering a possible pharmacokinetic variable due to the larger size of the human strips compared to the rat, a 4-fold increase in the toxin concentration (390 nM reduced 84-90 % the EFS-evoked contractions within 5 minutes and its subsequent relaxations in both tissue layers (Figure 1B and D). The toxin effect was reversible; 10 min after TTX, rinsing, the motor responses partially recovered evidencing again both motor components (Figure 1B and D). In view of the larger motor responses attained by the strips, ring experiments were discontinued as we focused on the strips.

**Studies with rat vas deferens strips**
Additional experiments aimed at determining the selectivity of the nucleotide response were performed in rat preparations. Applications of 30 mM ADP elicited only a minor contraction compared to ATP, while AMP was inactive. Adenosine applications caused a minor, yet significant relaxation; we never observed a motor, excitatory response with the nucleoside (data not shown). Moreover, to further test the receptor nature of the ATP-induced contractions, we examined the effect of increasing concentrations of suramin and its structural analog NF 499 on the motor activity elicited by 40 µM ATP. Both suramin and NF 449 reduced the ATP-induced contractions elicited by 40 µM ATP challenges, NF 449 proved at least 2 orders of magnitude more potent than suramin (Figure 3A). In a separate protocol, 10 nM NF 499 displaced to the right, in a parallel manner, the ATP concentration-response curve (Figure 3B), as expected for a competitive antagonism, a finding compatible with the notion that the ATP mediated responses are likely mediated by P2X1R activation.

**Desensitization of the ATP motor responses and cross desensitization to the electrically-evoked motor contractions; parallel studies with human and rat ductus**
Following repeated ATP applications we observed in 5 out of 5 separate human preparations that the tissues elicited progressively smaller contractions which finally failed to contract the tissues following additional nucleotide applications (Figure 4A). This finding was anticipated and was greatly reproducible in agreement with the desensitization protocol utilized. When ATP was replaced by the slowly hydrolysable analog α,β-mATP, the tissues only responded with a robust contraction to its first application, failing to reach further motor responses to additional sustained applications. ATP-induced desensitization was reversible (Figure 4 panels B and C). In one out of 3 experiments, after ATP-induced desensitization, we observed that a further nucleotide application elicited only a relaxation (recording not shown). Likewise, after desensitization of the α,β-mATP-induced motor responses, the application of ATP elicited in 2/3 cases a relaxation response (data not shown). Moreover, and in proof that desensitization is not due to a non-specific loss of the tissue motor responses, strips desensitized with α,β-mATP responded to the 70 mM KCl challenges with almost the same tension as attained prior to the application of 16 µM α,β-mATP (Figure 4 panel D). Notwithstanding, the 15 Hz EFS-evoked contractions were significantly reduced by 65 ± 7.7 % in α,β-mATP desensitized tissues (n=4, P< 0.01 Figure 4 panel B, C and D), indicating cross desensitization between purinergic agonists and the EFS-
evoked responses. Likewise, in α,β-mATP desensitized tissues, EFS evoked a small motor response that reached a slightly lower tone than the basal tissue tension (2 out of 5 experiments, data not shown).

Cross desensitization between α,β-mATP and ATP-mediated contractions; recovery time course
A single challenge application of 16 μM α,β-mATP to human or rat strips, elicited a fast non-sustained contracture that nullified the subsequent ATP-induced motor contractions. Full recovery of the ATP-induced contractions after changing the tissue buffer occurred within 40 min in the human tissue (Figure 5B) and even longer in the rat tissues where only 46.5 ± 8 % (n=10) recovery was observed within this time lapse (Figure 5E).

In addition, the contractions elicited by 1.6 Hz EFS were also markedly reduced following 16 μM α,β-mATP exposure either in human (Figure 5C) or rat tissues (Figure 5F); the motor responses recovered within 40 min of the α,β-mATP washout in the rat tissue (Figure 5F), in the human, 5 min after α,β-mATP washout, we observed only a 13.3 ± 13.1 % of the motor response recovery (Figure 5C), demonstrating significant cross desensitization between both tissue responses.

Oligomerization of the human P2X1R
Western blotting of human vas deferens extracts obtained from native tissue extracts, prepared in the absence of DTT revealed a single major band of slightly less than 200 kDa, plus a minor 70 kDa band (Figure 6A, left panel, labeled -DTT). In contrast, when the extracts were prepared and separated by electrophoresis in buffer, added with DTT (reducing conditions), a single band of approx 70 kDa was observed (middle panel, Figure 6A, labeled +DTT). When the 200 kDa oligomer was eluted from the first gel and ran in a second, separate gel prepared under reducing conditions, only the 70 kDa band was observed plus a minor smaller weight band (right panel, Figure 6A), verifying that 200 kDa band of the P2X1R was composed of 70 kDa monomers.

Similar findings were observed in triplicate experiments derived from separate human biopsies treated likewise.

Lipid raft micro regionalization of the human P2X1R
We next ascertained whether the human P2X1R was inserted into membrane micro regions, such as lipid rafts. To this aim, a human vas deferens extract treated with sodium carbonate was subjected to separation in a sucrose gradient. The gradient analysis revealed that 25-30 % of the P2X1R is localized in gradient fractions containing 15-29% sucrose (fractions 3-7), a finding compatible with lipid raft localization. Based on the almost identical flotillin-1 localization (Figure 6B), plus the 15-29% sucrose content of these fractions, in all likelihood they correspond to the P2X1R contained in lipid rafts. In addition, the blot also showed that the P2X1R is found in heavier gradient fractions, a non-lipid raft membrane distribution, not involved in motor responses. Similar findings were observed in the distribution of the P2Y1R in human vascular smooth muscles [20]. When the same analysis was extended to human vas deferens biopsies previously exposed to α,β-mATP, a protocol used to desensitize the P2X1R, the gradient from this particular tissue extract showed a similar P2X1R fractionation as with the control tissue (Figure 6C), suggesting that tissue desensitization is not likely due to P2X1R displacement out from lipid rafts or to P2X1R internalization. The immunoblots shown in the figure are representative of at least 3 identical protocols performed with different biopsy samples.
DISCUSSION
Several arguments strongly support our proposal that ATP is a motor transmitter of the longitudinal smooth muscle layer of the human vas deferens and acts through P2X1Rs to elicit the tissue contractile responses. ATP is released from the tissue nerve endings in a calcium-dependent manner [2] and elicits non-sustained rapid contractions as shown by the EFS myographs. The finding that EFS caused reduced motor responses after α,β-mATP-induced desensitization, is consistent with the role of P2X1R in the motor activity elicited by endogenous ATP, highlighting its role as a motor transmitter in the longitudinal smooth muscles of this tissue. In further support of the role of P2X1Rs in the human vas deferens transmission, we identified biochemically this receptor subtype in tissue extracts by immunoblots. Its molecular weight agrees with reported values from other P2XRs studies [14]; the present biochemical analysis is consistent with receptor oligomerization, consonant with the notion that the P2X1R is composed of three subunits similar to the topology reported for the crystallized P2X4R [21]. In addition, the lipid raft localization of the P2X1R was observed in different cell studies [13, 22] and agrees well with the co-transmitter role of ATP and NA in sympathetic transmission. In further sustenance of our contention that ATP through the P2X1R is involved in the contractile activity of the tissue, transgenic male mice lacking the P2X1R are infertile because of reduced vas deferens motility, rather than decreased spermatogenesis [23]. In addition pharmacological studies in the rat preparations indicated that ADP is a poor motor agonist, while AMP and adenosine failed to contract the rat tissue. Moreover, the fact that α,β-mATP potent and rapidly desensitized the P2X1 mediated responses plus the finding that the NF 499-induced antagonism of the ATP contractions, substantiate the role of the P2X1R as an ATP target in this neuroeffector junction. Altogether, these arguments strongly support the notion that the motor responses elicited by ATP in the tissue longitudinal smooth muscle are mainly mediated by P2X1Rs confirming the validity of our working hypothesis in the human tissue.

We used preferably α,β-mATP rather than ATP as a procedure to desensitize the P2X1R in the EFS-evoked motor response and examined the nature of the contractions elicited by exogenous ATP. Minor differences were found in the desensitization process and its recovery between human and the rat vas deferens preparation, an observation that agrees with the notion that ATP must play a preponderant motor transmitter role in the longitudinal muscles of both species. Although the molecular basis of desensitization is remains unknown, the present data indicates that desensitization is neither due to P2X1R internalization nor to its displacement out from raft membranes from the smooth muscle plasma membrane. In view that desensitization occurs within seconds, we infer that the P2X1R might adopt an inactive conformation or a similarly rapidly occurring membrane adaptation. P2X1R desensitization may involve additional regulatory mechanisms [24]. Within the past few years, the proposal that the intracellular amino terminus of the P2X1R, in the region just before the trans membrane domain, is actively involved in desensitization, and that the cytoskeleton and heat shock protein 90 dimers plays a role in P2X1R receptor regulation was has been discussed [13-25]. It is possible that rapid disruption of actin filaments also regulate P2X1R activity, a finding tentatively also linked to desensitization. We have not disregarded that α,β-mATP might also interact with other purinoceptors and involve other mechanisms in the search for the molecular basis leading to desensitization.
EFS-evoked contractions were blocked by TTX, albeit a higher toxin concentration was required likely due to the limited toxin distribution in the human compared to the rat tissue during the 5 min drug exposure. We conclude that the EFS-evoked contractions and its subsequent relaxations are of neuronal origin. Moreover, the EFS-evoked relaxations may be related to acetylcholine release; consistent with the commentary that atropine reduced this effect [11]. Therefore, we infer an inhibitory cholinergic modulation that regulates the human motor responses in both smooth muscle tissue layers, a finding in accordance with the dual autonomic innervation of peripheral organs. In addition, because α,β-mATP is several orders of magnitude more potent than ATP, we deduce that the tissue must be enriched in ectonucleotidases suggesting that the nucleotide must be rapidly degraded upon its synaptic release. In favor of this hypothesis, ARL 67156, an ectonucleotidase inhibitor, substantially increased the human tissue ATP-induced motor activity [12].

As to why the circular layer of the human vas deferens is poorly affected by purinergic agonists remains to be further examined. Either a different transmitter is secreted by the nerve endings of the circular layer, or the nucleotide is more avidly hydrolyzed in the circular layer. We have not ignored that the longitudinal and circular smooth muscle layer might have an uneven P2X1R distribution, consistent, perhaps, with a different transmitter operating in the two smooth muscle layers. In support of the latter hypothesis, less P2X1R immunoreactivity was observed in the circular smooth muscle layer as compared to the longitudinal layers of the human vas deferens [11]. The ATP-induced contractions of the tissue circular layer were markedly magnified in the presence of iberiotoxin, indicating that the P2X1Rs are expressed in the circular layer of the tissue, but its functional activity may be hindered by the large conductance calcium-activated K+ channels (BKCa) [12]. When this K+ channel was blocked by iberiotoxin, the magnitude of the α,β-mATP-induced contractions reached contractions of magnitudes comparable to those evoked in the longitudinal layer, suggesting a negative modulator role of this channel on the purinergic motor component of the human vas deferens.

Rafts have been associated with cell signaling platforms and/or intracellular cascades; a discrete cell membrane site suited for interactions with other receptors involved in the physiology of the smooth muscle, particularly receptors coupled to trimeric G proteins [26]. Among other receptors of interest, we suspect that cholinergic muscarinic receptors, the α1-adrenoceptors [6, 7] and vasopressin [8] all of which belong to the family of G-protein coupled receptors. The lipid raft localization of the P2X1R fosters direct and indirect possibilities of receptor interactions which can account for sympathetic co-transmission. In this regard, the view that adrenoceptors and other neurotransmitter receptors are associated to lipid rafts offers a plausible mechanistic model for co-transmission in the human vas deferens [21], an attractive working hypothesis opened for future cell biology and pharmacological studies.

On a comparative, applied, basis, we propose the rat vas deferens might be a relevant experimental model to study novel drugs of clinical importance in the treatment of human erectile dysfunction [27, 28]. Moreover, the present observations might have a clinical correlate linked to the human sexual response, since blockade of vas deferens motility through inhibition of P2X1R activity, might be considered a putative mechanism for an alleged male contraceptive opportunity.
In conclusion, the excitatory motor activity of ATP in the human vas deferens is exerted through the activation of the P2X1Rs, which are localized in rafts micro domains of the smooth muscle plasma membranes. Altogether, the present results highlight the functional role of ATP as a human motor transmitter in the longitudinal smooth muscle layer of the human vas deferens.

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REFERENCES

FIGURE LEGENDS
Figure 1. Representative recordings of contractions evoked by ATP, α,β-mATP, or electrical field stimulation of the human vas deferens nerve endings in preparations of the longitudinal and circular smooth muscle layer. A and B) traces from isometric smooth muscle tension recordings from a strip of the human vas deferens longitudinal smooth muscle preparation (note differences in calibration scales between A and B). C and D) Tracings from a ring preparation derived from the same biopsy as A and B. At the dots, applications of ATP or α,β-mATP spaced every 10
minutes as indicated for the strip or ring preparation. Squares denote the 30-sec 15 Hz electrical field depolarization (B and D) before and after tissue incubation with 390 nM tetrodotoxin (TTX). To examine the reversibility of the TTX effect, the toxin was washout (W); the preparations were stimulated again 10 min after tissue washout. Observe the difference in the calibration scales between strips and rings; the contractions of the longitudinal strips were 7-10-time stronger than those developed by the circular layer rings.

**Figure 2.** Quantification of the motor responses in the longitudinal and circular smooth muscle layers of the human vas deferens. Comparative study of the tension developed by 400 µM ATP, 16 µM α,β-mATP, 1 µM noradrenaline (NA), the 15 Hz-electrically-evoked contractions and the 70 mM KCl-induced tension. Columns denote the mean average tension generated by each agonist, or that elicited by electrical field stimulation; bars indicate the S.E.M. Grey columns denote the longitudinal muscle layer preparations, black columns indicate the rings used to obtain responses from the circular smooth muscle layer. Numbers inside the columns indicate the tissue samples examined with each stimulus. *P<0.05; ***P<0.001; Students t-test, compared tension generated by longitudinal versus circular layer preparations.

**Figure 3.** Suramin and NF 449 block the ATP-induced contractions in rat vas deferens preparations. A) Comparative concentration-response curves for the blockade of the 40 µM ATP-evoked contractions by suramin and NF 449. Columns indicate the magnitude of the ATP-evoked contractions in g tension in the control preparations that were treated with either suramin or NF 449; symbols indicate the tension ensued by 40 µM ATP applications in preparations treated with varying concentrations of either suramin (n=6) or NF 449 (n=6). Bars in columns or symbols indicate the mean values ± the S.E.M. B) 10 nM NF 449 displaced to the right the ATP-concentration response curve. Symbols indicate the mean ± S.E.M. (n= 6 for each curve).

**Figure 4.** Representative tracings of human longitudinal muscle strips of the vas deferens showing that repeated applications of 16 µM α,β-mATP resulted in loss of motor responses and cross desensitization to the application of 400 µM ATP additions or electrical nerve ending depolarization. A) Repeated ATP additions (closed dots) every 5 minutes elicited a complete desensitization of the nucleotide-induced motor responses. ATP was washout (W) from the tissues; ATP challenges were performed every 20 min; full recovery was attained after approx. 40 min of washout. B) In a separate strip following an ATP addition (closed dots) or the 15 Hz EFS (closed squares) spaced 10 min apart, three successive α,β-m-ATP applications (open dots), spaced each at regular 6 minutes intervals without washout in between, resulted in a complete loss of motor responses. Cross desensitization was observed to the motor response elicited by exogenous ATP (5 min after α,β-mATP washout) or to the EFS-evoked contractions, applied after a 5 minute washout and 16 min after the last α,β-mATP application. C) In a separate human vas deferens strip, 16 µM α,β-mATP caused complete loss of further motor contractions responses to successive α,β-mATP applications spaced every 6 minutes while the contraction to 70 mM KCl remained intact. D) Following α,β-m-ATP desensitization, as shown in B, a significant reduction in the motor contractions elicited by either ATP or the 15 Hz-electrical field stimulation was observed, but not to the KCl applications, evidencing that desensitization was not crossed to all contractile agents. Columns indicate the mean average tension developed by 400 µM ATP, the 15 Hz electrical-evoked contractions or the KCl-induced contractures, before (control, grey columns) or after the three applications of α,β-m-ATP used to desensitize the
Figure 5. α,β-mATP induced desensitization of the ATP-induced motor responses in human and rat vas deferens. Left panels (A, B, C) studies performed in human biopsies; right panel (D, E, F) studies from the prostatic end of the rat vas deferens. A and D) Representative tracings of the contractions induced by exogenous applications of 400 µM ATP (closed dots) before and 3-min after the application of 16 µM α,β-mATP, closed squares and subsequent ATP applications 5, 20 and 40 min following α,β-mATP washout (W). ATP was washout between successive ATP applications (W). B and E) Statistical analysis obtained from 6 separate experiments with human tissue and n=10 for the rat tissues. C and F) The cross desensitization between the motor response induced by the contraction evoked by transmural electrical nerve depolarization attained 5, 20 or 40 min after application of a desensitizing 16 µM α,β-mATP (n=5, human biopsies; n=7, rat vas deferens). *p<0.05; **p<0.01, as compared to the control motor contraction elicited by either ATP applications or electrical nerve depolarizations, Dunnett’s multiple comparison test.

Figure 6. Biochemical identification of P2X1R oligomers in native tissue extracts and its micro regionalization in lipid rafts. A) Left lane shows a representative P2X1R gel immunoblot of a human vas deferens extract prepared in the absence (-DDT) or an extract treated with dithiotreitol (+DTT, middle lane), under reducing conditions. The right panel shows an electrophoresis gel prepared with the 200 kDA material eluted from a native tissue extract (prepared in the absence of DTT) which was next treated with DTT and applied next to a gel prepared under reducing conditions (in the presence of DTT). Gel calibration on the left side shows the expected 70 and 200 kDa band molecular weight, according to the protein molecular weight markers used. B) Lipid raft micro regionalization of a human vas deferens extract following a sucrose gradient analysis which identified lipid rafts in fractions 4-6 of the gradient; these fractions correspond to 15-29 % of sucrose. Immunoblots were revealed with the same P2X1R antibody as used in panel A and with an antibody for flortillin-1, a well-recognized lipid raft marker. C) Immunoblot was prepared in the same conditions as in panel B, but the tissue was previously desensitized by three successive applications of 16 µM α,β-mATP. This blot is representative of three separate replicas.
Fig 1

**Longitudinal human vas deferens**

A

- 0.1 g
- 2 min
- 400 µM ATP

B

- 0.5 g
- 2 min
- 16 µM α,β-mATP
- 15 Hz
- 390 nM TTX

**Circular human vas deferens**

C

- 0.1 g
- 2 min
- 400 µM ATP

D

- 0.2 g
- 2 min
- 16 µM α,β-mATP
- 15 Hz
- 390 nM TTX
Fig 2

[Graphs showing tension (g) for various treatments: 400 μM ATP, 16 μM α,β-mATP, 1 μM NA, 15 Hz, and 70 mM KCl. The graphs compare tension in the longitudinal and circular layers.]
**Fig 3**

**A**

- Control
- Suramin
- NF 449

**B**

- Control
- 10 nM NF 449
Fig 4

A

400 μM ATP

B

400 μM ATP

15 Hz

16 μM α,β-mATP

400 μM ATP

15 Hz

C

70 mM KCl

16 μM α,β-mATP

70 mM KCl

D

[Graphs showing tension (g) for 400 μM ATP, 15 Hz EFS, and 70 mM KCl with control and + α,β-mATP conditions]
**Fig 5**

(A) Human

(B) 400 µM ATP
tension (g)

(C) 1.6 Hz tension (g)

(D) Rat

(E) 400 µM ATP
tension (g)

(F) 1.6 Hz tension (g)

- **Human**
  - 0.2 g
  - 2 min
  - 400 µM ATP
  - 16 µM α,β-mATP

- **Rat**
  - 0.2 g
  - 2 min
  - 400 µM ATP
  - 16 µM α,β-mATP
Fig 6

A

P2X1R

- DTT  + DTT  + DTT

200 kDa  oligomers  eluted oligomer

70 kDa  monomers

B

1  2  3  4  5  6  7  8  9  10  11

P2X1R  Flotillin-1

membrane rafts

C

Desensitization

P2X1R

membrane rafts