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# Probenecid directly impairs activation of the canine P2X7 receptor

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## Abstract

The current study aimed to determine if probenecid could directly impair the canine P2X7 receptor, a ligand-gated cation channel activated by extracellular adenosine 5'-triphosphate (ATP). Patch clamp measurements demonstrated that probenecid impairs ATP-induced inward currents in HEK-293 cells expressing canine P2X7. Flow cytometric measurements of ethidium + uptake into HEK-293 cells expressing canine P2X7 showed that probenecid impairs ATP-induced pore formation in a concentration-dependent manner, with a half maximal inhibitory concentration of 158 μM. Finally, ELISA measurements revealed that probenecid impairs ATP-induced interleukin-1β release in dog blood. In conclusion, this study reveals that probenecid can directly impair canine P2X7 activation.



## Disciplines

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# Probenecid directly impairs activation of the canine P2X7 receptor

Running head: Probenecid impairs canine P2X7 activation

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## ABSTRACT

The current study aimed to determine if probenecid could directly impair the canine P2X7 receptor, a ligand-gated cation channel activated by extracellular adenosine 5'-triphosphate (ATP). Patch clamp measurements demonstrated that probenecid impairs ATP-induced inward currents in HEK-293 cells expressing canine P2X7. Flow cytometric measurements of ethidium$^{+}$ uptake into HEK-293 cells expressing canine P2X7 showed that probenecid impairs ATP-induced pore formation in a concentration-dependent manner, with a half maximal inhibitory concentration of 158 μM. Finally, ELISA measurements revealed that probenecid impairs ATP-induced interleukin-1β release in dog blood. In conclusion, this study reveals that probenecid can directly impair canine P2X7 activation.

## KEYWORDS

Purinergic receptor, extracellular nucleotide, probenecid, gout, monocyte, cytokine, dog

## Introduction

The P2X7 receptor is a trimeric ligand-cation channel activated by extracellular adenosine 5'-triphosphate (ATP).[1] Activation of this receptor channel allows the rapid flux of $Na^+$, $K^+$ and $Ca^{2+}$, and the opening of a pore to allow the movement of organic cations, including fluorescent dyes such as ethidium$^+$, through the plasma membrane.[1] P2X7 is present on canine erythrocytes, lymphocytes and monocytes,[2, 3], as well as brain[4, 5] and kidney epithelial cells[6] of dogs. Activation of canine P2X7 leads to pro-inflammatory interleukin-1β (IL-1β) release from monocytes[7] and in whole blood,[8, 9] as well as to phosphatidylserine exposure on erythrocytes.[10] Other biological functions of canine P2X7 are yet to be reported, but it is likely that P2X7 activation mediates events similar to those observed in humans and rodents, where it is emerging as a potential therapeutic target in a range of disorders.[11, 12] Thus, for over a decade we have been studying the biology and pharmacology of canine P2X7 to determine if this receptor is a genuine therapeutic target in dogs.

Probenecid (*p*-(di-*n*-propylsulfamyl)-benzoic acid; Benemid® or Benuryl®), which inhibits organic anion transporters in renal proximal tubes to prevent reuptake of uric acid from urine, is predominately used as a second line treatment of gout in humans.[13] Probenecid can also be used in dogs to reduce renal excretion to increase the *in vivo* concentrations of some antibiotics,[14] although this seems to be rarely applied in veterinary settings. Probenecid can also impair the ATP release channel pannexin-1,[15, 16] which can directly interact with human and rodent P2X7.[17, 18] Notably, probenecid can directly impair human P2X7 but not rodent P2X7.[19] Moreover, the P2X7 antagonist profile of canine P2X7 is similar to that of human P2X7.[8, 20, 21] Thus, given the similar pharmacological profiles between these two receptors and the potential use of probenecid in dogs, the current study aimed to determine if probenecid could also directly impair canine P2X7.

## Materials and methods

### *Chemicals*

DMEM/F12 medium, GlutaMAX, penicillin-streptomycin, OPTI-MEM reduced serum medium, Lipofectamine 2000, RPMI-1640 medium and (water soluble) probenecid were from Thermo Fisher Scientific. Foetal bovine serum (heat inactivated before use) was from Bovogen Biologicals. ATP and lipopolysaccharide (LPS; *Escherichia Coli* serotype 055:B5) were from Sigma-Aldrich. Ethidium bromide and general chemicals were from Amresco.

### *Cell culture and transfections*

HEK-293 cells were maintained in DMEM/F12 medium containing 10% foetal bovine serum, 2 mM GlutaMAX, 100 U/mL penicillin and 10 µg/mL streptomycin at 37$^{o}$C/5%$CO_2$. HEK-293 cells were transiently transfected with canine P2X7 plasmid DNA using Lipofectamine 2000 diluted in OPTI-MEM Reduced Serum medium as described.[21]

### *Channel activity*

To assess P2X7 channel activity, ATP-induced inward currents in P2X7-transfected HEK-293 cells in low divalent solution (145 mM NaCl, 5 mM KCl, 0.2 mM $CaCl_2$, 13 mM glucose, 10 mM HEPES, pH 7.3) were recorded using whole-cell patch clamp as described.[19]

### *Pore formation*

To assess P2X7-mediated pore formation, ATP-induced ethidium$^{+}$ uptake into P2X7-transfected HEK-293 cells in NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM HEPES, pH 7.4) was determined by flow cytometry as described.[21]

### *IL-1β release*

Peripheral blood was collected into lithium heparin tubes (Greiner Bio-One) from three dogs (boxer bull terrier cross, bull mastiff cross, or Staffordshire bull terrier), and studied according to institutional guidelines (University of Wollongong). ATP-induced IL-1β release in whole blood diluted with an equal volume of RPMI-1640 medium and containing a final concentration of 100 ng/mL LPS, was performed as described.[9] The amount of IL-1β in cell-free supernatants was measured using a Canine IL-1β VetSet ELISA (Kingfisher Biotech) according to the manufacturer's instructions.

### *Statistical analysis*

Data are presented as mean ± SD. Differences between groups were compared with an one-way analysis of variance (using Tukey's post test) using Prism 5 for Mac OS X (GraphPad Software, San Diego, CA, USA) with $P < 0.05$ considered significant.

## Results

### *Probenecid impairs canine P2X7 channel activity*

To determine if probenecid could directly impair activation of canine P2X7, P2X7-transfected HEK-293 cells were treated with probenecid and/or ATP, and inward currents measured using whole-cell patch clamp. ATP induced robust inward currents, with similar sized currents observed following a second treatment with ATP (Figure 1), demonstrating, that under these conditions, the canine P2X7 channel does not undergo run-up or run-down. Following washout, co-treatment of cells with probenecid and ATP significantly reduced current density by 60 ± 6% (mean ± SD) (Figure 1). Following washout, pre-treatment of these cells with probenecid alone for 1 min significantly reduced ATP-induced current density by 83 ± 9%, an affect that was readily reversible following a final washout (Figure 1).

### *Probenecid impairs canine P2X7-mediated pore formation*

To determine if probenecid could impair canine P2X7-mediated pore formation, P2X7-transfected HEK-293 cells were incubated with increasing concentrations of probenecid for 15 min, and ATP-induced ethidium$^{+}$ uptake was assessed by flow cytometry. Probenecid impaired ATP-induced cation dye uptake in a concentration-dependent manner with near-maximal inhibition at 10 mM (97 ± 2%) and with a half maximal inhibitory concentration ($IC_{50}$) of 158 μM (Figure 2).

### *Probenecid impairs canine P2X7-mediated IL-1β release*

To determine if probenecid could impair canine P2X7-mediated IL-1β release, dog blood was pre-incubated with LPS for 2 h, and co-incubated in the absence or presence of probenecid during the final 15 min. Blood was then co-incubated for a further 30 min in the absence or presence of ATP, and the amount of IL-1β in cell-free supernatants assessed by ELISA. In the absence of ATP, IL-1β release was minimal and similar in either the absence or presence of probenecid (Figure 3). As expected, ATP incubation, in the absence of probenecid, significantly increased IL-1β release compared to blood incubated in the absence of ATP (Figure 3). Similar to P2X7 channel activity and pore formation, pre-incubation with probenecid reduced ATP-induced IL-1β release by 84 ± 13% (Figure 3).

## Discussion

Using whole-cell patch clamp measurements of P2X7 channel activity, the current study demonstrated that probenecid directly blocks canine P2X7 activation. This, finding is similar to the direct inhibition of human P2X7, but not rodent P2X7, by probenecid.[19] Using flow cytometric measurements of P2X7-mediated pore formation, the current study also showed

that probenecid impaired canine P2X7 with an $IC_{50}$ of 158 μM, which is similar to the $IC_{50}$ of probenecid against human P2X7 (203 μM).[19] The $IC_{50}$ value for probenecid against canine P2X7 was one to four logs greater than the $IC_{50}$ values observed for specific P2X7 antagonists against canine P2X7.[8, 20, 21] Similar differences in $IC_{50}$ values are also observed for human P2X7.[22-25] Finally, using a blood-based assay to study IL-1β release, probenecid impaired P2X7-mediated IL-1β release by 84%, which is slightly less to that observed previously for the P2X7 antagonist, A438079 (97%), using the same assay,[9] albeit at different concentrations (5 mM and 50 μM for probenecid and A438079, respectively). Combined, the data above indicates that probenecid is a direct but less potent inhibitor of canine P2X7 than conventional P2X7 antagonists.

The inhibition of both canine and human P2X7 by probenecid parallels the ability of conventional P2X7 antagonists to block this receptor in both these species.[8, 20, 21] This contrasts well-known differences in antagonist selectivity between human and rodent P2X7.[22-25] Moreover, probenecid does not impair rat or murine P2X7.[19] Collectively these findings suggests a high degree of similarity in the probenecid-binding site between canine and human P2X7 compared to that of rodent P2X7, which parallels the greater sequence identity between canine and human P2X7 than between rodent and human P2X7.[1] Although the probenecid binding site in P2X7 is unknown, crystal structures of giant panda P2X7 with structurally different P2X7 antagonists (A740003, A804598, AZ10606120, GW791343 or JNJ47965567) reveal a common antagonist-binding pocket in the large extracellular domain of P2X7, with antagonist binding mediated predominately by amino acid residues F88, F95, F103, M105, F293 and V312.[26] Notably, these amino acid residues are entirely conserved between canine and human P2X7, but not rat (L95 and A312), murine (A312) or guinea pig (L88 and L95) P2X7 (human P2X7 numbering),[27] providing a possible explanation for the similar antagonist profile shared by canine and human P2X7. Moreover, probenecid (286 Da)

is similar in size to the aforementioned P2X7 antagonists (315 to 489 Da) allowing the possibility that probenecid may also bind to this common antagonist-binding pocket,[26] which is also present in human P2X7.[28]

Findings from the current and previous[19, 29] studies of probenecid have implications for the experimental and clinical use of this compound. Experimentally, probenecid can be used at 2 to 2.5 mM *in vitro* to prevent the secretion and sequestration of calcium indicator dyes,[30, 31], and to block pannexin-1.[15, 16] Thus, caution should be applied when using probenecid to study canine and human P2X7-mediated $Ca^{2+}$ fluxes, or pannexin-1-mediated events associated with P2X7 activation in these species. Pannexin-1 has been identified in Madin-Darby kidney cells [32] but it remains to be determined if probenecid can inhibit canine pannexin-1. Clinically, standard oral therapeutic doses of probenecid are in the range 250 mg to 2 g twice per day,[33] with mean peak plasma concentrations of probenecid of 35, 70 and 149 μg/mL 3 to 4 h following single oral doses of 500 mg, 1 g or 2 g, respectively.[34] Thus, given that the $IC_{50}$ values of probenecid against canine and human P2X7 are approximately 160 and 200 μM (or 46 and 57 μg/mL), respectively, P2X7 may be an off-target of probenecid *in vivo*, at least in the circulation. It remains questionable if probenecid reaches sufficiently high enough concentrations to impair P2X7 activation within tissues. Curiously, monosodium urate crystals can induce ATP release from murine macrophages to promote IL-1β release, a process impaired by P2X7 antagonists,[35] suggesting a role for P2X7 activation in gout[36]. Thus, the possibility remains that probenecid may at least partially block this process in gout.

## Conclusion

In conclusion, the current study demonstrates that probenecid can directly impair canine P2X7 activation. Furthermore, probenecid can be added to the list of compounds that impair

both canine and human P2X7, which indirectly suggests that the antagonist-binding pocket may be quite similar between these two receptors. Finally, caution needs to be applied when using probenecid for experimental applications, such as reducing calcium indicator dye leakage, in studies relating to canine or human P2X7 activation.

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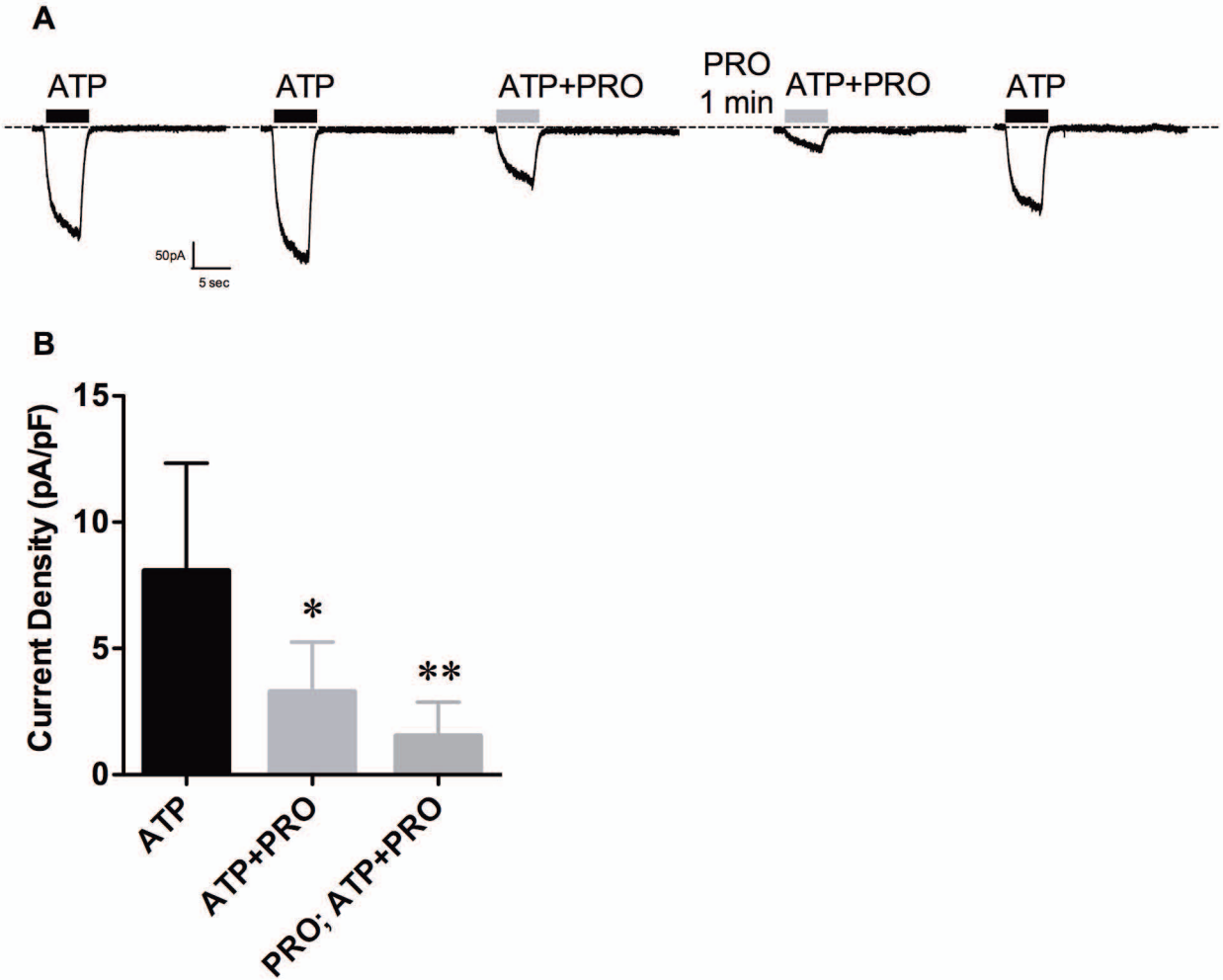
## Figure legends

**Figure 1.** Probenecid impairs canine P2X7 channel activity. (A, B) HEK-293 cells, transiently transfected with canine P2X7 plasmid DNA, were clamped at -60 mV at room temperature. Then 1 mM probenecid (PRO) and/or 250 μM ATP in low divalent solution (as indicated) was applied using a fast-flow delivery system and whole cell currents were recorded. (A) Inward currents to: ATP alone; ATP alone (repeat); ATP plus PRO; 1 min treatment with PRO followed by ATP plus PRO; and ATP alone, respectively. Bars indicate 5 s exposures; traces are representative of five to eight cells. (B) Results are mean ± SD of current density during first ATP exposure, ATP plus PRO exposure, and PRO (1 min pre-incubation) plus ATP exposure ($n$=6-8); *$P < 0.05$ and **$P < 0.01$ compared to ATP.

**Figure 2.** Probenecid impairs canine P2X7-mediated pore formation. HEK-293 cells, transiently transfected with canine P2X7 plasmid DNA, in NaCl medium were pre-incubated in the absence or presence of PRO as indicated for 15 min at 37$^{o}$C, before addition of 25 μM ethidium$^{+}$, and incubation in the absence or presence of 250 μM ATP for 5 min. Incubations were stopped and ethidium$^{+}$ uptake determined by flow cytometry. Ethidium$^{+}$ uptake is expressed as percent of maximal ATP response in the absence of PRO. Results are mean ± SD ($n$=3).

**Figure 3.** Probenecid impairs canine P2X7-mediated IL-1β release. Dog blood diluted with an equal volume of RPMI-1640 medium was incubated in the presence of 100 ng/mL LPS for 2 h at 37$^{o}$C, and during the final 15 min samples were incubated in the absence or presence of 5 mM PRO. Samples were then incubated for a further 30 min in the absence or presence of 6 mM ATP. The amount of IL-1β release in cell-free supernatants was determined by ELISA. Results are mean ± SD ($n$=3); *$P < 0.05$, compared to LPS alone; $^{\dagger}P < 0.05$ compared to LPS and ATP.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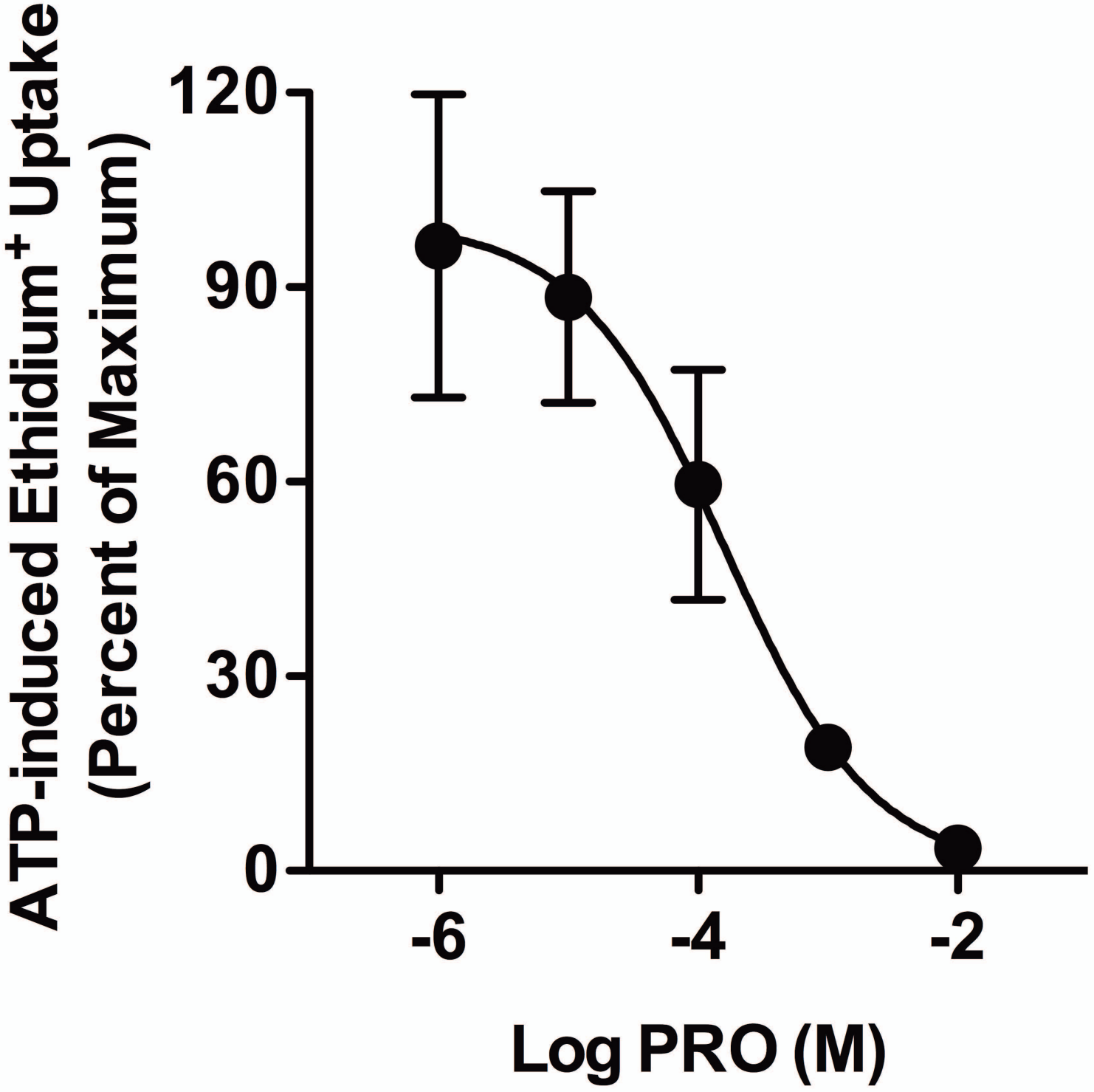
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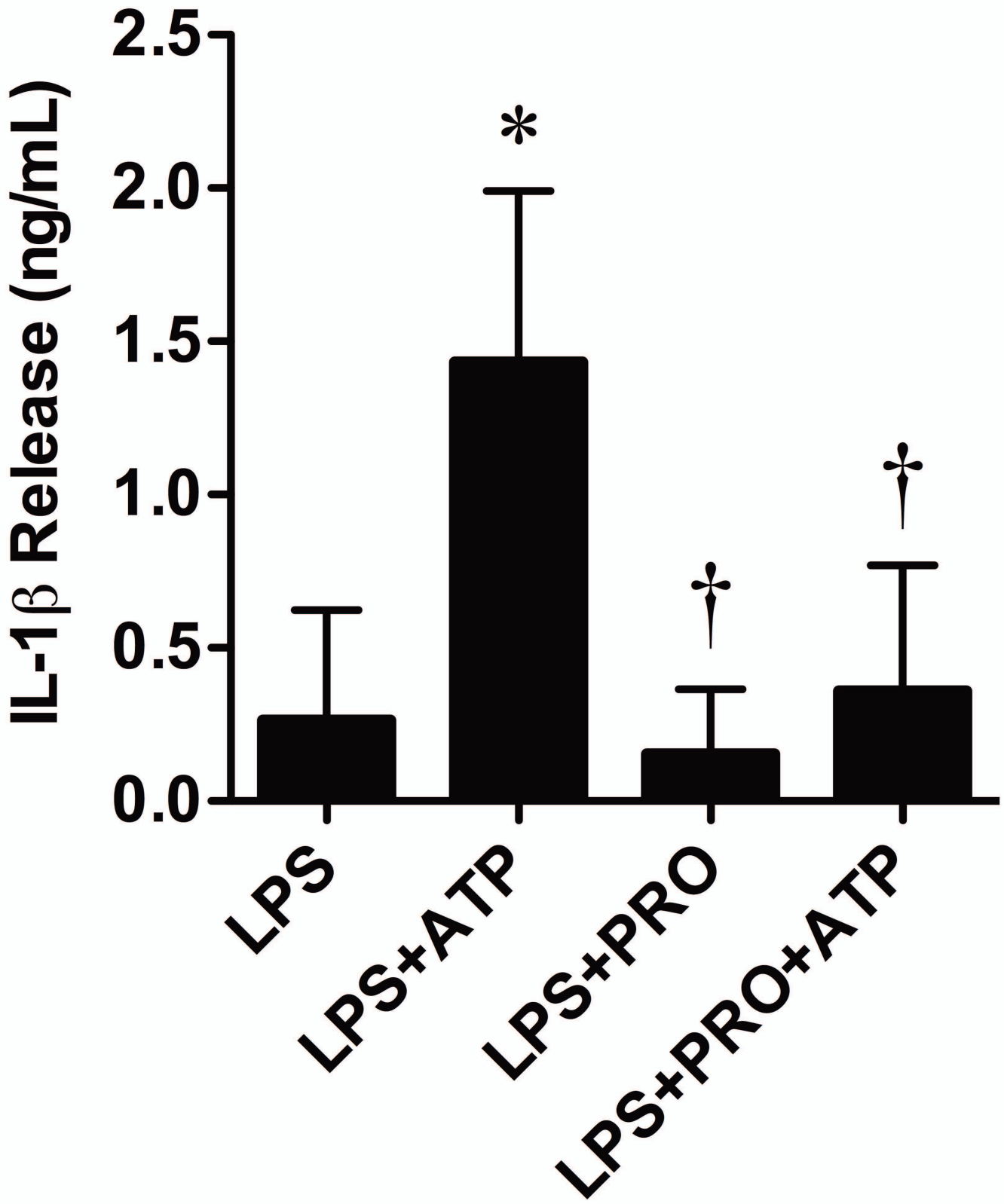
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