JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319

TITLE PAGE

Evidence for Multiple P2Y Receptors in Trabecular Meshwork Cells

Craig E. Crosson

Phillip W. Yates

Aruna N. Bhat

Yurii V. Mukhin

Shahid Husain

Departments of Ophthalmology (C.E.C., P.W.Y., A.N.B., S.H.), MUSC-Hewitt Laboratory of the Ola B. Williams Glaucoma Center, and Medicine (Y.V.M.), Medical University of South Carolina, Charleston SC

RUNNING TITLE PAGE

P2Y receptors in trabecular meshwork cells

Corresponding author and address for reprints:

Craig E. Crosson, Ph.D.

Storm Eye Institute

167 Ashley Ave

Charleston, SC 29425

Phone: 843-792-4193

Fax: 843-792-4096

E-mail: crossonc@musc.edu

No. of text pages (Abstract through Table): 25

No. of tables: 1

No. of figures: 6

No. of references: 31

No. of words in the *Abstract*: 246

No. of words in the *Introduction*: 372

No. of words in the *Discussion*: 1199

Section recommendation: Cellular & Molecular

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

The purpose of this study was to determine if functional purinergic P2 receptors are present in trabecular meshwork cells. The human trabecular cell line (HTM-3) and cultured bovine trabecular cells were used to assess the effects of P2 agonists on intracellular Ca^{2+} levels, extracellular signal-regulated kinase (ERK1/2) activation, and P2Y receptor expression. ATP, UTP, ADP and 2-methyl-thio-adenosine triphosphate (2-MeS-ATP) each produced a concentration-dependent increase in intracellular Ca^{2+} in bovine trabecular cells and the HTM-3 cell line. The addition of UDP did not produce any detectable rise in intracellular Ca^{2+}. Pretreatment with the $P2Y_1$ receptor antagonist, MRS-2179, blocked the ADP- AND 2-MeS-ATP-induced rise in intracellular Ca^{2+}. However, the ATP- or UTP-induced rise in intracellular Ca^{2+} was not inhibited by MRS-2179 pretreatment. The addition of ADP, 2-MeS-ATP, ATP or UTP were also found to activate the ERK1/2 signaling pathway. This activation of ERK1/2 was blocked by pretreatment with the MEK inhibitor, U-0126, or the PKC inhibitor, chelerythrine chloride, but not by MRS-2179. Analysis of mRNA from HTM-3 cells by RT-PCR revealed the expression of $P2Y_1$, $P2Y_4$, and $P2Y_{11}$ receptor subtypes. These data demonstrate that multiple P2Y receptors are present in trabecular cells. Our results are consistent with the idea that the mobilization of intracellular Ca^{2+}results from the activation of $P2Y_1$ and $P2Y_4$ receptors, while the activation of the ERK1/2 pathway results from the activation of $P2Y_4$ receptors alone. However, a role for the $P2Y_{11}$ receptors in mobilization of Ca^{2+}, or activation of the ERK1/2 pathway, cannot be discounted.

The existence of two families of purinergic receptors, P2X and P2Y that are activated by adenine or uracil nucleotides was proposed by Abbracchio and Burnstock (Abbracchio and Burnstock, 1994). To date, eight mammalian P2Y receptor subtypes and seven P2X receptor subtypes have been cloned and characterized pharmacologically. In general, mammalian P2Y receptors (i.e., $P2Y_{1,2,4,6,11}$) are coupled to the $G_{q/11}$ family of G proteins and stimulate the formation of inositol trisphosphate and diacylglycerol with subsequent mobilization of Ca^{2+} from intracellular stores (von Kugelgen and Wetter, 2000). Additionally, $P2Y_{11}$ receptors have been shown to stimulate adenyl cyclase. The $P2Y_{12\text{-}14}$ receptor subtypes are also G-protein coupled receptors, but have been found to primarily associate with Gi/o signaling systems (Abbracchio et al., 2003; Communi et al., 2001; Zhang et al., 2002). The P2X family of receptors function as ligand-gated cation channels (North, 2002).

The presence of adenine nucleotides in the humor of the eye has been known for some time (Greiner et al., 1991). Recent studies have also provided evidence that the activation of ocular P2 receptors can modulate IOP (Pintor et al., 2003). However, little is known about the expression and associated signaling events of P2 purinergic receptor subtypes in anterior segment tissues of the eye. The trabecular meshwork is a specialized region in the anterior chamber of the eye composed of connective tissue beams lined with smooth-muscle-like trabecular meshwork cells (Wiederholt et al., 2000). This meshwork forms the primary pathway for drainage of aqueous humor from the anterior chamber. Cells of the trabecular meshwork are thought to influence intraocular pressure (IOP) through their phagocytic actions (Tripathi and Tripathi, 1984), morphological changes altering intertrabecular space (Wiederholt et al., 2000), and influencing the extracellular matrix turnover (Shearer and Crosson, 2001; Yue, 1996). Consequently,

pharmacologic agents that target trabecular meshwork cells have the potential to regulate outflow resistance and IOP.

In these studies, we sought to determine if trabecular meshwork cells express receptors for adenine and uracil nucleotides, and begin to assess the signal transduction pathways coupled to these receptors. Our results show that trabecular meshwork cells express $P2Y_1$, $P2Y_4$, and $P2Y_{11}$ purinergic receptor subtypes. The activation of these receptors by P2 agonists leads to mobilization of intracellular Ca^{2+} and activation of the ERK1/2 MAP kinase pathway.

METHODS

Materials

Fetal bovine serum was obtained from HyClone Labs (Logan, UT) and DMEM was purchased from GIBCO-BRL (Grand Island, NY). Adenosine 5-triphosphate (ATP), 2-methyl-thio-triphosphate (2-MeS-ATP), adenosine 5-diphosphate (ADP), uridine 5-triphosphate (UTP), uridine 5-diphosphate (UDP), pyridoxal-phosphate-6-azophenyl-2,4-disulphonic acid (PPADS), MRS-2179, suramin, 8-sulfophenylthephylline (8-SPT), chelerythrine chloride and U-0126 were purchased from Sigma Chemical Company (St. Louis, MO). Fluo 3-AM was purchased from Molecular Probes (Eugene, OR).

Cell culture

Primary bovine trabecular cell cultures were established from trabecular meshwork explants by techniques described previously (Shearer and Crosson, 2001). Briefly, small strips of trabecular meshwork tissue were dissected from one or two eyes and homogenized by means of a Teflon hand-held homogenizer in DMEM containing 15% fetal bovine serum (FBS). The homogenized tissue was plated onto a 60 mm collagen-I-coated (Biocoat, Fort Washington, PA) cell culture plate and allowed to grow for 2 wk in DMEM containing 15% FBS. The resultant cells were harvested and plated onto polypropylene cell culture plates in DMEM containing 10% FBS. Second- or third-passaged cells were used in all studies. The transformed human trabecular meshwork cell line (HTM-3) was maintained on polypropylene cell culture plates and grown in DMEM containing 10% FBS (Pang et al., 1994). These cells were allowed to grow to approximately 80% confluence.

Determination of intracellular calcium

Intracellular free Ca^{2+} was determined using a fluorometric imaging plate reader (FLIPR) system (Molecular Devices, Sunnyvale, CA). Cells for intracellular Ca^{2+} measurements were sub-cultured into 96-well clear-bottom black microplates (Corning Costar; Cambridge, MA). On the day of each experiment, cells were incubated with 4 μmol/L fluo 3-AM (excitation at 488 nm, emission at 540 nm; Molecular Probes; Eugene, OR) in HEPES buffer (pH 7.4) containing 2.5 mmol/L probenecid for 1 hr at 37ºC. Cells were then washed four-times, placed in the FLIPR and each well of the microplate were monitored at 1.5 sec intervals over 6 minutes. Six wells were averaged for each individual value and experiments were repeated at least three times. In selected experiments, the contribution of extracellular Ca^{2+} to these responses was investigated by omitting Ca^{2+} from the incubation buffer and adding 2 mmol/L EDTA. In cross-desensitization studies, agonist treatments were separated by 2 minutes. For antagonist studies, cells were treated for 10 minutes with individual antagonists prior to the addition of P2 agonists.

Extracellular signal-regulated kinase (ERK) assay

Cells were maintained in serum-free medium for 16 hr before the addition of any agent. Cells were then treated with P2 agonists for 10 minutes. In experiments evaluating the P2 antagonist, U-0126, or chelerythrine chloride, cells were pretreated for 30 min with individual agents prior to the addition of the agonist. At the end of the incubation periods, cells were rinsed with ice-cold PBS and lysed by the addition of 0.5 mL of lysis buffer (50 mmol/L β-glycerophosphate, 20 mmol/L EGTA, 15 mmol/L $MgCl_2$, 1 mmol/L Na_3VO_4, 1 mmol/L dithiothreitol (DTT), and 1 μg/mL of a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). To determine the

level of ERK1/2 activation (phosphorylation), equivalent amounts of protein (15 μg) were loaded onto 12% SDS-polyacrylamide gels, proteins separated according to molecular weight using standard SDS-PAGE protocols, and transferred to a nitrocellulose membrane. Total ERK levels (phosphorylated and non-phosphorylated forms) were determined by immunoblot techniques using polyclonal anti-ERK1/2 antibodies (New England Biolabs, Inc., Beverly, MA). Bands were visualized by the addition of anti-rabbit HRP-conjugated secondary antibodies and ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Blots were then stripped by incubation in "stripping buffer" (62.5 mmol/L Tris [pH 6.7], 100 mmol/L β-mercaptoethanol, 2% SDS) for 30 min at 50ºC. The level of phosphorylated (activated) ERK1/2 was then determined by immunoblot analysis with polyclonal anti-phospho-ERK antibodies (New England Biolabs Inc., Beverly, MA) and visualized by the addition of anti-rabbit HRP-conjugated secondary antibodies and ECL reagents. Band densities were quantified by means of a BioRad Versa Doc Imaging System (Bio-Rad Laboratories, Hercules CA) and the level of phosphorylated ERK1/2 isoforms normalized for differences in loading, using band intensities from immunoblots of total ERK protein.

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from HTM-3 cells using a Trizol reagent RNA isolation kit (GIBCO BRL, Grand Island, NY, USA) according to the manufacturer's instruction. Two micrograms of total RNA were reversely transcribed for cDNA synthesis using SuperScript™ RNase H-Reverse Transcriptase and oligo (dT)-12-18 primer (Invitrogen, Carlbad, CA). Amplifications of targeted purinergic receptor cDNA were performed with specific primers that were designed based on GenBank nucleotide sequences. The PCR was allowed to proceed in a final volume of 20 μL in a

programmable Master Cycler Gradient Thermocycle (Eppendorf, Mansfield, TX) with the following settings: 5 min at 95ºC for initial denaturation followed by repeated cycles of denaturation at 95ºC for 3 min, primer annealing for 1 min at 55ºC, and extension at 72ºC for 1 min 30 sec. After the final cycle, further extension was allowed to proceed for another 10 min at 72ºC. The PCR products were resolved on a 1.0% ethidium bromide-stained agarose gel and then visualized under ultraviolet light transillumination. PCR product sizes were estimated from the migration of a DNA size marker run concurrently (1 kb plus DNA Ladder, Invitrogen Life Technologies, Carlsbad, CA). For each sample PCR was performed on RNA that had not been reversely transcribed to confirm that no genomic DNA was present in the samples. Positive reaction products were sequenced to confirm cDNA identity. Primers for each receptor were as follows: $P2Y_1$ (forward primer) TGTGGTGTACCCCCTCAAGTCCC (reverse primer) ATCCGTAACAGCCCAGAATCAGCA $P2Y_2$ (forward primer) GAGCATCCTGACCTGGAGAG (reverse primer) AGTGCATCAGACACAGCCAG, $P2Y_4$ (forward primer) CCACCTGGCATTGTCAGACACC (reverse primer) GAGTGACCAGGCAGGGCACGC, $P2Y_6$ (forward primer) CGCTTCCTCTTCTATGCCAACC (reverse primer) CCATCCTGGCGGCACAGGCGGC, and $P2Y_{11}$ (forward primer) ACAGAGCGTATAGCCTGGTG (reverse primer) ACTGCGGCCATGTAGAGTAG.

Statistical analysis

Data are presented as the mean, plus and minus the standard error (±SE), and were analyzed using analysis of variance followed by Duncan's multiple-range test for detecting differences, with $P < 0.05$ considered as significant. The dose–response curves were analyzed by nonlinear

regression analysis (GraphPad Software, Inc., San Diego, CA).

RESULTS

The effect of P2 agonists on Ca^{2+} mobilization. Exposure of HTM-3 or bovine trabecular cells to ATP, UTP or ADP (10^{-6} mol/L) produced a rapid increase in intracellular free Ca^{2+} concentration, peaking in 20-30 sec (Figure 1). The rise in intracellular Ca^{2+} was followed by a return to basal level in 60 to 80 seconds. At equivalent doses ATP and UTP produced similar increases in intracellular free Ca^{2+}; however, the rise in Ca^{2+} measured following ADP addition was consistently less than that observed for ATP or UTP. In cells incubated in Ca^{2+}-free buffer for 10 min, a rapid increase in intracellular free Ca^{2+} and subsequent decline in response to P2 agonists, was measured (data not shown). Figure 2 shows the concentration-response curve for peak rise in intracellular Ca^{2+} increase following the addition of various P2 agonists to BTM cells. The EC_{50} and response maxima for P2Y agonists in bovine primary cell cultures and the HTM-3 cell line are listed in Table 1. Except for UDP, all agonists produced a dose-related increase in intracellular Ca^{2+}; however, maximum response to ADP and 2-MeS-ATP was 35 to 45% lower than that observed for ATP and UTP.

To further characterize the P2 agonist-induced response in trabecular cells, the effects of $P2Y_1$-receptor antagonist, MRS-2179, and the nonselective P2 antagonists, suramin and PPADS, were evaluated. The increases in intracellular-free Ca^{2+} induced by 2-MeS-ATP and -ADP were blocked by the presence of MRS-2179 (10 μmol/L) (Figure 3). However, the ATP- and UTP-induced increase in Ca^{2+} mobilization was not altered by the presence of MRS-2179. Pretreatment of cells with nonselective P2 antagonists, suramin and PPADS (10 μmol/L) each significantly inhibited the ADP- and 2-MeS-ATP-induced rise in intracellular Ca^{2+} by 60 to 80%,

but did not significantly alter the responses to ATP or UTP. Pretreatment with the adenosine receptor antagonist, 8-SPT (10 μmol/L), did not significantly alter the rise in intracellular Ca^{2+} induced by any of the P2 agonists (data not shown).

To evaluate cross-desensitization between ATP and UTP, agonists were administered sequentially within a 2 minute interval. The addition of ATP (10 μmol/L) did not alter the subsequent addition of UTP. However, the addition of UTP (10 μmol/L) reduced the response to ATP by 26% (P <0.05).

Effects of P2 agonists on ERK1/2 activation. As shown in Figure 4, the addition of 10^{-7} mol/L ATP, UTP, ADP, and 2-MeS-ATP to bovine trabecular meshwork cells produced a significant increase in ERK1/2 phosphorylation. No increase in ERK1/2 activation was observed following the addition of UDP. In HTM-3 cells the addition of ATP, UTP, ADP and 2-MeS-ATP increased ERK1/2 activation by 233, 228, 247, and 190%, respectively. In both culture systems, the increase in ERK1/2 activation induced by each P2 agonist was not altered by pretreatment with MRS-2179, PPADS or suramin (data not shown).

To investigate the up-stream signaling events associated with P2 agonist-induced stimulation of ERK1/2, cells were pretreated with the MEK inhibitor, U-0126, or the PKC inhibitor, chelerythrine chloride. As shown in Figure 5, pretreatment of BTM cells with U-0126 (1.0 mol/L) blocked the ERK activation induced by ATP or UTP (10^{-7} mol/L). Pretreatment with the PKC inhibitor, chelerythrine chloride (20 μmol/L), also completely blocked the ATP- and UTP-induced ERK1/2 activation in these cells. In HTM-3 cells, pretreatment with U-0126 or

chelerythrine also completely blocked the ERK1/2 activation induced by ATP or UTP (10^{-7} mol/L).

Expression of P2Y receptor subtype mRNA in BTM and HTM-3 cells. To investigate the expression of P2Y-receptor subtypes, mRNA from human cell line (HTM-3) was analyzed by RT-PCR. As shown in Figure 6, mRNA for $P2Y_1$, $P2Y_4$, and $P2Y_{11}$ receptors was detected in HTM-3 cells. However, no message for $P2Y_2$ and $P2Y_6$ receptors could be detected in these cells.

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

Adenine nucleotides have been identified in the aqueous humor of animals (Greiner et al., 1991). However, our understanding of the role P2 receptors play in regulating anterior segment function has been limited. Although P2X receptors have been identified in the mammalian retina (Jabs et al., 2000; Wheeler-Schilling et al., 2001; Wheeler-Schilling et al., 2000), the expression of P2X receptors in anterior segment tissues have not been reported. In contrast, molecular and functional studies have provided evidence that P2Y receptors are expressed in a number of anterior segment tissues including corneal and ciliary epithelium, lens and conjunctiva (Collison and Duncan, 2001; Cowlen et al., 2003; Cullinane et al., 2001; Farahbakhsh and Cilluffo, 2002; Merriman-Smith et al., 1998). In this study, we investigated if trabecular meshwork cells express functional P2 receptors.

The addition of a P2 agonist to human or bovine trabecular cells produced a rapid rise in intracellular Ca^{2+}. This increase in intracellular Ca^{2+} did not appear to result from the activation of ionotropic P2X receptors, as it was not blocked by the incubation of cells in Ca^{2+}-free media. Although adenosine receptors have been identified on trabecular meshwork cells (Shearer and Crosson, 2002), the inability of the adenosine antagonist, 8-SPT, to alter the response to adenine and uracil nucleotides demonstrates that the activation of adenosine receptors did not contribute to responses observed in these studies. Taken together, these data support the idea that the responses induced by P2 agonists in trabecular cells are mediated by P2Y receptors.

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

The difference in response maxima (see Table 1) and the selective blockade of P2 agonists responses by MRS-2179 (see Figure 3), indicate that the activation of at least two P2Y-receptor subtypes can mobilize intracellular Ca^{2+} in trabecular cells. The moderately selective $P2Y_1$ agonists, ADP and 2-MeS-ATP, exhibited response maxima that were 35 to 45% lower than values determined for ATP and UTP. The EC_{50} values measured for ADP and 2-MeS-ATP are consistent with the EC_{50} for $P2Y_1$ receptors measured in other mammalian tissues (Nicholas et al., 1996; Pacaud et al., 1996; Palmer et al., 1998; Ralevic and Burnstock, 1996; Simon et al., 1995). In addition, the ADP- or 2-MeS-ATP-induced increase in intracellular Ca^{2+} was blocked by the pretreatment of $P2Y_1$ antagonist, MRS-2179. These data support the idea that both ADP- and 2-MeS-ATP stimulate the release of intracellular Ca^{2+} in trabecular cells through activation of the $P2Y_1$ receptor. The expression of $P2Y_1$ receptors in the HTM-3 cell line was confirmed by RT-PCR. The precise signal transduction pathways employed by $P2Y_1$ receptors in these cells are yet to be fully explored. Our results are consistent with other studies showing that $P2Y_1$ receptors are coupled to an increase in intracellular Ca^{2+} through a $G_{q/11}$/IP3 signaling system.

The increase in intracellular Ca^{2+} following the addition of UTP provides evidence that bovine and human trabecular cells also express one or more of the conventional uracil-sensitive P2Y receptors (i.e., $P2Y_2$, $P2Y_4$ or $P2Y_6$). The lack of any measurable increase in intracellular Ca^{2+} following UDP administration, and the absence of any detectable mRNA in RT-PCR analysis demonstrates that this response in not due to the activation of $P2Y_6$ receptors in these cells. As RT-PCR analysis also failed to detect $P2Y_2$ receptors in the human HTM-3 cell line, and that the calculated EC_{50} for UTP is similar to that reported for $P2Y_4$ receptors in other systems, the responses to UTP in these cells appears to result from $P2Y_4$ receptor activation. However, recent

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

studies have shown that UTP can mobilize intracellular Ca^{2+} by activating $P2Y_{11}$ receptors (White et al., 2003). As RT-PCR analysis did detect $P2Y_{11}$ receptor in HTM-3 cell line, we can not exclude the possibility that UTP mobilizes intracellular Ca^{2+} via the activation of $P2Y_{11}$ receptors.

Studies have shown that $P2Y_4$ receptors derived from rat and human cells can be activated by both ATP and UTP (von Kugelgen and Wetter, 2000). However, in $P2Y_4$ receptors derived from human cells ATP potency is normally less than that measured for UTP (von Kugelgen and Wetter, 2000). In this study, the calculated EC_{50}, response maxima, and antagonist sensitivity for ATP were very similar to that determined for UTP, for both human- and bovine-derived cells. Cross-desensitization studies also demonstrated that ATP treatment did not alter subsequent responses to UTP. These studies provide evidence that ATP and UTP responses are mediated by separate receptors. As ATP responses were not blocked by $P2Y_1$ antagonist, MRS-2179, the ATP-induced increase in intracellular Ca^{2+} appears to be mediated by $P2Y_{11}$ receptors expressed in these cells. However, the final determination will require the development of selective P2Y receptor antagonists.

Activation of the ERK1/2 pathway in trabecular cells has been shown to regulate matrix metalloproteinase secretion and cellular proliferation (Shearer and Crosson, 2001; Alexander and Acott, 2003; Jeon, 2003). In addition, the agents that alter IOP have been shown to regulate ERK1/2 activity in the trabecular meshwork (Shearer and Crosson, 2002). Hence, the activation of the ERK1/2 signaling pathway appears to play a central role in the regulation of trabecular function (Shearer and Crosson, 2002). The addition of ATP, UTP, ADP, or 2-MeS-ATP each

activated ERK1/2 in HTM-3 and bovine trabecular cells. The ATP- and UTP-induced activation of ERK1/2 was blocked by pretreatment with the MEK inhibitor, U-0126, or the PKC antagonist, chelerythrine chloride. These results are consistent with the idea that activation of P2Y receptors leads to mobilization of intracellular Ca^{2+}, activating calcium-sensitive PKC and eventually activating ERK1/2 in these cells. Unlike the Ca^{2+} mobilization studies, no significant difference in response magnitude was measured in these experiments. The activation of ERK1/2 by UTP in these studies indicates that uracil-sensitive $P2Y_4$ receptors are linked to ERK activation. Additionally, the inability of MRS-2179 to inhibit ERK activation induced by ADP or 2-MeS-ATP argues that the $P2Y_1$ receptor is not linked to this pathway. However, we cannot exclude the possibility that ERK activation observed following the addition of nucleotides also results from the activation of $P2Y_{11}$ receptors.

Original studies considered the trabecular meshwork a passive filter for aqueous humor, with changes in resistance to aqueous flow occurring indirectly through ciliary muscle contraction. Recent studies have demonstrated that trabecular cells are contractile in nature and are capable of modifying the extracellular matrix within the region, supporting the idea that these cells participate in the regulation of outflow resistance and IOP (Shearer and Crosson, 2001; Wiederholt et al., 2000; Yue, 1996). As the elevation in intracellular Ca^{2+} regulates trabecular cell contractility, and ERK has been shown to regulate matrix metalloproteinases, P2Y receptors may play an important role in regulation of trabecular function and aqueous outflow resistance. Recent studies have shown that P2 receptors modulate IOP (Pintor, 2003). Therefore, we speculate that pharmacological agents targeting trabecular P2Y receptors may prove to be efficacious agents for the treatment of glaucoma.

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

In summary, these data demonstrate that multiple P2Y receptors are present in human and bovine trabecular meshwork cells. Our results are consistent with the idea that the activation of $P2Y_1$, $P2Y_4$ and $P2Y_{11}$ receptors leads to the mobilization of intracellular Ca^{2+}. The stimulation of the ERK1/2 signaling pathway appears to result from the activation of $P2Y_4$ receptors via a PKC-dependent system. However, a role for the $P2Y_{11}$ receptor in the activation of this pathway cannot be excluded.

ACKNOWLEDGMENTS

The authors acknowledge critical review of the manuscript by L. Bartholomew, Ph.D.

REFERENCES

Abbracchio MP, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA and Burnstock G (2003) Characterization of the UDP-glucose receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* **24**:52-55.

Abbracchio MP and Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol Ther* **64**:445-475.

Alexander JP and Acott TS (2003) Involvement of the Erk-MAP kinase pathway in TNTalpha regulation of trabecular matrix metalloproteinases and TIMPs. *Invest Ophthalmol Vis Sci* **44**:164-169.

Collison DJ and Duncan G (2001) Regional differences in functional receptor distribution and calcium mobilization in the intact human lens. *Invest Ophthalmol Vis Sci* **42**:2355-2363.

Communi D, Gonzales NS, Detheux M, Brezillon S, Lannoy V, Parmentier M and Boeynaems J-M (2001). Identification of a novel human ADP receptor coupled to G_1. *J Biol Chem* **276**:41479-41485.

Cowlen MS, Zhang VZ, Warnock L, Moyer CF, Peterson WM and Yerxa BR (2003) Localization of ocular P2Y2 receptor gene expression by in situ hybridization. *Exp Eye Res* **77**:77-84.

Cullinane AB, Coca-Prados M and Harvey BJ (2001) Extracellular ATP effects on calcium signaling in cultured human non-pigmented ciliary body epithelium. *Curr Eye Res*, **23**:448-454.

Farahbakhsh NA and Cilluffo MC (2002) P2 purinergic receptor-coupled signaling in the rabbit ciliary body epithelium. *Invest Ophthalmol Vis Sci* **43**:2317-2325.

Greiner JV, Chanes LA and Glonek T (1991) Comparison of phosphate metabolites of the ocular humors. *Ophthalmic Res* **23**:92-97.

Jabs R, Guenther E, Marquordt K and Wheeler-Schilling TH (2000) Evidence for P2X(3), P2X(4), P2X(5) but not for P2X(7) containing purinergic receptors in Muller cells of the rat retina. *Brain Res Mol Brain Res* **76**:205-210.

Jeon JW, Lee SJ, Kim JB, Kang JJ, Lee JH, Seong GJ and Kim EK (2003) Cellular proliferative effect of dexamethasone in immortalized trabecular meshwork cell (TM5) line. *Yonsei Med J* **44**:299-306.

Merriman-Smith R, Tunstall M, Kistler J, Donaldson P, Housley G and Eckert R (1998) Expression profiles of P2-receptor isoforms P2Y1 and P2Y2 in the rat lens. *Invest Ophthalmol Vis Sci* **39**:2791-2796.

Nicholas RA, Watt WC, Lazarowski ER, Li Q and Harden K (1996) Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol Pharmacol* **50**:224-229.

North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* **82**:1013-1067.

Pacaud P, Feolde E, Frelin C and Loirand G (1996) Characterization of the P2Y-purinoceptor involved in the ATP-induced rise in cytosolic Ca2+ concentration in rat ileal myocytes. *Br J Pharmacol* **118**:2213-2219.

Palmer RK, Boyer JL, Schachter JB, Nicholas RA and Harden TK (1998) Agonist action of adenosine triphosphates at the human P2Y1 receptor. *Mol Pharmacol* **54**:1118-1123.

Pang IH, Shade DL, Clark AF, Steely HT and DeSantis L (1994) Preliminary characterization of a transformed cell strain derived from human trabecular meshwork. *Curr Eye Res* **13**:51-63.

Pintor J, Peral A, Pelaez T, Martin S and Hoyle CH (2003) Presence of diadenosine polyphosphates in the aqueous humor: their effect on intraocular pressure. *J Pharmacol Exp Ther* **304**:342-348.

Ralevic V and Burnstock G (1996) Discrimination by PPADS between endothelial P2Y- and P2U-purinoceptors in the rat isolated mesenteric arterial bed. *Br J Pharmacol* **118**:428-434.

Ralevic V and Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* **50**:413-492.

Shearer T and Crosson CE (2001) Activation of extracellular signal-regulated kinase in trabecular meshwork cells. *Exp Eye Res* **73**:25-35.

Shearer TW and Crosson CE (2002) Adenosine A_1 receptor modulation of MMP-2 secretion by trabecular meshwork cells. *Invest Ophthalmol Vis Sci* **43**:3016-3020.

Simon J, Webb TE and Barnard EA (1995) Characterization of a P2Y purinoceptor in the brain. *Pharmacol Toxicol* **76**:302-307.

Tripathi BJ and Tripathi RC (1984) Effect of epinephrine in vitro on the morphology, phagocytosis, and mitotic activity of human trabecular endothelium. *Exp Eye Res* **39**:731-744.

von Kugelgen I and Wetter A (2000) Molecular pharmacology of P2Y-receptors. *Naunyn Schmiedebergs Arch Pharmacol* **362**:310-323.

Wheeler-Schilling TH, Marquordt K, Kohler K, Guenther E and Jabs R (2001) Identification of purinergic receptors in retinal ganglion cells. *Brain Res Mol Brain Res* **92**:177-180.

Wheeler-Schilling TH, Marquordt K, Kohler K, Jabs R and Guenther E (2000) Expression of purinergic receptors in bipolar cells of the rat retina. *Brain Res Mol Brain Res* **76**:415-418.

White PJ, Webb TE and Boarder MR (2003) Characterization of a Ca^{2+} response to both UTP and ATP at human $P2Y_{11}$ receptors: evidence for agonist-specific signaling. *Mol Pharmacol* **63**:1356-1363.

Wiederholt M, Thieme H and Stumpff F (2000) The regulation of trabecular meshwork and ciliary muscle contractility. *Prog Retin Eye Res* **19**:271-295.

Yue BY (1996) The extracellular matrix and its modulation in the trabecular meshwork. *Surv Ophthalmol* **40**:379-390.

Zhang FL, Luo L, Gustafson E, Palmer K, Qiao X, Fan X, Yang S, Laz TM, Bayne M and Monsma Jr F (2002) $P2Y_{13}$: identification and characterization of a novel Galphai-coupled ADP receptor from human and mouse. *J Pharmacol Exp Ther* **301**:705-713.

FOOTNOTES PAGE

Supported in part by NEI grant EY-09741 and EY-014793 (C.E.C.), shared equipment grant S-10-RR-13005 (John Raymond), and an unrestricted grant to the Department of Ophthalmology from Research to Prevent Blindness, New York, NY.

LEGENDS FOR FIGURES

Figure 1. Examples of P2 agonists-induced changes in trabecular intracellular Ca^{2+} levels. Trabecular cells were loaded with 4 μmol/L fluo 3-AM in HEPES buffer (pH 7.4) for 1 hour at 37ºC and fluorescence monitored (see methods). Wells were treated with 10^{-6} mol/L of indicated nucleotide at t = 12 seconds. Panel **A** show the responses from primary cultures of bovine trabecular cells. Panel **B** shows responses from HTM-3 cell line.

Figure 2. Concentration-response curves for P2 agonist-induced increase in intracellular Ca^{2+}. Bovine trabecular meshwork cells were treated with a single P2 agonist and fluorescence monitored (see methods). For each agonist concentration, the difference between the peak response and pre-stimulus baseline was calculated as the percent of control. Values represent the means ± SE from 5 experiments.

Figure 3. Effect of the $P2Y_1$ antagonist, MRS-2179, on the P2 agonist-induced increase in intracellular Ca^{2+}. Bovine trabecular meshwork cells were pretreated with MRS-2179 (10 μmol/L) for 10 minutes prior to the addition of P2 agonists. Asterisks denote significant difference ($P < 0.05$) between agonist stimulation alone and agonist stimulation following pretreatment with MRS-2179 (n = 4).

Figure 4. Effects of P2 agonists on ERK 1/2 activation. Serum-deprived bovine trabecular cells were incubated for 10 minutes in the presence or absence (control) of individual P2 agonists (0.1 μmol/L). Panel **A** shows summary data from 5 experiments. Values are the means ± SE of

densitometry measurements from immunoblots cell lysates. Asterisks denote significant difference ($P < 0.05$) from control levels. Panel **B** shows representative immunoblots of phospho-ERK and total ERK from bovine trabecular cell lysates.

Figure 5. Inhibition of ERK1/2 activation by the MEK inhibitor U0126 and the PKC inhibitor chelerytherine. Representative immunoblots of phospho-ERK and total ERK from bovine trabecular cell lysates. Panel **A** shows responses from control cells, cells treated for 10 minutes with UTP (0.1 μmol/L) in the presence or absence of U0126 or chelerythrine for 30 minutes. Panel **B** shows responses from control cells, cells treated for 10 minutes with ATP (0.1 μmol/L) in the presence or absence of U0126 or chelerythrine for 30 minutes. Pathway inhibitors were added 30 minutes prior to the addition of ATP or UTP.

Figure 6. RT-PCR analysis of P2Y-receptor subtype expression in HTM-3 cells. For each panel, Lanes 1, 2 and 3 contain: RT product; non-RT product and genomic DNA, respectively.

TABLE PAGE

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Dose-response parameters for P2 agonist-induced mobilization of intracellular Ca^{2+} in trabecular meshwork cells.

	Bovine Trabecular Cells		**HTM-3 Cell Line**	
Agonist	**EC_{50}** (μmol/L)	**Response Maximum** (% Increase)	**EC_{50}** (μmol/L)	**Response Maximum** (% Increase)
ATP	0.58	428	0.27	287
UTP	0.37	419	0.20	267
ADP	0.35	269	0.053	177
2-MeS-ATP	0.070	225	0.048	172
UDP	ND	ND	ND	ND

Parameters for UDP were not determined (ND) as this agonist did not produce any significant elevation in intracellular Ca^{2+} at the concentrations tested.

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

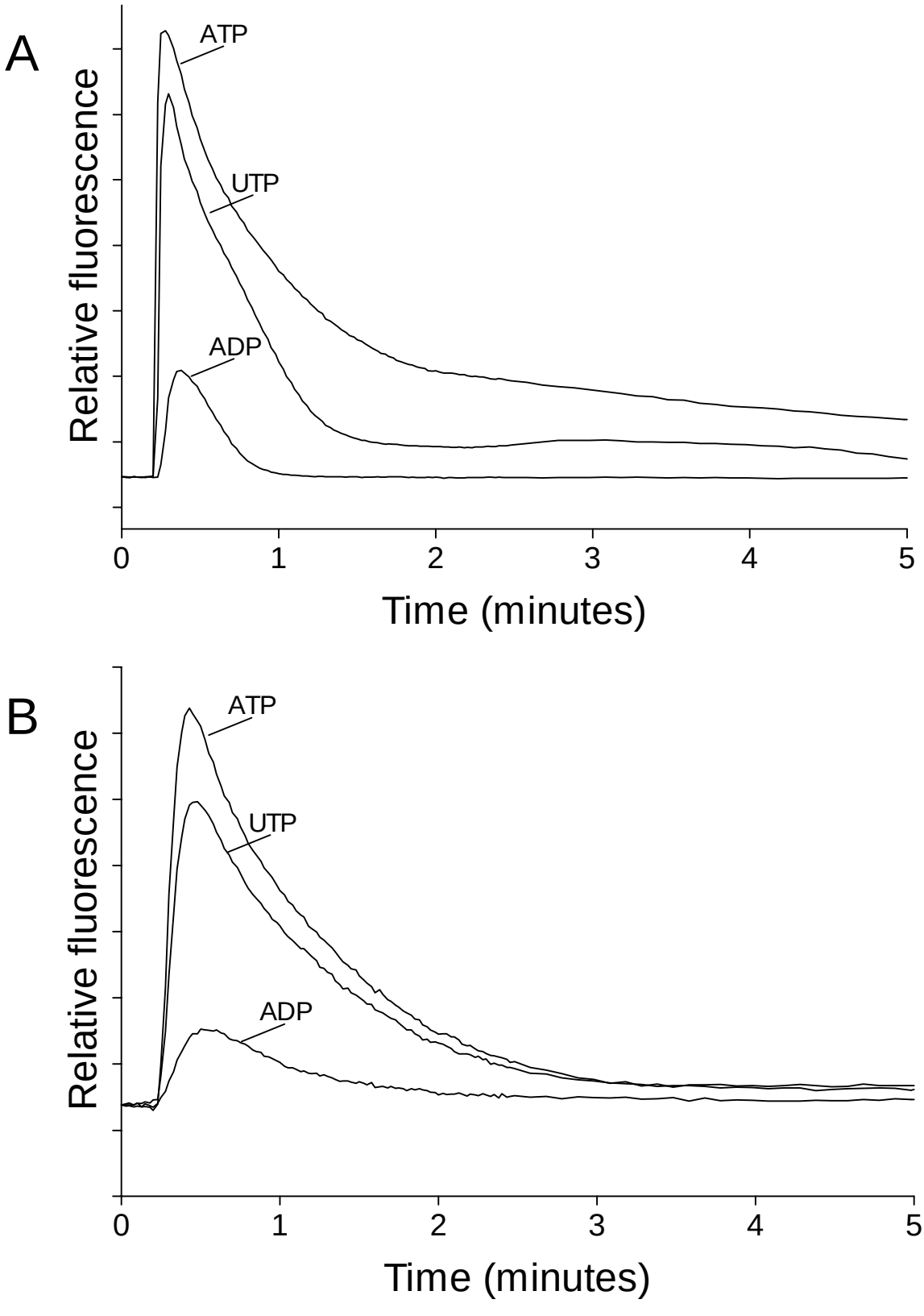

Figure1

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

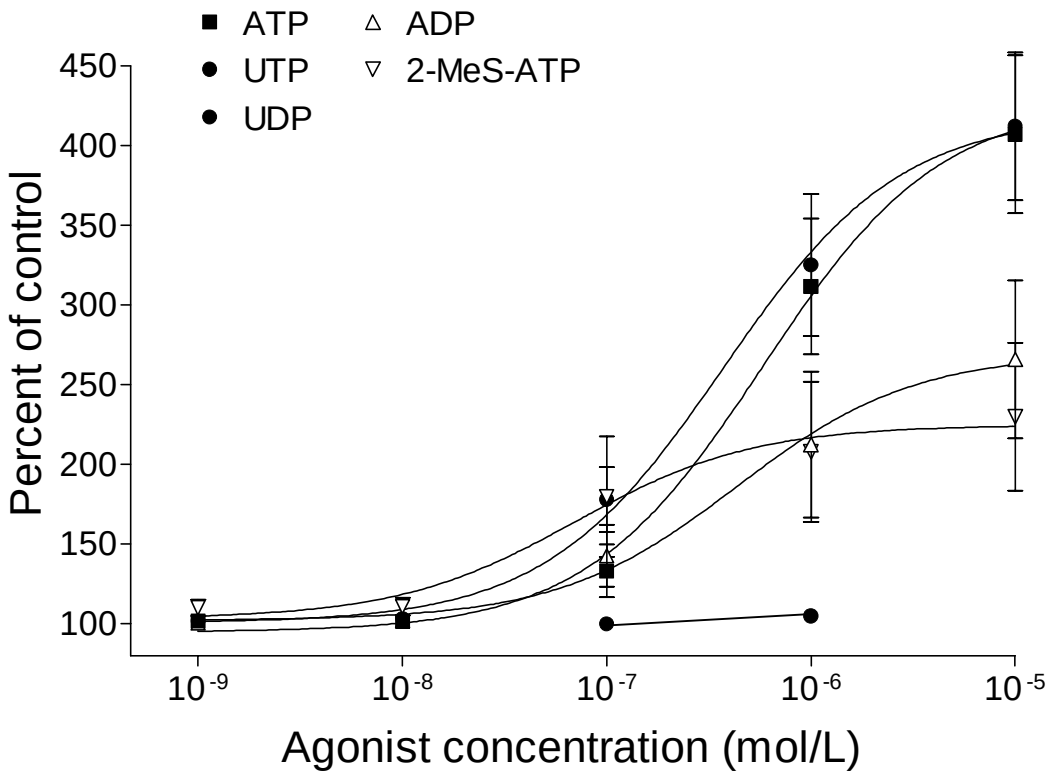

Figure 2

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

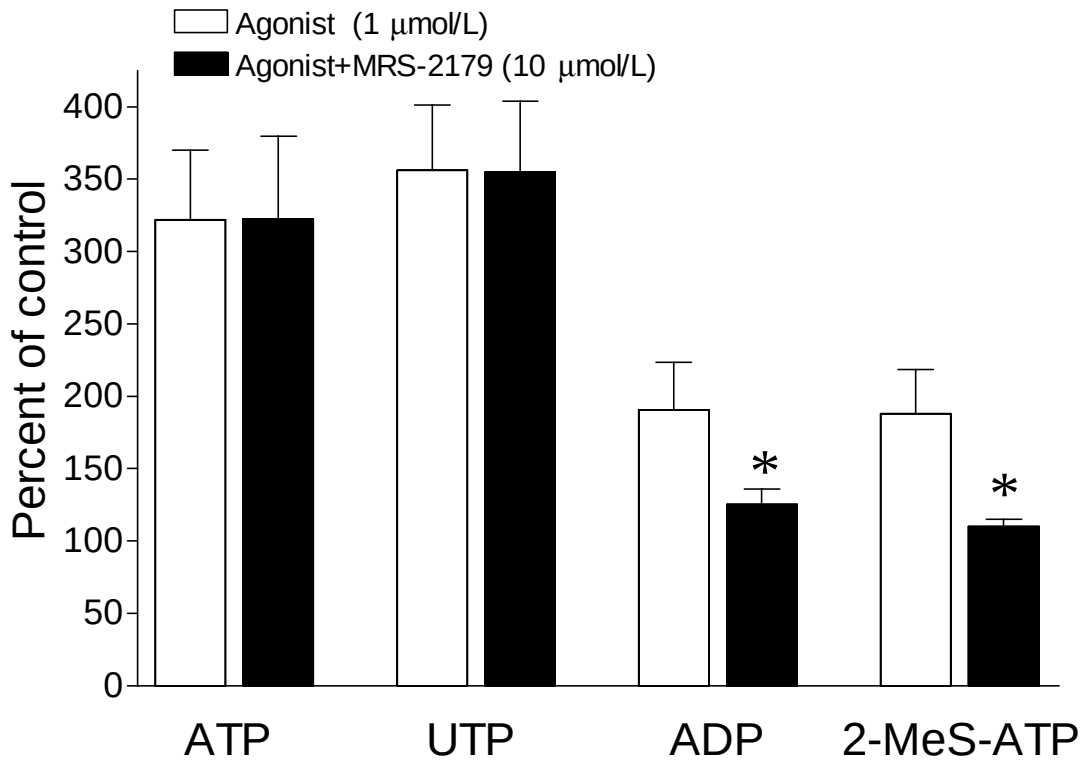

Figure 3

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

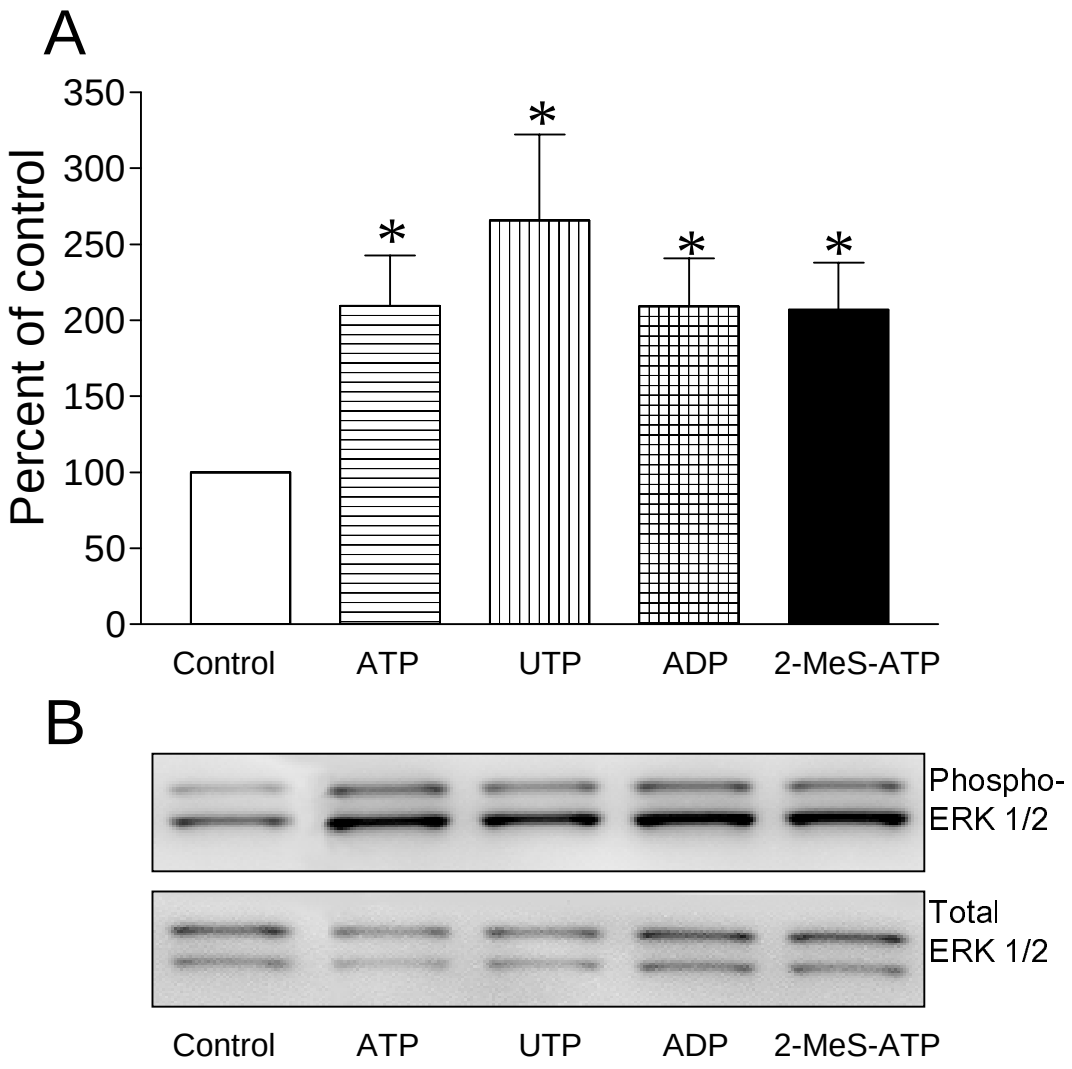

Figure 4

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

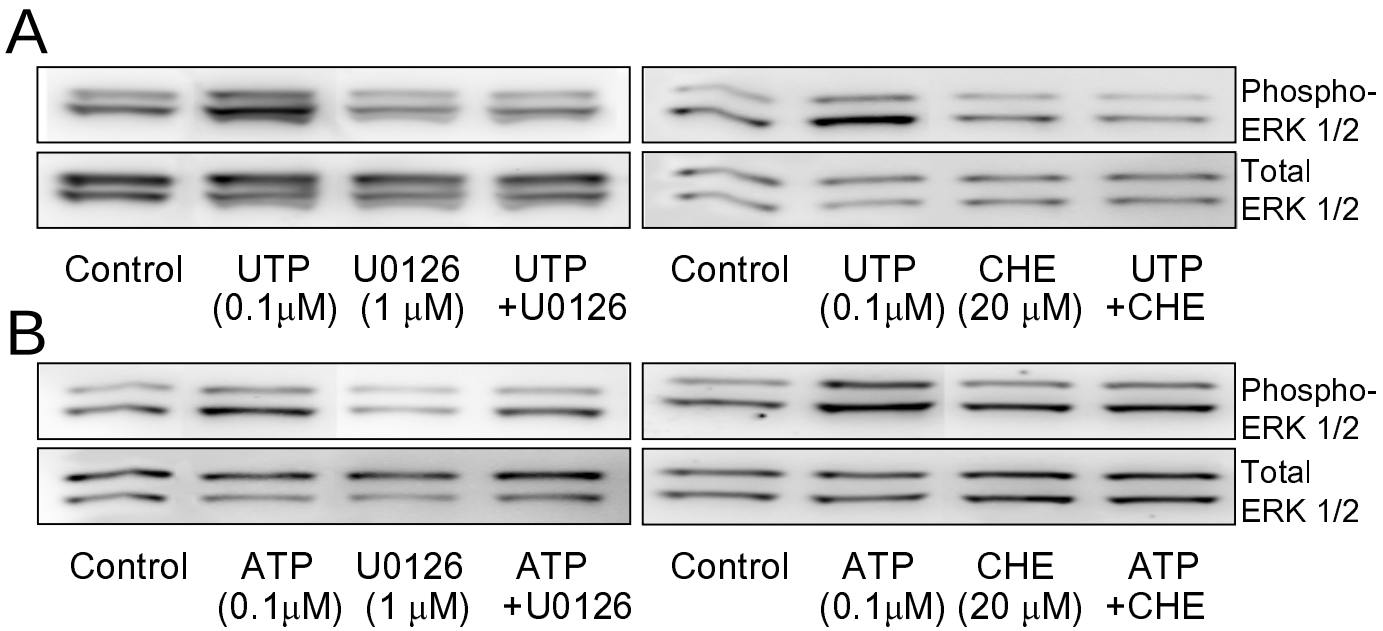

Figure 5

JPET Fast Forward. Published on January 23, 2004 as DOI: 10.1124/jpet.103.060319
This article has not been copyedited and formatted. The final version may differ from this version.

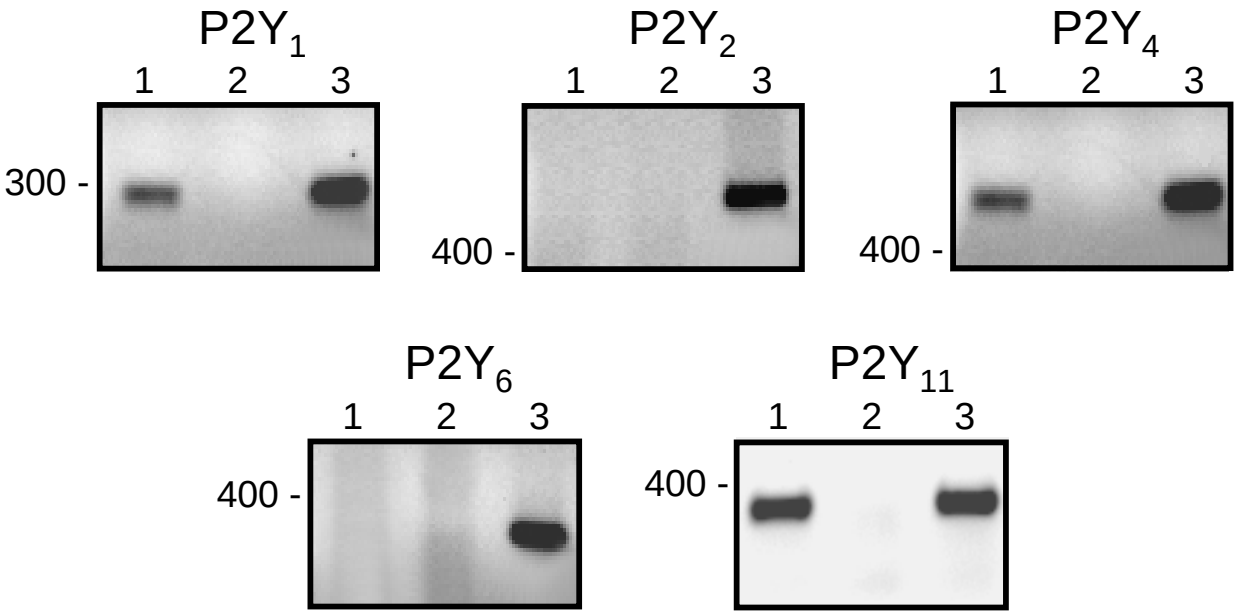

Figure 5