JPET Fast Forward. Published on June 9, 2005 as DOI: 10.1124/jpet.105.085274

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Effects of dinucleoside polyphosphates on trabecular meshwork cells and aqueous humor outflow facility

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Running title: dinucleotide effects in aqueous humor outflow

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Section: Cellular & molecular

Abbreviations: IOP, intraocular pressure; AH, aqueous humor; TM, trabecular meshwork; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle's Medium; *C*, outflow facility; Ap_3A, P^1,P^3-diadenosine triphosphate; Ap_4A, P^1,P^4-diadenosine tetraphosphate; Ap_5A, P^1,P^5-diadenosine pentaphosphate; 2-MeSADP, 2-(Methylthio) adenosine 5'-diphosphate; MRS2179, 2'-Deoxy-N^6-methyl adenosine 3',5'-diphosphate; Up_4U, diuridine tetraphosphate; PPADS, pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid; E-NPPase, ectonucleotide pyrophosphatase/phosphodiesterase.

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ABSTRACT

The most important risk factor for the development of glaucoma is elevated intraocular pressure (IOP). Hypotensive drugs decrease IOP preventing optic nerve damage and further vision loss. The balance between aqueous humor (AH) production and drainage determines IOP, and problems in AH outflow pathways are associated with open-angle glaucoma development. Previous studies have shown the presence of diadenosine tetraphosphate (Ap_4A) and pentaphosphate (Ap_5A) in the AH. Topic application of Ap_4A to the cornea decreased IOP while Ap_5A increased it. Since dinucleoside polyphosphates stimulate P2Y purinergic receptors, we studied their presence in trabecular meshwork (TM) cells. Additionally, the effects of diadenosine polyphosphates (Ap_nAs, n=3-5) and Up_4U (INS365) in outflow facility were tested. $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors were detected in TM cells by western blot and immunocytochemistry. In TM cells, Ap_3A, Ap_4A and Ap_5A induced discrete $[Ca^{2+}]_i$ mobilizations compared with higher and more sustained $[Ca^{2+}]_i$ mobilizations after Up_4U application. In bovine ocular anterior segments perfused at constant pressure, 1µM Ap_3A or Ap_4A increased outflow facility, whereas Up_4U or Ap_5A did not modify it. 2-MeSADP, a selective $P2Y_1$ agonist, induced outflow facility increases similar to those obtained after Ap_3A and Ap_4A, and these were prevented by addition of the selective $P2Y_1$ receptor antagonist MRS2179. Our results demonstrate that the hypotensive effect of Ap_4A and other dinucleotides is mediated, at least in part, by increasing trabecular outflow facility through activation of $P2Y_1$ receptors. The latter would appear to be an interesting target in the development of antiglaucomatous drugs to selectively increase AH outflow.

INTRODUCTION

In the eye, elevated intraocular pressure (IOP) due to impaired aqueous humor (AH) drainage is a major risk factor in the development of glaucoma. Glaucoma is the most important cause of irreversible non-traumatic blindness in the world and affects more than 66 million people. The main determinant of IOP is the volume of AH contained in the anterior and posterior chambers. AH is produced in the ciliary epithelium and eliminated through the outflow pathways in the anterior chamber angle. In several species, but especially in the primate eye, most AH flows through the trabecular meshwork (TM), a sponge-like filtering tissue, where it reaches Schlemm's canal and the collector channels before finally entering the venous system. The structure of the TM and its ability to modify its resistance to AH outflow play a key role in maintaining the IOP in the anterior chamber within its physiological range. TM cells express several receptors to substances released either in the AH from different ocular tissues (Mitchell et al., 1998; Coca-Prados et al., 1999) or from ocular innervation (Selbach et al., 2000) in physiological and pathological conditions (e.g. inflammation). Moreover, TM cells are thought to have autocrine/paracrine functions, releasing several agonists such as ATP or PGE_2 in response to various stimuli (Fleischhauer et al., 2003).

Several compounds have been identified as present in the aqueous humor, and among these, diadenosine polyphosphates have recently been found in rabbit aqueous humor (Pintor et al., 2003b). Diadenosine polyphosphates are a group of dinucleotides formed by two adenosine moieties bridged by a variable number of phosphates oscillating from 2 to 7 (Ap_nA, 2-7). Diadenosine tetraphosphate (Ap_4A) and diadenosine pentaphosphate (Ap_5A), in particular, have been found in similar concentrations to ADP and ATP (Pintor et al., 2003b). Moreover, Ap_4A degradation by phosphodiesterases is a source of other nucleotides such as

ATP (Pintor et al., 2003b). Diadenosine polyphosphates activate both P2X and P2Y purinergic receptors (Ralevic and Burnstock, 1998). The P2Y purinergic receptor subfamily comprises purine and pyrimidine nucleotide receptors coupled to G proteins. These receptors, cloned and characterized in different cell types, are known to activate phospholipase C (PLC), leading to IP_3 formation and intracellular Ca^{2+} mobilization (for review see (Ralevic and Burnstock, 1998). However, Ca^{2+} release from intracellular stores after P2Y activation has also been reported to stimulate a variety of signaling pathways including PKC, PLA_2, Ca^{2+}-dependent K^+ channels and nitric oxide synthase. In the eye, P2Y receptors have been found in different tissues including retina, ciliary body, cornea, conjunctiva, choroids and optic nerve head (Cowlen et al., 2003; Pintor et al., 2003b). Moreover, it is known that TM cells release ATP upon stimulation with hypotonic stimuli (Cui et al., 2001; Fleischhauer et al., 2003; Soto et al., 2004) or shear stress (Cui et al., 2001), and recently the presence of purinergic P2Y receptors has also been found in a human TM cell line (Crosson et al., 2004). However, a detailed study of the effects of purinergic receptor activation on outflow facility has yet to be conducted.

Recent studies by Pintor *et al.* (Pintor et al., 2003b) have found that application of certain dinucleoside polyphosphates to the eye modify IOP. In particular, Ap_4A has a hypotensive effect in the rabbit eye, while Ap_2A, Ap_3A and Ap_5A have the opposite effect. The ocular targets for dinucleoside polyphosphates have not been fully characterized. It is likely that some of these compounds modify aqueous humor production, but they may also affect outflow. From a physiological point of view, understanding how all these dinucleotides interact with ocular structures is important. Moreover, one of them, Ap_4A, presents interesting features from the therapeutic point of view, since it reduces IOP and therefore could be used in ocular hypertension and glaucoma treatment.

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In this study we identified the purinergic receptors present in TM cells and tested whether dinucleoside polyphosphates have any effects on intracellular calcium. Also, in a more physiological approach, we used a constant-pressure perfusion technique of ocular anterior segments to test the effects of dinucleotides on outflow facility. Our results show that Ap_4A and Ap_3A increase aqueous humor outflow, this not being the case of the others dinucleotides tested. Among the purinergic receptors identified in trabecular meshwork cells ($P2Y_1$, $P2Y_2$ and $P2Y_4$), the effects appear to be mediated by $P2Y_1$, since a selective $P2Y_1$ agonist (2-MeSADP) elicited similar effects.

METHODS

Bovine trabecular meshwork cell culture

Bovine TM cells were cultured using a modification of the technique described by Stamer *et al*. (Stamer et al., 1995). As described previously (Llobet et al., 1999), bovine TM strips were digested with 2mg/mL collagenase (Sigma, Madrid, Spain) and 0.5 mg/mL bovine serum albumin (BSA) (Sigma, Madrid, Spain) at 37^{o}C for 2 h. After mechanical digestion, the supernatant was collected, centrifuged and resuspended. The resuspended solution was seeded in culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM) (Bio-Whitaker, Barcelona, Spain) plus 10% fetal bovine serum, 100 mg/mL L-glutamine (Sigma, Madrid, Spain), 100 U.I./mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin-B (Bio-Whitaker, Barcelona, Spain). Cell growth was observed 2-4 days after seeding and cells reached confluence 12-15 days later. Cell passages were performed using Trypsin-EDTA (Bio-Whitaker, Barcelona, Spain). Cells from passages 1 to 3 were used.

Immunolabeling and western blotting with P2Y receptor antibodies

For the immunocytochemical study we used trabecular meshwork cells glued to coverslips pre-treated with poly-L-Lysine. Covers were then treated with 4% p-formaldehyde (w/v) for 15 min before being washed twice with phosphate buffered saline (PBS) medium. Cells were incubated overnight at room temperature in PBS containing 1% BSA and anti-P2Y primary antibodies (Alomone Labs., Israel). The dilutions of the primary antibodies were: anti-$P2Y_1$, 1/200; anti-$P2Y_2$, 1/500; anti-$P2Y_4$, 1/500, anti $P2Y_6$, 1/200 and anti $P2Y_{11}$ 1/1000. The covers were washed three times in PBS in the presence of 3% BSA and then incubated for 1 h with the secondary antibody which was also diluted in PBS/BSA solution. The secondary antibody used was goat anti-rabbit IgG-TRITC from Sigma (40 µg/mL). The

covers were washed three times with PBS and mounted following standard procedures. Controls were carried out by following the same procedures but substituting the primary antibody with the same volume of PBS/BSA solution. Cells were analyzed by confocal microscopy using a Zeiss Axiovert 200M microscope equipped with a LSM 5 Pascal confocal module. Trabecular meshwork cells were observed with a Zeiss 63X oil immersion lens, numerical aperture 1.40. TRICT was monitored by exciting at a wavelength of 543 nm. Differential interference contrast (Nomarski, DIC) was performed with the same 63X lens bypassed through the corresponding polarizers and analyzers. All the images were managed using the LSM 5 Pascal software.

For the western-blot analysis, trabecular meshwork cells were homogenized with a lysis buffer that contained Hepes 50 mM pH 7.5, Triton 2.5% (w/v), EDTA 10 mM, PMSF 0.2 mM and leupeptin 5 µg/ml. After homogenization, proteins were quantified by the Bradford method. Protein samples (40 µg) were separated by SDS-PAGE (10% acrylamide gel) using the Bio-Rad Mini-Protein® 3-Cell System. Proteins were transferred to nitrocellulose membranes. Following transfer, the membranes were washed with PBS and blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in PBS. Blots were then incubated overnight at 4ºC with primary antibodies in 5% (w/v) skimmed milk powder dissolved in PBS-Tween 20 (0.5% by volume). The dilutions of primary antibodies were as follows: anti-$P2Y_1$, 1/200, anti-$P2Y_2$, 1/500; anti-$P2Y_4$, 1/200, anti $P2Y_6$, 1/200 and anti $P2Y_{11}$ 1/1000. The primary antibodies were removed and the blots extensively washed with PBS/Tween 20. Blots were then incubated for 1 h at room temperature with the secondary antibody (mouse anti-rabbit IgG coupled to horseradish peroxidase, from Sigma, A-2074) at 1/1000 dilution in 5% (w/v) skimmed milk powder dissolved in PBS/Tween 20. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence detection system (Amersham).

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Cytosolic free Ca^{2+} measurement

Measurement of $[Ca^{2+}]_i$ was performed as described in detail previously (Llobet et al., 1999). Briefly, bovine TM cells were plated on 25 mm diameter glass coverslips (VWR Scientific Inc., Philadelphia, PA) and then loaded with 5 μM fura-2/AM (Calbiochem, San Diego, CA) for 25 min at 37°C in incubation buffer (121 mM NaCl, 4.7 mM KCl, 5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES and 0.01% BSA, at pH 7.4 with NaOH) (287±2 mOsm/Kg; mean±SD). Coverslips with fura-2 loaded cells were transferred into an open flow chamber (1 ml incubation buffer) mounted on the heated stage of a Nikon Diaphot-300 inverted epifluorescence microscope. Fluorescence images were obtained by a CCD camera (CH250, Photometrics, Tucson, AZ), and were digitized, stored and analyzed on an Apple Macintosh 840AV computer (Apple Inc, Cupertino, CA). After a stabilization period of 10 min, image pairs were obtained alternately every 4 s, and for a total of 8 min, at excitation wavelengths of 340 (λ1) and 380 nm (λ2; 10 nm bandwidth filters) in order to excite the Ca^{2+} bound and Ca^{2+} free forms of this ratiometric dye, respectively. The emission wavelength was 510 nm (120-nm bandwidth filter). Experiments were calibrated by measuring the minimum and maximum fluorescence ratio in fura-2 loaded cells in the absence of Ca^{2+} in the bath (R_{min}) and in the presence of Ca^{2+} + ionomycin (R_{max}). Calcium concentrations were calculated according to the formula $[Ca^{2+}]=K_d \cdot Q \cdot ((R-R_{min})/(R_{max}-R))$ where K_d is the Ca^{2+} dissociation constant of the dye, R is the fluorescence ratio $F_{\lambda1}/F_{\lambda2}$ and Q is the ratio of F_{min} to F_{max} at λ2. Typically, 10-20 cells were present in a field and $[Ca^{2+}]_i$ values were calculated and analyzed individually for each single cell from the 340- to 380-nm fluorescence ratios at each time point (Llobet et al., 1999). In both control and experimental groups, Ca^{2+} was recorded for 1 min before drug application

and for 7 min thereafter. Cells were considered as responders when $[Ca^{2+}]_i$ increased by more than 100% above the resting value. Drug responses in each field were homogeneous and several experiments with cells from different primary cultures were used to calculate dose-response curves. Dose-response curves for $[Ca^{2+}]_i$ were calculated using the maximum $[Ca^{2+}]_i$ increase in the first peak recorded after drug application.

Perfusion of anterior segments

Eyes from 3 to 6 month-old cows were obtained from the local abattoir 0.5 to 2 h after death and were kept in PBS at 4°C for not more than 1.5 h. Isolation of bovine anterior segments was performed as described previously (Gual et al., 1997). The perfusion technique has also been described elsewhere (Gual et al., 1997; Llobet et al., 1999). Briefly, bovine anterior segments were placed in a specially-designed perfusion chamber. The anterior segments, located in their respective chambers together with force transducers (Letica, Barcelona, Spain) and the tubing system, were placed in an incubator (Selecta, Barcelona, Spain) at 36°C and 5% CO_2. Perfusion was carried out with DMEM. The pressure of the artificial anterior chamber was monitored and recorded throughout the experiment with a pressure transducer (9162-0, Mallinckrodt, Northampton, U.K.) and was maintained with a suspended reservoir at 10 mmHg in bovine eyes. Outflow facility (C) was averaged over periods of 15 min (mean of 450 values). Baseline facility (C_0) was calculated during the first 90-min period of stable recording. When a drug was added to the perfusion medium, the tubes and anterior chamber were flushed and replaced with the new medium. This change was made by rapidly replacing the contents of the artificial anterior chamber by opening the exit needle until 200% of the volume had been exchanged; this exchange was always made at a pressure below 10 mm Hg. Recording of outflow facility measurements started after stabilization of flow.

The perfusion procedure was carried out using a protocol with three periods: perfusion with control isotonic DMEM for 90 min to establish the C_0 (baseline); 90 min perfusion with a drug (C_d); and finally, 90 min of perfusion with DMEM returning to the baseline conditions (C_{ret}). Outflow facility was calculated as the ratio between C_d/C_0 or C_{ret}/C_0.

Studies of the ecto-enzymatic degradation of diadenosine polyphosphates

Trabecular meshwork cells were plated in six-well dishes at a density of $3x10^6$ cells/well. DMEM was removed and cells were washed twice with PBS before commencing the degradation studies. To monitor the ecto-enzymatic degradation of diadenosine polyphosphates, cells were incubated with 3 mL, 1 µM of either Ap_3A, Ap_4A or Ap_5A at 37ºC. Aliquots of 50 µL were taken at 1, 5, 15, 30 and 60 min and analyzed by HPLC. The HPLC system consisted of a Waters 1515 Isocratic HPLC pump, a 2487 dual wavelength absorbance detector and a Reodyne injector, all controlled by the Breeze software from Waters. The column used was a NovaPak C18 (15 cm length, 0.4 cm diameter), also from Waters. The mobile phase consisted of 10 mM KH_2PO_4, 2 mM tetrabutyl ammonium, 17% acetonitrile, pH 7.5. Detection was monitored at a wavelength of 260 nm. In order to transform chromatographic peaks into concentrations, diadenosine polyphosphate peak areas were compared to external standards of known concentrations prepared from commercial dinucleotides.

Drugs

P^1,P^3-diadenosine triphosphate (Ap_3A), P^1,P^4-diadenosine tetraphosphate (Ap_4A), P^1,P^5-diadenosine pentaphosphate (Ap_5A), 2-(Methylthio) adenosine 5'-diphosphate (2-

MeSADP), 2'-Deoxy-N^6-methyl adenosine 3',5'-diphosphate (MRS2179), pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid (PPADS) and Suramin sodium salt were obtained from Sigma (Madrid, Spain). Diuridine tetraphosphate (Up_4U; INS365) was kindly provided by Inspire Pharmaceuticals.

Data analysis

Results are given as mean±SEM. Results were statistically analyzed using paired or unpaired Student's *t*-test, Fisher's exact test, and χ^2-test. Two-way ANOVA plus Bonferroni post tests were used to evaluate statistical differences between control and drug effects on outflow facility. P-values lower than 0.05 were considered significant.

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RESULTS

Localization of P2Y receptors in trabecular meshwork cells

Primary cultured trabecular meshwork cells were used 3-5 days after passage. Under the Nomarski interferential technique, the cells presented an elongated aspect as observed in Figure 1. Trabecular meshwork cells were incubated with commercially available P2Y receptor antibodies. Among the antibodies tested only $P2Y_1$, $P2Y_2$ and $P2Y_4$ positively labeled the cells (Fig. 1A). The other P2 antibodies used ($P2Y_6$ and $P2Y_{11}$) produced no positive result in these cells (Fig. 1A). Western blot analysis performed as described in the Methods section, gave similar results to those observed in the immunocytochemistry studies. Blots presented single bands for $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors as shown in Figure 1B. No bands were detected when the antibodies for $P2Y_6$ and $P2Y_{11}$ were assayed in the western blots (Fig. 1B), this being consistent with the immunocytochemical results described above. The band molecular weights of 70 kDa for $P2Y_1$, 50 kDa for $P2Y_2$ and 92 kDa for $P2Y_4$ are in line with those described in the literature (Pintor et al., 2004).

Effects of dinucleoside polyphosphates on intracellular calcium

In other cell types, dinucleoside polyphosphates are known to activate P2Y subtype purinergic receptors, which in turn stimulate PLCβ, IP_3 production and Ca^{2+} release from internal stores (Ralevic and Burnstock, 1998). Therefore, we tested this hypothesis by stimulating cultured TM cells with different dinucleotides while recording the intracellular calcium concentration ($[Ca^{2+}]_i$). Resting $[Ca^{2+}]_i$ in TM cells was found to be 85.5±3 nM (mean±SEM; n=120 experiments). In the control group, only 3% of cells (n=275) increased

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$[Ca^{2+}]_i$ after drug vehicle application.

As described in the Methods section, cells, when stimulated with dinucleotides, were considered as responders when $[Ca^{2+}]_i$ increased by more than 100% above the resting value. All the nucleotides tested (Ap_3A, Ap_4A, Ap_5A and Up_4U) mobilized $[Ca^{2+}]_i$ in TM cells, but the response patterns, the peak characteristics, and the potencies of each agonist varied between them.

Ap_3A and Ap_4A elicited similar $[Ca^{2+}]_i$ responses (Fig 2). Response percentages at each concentration are shown in Figure 3A and Table 1. At 10^{-5} M, Ap_3A induced one Ca^{2+} peak in 74% of responder cells (n=85), while Ca^{2+} oscillations were observed in the remainder. Ap_4A (10^{-5}M) elicited Ca^{2+} oscillations in 60% of responder cells (n=60) and only one peak in the rest. At the same concentration as the previous drugs, Ap_5A induced only one Ca^{2+} peak in all the responder cells (100%; n=14). Calculated EC_{50} from dose-response curves (Fig. 3A) were $1.1x10^{-5}$M for Ap_3A, $6.1x10^{-5}$M for Ap_4A and $4.5x10^{-5}$M for Ap_5A. Figure 3B and Table 1 show $[Ca^{2+}]_i$ increases induced by Ap_3A, Ap_4A and Ap_5A at concentrations ranging from 10^{-8} to 10^{-3}M. To further characterize the calcium responses, we also calculated T_{70}, as the time from the calcium peak until the cell has recovered 70% of the calcium increase. Ap_3A and Ap_4A (at 10^{-5}M) showed similar T_{70} values of 33.7±4.3 and 33.2±1.6 s (mean±SEM), respectively. Calcium peaks for Ap_5A were brief and recovered faster, within 15.0±2.1 s.

In contrast to the above results, Ca^{2+} mobilizations induced by Up_4U were consistently different from the effects induced by diadenosine polyphosphates. At 10^{-5}M, Up_4U increased $[Ca^{2+}]_i$ in 74% of cells (Table 1). Most of the responder cells (78%; n=102) elicited only one

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calcium peak. Ca^{2+} oscillations were rarely observed. Examples of these responses are shown in Fig. 2. Sigmoid fitting (Fig 3A) shows that Up_4U was the most potent agonist in mobilizing $[Ca^{2+}]_i$, it having an EC_{50} of $6.8x10^{-7}$M, about two orders of magnitude lower than the other dinucleotides tested. When comparing the amplitude of the calcium peaks (Fig 3B) Up_4U had an EC_{50} of $1.3x10^{-6}$M but a reduced maximal response. Finally, Up_4U calcium peaks where longer in duration, with a T_{70} of 123.4±7.5 s, as shown in Fig 2 and Table 1.

Taken together, these data and the individual Ca^{2+} responses shown in Figure 2 reveal a clear difference between the responses induced by Ap_3A, Ap_4A and Ap_5A and those elicited by Up_4U. These distinct response patterns suggest that Up_4U activates different intracellular mechanisms from the other drugs. We did not observe any correlation between cell morphology and responsiveness to the different dinucleotides or the calcium peaks elicited.

Effects of dinucleoside polyphosphates on outflow facility

We perfused ocular anterior segments at constant pressure, as described previously (Gual et al., 1997; Llobet et al., 1999), and evaluated the effects on trabecular outflow facility of the same dinucleotides tested in the calcium study. All the compounds were tested at 10^{-6}M since the physiological concentrations of dinucleotides found in rabbit aqueous humor were in the micromolar range (Pintor et al., 2003b). Control experiments with perfusion medium alone (DMEM; n=9) showed no significant variations during the 300 min perfusion protocol. In contrast, both Ap_3A (n=8) and Ap_4A (n=8) increased outflow facility significantly ($p<0.001$; two-way ANOVA *vs.* control perfusion; Fig 4A and B). Interestingly, Ap_4A increased outflow facility right after adding the drug to the perfusion medium while Ap_3A effects were slower to develop. Nevertheless, by the end of the perfusion experiments both drugs had increased outflow facility by a similar amount. In contrast, neither Ap_5A (n=8) nor

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Up_4U (n=8) increased outflow facility significantly (Fig. 4C and D). In fact, both drugs slightly decreased outflow facility, although no significant changes were found compared with the control perfusion or with their respective baseline perfusion periods. These results suggest that the reported hypotensive effects of Ap_4A in the eye (Pintor et al., 2003b) may be mediated, at least in part, by an increase in trabecular outflow facility.

Involvement of the $P2Y_1$ receptor in outflow facility modulation

Ap_3A and Ap_4A can activate both $P2Y_1$ and $P2Y_2$ purinergic receptors (Ralevic and Burnstock, 1998; Hoyle et al., 2001). In contrast, Up_4U is only active on $P2Y_2$. Since Up_4U did not induce significant changes in outflow facility, we tested the hypothesis that Ap_3A and Ap_4A effects on outflow facility may be mediated by $P2Y_1$ receptor activation. Stimulation of TM cells with the selective $P2Y_1$ agonist 2-MeSADP (1 μM) increased $[Ca^{2+}]_i$ in 80.3±8.2% (mean±SEM; n=234 cells) of cells ($p<0.001$ *vs.* control; Fig. 5A and B). The mean $[Ca^{2+}]_i$ increase was 384.5±10 nM (n=234) while T_{70} was 24.9±1.0 s, these values being similar to those observed for Ap_3A and Ap_4A. To further characterize the effects of $P2Y_1$ activation we tested $[Ca^{2+}]_i$ mobilizations induced by 2-MeSADP after pre-incubation of TM cells with two $P2Y_1$ antagonists, MRS2179 and PPADS. At 10 μM both compounds reduced significantly the percentage of cells responding to 2-MeSADP ($p<0.01$ and $p<0.001$, respectively; Fig. 5A). In contrast, suramin (100 μM), a non-selective $P2Y_2$ antagonist, did not significantly reduce 2-MeSADP effects on $[Ca^{2+}]_i$ (Fig. 5A). When 2-MeSADP (1 μM; n=10) was tested on trabecular outflow facility, it increased outflow facility significantly ($p<0.001$; two-way ANOVA *vs.* control perfusion; Fig 5C), thus providing evidence of $P2Y_1$ receptor involvement in outflow facility modulation. In agreement with this result, 2-MeSADP (1 μM) was unable to increase outflow facility in the presence of the selective $P2Y_1$ receptor

antagonist MRS2179 (10 μM; n=5; Figure 5C). Finally, we tested whether the effect of Ap_4A on outflow facility was also blocked by the $P2Y_1$ receptor antagonist. 10 μM MRS2179 partially blocked an Ap_4A-induced increase in outflow facility ($p<0.001$; two-way ANOVA *vs.* Ap_4A alone; Fig 5D; n=8) showing that at least part of the effect of this compound is mediated by activation of $P2Y_1$ receptors.

Ecto-enzymatic degradation of diadenosine polyphosphates

The lack of effect on outflow facility observed for Ap_5A could be due to the degradation of this dinucleotide by ecto-nucleotidases. To test this hypothesis, we studied the degradation of Ap_5A by TM cells compared with that for Ap_4A, a compound which had proven to have an effect on outflow facility. TM cells plated in multi-wells in a number of $3x10^6$ cells/well were studied in terms of their ability to cleave diadenosine polyphosphates. Incubation of 1 μM Ap_4A or Ap_5A showed that both were slowly degraded, although the former was more resistant to cleavage than the latter (Fig. 6A). The degradation time-course of both dinucleotides is shown in Figure 6B. It is clear that in the conditions under which the experiments were performed, and for all the times examined, Ap_5A was degraded faster than Ap_4A. After 1 h in the presence of the corresponding dinucleotide, TM cells were able to degrade 18% of Ap_4A and 44% of Ap_5A (Fig. 6B). It is important to emphasize that it was very difficult to detect any mononucleotide in the incubation medium due to the slow rate of degradation. This may reflect the fact that the rate of transformation of the generated mononucleotides by means of other ecto-nucleotidases, such as ecto-ATPase, ecto-ADPase, apyrase and ecto-5'nucleotidase, needs to be higher than the one that degrades diadenosine polyphosphates.

DISCUSSION

Diadenosine polyphosphates are natural compounds that control numerous physiological functions (Hoyle et al., 2001). For example, they have been found in tears (Pintor et al., 2002a), where they increase tear secretion (Pintor et al., 2002b). Thus, synthesized pharmacological compounds with related structures (e.g. Up_4U) have been proposed for dry eye treatment and topical application of Ap_4A accelerates the rate of re-epithelialization after corneal wound healing (Pintor et al., 2003a). In the retina, where P2X and P2Y purinergic receptors have been found, synthetic nucleotidic compounds such as INS37217 increase fluid absorption in rat models of retinal detachment (Maminishkis et al., 2002). Interestingly, micromolar concentrations of dinucleotides have been detected in rabbit AH, together with other mononucleotides (Pintor et al., 2003b). When tested in rabbit eyes, Ap_2A, Ap_3A and Ap_5A induced a dose-dependent increase in IOP, while Ap_4A produced the opposite effect (Pintor et al., 2003b). Here, the finding that Ap_3A and Ap_4A increase trabecular outflow facility leads us to hypothesize that, at least in part, the hypotensive effect of Ap_4A in rabbit eyes is mediated by an increase in aqueous outflow. When instilled topically, dinucleotides are likely to stimulate purinergic receptors present in the TM to increase AH outflow, as well as other purinergic receptors present in the eye, such as those in the ciliary processes (Farahbakhsh and Cilluffo, 2002), regulating inflow simultaneously. Since the effects of Ap_4A on IOP were tested in rabbits and our study has been performed in bovine eyes, the results cannot be fully extrapolated. Nevertheless, the hypotensive effect of Ap_4A seems to be well correlated with the increase in outflow facility found here. In contrast, Ap_3A, which also increased outflow facility, had an hypertensive effect in rabbit eyes. This effect may be due to the different selectivity of each agonist for the purinergic receptors stimulated and the receptor population present in the inflow and outflow pathways of the

rabbit.

We describe the presence of $P2Y_1$, $P2Y_2$ and $P2Y_4$ in bovine TM cells. In contrast, the immunocytochemistry and western blot results, appear to clearly indicate the absence of $P2Y_6$ and $P2Y_{11}$ purinoceptors. Using the same antibodies, it was possible to identify these receptors in rat ocular structures such as the corneal epithelium ($P2Y_6$) and the retinal pigmented epithelium ($P2Y_{11}$), and to confirm the existence of $P2Y_1$ and $P2Y_2$ in sections containing the TM (Pintor et al., 2004). Other authors have also reported the presence of $P2Y_1$ and $P2Y_2$ receptors in bovine TM cells (Cui et al., 2001) and $P2Y_1$, $P2Y_4$ and $P2Y_{11}$ in a human TM cell line (Crosson et al., 2004). Ap_nAs is known to activate several different purinergic receptors: Ap_3A and Ap_4A both activate $P2Y_1$ receptors with different selectivity , while Ap_5A is, in general, less effective at this receptor (Schachter et al., 1996). Furthermore, Ap_4A is a good agonist at $P2Y_2$ and $P2Y_4$ receptors, and although Ap_3A and Ap_5A can also activate these receptors, they do so with less affinity. Finally, Up_4U exhibits comparable potency with UTP as an agonist for $P2Y_2$ and $P2Y_4$ receptors, it being inactive at $P2Y_1$ (Pendergast et al., 2001). P2Y receptors act via a $G_{q/11}$ protein coupling to activate PLCβ, IP_3 formation and mobilization of $[Ca^{2+}]_i$, although coupling to adenylyl cyclase, PLA_2, PKC, NO synthase or BK_{Ca} channels activation has also been described (Ralevic and Burnstock, 1998).

In TM cells, all the Ap_nAs tested increased $[Ca^{2+}]_i$ in a dose-dependent manner (Figs. 2, 3) with similar EC_{50}. Compared with Up_4U, Ap_nAs exhibited a different pattern of $[Ca^{2+}]_i$ mobilization, with faster transients and frequent induction of Ca^{2+} oscillations. In contrast, Up_4U induced Ca^{2+} transients with longer durations, similar to the Ca^{2+} increases triggered by ATP (Crosson et al., 2004; Soto et al., 2004), bradykinin (Llobet et al., 1999) and carbachol (Shade et al., 1996). Interestingly, drugs that induce large and sustained Ca^{2+} mobilizations

either decrease outflow facility (bradykinin, carbachol and endothelin-1) (Wiederholt et al., 1995; Llobet et al., 1999) or do not modify it (Up_4U and ATP; current paper and Soto & Gasull unpublished observations). Large and long lasting Ca^{2+} increases may lead to cellular contraction or activation of different intracellular pathways, producing a decrease in TM permeability and thus reducing outflow facility. In fact, TM contractions have been described after application of carbachol or endothelin-1, known also to decrease outflow facility (Wiederholt et al., 1995; Wiederholt et al., 2000).

Our study suggests that Ca^{2+} mobilizations produced by Ap_3A and Ap_4A are not correlated with the observed outflow facility increase. Three observations support this hypothesis: 1) Up_4U increased intracellular Ca^{2+} but did not modify outflow facility; 2) Ap_5A mobilized intracellular Ca^{2+} with similar response patterns to those of Ap_3A and Ap_4A, but again, without modifying outflow facility; and 3) Ap_3A and Ap_4A increased outflow facility at a concentration (1 μM) at which only about 15% of the cells elicited Ca^{2+} peaks. Although we cannot rule out the possibility that even in a small population of cells these fast Ca^{2+} transients and Ca^{2+} oscillations could lead to rhythmic activation of certain enzymes such as Ca^{2+}/calmodulin-dependent protein kinase II (De Koninck and Schulman, 1998) or PKC (Oancea and Meyer, 1998) that may be involved in outflow facility regulation by TM cells, this explanation seems less plausible.

This and other studies (Wiederholt et al., 2000) suggest that *contractile* effects (e.g. cell contraction, PKC activation) would predominate after large Ca^{2+} mobilizations, while *relaxing* effects may be preferentially activated by discrete Ca^{2+} transients or by other signaling pathways (NO synthase, adenylyl cyclase, prostaglandin release). The balance between the *relaxing* (likely to increase outflow facility) and *contractile* effects (which would

lead to outflow facility reduction) would determine the permeability of the TM to the passage of aqueous humor (Wiederholt et al., 2000). In this regard, Ap_3A and Ap_4A appear to activate some of these *relaxing* mechanisms.

Other mechanisms proposed as playing important roles in TM function include changes in extracellular matrix (Tian et al., 2000), upregulation/downregulation of genes (Borras, 2003), and changes in cell shape (Gills et al., 1998) or cell volume (Al-Aswad et al., 1999; Soto et al., 2004). It is possible that dinucleotides could modify some of these parameters such as cell volume, as proposed for other compounds (Fleischhauer et al., 2003; Srinivas et al., 2004). Indeed, stimulation of adenosine A_1, A_{2A} and A_3 receptors reduces TM cell volume (Fleischhauer et al., 2003) and their agonists reduce IOP by increasing outflow facility and decreasing inflow (Crosson, 2001). This effect on outflow facility could be mediated by TM cell shrinkage (Fleischhauer et al., 2003). As seen in other tissues (Sumiyoshi et al., 1997), it is possible that dinucleotides raise cAMP concentration, which has also been linked to outflow facility increase (Erickson-Lamy and Nathanson, 1992; Gilabert et al., 1997). As previously shown, cAMP-mediated reduction in TM cell volume (Srinivas et al., 2004) could increase outflow facility. Finally, since activation of the NO/cGMP pathway has also been involved in aqueous outflow facilitation (Kee et al., 1994) we cannot rule out the possibility that Ap_nAs may induce NO release in TM cells as reported in other cell types (Hilderman and Christensen, 1998).

The finding that selective (2-MeSADP) and non-selective (Ap_3A and Ap_4A) $P2Y_1$ agonists increase outflow facility together with the lack of effect observed by the $P2Y_2/P2Y_4$ agonist (Up_4U), leads us to propose $P2Y_1$ as a specific receptor linked to an increase in TM permeability. This seems reinforced by the fact that the $P2Y_1$ antagonist MRS2179 blocks the

increase induced by 2-MeSADP and significantly reduces the effect of Ap_4A on outflow facility. Therefore, $P2Y_1$ receptors appear to be a suitable target for antiglaucoma therapy to improve aqueous humor outflow through the trabecular meshwork.

Independently of the receptor subtype activated by diadenosine polyphosphates, the existence of ectonucleotidases with the ability to cleave these molecules into mononucleotides has been preliminarily demonstrated in the present study. However, we can only speculate which of the cloned ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPase) is involved in the differential rate of hydrolysis of Ap_4A and Ap_5A (Vollmayer et al., 2003). This cleavage is slow, and does not allow the accurate measurement of the mononucleotides generated after dinucleotide cleavage. This reflects the fact that the rate of transformation of the generated mononucleotides by means of ecto-ATPase, ecto-ADPase, ecto-apyrase and ecto-5'nucleotidase needs to be higher than that which degrades diadenosine polyphosphates. Indeed, in chromaffin cells, the V_{max} for ATP degradation is almost 1000-fold higher than that for Ap_4A (Torres et al., 1990). It is important to note that either mononucleotides or adenosine, both of which are products of the dinucleotide cleavage, may contribute to the total facilitatory effect triggered by Ap_4A and Ap_3A. ATP, due to its fast degradation in comparison with the time necessary to measure the dinucleotide action in the facilitation studies, does not seem to be so relevant. In fact, we have not observed significant effects of ATP on outflow facility (Soto & Gasull, unpublished observations).

It will be interesting in the future to study the cross-talk between dinucleotides, mononucleotides and adenosine in TM cells to understand the importance of the purinergic system regulating aqueous humor physiology.

ACKNOWLEDGEMENTS

The authors thank Dr. Miguel Morales, Dra. Elisa Ferrer and Núria Comes for their support during the study and their helpful comments on the manuscript, as well as Dan Forsblade for his help with English grammar.

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FOOTNOTES

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Work supported by BFI2002-01202, BFI2003-01190, PI031495, SAF2003-00338, and FEDER UCOM03-33-024, Spain.

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LEGENDS FOR FIGURES

Figure 1

Detection of P2Y purinergic receptors in trabecular meshwork cells.

A. Immunolabeling of primary cultured trabecular meshwork cells. Cells were analyzed by confocal microscopy and differential interference contrast (Nomarski, DIC) with 63X objective. Antibodies against P2Y receptors showed positive staining to $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors and negative to $P2Y_6$ and $P2Y_{11}$. Goat anti-rabbit IgG-TRITC (40 μg/mL) was used as the secondary antibody. **B.** Western blotting showing the presence of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors in a trabecular meshwork cells. $P2Y_6$ and $P2Y_{11}$ provided negative results, as did the immunocytochemistry shown in A. The dilutions of primary antibodies were as follows: anti-$P2Y_1$, 1/200, anti-$P2Y_2$, 1/500; anti-$P2Y_4$, 1/200, anti-$P2Y_6$, 1/200; anti-$P2Y_{11}$, 1/1,000. The secondary antibody (mouse anti-rabbit IgG coupled to horseradish peroxidase) was at 1/1000. Bands at expected molecular weights are shown: 70 kDa for $P2Y_1$, 50 kDa for $P2Y_2$ and 92 kDa for $P2Y_4$.

Figure 2

Intracellular calcium mobilizations induced by dinucleoside polyphosphates in trabecular meshwork cells. Representative experiments of TM cells stimulated (arrow) with Ap_3A (A, B), Ap_4A (C, D), Ap_5A (E) and Up_4U (INS365; F) at 10^{-5}M. A, C, E, F show representative experiments in cells where these drugs only induced a single $[Ca^{2+}]_i$ peak. B and D show examples of cells that elicited multiple calcium peaks or calcium oscillations. Observe the different pattern of Ca^{2+} responses elicited by Up_4U (larger and more sustained Ca^{2+} increases) and diadenosine polyphosphates (smaller and briefer).

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Figure 3

Dose-response effects of dinucleoside polyphosphates on $[Ca^{2+}]_i$. Sigmoid fittings for the percentage of trabecular meshwork cells responding to each agonist (**A**) and the dose-response increase in Ca^{2+} in the responding cells (**B**). Up_4U (△) was the most potent agonist to mobilize $[Ca^{2+}]_i$ with an EC_{50} of 0.68 μM about two orders of magnitude lower than the other dinucleotides tested. Ap_3A (○), Ap_4A (●) and Ap_5A (□) each showed about the same potency in terms of stimulating $[Ca^{2+}]_i$ release with EC_{50} of 11 μM, 61 μM and 45 μM, respectively.

Figure 4

Effects of dinucleoside polyphosphates on outflow facility. Outflow facility ratio (normalized with baseline outflow facility) is plotted against time. Anterior segments were perfused with control medium (DMEM) for 90 min (baseline) and then perfusion medium was then exchanged for DMEM+drug (1 μM) for a further 90 min. Finally, a return period of 90 min with DMEM was carried out (same conditions as the baseline). Control perfusion group was done with DMEM in the absence of drugs (■; n=9). Ap_3A (**A**; ○; n=8) and Ap_4A (**B**; ◇; n=8) increased outflow facility significantly ($p<0.001$; two-way ANOVA *vs.* control perfusion). Neither Ap_5A (**C**; □; n=8) nor Up_4U (**D**; △; n=8) change outflow facility significantly.

Figure 5

Involvement of $P2Y_1$ receptor in outflow facility modulation. A. Stimulation of TM cells with the selective $P2Y_1$ agonist 2-MeSADP (1 μM) increased $[Ca^{2+}]_i$ in 80.3±8.2% of cells. Pre-incubation of TM cells with two selective $P2Y_1$ antagonists, MRS2179 (10 μM) and PPADS (10 μM), reduced significantly the percentage of cells responding to 2-MeSADP

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(**p<0.01 and ***p<0.001, respectively; Fisher's exact test). Suramin (100 μM), a non-selective P2Y antagonist, did not significantly reduce 2-MeSADP effects on $[Ca^{2+}]_i$. (results are mean±SEM). **B.** Representative $[Ca^{2+}]_i$ response to 2-MeSADP at 1 μM. **C.** 2-MeSADP (1 μM; ○; n=10) increased outflow facility significantly (p<0.001; two-way ANOVA) vs. control (■; n=11) in anterior segment perfusion experiments. When MRS2179 (10 μM; □; n=5) was present in the perfusion medium, it selectively blocked the effect of 2-MeSADP (1 μM) on aqueous humor outflow. **D.** Ap_4A (1 μM; ◇; n=8) increased outflow facility significantly (p<0.001; two-way ANOVA) *vs.* control (■; n=11) in anterior segment perfusion experiments. When MRS2179 (10 μM; ○; n=8) was present in the perfusion medium, it partially blocked the effect of Ap_4A (1 μM) on aqueous humor outflow (p<0.001; two-way ANOVA *vs.* Ap_4A alone).

Fig 6

Ecto-enzymatic degradation of diadenosine polyphosphates. TM cells plated in multi-wells in a number of $3x10^6$ cells/well were studied in terms of their ability to cleave diadenosine polyphosphates. Incubation of single concentrations of 1 μM demonstrated that both dinucleotides were slowly degraded, although Ap_4A was more resistant to cleavage than Ap_5A**. A.** Chromatographic composition of the corresponding profiles of Ap_4A and Ap_5A areas after the incubation with TM cells for 0, 1, 5, 15, 30 and 60 min (indicated by the labels on the corresponding peaks). The peaks occurred at the same retention time, but the starting point has been adjusted in the figure for each peak to allow comparison of peak magnitude. **B.** Time-dependent degradation of Ap_4A and Ap_5A. After 1 h in the presence of the corresponding dinucleotide, TM cells were able to degrade 18% of Ap_4A and 44% of Ap_5A.

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TABLE

Table 1. Effects of dinucleotides on $[Ca^{2+}]_i$ levels in cultured BTM cells

Drug	% Response (n)[1]	Resting $[Ca^{2+}]_i$ (nM)	$[Ca^{2+}]_i$ Increase (1^{st} peak) (nM)	T_{70} (s)	% Cells with oscillations
Control (Vehicle)	3% (275)	85	-	-	-
Ap_3A 10 μM	32% (269)	71	299	33.7	26%
Ap_4A 10 μM	20% (301)	66	257	33.2	60%
Ap_5A 10 μM	7% (188)	73	294	15.0	0%
Up_4U 10 μM	74% (137)	113	369	123.4	22%
2MeSADP 1 μM	80% (290)	88	384	24.9	25%

[1](n) total number of cells studied. BTM, bovine trabecular meshwork. $[Ca^{2+}]_i$ peaks were considered significant when they were at least two times baseline levels. $[Ca^{2+}]_i$ increase was measured in the first Ca^{2+} peak elicited after drug application.

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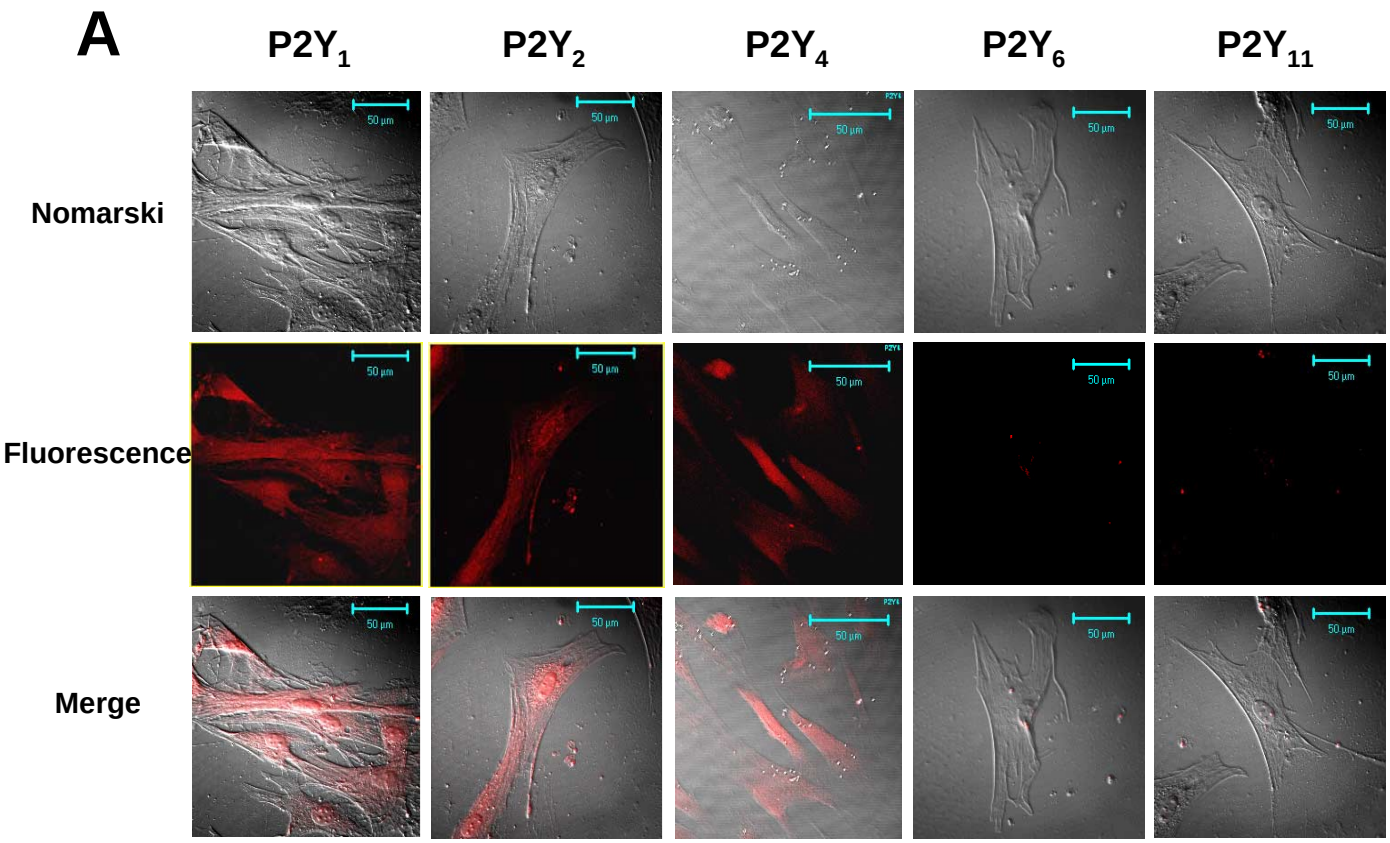


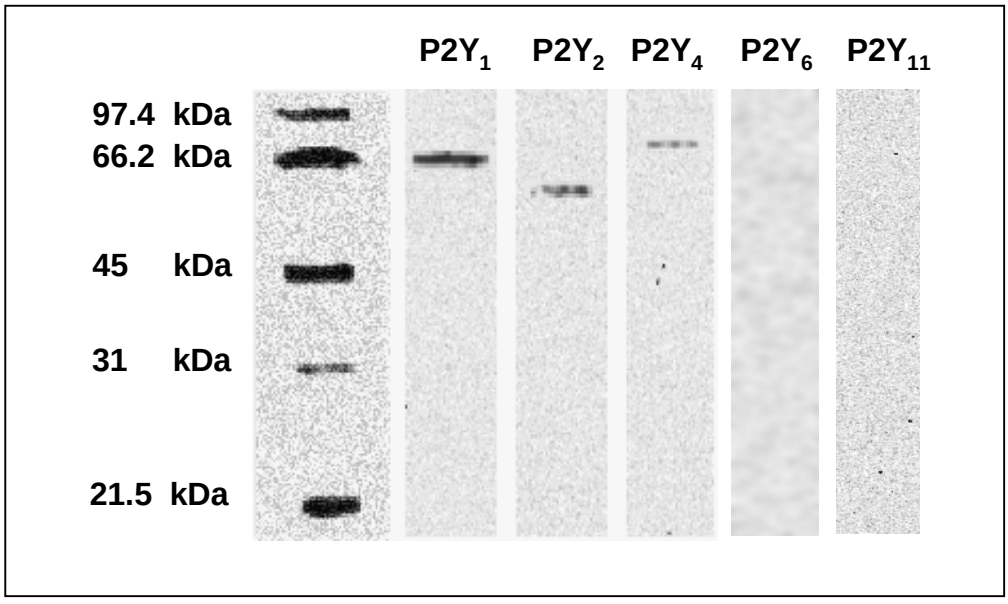


Fig 1

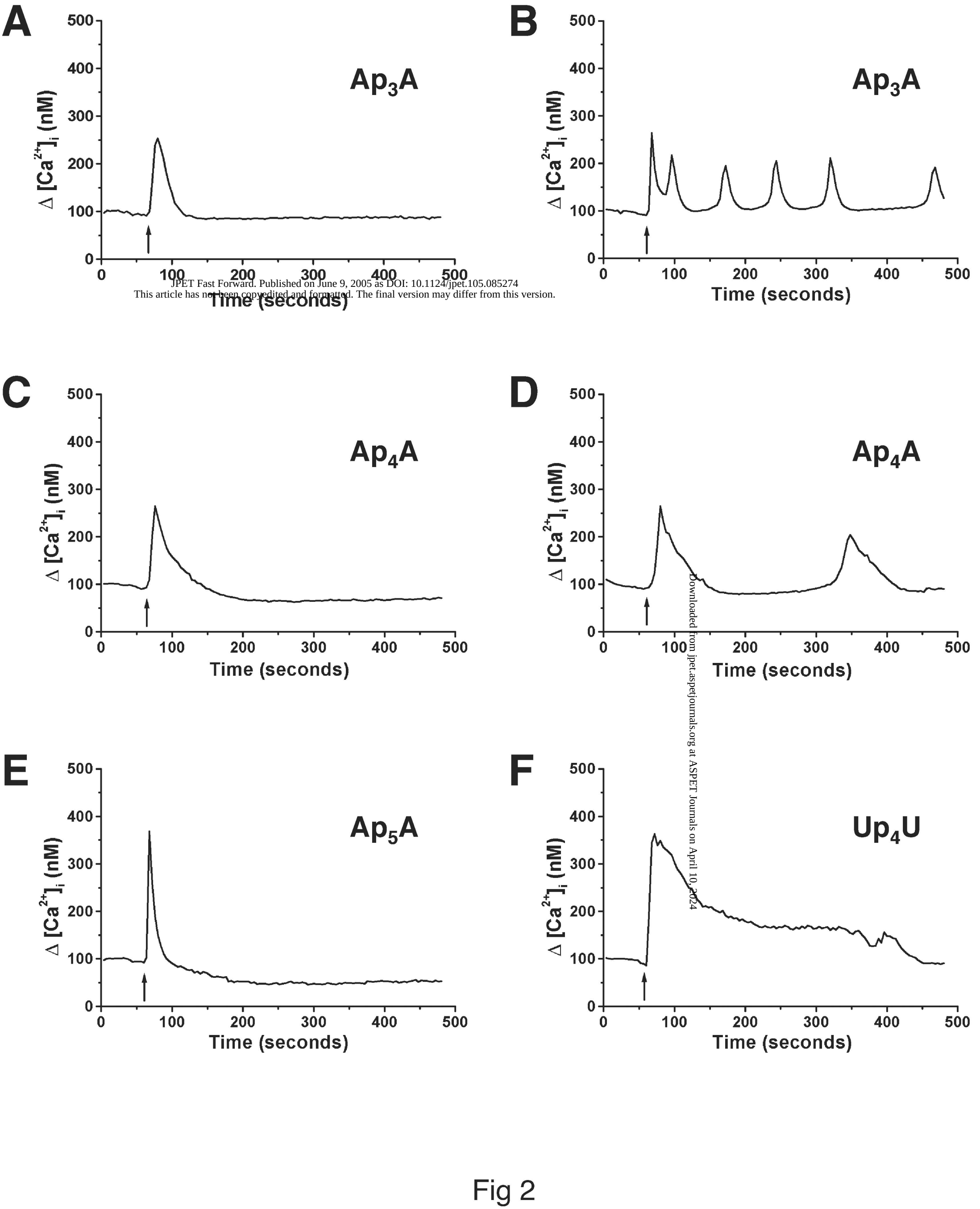
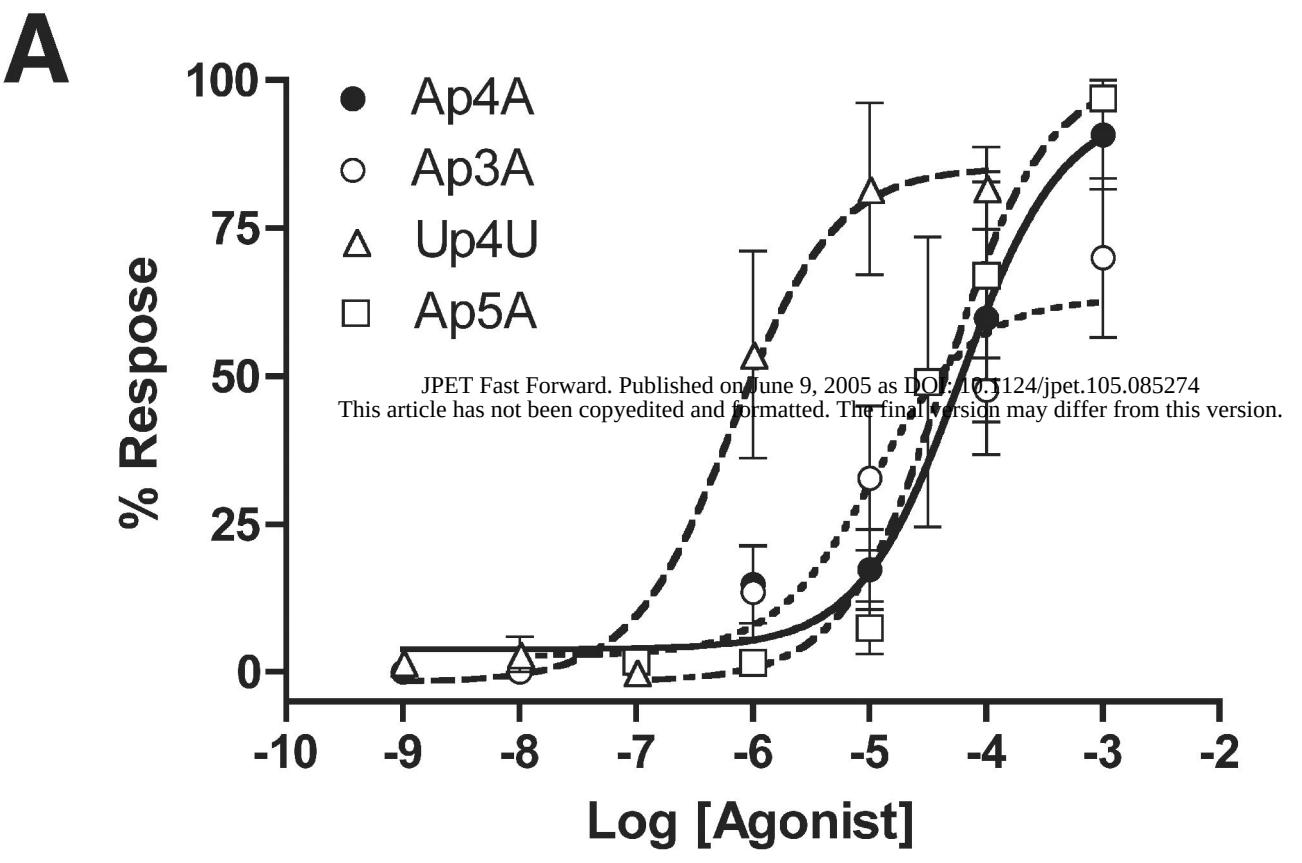


Fig 2



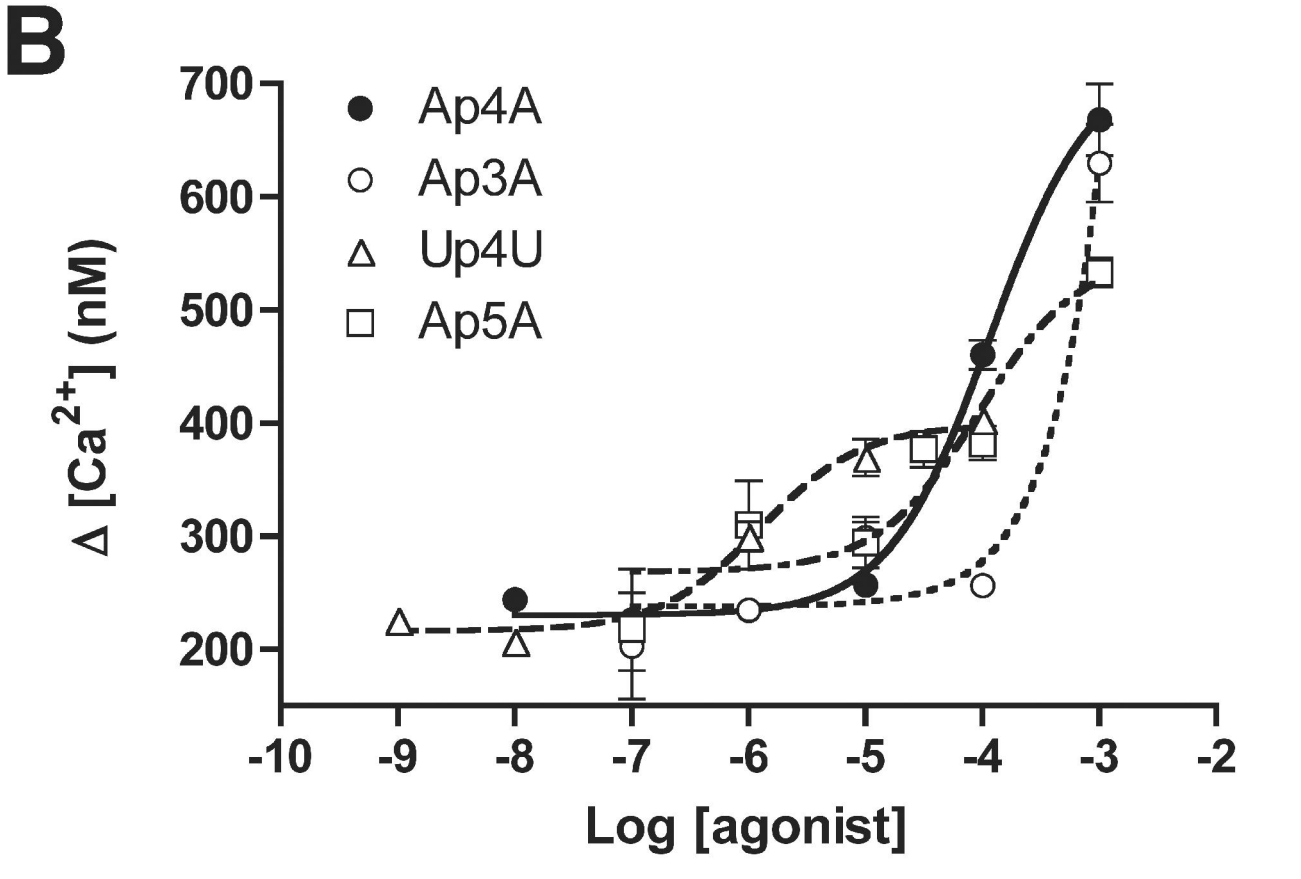


Fig 3

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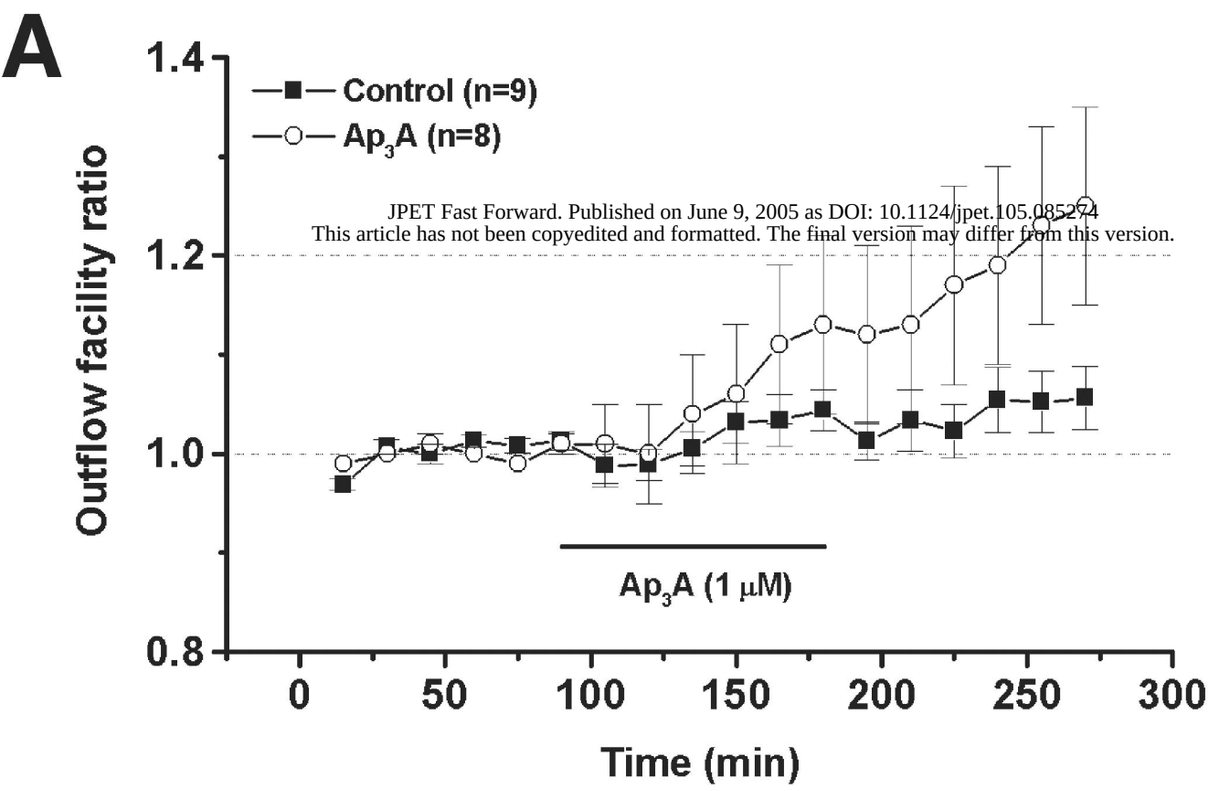

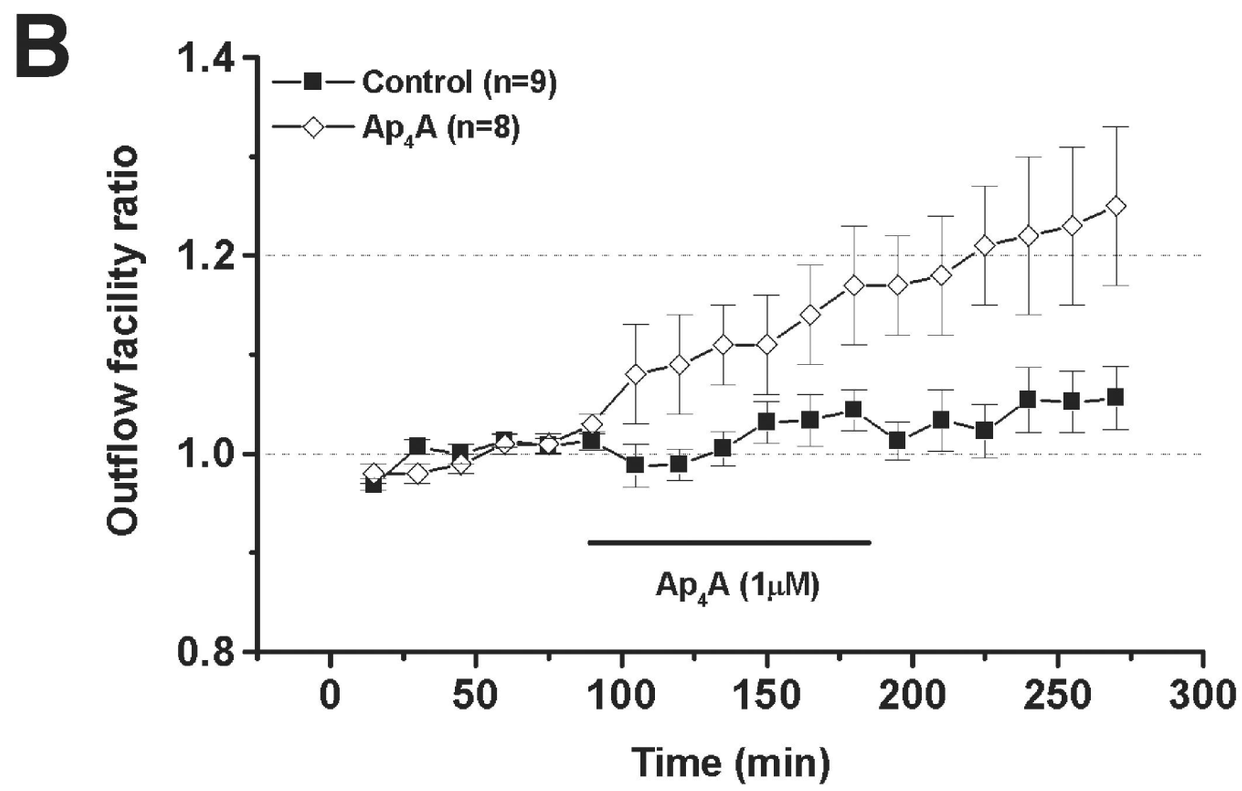

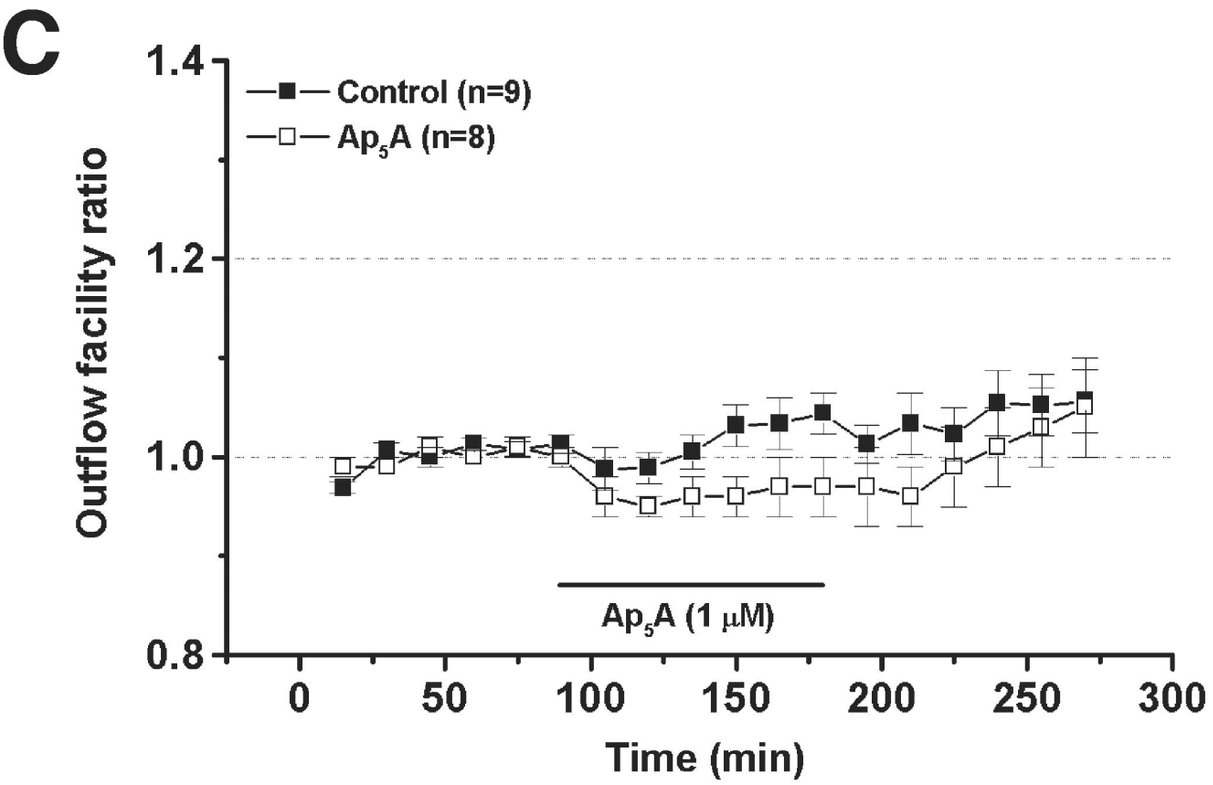

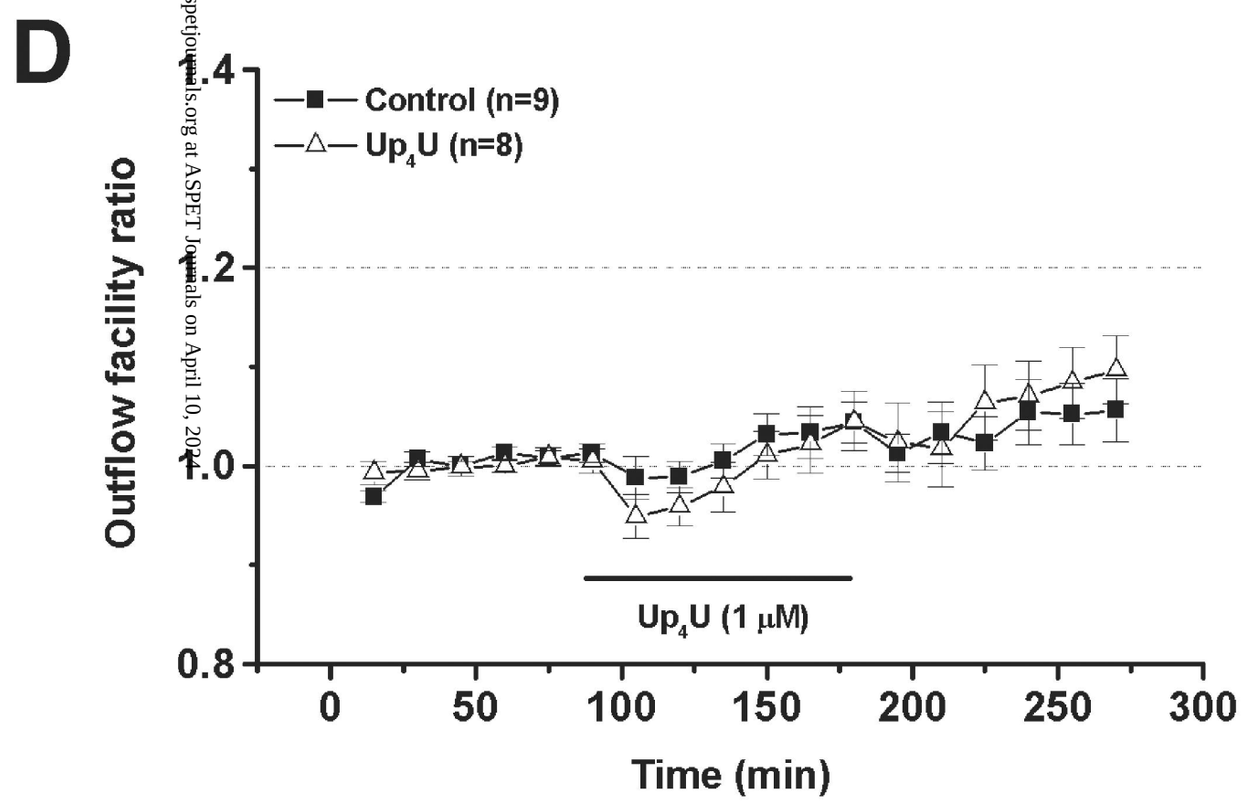

Fig 4

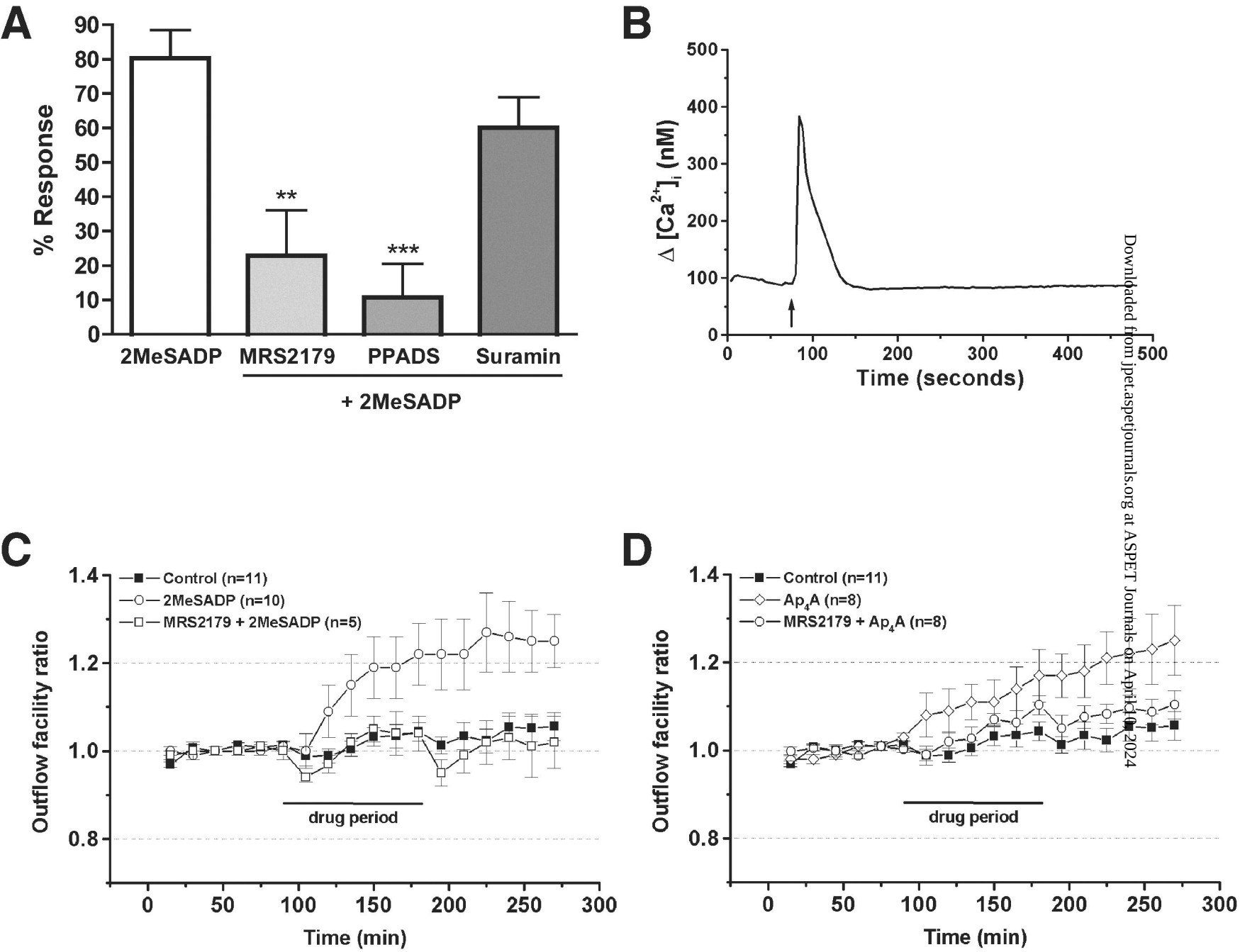

Fig 5

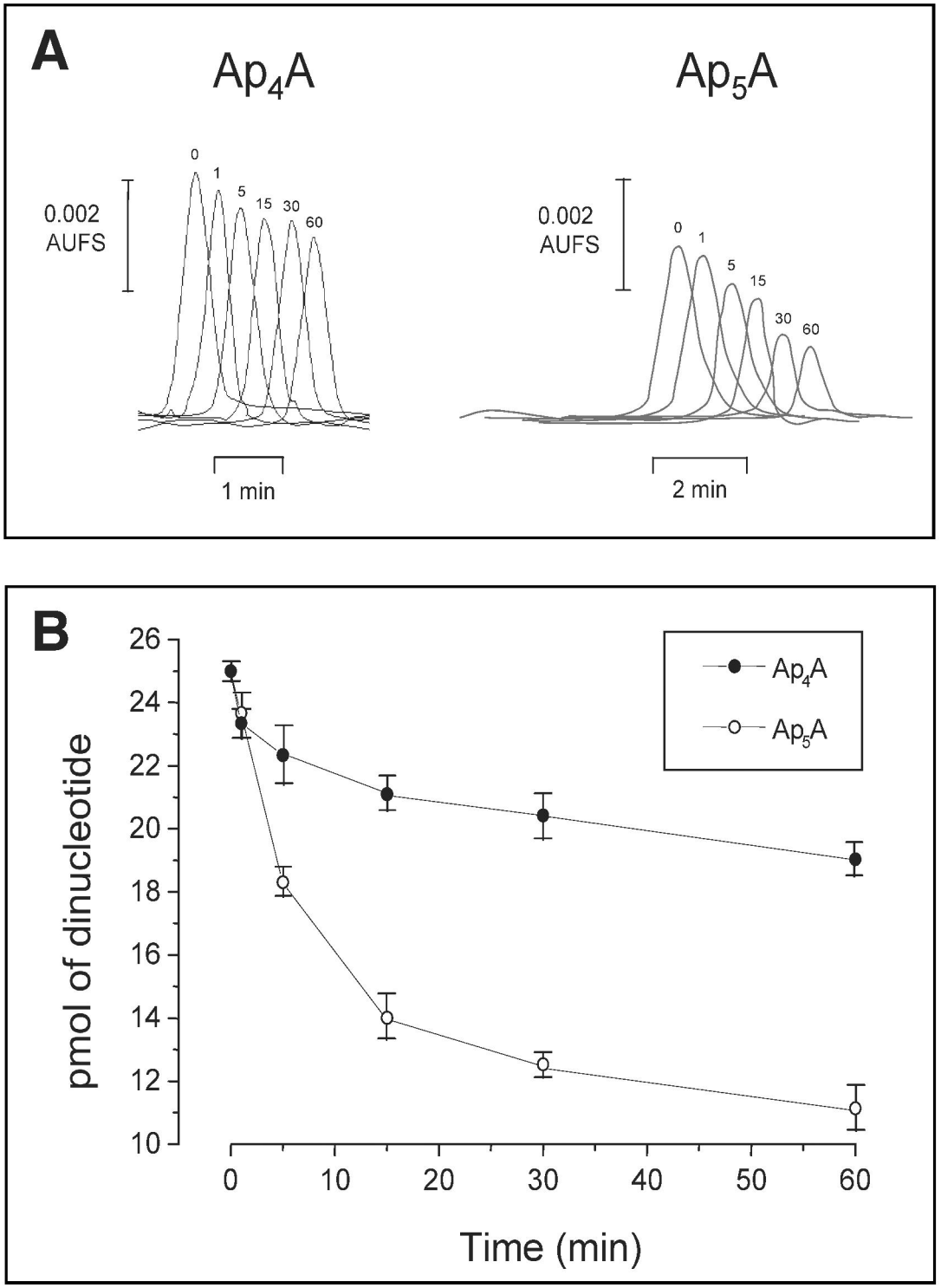
A
Ap4A
Ap5A
0.002
AUFS
0
1
5
15
30
60
1 min
2 min
B
pmol of dinucleotide
Time (min)
Ap4A
Ap5A
26
24
22
20
18
16
14
12
10
0
10
20
30
40
50
60

Fig 6