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Characterization of adenosine receptors in bovine corneal endothelium

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Abstract

Previous studies indicated that adenosine can increase $[cAMP]_i$ and stimulate fluid transport by corneal endothelium. The purpose of this study was to determine which adenosine receptor subtype(s) are expressed and to examine their functional roles in modulating $[cAMP]_i$, $[Ca^{2+}]_i$ and effects on Cl^- permeability in corneal endothelium. We screened bovine corneal endothelium (BCE) for adenosine receptor subtypes by RT-PCR and immunoblotting, and examined the effects of pharmacological agents on adenosine stimulated Cl^- transport, $[cAMP]_i$ and $[Ca^{2+}]_i$. RT-PCR indicated the presence of A_1 and A_{2b} adenosine receptors, while A_{2a} and A_3 were negative. Western blot (WB) confirmed the presence of A_{2b} (~50 kDa) and A_1 (~40 kDa) in fresh and cultured BCE. Ten micromolar adenosine increased $[cAMP]_i$ by 2·7-fold over control and this was inhibited 66% by 10 μM alloxazine, a specific A_{2b} blocker. A_1 activation with 1 μM N^6-CPA (a specific A_1 agonist) or 100 nM adenosine decreased $[cAMP]_i$ by 23 and 6%, respectively. Adenosine had no effect on $[Ca^{2+}]_i$ mobilization. Indirect immunofluorescence localized A_{2b} receptors to the lateral membrane and A_1 to the apical surface in cultured BCE. Adenosine significantly increased apical Cl^- permeability by 2·2 times and this effect was nearly abolished by DMPX (10 μM), a general A_2 blocker. Adenosine-induced membrane depolarization was also inhibited by 33% (n=6) in the presence of alloxazine. Bovine corneal endothelium expresses functional A_1 and A_{2b} adenosine receptors. A_1, preferentially activated at <1 μM adenosine, acts to decrease $[cAMP]_i$ and A_{2b}, activated at >1 μM adenosine, increase $[cAMP]_i$.

Keywords

adenosine receptors; cAMP; Cl^- permeability; corneal endothelium

1. Introduction

The effects of adenosine on corneal endothelial function have been studied since the early 1970s. Swollen corneas that were treated with adenosine deswelled more rapidly (Dikstein and Maurice, 1972), due to an increased rate of endothelial fluid transport (Fischbarg et al., 1977). More recent studies verified these effects and also showed that adenosine increased

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endothelial $[cAMP]_i$; that increasing $[cAMP]_i$ could stimulate fluid transport; and that A_2 receptor agonists could increase $[cAMP]_i$ and stimulate fluid transport (Riley et al., 1996). Furthermore, adenosine, cpt-cAMP or forskolin have been shown to increase endothelial cell Cl^- permeability (Bonanno and Srinivas, 1997). The increase in Cl^- permeability was predominantly at the apical membrane and mediated by the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent Cl^- channel (Sun and Bonanno, 2002). Since Cl^- (and HCO_3^-) are essential for endothelial fluid transport (Winkler et al., 1992; Riley et al., 1995), reviewed in Bonanno (2003), these physiological studies suggest that adenosine stimulates fluid transport by activating an A_2 receptor subtype linked to adenylate cyclase activation, which increases $[cAMP]_i$, activates PKA and apical cAMP dependent anion channels.

There are four subtypes of adenosine receptors: A_1, A_{2a}, A_{2b} and A_3. Activation of these receptors by their ligands initiates a signalling cascade via G-protein coupling that is both tissue and receptor specific. The activated A_1 receptor couples to the pertussin toxin sensitive $G_{i/o}$, which inhibits adenylate cyclase, leading to a decrease in the concentration of $[cAMP]_i$. The A_1 receptor can also mobilize intracellular calcium release from the endoplasmic reticulum (ER) store by coupling to the G_{q11} pathway (Feoktistov and Biaggioni, 1997; Linden, 2001). A_3 receptor activation also decreases $[cAMP]_i$ due to its coupling to G_i. Both A_{2a} and A_{2b} receptors couple to G_s, which activates adenylate cyclase, increasing $[cAMP]_i$. The A_{2b} receptor can also exhibit dual G-protein coupling in some cell systems leading to increased $[Ca^{2+}]_i$ by multiple pathways (Feoktistov and Biaggioni, 1997). The type(s) of signal transduction pathway(s) may depend on the co-localization of receptors and signalling molecules within a plasmalemmal microdomain (Ostrom et al., 2001) or the abundance of these G-proteins and the affinity of the activated A_{2b} receptor for either G_s or G_{q11}, as has been shown to be the case for α2-adrenergic receptors (Nasman et al., 2001).

The nucleoside adenosine is a general adenosine receptor agonist. It is formed from the degradation of ATP by ectonucleotidases. In the bovine eye, adenosine is present at submicromolar concentrations in the aqueous humour (Howard et al., 1998). The source of aqueous humour adenosine is not clear, but could include secretion from the ciliary body, ectonucleotidase catabolism of ATP released by ciliary epithelium (Mitchell et al., 1999; Matsuoka et al., 2002; Eltzschig et al., 2003), and similarly from ectonucleotidase catabolism of ATP release by the corneal endothelium (Soltau et al., 1993). Based on the known affinities of the receptor subtypes to adenosine (Klinger et al., 2002), the aqueous humour concentration of adenosine would favour A_1 receptor stimulation (decrease $[cAMP]_i$). However, the local concentration of adenosine at the endothelial surface is not known and could be much higher, in which case A_2 receptors could also be stimulated. When endothelial cells are stressed, the cells will release ATP (Srinivas et al., 2003). ATP can then be converted to adenosine by ectonucleotidases, suggesting that adenosine could exhibit autocrine and paracrine stimulation of fluid transport during stress conditions.

In the current study, we ask which adenosine receptor subtypes are expressed in bovine corneal endothelium and where they are localized; which of those expressed are coupled to adenylate cyclase; and does stimulation of the receptor lead to increased apical membrane

Cl^- permeability. We also investigated whether A_1 and A_{2b} receptors transduce signals by mobilization of $[Ca^{2+}]_i$. We found that lateral A_{2b} receptors and apical A_1 receptors, which only act to modulate $[cAMP]_i$, are expressed in corneal endothelium.

2. Materials and methods

2.1. Cell culture

Bovine eyes were obtained a few hours after death from a local slaughterhouse. Endothelial cells were harvested from dissected corneas as previously described (Bonanno and Giasson, 1992). Primary culture was established in T-25 flasks with DMEM supplemented with 10% fetal bovine serum and antibiotic/antimycotic (100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin, and 0·25 μg ml^{-1} fungizone) at 37°C in a cell culture incubator gassed with 5% CO_2, 95% air. The DMEM growth medium was changed every 3 days. The confluent cultures were then subcultured onto glass coverslips or 13 mm anodiscs and grown to confluency in 3–5 days.

2.2. RT-PCR screening

mRNA was extracted and purified using the Oligotex mRNA mini kit from Qiagen (cat. #70022) as per manufacturer's protocol. The purified mRNA was then used for cDNA synthesis and adenosine receptor PCR. cDNA synthesis was performed using Invitrogen Superscript III (200 U μl^{-1}), Oligo $dT_{12–18}$ primer and 1 μg mRNA as previously described (Sun et al., 2000). Hundred microliters of adenosine receptor PCR was performed in an Expand High Fidelity PCR reaction buffer (Roche, cat. #92760021) with 0·5 μl Taq polymerase (Roche, cat. #92877933), 5 μl of cDNA template, 8 μl of dNTP mix (2·5 mM each) and 0·3 μM (final concentration) adenosine receptor primers. The PCR parameters were: denaturation at 94°C for 3 min for one cycle, 30 cycles of denaturation at 94°C for 30 sec each, annealing at 50–60°C for 1 min, extension at 72°C for 2 min, and a final extension for one cycle at 72°C for 10 min. The PCR products were separated on 1·7% agarose electrophoresis gels and stained with 0·5 μg ml^{-1} ethidium bromide. The expected PCR bands were excised from the agarose gel, purified using a Qiagen gel purification kit, subcloned into pCR-TOPO downstream from a T7 sequence, and transformed into One Shot Chemically Competent *E. coli*. This plasmid was submitted to the Biochemistry Biotechnology Facility, Indiana University School of Medicine, Indianapolis, for sequencing using the Big Dye Termination process (Invitrogen). Confirmation of our PCR results was obtained by blasting our sequencing results against the NCBI database.

All four subtypes of adenosine receptor primers were designed to span at least one intron. The primers for PCR are as follows:

A_1 forward strand 5′ TGC TGA TGT GCC CAG CCT GT 3′, reverse strand 5′ TGC TGA TGA CCT TCT CGA ACT 3′, 556 bp product size, accession number XM_001687; A_{2a} forward strand 5′ GCT CCA TCT TCA GCC TTC TG 3′, reverse strand 5′ AGT GAC TTG GCT GCA TGG ATC T 3′, 789 bp product size, accession number AY136748; A_{2b} forward strand 5′ AAC CTG CAG AAC GTC ACC AAC T 3′, reverse strand 5′ GGA AGG TCT GGC GGA ACT C 3′, 438 bp product size, accession number NM_053294; A_3 forward

strand 5′ CTG GTC CCT TTG GCC ATT 3′, reverse strand 5′ AGG GTT CAT CAT GGA GTT GGC 3′, 649 bp product size, accession number BC013780.

2.3. cAMP assay

BCEC were subcultured to confluence on 25 mm petri dishes. The dishes were first incubated with serum-free, HEPES buffered bicarbonate-free DMEM for 2 hr. This was done to avoid activation of HCO_3^--stimulated soluble adenylate cyclase, which increases $[cAMP]_i$ and chloride transport by about 42% (Sun et al., 2003). This was followed by a 30 min DMEM incubation with 0·3 mM α,β-methyle-neadenosine 5′-diphosphate (AMP-CP, an ectonucleotidase inhibitor to reduce endogenous adenosine production), 50 μM rolipram (a PDE4 inhibitor), and relevant adenosine receptor antagonists. Control samples were subjected to only rolipram and AMP-CP in DMEM. After this 30 min incubation, the cells were stimulated with either specific or general adenosine receptor agonists (5 min), followed by two quick washes with bicarbonate-free phosphate free ringer. cAMP was then released from the cells by incubation in 0·1 N HCl at room temperature for 1 hr. The supernatant was removed and cAMP measured by ELISA (R&D Systems cat. DE0355). All measurements were in triplicate. The cells on the petri dishes were then lysed in RIPA buffer, centrifuged at 10 000 rpm for 10 min to remove cellular debris and the supernatant assayed for protein concentration.

2.4. Membrane protein isolation and immunoblotting

Total membrane protein was extracted from fresh and cultured BCE using the sulfo-NHS-biotin technique. This negatively charged biotin derivative bonds with primary amines present in all proteins, but cannot penetrate the cell membrane. Cultured and fresh bovine corneal endothelial surface proteins were labelled with 200 μg of EZ-link sulfo-NHS-biotin (Pierce) per ml of bicarbonate-free ringer that does not contain primary amines (pH 7·5) at room temperature for 30 min. The cells were washed several times, lysed in lysis buffer (50 mM Tris base, 150 mM NaCl, 0·5% deoxycholic acid, sodium salt, 2% SDS and 1% NP-40, pH 7·5) containing protease inhibitor cocktail, and then sonicated to solubilize remaining membrane protein. This was followed by centrifugation at 10 000 rpm to pellet cell debris. The supernatant was incubated with 50 μl of immobilized streptavidin at 4°C for overnight, rotated end over end. The streptavidin–biotinylated protein complex was pelleted at 10 000 rpm for 1 min and washed four times. Fifty microliters of 1× Laemmli sample buffer was then added and the mixture heated in a 95°C heating block for 10 min to denature the protein and break the streptavidin–biotinylated protein bond. The streptavidin beads were pelleted on a table-top microcentrifuge and the supernatant quickly removed. Pilot studies using an antibody against the α-subunit of the Na^+–K^+ ATPase as a control basolateral marker, indicated that under these experimental conditions both apical and basolateral membrane proteins were recovered.

The protein samples were fractionated on a 12% SDS-PAGE gel and transferred to a PVDF membrane for immunoblotting. Prior to antibody incubation, the PVDF membrane was blocked in 5% milk–0·5% TWEEN 20 solution at room temperature for 1 hr. All antibodies used were diluted in PBS/goat serum (1:1 ratio). The antibody concentrations were: (a) 1:500 polyclonal primary rabbit anti-human A_{2b} antibody (Chemicon cat. #Ab1589p); (b)

1:500 polyclonal rabbit anti-human A_{2a} antibody (Chemicon cat. #AB1559); (c) 8 μg ml^{-1} polyclonal rabbit anti-human A_1 antibody (Alpha Diagnostics cat. #A1R11-A).

All primary antibody incubations were performed at 4°C overnight while secondary horseradish peroxidase conjugated antibody incubation was for 1 hr at room temperature. Bands were visualized using Supersignal West Pico Chemiluminescent substrate (Pierce) and exposed to Kodak Bio Max Light film. Bovine brain (whole cell lysate) was used as positive control for all adenosine receptors.

2.5. Indirect immunofluorescence

2.5.1. Cultured BCE—Cultured Bovine corneal endothelial cells were seeded onto 25 mm glass coverslips. The confluent cells were washed with bicarbonate-free ringer before being fixed in PLP fixation buffer (2% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate, and 45 mM sodium phosphate (pH 7·4)) for 30 min. The cells were permeabilized in 0·01% saponin–PBS solution for 20 min, followed by blocking for 1 hr in PBS containing 0·2% BSA, 5% goat serum, 0·01% saponin, and 50 mM NH_4Cl. Cells were incubated with primary antibody for 1 hr at room temperature. Rabbit anti-A_{2b} polyclonal antibody was diluted 1:33 in equal mixture of PBS–goat serum. The A_1 primary antibody was diluted 1:10 for Cultured BCE samples, and 1:33 for fresh BCE samples. A_{2a} primary antibody concentration was 1:100 for both CBCE and FBCE. This was followed by three 5-min washes with PBS–0·01% saponin and three further 15-min washes. Secondary antibody, Alexa 488 anti-rabbit (1:1000 ratio in PBS–goat serum), was applied to the coverslips for 1 hr at room temperature. After three 15-min washes, 300 nM DAPI (5 min incubation followed by a PBS rinse) was used to stain nuclei. The stained cells were finally mounted with Prolong anti-fade medium (Molecular Probes). Parallel negative control was also performed by replacing the primary antibody with PBS–goat serum (1:1 dilution). Immunostaining was observed with a 40× oil objective lens using a standard epifluorescence microscope equipped with a CCD camera. A Bio-Rad 2000 scanning confocal microscope was used to confirm localization of the A_{2b} and A_1 receptors.

2.5.2. Frozen section A_{2b} staining with fresh bovine corneas—Bovine corneas were fixed for 5 min in PLP fixation buffer and washed with PBS. A piece of the cornea approximately 2 mm wide was embedded in OCT medium before being rapidly frozen in liquid nitrogen-chilled *t*-butyl butane. The frozen tissue was then sectioned at 4 μm thickness using a Tissue-TEK II Cryotome (Miles Scientific), transferred to superfrost microscope slides, and air-dried at room temperature. Indirect immunofluorescence for adenosine receptors was then carried out as stated for the cultured bovine samples with parallel negative controls.

2.6. Chloride permeability

Confluent layers of endothelial cells cultured on permeable anodiscs were placed in a double-sided chamber for independent apical and basolateral perfusions, as previously described (Bonanno et al., 1998). The chamber was seated on a 37°C brass stage warmer on a Nikon Diaphot inverted microscope equipped with a 40× water immersion lens, 2 mm working distance. All ringer solutions were placed in 60 cm^3 syringes hanging in a 37°C

temperature controlled Plexiglass box. Pharmed tubing connected the syringes to an eight-way valve that delivered the solutions to the microscope chamber.

Relative changes in chloride permeability in cultured bovine corneal endothelial cells were assessed with the halide-sensitive fluorescent dye MEQ, as previously described (Sun and Bonanno, 2002). Briefly, corneal endothelial cells on anodiscs were exposed to the nonfluorescent cell-permeant reduced quinoline derivative of MEQ (diH-MEQ) (Biwersi and Verkman, 1991), which is oxidized to MEQ within the cytoplasm. Cells were exposed to 10 μM diH-MEQ for 10 min at room temperature in Cl^- free Ringer's solution, and washed for 30 min with Cl^--free Ringer's solution. Cellular fluorescence was measured with a microscope spot fluorimeter (DeltaRam, Photon Technology International, Monmouth Junction, NJ). Fluorescence was excited at 365±10 nm and emission collected at 420– 450 nm. Synchronization of excitation with emission measurement and data collection (1 sec^{-1}) was controlled by Felix software (PTI). Relative differences in Cl^- permeability between control and experimental conditions in the same cells were determined by comparing the percentage change in MEQ fluorescence (F/F_0) after addition of Cl^- to the apical side, where F_0 is the initial fluorescence in the absence of Cl^-. The maximum slope of fluorescence change was determined by calculating the first derivative using Felix software.

2.7. DisBac$_{(2)}$3 membrane potential measurement

Relative changes in membrane potential were measured using the voltage sensitive dye bis-oxonol (DisBas$_{(2)}$3) as previously described (Bonanno et al., 1998). Cell confluent anodiscs were placed in the double-sided chamber for microscope perfusion. The ringer solutions used were bicarbonate-free and chloride-rich containing 500 nM DisBac$_{(2)}$3. The anionic DisBac$_{(2)}$3 binds immediately onto the cell membrane. The extent of DisBac$_{(2)}$3 binding is reversible, and is affected by membrane potential. Membrane hyperpolarization repels the dye from the cell membrane, leading to a decrease in fluorescence intensity and vice-versa for membrane depolarization. The maximum change in fluorescence (ΔF_m) following addition of adenosine agonists was divided by the baseline value (F) immediately prior to addition of drugs to give relative membrane potential change.

2.8. Fura-2 $[Ca^{2+}]_i$ measurement

To determine if activated adenosine receptors are coupled to the G_{q11}, we measured free cytosolic $[Ca^{2+}]_i$ using Fura-2 (Molecular Probes). Confluent coverslips were loaded with 1 μM Fura-2 AM for 30 min in the presence of 20% W/V pluronic acid, and washed for 30 min. Cells were perfused in bicarbonate-free Ringer while Fura-2 was excited at 340 and 380 nm, and emission at 510 nm was measured.

2.9. Ringer solutions and chemicals

The composition of the bicarbonate-free Ringer used throughout this study was (in mM) 150 Na^+, 4 K^+, 0·6 Mg^{2+}, 1·4 Ca^{2+}, 118 Cl^-, 1 HPO_4^{2-}, 10 HEPES, 28·5 $gluconate^-$, and 5 glucose. The pH was adjusted to 7·5 with sodium hydroxide after warming the solution to 37°C. Cl^--free ringer solution was prepared by equimolar replacement of NaCl and KCl with sodium nitrate and potassium nitrate, respectively. Osmolarity was adjusted to 295±5 mosM with sucrose.

Adenosine, alloxazine (AL), cycloplazionic acid (CPA), DPCPX, N^6-chloropentyl adenosine (N^6-CPA), and cyclopiezonic acid (CPA) were obtained from Sigma (St Louis, MO); DMPX from ICN; Fura-2, pluronic acid, MEQ and $DisBac_2(3)$ from Molecular Probes; cAMP ELISA kit from R&D Systems.

2.10. Statistical analysis

Data analysis is presented as mean values±standard deviations. Student's *t*-test was used to test for significance ($p<0{\cdot}05$).

3. Results

3.1. RT-PCR

Fig. 1 shows PCR results for adenosine receptors from cultured bovine corneal endothelial cells. Initial adenosine receptor RT-PCR screening using CBCE total RNA extract yielded a weak A_1 band. However, using purified mRNA for cDNA template generation, RT-PCR produced strong expected bandsizes for both A_1, 553 bp (Fig. 1A), and A_{2b} receptors, 438 bp (Fig. 1B). These A_1 and A_{2b} PCR results were confirmed by sequencing. Positive controls were bovine brain (for A_1) and a human respiratory epithelial cell line, Calu-3 (for A_{2b}). The 438 bp A_{2b} product from CBCE showed 92% homology with canine and 88% homology with human A_{2b}. CBCE A_1 sequence showed homology with published bovine (100%) and human (90%) A_1 sequences. Fig. 1C and D shows that A_{2a} and A_3 primers produced negative results for corneal endothelium, but were positive for cDNA derived from Calu-3 (789 bp A_{2a} PCR band) and bovine brain (649 bp A_3 PCR product), which were confirmed by sequencing.

3.2. cAMP

Fig. 2A shows that 10 μM adenosine (Ado) increased intracellular [cAMP] by 2·4-fold over control. Forskolin, a general activator of adenylate cyclase, produced nearly 13-fold increase in [cAMP] over control. Fig. 2B shows that the specific A_{2b} inhibitor alloxazine (AL), significantly decreased adenosine stimulated $[cAMP]_i$ by about 44%. DMPX (a general A_2 receptor antagonist), inhibited the increase in $[cAMP]_i$ by 26%. Fig. 2C shows that CGS21680 (a specific A_{2a} agonist) had no effect on $[cAMP]_i$, consistent with the negative PCR results. N^6-CPA, a specific A_1 receptor agonist would be expected to decrease $[cAMP]_i$ through activation of G_i. Fig. 2D shows that 1 μM N^6-CPA decreased $[cAMP]_i$ by ∼23% over control and that this was abolished in cells preincubated with 1 μM DPCPX, a specific inhibitor of A_1 receptors. Hundred nanomolar Ado also decreased $[cAMP]_i$ by a modest 6% over control. In summary, the cAMP assays, which are consistent with the PCR results, support the presence of A_1 and A_{2b} receptors, but not A_{2a} in bovine corneal endothelium.

3.3. Western blot

Fig. 3A shows Western blot of membrane protein for A_1 receptors using fresh and cultured BCE. A bandsize of ∼40 kDa was present in fresh BCE, cultured BCE, and the positive control (bovine brain). Fig. 3B shows that both cultured and fresh BCE cells were negative for the 45 kDa A_{2a} adenosine receptor, yet positive using bovine brain. Fig. 3C shows that using the rabbit anti-A_{2b} antibody, Western blot yielded a band ∼50 kDa for both cultured

and fresh bovine corneal endothelium, with bovine brain as positive control. This 50 kDa A_{2b} bandsize has been established in human, mouse and rat tissues of the thymus, colon, and small intestine (Puffinbarger et al., 1995). These data, which are consistent with the PCR and cAMP results, confirm the presence of A_1 and A_{2b} receptors in BCE.

3.4. Indirect immunofluorescence

Fig. 4 shows an immunofluorescence micrograph for the A_{2b} receptor using cultured bovine corneal endothelium. The apparent location is predominantly on the lateral part of the membrane (Fig. 4A). Negative control is shown in Fig. 4B.

In order to confirm the localization of A_{2b} receptors, confocal microscopy was performed. Fig. 4C shows a series of confocal fluorescence sections from cultured cells stained for A_{2b} receptors. The microscope focus was first positioned on the basal aspect of the confluent layer of cells. The *z*-axis motor then moved the focus in 0·5 μM steps in the apical direction. Green A_{2b} staining was first observed in a ringlike fashion at the lateral aspects of the cells. Further movement apically began to reveal the blue DAPI nuclear stain along with the lateral green stain. The green stain then disappeared while the nuclear stain remained strong. There was no apparent apical staining. Fig. 4D shows an *X–Z* projection of A_{2b} confocal staining. The *X–Z* projection also supports a lateral localization for the A_{2b} receptor. Furthermore, frozen sections of fresh bovine cornea (Fig. 4E, left panel) also indicates expression of A_{2b} receptors at the lateral membrane. Fig. 4E (right panel) shows the parallel negative control.

Fig. 5A shows A_1 staining in cultured BCE. The diffuse fluorescence all across the confluent sheet of cultured BCE cells suggests that A_1 staining is apical. Parallel negative control is in Fig. 5B. Confocal sections (Fig. 5C) and projection onto the X–Z plane (Fig. 5D) confirmed that A_1 is targeted to the apical surface. Fig. 5E (top panel) shows positive A_1 staining in fresh BCE, with parallel negative control (bottom panel).

3.5. Physiological evidence for A_{2b} receptor

3.5.1. MEQ quenching by chloride influx—If our hypothesis that A_{2b} receptors are present in BCE is true, then activation of A_{2b} receptors by adenosine is expected to stimulate chloride flux via the adenylate cyclase–PKA–CFTR pathway, while blocking A_{2b} receptors should reduce chloride flux stimulation by adenosine.

In Fig. 6, cultured cells were depleted of intracellular chloride by incubating them in chloride free ringer for at least 30 min. Chloride flux across the basolateral side, in which the $Na^+/K^+/2Cl^-$ (NKCC1) co-transporter resides, is blocked throughout the experiments by perfusing continually with chloride free ringer. When Cl^- is introduced to the apical side, there is a slow decrease in MEQ fluorescence indicating Cl^- entry. Fig. 6A shows that addition of 10 μM adenosine significantly increased the rate of MEQ quenching indicating an increase in Cl^- permeability. Using the same cell sample, Fig. 6B shows that DMPX, a general blocker of A_2 receptors, nearly abolished any stimulatory effect by adenosine. The bar graph in Fig. 6C summarizes the average relative effect of adenosine on chloride permeability over control, with and without DMPX. In the absence of DMPX, adenosine

increased chloride permeability by 2·5-fold over control. The specific A_{2b} receptor antagonist alloxazine interferes with MEQ fluorescence so could not be used in these experiments. Alternatively, we used alloxazine in a similar experiment while measuring membrane potential with $DisBac_2(3)$ (see below). Taken together, Fig. 6A–C provides confirmatory evidence for the existence of A_{2b} receptors in bovine corneal endothelium.

3.5.2. Alloxazine and $DisBac_2(3)$ membrane potential measurement—The resting condition of bovine corneal endothelium is conducive for chloride efflux. The resting membrane potential is ∼−50 mV (Watsky and Rae, 1991; Srinivas et al., 1998), and intracellular chloride concentration is ∼40 mM (Srinivas and Bonanno, 1997), a value above the chloride equilibrium potential (−28 mV when bath $[Cl^-]$=120 mM). Hence, under these conditions, if A_{2b} receptors are present, activation of this receptor should stimulate chloride efflux via cAMP dependent anion channels and result in membrane depolarization, which the $DisBac_2(3)$ dye registers as an increase in fluorescence. Partial inhibition of putative A_{2b} receptor by an antagonist should reduce the extent of membrane depolarization caused by A_{2b} receptor agonist.

Fig. 7A shows a representative trace of membrane potential changes due to application of 10 μM adenosine. Both basolateral and apical membranes were perfused with bicarbonate-free, chloride-rich ringer. In the presence of adenosine, membrane depolarization was recorded. Preincubating the cells with the A_{2b} receptor antagonist, alloxazine (AL, 10 μM) for 10 min reduced the extent of membrane depolarization from adenosine (Fig. 7B). Fig. 7C depicts the overall relative membrane potential depolarization caused by adenosine with and without alloxazine. Overall, alloxazine reduced the membrane potential depolarization by 67±15% (*n*=6).

3.6. Calcium measurement with Fura-2 AM—A_1 receptors appear to be coupled to G_i in BCE. In some cell types (Feoktistov and Biaggioni, 1997), A_1 can be coupled to G_q leading to increases in cytosolic $[Ca^{2+}]$. Fig. 8 shows that no change in cytosolic $[Ca^{2+}]$ occurred in response to either 200 nM adenosine, which would favour A_1 stimulation only or with 10 μM adenosine, however, Ca^{2+} stores could be mobilized by the ER Ca^{2+}-pump inhibitor, cyclopiezonic acid (Xie et al., 2002). This indicates that A_1 and A_{2b} receptors are not linked to G_q in cultured bovine endothelium under physiological conditions.

4. Discussion

Using the complementary approaches of PCR, Western blot, cAMP assay, Ca^{2+} assay, Cl^- flux, and membrane potential measurements, we conclude that bovine corneal endothelial cells express A_1 and A_{2b} adenosine receptors and not A_{2a} or A_3 receptors. Sequencing of the A_1 and A_{2b} PCR products matched the NCBI database, thus confirming that BCE express the A_1 and A_{2b} genes. PCR results were negative for A_{2a} and A_3 receptors, but positive with Calu-3 and bovine brain samples, respectively. The expected positive bovine brain and Calu-3 PCR results demonstrated that the A_{2a} and A_3 primers used here were specific.

cAMP assay and immunoblots support the presence of A_1 and A_{2b} receptor subtypes. 10 μM adenosine (a general adenosine receptor agonist) produced an increase in cellular cAMP

over control, indicating that either A_{2a}, A_{2b}, or both receptor subtypes are present. A_{2a} is ruled out as a candidate by: (a) negative A_{2a} RT-PCR, (b) negative Western blot, and (c) lack of $[cAMP]_i$ elevation when only the specific A_{2a} receptor agonist CGS 21680 was used. The net increase in $[cAMP]_i$ at micromolar levels of adenosine also suggests that either A_{2b} receptor activity is coupled to G_s more efficiently than to the A_1–G_i linkage, or the density of G_s is higher than G_i in the vicinity of the respective adenosine receptors. The absence of effects of adenosine on $[Ca^{2+}]_i$ suggests that the G_q pathway is not significant.

The presence of A_{2b} receptors in CBCE is also confirmed by functional studies. Chloride flux measurements using MEQ indicated that A_{2b} receptor activation by adenosine increased apical chloride permeability by ∼2·5-fold over control. This increased chloride permeability is explained by the A_{2b}–adenylate cyclase–PKA–CFTR pathway (Huang et al., 2001). The role of A_{2b} receptors in ion transport is further confirmed by the use of general A_2 antagonist (DMPX) to inhibit A_{2b} activation by adenosine. DMPX significantly reduced adenosine stimulated chloride influx. Additional experiments using the $DisBac_2(3)$ membrane potential probe with the specific A_{2b} receptor antagonist alloxazine also showed attenuation of membrane depolarization during adenosine stimulation.

Indirect immunofluorescence localized the A_{2b} receptor to the lateral membrane of cultured cell samples. In fresh bovine corneal endothelium, the A_{2b} receptor also had an apparent lateral localization. Cultured BCE showed fluorescence for A_1 receptors at the apical domain as determined by confocal microscopy. The significance of these separate locations for the A_1 and A_{2b} receptors is not clear. A_1 receptors are found on the apical membrane of MDCK II (Saunders et al., 1996) and LLC-PK1 (LeVier et al., 1992) renal epithelial cell lines. A_{2b} receptors are found on both apical and basolateral membranes of colonic epithelial T-84 cell (Wang et al., 2004). Corneal endothelium is bathed in aqueous humour which has submicromolar concentrations of adenosine (Howard et al., 1998) and should activate the apical A_1 receptors. A_{2b} receptors could be shielded from activation by their relatively lower affinity and by their lateral location. This will help regulate $[cAMP]_i$ at a low level in the normal healthy endothelium. On the other hand, during periods of stress, ATP release from endothelium, surrounding structures, or inflammatory cells, could increase the local concentration of adenosine which would diffuse across the relatively leaky endothelial tight junction to activate A_{2b} receptors or if the barrier function of the endothelial monolayer were compromised, directly expose lateral membranes to adenosine in aqueous humour.

One physiological consequence of A_{2b} receptor activation in corneal endothelium is an increase in anion flux especially across the apical membrane. This increases the fluid pump function of the endothelium (Fischbarg et al., 1977; Riley et al., 1996), which could be part of a response to counter deleterious effects of stress on endothelial function. In pulmonary artery endothelial cells, A_{2b}R activation (Stevens et al., 1997) also decreases paracellular permeability via cAMP dependent and cAMP independent pathways. Similarly, a recent study has indicated that adenosine decreases endothelial barrier permeability through a cAMP dependent pathway that induces myosin light chain dephosphorylation in cultured BCE (Srinivas et al., 2004). These data indicate that endothelial function is enhanced by increased $[cAMP]_i$ by a combination of increased ion flux and decreased barrier permeability.

In summary, BCE express both A_1 and A_{2b} purinergic receptors. A_1 activation at <1 μ_M adenosine will maintain low $[cAMP]_i$, while >1 μ_M will favour A_{2b} activation and an increase in $[cAMP]_i$. A_1 is linked to G_i while A_{2b} is coupled to G_s. There is no evidence that adenosine receptors are coupled to G_q.

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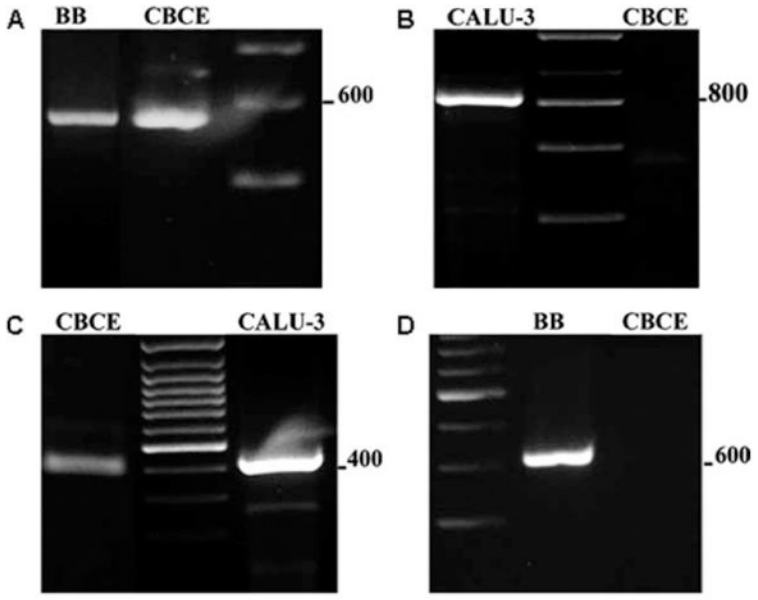

Fig. 1.

RT-PCR results for A_1, A_{2a}, A_{2b}, and A_3 adenosine receptors from cultured BCE. (A) Corneal endothelial cells are positive for A_1 receptors (556 bp); BB, bovine brain. (B) Cultured cells are negative for A_{2a}, but positive in Calu-3 cells (789 bp). (C) BCE are positive for A_{2b} (438 bp); Calu-3, positive control. (D) BCE are negative for A_3; BB is positive control (649 bp).

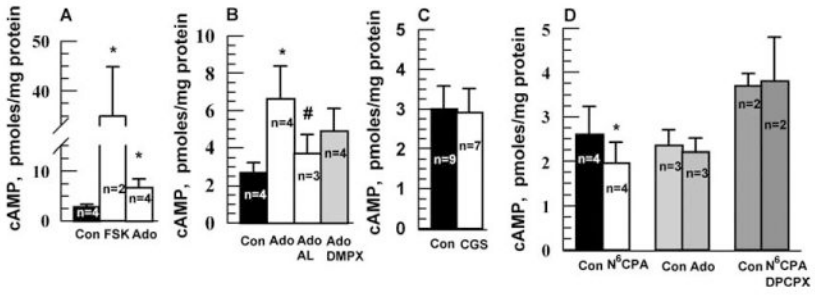

Fig. 2.

Effect of adenosine receptor (AR) agonists and inhibitors on $[cAMP]_i$. (A) Direct activation of adenylate cyclase by 10 μM forskolin and effect of 10 μM adenosine (Ado). (B) Co-incubating cells with 10 μM Ado and 10 μM AL (alloxazine, a specific A_{2b} blocker) or 10 μM Ado and 10 μM DMPX (a general A_2 blocker) decreased relative $[cAMP]_i$ by 44 and 26%, respectively. (C) 10 μM CGS21860, a specific A_{2a} receptor agonist, did not significantly affect $[cAMP]_i$. (D) N^6-CPA, a specific A_1 receptor agonist, reduced $[cAMP]_i$ over control, but this was inhibited by the A_1 receptor blocker DPCPX; 100 nM adenosine produced a small, but insignificant decrease in [cAMP]. *Values significantly different from control (con) at $p<0{\cdot}05$; #values significantly ($p<0{\cdot}05$) from Ado; error bars show standard deviation.

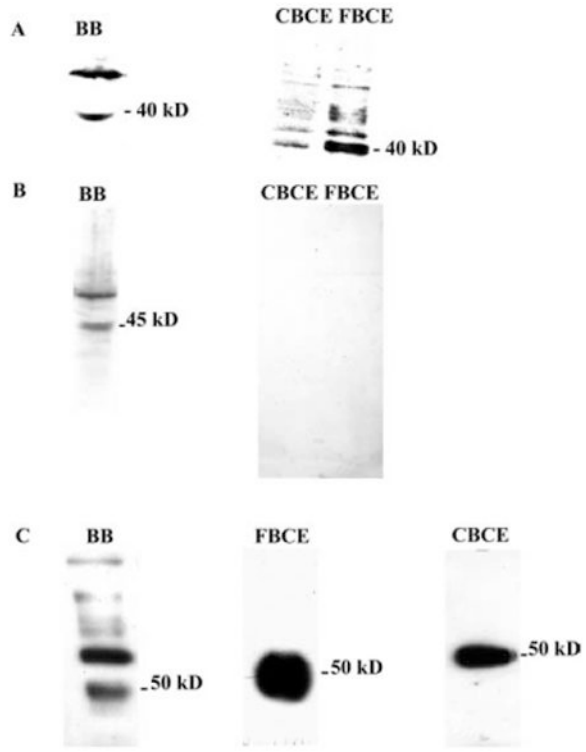

Fig. 3.

Western blot of fresh and cultured BCE for A_1, A_{2a} and A_{2b} receptors. (A) A_1 WB shows a ~40 kDa band for fresh BCE and cultured BCE. The positive control is bovine brain (BB). (B) A_{2a} WB showed the absence of ~45 kDa band for both fresh BCE and cultured BCE. Bovine brain exhibited the expected protein bandsize. (C) Cultured and fresh BCE, and BB all expressed the ~50 kDa A_{2b} protein.

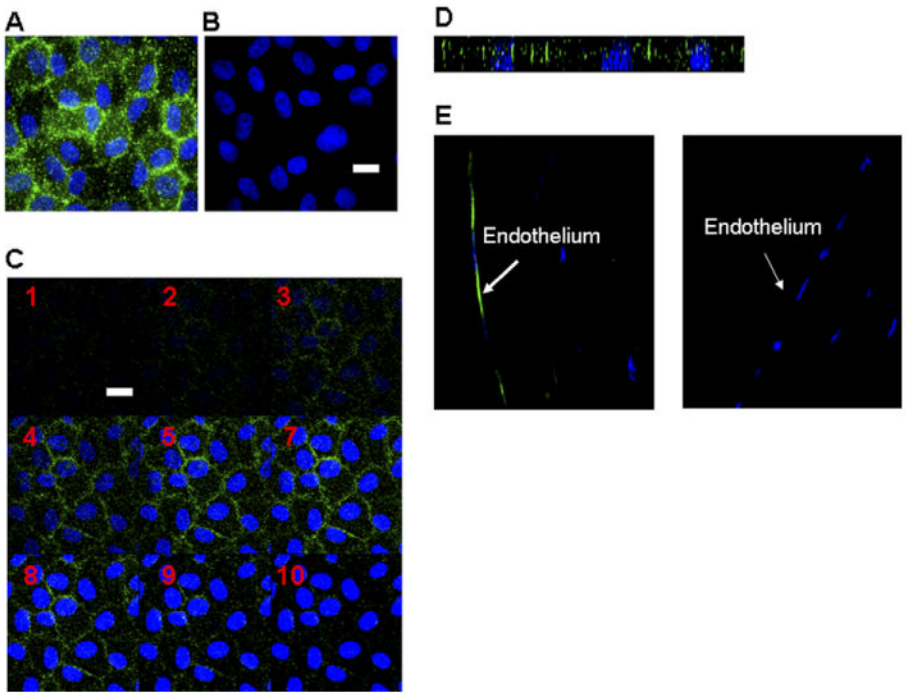

Fig. 4.

A_{2b} immunofluorescence staining. (A) Immunofluorescence micrograph shows apparent lateral membrane A_{2b} staining in cultured BCE. (B) Negative control acquired using the same exposure time, brightness and contrast parameters. Bar indicates 40 μM. (C) Confocal microscopy of A_{2b} staining in cultured BCE. Each *z*-axis panel is separated by 0·5 μM. The green staining (A_{2b}) gained significantly in intensity approximately 1·5–3 μM (panels 3–5) from the basal part of the cell (panel 1). Panel 9 is the most apical section. The nuclei are DAPI stained. (D) *X–Z* projections. (E) Fresh BCE A_{2b} immunofluorescence staining. Left panel shows positive lateral A_{2b} protein staining (green fluorescence). Bar indicates 40 μM. Right panel is the parallel negative control with primary A_{2b} antibody omitted.

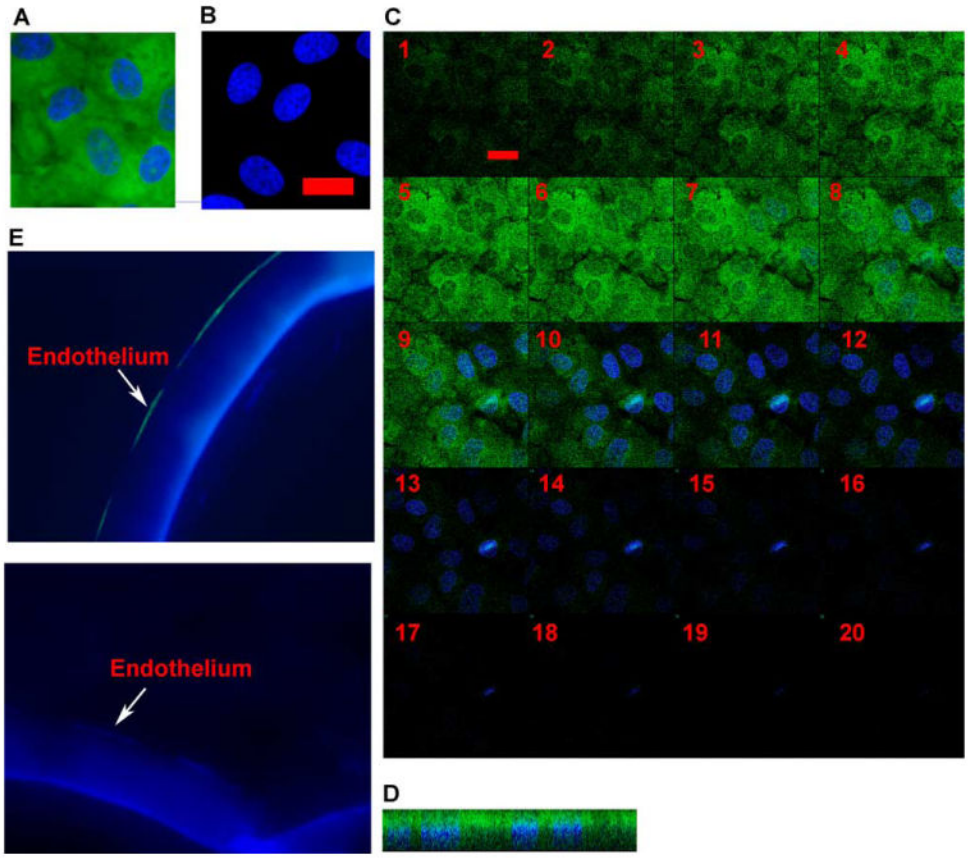

Fig. 5.

A_1R immunofluorescence staining in cultured BCE and fresh BCE. (A) Left panel, cultured BCE A_1R immunostaining. (B) Negative control for cultured BCE A_1R immunofluorescence. (C) Cultured BCE A_1 confocal micrograph. The z-axis motor is focused on the apical surface, and scanned 0·3 μM towards the basal surface. The uniform green fluorescence gained maximum intensity just as the nuclei became visible. Moving toward the basal surface, the A_1 staining disappeared as the nuclear fluorescence was most intense. (D) *X*–*Z* projection. (E) Right panel shows A_1 receptor staining in fresh BCE. Left panel is negative control.

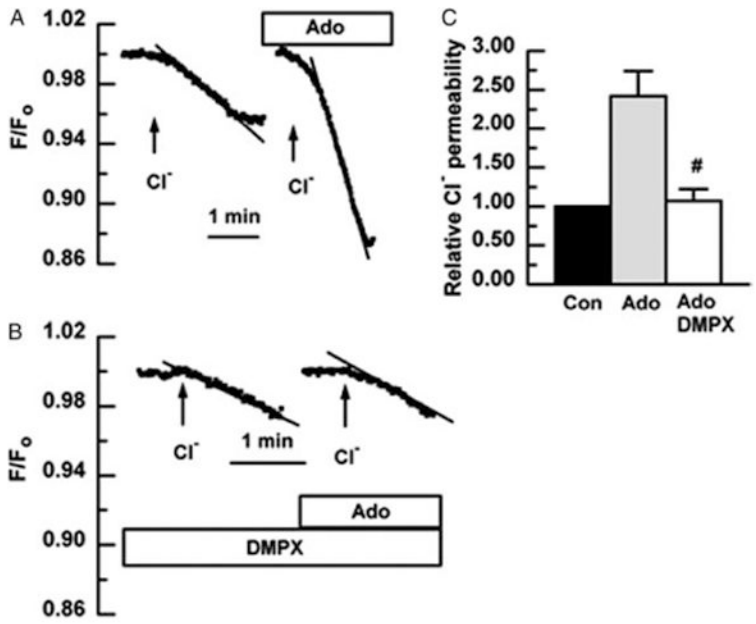

Fig. 6.

Effect of A_{2b} receptor activity on apical Cl^- permeability. Cells were depleted of Cl^-, loaded with the halide-sensitive fluorescent dye MEQ and perfused on basolateral and apical sides with Cl^- free ringer's solution. Relative apical Cl^- permeability is measured as the initial rate of MEQ fluorescence quenching upon addition of Cl^- to the apical perfusing solution. (A) 10 μM adenosine (ado) increased apical Cl^- permeability by ∼2·5-fold over control (con). (B) Preincubating the same sample with DMPX (a general A_2 blocker) almost completely blocked the stimulatory effects of Ado. (C) Bar graph summarizes the Cl^- permeability data (n= 5). #Mean value significantly different compared to Ado (p<0·05); error bars show standard deviation.

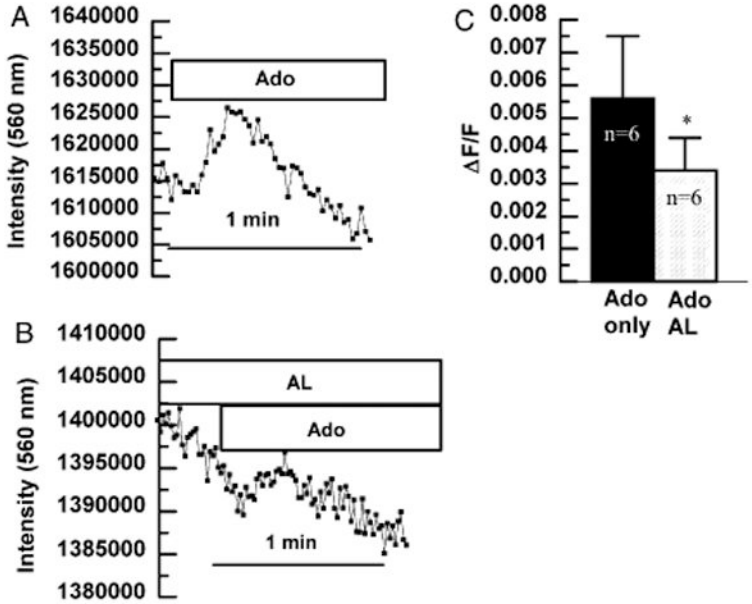

Fig. 7.

Effect of A_{2b} receptor activity on membrane potential. (A) 10 μM adenosine (Ado) induced a biphasic change in membrane potential in cultured BCE. Membrane depolarization (increase in $DisBac_2(3)$ intensity) reaches a plateau and is followed by a slower repolarization (decrease in $DisBac_2(3)$ intensity). (B) The use of alloxazine, a specific A_{2b} blocker, attenuated the biphasic response observed in (A). Data in (A) and (B) was corrected for background drift. (C) Bar graph summary of alloxazine's effect on membrane depolarization (Em). Data is plotted as $\Delta F/F$, which is the change in fluorescence divided by the baseline fluorescence measured at the peak of the change. *Value significantly different from Ado ($p<0{\cdot}05$); error bars show standard deviation.

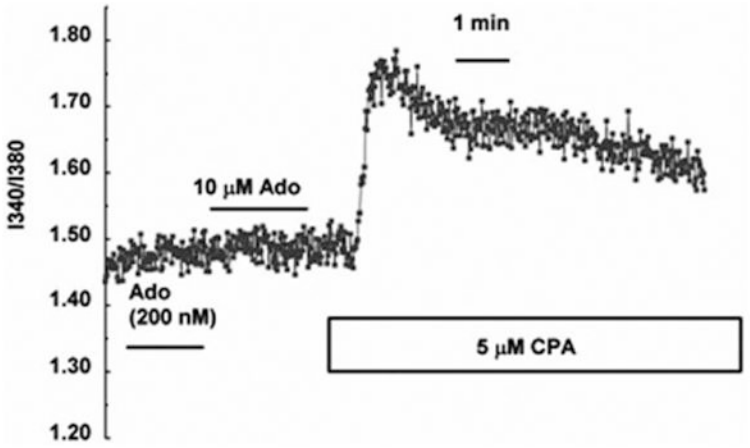

Fig. 8.

Fura-2 [Ca^{2+}] measurement. Effects of Ado (200 nM or 10 μM) and cyclopiezonic acid (CPA) (5 μM) on $[Ca^{2+}]_i$ mobilization. Positive control with CPA, which blocks the endoplasmic reticulum Ca^{2+} pump leading to increase cytosolic [Ca^{2+}].