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Common Actions of Adenosine Receptor Agonists in Modulating Human Trabecular Meshwork Cell Transport

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Abstract. A_1 adenosine receptors (ARs) reduce, and A_2ARs increase intraocular pressure, partly by differentially altering resistance to aqueous humor outflow. It is unknown whether the opposing effects of A_1AR and A_2AR agonists are mediated at different outflow-pathway cell targets or by opposing actions on a single cell target. We tested whether a major outflow-pathway cell, the trabecular meshwork (TM) cell might constitute the primary AR-agonist target and respond differentially to A_1, A_{2A} and A_3AR agonists. Receptor activation in human TM cells was identified by applying subtype-selective AR agonists: CPA and ADAC for A_1ARs, CGS 21680 and DPMA for A_{2A}ARs, and Cl-IB-MECA and IB-MECA for A_3ARs. Stimulation of A_1, A_{2A} and A_3ARs elevated Ca^{2+}, measured with fura-2. Whole-cell patch clamping indicated that AR agonists activated ion channels non-uniformly, possibly reflecting variability in magnitude of agonist-triggered second-messenger responses. A_1, A_{2A} and A_3AR agonists all reduced volume, determined by calcein cell imaging. The endogenous source of adenosine delivery to the outflow pathway could be the TM cells since these cells were stimulated to release ATP by hypotonic perfusion. We conclude that: (1) TM cells express functional A_1, A_{2A} and A_3ARs; and (2) the reported differential effects of AR agonists on aqueous humor outflow are not mediated by differential actions on TM-cell Ca^{2+} and volume, but likely by actions on separate cell targets.

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Introduction

Intraocular pressure (IOP) reflects a balance between rates of inflow and outflow of aqueous humor. The ciliary epithelium secretes the aqueous humor, which perfuses the avascular lens and cornea and eventually leaves the primate eye primarily through the conventional outflow pathway. This outflow pathway consists of the uveal and corneoscleral trabecular meshwork (TM), juxtacanalicular (JCT-TM) cells and Schlemm's canal (SC) in series (Krupin & Civan, 1995; Johnson & Erickson, 2000). Increased resistance to fluid movement through the outflow pathway can elevate IOP and is usually associated with glaucoma (Brubaker, 1998). Lowering IOP is the only strategy documented to reduce the rate of development and progression of blindness in glaucoma (Collaborative Normal-Tension Glaucoma Study Group, 1998, 1998b; The AGIS investigators, 2000; Kass et al., 2002).

Outflow resistance is likely regulated in vivo but the sensors and target cells involved are poorly understood. Among the mechanisms possibly contributing to the integrated regulation are (Johnson & Erickson, 2000; Mitchell et al., 2002): the contractile state of the trabecular meshwork cells and ciliary muscle (Wiederholt & Stumpff, 1998; Llobet et al., 1999; Tian et al., 2000; Wiederholt, Thieme & Stumpff, 2000), pore formation in the inner wall of Schlemm's canal (Johnson et al., 1992; Ethier et al., 1999), remodeling of the extracellular matrix of the JCT (Acott, 1992; Maepea & Bill, 1992; Lütjen-

Drecoll & Rohen, 1996; Johnson & Erickson, 2000), passive stretch and changes in shape (Epstein, Rowlette & Roberts, 1999) and swelling/shrinkage of the TM cells (Freddo et al., 1984; O'Donnell, Brandt & Curry, 1995; Al-Aswad et al., 1999). Remodelling of the cytoskeleton may be involved in both cellular contraction (Tian et al., 2000) and shrinkage (Henson, 1999).

Despite the complexity of the underlying cellular mechanisms, the purine adenosine triggers potentially important modulations of outflow resistance in vivo. Agonists of A_1 and A_2 adenosine receptors (ARs) have been reported to modulate outflow resistance. A_1AR agonists lower, and A_2AR agonists raise, IOP in rabbits (Crosson, 1995, 2001; Crosson & Gray, 1996; Crosson & Niazi, 2000), monkeys (Tian et al., 1997) and mice (Avila, Stone & Civan, 2001). The effect in monkeys has been ascribed primarily to actions on aqueous humor outflow (Tian et al., 1997). The ocular hypotensive effect of A_1AR agonists in rabbits has been postulated to consist of an initial fall in IOP mediated by a transient reduction in aqueous humor inflow, and a later reduction mediated by facilitating outflow (Crosson, 2001). Recently, A_3 receptor antagonists have been shown to lower and agonists to increase IOP in mouse (Avila et al., 2001), possibly reflecting both reduction in secretion (Mitchell et al., 1999) and enhancement of outflow (Avila, M.Y., C.H. Mitchell, R.A. Stone & M.M. Civan, Unpublished observations).

In the present work, we have tested whether the predominant cells of the outflow pathway, the TM cells, might mediate purinergic outflow effects by responding differentially to subtype-selective AR agonists.

Materials and Methods

Cell Preparations

Human TM (hTM) cells were isolated after collagenous digestion of trabecular meshwork explants (Stamer et al., 1995b). Cells obtained in this way are likely derived from uveal and corneoscleral TM cells and JCT-TM cells, and have been characterized with respect to their growth properties, morphology, presence of a cell-surface receptor for a low-density lipoprotein, and induction of myocillin protein upon dexamethasone treatment (Stamer et al., 1995b). They have also been used for studying aquaporin-1 (Stamer et al., 1995a), α_2-adrenergic (Stamer et al., 1996) and prostaglandin $F_{2\alpha}$ receptors (Anthony et al., 1998). The six cell lines studied and their donor ages were: #22 (55 years), #25 (66 years), #29 (stillborn), #36 (18 years), #47 (32 years) and 232-30 RC (55 years). The cell line and passage number of the cells studied are specified for each experiment in the figure legends. TM cells were grown to 80% confluence at 37°C in 5% CO_2 before study and split at a ratio of ≥1:4. The medium was low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (GibcoBRL).

Use of Calcein Fluorescence as an Index of Cell Volume

As previously discussed (Mitchell et al., 2002), our strategy has been to monitor cell area as an index of TM-cell volume. For this purpose, cells were studied after growth on coverslips (Fisher Scientific, Pittsburgh, PA) for 1–5 days. TM cells were loaded with 4 μM calcein-AM and 0.02% Pluronic F-127 at room temperature for 30–40 min. Coverslips were mounted in a chamber, and visualized with a 40× oil-immersion objective on a Nikon Diaphot microscope. Fields were chosen to include several cells of comparable diameter and displaying comparable loading, and contained between 1 and 4 non-confluent cells each. Focus was adjusted by maximizing the edge contrast between cells and bath displayed on the monitor, and thus maximizing the cell area, and was thereafter not changed during the experiment. Calcein was excited every 20 sec at 488 nm and light emitted at 520 nm was detected with an IC-200 CCD camera (Photon Technology International, Princeton, NJ). Cell area was defined as the number of pixels above threshold within a region of interest and determined using Imagemaster software (Photon Technology International). Threshold was automatically set at an intensity of 90 (out of a maximum grey scale of 256) as initial experiments showed this was optimal. Using this approach, we have observed that the TM cells display a regulatory volume decrease (RVD) in response to anisosmotic swelling, that the RVD can be inhibited by Cl^-- and K^+- channel blockers, and that isosmotic blockage of Na^+/H^+ exchangers with dimethylamiloride or of Na^+-K^+-$2Cl^-$ symports with bumetanide shrinks TM cells (Mitchell et al., 2002).

Intracellular Ca^{2+} Levels

Intracellular Ca^{2+} was measured in two ways. Experiments involving ionomycin and the subtype-selective adenosine agonists CPA (for A_1AR), CGS-21680 (for A_{2A}AR) and Cl- IB-MECA (for A_3AR) were performed on monolayers of confluent cells grown on coverslips for 1–10 days, using a photomultiplier tube. Cells were loaded in the dark with 5 μM fura-2 acetoxymethyl ester (AM) and 0.01% Pluronic F-127 for 30 min at 25°C, and perfused with fura-free solution for 30 min before beginning data acquisition (Mitchell et al., 2000). The cells were mounted on an inverted Nikon Diaphot microscope and visualized with a ×40 oil-immersion fluorescence objective. The emitted fluorescence (520 nm) from ~12 cells at ~90% confluency was sampled at 1 Hz with the photomultiplier following excitation at 340 nm and 380 nm, and the ratio determined with a Delta-Ram system and Felix software (Photon Technology International). Baseline levels from TM cells in the absence of fura-2 were subtracted from records to control for autofluorescence.

Experiments examining the effect of additional subtype-selective agonists ADAC (for A_1AR), DMPA (for A_{2A}AR) and IB-MECA (for A_3AR) were performed in essentially the same way, but the Ca^{2+} was measured in individual cells that had been grown on coverslips for 24 hours. Individual cells were chosen as described for the volume experiments. Intensity levels following excitation at 340 nm and 380 nm were detected with an IC-200 CCD camera and data were analyzed using Imagemaster software. Images were obtained every 5 sec.

The ratio R of light excited at 340 nm to that at 380 nm was converted into Ca^{2+} concentration using the method of Grynkiewicz et al. (Grynkiewicz, Poenie & Tsien, 1985). An in situ K_d value for fura-2 of 350 nM was used (Negulescu & Machen, 1990). R_{min} was obtained by bathing cells in a Ca^{2+}-free isotonic solution of pH 8.0 containing 10 mM EGTA and 10 μM ionomycin. R_{max} was obtained by bathing the cells in isotonic solution with 0.1 mM

Ca^{2+} and 10 μM ionomycin. Calibration was performed separately for each experiment.

Patch Clamping

Unless otherwise stated, whole-cell patch-clamp currents were recorded in the ruptured-patch mode. Micropipettes were pulled from Corning No. 7052 glass, coated with Sylgard, and fire-polished. The resistances of the micropipettes in the bath were usually 1–3 MΩ; successful seals displayed gigohm resistances. Following rupture of the membrane patch, the series resistance was measured to be only 5.6 ± 0.5 MΩ (N = 38), and was therefore not compensated; whole-cell capacitance was 52 ± 4 pF. Applied voltages were not corrected for the small junction potential [~−2.8 mV (Carré et al., 2000)] arising from the present micropipette-filling and external solutions (*see* Table 2). In a series of 9 experiments, currents were recorded in the perforated-patch mode as previously described (Anguita et al., 1995). In that case, we back-filled the micropipettes with solution containing amphotericin (168 μg/ml) and filled the tips with amphotericin-free solution.

Data were acquired at 2–5 kHz with either an Axopatch 1D (Axon Instruments, Foster City, CA) or a List L/M-EPC7 (Darmstadt, Germany) patch-clamp amplifier and filtered at 500 Hz. The membrane potential was held at either −40 or −80 mV and stepped to test voltages from −100 to +80 mV in 20 mV increments at 2 s intervals. At the more negative holding potential, depolarizations produced transient inward currents, consistent with L-type Ca^{2+} currents known to characterize these cells (Wiederholt & Stumpff, 1998). Otherwise, the current responses to voltage steps were similar at −40 and −80 mV. Each step lasted 300 ms with intervening periods of 1.7 s at the holding potential. Stimulatory responses were measured at peak levels and inhibitory responses at the nadirs. Baseline whole-cell currents were 766 ± 241 pA at +80 mV.

ATP Release

Cells were grown for 3–24 days to confluency on glass coverslips, washed in control solution (Isotonic, Table 1) and mounted on an inverted microscope (Fleischhauer et al., 2001). Bath ATP levels were measured continuously by including 2 mg/ml luciferin/luciferase in the assay mixture (Taylor et al., 1998). After recording background levels, isotonic or hypotonic solution (Table 1) containing luciferin/luciferase assay was carefully added to the cells. ATP released from cells into the extracellular bath reacted with the luciferase and led to photon production. Light was captured with a 20× objective, filtered at 520 nm, measured with a photomultiplier tube and recorded on-line using the Felix software suite (Photon Technologies International). The luminescence values were converted to concentrations of ATP using different calibration curves in analyzing isotonic and hypotonic data since some hypotonic solutions enhance the ATP signal (Mitchell, 2001).

Drugs and Experimental Solutions

All chemicals were reagent grade. The acetoxymethyl ester form of fura-2 and 0.005% Pluronic F-127 used to measure intracellular Ca^{2+} were obtained from Molecular Probes (Eugene, OR). Adenosine and ATP were purchased from Sigma Chemical (St. Louis, MO). The selective A_1AR agonists N^6-cyclopentyladenosine (CPA) and adenosine amine congener (ADAC), the selective $A_{2A}AR$ agonists 2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamido-adenosine (CGS-21680) and N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine (DPMA), and the selective A_3AR agonists 1-deoxy-1-(6-[([3-iodophenyl]methyl)amino]-9H-purin-9-yl)-N-methyl-β-D-ribofuranuronamide (IB-MECA) and 2-chloro-IB-MECA (Cl-IB-MECA) were obtained from RBI Sigma (St. Louis, MO). The Cl^--channel blocker [5-nitro-2-(3-phenylpropylamino)-benzoate] [NPPB, (Wangemann et al., 1986)] was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). The compositions of the isotonic and hypotonic solutions used for patch-clamp and fluorescence measurements are entered in Tables 1 and 2, respectively.

Table 1. Solutions for measurements of ATP release, volume and intracellular Ca^{2+} (in mM)

Component	Isotonic	Hypotonic	0.5 μM-Ca^{2+}	Calibration
Na^+	140.0	60.5	112.0	140.0
K^+	5.9	5.9	4.0	5.9
Cl^-	122.1	42.6	127.2	117.1
Mg^{2+}	1.2	1.2	1.2	1.2
Ca^{2+}	2.5	2.5	4.4	±0.1
HEPES	15.0	15.0	12.0	15.0
HCO_3^-	30.0	30.0	–	30.0
$H_2PO_4^-$	1.2	1.2	–	1.2
Glucose	10.0	10.0	5.0	10.0
Mannitol	–	–	60.0	–
EGTA	–	–	5.0	10.0

The osmolalities of the isotonic (and 0.5 μM-Ca^{2+}) and calibration solutions were 295–310 and 305–338 mOsm, respectively, while the hypotonic osmolality was 155–165 mOsm. The pH values of the isotonic and calibration solutions were adjusted to 7.4 and 8.0, respectively.

Data Analysis

Means ± SEM were calculated from analysis of N experiments. Unless otherwise stated, the probability P of the null hypothesis was estimated with the paired, 2-tailed Student's t-test.

Results

Adenosine Agonists and Intracellular Ca^{2+}

Recent evidence suggests that stimulation of adenosine receptors can lead to changes in the levels of intracellular Ca^{2+} in addition to the more frequently noted changes in cAMP (Kohno et al., 1996; Mitchell et al., 1999; Fredholm et al., 2001; Linden, 2001). We first tested whether adenosine could differentially alter intracellular Ca^{2+} concentration of human TM cells. In confluent cells, the baseline Ca^{2+} concentration was 136 ± 15 nM (N = 29), comparable to the 106 ± 0.5 nM previously reported for human TM cells (Howard et al., 1996). A transient increase in Ca^{2+} of ~40% followed by a sustained elevated plateau was triggered by 10 μM adenosine (Fig. 1). Subsequent application of ATP triggered a fresh response, suggesting ATP was activating a different population of receptors.

At a concentration of 10 μM, adenosine can stimulate A_1, A_{2A} and A_3 adenosine receptors, so

Table 2. Solutions for patch-clamp experiments

Component	Micropipette		External Bath Solutions		
	+K-MS	0K-MS	+K-BS	0K-BS	5 μM-Ca^{2+}
Na^+	12.2	25.0	112.0	116.0	112
Cl^-	25.0	25.0	122.0	122.0	118.4
K^+	122.5	–	4.0	–	4.0
NMDG	–	109.2	–	–	–
Aspartate	110.5	110.0	–	–	–
Mg^{2+}	1.0	1.0	1.2	1.2	1.2
Ca^{2+}	0.38	0.38	1.8	1.8	0.005
EGTA	1.0	1.0	–	–	–
HEPES	12.0	12.0	12.0	12.0	12.0
ATP	1.0	1.0	–	–	–
Glucose	–	–	5.0	5.0	5.0
Mannitol	–	–	±60.0	±60.0	60.0

Concentrations are given in mM. The osmolarities of the micropipette solutions, isotonic and hypotonic bath solutions were 265–287, 299–311 and 240–245 mOsm, respectively. The pH was adjusted to 7.2 and 7.4 for the micropipette and bath solutions, respectively. The free Ca^{2+} concentration of the micropipette solution was 100 nM.

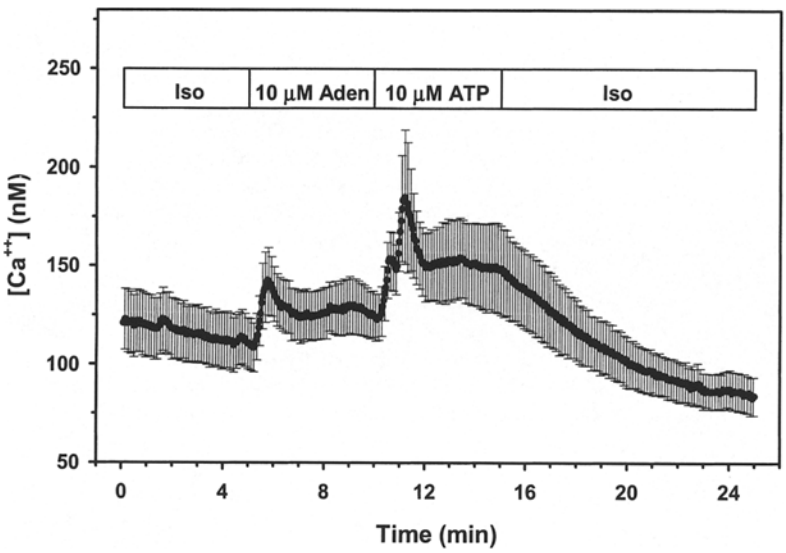

Fig. 1. Effects of adenosine and ATP on Ca^{2+} of TM cells in confluent monolayers. Following perfusion with 10-μM concentrations of adenosine (A), the cells were subsequently perfused with 10 μM ATP (hTM#22, p3). In this and subsequent graphs, averaged results are presented as means ± SEM.

agonists for each receptor were tested separately. Elevations in Ca^{2+} were triggered by CPA (A_1AR agonist, Fig. 2*A*), CGS-21680 (A_{2A}AR agonist, Fig. 2*B*) and Cl-IB-MECA (A_3AR agonist, Fig. 2*C*). Figure 2 illustrates that the first two applications of increasingly high concentrations of AR agonists characteristically resulted in graded increases in Ca^{2+}. However, the third application containing the highest agonist concentration (100 nM) produced a smaller Ca^{2+} response. To determine whether the relative magnitudes of the responses might depend on the sequence as well as concentration of the drug applications, we conducted a similar series of experiments, in which the order of addition of the final two drug concentrations was reversed. The 100-nM drug concentrations now produced increases in Ca^{2+} that were similar to or slightly higher than those triggered by the later intermediate concentrations [CPA (N = 3), CGS-21680 (N = 4) and Cl-IB-MECA (N = 3), *data not shown*]. We interpret the small increases in Ca^{2+} triggered by the 100-nM concentrations of Figure 2*A*–2*C* as likely reflecting receptor desensitizations, which can be variable in magnitude, partly as a function of cell line and passage number.

Although all agonists produced responses within receptor-specific concentrations, experiments to measure concentration-response relationships were complicated by: (1) the desensitization of ARs during individual experiments (Fig. 2), (2) the need to limit the total time of study during individual experiments with these cells to ~30 min to ensure technically satisfactory final Ca^{2+} calibrations and (3) substantial variability in the magnitudes of the Ca^{2+} spikes from preparation to preparation. To confirm further the pharmacologic identification of adenosine receptors on hTM cells, experiments were repeated

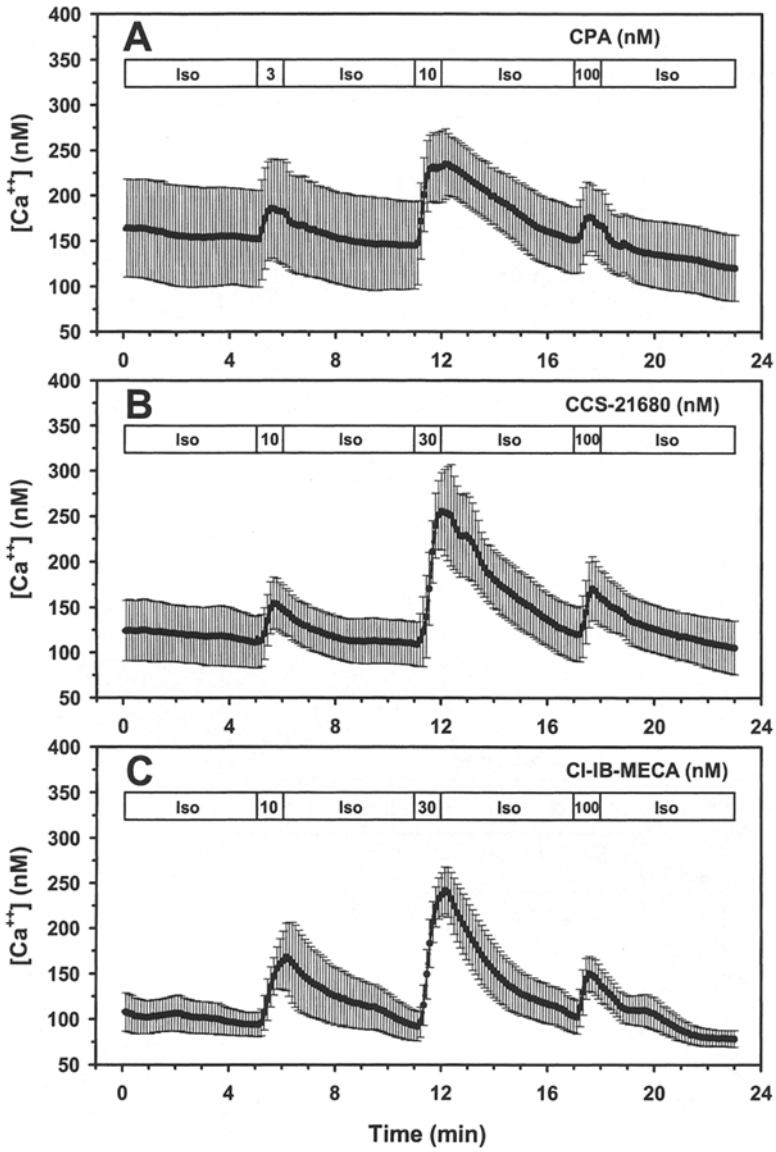

Fig. 2. Effects of increasing concentrations of subtype-selective AR agonists on intracellular Ca^{2+}. (*A*) CPA (A_1AR agonist): $N = 4$, hTM#29, p3–4. (*B*) CGS-21680 (A_{2A}AR agonist): $N = 4$, hTM#29, p3–4. (*C*) Cl-IB-MECA (A_3AR agonist): $N = 4$, hTM#29, p3.

using a second set of subtype-specific agonists. These measurements were obtained from single cells as opposed to confluent sheets to provide additional information about possible heterogeneity of cellular responses. Intracellular Ca^{2+} was increased by 30 nM ADAC (Fig. 3*A*), 50 nM DPMA (Fig. 3*C*) and 30 nM IB-MECA (Fig. 3*E*), agonists to the A_1, A_{2A} and A_3ARs, respectively. The concentrations were chosen to be large enough to ensure robust responses by the subtype-directed ARs and to be less than the published K_i values for the remaining putatively present receptors. The magnitude of the response varied considerably among different cells although all cells tested did respond to the agonists. The examples of responses by individual cells shown in Fig. 3*B*, 3*D*, and 3*F* illustrate this variation.

Table 3 summarizes the increases in Ca^{2+} triggered by AR agonists calculated as the paired differences between peak and baseline values measured just prior to perfusion with the new agonist concentration. Comparing the lowest effective concentrations with published estimates of K_i (Table 3) indicates that CPA and ADAC, CGS-21680 and DPMA, and Cl-IB-MECA and IB-MECA increased Ca^{2+} by selectively occupying A_1, A_{2A} and A_3ARs, respectively. It should be noted that the mean increases in Ca^{2+} concentrations entered in Table 3 were the mean paired peak values. Since the maximum response was not displayed by all cells at the same time, the apparent peak values in Figures 2–3 are somewhat less, resulting in a maximum underestimate of the peak response by ~12% in Figure 3*E*.

The subtype-selective AR agonists triggered surprisingly similar changes in intracellular Ca^{2+}. However, the agonists might also have triggered changes in multiple other signalling pathways, producing an integrated signal still specific for each adenosine receptor. To address this possibility, we

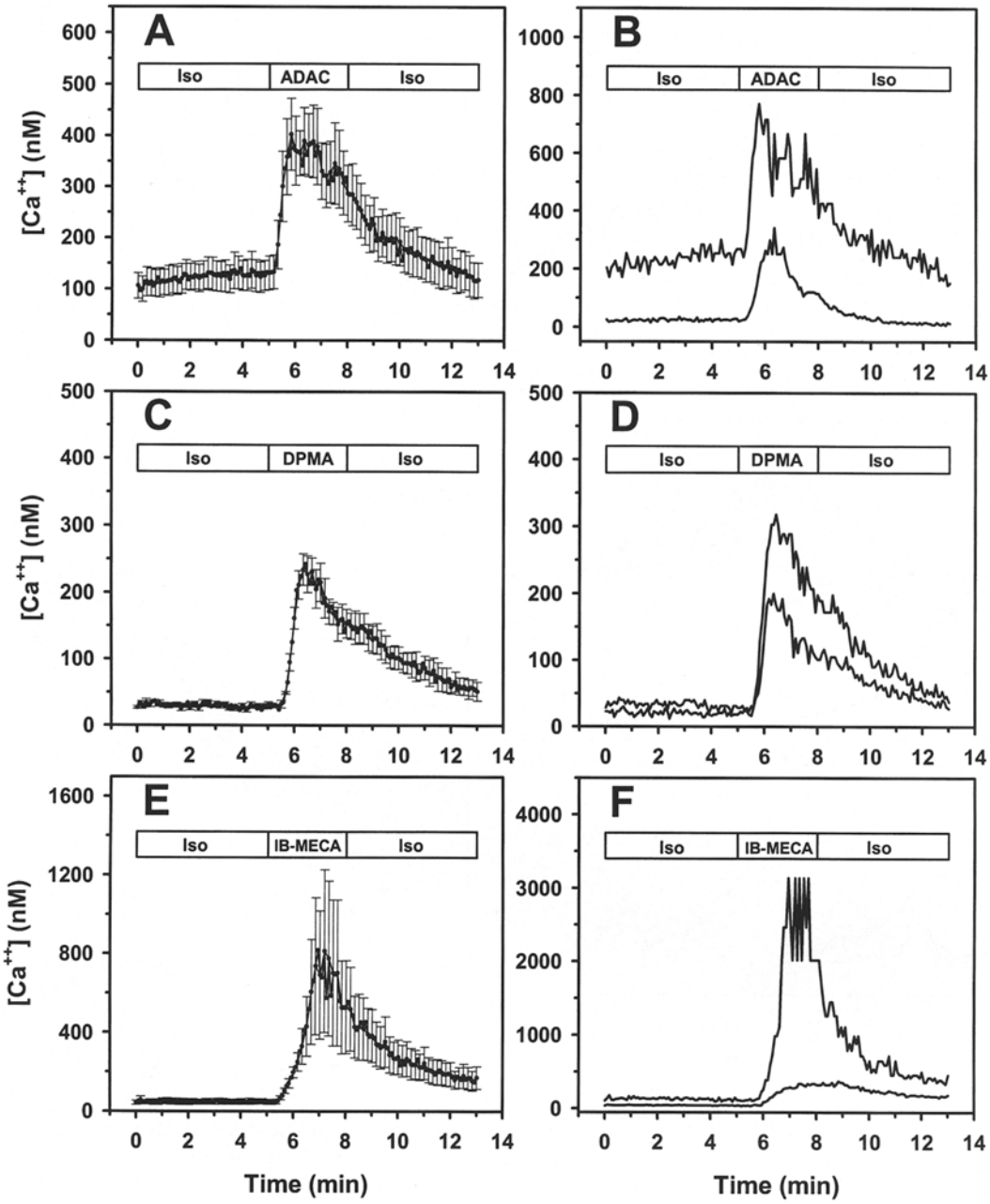

Fig. 3. Effects of adenosine receptor agonists on Ca^{2+} of single TM cells. Panels *A*, *C* and *E* show the mean Ca^{2+} responses in single attached cells to a second set of subtype-selective AR agonists. (*A*) ADAC (A_1AR agonist): 30 nM, N = 7, hTM#22, p5 and hTM#29, p3. (*C*) DPMA (A_{2A}AR agonist): 50 nM, N = 4, hTM#29, p4. (*E*) IB-MECA (A_3AR agonist): 30 nM, N = 8, hTM#29, p4 and hTM#22, p5. Panels *B*, *D* and *F* show the largest and smallest single response triggered by the agonists. (*B*): ADAC: hTM#29, p3. (*D*) DPMA: hTM#29, p4. (*F*) IB-MECA: hTM#22, p5.

measured the responses of a potential functional target of AR agonist-triggered signalling cascades, the TM-cell volume.

TM-Cell Volume

The volume of human TM cells has been shown to change in response to changes in tonicity and ion transport blockers (Mitchell et al., 2002; O'Donnell et al., 1995). Experiments were performed to determine if subtype-selective AR agonists produced opposite changes in cell volume, using calcein-detected cell area as an index of volume [(Mitchell et al., 2002); *see* also Discussion]. Volume is of particular interest since TM-cell shrinkage and swelling may play a role in regulating outflow (*see* Introduction). Following growth on coverslips for ~24 hrs, exposure to 100 nM CPA (A_1 agonist, Fig. 4*A*), 100 nM CGS-21680 (A_{2A}

Table 3. Measured increases in intracellular Ca^{2+} (nM) produced by subtype-selective AR agonists and published estimates of the agonist selectivities[a]

AR Target	A_1AR		$A_{2A}AR$		A_3AR	
Agonist Conc. (nM)	CPA	ADAC	CGS-21680	DMPA	Cl-IB-MECA	IB-MECA
3	36 ± 8 [7][e]	–	–	–	–	–
10	112 ± 19 [4][d]	–	44 ± 5 [8][g]	–	71 ± 19 [7][d]	–
30	–	344 ± 56[g]	173 ± 47 [4][b]	–	157 ± 18 [4][e]	885 ± 439[f]
50	–	–	–	219 ± 24[e]	–	–
100	49 ± 9 [9][d]	–	94 ± 37 [9][b]	–	90 ± 14 [7][g]	–
K_i (nM)[a]						
A_1	2	1	289	142	820	54
A_{2A}	94	210	27	4	470	56
A_3	18,600	281	67	3,570	0.3	1

Means ± SE were calculated from responses of cells exposed to no more than two different agonist concentrations, with an intervening washout period (as in Fig. 2). [a](Jacobson & Knutsen, 2001), [b]<0.05, [c]<0.025, [d]<0.01, [e]<0.005, [f]<0.005 by non-parametric coin test, [g]<0.001.

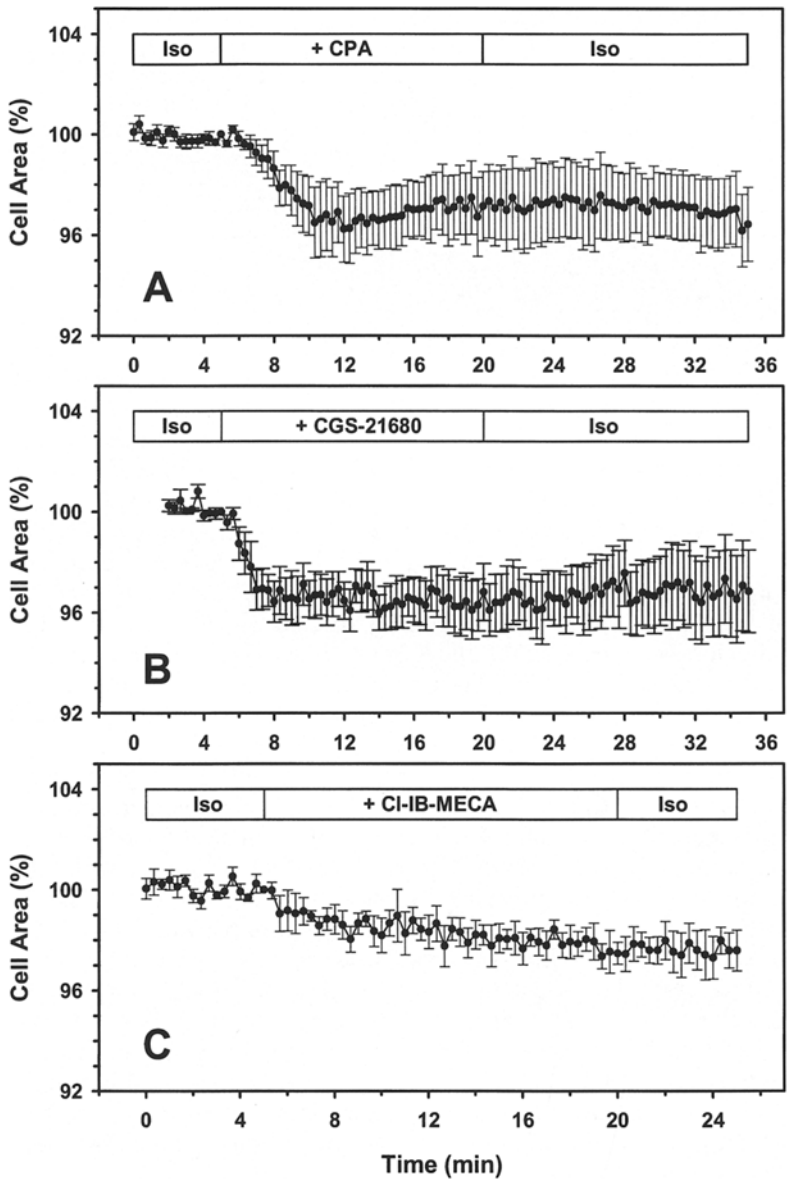

Fig. 4. Effects of AR agonists on area of TM cells grown on coverslips overnight. The A_1AR agonist CPA (panel *A*, 100 nM, $N = 6$, hTM#29, p4) and the $A_{2A}AR$ agonist CGS-21680 (panel *B*, 100 nM, $N = 6$, hTM#29, p4) both produced a sharp reduction in cell volume that did not readily return to pre-agonist levels. The A_3AR agonist IB-MECA (100 nM, $N = 6$, hTM#29, p4) produced a small reduction in area (panel *C*), although previous application of CGS-21680 may have attenuated the responses in this case.

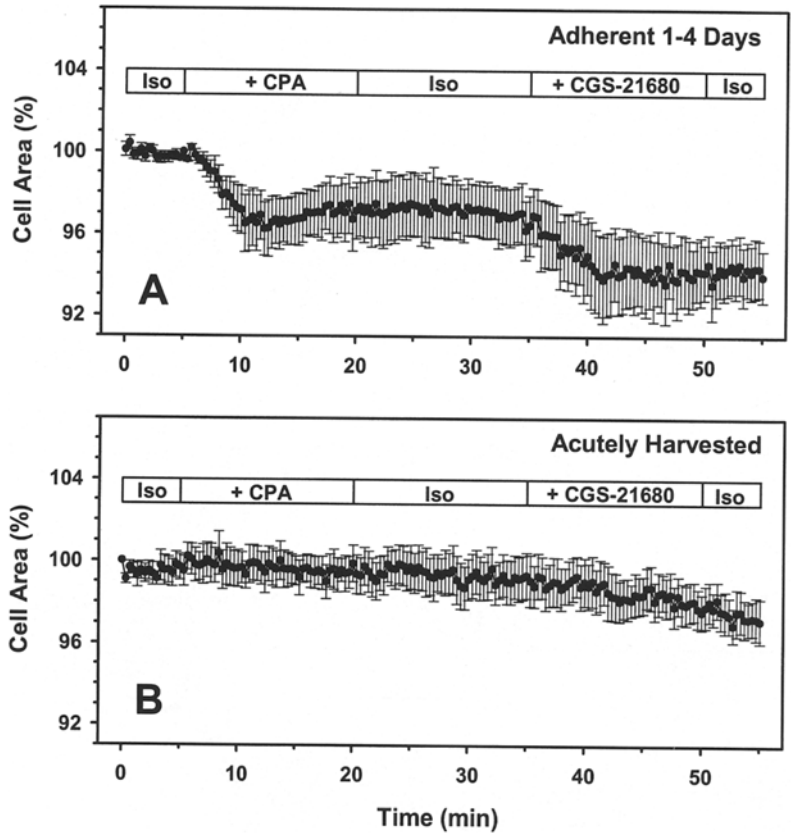

Fig. 5. Comparison of effects of AR agonists on cells acutely harvested and cells grown overnight on coverslips. (*A*) After growth on coverslips overnight, both 100 nM CPA and 100 nM CGS-21680 produced reductions in TM cell area. Data obtained from same experiments as those of Figure 4*A* ($N = 6$, hTM#29, p4). (*B*) Acutely harvested cells (adherent for 47–50 min) from same cell line and passage number ($N = 4$, hTM#29, p4) displayed no volume response to same AR agonists as in panel *A*.

agonist, Fig. 4*B*) and 100 nM Cl-IB-MECA (A_3 agonist, Fig. 4*C*) produced clear reductions in cell volume. The maximal changes in area (ΔA), were $-3.4 \pm 1.3\%$ following CPA ($P < 0.05$, $N = 6$), $-3.5 \pm 0.9\%$ following CGS-21680 ($P < 0.025$, $N = 5$) and $-2.1 \pm 0.5\%$ following Cl-IB-MECA ($P < 0.005$, $N = 5$). Subsequent addition of other agonists showed that single cells could respond to multiple agonists (Fig. 5*A*). The sensitivity of the assay was confirmed at the conclusion of each experiment when the characteristic swelling previously studied (Mitchell et al., 2002) was produced by hypotonic solution (*not shown*). Thus, stimulation of A_1, A_{2A} and A_3 adenosine receptors modified the volume of TM cells grown for one or more days. In contrast, freshly harvested cells (adherent for 47–50 min) from the same cell line and passage showed no volume response to AR agonists (Fig. 5), possibly because of partial internalization of AR receptors or remodelling of the cytoskeleton during the harvesting procedure.

To address whether the ability of adenosine agonists to elevate intracellular Ca^{2+} and reduce cell volume might be causally related, we elevated Ca^{2+} directly with the ionophore ionomycin while monitoring the volume of adherent cells. In the presence of 0.5 μM Ca^{2+}, 2 μM ionomycin produced clear reductions in cell volume (Fig. 6*A*). The same treatment elevated Ca^{2+} by 1.6 ± 0.8 μM in parallel experiments (Fig. 6*B*). Simultaneous application of a K^+-channel blocker (2 mM Ba^{2+}) and a Cl^--channel blocker (100 μM NPPB) blocked the volume response to ionomycin, but also reduced the magnitude of the rise in Ca^{2+} by ~80% to 372 ± 56 nM ($N = 4$, hTM#29, p3–4, *data not shown*), presumably by depolarizing the TM cell membrane. However, NPPB alone also blocked the volume response (Fig. 6*C*) without altering the increase in Ca^{2+} (Fig. 6*D*), so that the reduction in volume was likely mediated in part by the efflux of Cl^- ions. These results suggested that the TM cells might possess Ca^{2+}-sensitive ion channels whose opening can lead to reductions in cell volume.

The above results indicated that AR agonists trigger cell shrinkage through possible activation of ion channels. In many cells, reduction in cell volume reflects activation of K^+ and Cl^- channels, with fluid accompanying the movement of ions out of the cell. However, the K^+-Cl^- symport blocker [(dihydroindenyl)oxy]alkanoic acid (DIOA)] partially inhibits the regulatory volume decrease in the human TM cells studied here (Mitchell et al., 2002), so that electroneutral K^+-Cl^- symports might also mediate the shrinkage induced by AR agonists. Whether or not AR agonists differentially activated K^+ and Cl^- channels was tested directly with whole-cell patch clamping.

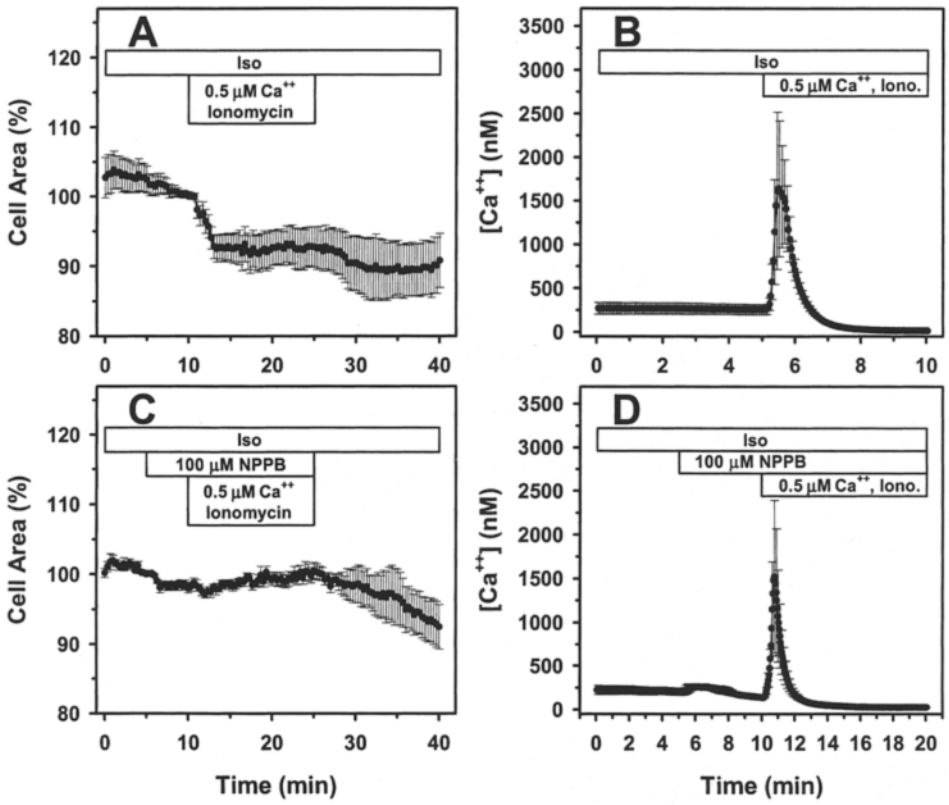

Fig. 6. Ionomycin decreases cell volume. (*A*) The Ca^{2+} ionophore ionomycin triggered a clear decrease in cell volume, as determined by measuring cell area (N = 6, hTM#29, p3–4). (*C*) The Cl^--channel blocker NPPB prevented this change in volume (N = 5, hTM#29, p4). Parallel experiments examining the intracellular Ca^{2+} levels illustrate that the large, sudden increase in Ca^{2+} produced by ionomycin was not altered by NPPB. (*B*) N = 4, hTM#47, p3–4 and hTM#29, p3. (*D*) N = 4, hTM#29, p4.

Effect of Adenosine Agonists on TM-Cell Currents

In the presence of K^+ (+K-MS micropipette solution and +K-BS bathing solution, Table 2), baseline currents were largely inhibitable by 7.5 mM TEA (Fig. 7*A*), indicating that K^+ channels contributed much of the baseline membrane conductance. With K^+ present, 10 μM adenosine increased baseline currents in 4/11 cells (Fig. 7) and these adenosine-activated currents were also largely inhibited by TEA (Fig. 7*A*). The time courses of the adenosine-difference currents following step changes in voltage are presented in Figure 7*B*, and the corresponding *I-V* relationship is presented in Figure 7*C*. The inhibition by TEA indicates that the depolarization-activated, outwardly rectifying adenosine-activated currents largely reflect operation of K^+ channels, likely corresponding to the BK channels previously noted in these cells (Wiederholt & Stumpff, 1998). However, in comparison to the TEA-inhibited difference currents, the adenosine-activated currents displayed a positive shift in reversal potential and persistence of inward currents at strong hyperpolarizations (Fig. 7*C*). Evidently, adenosine activated cation-nonselective and/or Cl^- channels in addition to the K^+ channels. The magnitude of the adenosine-triggered increase in current at +80 mV was 37–49% in 3 cells, and 201% in a 4th (mean = 82 ± 40%). Adenosine had no effect on 5/11 cells and actually reduced the K^+ currents of 2/11 cells. We also monitored the responses to adenosine in the absence of K^+, in order to enhance resolution for detecting activation of Cl^- and nonselective channels. However, 10 μM adenosine activated currents in only 1/9 cells without K^+.

The range of responses to adenosine perfusion might have reflected opposing effects of 10 μM adenosine on A_1, A_{2A} and A_3ARs. This possibility was addressed by separately perfusing selective agonists to each of these three ARs. The A_{2A}AR agonist CGS-21680 (100–150 nM) activated channels most consistently, increasing currents in 3/7 cells with K^+ (Fig. 8) and in 3/8 cells without K^+ (Fig. 9), without inhibiting any of the currents. The A_3AR agonist Cl-IB-MECA (100 nM) also activated currents in 3/7 cells with K^+ (Fig. 8) and in 2/7 cells without K^+ (Fig. 9), and did not trigger any inhibitions. The inhibitions produced by TEA (Fig. 8) and NPPB (Fig. 9) and the reversal potentials of the difference currents indicate that CGS-21680 and Cl-IB-MECA activated both K^+ and Cl^- currents. In contrast to the A_{2A}AR and A_3AR agonists, the A_1AR agonist CPA (100 nM) had little effect (Fig. 9), activating currents in 1/5 cells in the presence of K^+ and in 0/7 cells without K^+, and had no effect on the currents of the remaining cells. Figure 10 presents the magnitudes of the AR agonist-triggered effects (averaged only from cells displaying changes).

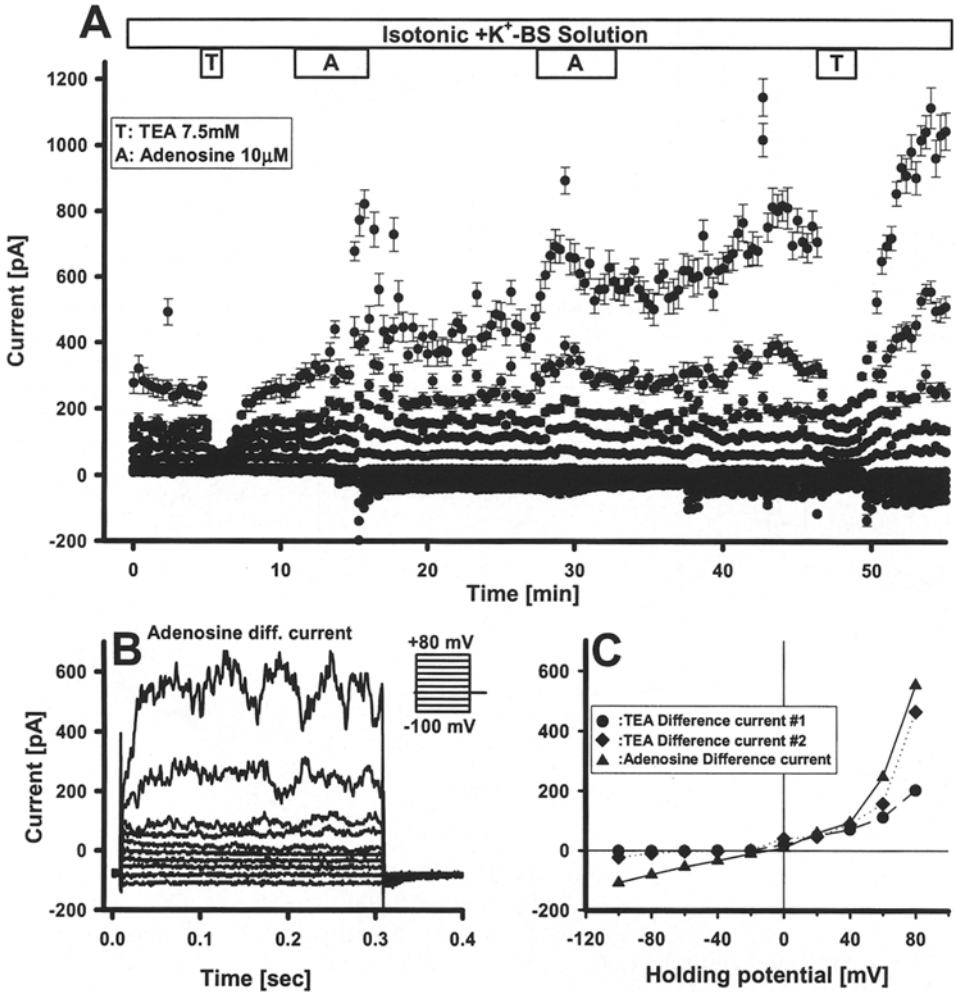

Fig. 7. Effects of adenosine on TM-cell currents in presence of K^+. (*A*) The time course of currents before, during and after transient sequential perfusion with 7.5 mM TEA, 10 μM adenosine twice successively, and TEA a second time at the conclusion of the experiment. Following the 2 periods of adenosine administration, the large TEA-sensitive currents were increased by ~130%, but the smaller TEA-insensitive currents were also increased by ~310% at +80 mV (hTM# 36, p3). (*B*) Responses of 10 μM adenosine-difference currents to step changes in voltage from the holding potential of −40 mV, In Figures 6–8, the difference currents were calculated by subtracting means of 1–3 sets of measurements just before change in perfusate from a similar set of records at the peak stimulation or maximal inhibition. (C) Current-voltage relationships for the initial adenosine- and the two TEA-difference currents.

In view of the range of responses to AR agonists, we wondered whether ruptured-patch whole-cell recording might also have been producing variable washout of cofactors potentially involved in channel activation and inactivation. We addressed this possibility by conducting 9 experiments in the perforated-patch whole-cell configuration. The probability of observing either excitatory or inhibitory effects was not increased. For example, 10 μM adenosine activated currents in 1/4 cells and had no effect on 3/4 TM cells in the presence of K^+. The results of the 9 experiments with perforated-patch recording are included in the reduced data cited above.

In part, the highly variable responses to AR agonists might also have reflected the variable magnitude of the Ca^{2+} elevation produced by adenosine agonists in different cells (Fig. 3). Consistent with this possibility, we verified that raising intracellular Ca^{2+} activity above the elevations triggered by AR agonists indeed triggered bursts of current activity. Application of the calcium ionophore ionomycin (10 μM) in the presence of 0.5–5.0 μM extracellular Ca^{2+} increased whole-cell current in each of four experiments by 716 ± 233% at +80 mV (*data not shown*). As noted above, superfusion with ionomycin and 0.5 μM Ca^{2+} raised the mean intracellular free Ca^{2+} to 1.5–1.6 μM (Fig. 6*B* and 6*D*).

Test of Signaling Role of Ca^{2+}

The effects of ionomycin on the volume and whole-cell currents of TM cells suggested that a rise in intracellular Ca^{2+} might mediate the observed responses to AR agonists. In order to test whether the

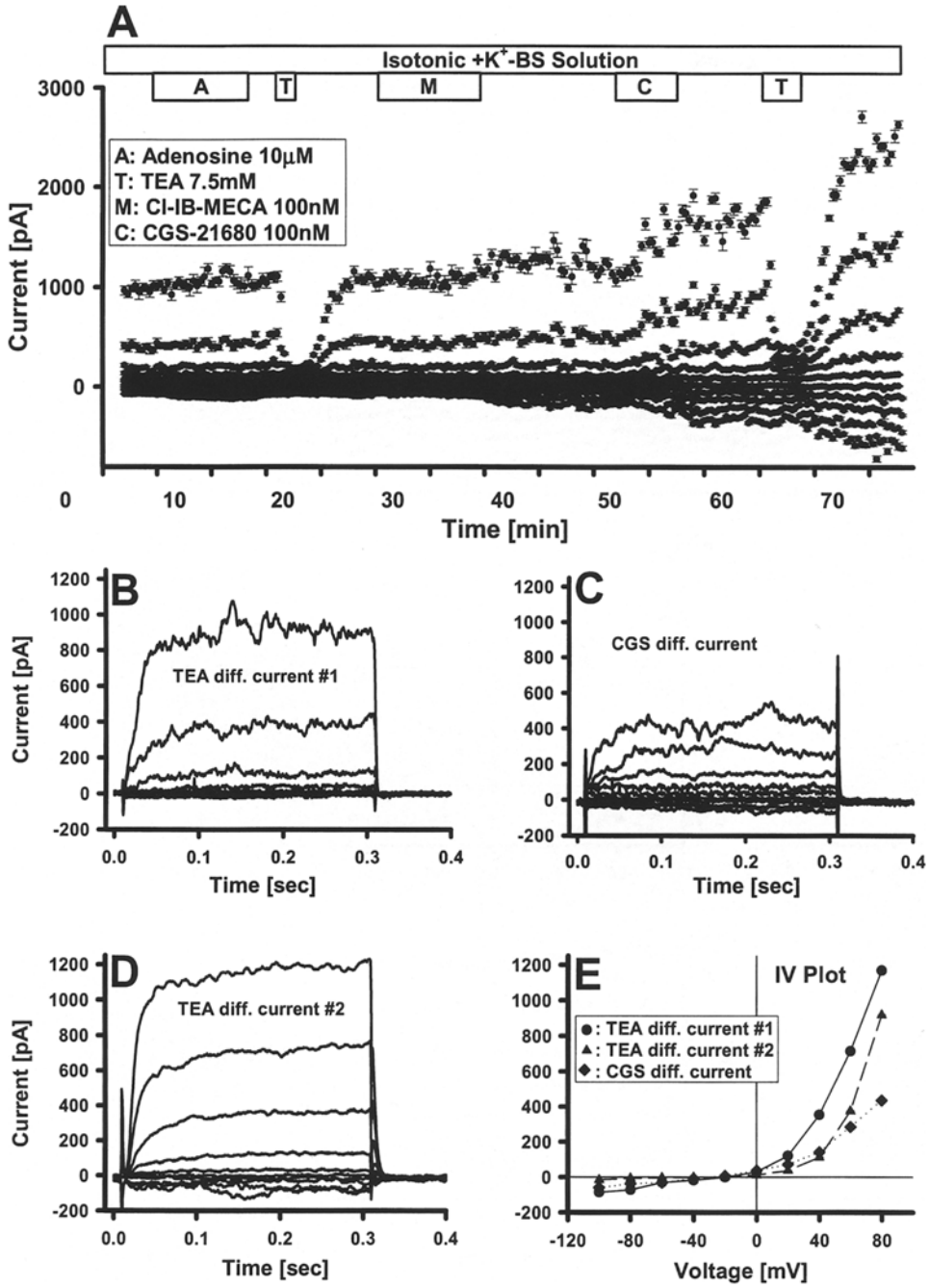

Fig. 8. Effects of subtype-specific adenosine agonists on TM-cell whole-cell currents in presence of K^+. (*A*) Initial application of 10 μM adenosine only slightly increased the currents, which were ~70% inhibitable by 7.5 mM TEA (hTM #36, p3). Subsequent perfusion with the selective A_3AR agonist IB-MECA (100 nM) produced a delayed increase in currents (10% at + 80 mV) beginning just before restoring the control solution (+K-BS, Table 1). The later application of the selective A_{2A}AR agonist CGS-21680 (100 nM) triggered the largest activation (36%) of currents, which were again ~70% TEA-inhibitable. (*B–D*) Responses of TEA- and CGS-21680-difference currents to step changes in voltage. (*E*) Current-voltage relationships for the TEA- and CGS-21680 difference currents.

AR agonist-induced shrinkage is mediated by the increase in Ca^{2+}, we preloaded TM cells with the Ca^{2+} buffer BAPTA. Duplicate measurements verified that 10 μM adenosine did not alter intracellular Ca^{2+} (hTM#26, p5, *data not shown*). Nevertheless, after incubating early-passage TM cells (hTM#232-30 RC, p2) in BAPTA-AM, the same concentration of adenosine reduced cell area by 6.3 ± 1.1% (N = 3, $P < 0.05$, *data not shown*). We conclude that AR agonists trigger both an increase in Ca^{2+} and a reduction in cell area, but the effects are not causally related.

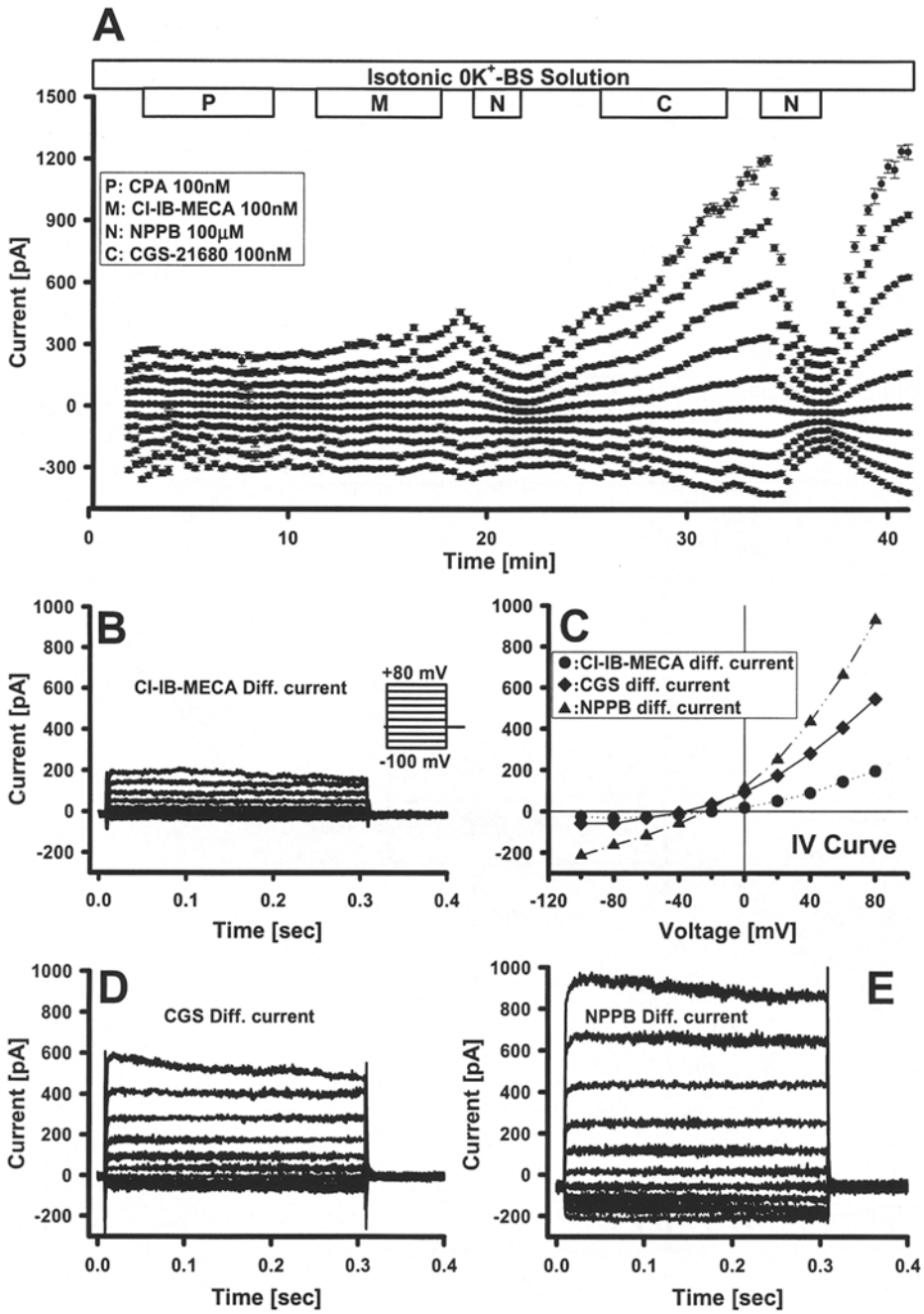

Fig. 9. Effects of subtype-specific adenosine agonists on TM-cell whole-cell currents in absence of K^+. (*A*) The selective A_1AR agonist CPA (100 nM) had no effect, but the selective A_3AR agonist IB-MECA (100 nM) increased the current by 75% at +80 mV (hTM # 29, p5). Subsequent application of the selective $A_{2A}AR$ agonist CGS-21680 (100 nM) further increased that current by 149%, All of the $A_{2A}AR$-mediated increase in current was NPPB-inhibitable since the residual NPPB-insensitive currents were unchanged by the CGS-21680. (*B*, *D*, *E*) Responses of Cl-IB-MECA-, CGS-21680- and NPPB-difference currents to step changes in voltage, respectively. (*C*) Current-voltage relationships for the difference currents of panels *B*, *D* and *E*.

Endogenous Source of Adenosine from Released ATP

Adenosine could be delivered to cells by ecto-enzymatic metabolism of ATP released into the extracellular fluid (Zimmermann, 2001). ATP is released into the aqueous humor by ciliary epithelial cells (Mitchell et al., 1998), but we wondered whether TM cells might provide an additional local source of ATP delivery to cells in the outflow pathway. ATP release was measured following isotonic and hypotonic perfusion of analogous fields of constant area containing

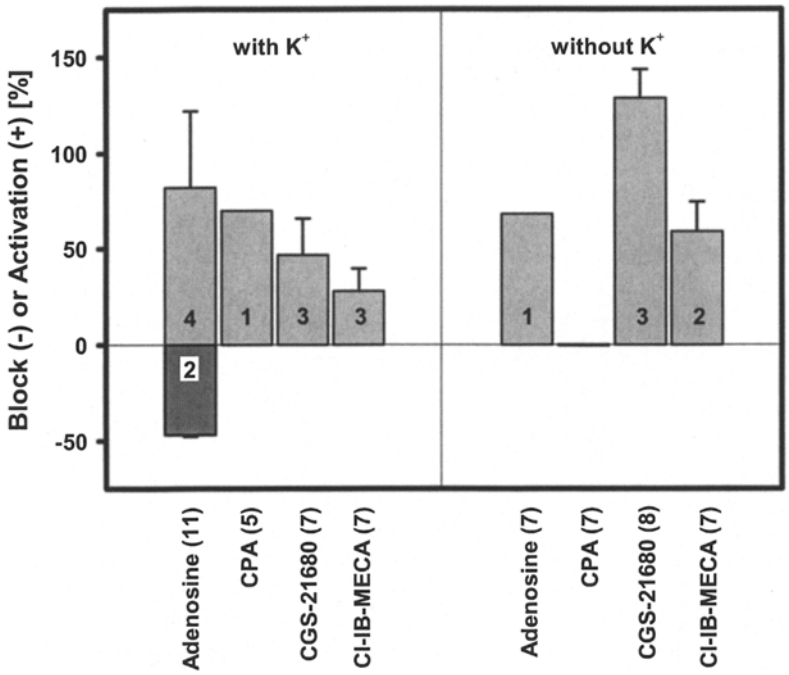

Fig. 10. Effects of ATP and AR agonists on TM-cell whole-cell currents in presence or absence of K^+. The heights and depths of the bars present the means ± SE of the percentage activations and inhibitions, respectively, averaged from those cells displaying responses. The numbers of cells responding to the agonists are indicated within the bars, and the total numbers of cells are included in parentheses along the abscissa.

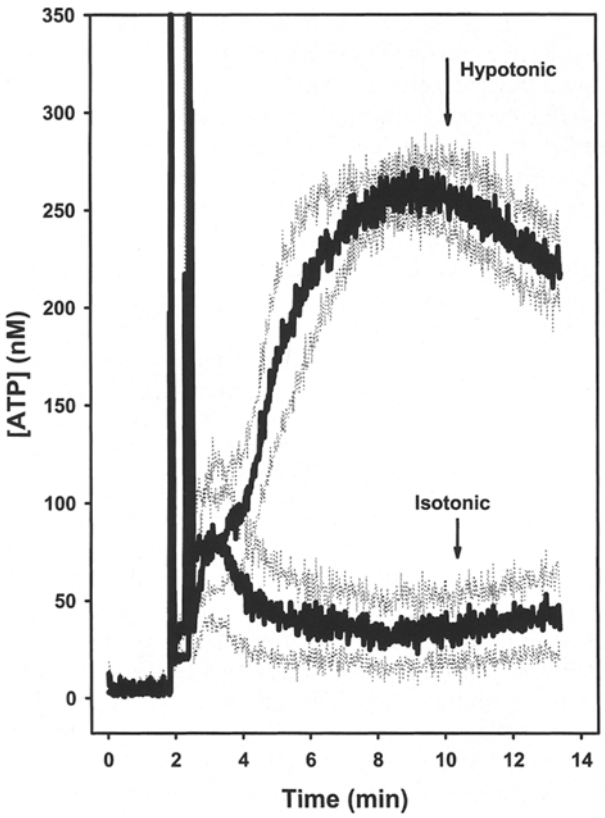

Fig. 11. Release of ATP by TM cells. Hypotonic perfusion triggered a 7-fold larger release of ATP than did isotonic perfusion of the human TM cells. The dark lines represent the mean of 4 experiments (hTM#29, p6) while the grey lines show the accompanying standard errors. The vertical lines at t ~ 2 min are caused by the opening of the chamber to change solutions to those containing the luciferin/luciferase assay system.

~90 confluent cells. As illustrated by Figure 11, hypotonic perfusion of TM cells triggered a seven-fold greater release of ATP than did isotonic perfusion. The ATP concentrations in hypotonic and isotonic solutions were 260 ± 12 nM and 34 ± 19 nM, respectively ($N = 4$, $P < 0.001$). These results suggest that TM cells may serve as a physiologic reservoir for ATP release, and by putative ecto-enzymatic activity, for physiologic delivery of adenosine to cells of the aqueous outflow pathway.

A very approximate estimate of the ATP release/unit area of confluent cells is provided by calculating the product of incubation volume (60 µl) and maximum measured ATP release. However, the released ATP is unlikely to diffuse evenly throughout the solution before reacting with the assay probe. More likely, the measured response largely reflects ATP reacting with probe immediately outside the unstirred layer. The presence of ecto-ATPases further complicates efforts to quantify precisely the amount of ATP released.

Discussion

The salient findings were that: (1) Human TM cells displayed functional responses to adenosine and subtype-selective agonists for A_1, A_{2A} and A_3 adenosine receptors; (2) all AR agonists triggered qualitatively similar changes in intracellular Ca^{2+} and volume; (3) the AR agonists produced highly variable Ca^{2+} increases and activation of K^+ and Cl^- channels in freshly harvested cells studied within ~2 hrs after being plated; and (4) human TM cells can be

stimulated to release ATP, an endogenous ecto-enzymatic precursor of adenosine.

Signalling Cascades

The agonist-triggered increases in intracellular free Ca^{2+} concentration are summarized in Table 3, together with published values (K_i) based on binding competition assays (Jacobson & Knutsen, 2001). The absolute magnitude of the Ca^{2+} response displayed great variability, reflecting differences not only in cell lines, passage numbers and sample preparations (monolayers or freshly harvested cells) but even between coverslips studied on the same day. However, the concentration dependence of the responses to AR agonists was revealed by applying increasing concentrations to cells on single coverslips. The first two concentrations (separated by a washout period) elicited graded responses; reduced responses were displayed thereafter, possibly because of desensitization and depletion of cell stores of calcium (Fig. 2). The significant Ca^{2+} elevations produced by low concentrations of CPA, CGS-21680 and Cl-IB-MECA and by single low concentrations of ADAC, DMPA and IB-MECA indicate that all of these subtype-selective agonists were acting at the targeted ARs. The current results provide clear documentation that human TM cells display functional A_1, A_{2A} and A_3ARs.

The results illustrated in Figs. 1–3 and Table 3 indicate that Ca^{2+} can serve as one second messenger for mediating the effects of AR agonists on hTM cells. However, the overall signalling cascades triggered by occupancy of adenosine receptors are complex and incompletely understood (Lorentzen & Schwabe, 2001). All four cloned and sequenced adenosine receptors activate G proteins, but the preference of G-protein coupling depends not only on receptor subtype but also on species and even on tissue, at least for the A_{2A} receptors (Fredholm et al., 2001). Furthermore, the relative roles of the G-protein α and $\beta\gamma$ subunits are not fully defined. The resulting effects on second messengers such as cAMP and Ca^{2+} are unpredictable, not only because of the complex G-protein signalling, but also because of multiple interactions with receptors to other hormones (such as histamine and bradykinin) and as yet poorly defined interactions with other uncloned adenosine receptors. In the case of Ca^{2+}, A_1 receptors can inhibit L-, N- and P-type Ca^{2+} channels, but also stimulate phospholipase C, so that the intracellular Ca^{2+} can be either increased or decreased in other cells (Lorentzen & Schwabe, 2001).

The present results give evidence of the complexity of the role of Ca^{2+} in the signaling cascades of TM cells. The ionomycin-produced increases in Ca^{2+} were sufficiently high both to activate whole-cell currents and to trigger shrinkage. However, the adenosine-induced shrinkage of BAPTA-buffered TM cells indicates that an increase in Ca^{2+} is not required for expression of the responses to AR agonists. Indeed, the shrinkage produced by adding 10 μM adenosine to BAPTA-buffered cells was significantly greater than that produced by applying Cl-IB-MECA to unbuffered cells, albeit not significantly different from CPA- and CGS-21680-treated unbuffered cells. At 10-μM concentration, adenosine likely stimulates A_1, A_{2A} and A_3ARs, but the second messenger mediating the TM-cell responses to the AR agonists is unclear.

Whole-Cell Currents

Hypotonic swelling of hTM cells triggers a secondary regulatory volume decrease (RVD) that largely reflects release of K^+ and Cl^- through parallel ion channels (Mitchell et al., 2002). It is likely that the AR agonist-triggered shrinkage was also mediated by KCl release through parallel K^+ and Cl^- channels. Interestingly, the activation of ion channels was far from uniform. CGS-21680 most commonly activated channels (in 6/15 cells) and CPA least commonly (1/12 cells). The basis for the heterogenous responses of membrane currents is unclear. The non-uniformity might have reflected not only partial internalization of adenosine receptors and reorganization of the cytoskeleton, but also cell heterogeneity. As discussed elsewhere (Mitchell et al., 2002), the cells studied were likely derived from both TM and juxtacanalicular cells. Furthermore, different TM cell populations have been identified on the basis of both morphological characteristics and resting membrane potential (Coroneo et al., 1991; Wiederholt & Stumpff, 1998) and by differential immunohistochemical staining for myosin (de Kater, Spurr-Michaud & Gipson, 1990).

Cell Volume

In view of the complexity of the signalling cascades triggered by AR agonists, we wondered whether A_1 and A_{2A}AR agonists might act differentially on hTM cell targets through multiple second messengers. One potentially important cell target is cell volume since changes in volume have been hypothesized to alter aqueous humor outflow (Freddo et al., 1984; O'Donnell et al., 1995; Al-Aswad et al., 1999). As discussed elsewhere (Mitchell et al., 2002), swelling and shrinkage of some cellular component of the outflow pathway reduces and increases aqueous humor outflow, respectively. Another potential target of the AR agonists could be the contractile state of the trabecular meshwork cells, which has been thought to alter outflow (Wiederholt & Stumpff, 1998; Llobet et fal., 1999; Tian et al., 2000; Wiederholt et al., 2000). Contraction of the TM cells has been associ-

ated with a reduction in outflow from the bovine eye (Wiederholt & Stumpff, 1998).

In principle, our measurements of cell area with calcein fluorescence could provide information on either of these potentially important targets of ARs, volume and contractile state. The results illustrated in Figure 6 bear on this issue. Increasing the intracellular Ca^{2+} with ionomycin would be expected to stimulate contraction of cells. However, the reduction in calcein-fluorescent area was entirely inhibited by blocking Cl^- channels with NPPB, without reducing the ionomycin-produced Ca^{2+} spike (Fig. 6), indicating that the ionomycin-triggered reduction in area reflected shrinkage and not contraction. Using the calcein-fluorescence technique, we have found that the subtype-selective AR agonists CPA (for A_1), CGS-21680 (for A_{2A}) and Cl-IB-MECA (for A_3) all reduced cell volume. Insofar as all AR agonists produced similar increases in Ca^{2+} and reductions in volume, it is unlikely that the differential effects of A_1ARs and A_{2A}ARs are mediated solely at the TM cells. A_1AR agonists might enhance outflow by shrinking TM cells, but at least one or more additional sites must be the targets for A_{2A}ARs. This is consistent with the suggestion that agonist occupancy of A_{2A}ARs can cause elevation of intraocular pressure by vascular expansion of the ciliary body (Tian et al., 1997).

Conclusions

The similarity of the responses of Ca^{2+} and cell volume and the highly variable responses of whole-cell currents to the subtype-selective AR agonists make it unlikely that differential effects of these agonists are mediated through the TM cells alone. The enhancement of aqueous humor outflow by A_1AR agonists may possibly be mediated by reduction of TM-cell volume. This possibility is supported by the observations that dimethylamiloride reduces TM cell volume (Mitchell et al., 2002), lowers mouse intraocular pressure (Avila et al., 2002), and enhances aqueous humor outflow in the mouse (Avila et al., 2003). However, the opposite action of A_{2A}ARs is likely mediated at one or more other cell targets or by volume-independent actions on the TM cells. One important purinergic role of TM cells may be to release ATP for ecto-enzymatic conversion and action on other cells further downstream in the outflow pathway.

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