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A3 adenosine and CB1 receptors activate a PKC-sensitive Cl^- current in human nonpigmented ciliary epithelial cells *via* a G$\beta\gamma$-coupled MAPK signaling pathway

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1 We examined A3 adenosine and CB1 cannabinoid receptor-coupled signaling pathways regulating Cl^- current in a human nonpigmented ciliary epithelial (NPCE) cell line.

2 Whole-cell patch-clamp recordings demonstrated that the A3 receptor agonist, IB-MECA, activates an outwardly rectifying Cl^-current ($I_{Cl,Aden}$) in NPCE cells, which was inhibited by the adenosine receptor antagonist, CGS-15943 or by the protein kinase C (PKC) activator, phorbol 12,13 dibutyrate (PDBu).

3 Treatment of NPCE cells with pertussis-toxin (PTX), or transfection with the COOH-terminus of β-adrenergic receptor kinase (ct-βARK), inhibited $I_{Cl,Aden}$. The phosphatidyl inositol 3-kinase (PI3K) inhibitor, wortmannin, had no effect on $I_{Cl,Aden}$; however, the mitogen-activated protein kinase kinase (MEK) inhibitor, PD98059, inhibited $I_{Cl,Aden}$.

4 Reverse transcription – polymerase chain reaction experiments and immunocytochemistry confirmed mRNA and protein expression for the CB1 receptor in NPCE cells, and the CB1 receptor agonist, Win 55,212-2, activated a PDBu-sensitive Cl^- current ($I_{Cl,Win}$).

5 Transfection of NPCE cells with the human CB1 (hCB1) receptor, increased $I_{Cl,Win}$, consistent with increased receptor expression, and $I_{Cl,Win}$ in hCB1 receptor-transfected cells was decreased after application of a CB1 receptor inverse agonist, SR 141716.

6 Constitutive activity for CB1 receptors was not significant in NPCE cells as transfection with hCB1 receptors did not increase basal Cl^- current, nor was basal current inhibited by SR 141716.

7 $I_{Cl,Win}$ was inhibited by PTX preincubation, by transfection with ct-βARK and by the MEK inhibitor, PD98059, but unaffected by the PI3K inhibitor, wortmannin.

8 We conclude that both A3 and CB1 receptors activate a PKC-sensitive Cl^- current in human NPCE cells via a $G_{i/o}/G\beta\gamma$ signaling pathway, in a manner independent of PI3K but involving MAPK.
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Abbreviations: CBE, ciliary body epithelium; Ct-βARK, carboxy terminus of β-adrenergic receptor kinase; hCB1, human CB1 receptor; $I_{Cl,Aden}$, IB-MECA-activated Cl^- current; $I_{Cl,Win}$, win 55,212-2-activated Cl^- current; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NPCE cells, nonpigmented ciliary epithelial cells; PDBu, phorbol 12,13 dibutyrate; PI3K, phosphatidyl inositol 3-kinase; PKC, protein kinase C

Introduction

The ciliary body epithelium (CBE) is responsible for aqueous humor production in the eye and is composed of both a pigmented ciliary epithelial (PCE) and a nonpigmented ciliary epithelial (NPCE) cell layer. The PCE cells face the stroma of the ciliary body and take up solute from the stroma, whereas the NPCE cells face the aqueous humor side and secrete solute into the posterior chamber of the eye. The extrusion of Cl^- ions, which occurs via Cl channels in NPCE cells, is a final and rate-limiting step for aqueous humor production (Jacob & Civan, 1996). Cell volume regulatory mechanisms together with G-protein-coupled receptors (GPCRs) participate in the regulation of Cl channels in NPCE cell (Jacob & Civan, 1996; Civan, 1998). Among the GPCRs identified in the CBE are A3 adenosine (Mitchell *et al*., 1999; Avila *et al*., 2001) and cannabinoid 1 (CB1) receptors (Porcella *et al*., 1998; 2000; Straiker *et al*., 1999; Stamer *et al*., 2001). Agonists for these receptors have been reported to alter intraocular pressure (IOP) (Crosson, 1995, 2001; Crosson & Petrovich, 1999; Pate *et al*., 1996; Tian *et al*., 1997; Porcella *et al*., 2000; 2001; Song & Slowey, 2000; Avila *et al*., 2001).

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Consistent with a regulatory role for A3 receptors in regulating IOP, A3 receptor knockout mice have decreased IOP (Avila *et al*., 2002), while administration of A3 agonists in animal models have been associated with rises in IOP (Avila *et al*., 2001). At the cellular level, studies of receptor-ion transport pathways in a human NPCE cell line have demonstrated that A3 agonists, such as *N*(6)-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine (IB-MECA), can stimulate a Cl^- current in NPCE cells. The A3 receptor-activated Cl^- current was associated with regulatory volume decrease (RVD) of NPCE cells (Carre *et al*., 1997; 2000; Mitchell *et al*., 1999). Taken together, these findings suggest that A3 receptor activation may modulate aqueous humor secretion, in part via the stimulation of Cl channels in NPCE cells. This may provide a mechanism for increasing solute secretion by the CBE. However, the signaling pathways that couple the A3 adenosine receptors to the activation of Cl^- channels in NPCE cells remain unclear. In other cell types, A3 receptors are coupled to the $G_{i/o}$ family of G proteins and are associated with adenylyl cyclase inhibition (Englert *et al*., 2002). The activation of $G_{i/o}$-protein-coupled receptors may also stimulate several other signaling pathways. These include phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) (Camps *et al*., 1992; Abbracchio *et al*., 1995; Touhara *et al*., 1995; Selbie & Hill, 1998; Schulte & Fredholm, 2000; Yart *et al*., 2000; Gao *et al*., 2001; Lee *et al*., 2001; Krugmann *et al*., 2002).

CB1 receptors are also present in the mammalian CBE (Porcella *et al*., 1998; 2000; Straiker *et al*., 1999; Stamer *et al*., 2001) and appear to be densely localized to NPCE cells. Endogenous cannabinoid receptor ligands, such as anantamide, and other synthetic cannabinoid receptor agonists such as Win55212-2 and CC-55,940 have been reported to induce a reduction in IOP (Pate *et al*., 1995; 1996; Song & Slowey, 2000; Porcella *et al*., 2001), although this is likely because of actions at multiple sites including both the CBE and trabecular outflow pathways. CB1 receptors in other systems couple to G_i proteins to activate downstream signaling pathways that include stimulation of MAPK (Rueda *et al*., 2000; Guzman *et al*., 2001). In NPCE cells, the coupled signaling pathways and actions of CB1 receptor activation of ion transporters are unknown, although it is possible that activation of these receptors, like A3 receptors, may share common signaling pathways that affect Cl^- channel activation.

The purpose of this study was: (1) to identify A3 receptor-coupled signaling pathways regulating Cl^- current in a human NPCE cell line and (2) to determine whether CB1 receptors can modulate Cl^- current in NPCE cells via activation of similar or distinct G-protein-coupled signaling pathways.

Methods

Cell culture

We used a well-established human NPCE (ODM) cell line (Coca-Prados & Wax, 1986). These SV40-transformed NPCE cells are functionally similar to both untransformed primary NPCE cells (Coca-Prados & Wax, 1986) and epithelial cells of the intact ciliary body (Yantorno *et al*., 1989), with preservation of ion transport systems (Delamere *et al*., 1993; Patil *et al*., 2001) and GPCRs (Coca-Prados & Wax, 1986; Crook *et al*., 1992; Mitchell *et al*., 1999; Crider & Sharif, 2002).

Human NPCE cells were maintained in Dulbecco's modified eagle's medium (DMEM; Life Technologies, Burlington, ON, Canada) plus 10% newborn calf serum (NCS) and 1% gentamycin in an atmosphere of 5% CO_2/95% O_2 at 37°C. Prior to electrophysiological experiments, cells were seeded onto 12 mm glass coverslips at a density of 10^5 cells ml^{-1} and incubated for another 24 – 48 h at 37°C in an atmosphere of 5% CO_2/95% O_2.

DNA constructs and transfection of NPCE cells

The human CB1 (hCB1) receptor construct in pRC/CMV expression vector (pRC/CMV-HCB1R) was provided by Dr William Bonner (National Institute of Mental Health, Bethesda, MD, U.S.A.). pIRES-EGFP and the $G\beta\gamma$ sequestrant vector pIRES-EGFP-βARK were received from Dr Gerald Zamponi (University of Calgary, Calgary, AB, Canada) and pEGFP-N1 was purchased from CLONTECH BD Bioscience, Mississauga, ON, Canada. Lipofectin® was purchased from Invitrogen (Life Technologies, Burlington, ON, Canada). Kanamycin and carbenicillin were purchased from Sigma Aldrich Canada (Missassauga, ON, Canada).

Aproximately 24 h prior to transfection, NPCE cells were subcultured using trypsin – EGTA and seeded onto glass coverlips at 10^5 cells ml^{-1}. DNA (5 μg) was used to transfect NPCE cells using Lipofectin® to facilitate the uptake of DNA constructs. For cotransfection, the ratio of pRC/CMV-HCB1R to pEGFP-N1 was 1 : 5, with pEGFP-N1 providing a control for transfection frequency, which was comparable between experiments. After incubation of NPCE cells overnight, DNA constructs were removed, and fresh medium containing 10% FBS was added. The cells were incubated for another 48 h prior to the electrophysiological experiments. Fluorescent transfected cells were selected for recording by viewing with a microscope equipped for epifluorescence (Nikon Diaphot).

Immunocytochemistry

Identification of CB1 receptor protein in transfected and untransfected NPCE cells was carried out using standard immunocytochemical techniques with a polyclonal antibody directed against the human cannabinoid CB1 receptor (Chemicon International, Temecula, CA, U.S.A.). The antibody was produced in rabbits using a synthetic peptide sequence close to the N-terminal region of the hCB1 receptor. For immunocytochemical staining, the antibody stock (2 μg/ml^{-1}) was diluted 1 : 300. Untransfected cells and cells transfected with pRC/CMV-HCB1R were fixed in methanol for 5 – 10 min at −20°C. Following fixation, cells were washed with phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton X-100. Following a blocking step with 10% donkey serum for 1 h, the cells were exposed to the CB1 primary antibody overnight at 4°C. Primary antibody labeling was visualized using a fluorescent secondary antibody (donkey anti-rabbit IgG secondary conjugated to Alexa 488, Molecular Probes, Eugene, OR, U.S.A.). Cells were then mounted in aqueous mount and staining was viewed using a confocal microscope (Nikon C1).

Whole-cell current recordings

Tight-seal patch-clamp recording methods were used to measure whole-cell currents from cultured NPCE cells. Coverslips with attached NPCE cells were placed in a 1 ml (total volume) recording chamber and positioned on the stage of a Nikon inverted microscope. During experiments, cells were continually superfused with external solution at a rate of $1-2\,ml\,min^{-1}$. The regular external solution contained (in mM): Trizma HCl, 70; $CaCl_2$, 1.5; $MgCl_2$, 0.8; HEPES, 10; TEA-Cl, 5; $BaCl_2$, 5; glucose, 10; sucrose, 105. The osmolarity of the external solution was $295\,mosmol^{-1}$. The pH of the external solutions was adjusted to 7.4 with CsOH. The intracellular electrode-filling solution was composed of (in mM): Trizma HCl, 60; Trizma base, 60; aspartic acid, 60; HEPES, 15; $CaCl_2$, 0.4; $MgCl_2$, 1; EGTA, 1; ATP (Mg), 2; GTP (Na_2), 0.1. The pH of the internal solution was adjusted to 7.2 with CsOH and the osmolarity was $264\,mosmol^{-1}$. The osmolarity of internal electrode solutions was routinely maintained at least $20\,mosmol^{-1}$ hyposmotic to the external solution in order to prevent any transient cell swelling during early time periods of whole-cell recording (Shi *et al.*, 1999).

We used pClamp6 software and an Axopatch-1D amplifier (Axon Instruments, Union City, CA, U.S.A.) to generate voltage commands and to record membrane currents. Patch electrodes had an external diameter of 1.5 mm and an internal diameter of 1.1 mm (Sutter Instruments, Novato, CA, U.S.A.), and were pulled from borosilicate glass using a two-stage vertical microelectrode puller (Narishige model pp83, Tokyo, Japan). Electrodes were coated with beeswax to reduce capacitance and had resistances of $3-5\,M\Omega$ when filled with internal solutions. A sealed electrode – salt bridge combination (Dri-ref-2, World Precision Instruments, Sarasota, FL, U.S.A.) was used as the reference electrode. Prior to seal formation, offset potentials were nulled using the amplifier circuitry. Current – voltage relations were corrected for the liquid junction potential, which was measured experimentally or calculated using a software program (JPCal, version 2.00; P.H. Barry, Sydney, Australia), and was aproximately 2 mV. Series resistance and cell capacitance values were obtained directly from the amplifier settings. For all recordings shown, the series resistance was $<15\,M\Omega$ and was compensated (80%). Experiments were conducted at room temperature (23 – 25°C).

Chemicals

Chemicals used for electrophysiological experiments including adenosine, IB-MECA, CGS-15943, Win 55,212-2, phorbol 12,13 dibutryrate (PDBu) and PD98059 were purchased from Sigma Aldrich Canada (Missassauga, ON, Canada). SR 141716A was donated from Sanofi Recherche (Toulouse, France). Wortmannin was purchased from Cedarlane (Hornby, ON, Canada). Drugs were prepared as stock solutions in dimethyl sulfoxide (DMSO) and were further diluted using regular isosmotic extracellular solution. The final DMSO concentration was $<0.01\%$, which has been previously shown to have no significant effects on whole-cell Cl^- currents (Shi *et al.*, 2002). The concentrations for all test agents used are cited in the results.

Reverse transcription – polymerase chain reaction (RT – PCR)

Reverse transcription – polymerase chain reaction (RT – PCR) techniques were used to detect mRNA expression of CB1 receptor in human NPCE cells and rat CBE tissue. The primers for amplification of the human CB1 receptor were: 5′-TGCAGGCCTTCCTACCACTTCATC-3′ (forward, bp 536 – 559) and 5′-GACGTGTGGATGATGATGCTCTTC-3′ (reverse, bp 1056 – 1033, GenBank accession number: XM_004350). PCR conditions for amplication of the CB1 receptor included: (1) 1 min at 94°C, (2) 1 min at 94°C, (3) 1 min at 56°C, (4) 1 min at 72°C for 1 min and (5) 7 min at 72°C, repeating steps 2 – 4, 35 times. RT – PCR amplification cyclophilin was also performed as an internal control (Bertolesi *et al.*, 2002; Hirooka *et al.*, 2002). PCR primers complimentary to bases 46 – 67 and 395 – 416 of human cyclophilin (GenBank accession number BC005320) were used. The PCR conditions were: (1) 1 min at 94°C, (2) 30 s at 94°C, (3) 30 s at 50°C, (4) 1 min at 72°C, (5) 10 min at 7°C, repeating steps 2 – 4, 28 times. PCR products of 520 and 371 bp were obtained using CB1 and cyclophilin primers, respectively. The PCR products for human CB1 receptors were cloned into HO1 *E. coli* bacterial cells. The oligonucleotide sequence was verified, using methods similar to those previously described (Bertolesi *et al.*, 2002; Hirooka *et al.*, 2002), using restriction enzyme analysis as well as sequencing.

Statistics and curve fitting

Data are presented as mean ± s.e.m. Student's *t*-test was used to compare differences between two groups. Differences between two groups were considered significant when $P<0.05$.

Dose – response curves were fit with the equation: $y = A2 + (A1 - A2)/(1 + 10\hat{}((\log IC_{50} - c)*n_H))$, where y is the difference current amplitude (normalized against capacitance), $A1$ is the maximum agonist-stimulated difference current, $A2$ is the difference current recorded with vehicle in the absence of agonist, c is the concentration of the agonist and n_H is the Hill coefficient.

Results

Activation of a Cl^- current by A3 adenosine receptor activation

The presence of A3 receptors, as well as A3 receptor activation of a staurosporine-sensitive Cl^- current, has been previously reported in an immortalized human NPCE cell line (Mitchell *et al.*, 1999; Carre *et al.*, 2000). We confirmed the effect of the selective A3 receptor agonist, IB-MECA, on whole-cell Cl^- conductance in the human NPCE (ODM) cell line under our experimental conditions. The voltage protocol used to record Cl^- currents is shown in the top left panel of Figure 1a. Cells were held at -62 mV (corrected for liquid junction potential, see Methods), and the membrane potential was then stepped from -102 mV to $+98$ mV in 20 mV increments. Figure 1a shows that IB-MECA (100 nM) application (5 min) produced an increase in the Cl^- current (IB-MECA, right panel) as compared to control (Control, left panel). The IB-MECA-stimulated current was reversible and the Cl^- current

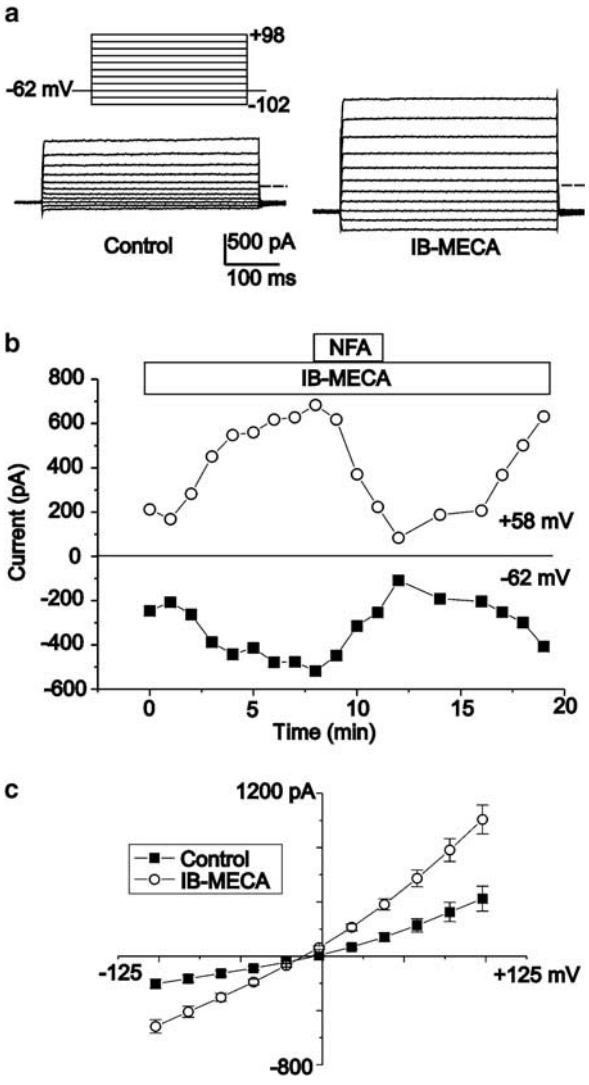

Figure 1 A_3 adenosine receptor agonist, IB-MECA activates a Cl^- current. (a) Representative current traces recorded in the absence (control, left panel) and presence of 100 nM IB-MECA (IB-MECA, right panel). The voltage step protocol is shown in the top left panel. (b) Time course for Cl^- current recorded at +58 and −62 mV in the presence of 100 nM IB-MECA (IB-MECA) and 0.5 mM niflumic acid (NFA). (c) Mean current–voltage relations for Cl^- current recorded in the absence (Control, $n=9$) and in the presence of 100 nM IB-MECA (IB-MECA, $n=9$).

recovered towards control values after washing out the A3 receptor agonist. Figure 1b shows a time course for whole-cell current recorded in the absence and presence of 100 nM IB-MECA, with and without the Cl channel blocker, niflumic acid (Mitchell *et al*., 1999; Shi *et al*., 1999). Current was measured at potentials negative (−62 mV) and positive (+58 mV) to E_{Cl} (−10 mV) in this and subsequent experiments to reveal the voltage-dependence of drug action on this rectifying conductance (Shi *et al*., 1999; 2002). Superfusion of 100 nM IB-MECA for 8 min increased Cl^- current from 212 to 684 pA at +58 mV and from −246 to −519 pA at −62 mV. Addition of 0.5 mM niflumic acid decreased the current to 83 pA at +58 mV and to −109 pA at −62 mV. Figure 1c shows the mean current–voltage (I–V) relations for Cl^- current recorded before (Control, $n=9$) and after application of 100 nM IB-MECA for 8 min (IB-MECA, $n=9$). At −62 and +58 mV, IB-MECA significantly increased Cl^- current by 105 and 153% ($P<0.01$), respectively. The IB-MECA-activated Cl^- current exhibited outward rectification, and reversed at approximately −11 mV, which is close to the calculated E_{Cl} of −10 mV.

IB-MECA increased the Cl^- current in a concentration-dependent manner. The Cl^- current shown in Figure 2a is the difference current ($I_{Cl,Aden}$) at +58 mV, obtained by subtracting the basal Cl^- current recorded in the absence of drug from the Cl^- current recorded in the presence of different concentrations of IB-MECA (10–1000 nM). At a concentration of 50 nM, IB-MECA significantly activated $I_{Cl,Aden}$ ($n=4$, $P<0.05$) compared to control basal current. The maximal $I_{Cl,Aden}$ activation by 1000 nM IB-MECA was 5.3 pA pF^{-1} at +58 mV ($P<0.05$, $n=4$), respectively. Based on these data, the EC_{50} for IB-MECA activation of $I_{Cl,Aden}$ was 69 nM.

Previous work in human NPCE cells demonstrated that the A3 specific antagonists, MRS-1097, MRS1191 and MRS-1523, selectively blocked Cl channel activity following IB-MECA stimulation (Mitchell *et al*., 1999). The IB-MECA-activated Cl^- current in NPCE cells was also inhibited by the adenosine receptor antagonist, CGS-15943. Cells were pre-incubated with 1 μM CGS-15943 for 5 min and superfused with 1 μM CGS-15943 throughout the recordings. Figure 2b shows that in the absence of CGS-15943, 100 nM IB-MECA increased the Cl^- current from −145±35 and 252±55 pA (Control, $n=10$) to −309±70 and 507±100 pA (IB-MECA, $n=6$) at −62 and +58 mV ($P<0.05$), respectively. However, in the presence of 1 μM CGS-15943 (IB-MECA+CGS, $n=4$), the Cl^- current was not increased by 100 nM IB-MECA. Taken together, these data confirm that A3 adenosine receptor activation stimulates a Cl^- current in human NPCE cells.

Previous studies have suggested that A3 receptor activation can stimulate a Cl^- current that produces isosmotic cell shrinkage (Carre *et al*., 1997; 2000; Mitchell *et al*., 1999). The A3-activated Cl^- current had properties similar to the volume-sensitive Cl^- current ($I_{Cl,vol}$) described in human (Civan *et al*, 1994; 1996; Carre *et al*., 2000) and rabbit NPCE cells (Shi *et al*., 1999; 2002). To further examine the relation between $I_{Cl,vol}$ and $I_{Cl,Aden}$, we examined the effect of the protein kinase C (PKC) activator, PDBu on the IB-MECA-activated Cl^- current. PDBu has also been previously demonstrated to activate PKC and inhibit $I_{Cl,vol}$ in rabbit SV40 NPCE cells (Shi *et al*., 2002). In experiments with PDBu, human NPCE cells were pre-incubated with 100 nM PDBu for 15 min prior to experiments and superfused with 100 nM PDBu throughout the recordings. Control current for PDBu-treated cells represents basal Cl^- current measured in the presence of PDBu. Figure 2c shows that in untreated cells, application of 100 nM IB-MECA increased mean Cl^- current measured at −62 and +58 mV from −171±51 and 255±67 to −303±29 and 572±61 ($n=9$, $P<0.001$), respectively. This represents an increase of 43 and 55% compared to control. In the presence of 100 nM PDBu, IB-MECA failed to significantly increase Cl^- current, with an increase in current measured at −62 and +58 mV of only 22 and 20% compared to the PDBu-treated control ($n=8$, $P>0.05$).

Activation of $I_{Cl,Aden}$ *occurs* via *a PTX-sensitive* $G_{i/o}$ *protein/*$G\beta\gamma$ *pathway*

To determine whether A3 adenosine receptor activation of $I_{Cl,Aden}$ in human NPCE cells involves signaling via PTX-

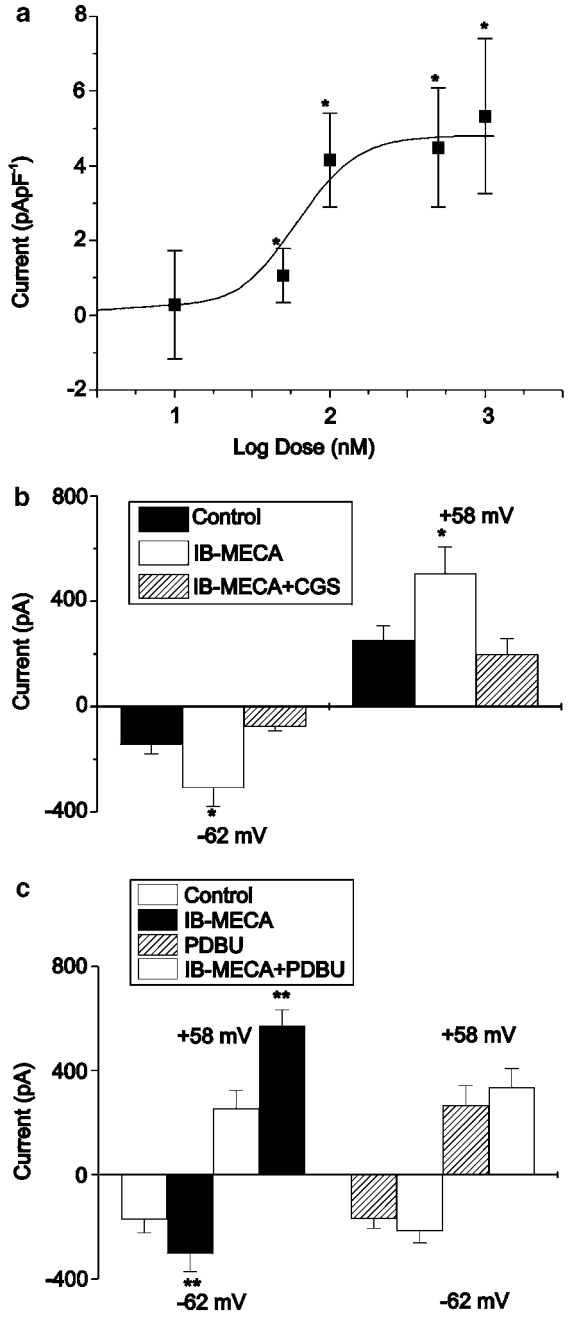

Figure 2 A_3 adenosine receptor activation stimulates a PKC-sensitive Cl^- current. (a) Dose–response curve for IB-MECA-stimulated Cl^- current ($I_{Cl,Aden}$) recorded at +58 mV. Concentrations of IB-MECA were 10 nM ($n=4$), 50 nM ($n=4$), 100 nM ($n=7$), 500 nM ($n=7$), and 1000 nM ($n=4$). $I_{Cl,Aden}$ represents the Cl^- difference current obtained by subtracting basal Cl^- current recorded in the absence of A3 receptor agonist from the Cl^- current recorded in the presence of IB-MECA. In the absence of agonist, $I_{Cl,Aden}$ is zero. (b) Mean Cl^- current recorded at −62 and +58 mV in the absence of agonist (Control, $n=10$), and in the presence of IB-MECA (IB-MECA, $n=6$), or IB-MECA plus CGS 15943 (IB-MECA+CGS, $n=4$). (c) Mean Cl^- current measured at −62 and +58 mV in the absence (Control, $n=9$) and presence of 100 nM IB-MECA (IB-MECA, $n=9$), with PDBu alone (PDBu, $n=8$) or with 100 nM IB-MECA plus 100 nM PDBu (IB-MECA+PDBu, $n=8$). *$P<0.05$.

sensitive $G_{i/o}$ proteins, we measured $I_{Cl,Aden}$ in untreated (IB-MECA) and PTX-treated (IB-MECA+PTX) cells. NPCE cells were incubated with DMEM containing 500 ng ml^{-1} PTX overnight at 37°C. Figure 3a shows that in the absence of PTX treatment (IB-MECA, $n=8$), mean $I_{Cl,Aden}$ was -145 ± 42 and 256 ± 64 at −62 and +58 mV, respectively, whereas $I_{Cl,Aden}$ recorded in PTX-treated cells (IB-MECA+PTX, $n=8$) was only -8 ± 8 and 31 ± 22 pA at the corresponding potentials ($P<0.01$). This represents an 88 and 96% reduction of $I_{Cl,Aden}$ at −62 and +58 mV, respectively, and confirms that A3 receptor stimulation of $I_{Cl,Aden}$ occurs via PTX-sensitive $G_{i/o}$ proteins.

The $G\beta\gamma$ subunits of G_i proteins have been implicated in the modulation of ion channels, either via direct interactions or via activation of downstream intermediaries (Wickman & Clapham, 1995a, b). To investigate if a $G\beta\gamma$ subunit-coupled pathway contributes to A3 receptor activation of $I_{Cl,Aden}$ in human NPCE cells, we transiently transfected cells with pIRES-EGFP-βARK, which encodes GFP as well as the COOH-terminal end of βARK (ct-βARK) containing the $G\beta\gamma$-binding domain of βARK1. ct-βARK can bind and sequester free $G\beta\gamma$ subunits, hence preventing $G\beta\gamma$-mediated signaling following receptor activation (Zou *et al*., 1998). $I_{Cl,Aden}$ was recorded in human NPCE cells transfected with 5 μg control plasmid pIRES-EGFP or 5 μg pIRES-EGFP-βARK. Figure 3b shows that $I_{Cl,Aden}$ was significantly decreased by expression of βARK ($P<0.05$). Mean $I_{Cl,Aden}$ recorded in pIRES-EGFP-βARK-transfected cells (IB-MECA+EGFP-βARK, $n=6$) was -33 ± 29 and 17 ± 38 pA at −62 and +58 mV, respectively. In constrast, $I_{Cl,Aden}$ in pIRES-EGFP-transfected cells (IB-MECA+EGFP, $n=6$) was -123 ± 25 pA at −62 mV and 142 ± 33 pA at +58 mV. These results indicate that the activation of $I_{Cl,Aden}$ by A3 adenosine receptors occurs via a PTX-sensitive $G_{i/o}$ protein/$G\beta\gamma$ signaling pathway in human NPCE cells.

PI3K is a common downstream target of G_i protein-coupled receptor-activated signaling pathways (Selbie & Hill, 1998; Krugman *et al*., 2002; Yart *et al*., 2002), and in rabbit NPCE cells, it was shown to be involved in the activation of a PKC-sensitive $I_{Cl,vol}$ (Shi *et al*., 2002). To examine whether PI3K is involved in the activation of $I_{Cl,Aden}$ by $G_{i/o}$ protein-coupled A3 adenosine receptors, we used the PI3K inhibitor, wortmannin, at a concentration previously shown to block $I_{Cl,Vol}$ in NPCE cells (Shi *et al*., 2002). Human NPCE cells were preincubated with 100 nM wortmannin for 30 min and superfused with 100 nM wortmannin during current recordings (Figure 3c). $I_{Cl,Aden}$, in the absence of wortmannin (IB-MECA, $n=3$), was -170 ± 35 and 288 ± 46 pA at −62 and +58 mV, respectively. At the corresponding potentials in the presence of wortmannin (IB-MECA+Wort, $n=5$), $I_{Cl,Aden}$ was -123 ± 33 and 229 ± 86 pA. Therefore, treatment with 100 nM wortmannin had no significant effect on $I_{Cl,Aden}$ ($P>0.05$), suggesting that adenosine A3 receptors activate $I_{Cl,Aden}$ via a PI3K-independent pathway.

We also examined whether A3 receptor activation of $I_{Cl,Aden}$ involved the MAPK pathway. MAPKs are serine/threonine protein kinases, which are regulated by phosphorylation cascades. Their immediate upstream activators are MAPK kinases or MEKs. MAPK can be activated by protein tyrosine kinase (PTK) in both PI3K-dependent and independent pathways (Pearson *et al*., 2001). We used PD98059 ($IC_{50}=5-10\ \mu M$), a highly selective MEK inhibitor that binds to the inactive forms of MEK and prevents its activation by upstream activators (English & Cobb, 2002). Figure 3d shows mean $I_{Cl,Aden}$ recorded from cells in the absence (IB-MECA,

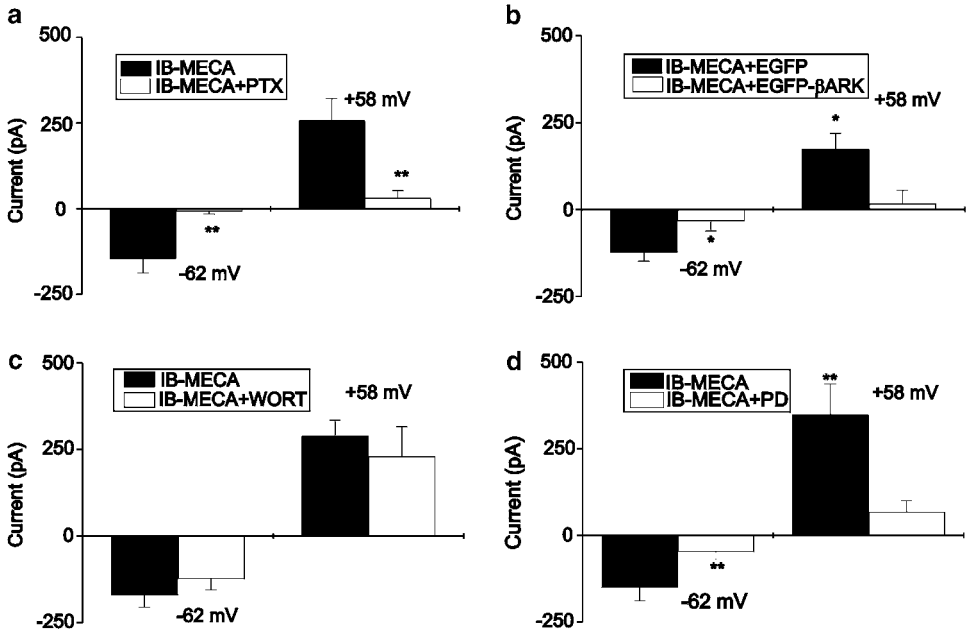

Figure 3 A PTX-sensitive $G_{i/o}/G\beta\gamma$ signaling pathway mediates activation of $I_{Cl,Aden}$. Mean $I_{Cl,Aden}$ (agonist-stimulated Cl^- current minus basal Cl^- current) recorded at −62 and +58 mV in: (a) NPCE cells before (IB-MECA, $n=8$) and after PTX treatment (IB-MECA + PTX, $n=8$), (b) NPCE cells transfected with control vector pIRES2-EGFP (IB-MECA + EGFP, $n=6$) or pIRES2-EGFP-βARK (IB-MECA + EGFP-βARK, $n=6$), (c) NPCE cells before (IB-MECA, $n=3$) and after 100 nM wortmannin treatment (IB-MECA + Wort, $n=5$) and (d) NPCE cells before (IB-MECA, $n=5$) and after 5 μM PD98059 treatment (IB-MECA + PD, $n=7$). $^*P<0.05$, $^{**}P<0.01$.

$n=5$) or presence of 5 μM of the MEK inhibitor, PD98059 (IB-MECA + PD, $n=7$). For those cells treated with PD98059, the drug was included in the internal electrode solution and current was measured 15 min after rupturing the membrane to form the whole-cell recording mode. In the presence of PD98059, $I_{Cl,Aden}$ was significantly reduced from -151 ± 39 and 349 ± 89 pA to -47 ± 22 and 68 ± 32 at −62 mV and +58 mV, respectively ($P<0.01$), confirming the involvement of MAPKs in $I_{Cl,Aden}$ activation.

Activation of a Cl^- current by CB1 receptors in human NPCE cells

We examined if activation of CB1 receptors, which are also reported to be G_i protein-coupled receptors (Bouaboula *et al.*, 1999; Guzman *et al.*, 2001), could lead to the activation of a Cl^- current in human NPCE cells. We first employed semiquantitative RT – PCR techniques to determine mRNA expression of CB1 receptor in human NPCE cells (Hirooka *et al.*, 2002). As shown in Figure 4a, RT – PCR amplification, with a primer pair specific for hCB1 receptor cDNA, detected a PCR product in NPCE cells with a predicted size of 520 bp. RT – PCR amplification of a housekeeping gene cyclophilin is also shown with a size of 371 bp. PCR product for the CB1 receptor in isolated rat CE tissue is shown for comparison in Figure 4b. Densitometry analysis, normalizing CB1 receptor mRNA expression to that of cyclophilin for the data shown in Figure 4a and b, indicated that CB1 receptor mRNA levels were greater in freshly isolated rat CE tissue than in cultured NPCE cells (Figure 4c). The PCR product obtained for the hCB1 receptor in NPCE cells was cloned and sequenced as described in Hirooka *et al.* (2002). The oligonucleotide sequence was identical to that of the hCB1 receptor cDNA reported in GenBank (GenBank number: XM_004350). The panels in Figure 4d show confocal photomicrographs of immunocytochemical staining of NPCE cells with an antibody against CB1 receptor protein. Control cells in the left panel have been exposed to the transfection-facilitating agent, lipofectin, only. Antibody staining indicates a low level of the endogenous receptor protein appearing as sparse punctate labeling of the plasma membrane in control cells. In NPCE cells transfected with pRC/CMV-HCB1R (right panel), in the presence of lipofectin, an increase in hCB1 receptor labeling is apparent, with dense receptor labeling distributed over the membrane surface and throughout the cell.

We next determined the effect of a CB1 receptor agonist, Win 55,212-2, on Cl^- current in human NPCE cells. Figure 5a shows representative current traces recorded in regular extracellular solution, either in the absence of agonist (Control, left panel) or after application of Win 55,212-2 (Win, right panel). The top left panel is the voltage protocol used. Application of 1 μM Win 55,212-2 increased the whole-cell current. The effect of Win 55,212-2 was partially reversable. Figure 5b shows the mean I – V relation for $I_{Cl,Win}$, obtained by subtracting basal Cl^- current recorded in regular extracellular solution from Cl^- current recorded in the presence of Win 55,212-2 (1 μM). Like $I_{Cl,Aden}$, $I_{Cl,Win}$ showed no inactivation at positive potentials and was outwardly rectifying. The reversal potential for $I_{Cl,Win}$, was −12 mV ($n=10$). This value is close to the calculated E_{Cl} (−10 mV).

The sensitivity of the Win 55,212-2-activated Cl^- current to PKC modulation was examined in human NPCE cells, using the PKC activator, PDBu (100 nM). Figure 5c shows Cl^- currents recorded in NPCE cells in the presence and absence of PDBu. In untreated NPCE cells, application of 1 μM Win 55,212-2 (Win, $n=5$) for 8 min increased Cl^- current from

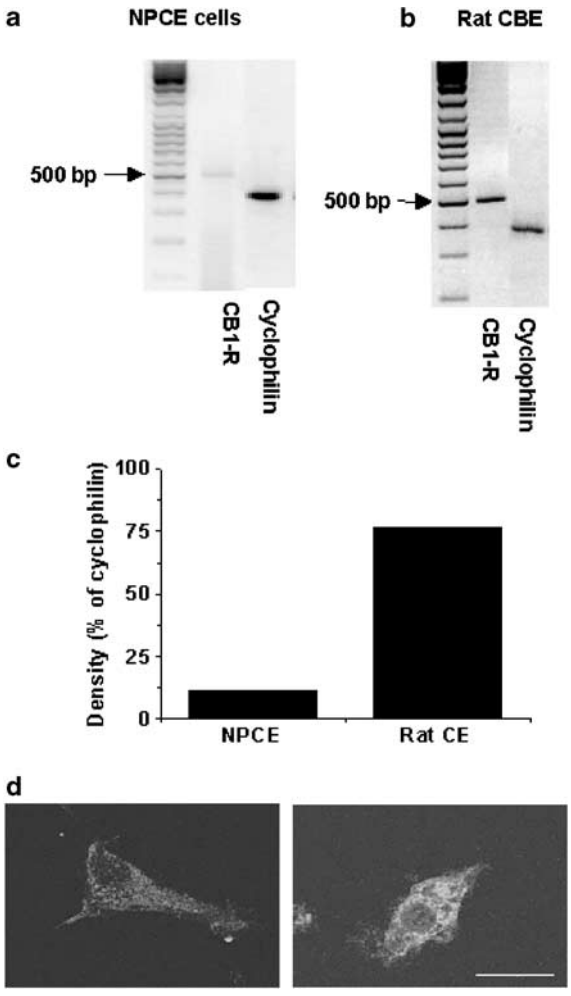

Figure 4 Expression of CB1 receptors in human NPCE cells. (a,b) Representative RT – PCR amplification of hCB1 receptor (CB1-R, 520 bp) and cyclophilin cDNA (Cycl, 370 bp) from SV40-transformed human NPCE cells (a) and rat CBE (b). (c) Densitometric analysis of the RT – PCR products for CB1-R from (a) and (b) normalized to the cyclophilin product. (d) Confocal photomicrographs of human NPCE cells immunoreactive for CB1-R in control cells (left panel) and cells transfected with pRC/CMV-HCB1R (right panel). Scale bar is 40 μM for both panels.

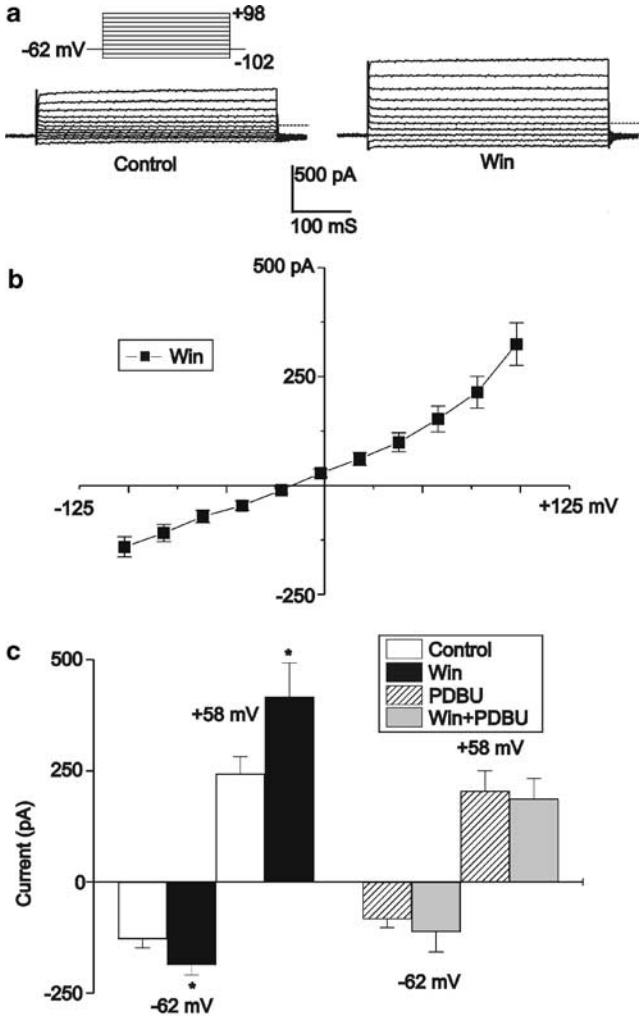

Figure 5 Win 55,212-2 activates a PKC-sensitive Cl^- current. (a) Typical current traces recorded in regular external solution (Control, left panel), and after application of 1 μM Win 55,212-2 (Win, right panel). The voltage protocol is shown in the left top panel. (b) Mean I – V relation for the Win 55,212-2-stimulated Cl^- current ($I_{Cl,Win}$), obtained by subtracting basal Cl^- current in the absence of agonist from Cl^- current recorded in the presence of 1 μM Win 55,212-2 ($n=10$). (c) Mean Cl^- current measured at – 62 and + 58 mV in the absence (Control, $n=5$) and presence of 1 μM Win 55,212-2 (Win, $n=5$), with 100 nM PDBu alone (PDBu, $n=5$) or with 1 μM Win 55,212-2 plus 100 nM PDBu (PDBu + Win, $n=5$). *$P<0.05$.

– 187 ± 23 and 244 ± 38 pA to 205 ± 45 and 416 ± ± 75 pA at – 62 and + 58 mV ($P<0.05$), respectively, compared to the unstimulated basal current (Control, $n=5$). In PDBu-treated cells, however, 1 μM Win 55,212-2 (Win + PDBu, $n=5$) had no stimulatory effect compared to basal Cl^- current recorded in the presence of PDBu (PDBu, $n=5$, $P>0.05$). These data suggest that like $I_{Cl,vol}$ and $I_{Cl,Aden}$, $I_{Cl,win}$ is also PKC-sensitive.

We next examined if the transfection of NPCE cells with hCB1 receptor could increase the Win 55,212-2-activated Cl^- current. To do so, NPCE cells were first transiently cotransfected with pRC/CMV-HCB1R and pEGFP-N1 as described in METHODS. Cells transfected with pEGFP-N1 alone were used as the control. Figure 6a shows the mean I – V relation for $I_{Cl,Win}$ recorded in hCB1 receptor-transfected cells (EGFP-CB1-R, $n=6$) and NPCE cells transfected with pEGFP-N1 only (EGFP, $n=5$). $I_{Cl,Win}$ was increased from −71 ± 22 to −125 ± 15 pA ($P<0.01$) and from 107 ± 37 to 267 ± 32 pA ($P<0.01$) at – 62 and + 58 mV, respectively, in cells transfected with hCB1 receptors as compared to cells transfected with control plasmid. This represents an increase in whole-cell conductance from 1.1 to 2.0 nS at – 62 mV and from 1.8 to 4.6 nS at + 58 mV, and suggest that transfection with pRC/CMV-HCB1R increases the functional expression of hCB1 receptors.

A dose – response relation for Win 55,212 – 2 (0.1 – 100 μM) activation of $I_{Cl,Win}$ was obtained in hCB1 receptor-transfected cells. Figure 6b shows that Win 55,212-2 activated the Cl^- current in a dose-dependent manner. $I_{Cl,Win}$ activated by 100 nM Win 55,212-2 was −0.9 ± 0.6 and 2.8 ± 1.0 pA pF^{-1} at – 62 and + 58 mV, respectively ($n=4$, $P<0.05$). Maximal activation of $I_{Cl,win}$ was observed following application of 5 μM Win 55,212-2 ($n=6$, $P<0.01$), with higher concentrations of agonist, producing no further activation of $I_{Cl,win}$. Based on

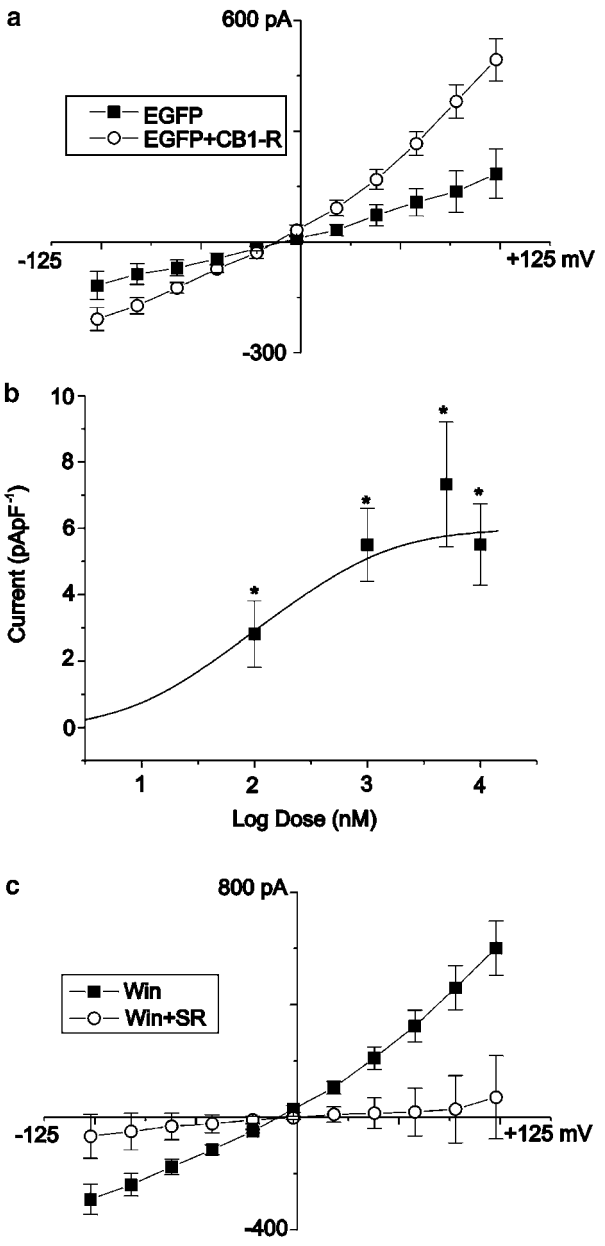

Figure 6 CB1 receptor mediates activation of $I_{Cl,Win}$. (a) Mean I – V relation for $I_{Cl,Win}$ (agonist-stimulated Cl^- current minus basal Cl^- current) following application of 1 μM Win 55,212-2 in NPCE cells transfected with control vector pEGFP-N1 (EGFP, $n=5$) and cells cotransfected with pEGFP plus pRC/CMV-HCB1R (EGFP-CB1-R, $n=6$). (b) Dose – response curve for $I_{Cl,Win}$ activation at +58 mV by 0.1 μM ($n=4$), 1 μM ($n=6$), 5 μM ($n=6$) and 10 μM ($n=3$) Win 55,212-2 in cells cotransfected with pEGFP plus pRC/CMV-HCB1R. (c) Mean I – V relation for $I_{Cl,Win}$ recorded in CB1-R-transfected cells before (Win, $n=6$) and after SR 141716 (Win + SR, $n=7$).

these data, the EC_{50} obtained for the activation of Cl^- current by Win 55,212-2 was 109 nM. To confirm that CB1 receptors mediate activation of $I_{Cl,Win}$ in hCB1 receptor-transfected cells, we used the specific CB1 receptor inverse agonist, SR 141716. Cells were pretreated with 1 μM SR 141716 for 5 min and superfused with 1 μM SR 141716 during the recordings. Figure 6c shows that in the presence of 1 μM SR 141716 (Win + SR, $n=6$), $I_{Cl,Win}$ was abolished as compared to $I_{Cl,Win}$ recorded in the absence of SR 141716 (Win, $n=7$). SR 141716 reduced $I_{Cl,Win}$ from -177 ± 27 to -32 ± 48 pA at – 62 mV ($P<0.05$) and from 324 ± 57 to 18 ± 84 pA at +58 mV ($P<0.05$). This result further confirms that CB1 receptors mediate $I_{Cl,Win}$ activation.

Increased CB1 receptor expression has been shown to be associated with enhanced constitutive activity of CB1 receptor-mediated signaling (Pan *et al*., 1998; Bouaboula *et al*., 1999; Vasequez & Lewis, 1999). Thus, we expected that with an increased expression of hCB1 receptor, the basal Cl^- current in human NPCE cells would also be increased. However, transfection with hCB1 receptors failed to significantly affect the basal Cl^- current in the absence of agonist ($P>0.05$, $n=5$, data not shown). Furthermore, 1 μM SR 141716 did not significantly inhibit the basal Cl^- current in either transfected or untransfected cells ($P>0.05$, $n=6$, data not shown). These results indicate that constitutive activity of CB1 receptors in the activation of Cl^- current is not significant in hCB1 receptor-transfected or untransfected NPCE cells.

Activation of $I_{Cl,Win}$ by CB1 receptor coupled signaling is via a PTX-sensitive $G_{i/o}/G\beta\gamma$ pathway

CB1 receptors have been shown to couple to both G_i as well as G_s proteins (Porter & Felder, 2001). To investigate the effector pathway responsible for activation of $I_{Cl,Win}$ in human NPCE cells, we first determined the effect of PTX on the $I_{Cl,Win}$ activation. Human NPCE cells were incubated with 500 ng ml^{-1}PTX overnight at 37°C. As shown in Figure 7a, activation of $I_{Cl,Win}$ by 1 μM Win 55,212-2 was inhibited by PTX treatment. $I_{Cl,Win}$ was decreased from -84 ± 16 and 141 ± 25 pA (Win, $n=5$) to -16 ± 6 and 37 ± 31 pA (Win + PTX, $n=4$, $P<0.05$) at – 62 and 58 mV, respectively, suggesting the involvement of $G_{i/o}$ protein-coupled signaling pathway(s) in $I_{Cl,Win}$ activation.

$G\beta\gamma$ subunits of PTX-sensitive G_i proteins are major effectors for CB1 receptor-coupled signaling (Bouaboula *et al.*, 1999; Gomez del Pulgar *et al*., 2000; Rueda *et al.*, 2000; Derkinderen *et al*., 1996; Guzman *et al*., 2001). We therefore, explored whether the activation of $I_{Cl,Win}$ is mediated by $G\beta\gamma$ subunits. $I_{Cl,Win}$ was recorded in cells transfected with 5 μg ml^{-1} of control vector pIRES2-EGFP or pIRES2-EGFP-βARK. As shown in Figure 7b, transfection with pIRES2-EGFP-βARK significantly decreased $I_{Cl,win}$. The current was reduced from -144 ± 35 and 304 ± 88 pA (Win + EGFP, $n=5$) to -49 ± 17 and 105 ± 28 pA (Win + EGFP-βARK, $n=4$, $P<0.05$) at – 62 and +58 mV, respectively. These results suggest that a $G_{i/o}/G\beta\gamma$ pathway is responsible for the activation of $I_{Cl,Win}$ by CB1 receptors in NPCE cells.

PI3K, a signaling intermediary involved in the activation of the $I_{Cl,vol}$ in rabbit NPCE cells (Shi *et al*., 2002), is also an activated downstream target of CB1 receptors in certain cell types (Gomez del Pulgar *et al*., 2000). We investigated whether PI3K is also involved in $I_{Cl,win}$ activation using the PI3K inhibitor, wortmannin. As shown in Figure 7c, $I_{Cl,win}$ recorded in the the absence of wortmannin (Win, $n=7$) was -58 ± 16 and 173 ± 46 pA at – 62 and +58 mV, respectively. This was not significantly different to $I_{Cl,win}$ recorded in the presence of 100 nM wortmannin (Win + Wort, $n=6$), which was -53 ± 21 and 131 ± 63 pA at – 62 and +58 mV, respectively ($P>0.05$). In constrast, Figure 7d shows that treatment of NPCE cells with the MEK inhibitor, PD98059 (5 μM), completely abolished $I_{Cl,Win}$ ($P<0.001$; $n=6$) reducing mean $I_{Cl,Win}$ from – 80.0

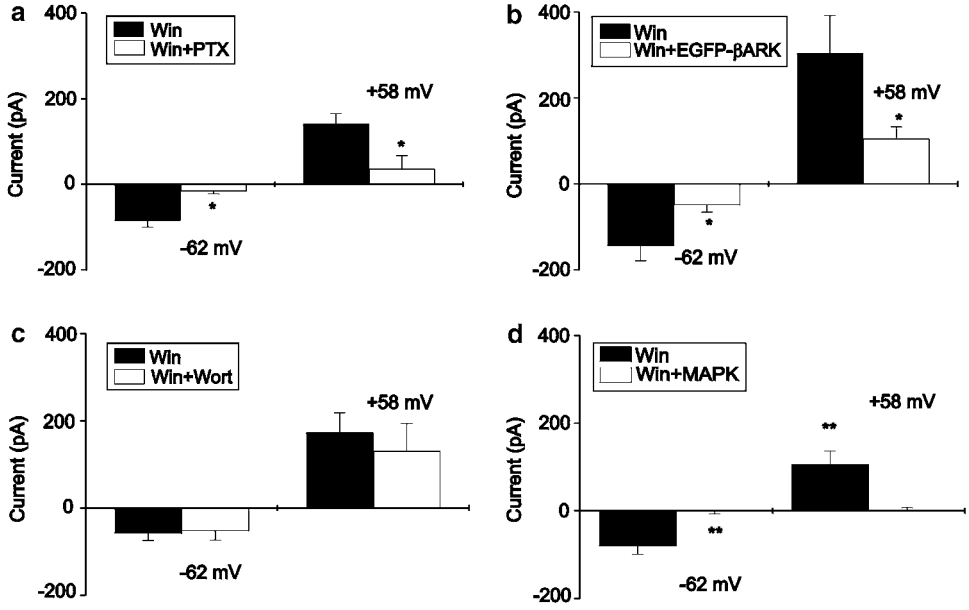

Figure 7 Activation of $I_{Cl,Win}$ is mediated via a $G_{i/o}$ protein/G$\beta\gamma$ signaling pathway. Mean $I_{Cl,Win}$ (agonist-stimulated Cl^- current minus basal Cl^- current) following application of 1 μM Win 55,212-2 at – 62 and + 58 mV in: (a) NPCE cells before (Win, $n=5$) and after PTX treatment (Win + PTX, $n=4$), (b) NPCE cells transfected with control vector pIRES2-EGFP (Win + EGFP, $n=5$) or pIRES2-EGFP-βARK (Win + EGFP-βARK, $n=4$), (c) NPCE cells before (Win, $n=7$) and after 100 nM wortmannin treatment (Win + Wort, $n=6$), and (d) NPCE cells before (Win, $n=6$) and after 5 μM PD98059 treatment (Win + PD, $n=6$). *$P<0.05$, **$P<0.001$.

$\pm$19.4 and 106.0 $\pm$ 30.0 pA to – 2.0 $\pm$ 4.9 and 3.16 $\pm$ 3.6 pA at – 62 and + 58 mV, respectively. Therefore, these data suggest that CB1 receptor activation of Cl^- current in human NPCE cells, like that of A3 adenosine receptor activation of Cl^- current, is PI3K-independent but MAPK-dependent.

Discussion

Both A3 adenosine receptors and CB1 receptors can couple to $G_{i/o}$ proteins, and signaling pathways activated by both these receptors have been reported to share similar characteristics, including inhibition of AC, and activation of PI3K and MAP kinases (Bouaboula *et al*., 1997; Gomez del Pulgar *et al*., 2000; Gao *et al*., 2001; Graham *et al*., 2001; Englert *et al*., 2002;). In this study, we investigated the signaling pathways linking activation of A3 adenosine receptors and CB1 receptors to stimulation of a Cl^- current in SV40-transformed human NPCE cells. Our results demonstrate that agonists for both A3 adenosine and CB1 receptors activated an outwardly rectifying PKC-sensitive Cl^- current. A3 receptor mRNA expression has been previously reported in an immortalized human NPCE cell line (Mitchell *et al*., 1999), and in this present study we demonstrate that the human NPCE (ODM) cell line also expresses endogenous CB1 receptor mRNA and protein. In addition, our data also show, for the first time, that both A3 and CB1 receptors are coupled to Cl^- current activation via a PTX-sensitive $G_{i/o}$/G$\beta\gamma$/ MAPK pathway, which is independent of PI3K.

Previously, we reported that cell swelling activated a PKC-sensitive Cl^- current ($I_{Cl,Vol}$) in rabbit NPCE cells via a PI3K and protein phosphatase signaling pathway (Shi *et al*., 2002). In human NPCE cells, a swelling-activated Cl^- current that is enhanced by PKC inhibitors, such as staurosporine, has also been described (Civan *et al*., 1994; 1996). The reported properties for $I_{Cl,Vol}$ identified in rabbit and human NPCE cells include outward rectification, inactivation at positive potentials, inhibition by PKC activation and increased current activation in the presence of PKC inhibitors. Although $I_{Cl,Aden}$ and $I_{Cl,Win}$, in this present study, exhibited no apparent inactivation at positive potentials, they did show outward rectification and were sensitive to PDBu inhibition. This suggests that cell swelling and $G_{i/o}$-coupled receptors, such as A3 and CB1, could activate the same PKC-regulated Cl^- current in human NPCE cells. Variation in current inactivation between $I_{Cl,Vol}$ and $I_{Cl,Aden}$ as well as $I_{Cl,win}$ could be because of different experimental conditions such as cell swelling-associated cytoskeleton changes and alterations in intracellular ionic strength. In support of this, Mitchell *et al.* (1999) reported that the adenosine-activated Cl^- current in a human NPCE cell line showed slight inactivation at positive potentials under their experimental conditions. This study and a subsequent report (Carre *et al*., 2000) also suggested that adenosine A_3 receptors may mediate the activation of $I_{Cl,Vol}$ in mammalian NPCE cells, because of the fact that both IB-MECA and adenosine stimulated cell shrinkage under isosmotic conditions. Furthermore, it was suggested that activation of A_3 adenosine receptors in NPCE cells could involve a mechanism whereby swelling induced release of ATP, a precusor of adenosine, leads to A3 adenosine receptor activation (Mitchell *et al*., 1998; 1999; Carre *et al*., 2000).

Based on these studies, we hypothesized that $G_{i/o}$ protein-coupled receptors such as A_3 adenosine and CB1 receptors, as well as cell swelling, could activate a common Cl channel(s) in human NPCE cells via a signaling pathway involving PI3K and PKC. However, in contrast to $I_{Cl,Vol}$ described in rabbit NPCE cells,where the PI3K inhibitor wortmannin abolished $I_{Cl,Vol}$ and the MAPK inhibitor, PD98059, had no significant

effect, this study found that activation of both $I_{Cl,Aden}$ and $I_{Cl,Win}$ was independent of PI3K but did require MAPK activation. Although the molecular pathways leading to activation of PKC-sensitive $I_{Cl,Vol}$ in human NPCE cells need to be confirmed to rule out species variations in signaling pathways, these present results suggest that while cell swelling and $G_{i/o}$-coupled receptor signaling pathways may activate common population(s) of Cl channels in NPCE cells, this occurs via different downstream signaling molecules. Other possible signaling cascades involved in GPCR-mediated activation of Cl^- currents in human NPCE cells may include direct $G\beta\gamma$ interaction with Cl channels or activation of MAPK signaling pathways. While MAPK activation has been generally associated with activation of PI3K (Touhara *et al.*, 1995; Yart *et al.*, 2002), PI3K-independent activation of MAPK has been documented (Danilovitch *et al.*, 2000), and in cerebellar granule cells AMPA receptor-induced activation of MAPK pathways is independent of PI3K and dependent on PTX-sensitive G proteins (Limatola *et al.*, 2002). Recently, it was reported that PTX-sensitive G proteins regulate the Cl^- permeability of HTC cells through Ca^{2+}-dependent stimulation of COX activity (Kilic & Fitz, 2002), and in isolated rabbit CBE, A1 agonists are able to stimulate an increase in $[Ca^{2+}]_{in}$ via a PTX-sensitive pathway (Farahbaksh & Cuillufo, 1997). Further experiments need to be carried out to determine if similar Ca^{2+}-dependent signaling pathway(s) mechanisms may contribute to the activation of a Cl^- current by A3 and CB1 receptors in human NPCE cells.

Constitutive activation of CB1 receptors coupled to G_i proteins has been reported. According to the model developed by Bouaboula *et al.* (1997), the CB1 receptor could exist in three states with inverse agonists such as SR 141716 promoting or stablizing the active negative state of the receptor. Overexpression of CB1 receptors has been suggested to increase constitutively active receptors and is associated with increased sequestration of G_i proteins from a common pool (Pan *et al.*, 1998; Vasquez & Lewis, 1999). We examined if constitutively active CB1 receptors were present in NPCE cells, especially in cells transfected with hCB1 receptor. We expected that in the absence of a CB1 receptor agonist, a portion of the basal Cl^- current could be contributed to the constitutive activity of CB1 receptors, which should be inhibited by SR 141716. However, our data showed that despite increased agonist-activated Cl^- current, transfection with hCB1 receptors appeared to have no measurable effect on the basal Cl^- current in the absence of agonists. Similarly, the inverse agonist SR 141716 did not decrease the basal Cl^- current in the transfected or untransfected cells. While we cannot rule out insufficient CB1 receptor overexpression to account for the lack of constitutive receptor activity, our data suggest that even in transfected NPCE cells, the amount of hCB1 receptor constitutive activity is not significant and cannot stimulate Cl^- current in the absence of agonist.

Our study, which demonstrates activation of Cl^- current in NPCE cells by the A3 adenosine receptor agonist, IB-MECA, is predictive of an increase in transepithelial secretion and is consistent with the observed increase in IOP which has been reported following *in vivo* administration of A_3 receptor agonists (Mitchell *et al.*, 1999; Carre *et al.*, 2000; Avila *et al.*, 2001). A recent study describing a decrease in IOP in the A3 knockout mouse model is also supportive for a role for A3 receptors in the NPCE in mediating increases in transepithelial ion transport and aqueous secretion (Avila *et al.*, 2002).

The activation of CB1 receptors in the mammalian eye has been previously associated with decreased IOP (Green & Roth, 1982; Pate *et al.*, 1995; 1996; 1998; Porcella *et al.*, 2001), an observation which cannot be explained by the stimulatory action of CB1 receptors on the Cl^- current in human NPCE cells. The high level of CB1 receptor expression in the NPCE (Porcella *et al.*, 1998) suggests that these receptors are physiologically relevant in the regulation of aqueous humor secretion, however, RT – PCR techniques and immunostaining have demonstrated that CB1 receptors are present at multiple sites within the human eye including the ciliary body blood vessels, CBE, ciliary muscle, trabecular meshwork and Schelmm's canal (Straiker *et al.*, 1997; Stamer *et al.*, 2001). Thus, the activation of CB1 receptors at these various sites could affect both aqueous humor inflow and outflow with net alterations in IOP reflecting contributions at all sites. In addition, it has been suggested that CB1 receptor activation could lead to a decrease in the release of noradrenaline in ocular tissues via activation of Ca channels (Porcella *et al.*, 2001), resulting in a decrease in aqueous humor formation. Furthermore, although the present study showed that activation of CB1 receptors in NPCE cells stimulated Cl^- current, which would favor an increase in solute secretion, CB1 receptors may also be negatively coupled to other ion transporters in the CBE, such that the net consequence of CB1 receptor activation in the CBE may be a decrease in aqueous humor production. Alternatively, interactions between CB1 receptors and other G protein-coupled receptors in the CBE may be important in circadian regulation of aqueous humor production and IOP regulation.

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