British Journal of Pharmacology (1998) 124, 1115–1122 © 1998 Stockton Press All rights reserved 0007–1188/98 $12.00
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Activation of a nonspecific cation current in rat cultured retinal pigment epithelial cells: involvement of a $G_{\alpha i}$ subunit protein and the mitogen-activated protein kinase signalling pathway

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1 Whole-cell patch-clamp recording techniques were used to investigate the G protein subtype and related signalling molecules involved in activation of a nonspecific cation (NSC) current in rat cultured retinal pigment epithelial (RPE) cells.

2 Under control conditions, in 130 mM NaCl with K^+ aspartate in the pipette, cytosolic dialysis with guanosine-5′-*O*-(3-triphosphate) (GTPγS, 0.1 mM) activated a large non-inactivating NSC current in 80% of the cells recorded from.

3 Loading RPE cells with antibodies (10 μg-ml^{-1}) against the α subunit of all PTX-sensitive G proteins ($G_{\alpha i/o/t/z}$) reduced NSC current activation to 11%, while loading RPE cells with antibodies directed specifically against the α subunits of the G_i subclass ($G_{\alpha i\text{-}3}$) completely abolished current activation. In RPE cells loaded with anti-$G_{\alpha s}$ activation of the NSC current was unaffected.

4 Investigation of the potential downstream mediators in the $G_{\alpha i}$ NSC channel pathway revealed that activation of the cation conductance was unaffected by treatment of RPE cells with the selective protein kinase C inhibitor GF 109203X (3 μM) or the selective CaM kinase II inhibitor KN-93 (50 μM). However, NSC current activation was delayed and the current amplitude reduced in the presence of the nonselective kinase inhibitor H-7 (100 μM) or the selective inhibitor of MAPKK (MEK) activation, PD 98059 (50 μM).

5 In the absence of GTPγS, the NSC current was not activated by superfusion of the cells with the cyclic GMP kinase activator dibutyryl-cyclic GMP or with the adenylate cyclase activator forskolin.

6 These results support the involvement of a G protein of the $G_{\alpha i}$ subclass in the activation of a NSC current in rat RPE cells, and suggest a potential modulatory role for MAP kinase-dependent phosphorylation in current regulation.

Keywords: Retinal pigment epithelium; nonspecific cation current; GTPγS; G protein; MAP kinase

Introduction

The retinal pigment epithelium (RPE) is a specialized monocellular layer of epithelial cells that lies external to the neurosensory retina. The RPE serves a number of functions essential to the viability of the outer retina and the photoreceptors including: acting as a pigment barrier to absorb stray light, uptake and storage of retinoids, ion and metabolite transport between the retina and choroidal circulation and phagocytosis of detatched portions of rod and cone outer segments (Zauberman, 1979). Fluid and ion transport by the RPE are thought to be essential for the maintenance of retinal attachment and optical clarity, as well as being integral components in the regulation of ion homeostasis in the subretinal space between the photoreceptors and the RPE (Steinberg & Miller, 1979; Zauberman, 1979).

Patch-clamp recordings from freshly isolated and cultured RPE cells in both amphibians and mammals have identified a variety of ion channels. Several voltage-dependent K^+ selective (Fox *et al*., 1988; Strauss *et al*., 1993; 1994; Tao *et al*., 1994) and Ca^{2+} selective ion channels (Ueda & Steinberg, 1993; Strauss & Wienrich, 1993) as well as anion channels have now been described (Botchkin & Matthews, 1993; Ueda & Steinberg, 1994). Recently, we have reported the existance of a nonspecific cation (NSC) current in rat RPE cells that is activated by a pertussis toxin (PTX) sensitive G protein pathway (Poyer *et al*., (1996)).

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GTP-binding proteins (G proteins) are known to couple directly a variety of receptors to intracellular effectors and considerable evidence now confirms that ion channels are common effector proteins in G-protein mediated signalling pathways (for review see Breitwieser, 1991; Rodbell, 1992). Ion channels can be modulated indirectly by G proteins, via activation of soluble second messengers of protein kinase phosphorylation pathways. A pathway independent of soluble second messengers involving direct membrane delimited G protein regulation of ion channels has also been demonstrated (Brown, 1993; Wickman & Clapham, 1995).

Several G protein alpha subunits (G_α) have been shown to be expressed in RPE cells (Jiang *et al*., 1991). However, the roles these G proteins may play in regulating functions of the RPE, such as fluid and ion transport, have not been fully elucidated. The purpose of this study was to identify the specific G protein subclass involved in the regulation of a NSC current that we have previously described in rat RPE cells (Poyer *et al*., 1996), and to investigate the potential role of other downstream signalling components in the regulation of this channel.

Methods

Cell dissociation and culture

RPE cells were dissected from Long Evans rats (8–12 days old) as previously described (Poyer *et al*., 1996). Rats were maintained and killed in accordance with the ARVO

Statement for the Use of Animals in Ophthalmic and Vision Research. Briefly, after removing any connective tissue, enucleated globes were bisected along the equator and the anterior portions were discarded. The posterior eye cups were placed in calcium-free Hank's EDTA (CFHE) containing 220 μ ml^{-1} hyaluronidase type III and 65 μml^{-1} collagenase A for 10 min at 37°C. Eye cups were then transferred into fresh CFHE, following which the neural retina was peeled off and discarded and the remaining posterior segments were placed in the enzyme solution for a further 5 min at 37°C. RPE tissue was gently peeled away from the choroid under view of a dissecting microscope (Zeiss) and then collected in fresh CFHE. RPE tissue pieces were then triturated through a fire-polished Pasteur pipette to yield a suspension of tissue clumps and single cells. The cell suspension was washed and resuspended in Dulbecco's Modified Eagle's medium (DMEM) plus 20% new born foetal calf serum (NCS), 0.5% penicillin-streptomycin and 50 μg ml^{-1} gentamicin. Isolated cells were seeded onto glass coverslips and maintained in culture at 37°C with 5%CO_2/95% O_2 for 2–5 days before electrophysiological recording.

As previously described (Tao *et al*., 1994; Gupta *et al*., 1997), RPE cells were clearly identified by their dense pigmentation, the presence of apical microvilli and positive staining for cytokeratins and the rat RPE-specific antibody, RET-PE10 (Neill *et al*., 1993).

Loading of RPE cells with G_α antibodies

To load RPE cells with antibodies against G_α subunits, cells were grown for 3–5 days in culture and then replated in 10 μg ml^{-1} of either $G_{\alpha i/o/t/z}$, $G_{\alpha i\text{-}3}$ or $G_{\alpha s}$ antisera. The $G_{\alpha i/o/t/z}$ antibody used in these experiments was a rabbit polyclonal antibody raised against a conserved sequence near the carboxy terminus of rat $G_{\alpha z}$. This antisera is cross-reactive with all members of the G_i family but not other G_α subunit proteins. The rabbit polyclonal $G_{\alpha i\text{-}3}$ antibody used was raised against amino acids 345–354 at the C-terminus of rat $G_{\alpha i\text{-}3}$. This antisera is reactive with $G_{\alpha i\text{-}1}$, $G_{\alpha i\text{-}2}$ and $G_{\alpha i\text{-}3}$ but not cross reactive with other G_α subunit proteins. Cells were loaded with antibodies using a variation of the scrape loading technique of McNeil *et al*. (1994). Replating was assumed to be physically equivalent to scrape loading in that during the replating process transient membrane pores are created through which antibodies can enter.

Briefly, cells were incubated with trypsin/EDTA for 5 min and then washed with an excess of DMEM plus 10% NCS. The cells were then centrifuged at 5000 *g* for 5 min, washed, resuspended and plated in DMEM/NCS containing 10 μg ml^{-1} of antibody solution. Resuspended cells were allowed to replate onto glass coverslips for 12–14 h. Control groups were: (1) cells resuspended in the absence of antisera; (2) cells replated with 10 μg ml^{-1} of non-reactive monoclonal antisera against glial fibrillary acidic protein (anti-GFAP) and (3) non-replated cells incubated with 10 μg ml^{-1} G_α antisera. Following electrophysiological recording, cells were washed, fixed and incubated with fluorescent secondary CY3 antibody to confirm G protein antibody loading.

Superfusion and solutions

Isolated cells attached to glass coverslips were positioned on the stage of a Nikon inverted microscope in a shallow recording chamber (1 ml). The recording chamber was superfused with the extracellular solution at a rate of 1–2 ml min^{-1}. The standard extracellular solution contained (in mM): NaCl 130, KCl 5, Na^+-HEPES 10, $NaHCO_3$ 10, $MgCl_2$ 1, $CaCl_2$ 1 and glucose 10. The intracellular solution contained (in mM): K^+-aspartate 100, KCl 10, HEPES acid 20, $MgCl_2$ 1, $CaCl_2$ 0.4, EGTA 1, ATP 1 and GTPγS 0.1. In several experiments intracellular K^+ was replaced by Cs^+ and the extracellular Cl^- concentration was reduced by equimolar substitution of NaCl with Na^+-asparate. Solutions were adjusted to pH 7.3–7.4. The extracellular solution was continuously bubbled with 5% CO_2/95% O_2 during electrophysiological recording.

To investigate the potential involvement of protein phosphorylation in the regulation of the NSC current, RPE cells were pre-incubated (20 min) and superfused during electrophysiological recording with 4 different membrane permeant protein kinase inhibitors: the non-specific kinase inhibitor, H-7; the PKC inhibitor, GF 109203X; the CaM kinase II inhibitor, KN-93 and the MEK inhibitor, PD 98059 at the concentrations cited in the Results section.

To explore further the involvement of cyclic nucleotides in NSC current activation in rat RPE cells, the cells were superfused with the membrane permeant analogue of guanosine 3′: 5′-cyclic monophosphate (cyclic GMP), dibutyryl cyclic GMP (300 μM), or the membrane permeant adenylate cyclase activator forskolin (50 μM) to generate adenosine 3′: 5′-cyclic monophosphate (cyclic AMP). For these experiments, drugs were superfused in low Cl^- (Na^+-asparate) external Ringers with Cs^+-asparate internal electrode solution, to block the K^+ or Cl^- currents that may be activated by the cyclic nucleotide analogues.

Electrophysiological recording techniques

We used whole-cell patch-clamp techniques to measure currents in isolated RPE cells (Hamill *et al*., 1981). Membrane potential and currents were recorded with an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA, USA) and pClamp software (Axon Instruments) was used to generate voltage commands. A two-stage vertical microelectrode puller (Narishige model PP83, Tokyo, Japan) was used to pull patch electrodes from borosilicate glass micropipettes with diameters of 1.5 mm outside and 1.1 mm inside. Electrodes were coated with beeswax to reduce capacitance and had resistances of 2–3 MΩ. The reference electrode was a sealed electrode-salt bridge combination (Dri-ref-2; World Precision Instruments, Sarasota, Fl). Before seals were formed on the cells, offset potentials were nulled using the amplifier circuitry. Capacitance subtraction and series resistance compensation (80%) was used in all recordings. The average resting membrane potential for cultured RPE cells determined under standard recording conditions immediately after break-in was -47 ± 2 mV ($n = 33$) and cell capacitance averaged 24 ± 9 pF ($n = 33$). Liquid junction potentials (LJP) between the bath and the electrode were measured experimentally and defined as the potential of the bath with respect to the electrode solution (Barry & Lynch, 1991). All data shown have been corrected for an LJP of 9 mV in standard K^+-aspartate intracellular and NaCl extracellular solutions and an LJP of 4 mV in Cs^+-aspartate intracellular and Na^+-aspartate extracellular solutions.

Statistical analysis

Data are presented as mean ± s.e.mean and analysed by use of Student's unpaired *t* test unless otherwise noted. A significance level of $P \leqslant 0.05$ was accepted.

Materials

Hank's EDTA and DMEM were purchased from Gibco BRL (Burlington, Ontario, Canada). G protein α subunit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, U.S.A.) and CY3 was purchased from Biocan Scientific (Missassauga, Ontario, Canada). H-7 (1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine), GF 109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide), KN-93 (2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]-amino-N-(4-chlorocinnamyl)-N-methylbenzylamine), PD 98059 (2′-Amino-3′-methoxyflavone) cyclic GMP and forskolin were all purchased from Calbiochem (LaJolla, California, U.S.A.). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Investigation of the G_{α} subunit involved in NSC current activation

As previously reported (Poyer *et al.*, 1996), the introduction of the non-hydrolyzable GTP analogue GTPγS (0.1 mM) into the cytoplasm of rat cultured RPE cells activates a NSC current. In this study, replated RPE cells attached readily to the substrate, resumed their normal morphology and exhibited whole-cell currents similar to those observed in non-replated cells. Immunofluorescent detection confirmed that all cells replated with G_{α} antisera appeared to contain antibody, indicative of a loading efficiency approaching 100%. No staining was observed in cells replated in the absence of antisera, anti-GFAP or non-replated control cells (data not shown).

Figure 1a shows macroscopic currents recorded from a representative cell replated in the absence of antibody 1 min and 15 min after assuming the whole cell configuration, in 130 mM NaCl and low chloride K^+-aspartate solution with 0.1 mM GTPγS in the pipette. Cells were held at a holding potential (V_h) of −69 mV and stepped to potentials of −129 to +51 mV in 20 mV increments for 500 ms. Immediately after break-in, the cell exhibits both inwardly and outwardly rectifying currents that resemble K^+ currents observed under normal conditions in the absence of GTPγS (Poyer *et al.*, (1996). However, 15 min after break-in, large noninactivating currents are apparent at potentials hyperpolarized and depolarized to the holding potential (−69 mV). The activated current was present in 76% of cells tested within a 15 min period following cytosolic dialysis with GTPγS. The current-voltage relationship shown in Figure 1b for the GTPγS-activated current, obtained by subtracting control current at 1 min after break-in from current at 15 min after break-in, was

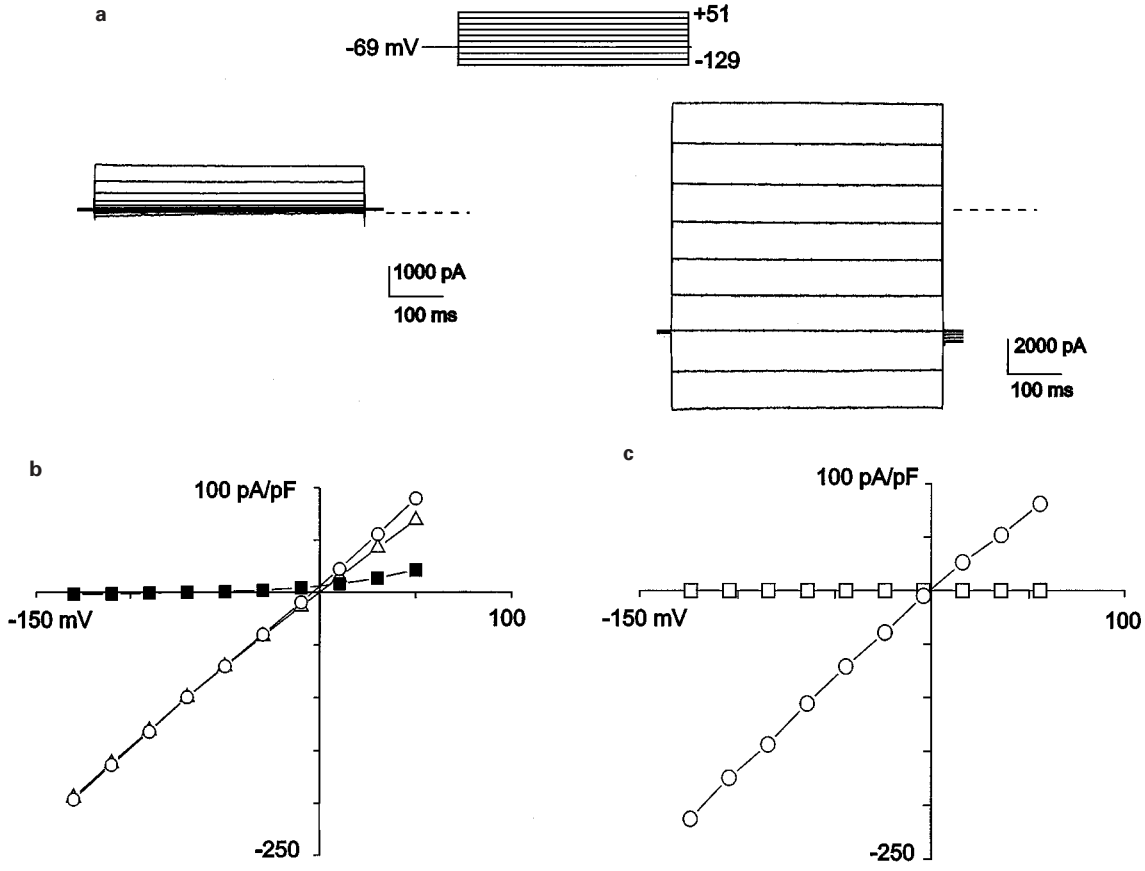

Figure 1 (a) Patch clamp recording from a representative RPE cell taken 1 and 15 min after assuming the whole-cell configuration, in standard 140 NaCl extracellular solution and K^+-aspartate intracellular solution with 0.1 mM GTPγ S in the pipette. The voltage protocol is shown at the top of the figure. (b) Current-voltage plot for the traces shown in (a) for 1 (closed squares) and 15 min (open circles) after break-in. The GTPγS-activated current (open triangles) was calculated by subtracting current at 1 min from those recorded at 15 min. (c) Current-voltage plot for a representative RPE cell in Na^+-aspratate extracellular and Cs^+-aspartate intracellular solution with 0.1 mM GTPγS in the pipette. Currents were measured 1 (open squares) and 15 min (open circles) after assuming whole-cell configuration. All currents have been normalized for cell capicitance. Dashed lines indicate the zero current potential in this and subsequent figures.

linear and reversed in this cell at 0 mV in standard recording solution. In 25 other cells the mean reversal potential of the GTPγS-activated current was -1.6 ± 1. Figure 1c shows the current-voltage relationship from a representative cell, recorded in solutions designed to eliminate K^+ and minimize Cl^- conductances (Cs^+-aspartate internal and Na^+-aspartate external solution), 1 and 15 min after break-in with 0.1 mM GTPγS in the pipette. Very little current was apparent immediately after break-in. However, 15 min after break-in with GTPγS, a large cation current was activated, the current-voltage relationship of which was linear and reversed at 0 mV ($+5.7\pm1.4$ mV; $n=18$). Activation of this current under conditions where contributions from K^+ and Cl^- currents are reduced confirms the nonselectivity of the current (Poyer *et al*., 1996).

Figure 2, panels a–c show macroscopic currents from representative cells loaded with anti- $G_{\alpha s}$ (a), anti-$G_{\alpha i/o/t/z}$ (b) or anti-$G_{\alpha\ i\text{-}3}$ (c), under the same recording conditions as replated control cells (see Figure 1a). The experimental protocol is shown at the top of Figure 2, with cells held at $V_h=-69$ mV

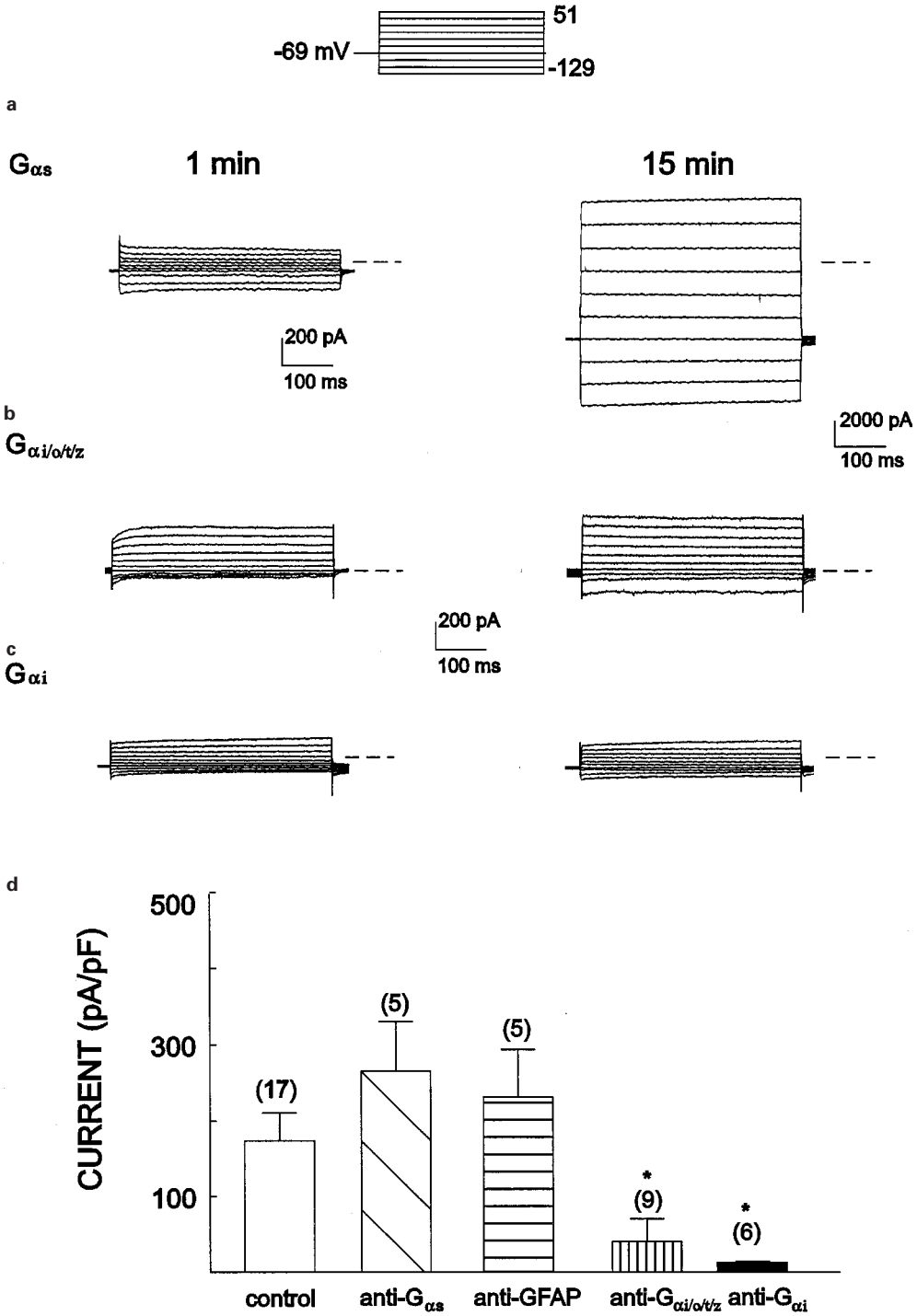

Figure 2 Whole-cell currents recorded from three representative RPE cells loaded with anti-$G_{\alpha s}$ (a), anti-$G_{\alpha\ i/o/t/z}$ (b) or anti-$G_{\alpha\ i-3}$ (c). Currents were recorded 1 and 15 min after assuming whole-cell configuration in standard recording solutions with 0.1 mM GTPγS in the pipette. (d) Mean (± s.e.mean) current amplitude measured at the end of a 500 ms step to +51 mV from $V_h=-69$ mV in control RPE cells (open column), RPE cells loaded with anti-GFAP, anti-$G_{\alpha s}$, anti-$G_{\alpha\ i/o/t/z}$ or anti-$G_{\alpha\ i-3}$. Current has been normalized for cell capacitance. (*$P<0.05$).

followed by voltage steps of 500 ms from −129 to +51 mV in 20 mV increments. As observed in control cells, both inwardly and outwardly rectifying currents were recorded 1 min after assuming the whole-cell configuration in all antibody-loaded cells (Figure 2 (a–c); left panel). In those RPE cells loaded with $G_{\alpha s}$, GTPγS- activation of the NSC current occurred in 74% of the cells tested ($n=7$) by 15 min after break-in (Figure 2a; right panel). In contrast, 15 min after break-in activation of the NSC current was observed in only 11% of the $G_{\alpha i/o/t/z}$ treatment group ($n=9$; Figure 2b; right panel), and the NSC current was not activated by GTPγS in any cells loaded with $G_{\alpha i\text{-}3}$ ($n=6$; Figure 2c; right panel).

The GTPγS-activated NSC current in control cells had a mean current amplitude at +51 mV of 174±37 pA pF^{-1} (replated control; $n=17$) and 233±62 pA p^{-1} (anti-GFAP; $n=5$) 15 min after break-in. Similarly, in RPE cells loaded with anti-$G_{\alpha s}$, the GTPγS-activated current measured at +51 mV had a mean current amplitude of 309±109 pA pF^{-1} ($n=7$) at 15 min after break-in. In contrast, current amplitude measured 15 min after break-in at +51 mV in RPE cells replated with anti-$G_{\alpha i/o/t/z}$ or anti-$G_{\alpha i\text{-}3}$ had mean current amplitudes of 40±29 pA pF^{-1} ($n=9$) and 9.9±2 pA pF^{-1} ($n=6$), respectively (Figure 2d). These results confirm that a G protein of the $G_{\alpha i}$ subtype is involved in the activation of the NSC current in rat RPE cells.

Involvement of protein kinases in current modulation

Figure 3a shows the time course for currents recorded in a control cell and 4 other representative cells, following pre-incubation and superfusion with the protein kinase inhibitors H-7, GF 109203X, KN-93 or PD 98059. H-7 is an isoquinolinesulphonamide that, at the concentration used in this study (100 μM), is known to be a nonselective inhibitor of a number of protein kinases including: protein kinase C (PKC), cyclic AMP-dependent protein kinase, myosin light chain kinase and cyclic GMP-dependent protein kinase (Hidaka *et al.*, 1994). The bisindolylmaleimide GF 109203X is a selective inhibitor for PKC and inhibits PKC in intact cells at low micromolar concentrations (Toullec *et al.*, 1991) Similarly, at micromolar concentrations, the Ca^{2+}/calmodulin-dependent protein kinase inhibitor, KN-93, inhibits a number of physiological responses associated with activation of CaM kinase II (Mamiya *et al.*, 1993; Marley & Thomson, 1996). We used PD 98059, a specific inhibitor of the MEK (Dudley *et al.*, 1995; Alessai *et al.*, 1995), to explore the possibility that mitogen-activated protein kinases (MAPKs) may be involved in NSC current activation.

To generate the time course of NSC current activation shown in Figure 3, current was measured every 20 s from $V_h=-69$ mV, using a 100 ms voltage pulse to +51 mV in standard intracellular solution containing 0.1 mM GTPγS. In the absence of kinase inhibitors, the NSC current was activated in 17/26 cells tested. The NSC current was also activated in 8/9 cells treated with either 100 μM H-7 or 3 μM GF 109203, in 3/4 cells treated with 50 μM KN-93 and in 3/3 cells treated with 50 μM PD 98059. The NSC current activated in the presence of the kinase inhibitors GF 109203X and KN-93 had properties similar to control cells. However, current activation in H-7 and PD 98059-treated cells was delayed compared to control, GF 109203X or KN-93-treated cells. In control cells activation of the NSC current significantly increased the current amplitude measured at +51 mV from 12±2 pA pF^{-1} at break-in to 135±42 pA pF^{-1} at 7 min post break-in in RPE cells pretreated with the kinase inhibitors GF 109203X (from 17±6 pA pF^{-1} to 100±30 pA pF^{-1}; $P<0.05$) or KN-93 (from 16±13 pA pF^{-1} to 148±66 pA pF^{-1}; $P<0.05$). In contrast, current amplitude measured at +51 mV at 7 min after break-in was not significantly different from break-in current amplitude in cells pretreated with H-7 (10±4 and 30±15 pA pF^{-1}; $P>0.05$) or PD 98059 (22±15 and 32±19 pA pF^{-1}; $P>0.05$). The decrease in current amplitude at 7 min with H-7 and PD 98059 is consistent with the delay in activation of the NSC current observed in the presence of these kinase inhibitors.

Figure 3b shows the mean current amplitude for the GTPγS-activated current in control cells and in cells pretreated with kinase inhibitors. Current amplitudes measured in control cells at +51 and −129 mV at 15 min after break-in were 195±32 pA pF^{-1} and −423±85 pA pF^{-1}, respectively ($n=17$). The mean amplitude of the current at ±51 and −129 mV in cells pretreated with H-7 (65±19 and −169±43 pA pF^{-1}; $n=8$) was significantly reduced compared to current amplitude measured in control cells ($P<0.05$). NSC current amplitude at +51 and −129 mV was unaffected in RPE cells treated with GF 109203X (193±52 and −625±212 pA pF^{-1}; $n=8$) or KN-93 (238±86 and −528±193 pA pF^{-1}; $n=4$). However, like H-7, pretreatment of RPE cells with the MEK inhibitor PD 98059

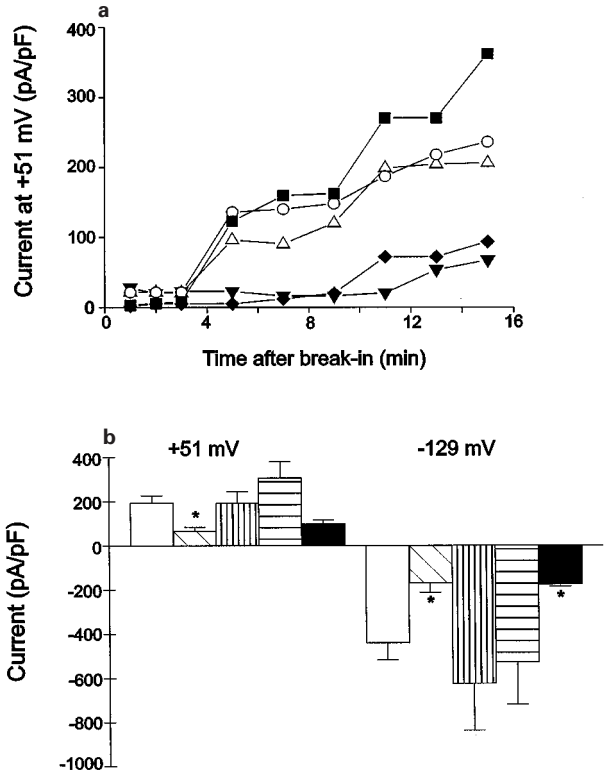

Figure 3 Time course for GTPγS-activation of the NSC current. (a) Current was measured every 20 s from a $V_h=-69$ mV using a 100 ms step to +51 mV, in standard K^+-aspartate intracellular solution containing 0.1 mM GTPγS and standard 130 NaCl extracellular solution. Current is shown for a representative control RPE cell (open triangles) and for four other cells that were pre-incubated and superfused with the protein kinase inhibitor H-7 (solid triangles), GF 109203X (open circles), KN-93 (solid squares) or PD 98059 (solid diamonds). (b) Mean (± s.e.mean) current amplitude measured at +51 and −129 mV at 15 min after assuming whole-cell configuration in control RPE cells (open columns) and in RPE cells pretreated and superfused with the kinase inhibitors H-7 (hatched columns), GF 109203X (stripped columns, vertical), KN-93 (stripped columns, horizontal) or PD 98059 (solid columns). All data have been normalized for cell capacitance. (*$P<0.05$).

also reduced the current amplitude measured at +51 and −129 mV (98±20 and −176±10 pA pF^{-1}; $n=3$) compared to control.

The lack of effect of the selective kinase inhibitors GF 109203X or KN-93 suggests that neither PKC nor CaM kinase II play a role in modulating NSC current activation in rat RPE cells. However, the delay in NSC current activation and the reduced current amplitude in the presence of the nonselective kinase inhibitor H-7 and the selective inhibitor of MEK, PD 98059, suggests that some phosphorylation event, potentially mediated by a MAP kinase, plays a role in NSC current modulation.

Involvement of cyclic nucleotides

Figure 4a shows the mean current amplitude measured at break-in (1 min) and 10 min after superfusion of RPE cells with the membrane permeant adenylate cyclase activator forskolin or the membrane permeant cyclic GMP analogue dibutyryl cyclic GMP. Cells were recorded with Na^+-aspartate external and Cs^+-aspartate internal solution, in the absence of GTPγS. Activation of the NSC current failed to occur in any of the tested RPE cells exposed to 10 μM forskolin for 10 min ($n=6$). The mean current amplitudes in forskolin-treated cells measured 1 and 10 min after break-in at +56 mV and −124 mV were 10±4 and −13±3 pA pF^{-1} and 13±10 and −19±15 pA pF^{-1} ($n=6$), respectively. The inability of forskolin to mimic activation of the NSC current in the absence of GTPγS, suggests that cyclic AMP is not involved in current activation and further confirms the lack of involvement of a $G_{\alpha s}$-coupled signalling pathway.

Superfusion of RPE cells with 300 μM dibutyryl cyclic GMP also failed to activate the NSC current in any cells tested Figure 4b; $n=6$). Mean current amplitudes measured at +56 and −124 mV at 1 min after break-in were 8±2 and −6±3 pA pF^{-1}, respectively. Similarly, 15 min after break-in, in the presence of dibutyryl cyclic GMP, the mean current amplitude at +56 mV (11±4 pA pF^{-1}) was not significantly different from break-in ($P>0.05$). However, in all 6 cells examined, superfusion with dibutyryl cyclic GMP significantly increased the mean current amplitude measured at −124 mV (−34±106 pA pF^{-1}; $P<0.05$; Student's paired t test) compared with break-in current. The mean current activated by dibutyryl cyclic GMP (see inset, Figure 4b) was obtained by subtracting I-Vs generated under control conditions from those obtained in the presence of cyclic GMP. The cyclic GMP-activated current showed rectification at negative potentials, with little outward current at depolarized potentials. The cyclic GMP-activated current had a mean current reversal of 1.2±3 mV and a mean whole-cell conductance of 2855 pS at −124 mV. This cyclic GMP-activated current in RPE cells may be similar to the cyclic GMP-activated nonselective cation currents described in other retinal cell types (Kusaka *et al*., 1996; Wei *et al*., 1996), but was distinct from the GTPγS-activated NSC current being investigated in this paper.

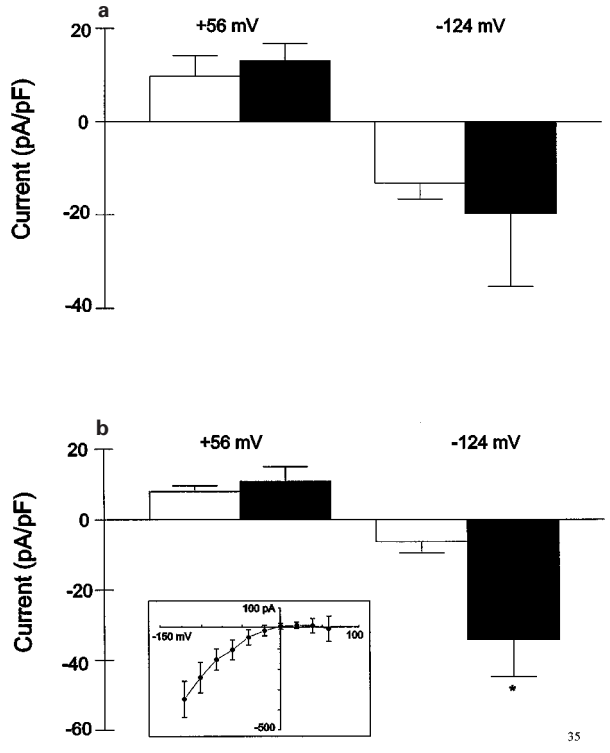

Figure 4 Mean (± s.e. mean) current amplitude measured in RPE cells with Cs^+-aspartate intracellular solution in the absence of GTPγS and Na^+-aspartate extracellular solution containing either forskolin (a) or dibutyryl-cyclic GMP (b). Current was measured at +56 and −124 mV at 1 (open columns) and 15 min (solid columns) after assuming whole-cell configuration. All current values have been normalized for cell capacitance. (b) Inset: mean current-voltage relationship for dibutyryl-cyclic GMP activated current in RPE cells, obtained by subtracting whole cell currents measured after 15 min superfusion with dibutyryl-cGMP from those measured immediately after break-in; vertical lines show s.e.mean (*$P<0.05$; Student's paired t test).

Discussion

We previously described a nonspecific cation (NSC) current in rat retinal pigment (RPE) cells that is activated by a PTX-sensitive G protein (Poyer *et al*., 1996.). This present study extends our previous findings by identifying the G_α protein subtype involved in NSC current activation. Furthermore, we have also demonstrated that the downstream events in NSC channel activation require phosphorylation, and may be mediated via a MAP kinase signalling pathway.

Our results indicate that loading RPE cells with an antibody which recognizes the α subunit of all PTX-sensitive G_i proteins ($G_{\alpha i\text{-}1}$, $G_{\alpha i\text{-}2}$ and $G_{\alpha i\text{-}3}$), completely abolishes GTPγS activation of the NSC current. This finding, combined with the lack of effect of antibodies directed against the PTX-insensitive $G_{\alpha s}$ subunit proteins, confirms that a G protein of the $G_{\alpha i}$ subtype is involved in NSC current activation in rat RPE cells.

In support of our findings, studies in mammalian RPE cells have identified multiple G protein species, including all 3 subtypes of $G_{\alpha i}$ proteins (Jiang *et al*., 1991), and demonstrated that a number of light-adaptive responses, ion transport pathways and second messenger activity in this epithelium are modulated by ligands that are known to couple to G protein signalling pathways (Hughes *et al*., 1988; Dearry *et al*., 1990; Nash & Osborne, 1995). More recently, a PTX-sensitive nonselective cation channel has also been described in human RPE which is activated by lysophosphatidic acid (Thoreson & Chako, 1997). Evidence from other studies in a variety of cell types also supports the involvement of G proteins, including PTX-sensitive ones, in the activation of nonselective cation channels. For example, muscarine-mediated activation of a nonselective cation channel in smooth muscle is known to involve G_i/G_o proteins (Wang *et al*., 1997) and in Hl-60 cells, a nonselective cation channel has also been described that is

activated via PTX-sensitive G_i proteins (Hageluken *et al*., 1995).

Ion channels coupled to G protein signalling pathways can be modulated both directly by activated G protein subunits and indirectly by a number of downstream signalling intermediaries (Wickman & Clapham, 1995). Second messengers like Ca^{2+} and ATP modulate the activation of a number of different ion channels (for review see, Ghosh & Greenberg, 1995), including nonselective cation channels (Inoue *et al*., 1996; Lidofsky *et al*., 1997). In addition to Ca^{2+} and ATP, multiple protein kinases, under the control of several signalling pathways, are responsible for site-specific phosphorylation of ion channels, which result in changes in channel gating properties (for review see, Smart, 1997). The GTPγS-activated NSC current in rat RPE cells did not require rises in intracellular Ca^{2+} or internal ATP for its activation (Poyer *et al*., 1996). Our current results, examining the involvement of protein kinases in NSC channel activation, indicate that neither CaM kinase or PKC-mediated phosphorylation are involved in NSC current regulation, as inhibitors of these kinases (KN-93 and GF 109203X) had no effect on NSC current activation or amplitude. However, in the presence of the nonselective kinase inhibitor H-7 or a selective inhibitor of MEK activation (PD 98059), NSC current activation was delayed and current amplitude reduced. These results, therefore, support a role for protein kinases in NSC current regulation and suggest that MAPK(s)-dependent phosphorylation may be involved in modulating NSC channel activity in rat RPE cells

Modulation of nonselective cation currents by kinase-mediated phosphorylation events have now been described in a variety of cell types. For example, in human T84 epithelial cells, phosphorylation mediated by CaM kinase was demonstrated to be required for activation of a Ca^{2+}-activated nonselective cation current (Braun & Schulman, 1995). PKA-dependent phosphorylation is also involved in the activation of a nonselective cation current in renal epithelial cells (Marunaka *et al*., 1997) and tyrosine phosphorylation by the endogenous tyrosine kinase Src regulates the activity of N-methyl-D-aspartate receptors in mammalian central neurones (Yu *et al*., 1997). A role for MAPK(s)-dependent phosphorylation in nonselective cation channel activation has not yet been described. However, a recent study has identified that MAPK(s) are involved in the activation of a volume-activated chloride current in rat astrocytes (Crepel *et al*., 1998). Furthermore, recent evidence has demonstrated that the PTX-sensitive G proteins $G_{\alpha i\text{-}2}$ and $G_{\alpha i\text{-}3}$ can activate the mitogenic cascade leading to the activation of MAPKs (Kinane *et al*., 1997), supporting our finding that MAPK(s) may be downstream mediators of the $G_{\alpha i}$ signalling pathway coupled to NSC current activation.

Nonselective cation channels have been described that can be activated or modulated indirectly or directly by cyclic nucleotides, such as cyclic GMP or cyclic AMP (Fin *et al*., 1996; Kusaka *et al*., 1996). We found no evidence for the involvement of cyclic AMP in NSC current activation, as forskolin failed to activate the current in all RPE cells examined. This result supports our previous finding demonstrating the lack of effect of anti-Gαs on current activation and confirms that a G_s-stimulated adenylate cyclase/cyclic AMP pathway is not involved in NSC current regulation in rat RPE cells. We also explored the possibility that the cyclic nucleotide cyclic GMP may activate the NSC current in rat RPE cells as other mammalian retinal cells, including Muller (glial) (Kusaka *et al*., 1996) and rod photoreceptor cells (Wei *et al*., 1996), are known to possess cyclic GMP-activated nonselective cation currents. The cyclic GMP analogue dibutyryl cyclic GMP failed to activate a current with the characteristics previously described for the GTPγS-activated NSC current in rat RPE cells. However, we did identify that dibutyryl cyclic GMP activates an inwardly rectifying conductance that reversed close to 0 mV, suggesting that rat RPE cells do possess a cyclic GMP-gated cation current. More detailed studies on the ion permeation of this channel will be necessary in order to elucidate fully the characteristics of this current in rat RPE cells.

In summary, this present study demonstrates that activation of the NSC current in rat RPE cells is mediated by a $G_{\alpha i}$ protein. We also show that kinase-dependent phosphorylation, potentially mediated by MAPK(s), is a component of the signalling pathway activated by $G_{\alpha i}$ and is involved in regulating NSC channel activity. Although the factor(s) and receptor(s) responsible for physiological initiation of the $G_{\alpha i}$ signalling pathway leading to NSC current activation has not yet been identified, activation of this current *in vivo* could provide a pathway for significant Na^+ and cation influx. The resulting membrane depolarization could activate other ionic conductances, such as voltage-dependent K^+ or Cl^- channels, which could have important functional consequences for ion transport across this epithelium.

We thank Christine Jollimore for editorial assistance and technical support. This study was funded by the Natural Science and Engineering Council (NSERC) of Canada. JSR holds an NSERC PGSB postgraduate Studentship award.

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(Received February 17, 1998
Revised April 3, 1998
Accepted April 9, 1998)